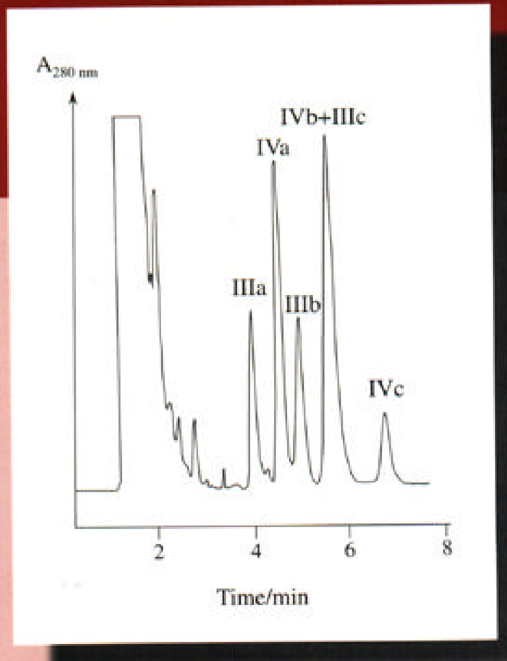
Food Analysis by HPLC

Second Edition
Revised and Expanded



edited by

Leo M. L. Nollet

Food Analysis by HPLC

Second Edition
Revised and Expanded

edited by
Leo M. L. Nollet
Hogeschool Gent, Ghent, Belgium

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ISBN: 0-8247-8460-X

This book is printed on acid-free paper.

Headquarters
Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
tel: 212-696-9000; fax: 212-685-4540

Eastern Hemisphere Distribution
Marcel Dekker AG
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
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World Wide Web
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Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

Preface

High-performance liquid chromatography (HPLC) is a fast technique that, with high precision and specificity, separates mixtures into individual ingredients. Used as a routine procedure, it has several advantages: It can be completely automated; sample cleanup and preparation are simple; and the reproducibility of the packing material means that the analytical conditions remain the same for a new column.

Different kinds of HPLC exist. Many kinds of column packings and solvents are available. Retention behavior and resolution are affected by column characteristics (C-loading, chain length, porosity, etc.) and by elution scheme characteristics (mobile phase, pH, organic modifier, etc.). The samples can be separated on the basis of solubility and polarity of the sample components.

For normal-phase HPLC the solvent (mobile phase) is nonpolar (hexane, methylene chloride, etc.) and the column packing (stationary phase) is polar (silica). Sample molecules are more or less attracted to the particles in the column as opposed to the solvent. The less polar molecules are eluted first. Reversed-phase HPLC (RP HPLC) uses polar solvent (water, polar solvents, or mixtures) and nonpolar packing (C-18 or phenyl). In RP HPLC the elution order is reversed. The stationary, phases are mostly derivatized with alkylsilane such as octadecyl groups (C-18 or ODS) or octyl-groups (C-8). In polar-bonded phases the silica surface is derivatized with polar functional groups. These packings are used in the normal and reversed mode. In gel permeation chromatography (GPC) the separation of large molecules, such as proteins, is based on their effective size. Ion-exchange chromatography separates samples based on their charge (anion or cation exchange). Tailor-made or specialty columns provide high-resolution separations of well-defined components, such as fatty acids or hydrocarbons.

Applications of HPLC in food technology involve, on one hand, an analytical and quantitative testing of the product composition and, on the other hand, an assurance of product quality with increased productivity. HPLC is used in the food industry for the analysis of components in both raw and processed products. In a new food product, analysis of the raw materials, the intermediates, and the final products is necessary. Changes during processing or storage are to be followed as well. Foods and beverages may be tested on contaminants or additives, so that governmental regulations may be followed.

In recent years HPLC technique has been perfected. A large number of food components may be separated with HPLC. Many excellent books have been published on HPLC theory and thousands of articles have been written on chromatographic separations of food-related components.

The first edition of *Food Analysis by HPLC* fulfilled a need because no other book was available on all major topics of food compounds for the food analyst or engineer. In this second edition, completely revised chapters on amino acids, peptides, proteins, lipids, carbohydrates, vitamins, organic acids, organic bases, toxins, additives, antibacterials, pesticide residues, brewery products, nitrosamines, and anions and cations contain the most recent information on sample cleanup, derivatization, separation, and detection. New chapters have been added on alcohols, phenolic compounds, pigments, and residues of growth promoters.

The reader should find it helpful that the most recent published articles of the last few years are discussed. As in the first edition, special attention is paid to reversed-phase separations without neglecting other HPLC techniques. Specialists describe in detail, step by step, sample preparation and separation conditions. The applications to food chemistry are specific and practical. The book will once again find a large audience in the fields of chromatography, analytical chemistry, and, especially, food chemistry and food technology.

Sincere thanks are given to everyone who has contributed. I would like to thank all the contributors for their excellent efforts.

This work is dedicated to my daughter, Annemarie, and her unknown and mysterious friend whom, I'm sure, she will meet.

Leo M. L. Nollet

Learning is the fountain of youth.
No matter how old you are,
You mustn't stop growing.

365 Tao: Daily Meditations
Deng Ming-Dao
Harper San Francisco, 1992

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Food Analysis by HPLC

1
HPLC

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I. INTRODUCTION

Analysis is the separation of a whole into parts. It is the qualitative and quantitative investigation and evaluation of samples. Chromatography and electrophoresis are the most powerful techniques of separation. Thousands of papers are published, as can be seen from the biannual reviews from *Analytical Chemistry*. Efficiency, selectivity, and sensitivity are ever-increasing, and a simple comparison between chromatograms from the 1980s to those from the '90s is impressive. Manufacturers have made tremendous efforts to give analysts the opportunity to achieve difficult separations with reliable, easy-to-handle instruments.

The trend is toward "friendly," increasingly miniaturized instruments that can produce efficient and selective separations. Many instruments are controlled by insertable PC cards so the analyst can automatically set up the desired procedure. Moreover, diagnostic software is helping the user to identify failures. Costs are minimized, especially solvent costs. Optimization procedures currently in use save time (and time is money). Methods and instruments are validated for compliance with, for example, ISO recommendations.

An enormous amount of literature is available on the topic. Readers may refer to such textbooks as *Practical HPLC Method Development*, edited by L. R. Snyder, J. J. Kirkland, and J. L. Glajch; *HPLC, Fundamental principles and Practice*, edited by W. J. Lough and I. W. Wainer; *Handbook of HPLC*, edited by E. Katz, R. Eksteen, P. Schoenmakers, and N. Miller; or other textbooks previously published. Useful information can be retrieved from specialized journals on chromatography such as *Journal of Chromatography*, *Chromatographia*, *LC-GC International*, and *Journal of Liquid Chromatography*, which publish special issues on selected topics. *Analytical Chemistry* has many articles on chromatography, together with the biannual reviews already noted.

According to the IUPAC definition, (1) chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), and the other of which (the mobile phase) moves in a definite direction. The mobile phase is a fluid that percolates through or along the stationary bed. Three types of fluid are in current use: liquid, gas, and supercritical. Chromatography is named mainly by the nature of the mobile phase (liquid chromatography, or LC; gas chromatography, or GC; supercritical fluid chromatography, or SFC). Since its advent in 1974–75, high-pressure liquid chromatography (HPLC) has expanded very quickly. The acronym has come to mean high-performance liquid chromatography. Since HPLC is fully instrumentalized, LC is often used in place of HPLC.

II. DEFINITIONS—RETENTION

Thanks to progress achieved in solid support, LC is utilized mainly as liquid/solid chromatography. Liquid/liquid chromatography is restricted to countercurrent chromatography (CCC).

In LC we can distinguish packed columns and open tubular columns. For the mobile-phase velocity, Darcy's law (2) is valid. For packed columns it is written

$$u = \frac{-B}{\eta}\frac{dp}{dx}$$

where u is the local velocity at point x along the column, the local pressure is p, dp/dx is the pressure gradient, η is the viscosity of the mobile phase, and B is the column permeability. $B = B_o d_p^2$, where B_o is the Karman–Koszeny permeability and d_p is the particle size. The flow resistance parameter is $\phi = d_p^2/B_o$.

Particles are now almost exclusively spherical. Most particles used in HPLC are not monodisperse. They have a finite particle size distribution, which is typically in the range of the mean particle diameter $\pm 50\%$. The trend is toward monodisperse particles.

The viscosity of a liquid usually increases with increasing pressure. In the pressure range up to 200 bars this variation is practically linear.

The observed linear velocity of eluent is $u = F/\epsilon S$, where F is the flow rate, ϵ is the porosity, and S is the tube section.

With open tubular columns, permeability is given by Poiseuille's law, $B = d_c^2/32$, where d_c is the column internal diameter.

The time that elapses between injection and elution of a solute is called the *retention time*. When a solute is not retained, we write $t_o = L/u$, where L is the column length.

Particles are either porous or nonporous. With porous particles, fluid can go through pores where the velocity is less than that outside the pores. It means that unretained solutes travel at different velocities whether they can penetrate into pores or not. We shall also see that the size exclusion principle applies, meaning that "true" t_o does not exist and the analyst must carefully indicate what solute is considered as unretained. On a recorder chart, the analyst reads d_0, the retention distance of the unretained solute; V_0 is the retention volume of the unretained solute.

A solute that exhibits some affinity toward the stationary phase jumps from one phase to another. It is retained for a while in the stationary phase, with the consequence that its average speed of travel is less than that of the unretained solute. Retention time is $t_r > t_o$. We thus can write $t_r = t_o(1 + k)$, where $k = (t_r - t_o)/t_o$. k is the *retention factor* (formerly called *capacity factor*). The retention factor is a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase.

Molecules of solute travel as a zone in the chromatographic system. Recording of molecules eluting from the column yields a chromatogram (Fig. 1), whose characteristics are peaks. When peaks are symmetrical (Gaussian shape), retention times are taken at peak height. Since k is dimensionless, one can record retention distances or retention volumes on the chromatogram and

$$k = \frac{t_r - t_o}{t_o} = \frac{d_r - d_o}{d_o} = \frac{V_r - V_o}{V_o}$$

where d_r is the retention distance on the recording chart and V_r is the volume of retention. The retention factor is also equal to the ratio of amounts of sample component in the stationary and mobile phases at equilibrium:

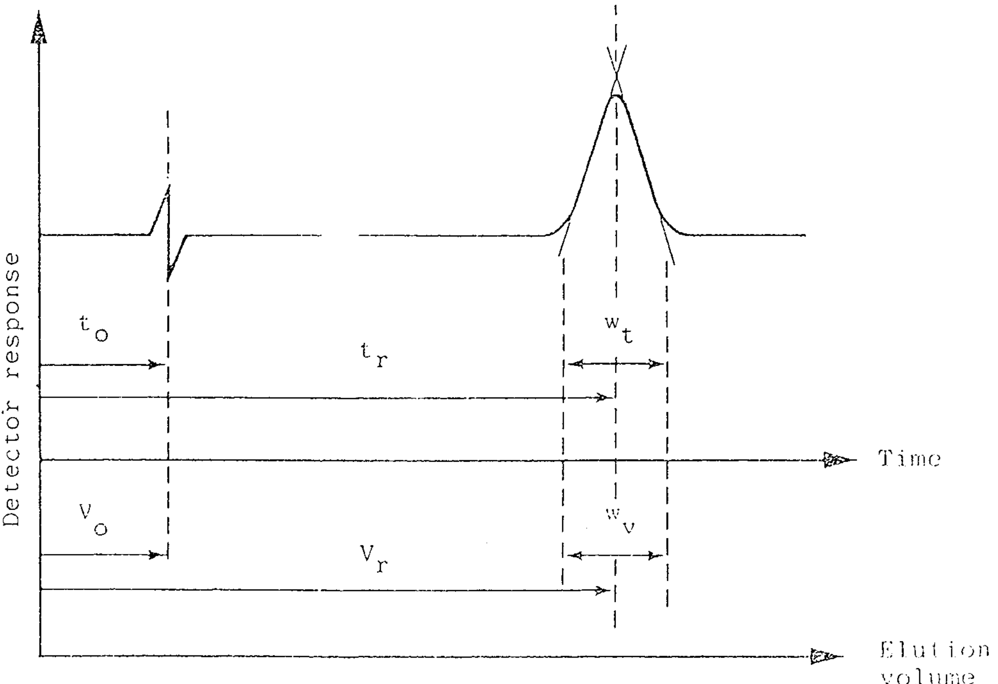


Fig. 1 A recorded chromatogram. t_o, V_o are retention time and retention volume of the unretained (inert) solute. t_r, V_r are retention time and retention volume of a retained solute. ω_t, ω_v are peak width.

$$k = \frac{\text{Amount of component in stationary phase}}{\text{Amount of component in mobile phase}}$$

Because a chromatographic peak is assumed to result from the spreading of the sample zone with a Gaussian distribution of sample concentrations in the mobile and stationary phases, the calculation of the column efficiency is related to the associated variance, σ^2. The efficiency of the process is thus the ratio t_r^2/σ^2; σ^2 is the variance in time units (in this case). When d_r is measured on the recorder chart, the ratio d_r^2/σ^2 is considered, with σ in distance units.

With Gaussian peaks (Fig. 2), the width of a zone on the record baseline is $\omega = 4\sigma$ (σ is the standard deviation).

$$N = t_r^2/\sigma^2 = 16(t_r/\omega)^2$$

N is the also called the number of theoretical plates.

$H = L/N$ is the height equivalent to a theoretical plate (HETP). It can be considered as the thickness of a transverse slice of column. Since it is often difficult to measure accurately the peak width ω, one can use the peak width at half height, and $N = 5.54(t_r/\partial)^2$. ∂ is the thickness of the peak at half height, it is different from 2σ.

For a discussion on efficiency measurement, see Ref. 3.

When a peak is not symmetrical, the asymmetry factor is calculated via $A_s = A/B$, as defined (in Fig. 3) by drawing a line parallel to the baseline at 10% of peak height. With non-symmetrical peaks, it is necessary to use statistical moments. The zero moment, $M_0 = \int_{-\infty}^{+\infty} h(t)\, dt$, is the peak height at time t. The first moment, $M_1 \int_0^{\infty} th(t)\, dt$, expresses the true retention time as

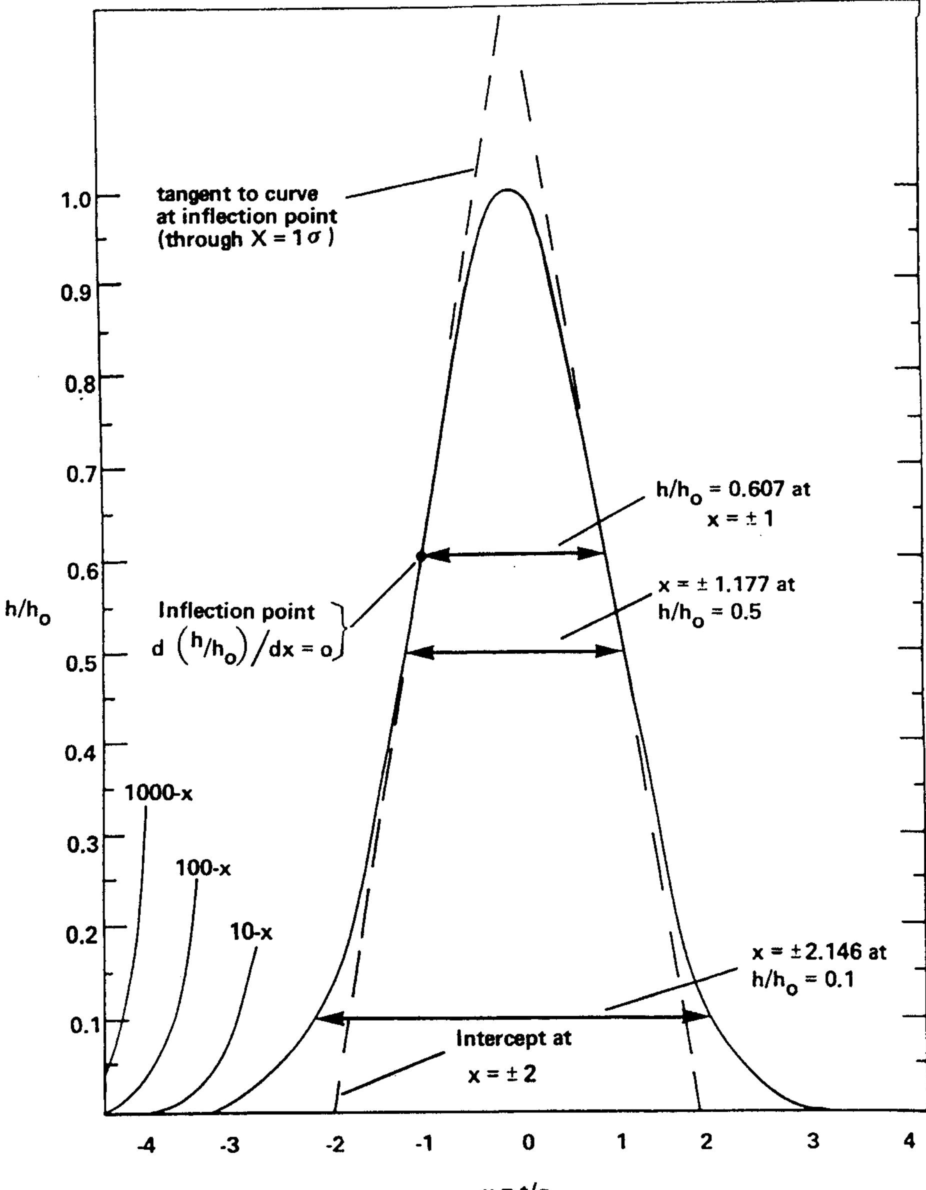


Fig. 2 Gaussian curve and related figures of significance.

it corresponds to the elution time of the center of gravity of the peak. The second moment is the peak variance, and the third moment is the peak skew.

When two Gaussian peaks (i and j) are close, resolution (R_s) is defined as the peak separation divided by the mean peak width:

$$R_s = \frac{2(d_{rj} - d_{ri})}{\omega_i + \omega_j}$$

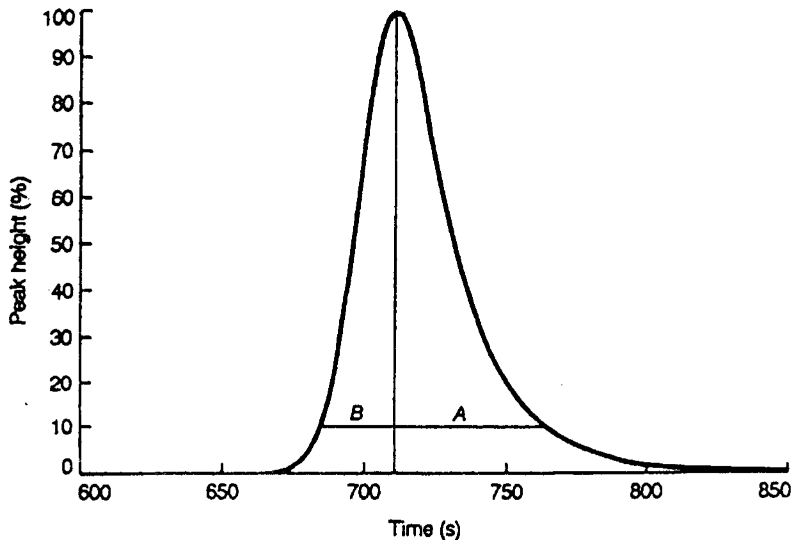


Fig. 3 Asymmetrical peak. Asymmetry is the ratio A/B. A and B are determined from a line parallel to the baseline at 10% of the peak height.

When two peaks are baseline resolved, $R_s > 1.25$.

Selectivity is $\alpha = k_j/k_i$, with $k_j > k_i$, which means $\alpha > 1$. When $\alpha = 1$, no separation occurs.

From the foregoing equations it is readily shown that the necessary number of plates to achieve a separation with a given resolution (e.g., $R_s = 1.25$) is given by

$$N_{\mathrm{ne}} = 16R_s^2 \frac{(\alpha)^2}{(\alpha - 1)^2} \frac{(1 + \bar{k})^2}{\bar{k}^2}$$

with $\bar{k} = (k_i + k_j)/2$. To emphasize the selectivity we can write

$$\alpha = \left[1 - \frac{4R_s}{\sqrt{N}} \frac{1 + \bar{k}}{\bar{k}}\right]^{-1}$$

For a given 12,000-plate column, a slight change of α from 1.030 to 1.044 yields a complete resolution.

The area of a Gaussian peak A_p is given by

$$A_p = \frac{\sqrt{2\pi}}{4} \omega h_p$$

where h_p is the peak height. The peak capacity n_p is the number of peaks that can be observed on a chromatogram with baseline resolution:

$$n_p = 1 + \frac{\sqrt{N}}{4} + \ln \frac{t_{r1}}{t_{rz}}$$

where t_{r1} is the retention time of the first eluted solute and t_{rz} is the retention time of the last eluted solute.

To compare columns of different sizes packed with different packings we use reduced dimensions:

Reduced plate height is:

$h = H/d_p$ with packed columns

$h = H/d_c$ with open tubular columns

Reduced length of column is $l = L/d_p$ or L/d_c.

Reduced velocity of the mobile phase is $\nu = ud_p/D_m$, where D_m is the diffusion coefficient of the solute in the mobile phase.

As in gas chromatography, there is a relationship between the reduced velocity of the mobile phase and the reduced plate height. It is the Knox equation (4):

$$h = A\nu^{1/3} + B/\nu + C\nu$$

where A, B, C are dimensionless coefficients. With good columns, $A = 0.8–1.0$; $C = 0.01–0.03$. A is a packing term related to particle diameter and permeability of the column. When a column is properly packed, A is minimum. Guiochon (5) has pointed out that the packing of chromatographic columns is often heterogeneous, being denser and less organized close to the column wall. A is zero with open tubular columns. B is the effective diffusion coefficient; it depends on the solute. B is around 1.5–2.0. C is connected to mass transfer. Plots of h versus ν exhibit a minimum (Fig. 4) that corresponds to the optimum experimental conditions to achieve the highest number of plates. With very small particles (e.g., less than 3 μm), C is very low and the HETP curve is flat at high velocities.

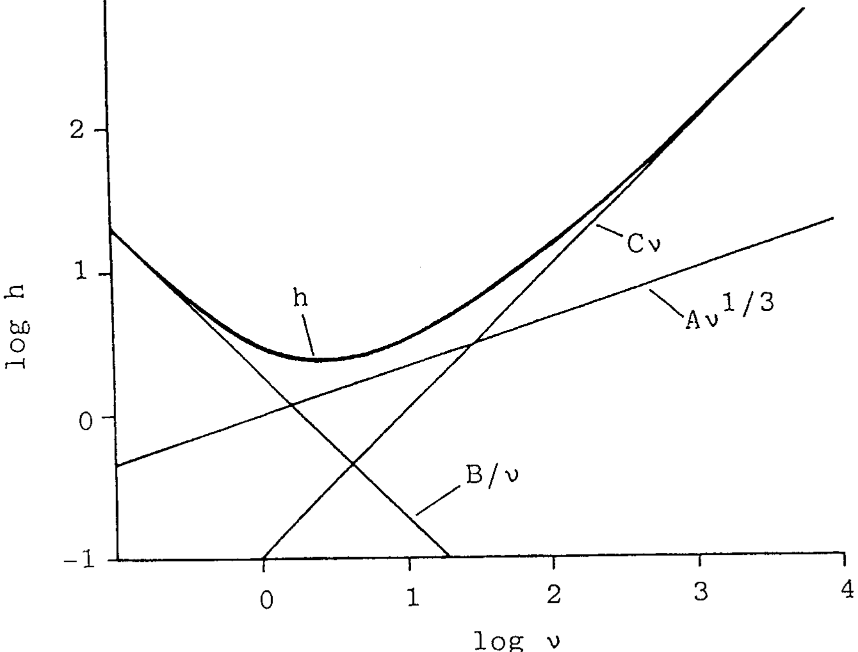


Fig. 4 The Knox plot: h (reduced plate height) versus (reduced velocity). (Reproduced with permission from Preston Publications.)

According to Guiochon (6), when C is negligible, the optimum value of ν, ν_{opt}, is

$$\nu_{opt} = (3A)^{4/3}/(B)$$

With good columns, $h = 2$, which permits the calculation of column length.

When measuring the recorded peak variance we must keep in mind that the observed width of a peak consists of two parts: the width due to the distribution process in the column and the undesirable dispersion outside the column (injector, connecting tubing, detector, everything that comes from the instrument). From the theorem of variance additivity of independent processes we can write

$$\sigma^2_{observed} = \sigma^2_{inj} + \sigma^2_{chromato} + \sigma^2_{conn} + \sigma^2_{detec} + \sigma^2_{rec}$$

where the subscripts are variances associated, respectively, with dispersion in the injection device, the chromatographic process, the connecting tubing, the detector, and the recorder, and σ^2_{det} and σ^2_{rec} include time constants. The contribution from the extra column band broadening is calculated from the following relationship:

$$N = 16(V_r)^2/(\omega)^2 = 16V_o(1 + k)^2/\omega^2_{col}\,[1 + (\omega_{ext\text{-}col}/\omega_{col})]^2$$

One way to calculate the extra column band broadening $\sigma^2{}_{extr}$ is to consider that extra column variance is rather low or negligible for solutes of high capacity. In this way we can write

$$\sigma^2_{observed} = t_o^2/N(1 + k)^2 + \sigma^2_{extr}$$

where $\sigma^2_{observed}$ is the observed variance. With solutes of different k values, one plots $(1 + k)^2$ versus $\sigma^2_{observed}$. The intercept represents σ^2_{extr}.

Column impedance E has been introduced by Bristow and Knox (7):

$$E = t_o\Delta P/N^2\eta = h^2\phi$$

where h is the reduced plate height and ϕ is the flow resistance parameter. E is around 3000–4000.

Analysis time increases in proportion to the square of the plate number, i.e., to the fourth power of the resolution aimed at. With open tubular columns the reduced HETP h is given by the Golay (8) equation:

$$h = \frac{2}{\nu} + \frac{1 + 6k + 11k^2}{96(1 + k)^2} \times \nu + \frac{2}{3}\frac{k}{(1 + k)^2}\frac{D_m}{D_s}\frac{e_f}{d_c} \times \nu$$

where ν is the reduced velocity of the mobile phase and k is the retention factor. From the derivation of this equation it can easily be demonstrated that

$$h_{min}\nu_{opt} = 4$$

In the Golay equation, e_f is the phase thickness, D_m and D_s are diffusion coefficients in the mobile phase (m) and the stationary phase (s), respectively.

III. MOBILE PHASES

In GC, the mobile phase acts only as a carrier. In LC, solute undergoes interaction with liquids or mixtures of liquids used as the mobile phase. Selection of the mobile phase is critical. The most useful criteria are the solubility parameter concept, Snyder's selectivity triangle, and solvatochromic parameters.

The Hildebrand solubility parameter δ_t (total solubility parameter) is a rough measure of solvent strength:

$$\delta_t^2 = -E_s/V_s$$

where V_s is the molar volume of the solvent and E_s is the cohesion energy, which is identical to vaporization energy.

$$\delta_t^2 = \delta_d^2 + 2\delta_{\text{in}}\delta_d + \delta_o^2 + 2\delta_a\delta_b$$

where:

δ_d is a measure of the ability of a solvent to participate in dispersive interactions,
δ_o is a measure of the ability of a solvent to participate in orientation interactions,
δ_{in} is a measure of the ability of a solvent to induce a dipole moment in surrounding molecules,
δ_a is a measure of the ability of a solvent to act as a proton donor,
δ_b is a measure of the ability of a solvent to act as a proton acceptor.

From Hildebrand's solubility parameter, heptane is less polar than toluene, which in turn is less polar than methylene chloride, etc., to water, the most polar. Unfortunately, toluene and ethyl acetate exhibit similar δ_t, which does not account for their chemical properties; moreover, Hildebrand's solubility parameters are not known for mixtures.

The solvent triangle classification of Snyder (9) is widely used by chromatographers. The solvent polarity index, P', and selectivity factors X_i were based on GC measurements of three probe solutes (nitromethane, ethanol, and dioxane) on different liquid stationary phases. Snyder was able to draw the selectivity triangle displayed in Fig. 5. It is clear from this figure that chloroform and methylene chloride are not equivalent. Similarly it must be kept in mind that acetonitrile and methanol are different, and the solubility of solutes will not be the same in both solvents. The most striking feature of the scheme is the obvious selection of a solvent. If a solvent from a given class is unable to achieve the desired selectivity, one must select another one from an opposite class. Change of methanol to ethanol does not bring anything, but change from methanol to acetonitrile will dramatically change interactions.

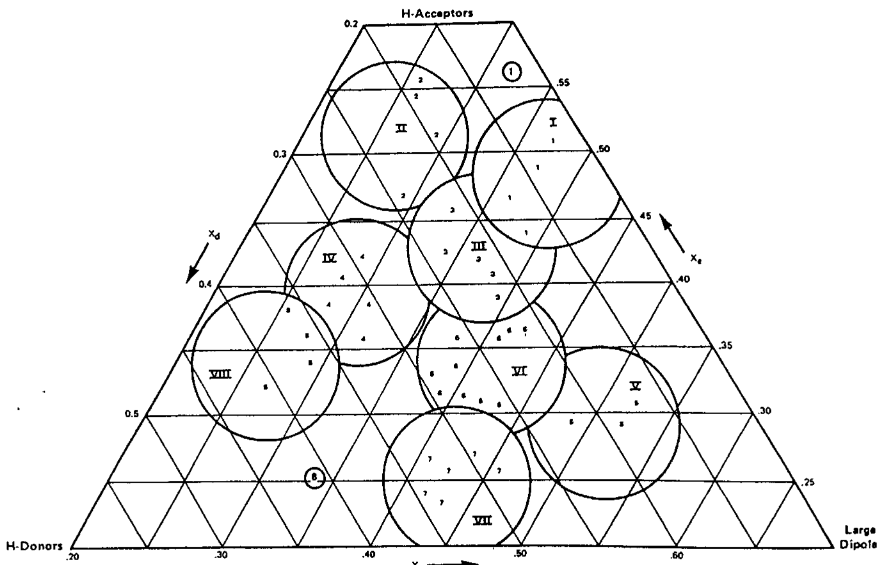
The method of linear solvation energy (LSER), based on the Kamlet–Taft multiparameter scale (10) has been successfully exploited to study retention in LC. The LSER approach, when applied to phase-transfer processes, correlates a general solute property (SP), such as logarithmic capacity factor, with parameters of the solute and both the mobile and stationary phases:

$$SP = \ln k = S_{po} + m(\delta_s^2 - \delta_m^2)V_i/100 + s(\pi_s^* - \pi_{\text{m}}^*)\pi_2^* a(\beta_s - \beta_m)\alpha_i + b(\alpha_s - \alpha_m)\beta_i$$

(cavity term + dipolar term + hydrogen bonding term s), where:

S_{po} is a solute-dependent intercept,
δ is the Hildebrand solubility parameter,
π^* is related to the solvent ability to interact with a solute by dipolar and polarization factor,
α is the hydrogen bond donor ability toward a basic acceptor,
β is the hydrogen bond acceptor toward a protic solute,
m, s, a, b are related to the test solute size, dipolarity/polarizability, basicity, and acidity, respectively.

Solute descriptors are available for more than 2,000 compounds. The magnitude of the coefficients is due to a difference between mobile and stationary phase properties. According to Sny-



Group Designation	Solvents
I	Aliphatic ethers, *methyl t-butyl ether*, tetramethylguanidine, hexamethyl phosphoric acid amide (trialkylamines)
II	Aliphatic alcohols, *methanol*
III	Pyridine derivatives, *tetrahydrofuran*, amides (except formamide), glycol ethers, sulfoxides
IV	Glycols, benzyl alcohol, *acetic acid, formamide*
V	Ethylene chloride
VI	a) Tricresyl phosphate, aliphatic ketones and esters, *dioxane*, polyesters b) Sulfones, nitriles, *acetonitrile*, propylene carbonate
VII	Aromatic hydrocarbons, *toluene*, halosubstituted aromatic hydrocarbons, nitro compounds, *methylene chloride*, aromatic ethers
VIII	Fluoroalcohols, m-cresol, water, (*chloroform*)

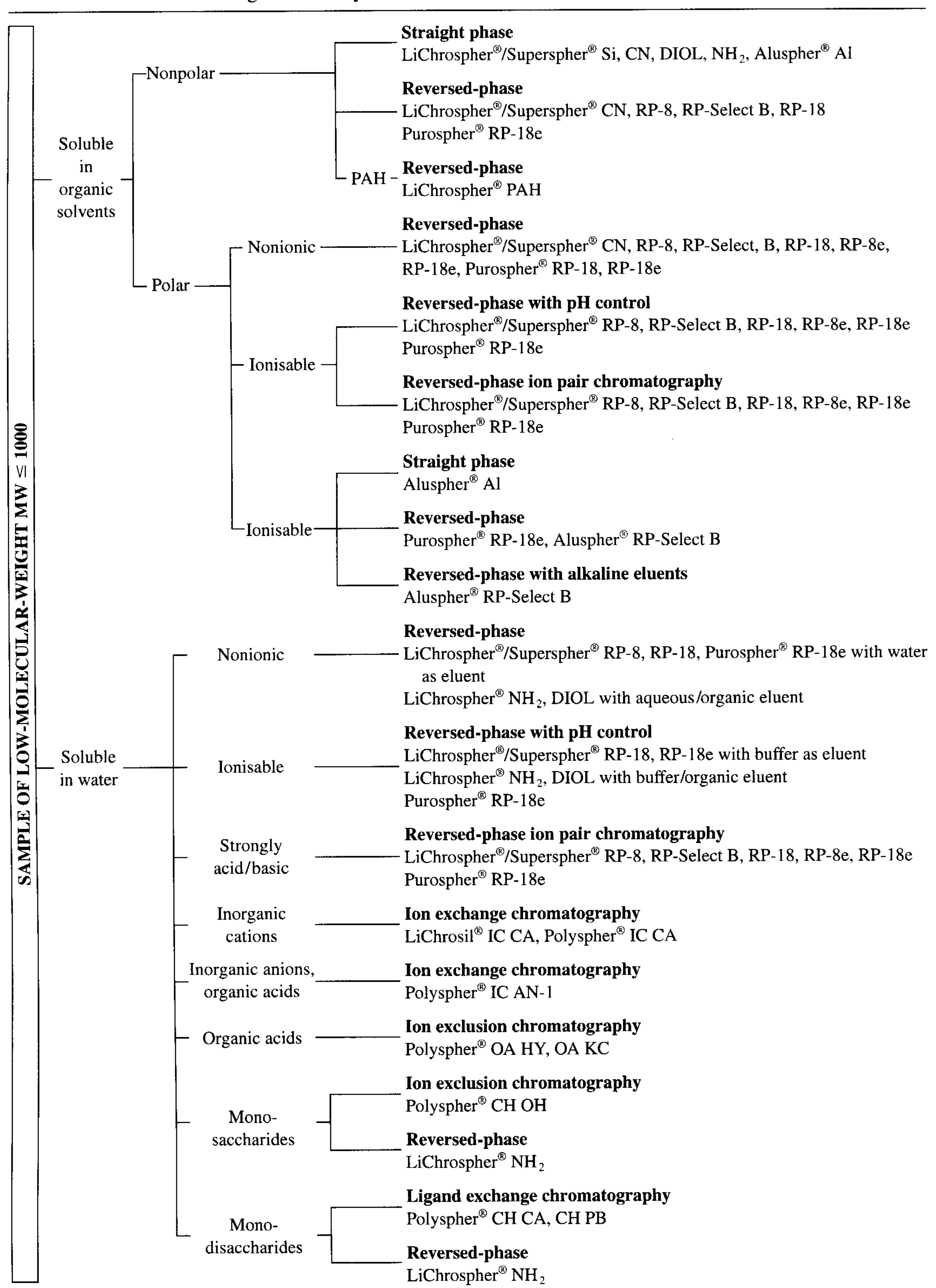
Fig. 5 Solvent classification scheme according to Snyder (Ref. 9). (Reproduced with permission from Preston Publications.)

der's conclusion (11), the solvatochromic solvent selectivity triangle provides a better qualitative classification, but it should not be used for quantitative predictions of selectivity.

IV. STATIONARY PHASES

Solutes undergo retention when weak interaction occurs with the stationary phase. The analyst must select a stationary phase according to the nature of the solute (apolar, polar, ionizable). For convenience we shall consider the following different mechanisms: adsorption, partition, chromatography of ionizable substances, chiral separations, exclusion. Table 1 is a quick selection guide.

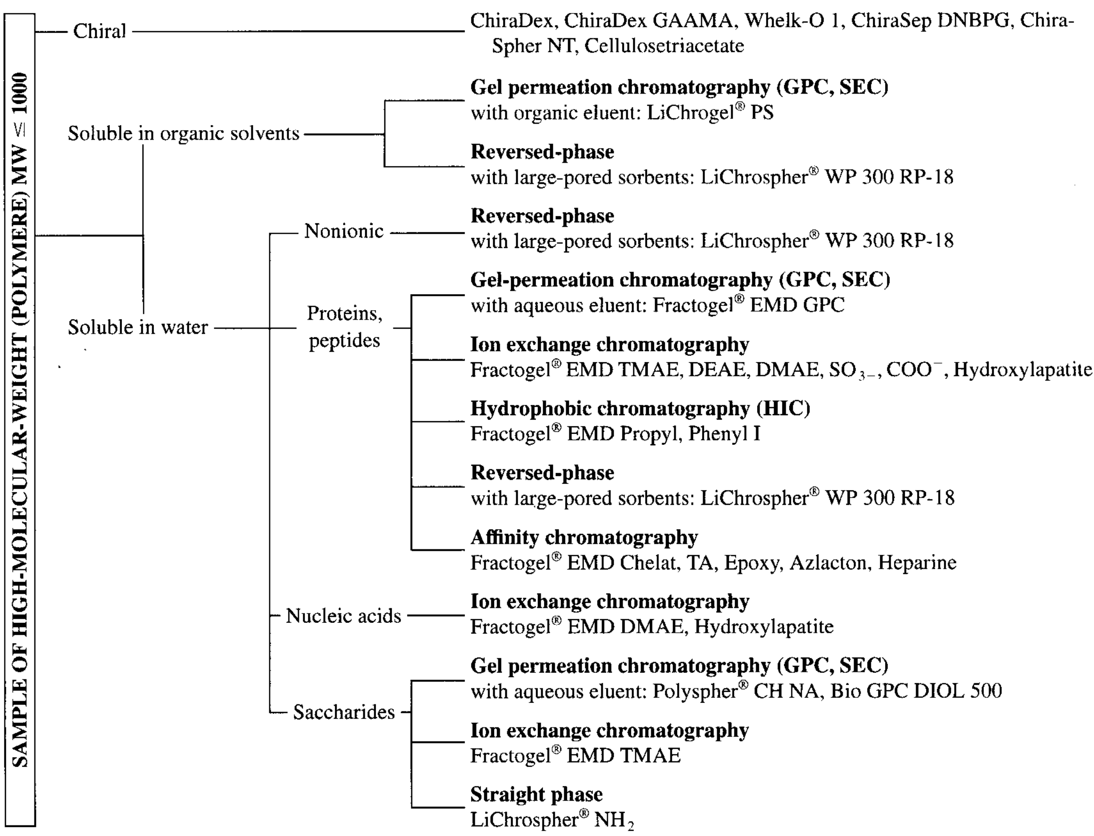
Table 1 Selection of the Right Stationary Phase



SAMPLE OF LOW-MOLECULAR-WEIGHT MW $\leq$ 1000

Solubility	Sample type	Subtype	Method	Stationary phase
Soluble in organic solvents	Nonpolar		**Straight phase**	LiChrospher®/Superspher® Si, CN, DIOL, NH_2, Aluspher® Al
	Nonpolar		**Reversed-phase**	LiChrospher®/Superspher® CN, RP-8, RP-Select B, RP-18 Purospher® RP-18e
	Nonpolar	PAH	**Reversed-phase**	LiChrospher® PAH
	Polar	Nonionic	**Reversed-phase**	LiChrospher®/Superspher® CN, RP-8, RP-Select, B, RP-18, RP-8e, RP-18e, Purospher® RP-18, RP-18e
	Polar	Ionisable	**Reversed-phase with pH control**	LiChrospher®/Superspher® RP-8, RP-Select B, RP-18, RP-8e, RP-18e Purospher® RP-18e
	Polar	Ionisable	**Reversed-phase ion pair chromatography**	LiChrospher®/Superspher® RP-8, RP-Select B, RP-18, RP-8e, RP-18e Purospher® RP-18e
	Polar	Ionisable	**Straight phase**	Aluspher® Al
	Polar	Ionisable	**Reversed-phase**	Purospher® RP-18e, Aluspher® RP-Select B
	Polar	Ionisable	**Reversed-phase with alkaline eluents**	Aluspher® RP-Select B
Soluble in water	Nonionic		**Reversed-phase**	LiChrospher®/Superspher® RP-8, RP-18, Purospher® RP-18e with water as eluent LiChrospher® NH_2, DIOL with aqueous/organic eluent
	Ionisable		**Reversed-phase with pH control**	LiChrospher®/Superspher® RP-18, RP-18e with buffer as eluent LiChrospher® NH_2, DIOL with buffer/organic eluent Purospher® RP-18e
	Strongly acid/basic		**Reversed-phase ion pair chromatography**	LiChrospher®/Superspher® RP-8, RP-Select B, RP-18, RP-8e, RP-18e Purospher® RP-18e
	Inorganic cations		**Ion exchange chromatography**	LiChrosil® IC CA, Polyspher® IC CA
	Inorganic anions, organic acids		**Ion exchange chromatography**	Polyspher® IC AN-1
	Organic acids		**Ion exclusion chromatography**	Polyspher® OA HY, OA KC
	Mono-saccharides		**Ion exclusion chromatography**	Polyspher® CH OH
	Mono-saccharides		**Reversed-phase**	LiChrospher® NH_2
	Mono-disaccharides		**Ligand exchange chromatography**	Polyspher® CH CA, CH PB
	Mono-disaccharides		**Reversed-phase**	LiChrospher® NH_2

Table 1 Continued



Source: Merck, Darmstadt, Germany.

A. Adsorption or Normal Phase (NP) Chromatography

Normal phase refers to systems in which the stationary phase is more polar than the mobile phase. Historically, it was the first mode of operation, and the development of theory on the retention mechanism has been under way for several decades. Solid supports of this type are: silicagel, alumina, and zirconia.

1. *Silicagel*

Silicagel is also called silica or bare silica. Its adsorptive properties depend on the hydroxyl groups attached to surface silicon atoms. Silicagel has a maximum silanol density of 8.0 μmoles/m^2. Many of these silanols are buried deep in the porous structure and are available only to the smallest analytes. Silanols are either isolated, geminal, or vicinal; they can be distinguished by means of Si solid-state nuclear magnetic resonance (NMR). The surface also contains siloxane bonds (Si–O–Si), which are considered hydrophobic.

From the Snyder–Soczewinski model (12, 13), the entire adsorbent surface is covered by an adsorbate monolayer that consists of mobile phase. Retention is assumed to occur as a displacement process in which an adsorbing solute molecule X displaces some number n of previously adsorbed mobile-phase molecules S

$$X_m + nS_{ad} \leftrightarrow X_{ad} + nS_m$$

The subscripts *m* and *ad* refer to a molecule in the nonsorbed and adsorbed phases, respectively. It is assumed that the rate of equilibration is fast. The net reaction free energy (actually, dimensionless) is

$$\Delta E = E_{\mathrm{Xad}} + nE_{\mathrm{S}} - E_{\mathrm{X}} - nE_{\mathrm{Sad}}$$

To a first approximation the mobile-phase terms can cancel, leaving

$$\Delta E = E_{\mathrm{Xa}} - nE_{\mathrm{S}}$$

This equation leads to the basic equation of retention:

$$\ln k = \ln \frac{(V_a \times W)}{V_m} + \beta(S^\circ - \epsilon^\circ \times A_S) + \Sigma Q_i$$

where:

k refers to retention factor of the solute in a given system,
V_a is the approximate volume of the adsorbed monolayer per gram of adsorbent,
W is the weight of adsorbent in the column,
V_m is the column void volume,
β is the activity of the adsorbent (related to water at the surface: water binds to silica either as capillary water or as water associated with the hydroxyl groups of the surface),
S° is a parameter that reflects the relative interaction energy of the solute molecule with the adsorbent surface,
ϵ° is the solvent-strength parameter that reflects the relative interaction energy of mobile-phase molecules with the adsorbent surface (values are equal to the adsorption energy of the solvent molecule when the latter is injected as a sample divided by the molecular area of the solvent molecule),
A_s is the relative area of the solute molecule when adsorbed,
ΣQ_i is a second-order term.

The most important features of this equation are the emergence of a eluotropic series, i.e., an eluent-strength series based on ϵ°. The key solvent-strength parameter, ϵ°, which relates to the eluotropic series, was calculated by Snyder (14) for a wide variety of pure solvents. Table 2 lists some values.

One single eluent is often not convenient to achieve the desired separation. Intermediate values of ϵ° are required. For that purpose the simplest thing is to use binary mixtures of eluents: an apolar diluent A and a modifier B. Calculation of eluotropic strength is not straightforward. It can be achieved through the formula derived by Snyder. The easiest way is to draw plots of ϵ° versus volume of the strongest eluent B (Fig. 6).

It has been demonstrated by Soczewinski (15) that in most cases

$$\ln k = \mathrm{Cte} - n \ln \mathrm{Xs}$$

where Xs is the molar fraction of the modifier. When a binary mixture is inefficient, a more complex mixture is required. The Prisma optimization method from Nyiredy (16), first developed with Planar Chromatography, works pretty well.

The activity of the silica is an important parameter. When silica is "wet" by several layers of water, pores are filled and solutes cannot undergo adsorption. As a consequence these are retained only slightly. They can even undergo partition mechanism. Figure 7 demonstrates the dramatic influence of water on retention. Reproducible retention can be achieved by Engelhardt's method (17): One tank is filled with dry solvent and another tank is filled with fully wet solvent. A proportioning valve allows for the exact mixing, and the water content is controlled. A moisture

Table 2 Eluotropic Series for Different Adsorbents

Solvent	Solvent-strength parameter					
	Alumina	Silica	Carbon	Aminopropyl	Cyanopropyl	Diol
Pentane	0.00	0.00				
Hexane	0.01	0.01	0.13–0.17			
Carbon tetrachloride	0.17	0.11		0.069		
1-Chlorobutane	0.26	0.20	0.09–0.14			
Benzene	0.32	0.25	0.20–0.22			
Methyl-tert. butyl ether	0.48			0.11–0.124	0.049–0.085	0.071
Chloroform	0.36	0.26	0.12–0.20	0.13–0.14	0.106	0.097
Dichloromethane	0.40	0.30	0.14–0.17	0.13	0.120	0.096
Acetone	0.58	0.53		0.14		
Tetrahydrofuran	0.51	0.53	0.09–0.14	0.11		
Dioxane	0.61	0.51	0.14–0.17			
Ethyl acetate	0.60	0.48	0.04–0.09	0.113		
Acetonitrile	0.55	0.52	0.01–0.04			
Pyridine	0.70					
Methanol	0.95	0.70	0.00	0.24		

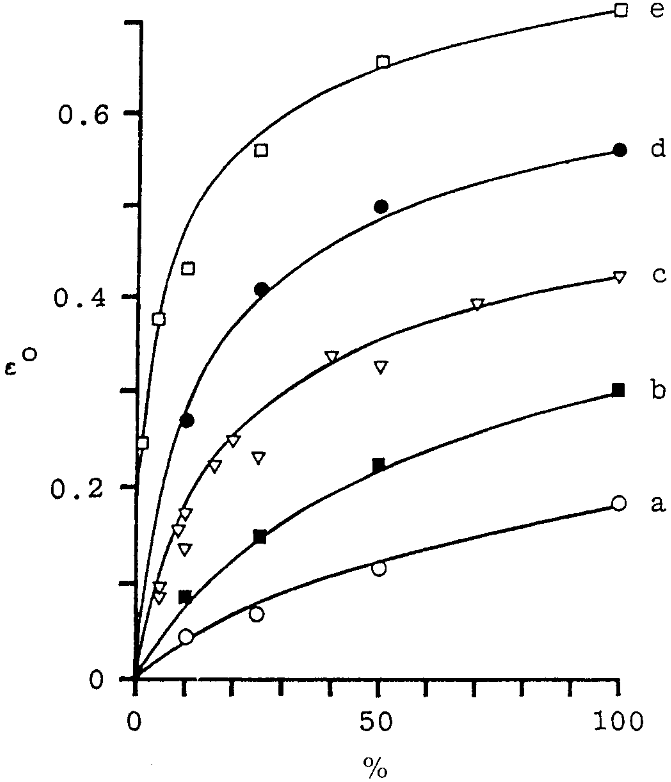


Fig. 6 Dependence of ϵ° versus the percentage of modifier in binary mixture A/B. A = apolar diluent (hexane), B = modifier, a = carbon tetrachloride, b = propyl chloride, c = methylene chloride, d = acetone, e = pyridine (normal phase chromatography).

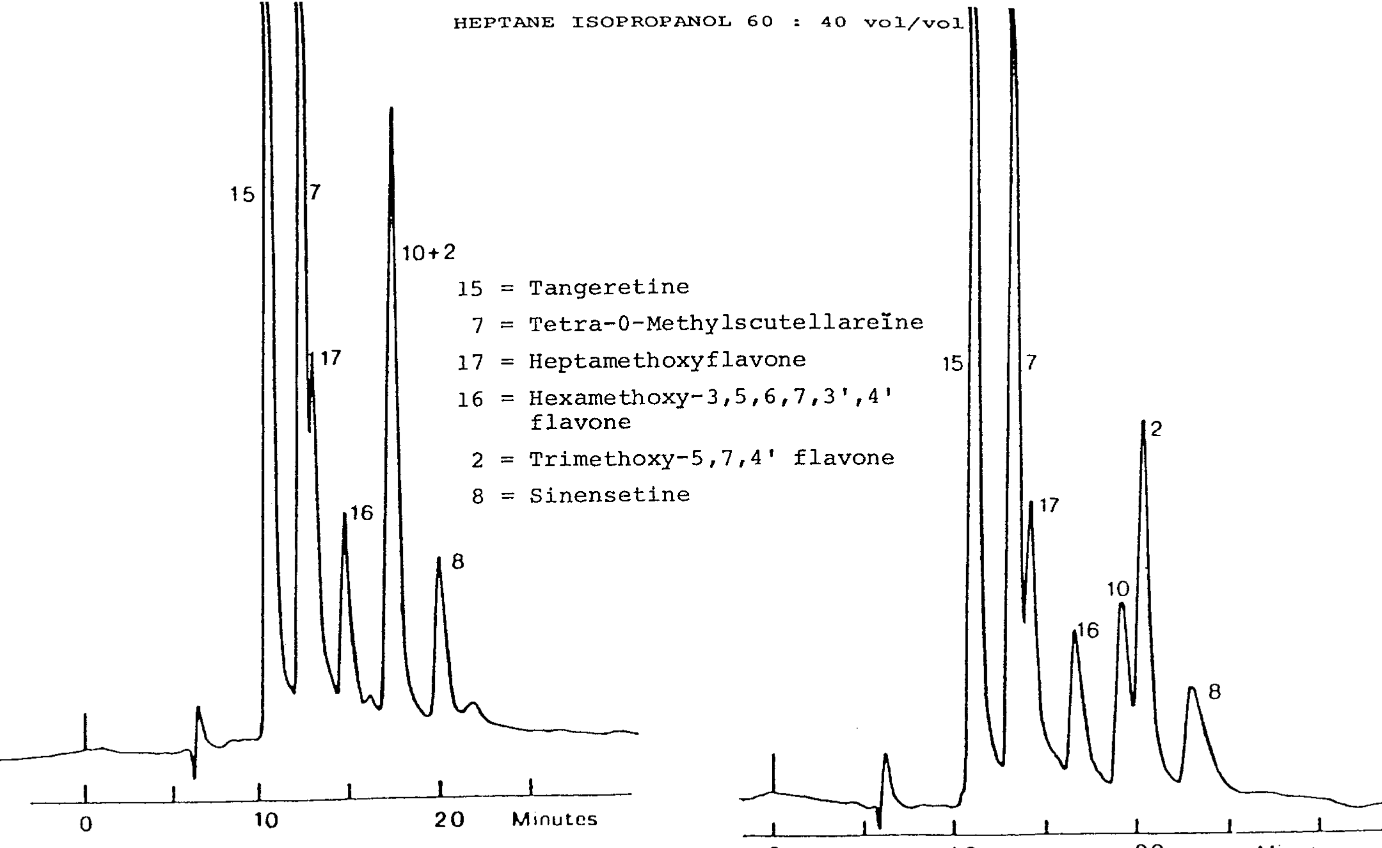


Fig. 7 Influence of water content on the sparation of some flavones. Column: Si 60 250 × 4.6 mm (5 μm) from Merck; eluent heptane/isopropanol 60:40 (vol:vol) 1 mL/min. (a) 0.4% water; (b) 0.05% water. Detection UV 280 nm. (From J-P Bianchini, Les Cahiers de Chromatographie, Vol. 4, 1977. Reproduced with permission.)

control system is inserted before the pump, and the eluent can be recycled through the column and the system.

A more convenient form of the earlier retention equation is

$$K = K_{ads}(W/V_m)$$

where:

W is the weight of adsorbent,
K_{ads} is the sample adsorption coefficient,

K_{ads} is given by the adsorption isotherm, which may be linear, convex, or concave (Fig. 8). K_{ads} is the slope of the line or the tangent to the curve at very low concentrations of solute. When a

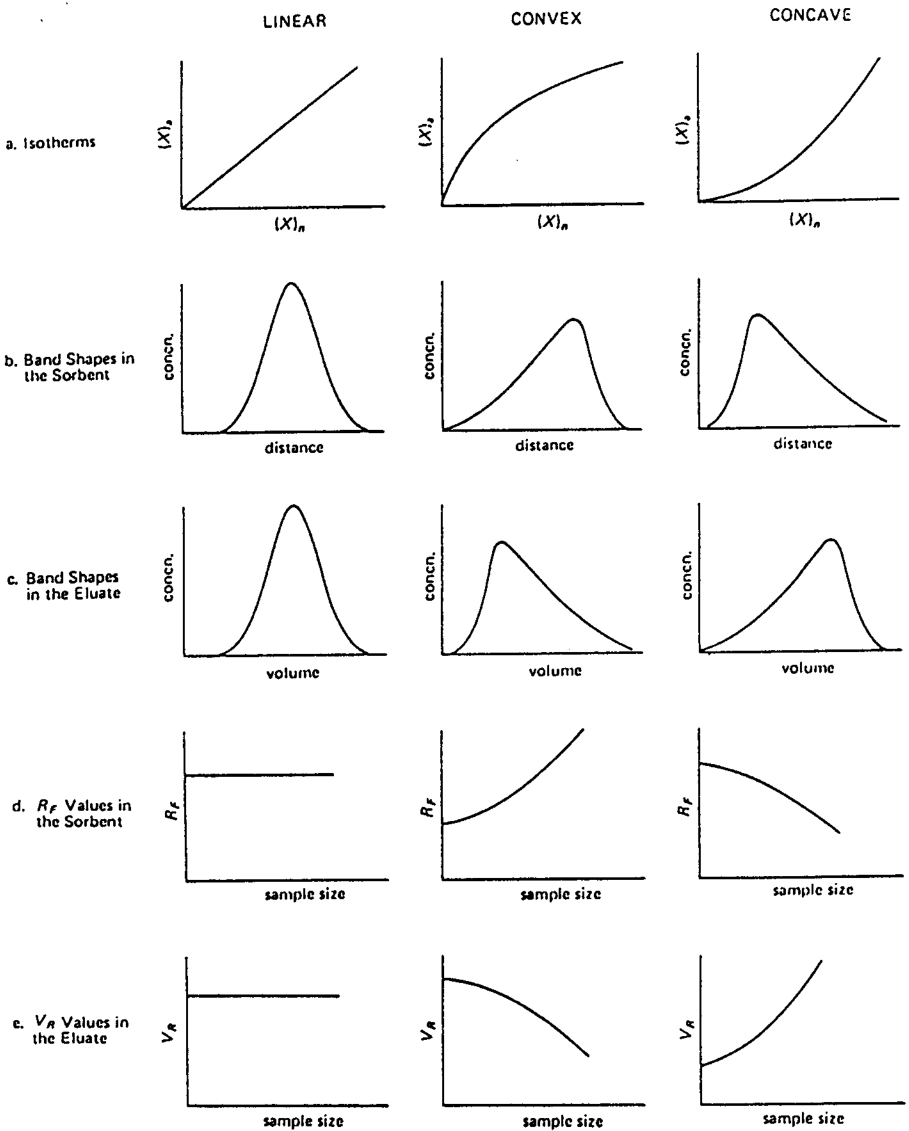


Fig. 8 Shapes of adsorption isotherms and some of their chromatographic consequences. (Reproduced with permission from Van Nostrand–Renhold.)

nonlinear region of the isotherm is reached, the chromatographic peak is distorted and exhibits a tailing.

In normal-phase or adsorption chromatography, the chromatographic properties are functions of the specific area. Retention factors increase with the specific surface area. The parameter of the specific surface area of the packing could be of great importance when selectivity and efficiency have to be improved. One very short column packed with a silica of high specific surface area will yield the same results as a long column packed with a silica of low specific surface area (18).

Specific surface area is dependent on the reciprocal of pore diameter. However, with a given pore diameter the measured specific surface may be different. A silicagel should be defined with mean particle diameter, specific surface area, pore diameter, and pore volume.

2. *Alumina*

Alumina is an alternative to silica, owing to its inherent tolerance toward wider pH variation ranging from 1–13. However, it is not in current use.

3. *Zirconia*

Zirconia is an amphoteric metal oxide. Zirconia has the desirable physical and mechanical properties of silica, and its chemical stability is high. Zirconia supports have been pioneered by Carr and co-workers (19). It can be coated with butadiene or reacts with silanes to produce alkyl-bonded phases.

Chromatographic properties of silica, alumina, titania, and zirconia have been compared (20). In contrast to silica, alumina, titania, and zirconia surfaces are basic.

V. POLAR-BONDED PHASES

Three types are widespread: cyano, amino, and diol, according to the functionality terminating the spacer arm bonded to the silica matrix:

$Si—O—Si\,(CH_2)_3—NH_2$ Amino
$Si—O—Si—(CH_2)_3—CN$ Cyano
$Si—O—Si—(CH_2)_3—OCH_2—CHOH—CH_2OH$ Diol

Retention on these supports is adaquetely described by the adsorption displacement model. Nevertheless, the adsorption sites are delocalized due to the flexible moiety of the ligand, and secondary solvent effects play a significant role. The cyano phase behaves much like a deactivated silica toward nonpolar and moderately polar solutes and solvents. Cyano propyl columns appear to have basic tendencies in chloroform and acidic tendencies in methyl tertiobutyl ether (MTBE) (21). Acidic solutes can localize on cyano groups of the bonded phase. The cyano phase is very versatile and has been proposed as the unique stationary phase in an expert system (22). In reversed-phase mode, CN-bonded silica exhibits better selectivities than alkyl-bonded silica toward proteins and peptides.

Aminophase behavior has been studied (23), and the lone pair of electrons of the amino group accounts predominantly for retention. $\pi-\pi$ interactions are prominent when moderately polar solutes are chromatographed with apolar diluent polar modifier. The chromatographic properties are governed largely by the basic nature of the NH_2 group.

A mixture of acetonitrile and water is capable of separating sugars on amino phase. Problems with the determination of reducing sugars can occur, since the reaction of the keto group with the amino moiety is well known. In a similar way, an amino phase in normal-phase mode should not be used with acetone. NH_2 packing can be used as a weak anion exchanger. The diol

phase is much less hydrophilic than plain silica. Separations performed on silica can be carried out on a diol phase with a weaker eluent.

VI. REVERSED-PHASE PACKINGS

From surveys published in many journals, reversed-phase mode is utilized by a vast majority of users. In this mode the mobile phase is more polar than is the stationary phase. Usually, stationary phase is hydrophobic in nature. Silicagel is still the base material onto which is bonded an alkyl moiety. Silica is rigid and can easily react through the surface silanols. Commercially available alkyl-bonded phases are derived from reactions between silanols and organochlorosilanes or alkoxysilanes. Most manufacturers use monofunctional chlorosilanes. Reaction is carried out in a water-free medium, and a nitrogen base acts as a proton acceptor or is involved in the mechanism of the reaction. The final surface coverage lies within the range 2.8–4.0 μmole/m^2, which is far from complete. Specifications of reversed-phase material should be as complete as possible, with specific surface area before and after reaction, carbon loading, and calculation of the surface coverage.

Residual silanol groups may confer undesirable adsorptive properties on the material. End capping is an additional treatment of the bonded silica with a short chain, for example, with trimethylsilyl groups. It is not totally successful, and manufacturers make dramatic efforts to improve peak shape and hydrophobicity. Surface silanols are known to undergo self-ionization leading to fixed negative charges on the surface of the material. The tendency to undergo self-ionization depends on the electronic environment and is strong when the adjacent silicon atom is substituted by a metallic impurity such as sodium or iron. The ultrapure starting silica has almost no metal impurities, for exceptional inertness. Steric protection through bulky difunctional silane groups such as diisopropyl and diisobutyl silanes is promoted by Hewlett Packard in the Zorbax line. Such a moiety is less vulnerable to destruction at low pH (pH 2 is often used in peptides and protein separations). Waters utilizes an embedded carbamate polar group that interacts with the surface silanols in such a way that their activity is greatly reduced. In RP chromatography it is possible to select a column out of 600 available stationary phases with different selectivities and efficiencies. Only few systematic approaches have been published to characterize stationary phases. Two tests are popular: Tanaka's test and Engelhardt's test. The main differences are in the use of buffered or unbuffered eluents, buffer concentrations, and the basicity of test solutes. According to Tanaka (24), a set of seven substances is used to describe capacity, hydrophobicity, steric selectivity, shielding at pH beyond 7 or below 3, symmetry, and silanophilic properties. To facilitate the illustration and to check the quality of a sorbent at one glance, the values of these parameters are outlined on the six axes of a hexagon. The more symmetrical the hexagon appears, the more balanced the sorbent as far as chromatographic properties are concerned (Fig. 9). In Engelhardt's test (25), 10 compounds are considered. The most important criterion is the coelution of the isomers of ethylaniline, which indicates a good suppression of the silanophilic activity. Base-deactivated silica (RP Select B from Merck, for example) does not exhibit peak tailing with some solutes, such as benzodiazepines.

Relevant parameters for column-to-column reproducibility data are: plate number, peak symmetry, selectivity, and adsorption phenomena checked with chromatography of amines and acids. For column life, the analyst must periodically check: k loss, change in asymmetry factor, and plate number.

Many of the early problems of reproducibility of columns from lot to lot have by now been solved. C18, C8 alkyl-bonded chains are currently advocated, but some others are available, such as C30, phenyl, and cyclohexyl. Since the alkyl phase is hydrophobic, apolar eluent exhibits the strongest eluting strength and water the least. This is the reverse of what is observed on plain sil-

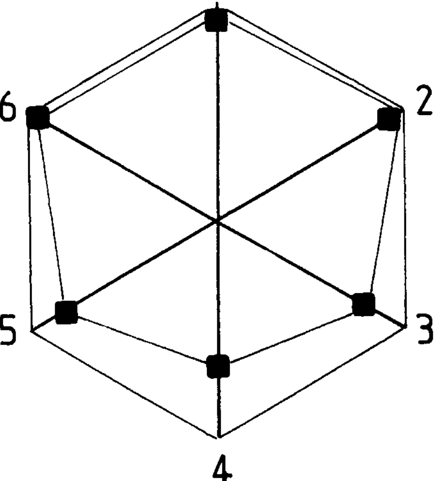


Fig. 9 Hexagon visualizing the desired properties of a C_{18}-bonded silica column: efficiency (1), symmetry (2), capacity (3), hydrophobicity (4), steric selectivity (5), metal shielding (5), and silanophilic properties (6). (Reproduced with permission from Merck, Darmstadt, Germany.)

ica and it gave the abbreviation RP (reversed phase). Reversed-phase liquid chromatography (RPLC) is performed with polar mobile phases. Mixtures of water (the diluent) and organic modifier(s) are utilized. Methanol (MeOH), acetonitrile (ACN), and tetrahydrofurane (THF) are utilized the most. It must be kept in mind that these modifiers are in different places in Snyder's selectivity triangle; consequently, retention order can be affected when passing from methanol to acetonitrile or THF. From solubility parameter theory, isoeluotropic compositions of binary mixtures can be determined. For example, MeOH/water 50/50, ACN/water 30/70, and THF/water 25/75 will produce same retention of benzene, a solute that does not undergo hydrogen bonding. Some rules for the selection of isoeluotropic mixtures for RPLC have been given by Schoenmakers (26).

Increasing the volume percent of water increases retention. It has been observed with binary mixtures that ln k is linearly dependent on the volume fraction of the modifier according to

$$\ln k = \ln K_w - S\phi$$

In this relationship, ϕ is the volume percentage of organic modifier. The slope value (S) shows the sensitivity of the retention of a given compound caused by a change of the percentage of organic modifier in the mobile phase. ln K_w is the hypothetical retention in pure water. This linear relationship is valid only within a range of ϕ. This range is wide whether or not solute can undergo hydrogen bonding. More generally the relationship is quadratic:

$$\ln k = \ln K_w - S\phi - a\phi^2$$

where a depends on the solute.

The retention mechanism is not yet fully understood. The solvophobic theory does not account for any interaction in the stationary phase, which plays a passive role. The partition mechanism as described by Dill and Dorsey (27) is generally accepted. The most relevant feature is the linear plot of ln k versus carbon number in a homologous series (Fig. 10), which is similar to what is observed in isothermal gas-liquid chromatography. Retention is governed mainly by hydro-

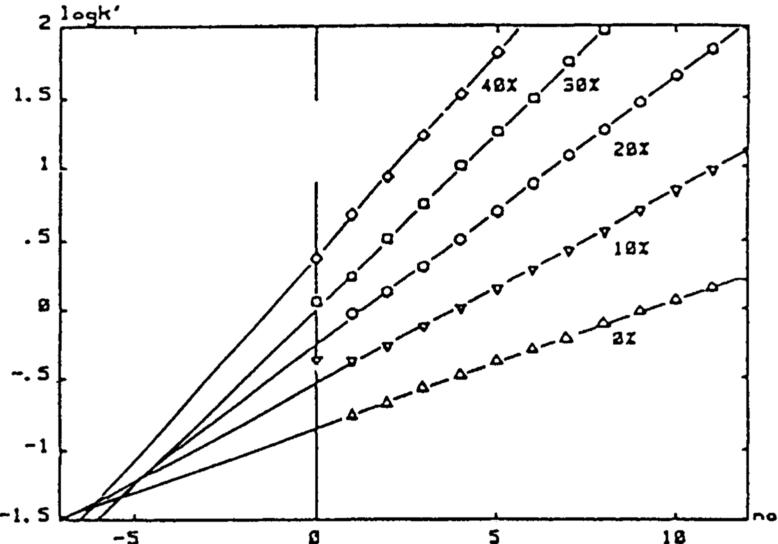


Fig. 10 Plot of log k versus the number of carbon atoms n_c (methanol : water mixtures) (vol : vol), column Lichrosorb RP18 solutes; alkylbenzenes. (From H. Colin, PhD dissertation, 1980.)

phobicity. The more hydrophobic the solute, the more it is retained. A correlation with the partition coefficient between octanol and water, log $P_{\text{octanol/water}}$ is observed. However, it is a correlation with some scatter. The value of ln K_w is only a rough measure of hydrophobicity. When extrapolating from plots of retention versus ϕ it is often observed that values of K_w are different from methanol/water mixtures and acetonitrile mixtures.

Since the early works of Hansch and Leo (28), and the fragmental constants of Rekker (29), many papers and a book (30) have appeared on how to predict retention from molecular structure. Retention increases with an increase in hydrophobicity. When hydrophobicity order is not straightforward, a rule of thumb is to consider that elution is correlated with the solubility in the mobile phase.

In extension, some retention index scales were proposed to mimic the Kovats index in GC. Alkanes, n-alkylbenzenes, alkan-2ones, alkylaryl ketones, nitroalkanes, or polynuclear aromatic hydrocarbons were the advocated solutes. None of these scales is reliable, and observed indexes are not stable with variation in eluent composition, which precludes their use as a "Kovats" scale.

Van't Hoff plots of ln k versus the inverse of temperature (generally $1000/T$ for convenience) are very often linear, especially with monomeric bonded phases. They can exhibit nonlinear behavior, and the transition temperature is often close to the undefined "room temperature." Temperature optimization is one trend in LC. A rising temperature increase reduces viscosity and increases the diffusion rate, thereby enhancing mass transfer, which flattens the HETP curve at high velocities (31). Conversely, Sander and Wise (32) investigated the influence of temperature reduction.

Change in selectivity, obviously connected with phase conformational change, is observed when phosphate buffer is utilized. It is not possible to go back to the manufacturer's chromatogram even after thorough rinsing (33).

The drawback of silica based material is the pH stability. Silica begins to dissolve at pH beyond 8, and the higher the pH the higher the solubilization. Polymeric phases with polystyrene

crosslinked with divinyl benzene (PS-DVB) are stable from pH 0 to 14, whereas silica-based columns have a pH range from 2 to 7. To overcome the shrinking and swelling observed with high pressures, manufacturers increase the DVB percentage (up to 100%!). Alkaline mobile phases can be used to separate amines in their neutral state, and acidic mobile phases down to a pH of 0 can be used to protonate strong acids without harming the packing. The column can be run at higher temperatures without fear of stripping the phase. Unfortunately, a slow mass transfer is usually observed, and the plate count of best polymeric PS/DVB material does not compare with what is obtained with silica.

Carbon-based material on a silica template has been pioneered by Knox (34). It can be used at any pH. However, the mechanism of retention on this support is quite different from that for the average alkyl-bonded silica (35). Further information on reversed-phase retention can be found in Ref. 36.

VII. SYSTEM PEAKS

The injection of a sample in a chromatographic column may result in more peaks than there are components in the mixture if the mobile phase contains one or several additives. These additional peaks result from the perturbation of the additive equilibrium between the two phases caused by the injection of a sample. It may be assumed that there is a competitive equilibrium of the sample and the modifier. Solutes enter the column, moving with the velocity of the mobile phase and not with the equilibrium velocities dictated by the equilibrium between mobile and stationary phases. System peaks are visualized with an appropriate detector, particularly a refractive index detector. This may cause trouble for the analyst, since the system peak may exhibit k values more than 1 (37).

VIII. POSITIONAL AND OPTICAL ISOMERS

A. Silver Ion LC

This technique is used extensively in thin-layer chromatography (TLC). Silver ion LC exhibits a great potential for the separation of positional and configurational isomers of fatty acids and for the determination of trans unsaturation. Attempts to load an LC column with silver nitrate or to use silver nitrate as an additive in the mobile phase were not very successful. A breakthrough in the field was made by Christie (38), who converted an ion exchange packing (sulphonic acid bonded) to silver ion form by injection of silver nitrate through the Rheodyne valve while pumping water through the column before the aqueous phase was replaced by organic solvents. In this mode, separation of monoenic, dienic, and trienic acids (oleic, linoleic, and linolenic) is easily performed with the cis/trans resolution as well. An example of positional isomer separation is given in Fig. 11.

B. Chiral Phases

Chiral resolution is a matter of great importance. For example, the food and beverage industry is increasingly concerned with enantiomeric separations, because they can affect flavor, fragrance, and nutrition and can be used to monitor fermentation or product adulteration. Separations are carried out mainly on chiral stationary phases (CSPs).

Enantiomeric separation is a great challenge. Every separation requires a dedicated column. Some packings are more able to separate many racemates. When a chiral selector is used as the stationary phase, the primary retention mechanism is complexation with the surface-

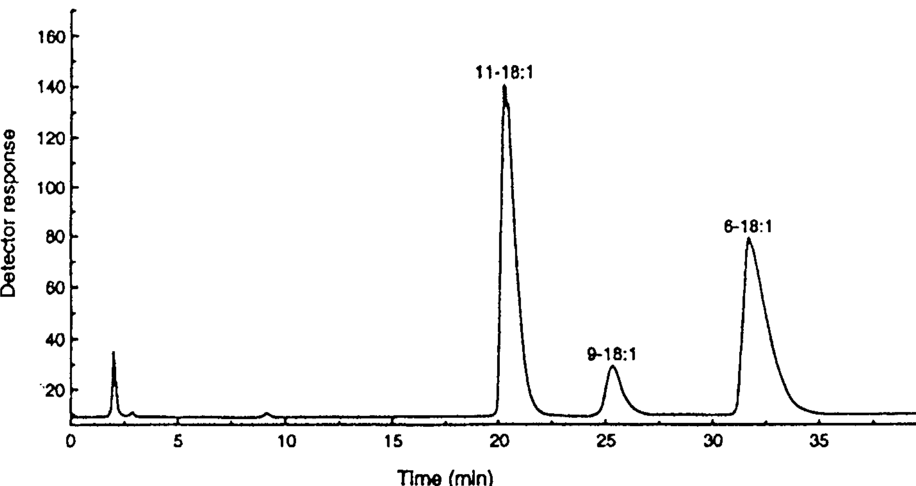


Fig. 11 Separation of phenacyl esters of the isomeric octadecenoic acids, petroselinic acid (6–18:1), oleic acid (9–18:1), and vaccenic acid (11–18:1) by HPLC on silver ion column (Nucleosil 5SA) loaded with silver ion eluted with dichloromethane-dichloroethane-acetonitrile 50:50:0. 25 (vol:vol) detection light-scattering detector. (From W.W. Christie, Analusis 26:M38 (1998), reproduced with permission.)

immobilized chiral selector. When 1/1 solute selector complexation occurs, the equilibrium constant K_{Comp} is related to the retention factor k by

$$K_{\mathrm{Comp}} = \frac{(\text{solute}-\text{selector})}{(\text{solute}) \times (\text{selector})} = \frac{k}{(\text{selector}) \times \phi_{s/m}}$$

where $\phi_{s/m}$ is the phase ratio and (solute), (selector), and (solute–selector) represent the equilibrium concentrations of free solute, free selector, and solute–selector complex, respectively.

Pirkle-type CSPs were developed from the pioneering work of Pirkle (39), who discovered in 1966 the nonequivalence of NMR signals arising from enantiomers in the presence of a chiral solvating agent. Immobilization on a silica support afforded enantiomeric resolution, and it was the beginning of a fruitful development of bonded phases. In the thorough review by Welch (40), many types of Pirkle's CSPs are displayed: dinitrobenzoyl aminoacid; hydantoin-derived; 2-arylamidoalkane-based; *N*-aryl aminoacid-derived; phtalide-derived; etc.

Ligand exchange has proved to be very successful in the separation of several enantiomers. Davankov and Rogozhin (41) used chiral copper complexes bonded to silica. The enantiomeric separation is based essentially on the formation of diastereomeric mixed complexes with different thermodynamic stabilities. It is generally accepted that chiral discrimination proceeds via the substitution of one ligand in the coordination sphere of the metal ion. Ligand exchange technique is especially effective for the enantiomeric resolution of aminoacids, aminoacids derivatives, and hydroxy acids (42).

Cyclodextrins (CDs) are cyclic oligosaccharides composed of several (D+) glucopyranose units with a shape similar to a truncated cone (Fig. 12). The exterior of CD is hydrophilic due to the presence of hydroxyl groups. The interior of the cavity consists of CH groups. The cavity is thus hydrophobic. According to the number of glucose units forming the cyclodextrin ring (6, 7, or 8) one differentiates between alpha, beta, or gamma CD. The cavity acts as a chiral selector. Additional kinds of multimodal interactions (hydrogen bonding and dipole–dipole interactions) at the periphery of the tore also play a significant role. Commercially available β CD bonded sta-

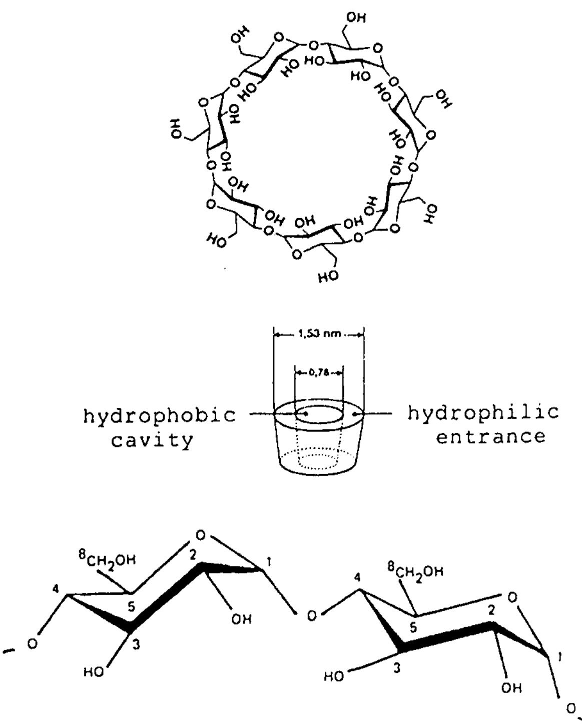


Fig. 12 Cyclodextrin: chemical structure and geometrical arrangement of the glucose units.

tionary phases for HPLC have been successfully used for the separation of a variety of racemates. Most separations have usually been obtained in the reversed-phase mode since the recognition mechanism was elucidated (43).

Permethylation of CD hydroxyl groups markedly changes the chromatographic selectivity in comparison to native CDs. Various CD derivatives are described. The various moieties used to functionalize the CDs greatly affect the enantioselectivity. Since the pioneering work of Armstrong, the topic of separations with cyclodextrins or cyclodextrin derivatives has been well documented (44).

The mechanism of chiral discrimination on cellulose triacetate is not fully understood. Cellulose ester derivatives and cellulose carbamate derivatives (Table 3) are able to separate compounds possessing an amide group, aromatic group, or nitro group in the normal-phase mode. These chiral polymers are coated on silanized, wide-pore silicagel. They are used most often in the normal-phase mode. Francotte (45) has investigated the molecular recognition mechanism and evidenced the supramolecular effects from the parallel or antiparallel stacking. Amylose derivatives such as 3,5-dimethylphenyl carbamate derivative exhibit a wide range of resolution capabilities (46).

Glycopeptide macrocyclic antibiotics such as vancomycin and teicoplanin have recently appeared. A great deal of interest is attached to these compounds, since they can be used in both the normal-phase mode and the reversed-phase one. Vancomycin contains 18 chiral centers surrounding three cavities. Five aromatic ring structures bridge these cavities. Hydrogen donor and acceptor sites are readily available close to the ring structures. Bonded vancomycin has the trade name Chirobiotic V. Teicoplanin contains 23 chiral centers surrounding four cavities. It can act through $\pi-\pi$ interactions, inclusion complexation, hydrogen donor or acceptor (Chirobiotic T).

Table 3 Structures of Cellulose Derivatives

R =	name
CH_3	Cellulose triacetate
	Cellulose tribenzoate
H_3C	Cellulose *meta*-methylbenzoate
H_3C–	Cellulose *para*-methylbenzoate
–N(H)–	Cellulose phenylcarbamate
CH_3, CH_3, –N(H)–	Cellulose 3,5-dimethylphenylcarbamate

Proteins such as bovine serum albumin (BSA), human serum albumin (HSA), and ovomucoid are either bonded or coated. They are used in the reversed-phase mode with aqueous buffers or hydroorganic solvent systems.

Polymeric methacrylates are not commercially widespread.

When dealing with a particular enantiomeric separation, the analyst is confronted with the crucial question of which CSP to select. Furthermore, the ever-increasing number of CSPs and dispersed data make the decision even more difficult. The graphical molecular database Chirbase covers information (structural, bibliographic, and chromatographic data) on every chromatographic mode (47). It provides integrated responses from single questions as well as from combinatorial questions using graphical molecule-oriented searching strategies:

What are the most efficient stationary phases?
What conditions are most suitable?
How to optimize a separation?
How to maximize a parameter?

The graphical visualisation of the molecular structures of both analyte and CSP in the reaction database gives the chemist a bettter idea of the possible intermolecular interactions.

IX. SEPARATION OF IONIZABLE SPECIES

Many methods are available to the analyst: ion interaction, ion exchange, ion exclusion, ion suppression, and ion pairing.

A. Ion Exchange Chromatography

Ion exchange chromatography with eluent suppression and conductometric detection is referred to as ion chromatography (IC). The ion exchanger consists of a basic structure (the matrix) and

active groups (the fixed ions). The basic structure is a polystyrene crosslinked with divinyl benzene or polyacrylate resins or silica. The active group consists of fixed ions and counter ions. The fixed ion is covalently bound to the basic structure (the matrix); the counter ion is exchangeable.

A typical ion exchange equilibrium is written, for example, as follows:

$$\text{matrix SO3}^- \text{H}^+ + \text{X}^+\text{A}^- \leftrightarrow \text{matrix SO}_3^-\text{X}^+ + \text{H}^+\text{A}^-$$

where matrix $SO3^-H^+$ represents an ion exchange site, the $SO^-{}_3$ being fixed to the matrix. While the associated counter ion H^+ is in the liquid phase, the counter ion can be displaced by a solute ion X^+. With K_{ie} being the exchange constant, the retention factor is

$$k = K_{ie}(\text{matrix SO}_3^-, \text{X}^+)/(\text{X}^+) = K_{ie}(\text{matrix SO}_3^-, \text{H}^+)/(\text{H}^+)$$

Since (matrix $SO3^-$) is constant, k is inversely proportional to (H^+).

We can distinguish cation exchangers as either SCX (strong cation exchanger), with the sulphonic acid group $SO^-{}_3$ as the fixed ion, or WCX (weak cation exchanger), with COO^- as the fixed ion. In SCX, the binding force decreases with decreasing diameter of the ion:

$$Li^+ < H^+ < Na^+ < K^+ < Ag^+$$
$$Mg^{2+} < Ca^{2+} < Ba^{2+}$$
$$Cu^{2+} < Ni^{2+} < Pb^{2+}$$

Determination of cations is one of the most important analyses for the routine quality control of fruit juices and purées. Weak cation exchangers are in general highly selective with respect to the H^+ ion:

$$Na^+ < Mg^{2+} < Ca^{2+} < H^+$$

Similarly, anion exchangers are either strong (SAX) or weak (WAX). The general trend for SAX is as follows:

$$F^- < OH^- < CH_3COO^- < H_2PO_4^- < Cl^- < NO_2^- < NO_3^- < SO_4^{2-}$$

Chelating resins are iminodiacetate type, polyamine type, or glucamine type. They exhibit high retention of some metals, such as heavy metals from alkali metal ion solutions.

A breakthrough in ion chromatography came from pellicular packings, which were introduced by Dionex. Packing consists of an inert, nonporous, chemically and mechanically stable core, a surface sulfonated area covering the core, and an outer layer of permanently attached submicron ion exchange.

Investigation of the retention mechanism has been reconsidered. Kovats (48) treated the strong ion exchanger as a condenser, Stahlberg (49) used the Gouy Chapman double layer. A discussion of retention models can be found in Refs. 50 and 51. Plots of ln k versus the logarithm of salt concentration in the eluent generally yield straight lines. With the separation of (for example) anions A^{x-} and B^{y-} on a sorbent with functional group R^+ with eluent anion E^{z-}, the retention factor k_A is

$$k_A = k_{xE}^{zA\,1/x} \left[\frac{Q_g/z}{c_E} \right]^{x/z} \frac{m_s}{V_m}$$

where Q_g is the specific ion exchange capacity of the sorbent, c_E is the eluent concentration, m_s is the mass of sorbent, and V_m is the volume of mobile phase in the column. Selectivity is given by

$$\alpha_{A,B} = (K_{xB}^{yA})^{1/x} K_A^{(x-y)/x} \left[\frac{V_m}{m_s} \right]^{(x-y)/x}$$

B. Ion Exclusion

Separation is dependent on three different mechanims: Donnan exclusion, steric exclusion, and adsorption/partitioning. Donnan exclusion causes strong acids to elute in the void volume of the column. Weak acids that are partially ionized in the eluent are not subject to the Donnan exclusion and can penetrate into the pores of the packing. Separation is achieved through differences in acid strength, size, and hydrophobicity. The major advantage of ion exclusion is the ability to handle samples that contain both strong and weak acids.

C. Ion Pairing

Ion pair extraction is very useful in liquid–liquid partition techniques:

$$(A^{n+}_{aq} + B^{n-}_{aq}) \leftrightarrow (nA^{M+} + B^{M-})_{org}$$

The complex species is more soluble in organic solvents than in water. Many examples are quoted in the literature, with special emphasis on metal chelates extraction.

In most LC procedures an alkyl-bonded phase is utilized as stationary phase. The mobile phase is a hydro-organic mixture to which is added an ionic additive (hetaerion) capable of exchanging one ion to form a complex with one ion of the solute. Concentration of the ion pairing agent is usually 10^{-4}M to 10^{-5}M. Ion pair formation is favored when the concentration of the organic component in the mobile phase is increased due to the decreasing dielectric constant of the medium. The mechanism of retention is still not totally clear, despite the many published papers. The most probable is that the hetaerion is coating the hydrophobic surface of the stationary phase through its hydrophobic moiety. It thus forms a dynamic ion exchange packing. Since the electric potential at the surface is modified by the coating of the hetaerion, an electrical double layer is formed and coulombic interactions are involved in retention. However, it is well known that retention follows the guidelines of liquid–liquid partition experiments. The higher the organic/water *P* coefficient, the higher the retention. Retention increases with the hydrophobic character of the hetaerion. The selection of the proper hetaerion is critical. Table 4 displays some in current use.

Table 4 Anionic and Cationic Counterions Useful in Reverse-Phase HPLC

Anionic counterions	
Chloride	C_1–C_{16} sulfonate
Bromide	Toluene sulfonate
Iodide	Naphthalene sulfonate
Perchlorate	Camphor sulfonate
Phosphate	C_1–C_{16} sulfate
Acetate	Butylphosphate
Propionate	Citrate
Picrate	Tartrate
	Trifluoroacetate
Cationic counterions	
(a) R_4NH^+, where R = C_1–C_7	
(b) R_1NH^+, where R = C_2, C_8, or C_{10}	
(c) $R_2NH^+{}_2$, where R = C_1 or C_2	
(d) where R = C_1–C_{12} or 2-hydroxyethyl-	
(e) Inorganic ions, e.g., Na^+, K^+, Li^+, Cs^+, Mg^{2+}, Ca^{2+}	

Ion suppression is used mainly with weak acids. For example, for an acid of type AH, we can write

$$AH \leftrightarrow A^- + H^+$$

Buffering the mobile phase with appropriate buffer solution will result in the sole AH as chemical species. In this mode we can easily use RPLC (provided the phase will stand the pH!), and acids are eluted according to their hydrophobicity.

X. MICELLAR LIQUID CHROMATOGRAPHY (MLC)

In MLC, the mobile phase consists of surfactants at concentrations above their critical micelle concentration (CMC) in an aqueous solvent with an alkyl-bonded phase (52). Retention behavior in MLC is controlled by solute partitioning from the bulk solvent into micelles and into stationary phase as well as on direct transfer from the micelles in the mobile phase into the stationary phase. Eluent strength in MLC is inversely related to micelle concentration. A linear relationship exists between the inverse of retention factor and micelle concentration. Similar to what is observed in RPLC, a linear relationship exists between retention in MLC and ϕ, the volume fraction of the organic modifier. Modeling retention in MLC is much more complicated than in RPLC. The number of parameters is important. Micelles are obviously a new domain in both liquid chromatography and electrophoresis. Readers interested in the topic will appreciate Ref. 53, a special volume on it.

XI. GRADIENT ELUTION

In isocratic elution, the mobile phase composition is held constant during the whole run. To solve the general problem of the last eluting compounds, which are strongly retained, it is necessary to increase the solvent strength. Linear solvent strength (LSS) theory (54) predicts that RPLC retention for a binary solvent pair will vary linearly with mobile phase composition. In LSS gradient, the retention factor for each solute at the column inlet, k_i, decreases during gradient elution according to

$$\ln k_i = \ln k_o - b(t/t_o)$$

where:

t_o is the column dead time,
b is the gradient steepness parameter (slope value),
k_o is the value of k in gradient elution at the start of separation.

If we consider the simplest relationship in RPLC, then

$$\ln k = \ln K_w - S\phi$$

In gradient elution, retention time t_r is

$$t_r = (t_o/b) \log (2.3k_o b) + t_d + t_o$$
$$b = V_m \Delta\phi\, S/(t_g F)$$

where V_m is the column dead volume in mL, $\Delta\phi$ is the change in ϕ from the start to the end of the gradient, t_g is the gradient time in minutes, F is the flow rate in mL/min, t_d is the delay time between the pump and the column or gradient dwell time, and t_o is the column dead time. To check

the delay time and the accuracy of the gradient step, the analyst can use methanol with toluene as modifier and record on a UV detector.

A gradient elution should be described by the initial and the final mobile-phase compositions, by the gradient shape, and by the gradient steepness. A step gradient is often convenient when strongly retained solutes are not the solutes of interest.

The combined use of temperature and solvent strength has been thoroughly investigated by Dolan and Snyder (55, 56).

XII. SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Size exclusion chromatography is devoted to macromolecules. In SEC, the packing is a porous material that is either organic (a crosslinked polymer such as PS-DVB) or inorganic (silica or diol-bonded silica). A solute traveling through the packing may or not enter a pore. Liquid flowing through the column exhibits different linear velocities depending on whether it goes inside or outside the pores. When no interaction of the solute with the stationary phase occurs (unretained solute), a molecule of solute travels with different velocities according to its size. When small, it can penetrate inside the pores; when large, it cannot. When penetrating into one pore of similar dimension, a solute molecule experiences an entropy decrease. The number of conformations allowed to a flexible polymer decreases when it comes close to a solid wall. To avoid this region of low entropy, the macromolecule keeps a distance between its gravity center and the sol gel interface, which is roughly the hydrodynamic radius of the molecule. The hydrodynamic radius R_h is the radius of a sphere with hydrodynamic properties similar to the macromolecule. $R_h = 0.7R_g$, where R_g = gyration radius. Hydrodynamic volume is $V_h = (\eta)M$ where (η) is the intrinsic viscosity and M is the molecular weight of the polymer.

The volume available to the molecule in a cylindrical pore is

$$V_{acc} = (1 - 2R_h/d_{po})2V_p$$

where d_{po} is the pore diameter and V_p is the pore volume.

Macromolecules partition between the mobile phase outside and inside the pores. K_{SEC} is given by

$$K_{SEC} = V_{acc}/V_p$$

Retention is dependent on three variables: K_{SEC}, V_o (the interparticle volume), and V_p. Retention volume is

$$V_r = V_o + V_{acc} = V_o + K_{SEC*}V_p$$

$K_{SEC} = 0$ when the molecule hydrodynamic radius is higher than the mean pore diameter. K_{SEC} is 1 with small molecules, which can easily penetrate into the pores. The most important parameters influencing resolution are the pore volume, pore size distribution, and particle size. The separation domain is between the exclusion volume V_o and the inclusion volume $(V_o + V_p)$.

Since columns separate molecules based on their size, not their molecular weight, calibration is necessary. Two solutes with the same molecular weight but different conformation (rod shape vs. globular) do not exhibit the same retention. A typical calibration curve from a manufacturer is displayed in Fig. 13. To draw the calibration curve, 8–10 molecular weights that bracket the molecular weight of the sample are necessary. The hydrodynamic volume of a polymer molecule in solution is proportional to the intrinsic viscosity and molecular weight divided by Avogadro's number, N_{AV}. The Mark–Houwink plot is a log-log plot of intrinsic viscosity versus molecular weight for a polymer sample. A viscosimeter detector is proportional to the intrinsic viscosity and concentration: $(\eta) = f(V_r)$. The low-angle laser light-scattering detector gives a

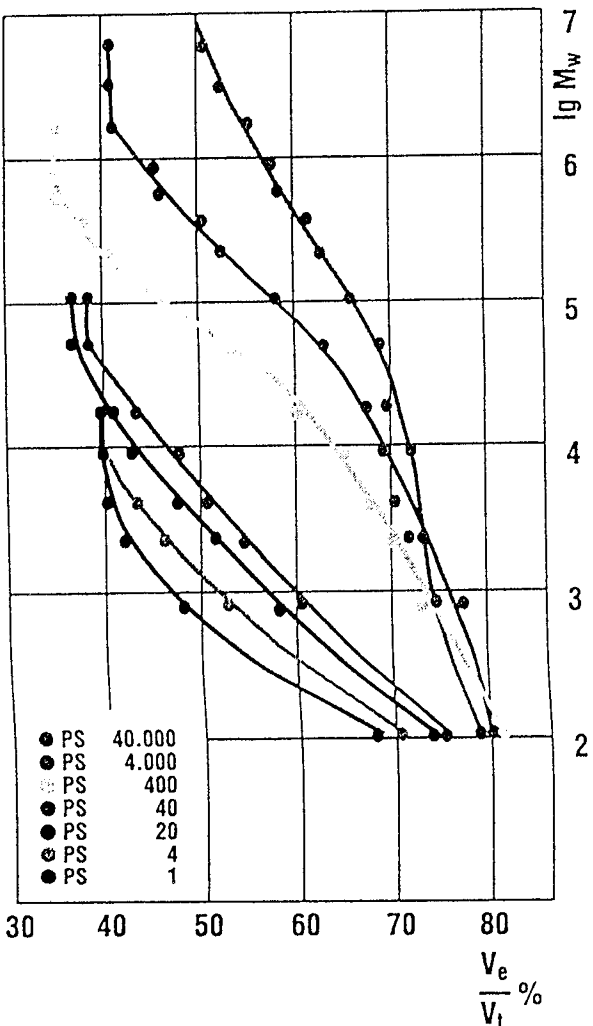


Fig. 13 Calibration curves for Lichrogel PS1, PS4, PS40, PS400, PS4000, and PS 40000, determined with polystyrene standards and tetrahydrofuranne as eluent. (Courtesy of Merck, Darmstadt, Germany.)

response proportional to molecular weight and concentration. From the data obtained with this triple detection one can draw universal calibration curves.

For applications directed at the analysis of oils and fats, PS-DVB copolymers are often selected. The selected porosities for separations in the range 1,000–20,000 MW are 50, 100, and 500 Å, and 5–10 μm particle sizes are common. Tetrahydrofuran (THF) is the most popular solvent.

XIII. INSTRUMENT

Basically, the LC instrument is made of solvent reservoirs, pumps, an injection device, a detector, and a data acquisition system (Fig. 14). It must be kept in mind that two solutes cannot be electronically resolved.

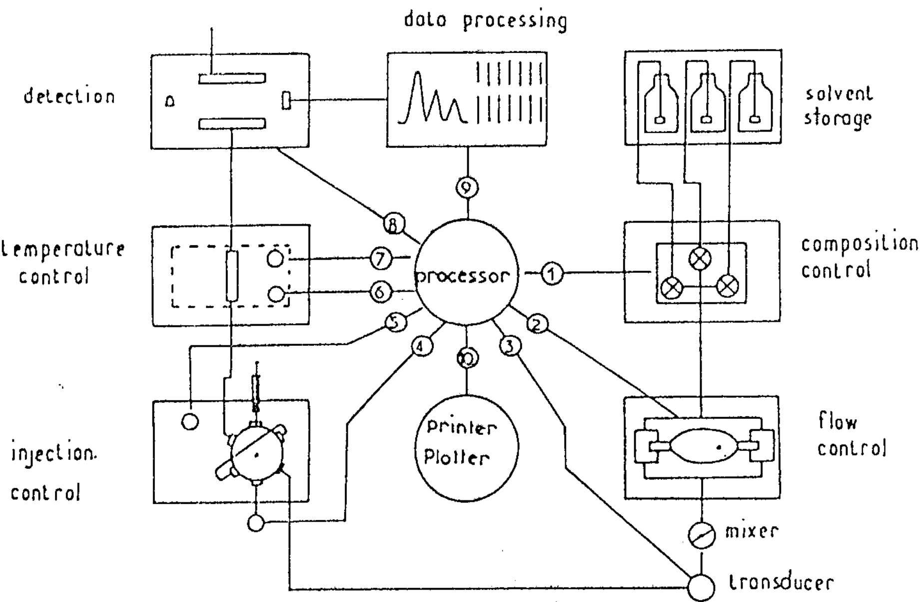


Fig. 14 Schematic of a typical LC instrument. (Courtesy of Hewlett Packard.)

A. Columns

The heart of any chromatographic system is the column. Packed columns (Fig. 15) are by far the most utilized, especially in conventional columns, which are available in different lengths (5, 10, 25 cm). Despite the introduction of many column designs and shapes, the most popular columns are either the standard 250 × 4.6 mm (length × internal diameter ID) or 100 × 4.6 mm. Short columns (5 cm) are often used in LC-MS. Tubing for conventional columns is generally stainless steel, which can withstand high pressures, high temperatures, and corrosive chemicals. With metal-sensitive compounds such as some proteins or chelating agents, polytetrafluoroethylene-(PTFE-) lined stainless steel or glass-lined stainless steel is required. PEEK (polyetherether ketone) tubing is an inert material allowing for sample exposure of both small and large molecules. This material is ideal for ion chromatography. Cartridges that can be connected without void volume are available. Merck (Darmstadt, Germany) introduced a system where the column itself is grooved and the reusable end fittings are retained by nuts at each end of the column. Analysts can add a small guard column without using additional fittings or a second holder. Guard columns are inserted between the injector and the analytical column. It extends column life, since it collects debris from pump seals or solvents and strongly retained compounds. When the plate count for a required separation is low, the guard column can be used as the analytical column.

There is a need for smaller columns in order to reduce solvent consumption and analysis time. Following Vissers et al. (57), we suggest different names for packed micro LC columns according to the internal diameter:

0.5–1.0-mm ID: micro LC.
100–500-μm ID: capillary LC
10–100-μm ID: nanoscale LC

One can find 1-mm glass-lined stainless steel columns or fused silica capillary columns. Fused silica is available with 75-μm, 100-μm, or 320-μm internal diameter by 20-cm, 25-cm, or 30-cm length. Fused silica is a glass tubing with an outside polyimide coating to prevent breakage.

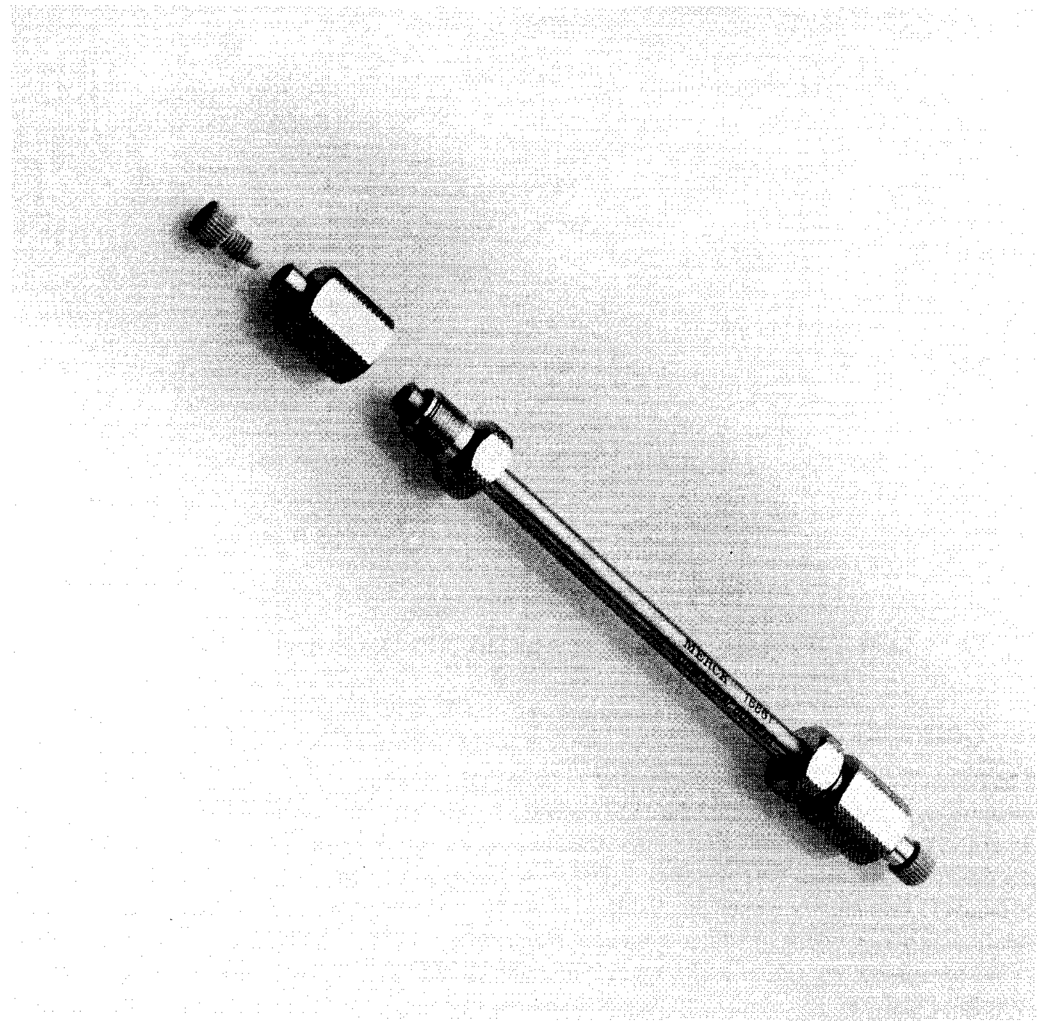


Fig. 15 A classical LC column. (Courtesy of Merck.)

To ensure optimal sample distribution and low dead volume, much attention has been paid to frits that close the tubing; stainless steel mesh performs better than conventional frits.

B. Packings

Due to theoretical work, there has been a trend toward smaller and smaller particles. At present, the 3–5-μm particles are compatible with standard equipment without producing excessive band broadening. The 3–5-μm particles permit performances of more than 12,000 plates on 10 cm with a good column lifetime. Particles are either porous or nonporous. Manufacturers make dramatic efforts to guarantee performance, selectivity, and capacity. They supply excellent columns. However, analysts should take care with columns. In RPLC mode, for example, it is a bad practice to use a single column for runs with different mobile phases containing buffers. Guard columns are of primary importance. Excellent troubleshooting can be found in LC-GC (58).

C. Pumps

A solute eluting from the column is recorded as a peak of width measured in time units:

$$\omega = V_r/F \times N$$

where V_r is the retention volume, F is the flow rate and N the plate count. Control of the flow rate is of the greatest importance, especially when a computer is utilized to check retention times.

1. Desirable Characteristics

Good pump units have the following characteristics.

a. Flow Rate Stability

Flow noise is characterized by fluctuation periods that are short compared to the peak width. It affects mainly the detector baseline. Flow noise arises from insufficient pulse dampening of reciprocating pumps or from poor check valve functioning. *Flow wander* is characterized by longer fluctuation periods (up to 10 times the peak width). It affects peak area precision directly. It may come from piston seals, but very often comes from very small particles or bubbles in the mobile phase. Continuous degassing of solvents with helium at too high a flow rate may produce slugs that cause flow wander. *Flow drift* is characterized by periods larger than peak retention times and affects both retention times and peak areas. It often occurs in gradient runs with changes in viscosity.

b. Flow Precision and Accuracy

Flow rate variations should be less than 0.2%. According to Foley (59), the sources of flow rate errors in LC can be classified into four categories: nonideal mobile-phase behavior, nonideal stationary phase, mechanical limitations of the delivery system, and operator error. This last category does not exist any longer since the advent of computer-controlled systems. However, the volumes of mixing of methanol-water, acetonitrile-water, and tetrahydrofuran-water are far from zero. Furthermore, the use of very small packing particles induces high pressures, and viscosities are pressure dependent. Manufacturers have made great efforts to take into account all these sources of error.

c. Reproducibility

Day-to-day reproducibility depends on the lifetime of the different parts of the pumping unit. Corrosion with some additives, such as cetrimide, is well known. Metal particles from frits or leaking from the pump hardware may dramatically affect peak shape or baseline in ion chromatography.

d. Flow Rate Range

Flow rate range is rather large. A pump is capable of delivering 0.001–9.99 mL/min in analytical mode. Some pumps are also capable of delivering up to 50 mL/min in preparative mode. In micro LC, the mobile phase is usually delivered at very low flow rates (0.1–100 μL/min). All pumps are equipped with an automatic device that stops the motor when pressure reaches a given value.

D. Pumping Systems

At present, reciprocating pumps are in use for conventional column LC. In micro LC both syringe and reciprocating pumps are the favored solvent delivery systems.

Reciprocating pumps are either single head or double head. In a single-head pump the liquid enters the chamber through the inlet valve and is expelled through a check valve. The piston

is usually cam driven, and the flow profile is sinusoidal. An efficient pulse dampener is required. Modern single-piston pumps are self-priming and of the rapid-refill type. They are not very expensive and require reduced maintenance.

In two-parallel-head pumps the pistons are driven by two identical cam discs having a phase shift of 180°. The liquids displaced from both heads are combined in the outlet manifold assembly to produce a continuous solvent delivery. The two-heads-in-series design is used by most manufacturers. In this mode the second piston displaces a volume that is one-third or one-half of the volume displaced by the first piston (the working piston). During the working piston's forward stroke, the downstream piston is moving backward and its chamber is refilled at high pressure so that the flow rate delivered to the column is constant.

In the syringe-type pump the liquid is enclosed in a cylinder. A piston moves at a constant speed to push the liquid. Eluent compressibility induces time-consuming flow equilibrium. Nevertheless, the flow from a syringe pump is pulse free. For micro LC, flow rates of 50 μL/min are utilized in spite of the drawback of column pressurization. With very low flow rates (in the nanoliter range) the use of pumps is tedious, and split-flow techniques are required.

Pumping systems are apparently well established, and nothing really new appeared in the different reviews on instrumentation. Standard 316 stainless steel wetted components provide superior corrosion resistance to most system fluids.

Tests must be performed to ensure that the pump is working well: pressure, flow rate, and gradient linearity.

1. Gradient Generation

a. Low-Pressure Gradients

Solvents from reservoirs are mixed prior to entering the single reciprocating pump. Compositional accuracy is achieved through solenoid valves that operate on a time cycle. To prevent a nonuniform aspiration cycle or siphoning, optical or electrical encoders can make the proper adjustment of solvent composition. A very short aperture time of the solenoid valve is required.

b. High-Pressure Gradients

High-pressure gradients require two pumps. They independently deliver two stream flows to a mixing chamber on the high-pressure side of the LC system. The main requirement is compositional accuracy, since one pump speeds up while the other slows down. This is especially critical when starting a gradient in which the modifier is present at a very low percentage. Any pump manufacturer should specify the flow rate increment (typically 0.1 μL/min in micro LC, 1 μL/min in conventional LC. Furthermore, gradient-proportioning precision is of primary importance (typically 1% in micro LC, 0.5% in conventional LC). In performing gradients and especially in micro LC, the mixer design is of critical importance. Every manufacturer provides its own design. They all perform well.

2. Gradient Measurements

The gradient volume V_G is the volume of eluent flushed through the column during the gradient run (60):

$$V_G = Ft_{gr}$$

where t_{gr} is the gradient time. Separation is identical whether $F = 2.0$ mL/min within $t_{gr} = 10$ min or $F = 1.0$ mL/min within 20 min.

The response volume is the volume it takes to reach a given concentration once the gradient has started. The elapsed time is the response time. It is experimentally determined by measuring the volume from the first appearance of a step gradient at the detector until an arbitrary

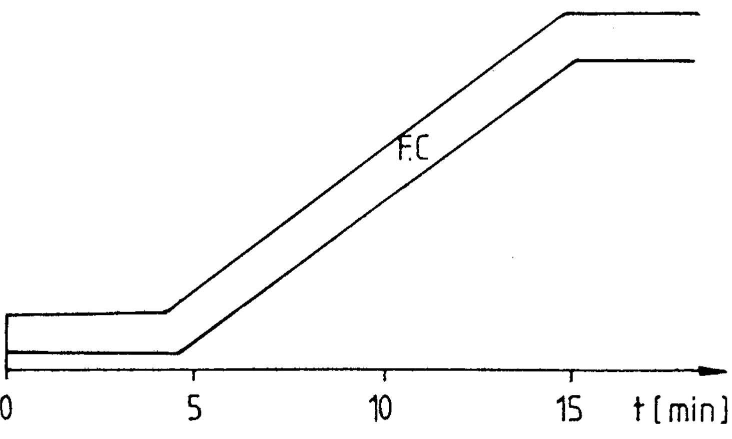
final composition is obtained. Since with dynamic mixers the composition of the solvent leaving the mixing chamber varies exponentially, the response time can be defined at the point where the gradient has progressed to 63% of final composition.

Delay volume is the volume found between the time the gradient is started and when it reaches the column. The corresponding time is the delay time t_D (see earlier). By running a gradient from pure methanol to methanol containing 100 ppm of UV-absorbing toluene, the delay volume can be measured.

Compositional accuracy is the ability to generate the same solvent composition from run to run.

3. Gradient Linearity

Observed profiles are not truly linear. There is some rounding (Fig. 16) at very low flow rates due to the very small increments and to the mixing in the connecting tubing from the mixing chamber to the column inlet.



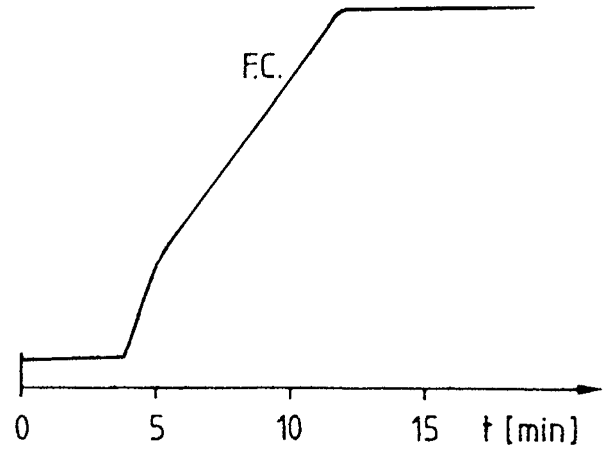


Fig. 16 Two different gradient profiles obtained with different instruments. Gradient from methanol (eluent A) to methanol containing 100 ppm toluene. FC: final conditions. (From Ref. 60. Reproduced from Vieweg with permission.)

Injection devices are all six-port valves. Sample loops have different volumes. Volumes of the sample loop must be adapted to the column diameter (61). Increases in the volume of very dilute solutions do not destroy efficiency, but increases in both the volume and amount of solute are detrimental.

E. Detectors

Detectors can be divided in three categories according to:

The response: we can distinguish differential detectors, which monitor the difference in the composition of the column effluent, and integral detectors, which measure the amount of sample component passing through the detector.

The selectivity: universal detectors exhibit a response to every sample (except the mobile phase), whatever the chemical species. Selective detectors respond to samples that exhibit a physical or chemical property (UV absorbance, for example). In this mode the mobile phase should not interfere.

Concentration-sensitive or mass flow detectors: whether the response is proportional to the concentration of the sample or to the amount of the sample.

Detectors of all types exhibit noise (N). *Noise* is the amplitude (in detector response units) of the envelope of the baseline, which includes all random variations of the detector signal, whose frequency is on the order of one or more cycles per minute. *Short-term noise* is defined as that portion of the signal that consists of random periodic variations in the detector signal with a frequency of 1/min or greater. *Long-term noise* is similar to short-term noise except that the frequency range is between 6 and 60 cycles per hour.

Drift is the average slope of the noise, expressed in units per hour.

Flow sensitivity is the rate of change of signal displacement with flow rate resulting from step changes in flow rate. Flow sensitivity is solvent dependent.

The *time constant* of the detector is related to the electronics and should not be confused with the time constant of the recorder (or any data acquisition).

The *calibration curve* is the plot of the recorded detector signal (the peak area in mV/min or absorbance unit/min) versus concentration or mass, according to the type of detector (Fig. 17). Any calibration curve exhibits a linear portion and then a curvature to a plateau.

Sensitivity is defined via the equation $R = R_0 + SC$, where R is the detector output, S is the response factor or sensitivity, and C is the concentration (or mass flow rate if applicable). R_0 is the response at zero concentration. It is obvious that when S is high, a slight change in concentration will produce a more detectable signal.

According to the ASTM definition, *linear range* and *dynamic range* are two different concepts. The *dynamic range* is that range of concentration or mass flow rate of a substance over which an incremental change in concentration (or mass flow rate) produces an incremental change in detector signal. The lower limit of the dynamic range is the concentration producing a detector output signal equal to a specified multiple of the detector short-term noise level. It is the minimum detectability. It can be be calculated from the sensitivity (S) and the noise (N): $D = 2N/S$. The upper limit of the dynamic range is the concentration at the point where the slope of the calibration curve becomes zero.

The *linear range* of a detector is defined as the range of concentration over which the sensitivity is constant to within a defined tolerance. S is obtained by performing a linear regression analysis of the data. A simple way to determine the linear portion is to draw a second line having a slope equal to 95% of that of the best-fit line. It intersects the response curve and determines the linear range (Fig. 17).

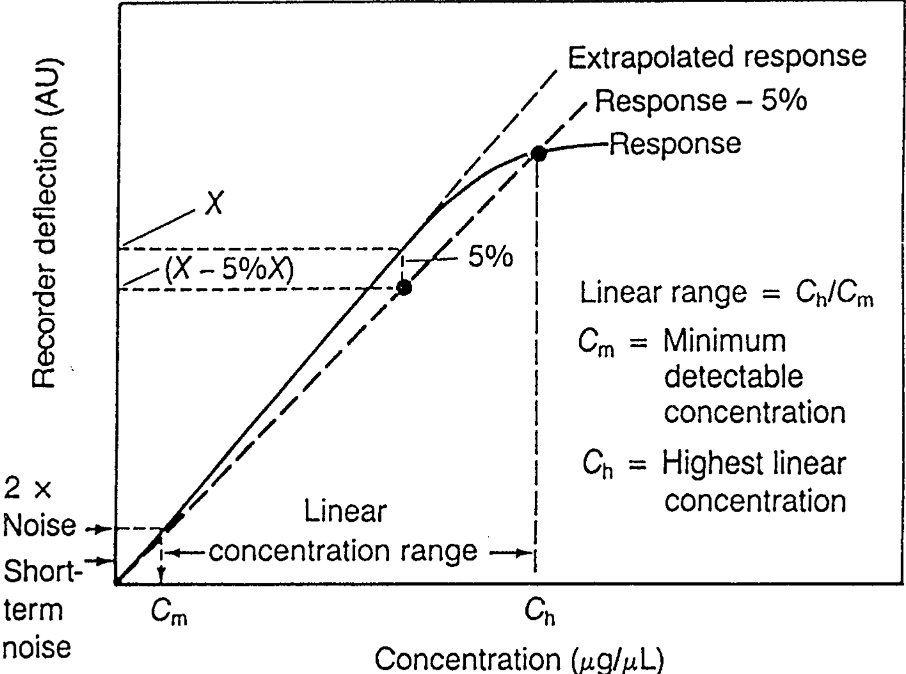


Fig. 17 Plot of recorder deflection versus concentration to determine the linear range of a detector (photometric).

The *detection limit* (DL) is the smallest quantity of sample that can be detected qualitatively but not quantitatively. It is determined by measuring the maximum amplitude of the signal on a distance equal to (at least) 20 times the peak width of the sample. The detection limit is $DL = 3S/N$.

The *quantification limit* (QL) is the smallest quantity of a sample that can be accurately determined with a given accuracy and reliability: $QL = 10S/N$.

It is of great importance to remember that detector response is influenced by the chromatographic conditions. With a concentration-sensitive detector, for example, the peak area is

$$A_p = \int_{t1}^{t2} R\,dt = S \int C\,dt$$

The amount of solute is

$$M = \int C\,dV = \int CF\,dt$$

where F is the flow rate and C is the concentration. Thus,

$$A_p = (S/F)M$$

The peak area is reciprocally connected to the column flow rate. The efficiency is $N = 16(t_r/\omega)^2$, so (with a gaussian peak)

$$A_p = \sqrt{2\pi/4}\,\omega h_p$$

where h_p is the peak height.

If the mobile phase is not compressible, then

$$t_r = L/u(1 + k) \qquad \text{and} \qquad A_p = \sqrt{2\pi}\, Lh_p(1 + k)/4u\sqrt{N}$$

The peak height is thus proportional to N and is highest when the optimum flow rate is achieved. Any comparison between quantitative data should indicate what the unretained solute was, what the flow rate of the mobile phase was, and what the plate count was.

1. Photometric Detectors

a. UV-VIS Absorption

From published papers and proposed methods, this type of detection is very popular because of its ease of use, broad application area, and low purchase price. The detector measures the change in absorbance of light in the 190–350-nm region (UV) or the 350–700-nm region (visible).

Ultraviolet light sources are either mercury lamps with strong emission lines at 254, 313, and 365 nm, cadmium at 229 and 326 nm, zinc at 308 nm, or deuterium and xenon lamps, which exhibit a continuum in the 190–360-nm region. Halogen lamps are used in VIS region. A filter or grating is used to select the required wavelength for measurement. Cutoff filters pass all wavelengths of light above or below a given wavelength. Bandpass filters pass light in a narrow range (e.g., 5 nm). Specifications include wavelength accuracy (e.g., 2 nm), wavelength reproducibility (e.g., 1 nm), spectral resolution (e.g., 2 nm), and drift (less than 10–5/hr). It is possible to change the detection wavelength during measurements, for the filters can be switched via keyboard, time program, or external analog control.

On commercially available instruments, the flow cell is typically 2–8 μl, with a 10-mm path length. According to Beer's law, which states that log 1/T = $A = \epsilon lC$, where A is the absorbance, ϵ is the molar absorptivity, l is the path length, and C is the concentration, the higher the path length, the higher the transmitted light. UV-VIS detectors are concentration sensitive. Most cells are Z shaped to increase the path length. With capillaries the path length is very limited, and the curvature of the wall makes the transmission more difficult. When the aperture of the source is adjusted to the inside diameter of the capillary, the effective light path is

$$l_{\text{eff}} = \frac{1}{2}\pi r$$

where r is the radius of the capillary. The limits of detection are dependent on the molar absorptivity of the solutes (ϵ); the higher the ϵ, the lower the limit of detection.

b. Photodiode Array (PDA) Detector

The PDA detector passes the total light through the flow cell and disperses it with a diffraction grating (Fig. 18). The dispersed light is measured by an array of photosensitive diodes. The array of diodes is scanned by the microprocessor (16 times a second is usual). The reading for each diode is summed, and the total is averaged. Photodiode array detectors can simultaneously measure the absorbance at all wavelengths versus time. The amount of data storage is a key feature in the PDA. A run can easily take several megabytes for data storage.

Peak purity is based on the proprietary spectral contrast algorithm, which converts spectral data into vectors that are used to compare spectra mathematically. This comparison is expressed as a purity angle that is compared to the purity threshold. Spectral deconvolution techniques are used when two peaks coelute.

It seems that the PDA is not fully utilized. When quantitative measurements are performed, most of the spectral information is often ignored, since detector's response is locked on a single wavelength (or two). Multicomponent analysis uses multiple wavelengths to deconvolute the unresolved peaks and provides accurate quantitation independent of peak resolution and peak shape.

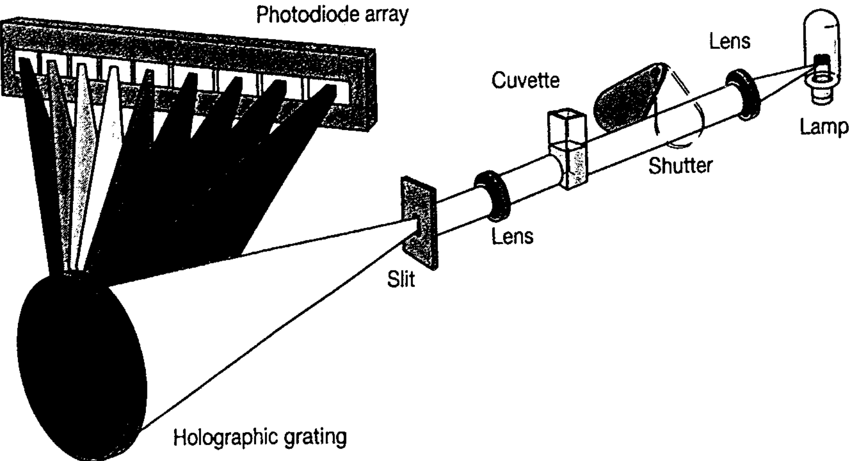


Fig. 18 Diagram of a photodiode array.

These detectors obviously require a careful selection of solvents utilized as mobile phases. Tables of UV cutoffs are readily available. UV-VIS detectors can be used with gradients. An interesting feature is the use of derivatives of the spectra.

c. Fluorescence Detector

Fluorescence is a three-stage process: excitation via the absorption of a radiation, excited state for a very short time $-(10^{-9}$ sec), and emission. The excitation of a molecule is achieved by the absorption of a light quantum of an appropriate wavelength, promoting, in a simplified view, a π or nonbonding (n) electron to a π antibonding orbital. The quantum yield ϕ is a fundamental molecular property that describes the ratio of the number of emitted photons to the number of photons absorbed.

Signal intensity is given by Beer's law:

$$I_f = I_o(1 - e^{\epsilon lc})\phi_F k$$

where ϕ_F is the quantic yield. It is, consequently, a concentration-sensitive detector.

Fluorescence emission provides more selectivity and increased sensitivity compared to UV absorption. Basically, the instrumentation is composed of an excitation source, an excitation-wavelength selector, a flow cell, an emission collector, and an emission-wavelength selector. Due to their highly collimating nature, lasers are utilized as sources (He/Ne, diode, argon ion). The diode laser seems the best choice.

Filters or monochromators are generally used for the selection of either excitation and/or emission. Other, undesired sources of radiation (Raman scattering, Rayleigh scattering, solvent impurity emission) must not reach the photomultiplicator.

Laser-induced fluorescence or detection of fluorescence emission in the packing is utilized in capillary LC.

d. Chemiluminescence

Briefly, chemiluminescence is the production of light by means of a chemical reaction. It is an area of increasing interest for sensitive detection in HPLC. Chemiluminescence is the production of electromagnetic radiation (UV, VIS, or IR) by a chemical reaction between at least two reagents, A and B, in which an electronically excited intermediate or product, C*, is obtained and

subsequently relaxes to the ground state, with the emission of a photon or by donating its energy to another molecule that then luminesces. A number of different chemiluminescent systems based on peroxyoxalate, luminol, or acridinium esters have been described. It does not seem from the available literature that the thermal energy analyzer (TEA) used in GC detection of nitrosamines can be easily interfaced with HPLC.

e. Derivatization

Many solutes do not exhibit UV absorption or any fluorescence. They can be converted in UV-absorbing derivatives or fluorescent derivatives by pre- or postcolumn derivatization. The postcolumn procedure is very convenient, since it does not change the chromatographic system and permits the detection of the solute of interest without interference. Prechromatographic derivatization involves a change in the selection of the chromatographic procedure. It is convenient with, e.g., fatty acids. In postcolumn derivatization there is no need for completion of the reaction; all that is required is knowledge of the kinetics. With the reversed-phase (RP) type of eluents, reactions may be quite tedious, since most are in nonaqueous media. Adding a reagent to the eluent is simple. Another pump is delivering the reagent, which is mixed with the eluent in a tee. Then, according to kinetics, a reactor ensures a thorough mixing and achievement of the reaction if not immediate.

Reactors are of several varieties. With *coiled open tubes* (a standard capillary tube is well suited), band broadening is calculated via the Golay equation. To reduce it, knitted open tubes or stitched open tubes have been designed, though they are not commercially available yet. *Packed bed reactors* are either those packed with inert solid supports or those packed with reactive supports. In the second type, enzyme-supported materials are generally utilized. To prevent dilution in the reagent, an elegant solution is to perform photochemical irradiation from a UV lamp (62). Teflon tubing is UV transparent and can be coiled around an appropriate lamp to generate electroactive species.

Since the early detection of amino acids with ninhydrin, many derivatization procedures have investigated these solutes. 4-Dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) is performing well, but the best seems to be FMOC (9-fluorenyl chloroformate) (63). More generally, each class of reactions replaces the active hydrogens of the OH, NH, and SH groups.

2. *Light-Scattering Detector*

Operation of the light-scattering detector (Fig. 19) involves a three step process: (1) nebulization of the eluent to a fine mist from which the solvent can be vaporized, (2) evaporation of the solvent molecules from the mist using a heated drift tube to provide particles of pure solute, (3) measurement of the scattered light by means of solute particles, related to the concentration of the compound of interest in the sample. In most cases, nebulization incorporates a Venturi type of flow of inert gas, coaxially wrapping the flow of column eluent through a small aperture. Evaporation takes place as the droplets are carried by the gas flow from the nebulization chamber to a heated drift tube. The main requirement is to maintain the uniformity of particle size. The larger the solute particle, the higher the intensity of the light scattering. The amount of light scattered by the solute particles is measured using a photomultiplier or a photodiode. All solutes are detected. It is a universal detector. The measured peak area A is related to the sample mass by the relationship $A = am^b$, where a and b are coefficients dependent on droplet size, concentration, flow rate, vaporization temperature, etc. The response is nonlinear: $\log A = b \log m + \log a$; the calibration curve is log-log. The limit of detection is about 10 ng.

The temperature used to evaporate the mobile phase is critical. The flow tube is designed for the evaporation of high-boiling eluents (e.g., water) at low temperatures (40°F). Baseline noise is also critical, and the eluent stream must be particle free.

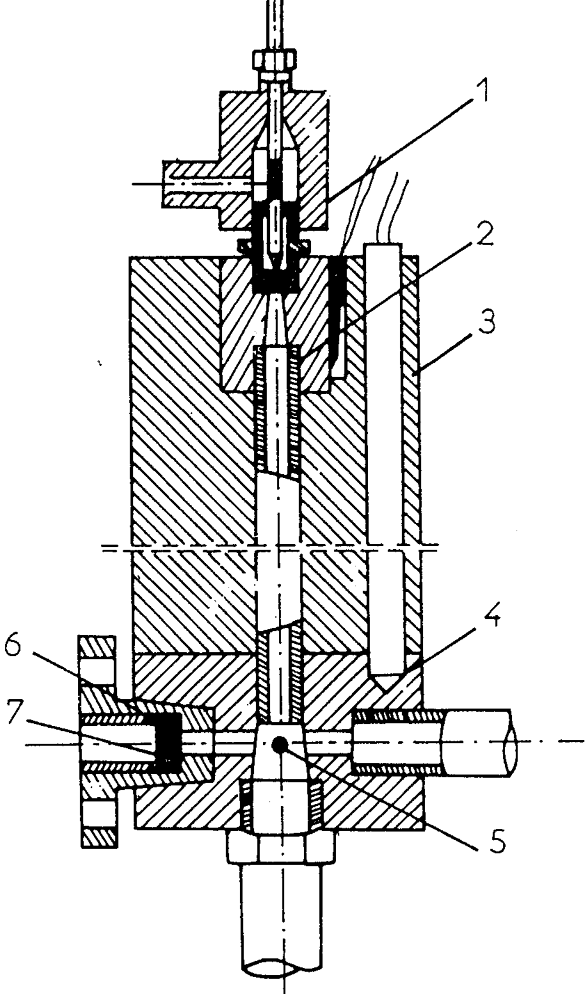


Fig. 19 Detector block for the light-scattering detector. (1) nebulizer, (2) drift tube, (3) heated copper block, (4) light-scattering cell, (5) glass rod, (6) glass window, (7) diaphragm. (Reproduced from A. Stolyhwo, H. Colin, and G. Guiochon, J. Chromatogr. 265:1 (1983) with permission.)

Modern LSD detectors yield good results even under gradient elution. No disturbance is observed when solvent composition changes. Organic solvents (acetone, propanol, chloroform) can be used in the mobile phase. In reversed-phase mode, water content up to 25% and small amounts of buffers are not a problem. Typical applications are lipids, phospholipids, sugars, and vitamins.

3. LC-MS (Mass Spectrometry)

Mass spectrometry (MS) is now an integrated detector for liquid chromatography. This is due to the advent of atmospheric pressure ionization (API) interfaces. In an API interface, the column effluent is nebulized into an atmospheric pressure ion region. Nebulization is performed pneumatically in atmospheric pressure chemical ionization (APCI) by a strong electrical field in electrospray or by a combination of both in ion spray. Ions are produced from the evaporating droplets

either by gas-phase ion–molecule reactions initiated by electrons from a corona discharge or by the formation of micro droplets by solvent evaporation followed by desolvation of ions from the droplets. The mass spectrometer is a mass-flow-sensitive detector. The thermospray interface, which was originally developed by Vestal (64), is declining. The thermospray was interesting because it can accommodate reversed-phase solvents and any volatile buffer. However, it is analyte dependent and often does not display fragment ions.

Electrospray MS (ESI-MS) is divided into three steps: nebulization of a sample solution into electrically charged droplets, liberation of ions from droplets, and transportation of ions from the atmospheric pressure ionization source region into the vacuum and mass analyzer of the mass spectrometer. The operating principle is displayed in Fig. 20. A voltage of 2–3 kV is established between the outlet of the capillary and the grounded entrance of the mass spectrometer. When the capillary is the positive electrode, source positive ions in the liquid will drift toward the liquid surface, and some negative ions drift away from it until the imposed field inside the liquid is essentially removed by this charge distribution. However, the accumulated positive charge at the surface leads to destabilization of the surface, because the positive ions are drawn downfield but cannot escape from the liquid. A liquid cone is produced. At a sufficiently high electric field, the liquid cone vanishes and a fine mist of small droplets is generated. Each droplet's surface is enriched with positive ions for which there are no negative counter ions. Under good conditions, the droplets are small and exhibit a narrow distribution of sizes. The droplets shrink by evaporation of solvent molecules until they come close to the Rayleigh limit, which gives conditions in which the charges become sufficient enough to overcome the surface tension (γ) that holds the droplets together. They undergo fission into smaller droplets. Evaporation of these droplets is stimulated by the use of a countercurrent heated gas or by a heated sampling capillary. Extremely small droplets containing a single ion will give rise to a gas-phase ion. In the ESI process gas-phase ions are softly generated, leading to the formation of $(M+H)^+$ (positive-ion mode) or $(M-H)^-$ (negative-ion mode).

With high concentrations of salts, signal instability is observed; the small orifice of the cone can become rapidly plugged. Since the electrical field is formed along the whole spray, high flow rates may lead to electrical arcing or overheating. The ESI interfaces are limited to only a few μl/min. To overcome this drawback, a makeup gas is added that shields the spray from arcing. In most API sources the spray device is in axial position. An orthogonal spray device is available from Hewlett Packard (Fig. 21). The orthogonal sprayer reduces the contamination of the sampling orifice.

The APCI technique (Fig. 22) is based on the vaporization of the LC eluent by means of a soft flow of helium and by heating to facilitate the formation of droplets. Helium acts as a carrier gas and helps the formation of small droplets by evaporation. The spray leaves the desolvation chamber and enters a region where a high electrical field is produced by a fine corona-discharge needle running at voltages of about 3–5 kV. Mobile-phase molecules are ionized and serve as a chemical reagent gas to form an ion–molecule reaction with the analyte molecules. The resulting product molecules are thus ionized in a soft way and normally show only the addition of a proton [for example, ammonia $(M+18)^+$ or sodium $(M+23)^+$] coming from ubiquitous sources. In negative mode mostly a proton is abstracted to form an M-1 ion. Improvements in LC-MS have been tremendous. Now available is LC-TOF (time of flight) MS. Reference 65 is an excellent tutorial on the latest in LC-MS for the beginner and the more experienced.

4. *Refractive Index (RI) Detector*

This is a universal detector. Before the advent of LC-MS it was considered the only universal detector easily available. The RI detector monitors both the eluent and the analyte. The output reflects the difference in refractive index between a sample flow cell and a reference cell. The

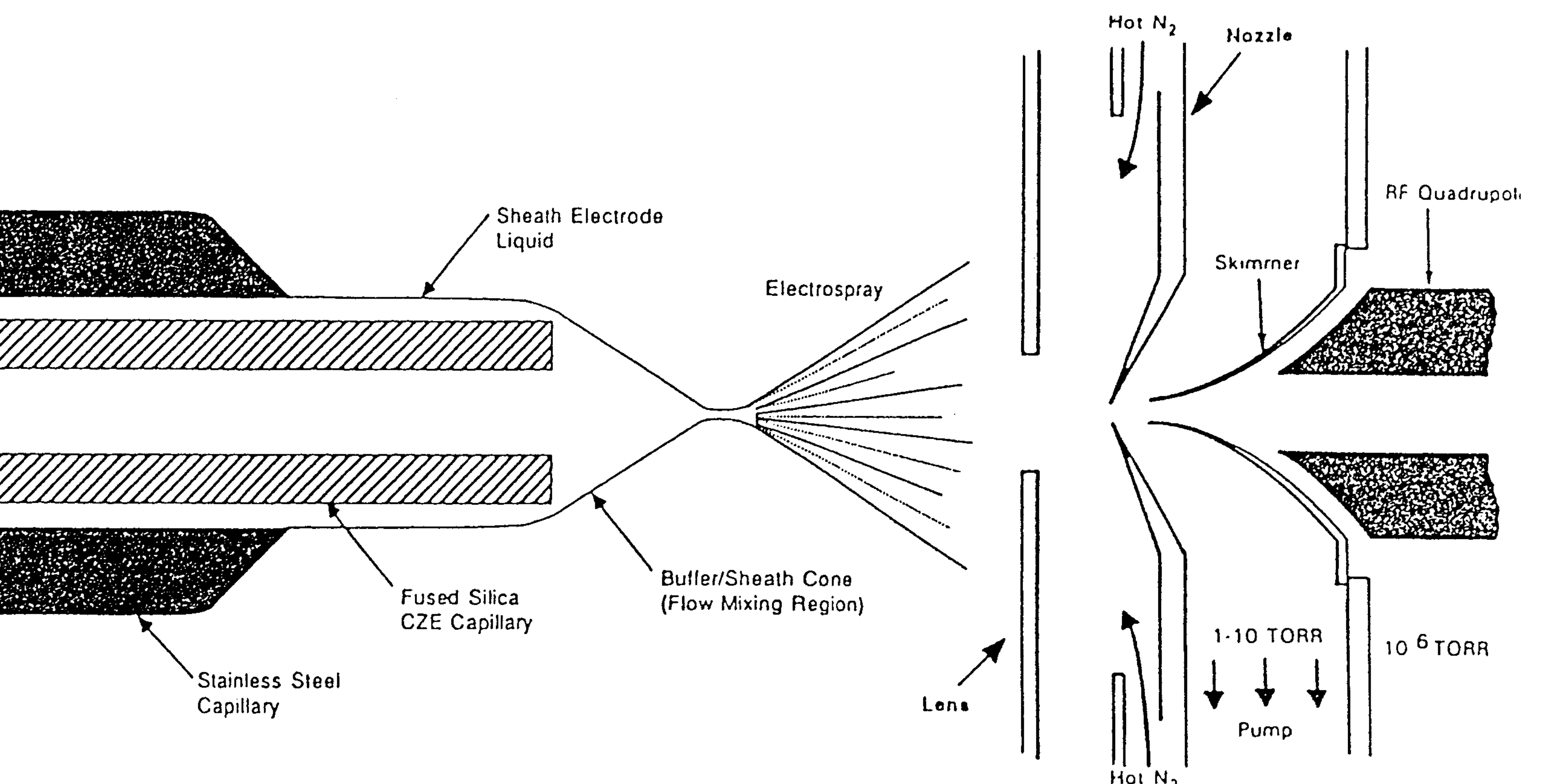


Fig. 20 Principle of the electrospray interface.

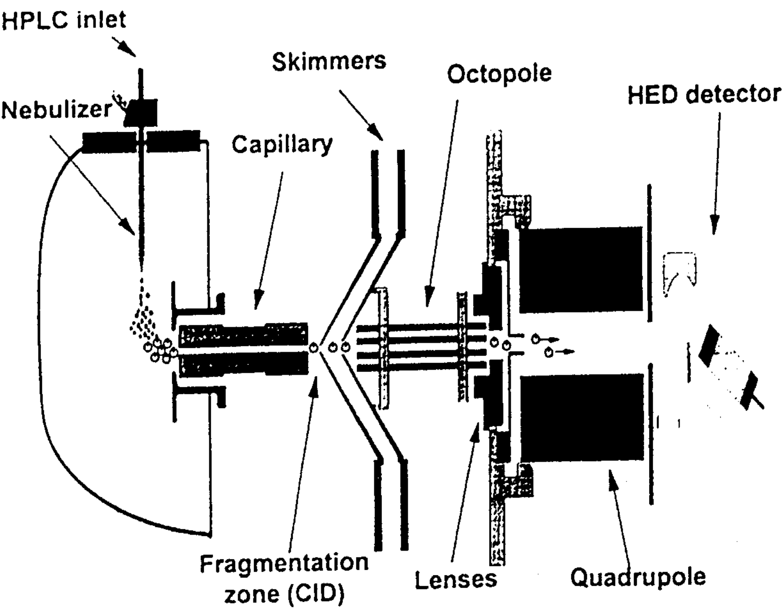


Fig. 21 Schematic of the Hewlett Packard orthogonal electrospray system. (Courtesy of Hewlett Packard.)

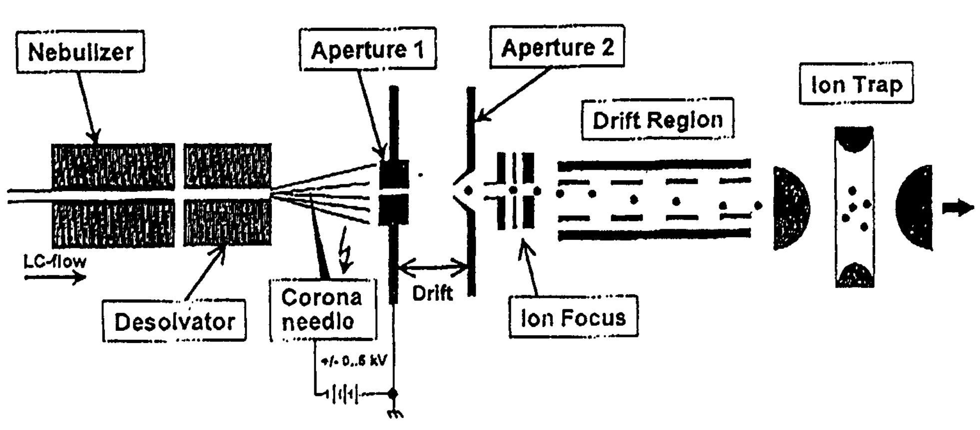


Fig. 22 Atmospheric pressure chemical ionization LC-MS interface (model M-8000 from Hitachi). (Courtesy of Hitachi.)

reference side of the cell contains static solvent; the sample side contains the chromatographic eluent. When both sides of the cell contain solvent, the net deflection is zero. When the sample solution enters the sample side of the cell, the refractive index is changed, giving a net deflection of the beam, which is electronically measured by the beam splitter and matched pair of photodiode detectors. The measured RI response is determined by the volume fraction of the analyte in the flow cell (x) and the volume fraction of the eluent in the other flow cell ($1 - x$):

$$\eta - \eta_2 = V_1(\eta_1 - \eta_2)$$

where

V_1 = volume fraction of analyte
η_1 = refractive index of pure analyte
η_2 = refractive index of pure solvent
η = refractive index of solution in sample cell

The average cell volume is 8 μl; with capillary LC, nl to pl volumes must be used. Progress in lasers permit such determinations. Currently available detectors include the following.

Snell type: Deflection-type refractometers are the most popular type of RI detectors. A collimated light beam is projected through a dual, flow-through cell, intersecting the interior glass/liquid interfaces at a given angle (usually 45°). Any difference in refractive index between the glass and the liquid results in a deflection of light according to Snell's law. Snell's law governs the angles of incidence and refraction at an interface: $\eta_1 \sin \theta_1 = \eta_2 \sin \theta_2$, where θ_1 is the angle of the beam with respect to the normal of the interface in the medium with RI of η_1 (Waters RI detector is of this type).
Fresnel type: $\Delta\eta$ is a measure of change in reflectivity. A laser source is now used instead of a tungsten lamp.
Interference type: This technique has been proposed by Tarigan (66) for RI detection in capillary separation.

The refractive index is very sensitive to temperature and pressure. To overcome this drawback, the two cells are close. Any change in temperature affects both cells. The main specifications are refractive index range, flow rate range, and temperature settings. Gradients cannot be used with RI detectors, but some devices to do so have been described.

5. *Electrochemical Detectors*

Electrochemical detection is a concentration-sensitive technique. In amperometric mode, solutes undergo oxidation or reduction reaction through the gain or loss of electrons at the working electrode. All electrochemical detectors depend on Faraday's law, $Q = znF$, where Q is the number of coulombs required to convert n moles of reactant to product by a reaction involving z electrons per ion or molecule of reactant and F is the Faraday constant. Equations for current have been described. They are complex, but the main parameters are cell geometry and electrode dimensions. A thin-layer cell design is displayed in Fig. 23. The working electrode is kept at constant potential versus a reference electrode. An auxiliary electrode closes the circuit. Electrodes are made of glassy carbon, and the surface flatness is the main parameter. Polishing and cleaning of the electrode surface is critical. With ion chromatography, a platinum electrode or a silver electrode is also advocated.

The use of dual-electrode amperometric detectors provides advantages in sensitivity and detection limits. Series configuration and parallel configuration are both possible. Ion-selective electrodes allow the selective quantification of selected analytes even in complex matrices.

Suppressed conductivity detection in ion chromatography involves the use of eluents like sodium hydroxide or carbonate-based buffers that are converted into species of low conductance like water or H_2CO_3 after exchanging the cations of the eluent for hydrogen ions by means of a suitable device. One significant advance in the field is the self-regenerating device from Dionex. Water is electrolyzed to generate protons that can cross the ion exchange membrane and neutralize the eluent (Fig. 24). However some byproducts may be generated such as the oxidation of chloride ions.

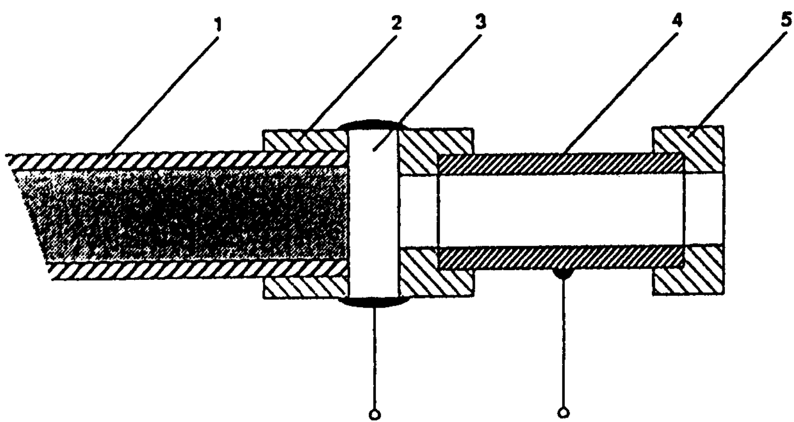


Fig. 23 Cell construction of an amperometric detector for capillary LC. 1: column; 2 and 5: fluoroplastic body; 3: working electrode; 4: reference electrode. (Reproduced with permission from Elsevier.)

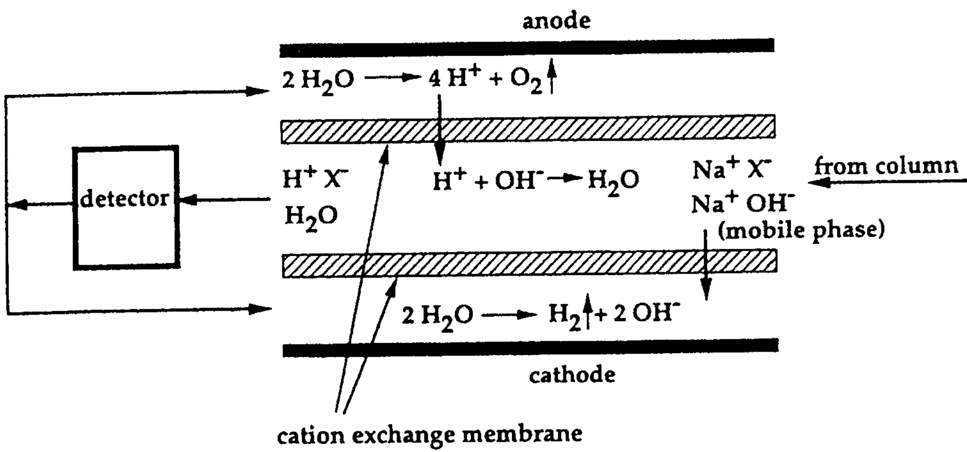


Fig. 24 Schematic of the self-regenerating suppressor in a recycle mode. (Courtesy of Dionex.)

6. *LC-NMR (Nuclear Magnetic Resonance)*

Hyphenation of LC with NMR is gaining acceptance, and an increasing number of applications have been published. In a typical online coupling, the LC instrument is located at a distance of 1.5–2.0 m beside the NMR cryomagnet. With the high-dynamic-range receivers now available, the intense solvent signals can easily be handled. The solvent signal can be reduced by a factor of 10^4. Nuclear magnetic resonance flow cell detection volumes are 60–100 μl, which are rather large and detrimental to the chromatographic separation. Stopped-flow NMR is usually performed by triggering the recording via the signal of a UV detector. A careful determination of dead volumes is required. Since the trend is toward capillary separations, some hyphenations with capillaries are under development, with the advantage of possible use of fully deuterated solvent. One possibility is to fix the NMR detection coil directly to the horizontal capillary, resulting in a so-called solenoidal design. The other approach is to minimize the conventionnally used double-saddle Helmholtz coils and to insert the capillary in a vertical direction. Albert (67) inserted a

fused silica capillary within a specially designed microprobe. The exchange of capillaries is performed without disrupting the NMR detection coil. Detection volume is 200 nl.

7. LC-ICP-MS

Basically, an inductively coupled plasma (ICP) is an electrodeless discharge created in a continuous stream of argon. An ICP operates at atmospheric pressure and is sustained by energy generated in a radio frequency (RF) generator. Radio frequency energy of typically 1.25 kW at 27–12 MHz is coupled to the plasma by means of a cooled three-turn coil. Sample introduction is usually performed by pneumatic nebulization: A stream of argon expanding with high velocity from an outlet converts a stream of liquid into an aerosol of droplets with a wide spread in diameter. Only 1–2% of the droplets are fit to enter the ICP. The sample material introduced is exposed to hostile conditions of the ICP (5000–9000° K) and is successively desolvated, vaporized, and dissociated, after which the atoms are ionized (Fig. 25). The dwell time in ICP is a few milliseconds. Nebulizer designs have made great progress in handling microflows and macroflows (68).

XIV. COLUMN SWITCHING

The column-switching procedure is similar to heart cutting in GC. It is easier to handle, since there is no problem of leaking and a simple tee is able to direct the fluid containing the solutes of interest toward another LC column. This column must operate in another chromatographic mode. Switching from a water/organic eluent toward an apolar diluent may be tedious, and very small volumes should be handled. It must fulfill the following requirements: (a) the chromatographical results of the first column must be judgable qualitatively and quantitatively; (b) the switching times must be determinable directly; (c) high switching speeds must be achieved.

XV. SAMPLE PREPARATION

A sample preparation is often necessary to isolate the components of interest from a sample matrix. Removal of interfering compounds prevents blocking of the HPLC column in spite of the

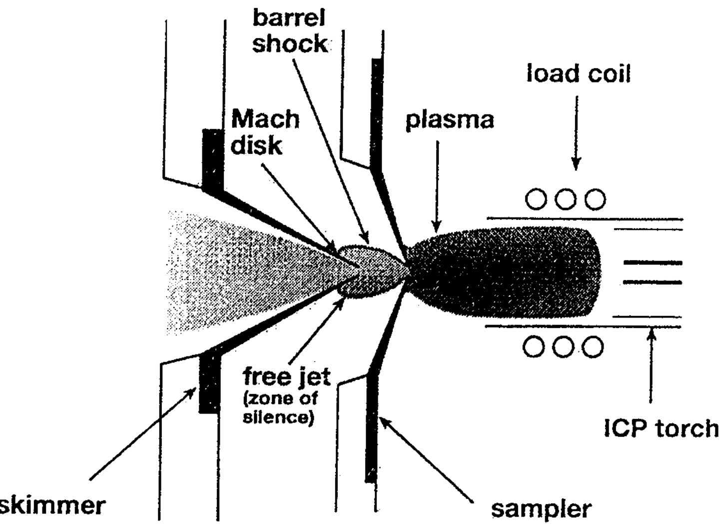


Fig. 25 Schematic of an inductively coupled plasma–MS interface during ion sampling. (Reproduced with permission from Elsevier.)

presence of a guard column. Moreover, in many cases trace amounts of solute are to be detected and an enrichment procedure is required. Sample preparation is often the major source of error in analytical procedures, for practitioners are often required by law to use traditional (obsolete) methods. Ideal sample pretreatment should be online and solvent free. This goal is difficult to achieve in practice, but some devices are under development. Liquid–liquid extraction (LLE) is declining. Solid-phase extraction (SPE) on cartridges or discs is gaining wide acceptance. It is becoming more and more miniaturized (solid-phase microextraction (SPME).

A. Liquid–Liquid Extraction (LLE)

This sample pretreatment is devoted mainly to organic compounds that can be removed from an aqueous solution by extracting them into a water-immiscible solvent. Chelation or ion pairing between large and poorly hydrated ions and chelating agent may form neutral compounds that can be extracted by organic solvents.

When two immiscible solvents are placed in contact, any substance soluble in both of them will distribute or partition between the two phases in a definite proportion. According to the Nernst partition isotherm, the following relationship holds for a solute partitioning between two phases a and b:

$$\frac{(A)_a}{(A)_b} = K_d$$

where K_d is the partition coefficient.

$$(A)_a \leftrightarrow (A)_b \qquad \text{very often} \qquad (A)_{\text{aq}} \leftrightarrow (A)_{\text{org}}$$

This assumes that no significant solute–solute interactions or strong specific solute–solvent interaction occurs. The K_d value is constant when the distributing substance does not chemically react in either phase and temperature is kept constant.

The fraction R extracted is related to K_d by

$$\frac{C_o V_o}{C_o V_o + C_w V_w} = \frac{K_d V}{1 + K_d V}$$

where C_o and C_w represent the solute concentration in organic (o) and water (w) phases, respectively, and V_o and V_w are the volumes of organic and aqueous phases with $V = V_o/V_w$.

It is possible to increase the extent of extraction with a given K_d by increasing the phase/volume ratio. When performing micro-LLE, the analyst works with an extreme ratio of extracting solvent/extracted liquid (for example, 1/800). Another way is to carry out a second and a third extraction. After n extractions the final concentration of the compound in the aqueous phase is

$$C_{w_n} = C_w \left[\frac{V_w}{V_w + K_d V_o} \right]^n$$

An extraction process is more efficient if it is performed with several small portions of solvents.

Solubility increases as the values of the solubility parameter δ of the solute and the solvent are close:

$$\ln K_d = \frac{\bar{V}_s}{RT}[(\delta_s - \delta_i)^2 - (\delta_j - \delta_s)^2]$$

where $\bar{V}_s$ is the molar volume of the distributing solute, δ_s its solubility parameter, and δ_i and δ_j are the solubility parameters of the pair of immiscible solvents.

It must be kept in mind that some species may exist under different forms in aqueous media. An acid, for example, must be written in this form:

$$HA \leftrightarrow A^- + H^+$$

$$K_d = \frac{(HA)_o}{(HA)_w} = \frac{(HA)_o}{(HA)_{aq} + (A^-)_{aq}}$$

Selective extractions can be carried out by careful selection of pH. When extracting organic species it must be remembered that compounds may exist as different species, depending on the pH. P_{ow}, the partition coefficient of a solute between octanol and water, is a common measure of hydrophobicity. High values of log P_{ow} are favorable for extraction. Reversed-phase HPLC has also been extensively used.

B. Solid-Phase Extraction

Solid-phase extraction (SPE) is the most widely used method. Analytes (mainly organics) are trapped by a suitable sorbent by passing through a plastic cartridge containing an appropriate support. A selective organic solvent is used to wash out the target analytes. Solid-phase extraction is rapid and relies upon chromatographic retention and log P_{ow}. It can be easily automated. Offline procedures are inexpensive. Online devices are readily available from many companies.

1. *Offline*

A typical SPE cartridge is displayed in Fig. 26. Sorbents are very similar to those for liquid chromatography stationary phase. The analyst can take advantage of the following

Nonpolar interactions (hydrophobic): typical octadecyl-modified silica, polystyrene-divinyl benzene copolymers, or carbon-based sorbent.

Polar interactions through hydrogen bond, for example: In this mode sorbents are bare silica, polar-bonded silica, or polyamide.

Ion exchange: benzene sulphonic acid (cation exchange) or quaternary amine (anion exchange).

Immunosorbents: The lack of selective sorbents to trap organic analytes in water is certainly the most important weakness of the SPE technique. Selective interactions are involved with immunoaffinity sorbents.

An alternative to SPE columns is to use disks. Disks do not exhibit bed channeling. Samples can be applied to the disks using either a syringe or a vacuum manifold. Flow rates can be made faster by pushing samples through the discs with increasing pressure on the syringe. Conditioning is carried out with methanol and water.

The SPE technique is as follows: conditioning, sample application, washing, and elution.

Capacity is the quantity of sample molecules retained per unit quantity of adsorbent. Capacity depends on solute size. It lies in the range of 4–60 mg/g of packing.

Breakthrough of solutes occurs when they are no longer retained by the sorbent. Overloading beyond the sorbent capacity may also lead to breakthrough of analytes. The breakthrough volume can be measured from the breakthrough curve obtained by monitoring the signal of the effluent from the extraction column (Fig. 27). V_b is usually defined at 1% of the initial absorbance and corresponds to the sample volume that can be handled without breakthrough. V_r is the retention volume of the analyte. V_m is defined at 99% of the initial absorbance. Prediction of the break-

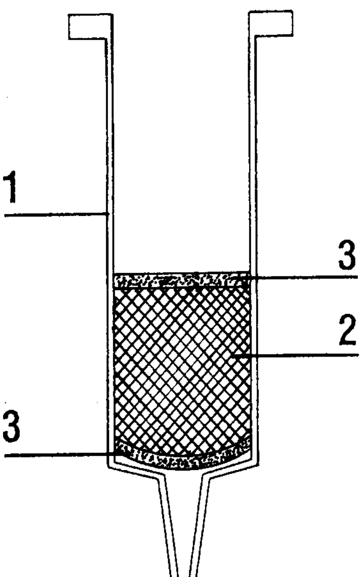


Fig. 26 Schematic of a Lichrolut extraction cartridge. 1: polypropylene housing; 2: packing material bed; 3: 20-μm frits. (Courtesy of Merck.)

through volume is important for selecting a convenient sorbent and, consequently, the amount of sorbent.

a. Hydrophobic Sorbents

n-Alkyl silicas are by far the most utilized hydrophobic sorbents. A large number of applications on such sorbents has been published. The drawback (as with every bonded silica) is the poor stability in very acidic or basic media.

From liquid chromatography we know that in reversed-phase mode

$$\ln k = \ln k_w - S\phi \qquad \text{or} \qquad \ln k = \ln k_w - b\phi - a\phi^2$$

Here, $\ln k_w$ can be estimated by a graphical extrapolation to zero modifier content. Values of $\ln k_w$ may be as high as 3–4, which means that large sample volumes with trace amounts of solutes can be handled.

Styrene divinyl benzene copolymers (PS-DVB), either porous or rigid, are stable over the whole pH range. Calculated $\ln k_w$ values on these sorbents are higher than those on C_{18}. Consequently, moderately polar compounds that are not retained by C_{18} silica are more readily concentrated on these sorbents.

Selection of eluting solvent is based on knowledge of the eluting strength $\epsilon°$ of the solvent in the chromatographic mode.

b. Procedures with Polar Adsorbents

These procedures rely on chromatographic knowledge of solutes. This is well documented, and huge amounts of data can be retrieved from thin-layer chromatography experiments.

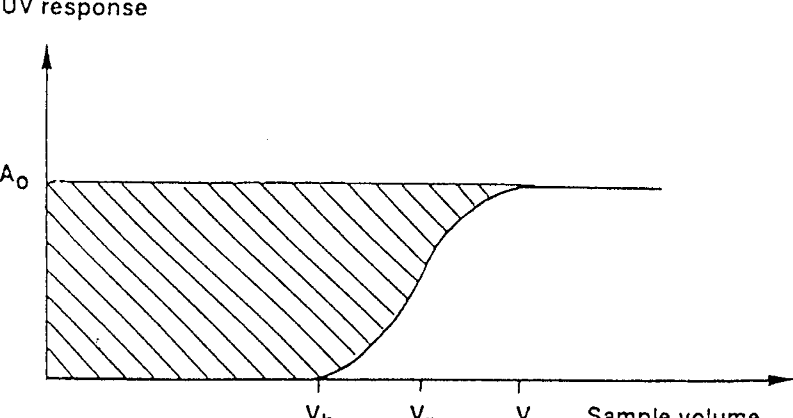


Fig. 27 A breakthrough curve obtained by percolation of a spiked sample (UV absorbance A_0) through a precolumn. [From M-C. Hennion and V. Coquart, J. Chromatogr. 642:211 (1993), with permission from Elsevier.]

c. Adjusting pH Values

Via pH adjusting, many solutes can be ionized (e.g., carboxylic acids) and trapped on ion exchangers. Owing to pH stability, resins are widely used. The drawback comes from high amounts of inorganic ions (e.g., seawater), which easily overload the capacity of the sorbent.

d. Immunosorbent

It is now possible to produce immobilized antibodies that are able selectively to extract a group of structurally related analytes from the matrix. Some target compounds, including some small molecules such as pesticides, can thus be extracted from carrots, potatoes, or strawberries.

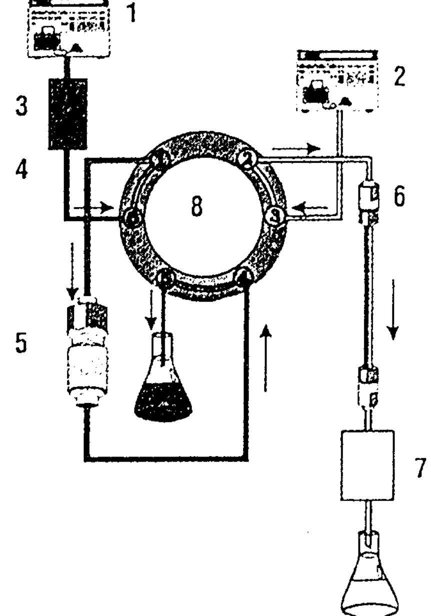
Solid-phase microextraction uses a 1-cm length of focused silica fiber, coated on the outer surface with a stationary phase and bonded to a stainless steel plunger holder that looks like a modified microliter syringe. The fused-silica fiber can be drawn into a hollow needle by using the plunger. In the first process, the coated fiber is exposed to the sample and the target analytes are extracted from the sample matrix into the coating. The fiber is then transferred to an instrument for desorption. The technique has been promoted by Pawliszyn (69).

2. *Online*

Online coupling SPE to either LC or GC is easily performed. In the simplest method, a precolumn is placed in the sample loop position of a six-port switching valve. After conditioning, sample application, and cleaning via a low-cost pump, the precolumn is coupled to an analytical column by switching the valve into the inject position. The solutes of interest are eluted directly from the precolumn to the analytical column by an appropriate mobile phase. The sequence can be fully automated (Fig. 28). It is also a simple matter to enhance the gap between two solutes in elution from a precolumn (70).

3. *Cleanup*

Extracts obtained from either LLE or SPE contain analytes and other compounds that may interfere in the chromatographic separation. A cleanup is required. The most widely used is fractionation



The sample is injected directly onto the precolumn. In an ideal situation the precolumn packing only extracts and enriches the analyte(s), while all other sample components (unwanted matrix) are discharged to waste with the eluent delivered by Pump 1.

1 ▶ Pump 1
2 ▶ Pump 2
3 ▶ Sample injector (Autosampler)
4 ▶ On-line filter
5 ▶ Precolumn LiChroCART® 25-4 LiChrospher® RP ADS
5 ▶ Analytical HPLC column
6 ▶ Detector
7 ▶ Six-port-valve

Fig. 28 An LC integrated sample preparation. (Courtesy of Merck.)

by LC. The extract is loaded onto a chromatographic column packed with an appropriate sorbent (silica, alumina, florisil, bonded silica), and step elution with solvents is carried out. Each fraction is collected and submitted to chromatography. Derivatization prior to fractionation is sometimes performed. Coupling two sorbents in an SPE procedure—for example, hydrophobic sorbents and ion exchange—in series is efficient.

XVI. OPTIMIZATION STRATEGIES

Efforts have been made to simplify procedures. Since RPLC is most often advocated, it is not surprising that optimizations are focused on this type of chromatographic mode. However, a computer-assisted technique for the retention prediction of inorganic cyclic polyphosphates under gradient elution has been proposed by Baba and Kura (71). In the 85-90s optimization strategies made use of complex mixtures of three to four solvents. Overlapping resolution mapping (ORM) has been successfully applied. This technique uses a single valued response function of the usual resolution functions evaluated for all adjacent peaks from an experimental run. A series of experimental runs is performed for different mobile-phase compositions, and the maximum value is found by overlapping the contours of resolution function values for all adjacent peaks. Smet et al. (72) used information from the theory to optimize the separation of drugs on CN-bonded packing. Initially a gradient elution with two separate solvents is performed, from which a starting isocratic mobile-phase composition is selected. An expert system (LABEL) was derived from this approach. The most powerful and widespread approach is Drylab (I for isocratic and G for gradient) software (73). As the first step in the method development process, two linear gradients of different steepnesses are run, varying the percentage of component B of the mobile phase in the range 0–100%. The information from these two runs is entered in the computer, and different gradient conditions can be explored via computer simulation. The best conditions are selected (for example, with a relative resolution map). Drylab has been improved many times (74) and can be extended to ion chromatography. Other optimization software exists. A comparison between Drylab, Eluex, and Prisma has been performed by Outinen (75). ChromDream from Galushko (76) predicts starting conditions for RPLC from chemical formulas without initial experiments. Structures are translated into fragments; volume and energy parameters are entered, and the simulation of chromatographic behavior is performed. In normal-phase gradient, elution optimization has been thoroughly studied by Jandera (77). Most of the software available is proposed to purchasers of an instrument by manufacturers.

XVII. VALIDATION

Any analytical method should be validated. The U.S. Food and Drug Administration has proposed guidelines on submitting samples and analytical data for validation (78). In LC one should check at least: specificity, selectivity, accuracy, precision, linearity, reproducibility, limit of detection, limit of quantitation, and robustness.

Problems encountered in HPLC analysis most often stem from a lack of knowledge of the influence of the slight variation of the experimental parameters (pH, temperature, solvent composition, flow rate, etc.). The analyst has to set up the list of parameters and their possible interactions. There are hardware parameters (e.g., flow control, temperature control, lamp current) and software parameters used to interpret and report the results from stored data. The use of factorial designs is of great help. Software such as Validation Manager, from Merck, produces, in a table for each parameter and interaction, its percentage and confidence interval as well as information to help the analyst in concluding the study.

For the less experienced user who is unfamiliar with the method, selection of initial parameters can be daunting. Expert systems have been developed that guide the user and utilize results from successful or failed experiments to move the analyst toward a satisfactory separation.

XVIII. CONCLUSION

Liquid chromatography is now a mature technique. Instruments are reliable and increasingly computer assisted. Column-to-column reproducibility is ensured by most manufacturers. The quest for the "universal" detector is about to end with the advent of a sophisticated and miniaturized MS detector. Development of a method can be achieved in a rather short period with available software. The emphasis is on validation more than on "how to handle it." Capillary columns are sure to improve, and the trend will be toward many parallel analyses.

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2
Amino Acids

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I. INTRODUCTION

A. Amino Acid Properties

The overwhelming majority of foods contain amino acids, either in the free form (e.g., fruit juice) or in the form of protein (partially hydrolyzed or intact). Proteins are polymers, and their monomeric units are amino acids. Predominantly, proteins are comprised of 20 amino acids (see Fig. 1). In addition, some structural proteins contain large amounts of hydroxyproline (e.g., collagen). Far less abundant is the amino acid hydroxylysine. The principal sources of dietary amino acids for humans are proteins, which are enzymatically digested to liberate their constituent amino acids.

Amino acids share common aspects of their chemical structure. Almost all amino acids are α-amino carboxylic acid (IUPAC: 2-aminoethanoic acid) derivatives in which there is an additional substitution on the α-carbon (see Fig. 2). Proline, an exception to the rule, is an α-imino carboxylic acid. Thus, proline possesses a secondary rather than a primary amine group. The R group (also referred to as the "side chain") can take the form of many different functional groups. These include alkyl chains (linear or branched), alcohols, thiols, amines, amides, carboxylic acids, aromatic hydrocarbons (benzene, phenol), and heterocyclic moieties. Again, proline is unusual in that its side chain is cyclized back to include the α-imino group as part of a heterocyclic ring. In nature, the α-carbon is almost always substituted to give the stereochemical L-isomer. Glycine, which is simply unsubstituted 2-aminoethanoic acid, is achiral. Some D-isomers do occur in nature and usually have very specialized functions. These include various D-amino acids in antibiotics and also D-alanine and D-glutamic acid in some bacterial cell walls (presumably to make them resistant to enzymic attack).

The similarities and differences just mentioned are both a blessing and a curse to the analytical chemist. Very few of the amino acids have a chromophoric moiety that allows for facile detection after separation. Fortunately, the presence of common functional groups (carboxylic acid and amine) allows for derivatization schemes that render the amino acids amenable for spectrophotometric detection after separation.

The vast differences in the functional groups possessed by the side chains allows for their chromatographic separation. However, closely related compounds can still be tough to resolve. For example, leucine and isoleucine tend to afford very tight resolution. The chemical differences in the side chains can be very problematic when it comes to sample preparation. This is especially true for the acid hydrolysis of proteins to liberate their constituent amino acids in the free form. The different functional groups exhibit different chemical stabilities in an acid environment.

Glycine, Gly, G

Lysine, Lys, K

Alanine, Ala, A

Arginine, Arg, R

Valine, Val, V

Histidine, His, H

Isoleucine, Ile, I

Aspartic Acid, Asp, D

Glutamic Acid, Glu, E

Leucine, Leu, L

Asparagine, Asn, N

Methionine, Met, M

Glutamine, Gln, Q

Cysteine, Cys, C

Serine, Ser, S

Phenylalanine, Phe, F

Threonine, Thr, T

Tryptophan, Trp, W

Proline, Pro, P

Tyrosine, Tyr, Y

Fig. 1 Amino acids.

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       NH2                        NH3+
       |                          |
  R----C----COOH             R----C----COO-
       |                          |
       H                          H

Generic Structure            Zwitterion
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Fig. 2 Chemical structure of amino acids.

Finally, chemists are generally aware that amino acids are zwitterions at most pH values (except at the extremes). Consequently, many chemists mistakenly assume that the doubly charged (1 cationic-amine, 1 anionic-carboxylic acid) molecules all exhibit high solubilities in water. However, the solubilities of amino acids in water are fairly limited and widely variable. Solubilities in water (25°C) span a range from the least soluble (tyrosine) at 0.453 grams per 100 grams of water to a high (proline and lysine) of >1,000.0 grams per 100 grams of water. The median solubility for the 20 common amino acids is 47.8 grams per 100 grams of water. Additionally, the rates of dissolution for the various amino acids can be vastly disparate. Tyrosine and tryptophan exhibit notoriously slow dissolution kinetics, whereas lysine rapidly disappears into solution. It is very important for the analyst to keep this in mind when developing methods that involve extractions as part of the sample preparation.

B. Biological Importance of Amino Acids

In 1906, Willcock and Hopkins demonstrated that tryptophan was a dietary essential amino acid. This meant that if tryptophan was not supplied in the diet, animals were unable to grow or maintain a positive nitrogen balance. This discovery spurred investigation for other essential amino acids. In 1914, Osborne and Mendel had shown that lysine was essential. By the 1940s, the entire set of what is now considered the "essential amino acids" had been elucidated. In one of the earlier reviews on this topic, Rose (1) outlined the core of eight essential amino acids: tryptophan, phenylalanine, lysine, threonine, methionine, leucine, isoleucine, and valine. These eight essential amino acids are ones that cannot be synthesized in the body and must be provided dietetically for humans to maintain nitrogen balance. The nonessential amino acids could be synthesized if an adequate quantity of fixed nitrogen was available (i.e., the human body could utilize the skeletons of other amino acids for modification and subsequent incorporation as needed). Rose also noted the "sparing effect" of cysteine and tyrosine. It turns out that these two amino acids can be synthesized in the body in adequate amounts, but only from essential amino acid precursors. Cysteine can be created only from methionine, and tyrosine can be created only by modification of phenylalanine. Thus, cysteine and tyrosine are indirectly members of the essential amino acid family. It is now common to report the total requirement of cysteine and methionine in combination. This is also true for the tyrosine/phenylalanine combination. Finally, there is another member of the essential amino acid family, histidine. This amino acid apparently can be synthesized in the body, but not at a rate sufficient for anabolic needs.

It should be noted that there is a growing list of "conditionally essential" amino acids. These are amino acids that must be augmented through the diet under a variety of special circumstances. Often these circumstances include infancy (where the various enzyme systems for metabolism have not been fully expressed) or periods of metabolic stress due to injury or illness. Conditionally essential amino acids include taurine (infants) and glutamine (metabolic stress).

C. Why Analyze for Amino Acids?

For many populations, the exact amino acid composition of a particular food is not particularly important. The daily intake of total protein is very high, and the protein sources are varied enough that limiting amino acids do not come into play. However, for a variety of specific situations, determination of amino acid content is important. A list of these situations was first articulated by Baxter (2) and will be partially recapitulated here.

1. Sole Source for Nutrition

When a particular food product is the sole source for nutrition, it is imperative that the amino acid profile be known. This information must be used to determine that the limiting (relative to metabolic need) amino acid is being supplied in sufficient quantity. A prime example of this category is infant formula.

2. Fortified Nutritional Product

Specialized nutraceutical-type products often are targeted at persons with unusual metabolic needs. To meet these elevated requirements, products are fortified with specific, free amino acid(s). These products must be examined to ensure that the amino acid in question has been properly supplemented. An example of this category is nutritional products supplemented with glutamine to promote healing after gut trauma.

3. Absence Verification

People can suffer from a variety of inborn metabolic errors that can result in various amino acids exhibiting toxic effects if ingested above certain threshold levels. Specialty products, intended to be absent these amino acids, must be rigorously tested for verification that threshold levels are not exceeded. Examples of this situation include phenylketoneuria (intolerance of phenylalanine) and maple syrup urine disease (intolerance for leucine, isoleucine, and valine).

4. Acceptance Testing for Commodities

This is self-explanatory. Commodities intended for eventual use in a product should be tested on an incoming basis. This discovers any problems before commodities have been incorporated into a product and resulted in unnecessary loss of money.

5. Regulatory Concerns

Legal requirements vary from country to country and are beyond the scope of this discussion. In general, if a product falls into the first three categories just listed, one can expect legal requirements for amino acid determination. If label claims (also product literature) are made regarding the amino acid content of any food product, it will be necessary to document adequately that the product meets those claims. Additionally, during regulatory audits, companies are expected to have documentation showing that the analytical methods employed are valid.

D. HPLC vs. Other Analytical Techniques

By far, the predominant methods for determination of amino acids in foods are based on HPLC. However, alternative methods for amino acid analysis do exist. Many of the earliest determinations for certain amino acids were based on microbiological tests (and other bioassays), but these are no longer widely employed. Cost and analysis time are obvious factors in the demise of these type of methods. Also, these types of methods are very prone to biased results and high variance.

The newest alternative to HPLC is capillary zone electrophoresis (CZE). Perhaps the greatest advantage to CZE is the ultralow detection limits and the extremely small samples necessary

to afford quantitative results. However, when analyzing foods for amino acids, sample size is almost never an issue. Thus, there has been little incentive for the food industry to learn a new (and relatively exotic) technology. Also, it is much less expensive to buy new types of columns and/or derivatization chemistries targeted at new applications than it is to buy entire CZE instruments. The future for CZE in the food industry clearly lies in the determination of trace levels of bioactive peptides.

Gas chromatography (GC) for amino acid analysis is the alternative to HPLC that has found the greatest acceptance. It requires the preseparation derivatization of the amino acids to render them volatile. For this purpose, amino acids are frequently converted into acylated esters. *N*-Trifluoroacetyl-*n*-butyl esters and *N*-heptafluorobutyrylisobutyl esters are most commonly employed. There have been comparative studies (3,4) that document similar (if not equivalent) analytical results for GC and the classic ion-exchange chromatographic method applied to a variety of food samples. Comparison (5) of GC to the reversed-phase HPLC determination of amino acids (phenylisothiocyanate derivatized) also shows excellent agreement.

Although GC has been demonstrated to yield accurate results, it is still relatively little used compared to HPLC. This is largely for practical reasons relating to the derivatization procedures necessary for GC. Derivatization kinetics differ from one amino acid to another. Thus, individual amino acid recoveries are highly dependent on the exact reaction time and conditions. Highly experienced analysts are necessary for good precision.

II. SAMPLE PREPARATION (HPLC)

The amino acid profile method can be viewed as a two-stage process. The first stage involves the sample preparation. The second stage involves chromatographic analysis. The greatest challenge to the accurate and precise determination of amino acid content of foods is presented by the initial stage, the sample preparation. A plethora of chromatographic techniques have been developed that more than adequately address all the needs of the typical analysis. These chromatographic techniques will be discussed in a later section of this chapter. Sample preparation, however, is still very problematic.

There are two major categories for amino acid analysis: (a) free amino acid analysis and (b) determination of total amino acid content. The total amino acid content includes contributions from the free amino acids and the amino acids that are originally protein bound. These protein-bound amino acids must first be liberated before chromatographic analysis. This necessitates a more extensive, and problematic, sample preparation. Because the sample preparation procedures are so disparate, it is convenient to address these two categories of amino acid analyses separately. It should be noted that while the sample preparations for these analyses are quite different, both utilize essentially the same chromatographic techniques for the second stage of amino acid analysis.

A. Free Amino Acids

It has long been commonplace to determine the concentration of free amino acids in the beverage industries (e.g., wine, beer, fruit juice). In recent years, it has become more common to analyze for free amino acid content in other food and nutritional products. This is due, in part, to the growing practice of supplementing nutritional products with added free amino acids. Since these free amino acids are often being added in response to specific/special nutritional requirements of a target population, it is very important to ensure the fortification levels in these products.

The sample preparation for analysis of free amino acids generally involves two steps. The first step is to ensure complete dissolution of the target amino acids. For beverages, this might al-

ready be the situation. For solids, a little more effort is required, which usually includes homogenization of the sample and then extraction of the amino acids. The second step in the sample preparation for the analysis of free amino acids is a cleanup step designed to remove matrix components that might interfere with the subsequent analysis.

1. Extraction

For solid materials, it is first necessary to homogenize the sample. This can be accomplished only after the physical structure of the sample is destroyed. This can be as simple as grinding seeds in a mortar and pestle or using a food blender to create a puree from a sports nutritional bar. Additionally, one may wish to sonicate the sample to rupture cells and release their content. A review that also discusses more stringent methods for homogenization (e.g., hammer milling, CO_2 milling) is presented by Lichon (6).

Having accomplished the homogenization of the sample, extraction of free amino acids is usually a simple process of stirring the sample in an appropriate solvent. This is typically a dilute solution (0.1N) of hydrochloric acid (2,7). Elevated temperatures may be used to assist dissolution, but care must be taken not to damage the more acid-labile amino acids. More recently, Moret and Conte (8) report that 0.1N HCl was not a suitable solvent for all types of food samples. For some types of meat samples, 5% trichloroacetic acid is reported to afford superior performance (for biogenic amines, also amino acids?). It is not unusual to see perchloric acid employed for extraction from meat tissues (also more commonly for biogenic amines).

There are not a lot of detailed published procedures for the homogenization and extraction of solid foods. This is not surprising, since there is such a wide array of distinct sample matrices. No single procedure can be expected to work for all sample types. Development of homogenization/extraction procedures must necessarily be on a case-by-case basis. It is important for the investigator to verify the efficiency of the procedure with respect to the overall recoveries afforded and the kinetics of the extraction.

2. Cleanup

Food sample matrices contain a variety of components capable of interfering with the analysis of free amino acids. Not surprisingly, proteins/peptides are a frequent source of problems. Proteins will progressively degrade chromatographic performance of a column by loss of resolution and by clogging the column, resulting in excessive back pressure. Also, deposition of protein on the column frits or at the top of the column packing will eventually lead to split/double peaking.

Deproteinization of samples can be accomplished by precipitation with picric acid, sulfosalicylic acid, perchloric acid, tungstic acid, ammonium sulfate, or many organic solvents (acetonitrile is most efficient). An excellent study comparing the relative efficiency of various precipitating agents (for plasma proteins) is reported by Blanchard (9). Interestingly enough, Blanchard does not include the most popular precipitating agent, sulfosalicylic acid (SSA) (10), in his study.

Sulfosalicylic acid has most commonly been used to precipitate proteins prior to ion-exchange amino acid analysis (11). In this mode, SSA allows for a very simple sample preparation that requires only centrifugation of the precipitated sample and then direct injection of the resulting supernatant solution. The supernatant solution is already at an appropriate pH for direct injection. Also, the SSA does not interfere chromatographically since it elutes essentially in the void volume of the column. It has been noted that, if an excessive amount of SSA is employed, resolution of the serine/threonine critical pair can suffer (12). The use of SSA prior to reversed-phase HPLC can be more problematic, since its presence can interfere with precolumn derivatization. For example, Cohen and Strydom (13) recommend the separation of the amino acids from the SSA solution on a cation-exchange resin prior to derivatization with phenylisothiocyanate (PITC).

The increasing popularity of reversed-phase HPLC with precolumn derivatization of amino acids has resulted in more studies in which organic solvents are being used as precipitating agents (14,15). This results in supernatant solutions that are more friendly, although much more diluted, to precolumn derivatization with chromophoric agents. This same trend is causing the increased use of ultrafiltration as a physical means for removing protein from solutions. However, there can be serious problems with ultrafiltration. On the practical side, the pores of ultrafiltration membranes have a tendency to clog for samples with high levels of fats and/or protein. Additionally, losses of certain amino acids due to physiochemical adsorption to the membrane is not uncommon. This is especially problematic for the basic amino acids.

In a more recent comparative study, Aristoy and Toldra (16) compare the efficacy of a variety of chemical precipitating agents (including SSA) and also include the physical technique of ultrafiltration. Some interesting results from this study are shown in Table 1, which lists the recoveries from a standard solution of amino acids after various deproteinization procedures have been applied. The precipitating agents investigated included perchloric acid (PCA), phosphotungstic acid (PTA), picric acid (PA), trichloroacetic acid (TCA), acetonitrile (ACN), and sulfosalicylic acid. There are two trends that warrant special attention. First, SSA is shown to result in very poor recoveries for aspartic acid and glutamic acid. The second trend is the severe losses of the basic amino acids (lysine, histidine, arginine, and ornithine), which can occur during ultrafiltration. Recoveries of the basic amino acids by ultrafiltration can be highly variable and depend on many factors, including the choice of membrane and the chemical pretreatment prior to ultrafiltration (17).

A note of caution is that the precipitating agents just mentioned all result in fairly complete precipitation for large (intact) proteins only. Remember that these deproteinization studies usually involve plasma proteins. Increasingly, many nutritional products are employing partially hy-

Table 1 Percent Recovery of Standard Amino Acids after Deproteinization Pretreatment

	Amino acid	Chemical precipitants						Ultrafiltration	
		PCA	TCA	PTA	SSA	PA	ACN	10 kDA	1 kDa
1	Asp	99.5	97.0	52.7	70.0	98.0	97.7	97.0	97.1
2	Glu	99.1	99.0	77.6	73.5	98.3	99.2	97.7	98.8
3	Asn	101.0	102.3	71.4	100.9	101.8	97.3	99.3	95.3
4	Ser	98.5	94.5	92.4	95.0	99.3	100.6	101.2	98.7
5	Gln	98.6	96.0	81.4	92.3	101.2	97.2	98.2	94.5
6	Gly	103.6	104.3	96.5	113.1	103.2	100.4	101.2	105.7
7	His	96.7	92.2	93.1	89.7	99.7	100.0	100.0	55.2
8	Tau						101.3	99.1	101.6
9	Arg	90.8	92.1	35.4	83.3	100.3	99.0	103.5	69.5
10	Thr	97.1	96.4	95.5	97.9	100.2	98.5	98.3	94.6
11	Ala	103.8	104.5	100.5	96.4	102.2	100.7	100.9	99.5
12	Pro	104.5	108.2	104.2	150.0	104.2	100.0	99.3	102.1
13	Tyr	92.8	98.2	102.8	96.2	101.5	101.6	102.9	104.7
14	Val	97.5	98.5	98.5	89.2	100.0	98.1	98.8	98.8
15	Met	103.6	106.2	91.5	106.7	105.3	101.2	99.5	100.4
16	Ile	98.5	97.9	95.7	88.7	99.8	98.7	101.7	98.7
17	Leu	98.6	97.3	101.8	84.5	96.1	100.0	103.9	98.3
18	Phe	99.3	99.4	97.6	92.9	106.0	99.4	100.0	101.4
19	Trp	72.9	92.8	83.7	97.5	100.8		98.5	103.5
20	Orn	95.5	94.1	38.7	96.2		97.5	101.2	34.3
21	Lys	99.9	96.6	10.4	88.0	102.1	98.6	99.2	51.6

drolyzed protein commodities. As a protein commodity becomes progressively more hydrolyzed, a larger percentage of its peptides will survive attempts to deproteinize by chemical precipitation.

Finally, sample cleanup can be achieved by pseudo-chromatographic methods employing small, disposable cartridges filled with various resins. This has the added advantage of allowing for simultaneous sample concentration. One of two general schemes tends to be employed. The first involves the use of a cation-exchange resin [e.g., Amberlite IR120 (18), Rexyn 101 (19), Biorex 70 (20), Dowex 50W (21)], in which the amino acids are initially retained on the column under acidic conditions. After interfering matrix components have been washed through the column, elution of the amino acids is achieved by employing a strong, volatile base that can easily be roto-evaporated away.

The opposite scheme is to employ a C_{18} resin (e.g., C_{18} Sep-Pak from Waters Associates) in which the eluent will contain the amino acids while lipids and large proteins will be retained on the column. This procedure has been called into question, however (22). It has been suggested that partial retention of the hydrophobic amino acids can occur. This is especially a problem if norleucine (or any other hydrophobic amino acid, e.g., norvaline) is employed as an internal standard. Its partial retention on the C_{18} column will skew the apparent recoveries for all the other amino acids. This points to a particular problem for amino acid analysis: There is no ideal choice for an internal standard. A subsequent section of this chapter will address the issue of internal standards in more detail.

Another short note: For samples that are relatively clean, one could always resort to employing (and frequently replacing) a guard column. This might be a bit more costly, but it greatly simplifies any sample preparation procedure and will probably lead to better method precision. One should always keep in mind that the largest contributor to poor method precision is usually manual sample preparations.

B. Total Amino Acid Profile

The nutritional values of foodstuffs are better correlated with the overall amino acid profile (compared to free amino acid composition). Not surprisingly, then, total amino-acid profile is much more commonplace in the food industry than is the analysis for free amino acids. In order to determine the amino acid composition of a foodstuff, proteins must first be hydrolyzed into their constituent free amino acids.

1. *Acid Hydrolysis*

a. Conventional Oven

Liberation of the protein-bound amino acids is most commonly achieved by acid digestion. Typically, the sample is combined with 6M hydrochloric acid into a glass ampule. The glass ampule is nitrogen purged and then flame sealed. This is then placed in a 110°C oven for approximately 22 hours. While the overwhelming majority of acid digestions are carried out in this fashion (or something very similar), one should not infer that this procedure is ideally suited for the accurate determination of all the amino acids of interest. Rather, it is a compromise of conditions that offers the best overall estimation for the largest number of amino acids. No single set of conditions will yield the accurate determination of all the amino acids.

The problems encountered are numerous. Tryptophan is highly prone to degradation in acid digestions. This is especially the case in food analysis, where samples often contain significant quantities of carbohydrates that greatly exacerbate tryptophan's degradative tendencies. Cyst(e)ine is partially oxidized during acid hydrolysis and will likely be found in several forms: cystine, cysteine, cysteine sulfinic acid, and cysteic acid. Methionine can be partially lost in simi-

lar fashion. When conditions are more harsh, significant losses of threonine and serine are observed (presumably by dehydration). Finally, glutamine and asparagine are deamidated, resulting in glutamic acid and aspartic acid, respectively.

The final complicating factor is the disparity among the various peptide bonds in their stability toward acid hydrolysis. While most of the peptide bonds are very labile toward acid hydrolysis, those peptide bonds formed by the hydrophobic amino acids can be resistant to acid hydrolysis. This is especially true for the peptide bonds formed by combinations of valine, leucine, and isoleucine.

Here is the essential problem faced when preparing a sample for total amino acid hydrolysis: The chemical instability of the more labile amino acids results in their partial degradation under conditions sufficiently harsh to ensure the complete hydrolysis of the more stable peptide bonds. An early academic approach to this problem was to analyze samples at multiple hydrolysis times. Then the analyst could extrapolate back to zero time for the labile amino acids, or extrapolate to infinite time to quantify accurately the amino acids involved in hydrolysis-resistant peptide bonds. It has been common for three different hydrolysis times (24, 48, and 72 hours) to be employed. Hirs et al. (23) indicated that two hydrolysis times, 20 and 70 hours, are sufficient for accurate extrapolation. Gehrke et al. (24) confirm this by comparing the use of four hydrolysis times versus two and find that extrapolation to zero time gives equivalent results for either procedure. To save some time, it is possible to use multiple hydrolysis times on a particular sample and use this data to derive correction factors that can subsequently be used for that sample when doing routine 24-hour acid hydrolyses (24,25). If a very high degree of accuracy is needed to find the amino acid content of a pure protein, 10 hydrolysis intervals may be employed along with nonlinear regression analysis of the resulting data (26). Naturally, this approach is avoided by the food industry due to its cost and, more importantly, time consumption.

As a practical matter in the food industry, total amino acid analysis is achieved by the parallel application of several techniques. Then the overall amino acid profile is achieved as a retrospective composite of the results from these techniques. The general acid hydrolysis mentioned earlier yields acceptable results for most of the amino acids. Recoveries for serine, threonine, valine, leucine, and isoleucine tend to be a bit low (85–95%) but are usually tolerable. Glutamine and glutamic acid are determined together as Glx. Similarly, asparagine and aspartic acid are codetermined as Asx.

Cyst(e)ine, and sometimes methionine, require an alternative sample preparation. Prehydrolysis oxidation of the sample with performic acid yields cysteic acid and methionine sulfone, respectively. These are chemically stable entities that afford improved recoveries. The disadvantage of this procedure is that it is unable to distinguish the original status of the cyst(e)ine and methionine being quantified. Oxidation is a common degradative route for these amino acids (especially in the free form) in aged products. Highly oxidized species of these amino acids are known to be biologically unavailable.

Tryptophan is commonly determined by one of two general categories of sample hydrolysis: (a) chemical hydrolysis in base (NaOH, LiOH, etc.) or (b) enzymatic hydrolysis. Attempts to adapt the standard acid hydrolysis to accommodate the problematic amino acids will be addressed in later subsections of this chapter.

There have been many studies for the optimization of conditions for the standard acid hydrolysis, but only a few of the more recent examples (24,27–29) are referenced here. These studies address the influence of various hydrolysis parameters on the accuracy of amino acid recoveries. Topics include acid-to-protein ratio, hydrolysis time, hydrolysis temperature, and the use of sealed tubes vs. open reflux. There is also evidence of a wide variety of techniques for the deaeration (very important!) of the sample, including vacuum, nitrogen purging, freeze/thawing, and combinations thereof. All of these issues have already been thoroughly reviewed in earlier

books (30–32) and will not be recapitulated here. Additionally, there have been large collaborative studies (33–36) to characterize the inter- and intralaboratory precision for amino acid analysis. These focus largely on the sample hydrolysis as the largest contributor to the overall variance observed.

b. Accelerated Hydrolyses

Since the classic acid hydrolysis takes 18–24 hours, there exists significant incentive to find ways to shorten this procedure. Not surprisingly, the effort to shorten the digestion time for acid hydrolysis has focused on raising the digestion temperature. Early efforts (24) included using glass tubes (with Teflon-lined screwcap) as digestion vessels and then heating them in conventional ovens to 145°C. This shortened digestion times down to 4 hours.

Physical strength of the digestion vessels has been a problem limiting how high a temperature may be used during digestion. Glass ampules will explode at sufficiently high temperature. Liu and Boykins (37) report using sealed microcapillary tubes to achieve higher temperatures. They also claim less degradation of methionine due to exclusion of air (oxygen) from the microcapillary tubes.

Gas-phase hydrolyses have become more popular. Initially, these were performed using conventional heating and, therefore, offered no time savings (38,39). In this case, the main advantages to gas-phase hydrolyses were the extremely small sample size that could be employed and the reduction in sample contamination by exogenous sources of amino acids. Naturally, there have been efforts to improve on this scheme by using elevated temperatures (40). Gas-phase hydrolysis has more recently been reported to offer the advantage of improved amino acid recoveries from glycoproteins (41). This is presumed due to the avoidance of Maillard browning types of reactions (although Maillard browning reactions are typically associated with more basic solutions).

Microwave heating is often associated with gas-phase hydrolysis. Perhaps the earliest investigation into the performance of vapor-phase hydrolysis via microwave heating is reported by Gilman and Woodward (42). More recently, the performance of microwave heating for vapor-phase hydrolysis at elevated temperatures was compared to the Pico Tag workstation, which employed conventional vapor-phase hydrolysis (43). Amino acid recoveries were slightly inferior with microwave heating. If the analyst needs to determine the amino acid composition for a purified protein but possesses only very small quantities of material, vapor-phase hydrolysis appears to be the method of choice (conventional or microwave heating). However, a general problem associated with gas-phase hydrolyses is that they can be rather labor intensive and handling such small samples can require a highly skilled technician.

In the food industry, availability of material is seldom an issue, and the extra problems handling extremely small sample sizes hardly seems worthwhile. Indeed, Refs. 44 and 45 have reported the application of microwave heating to the classic acid hydrolysis (although now at elevated temperatures) in which the sample is combined directly with the hydrochloric acid. Many (46–48) employ some type of Teflon vessel with two-piece construction (body with threaded top piece). These vessels are mechanically strong enough to allow heating to elevated temperatures (~180°C maximum). Earlier microwave systems were pressure-controlled units in which the digestion cells are manifolded to a pressure transducer. Newer systems allow for control via temperature measurement. This is accomplished by a fiber-optic probe with a tip that has been doped with a fluorophore. The luminescent properties of this fluorophore, such as fluorescence lifetime, change with temperature.

As a whole, the articles referenced previously report the same problem that has always plagued acid digestions of proteins at elevated temperatures. Optimization for recovery of hydrophobic amino acids (Leu, Ile, Val) and optimization for recovery of the alcohol amino acids (Ser, Thr) appear to be competing concerns. Investigators usually try to find a balance that is hot

enough to liberate most of the hydrophobic amino acids while limiting destruction of threonine and serine to "acceptable" levels. Of course, the big advantage of microwave heating to elevated temperatures is digestion times as short as 10 minutes!

c. Nontraditional Acid Hydrolyses

As mentioned previously, the classic acid hydrolysis using 6N HCl results in at least the partial destruction of several amino acids. There have been many attempts to ameliorate this problem. Clearly, it would be highly advantageous to determine all the amino acids with a single hydrolysis. Attempts to develop a single universal hydrolysis generally fall into two strategic categories. The first involves the addition of protective agents to the hydrochloric acid digestions. The second involves eliminating HCl and using alternative organic acids in the hope that they would not be as harsh and destructive as hydrochloric acid.

There are many papers detailing a wide array of protective agents and alternative acids. Only a subset will be referenced herein. The results from these studies can be difficult to reconcile. Often one investigator will report that a particular strategy spared the labile amino acids, only to have another investigator later report that that particular strategy offered insufficient or no protection whatsoever. It appears that the underlying determinant is the type of sample matrix that one is dealing with. For highly purified proteins, many of the strategies listed here offer significant improvement over the plain hydrochloric acid hydrolysis. Significant sparing effect is evidenced, and amino acid recoveries show a high degree of accuracy. At the other end of the spectrum, however, complex sample matrices that possess large quantities of carbohydrate (and fats, metals, etc.) rarely, if ever, yield acceptable recoveries for the more labile amino acids (e.g., tryptophan). If an investigator does report high amino acid recoveries in a complex matrix, it is often because that study included laborious and time-consuming sample cleanup steps to remove carbohydrates/fats prior to acid hydrolysis.

Protective Agents. The protective agents generally fall into two categories: (a) halogen scavengers, and (b) free-radical scavengers meant to prevent degradation due to the presence of oxygen. Phenol is an example of an additive that can act as a halogen scavenger. Many hydrolysis procedures involve the use of performic acid to oxidize cyst(e)ine to the stable cysteic acid prior to acid hydrolysis. Residual peroxide in the hydrochloric acid digestion could promote the unwanted halogenation of various amino acids. It has been reported that phenol (1% w/w) offers sufficient protection to allow the accurate quantitation of most amino acids including phenylalanine (49). However, Elkin and Griffith (50) claim to see the widespread losses of both phenylalanine and tyrosine in spite of using phenol as an additive. This is presumed due to the halogenation of these two amino acids, as evidenced by the appearance of extraneous chromatographic peaks that are purported to be identified as halogenated by-products.

Perhaps the most studied protective agent is tryptamine. It is usually intended as an oxygen scavenger to protect tryptophan from degradation. Results run the gamut. However, for complicated food matrices, one should not expect tryptophan recoveries to improve above 80% (and often much worse) in acid hydrolyses. For more examples, see Table 2.

Organic Acids. Hydrochloric acid will show a propensity to cause degradation of amino acids by halogenation (especially in procedures that employ peroxide treatment). This can be especially troublesome for tyrosine and phenylalanine. Thus, alternative acids that do not pose a risk for halogenation have been investigated.

Many of the organic acids employed possess a thiol group. This is meant to create an overall reducing environment in the acid digestion media. This strategy is successful for preventing the oxidation of methionine. Troubles are encountered, however, due to the reduction of cystine to cysteine. It is reported (60) that proline recoveries are artificially high due to the coelution of cysteine with proline for ion-exchange separations. Additionally, none of the organic acids yield acceptable recoveries for tryptophan. Perhaps because of this, many studies also add protective

Table 2 Protective Agents for Hydrochloric Acid Hydrolysis

Protective agent	Conditions	Ref.	Notes
Ethanedithiol	20 h, 110°C	51	Tryptophan recovery from lysozyme was 75%
Thioglycolic acid	4 h, 145°C	52	80% recoveries from model proteins
	15 min, 165°C	53	Vapor phase with 20% thioglycolic acid and 10% trifluoroacetic acid; trp recoveries exceed 80% for pure proteins only
	18 h, 118°C	54	5% thioglycolic acid and 0.1% phenol; tryptophan recoveries from pure proteins ~ 80%; also excellent recoveries of cysteine (>90%)
β-Mercaptoethanol	24 h, 110°C, 0.4% β-mercaptoethanol	55	Excellent trp recoveries for pure proteins
Phenol	25 min, 166°C, 1–3% phenol (w/v)	56,57	80% tryptophan recoveries for pure proteins
Tryptamine	4 h, 145°C	58,59	Tryptophan recoveries from 76–98% for various proteins; added at ~1:1 (w/w) tryptamine: protein
	Various temperatures and times	43	Microwave heating; recoveries similar to preceding

Table 3 Alternative Organic Acid Hydrolyses

Organic Acid	Conditions	Ref.	Notes
Methane sulfonic acid (4M)	180°C, 4 min, 0.2% tryptamine	44	Microwave heating; tryptophan recoveries ~98% for pure proteins
	115°C, 22 h, 0.2% tryptamine	61	High recoveries of tryptophan from cytochrome C
p-Toluenesulfonic acid (3M)	Unknown temperature, 15 min.	62	98.6% Tryptophan recovery, but only for a model amino acid solution; microwave heating
	110°C; 22-, 48-, and 72-h; 0.2% tryptamine	63	Recoveries comparable to methanesulfonic acid
	110°C, 22 h	64	Determination of methionine sulfoxide
Mercaptoethanesulfonic acid (3M)	160°C, 30 min	65	Trp recoveries >90% for pure proteins
	110°C, 24 h	66	Compares p-toluenesulfonic acid to mercaptoethanesulfonic acid

agents (e.g., tryptamine) to the acid digestion milieu. Even these combinations, however, have not resulted in acceptable recoveries for tryptophan from samples other than purified proteins. For examples, refer to Table 3.

2. *Special Chemical Pretreatments for Problem Amino Acids*

a. Tryptophan

Tryptophan is a metabolic precursor for many important biochemical compounds, such as nicotinic acid, kynurenic acid, serotonin, and melatonin (67). The tryptophan content of various food-

stuffs can vary greatly. For example, corn is notorious for its low tryptophan content. In countries/cultures where corn is the primary dietary protein source, the niacin deficiency syndrome, pellagra, is common. Because of this variability in content and because tryptophan is an essential amino acid with such important metabolic destinations, it will always be very important to monitor the tryptophan content of foodstuffs.

The determination of total tryptophan presents analytical difficulties due to the chemical behavior of its indole side chain moiety. As discussed previously in this chapter, tryptophan is largely destroyed by the standard hydrochloric acid digestion. Furthermore, attempts at modifying the standard hydrolysis by adding protective agents or using alternative organic acids have all failed to give acceptable recoveries of tryptophan in most food matrices. Because tryptophan is so labile in an acid environment, base hydrolysis has become a widely accepted method for liberation of tryptophan in proteins.

Base Hydrolysis. Protein digestions in basic media have proven to be no panacea. Early studies employed 4.2N sodium hydroxide and saw success with pure proteins (68). Collaborative studies (69,70) have since shown optimized recoveries of ~85% for NaOH hydrolyses in complex food samples. In general, for samples rich in fats and carbohydrate, attempts to use starch or ascorbic acid as protective agents have shown little improvement in recoveries.

Improved recoveries have been reported using barium hydroxide. In the first of a long series of studies by Delhaye and Landry (71) (all examining slight variations in procedures for $Ba(OH)_2$ hydrolyses), barytic hydrolysates are claimed to yield >99% recoveries of tryptophan for pure proteins and for lysozyme spiked into various feedstuffs. However, Lucas and Sotelo (72) previously reported a comparative study of the yields afforded by NaOH, $Ba(OH)_2$, and LiOH at various concentrations. The results indicate that LiOH hydrolysis might afford tryptophan recoveries that are significantly higher (~10%) than those of NaOH and $Ba(OH)_2$. This apparent contradiction seems due to the type of chromatography employed. Barium cations are a problem for ion-exchange columns and must be removed as part of the sample preparation. It is thought that some tryptophan is lost as a coprecipitate with the barium salts. This is not a problem if reversed-phase separations are employed. An examination of recent collaborative studies reported by Delhaye and Landry (73) yields disappointing results. The average tryptophan recovery for 23 participating laboratories was $86.5 \pm 5.6\%$.

An alternative, and less common, approach is to employ an internal standard. If the degradation of tryptophan cannot be prevented, then one could (at least theoretically) normalize their recovery data vs. an internal standard whose chemical behavior closely mimics that of the principal analyte. 5-Methyltryptophan has been employed with good results (74). In as-yet-unpublished results, we also have been able to achieve excellent normalized recoveries using 5-methyltryptophan as an internal standard. Another possible candidate for an internal standard is α-methyltryptophan. In fact, Bech-Anderson (75) conducted a comparison between these two internal standards and concluded that α-methyltryptophan yielded more accurate recoveries. This makes sense. 5-Methyltryptophan is substituted on the indole ring, whereas α-methyltryptophan is not. If we assume that degradation of tryptophan occurs primarily at the indole ring, then we would expect any substitution on the indole ring to affect degradation kinetics more strongly than a substitution at the α-carbon of the amino acid (see Fig. 3).

It should be noted that, when using an internal standard, we have typically seen normalized tryptophan recoveries in the range of 100–105%. The greater than 100% recoveries are not a surprise to us. It seems that free amino acids are more labile than their protein-bound counterparts. This could be due to steric exclusion effects (protein folding), or the peptide bonds themselves might somehow confer a degree of added chemical stability to the amino acids. The internal standard is present as the free amino acid right from the beginning of the digestion. To the degree that tryptophan will spend part of its time initially protein-bound, its degradation rate will be somewhat diminished compared to that of the internal standard.

5-Methyltryptophan   Alpha-methyltryptophan

Fig. 3 Chemical structures of tryptophan internal standards.

Enzymic Hydrolysis. Rather than employ a chemical hydrolysis of protein, enzymatic digestions of protein have been demonstrated to be a viable means for liberating tryptophan with acceptable recoveries. Various proteases, or mixtures thereof, have been employed for these enzymatic digestions. Papain (76) and pronase (77) are fairly typical examples. Baxter and Garcia (78) have reported drawbacks to employing these two enzymes. Among these are the high background signal from papain due to autodigestion and the excessively long digestion times necessary for pronase digestion under the conditions specified in Ref. 77. Baxter further reported a modified pronase digestion method, which has typically afforded recoveries of 95–99% and a relative standard deviation (RSD) of 1–2%. The main drawback to this method is the 6-hour digestion time required. The complexity of the method and the need for lab assistants to handle pronase enzyme are also ancillary issues.

b. Cysteine and Methionine

In addition to their importance as essential amino acids for humans, the quantitative determination of cysteine and methionine seems to be growing in importance in the animal feed industry. The dietary requirements for the sulfur amino acids tend to be very high in many animals. This is presumably due to the magnitude of hair/feather growth and the fact that the structural proteins that comprise hair/feathers often have high cyst(e)ine content.

The analysis of methionine and cysteine is problematic. The sulfur containing side chains of these amino acids are prone to oxidation. The standard hydrochloric acid hydrolysis will cause the partial conversion of these amino acids into cystine, cysteine, cysteine sulfinic acid, cysteic acid, methionine, methionine sulfoxide, and methionine sulfone. The classic strategy (79) for dealing with this problem is simply to drive the oxidative process to completion (i.e., convert all the cyst(e)ine to cysteic acid) and then to analyze chromatographically for cysteic acid and/or methionine sulfone. This is traditionally accomplished by a prehydrolysis treatment of the sample with performic acid. While this method has sufficed over the years, the typical recovery (85–90%) and precision (4% intra- and 15% interlaboratory) have been poor (80).

More recently, there have been numerous collaborative studies (81–84) that have attempted to improve the accuracy and precision of this method. A typical example by MacDonald et al. (81) reported a collaborative study by seven laboratories. Samples were oxidized with performic acid for 16 hours over ice bath. After oxidation, HBr was used to destroy excess performic acid. Samples were then roto-evaporated to dryness, dissolved in 6N HCl, nitrogen purged, and then hydrolysed for 18 hours at 100°C. Interlaboratory precision for cysteic acid determination in six food ingredients ranged from 7 to 10%. For methionine sulfone, interlaboratory precisions ranged from 1 to 13% for the same six food ingredients. The mean recovery of cysteine was 95% and of

methionine was 101%. However, recoveries were based on the analysis of pure β-lactoglobulin rather than a complex food matrix.

It has been presumed that there are two possible causes for the poor recoveries of cyst(e)ine as cysteic acid. The first is the incomplete conversion of cyst(e)ine to cysteic acid by the performic acid oxidation. The second is the oxidative destruction of cysteic acid during the HCl digestion due primarily to the presence of residual performic acid at elevated temperatures. In response to this possibility, many studies have employed the addition of HBr after the oxidative pretreatment to consume excess/residual peroxide. An interesting collaborative study reported by Llames and Fontaine (84) compares the use of HBr vs. metabisulfite for the purpose of scavenging leftover peroxide. It appears the use of hydrobromic acid yields slightly better results.

An interesting study (85) explores the use of sodium azide as an oxidative agent instead of performic acid. The big advantage offered here is that the oxidation of cysteine to cysteic acid is effected concurrent with the hydrochloric acid hydrolysis. The authors claim that the presence of 0.2% (w/v) NaN_3 in the HCl digestion does not represent an explosion risk. Recoveries of cysteine as cysteic acid were typically ~90% for pure proteins.

An alternative strategy to the performic acid oxidation of cysteine is to convert cysteine into an acid-stable species by alkylating its side chain. Examples of alkylating agents used for this purpose are 3-bromopropionic acid (86), 4-vinylpyridine (87), 3,3′-dithiodipropionic acid (88,89), and 3-bromopropylamine (90). In general, all of these methods yielded good recoveries for cysteine (>95%) in pure proteins. There is scant data for recoveries in complex food matrices, however. Additionally, little or no data regarding the precision of these methods is currently available. A serious drawback to these methods is that they tend to require additional sample preparation to remove excess alkylating reagent prior to the hydrolysis or prior to analysis by HPLC. An advantage to these methods is the improved chromatography compared to that for cysteic acid.

A problem with the chromatographic determination of cysteic acid is that there is almost no retention of cysteic acid. For both reversed-phase HPLC and ion-exchange amino acid analyzers (usually employing cation-exchange resins), cysteic acid is essentially eluted within or near the void volume of the column. This makes it more susceptible to unknown chromatographic interferences from various matrices. When cysteine is alkylated by 3-bromopropylamine, the product (*S*-3-aminopropylcysteine) looks very similar to lysine in structure. Hale et al. (90) show that this alkylated species affords excellent chromatographic separation on four different commercially available amino acid analysis systems and that, indeed, it does elute very near lysine in each case (see Fig. 4).

Finally, it is very common for methionine to be determined as part of the standard hydrochloric acid hydrolysis. Indeed, methionine is not nearly as labile to oxidation as cysteine is. While this is appropriate for many samples, there are studies (27,91) that indicate that seriously flawed recoveries (10–40% low) may result from methionine determination by standard acid hydrolysis if the samples contain high levels of carbohydrate. For these sorts of samples, it is recommended that determination of methionine as methionine sulfone (by performic acid oxidation) be pursued.

c. Glutamine and Asparagine

While neither glutamine nor asparagine is an essential amino acid, there is still interest in quantifying these two amino acids, especially glutamine, which is recognized to improve healing for patients suffering from gut trauma (92). Additionally, glutamine is becoming recognized as a conditionally essential amino acid during periods of general catabolic stress (93). Unfortunately, during standard acid hydrolysis, the side chains of these amino acids are deamidated to become carboxylic acids. Thus, asparagine is converted into aspartic acid and glutamine into glutamic acid.

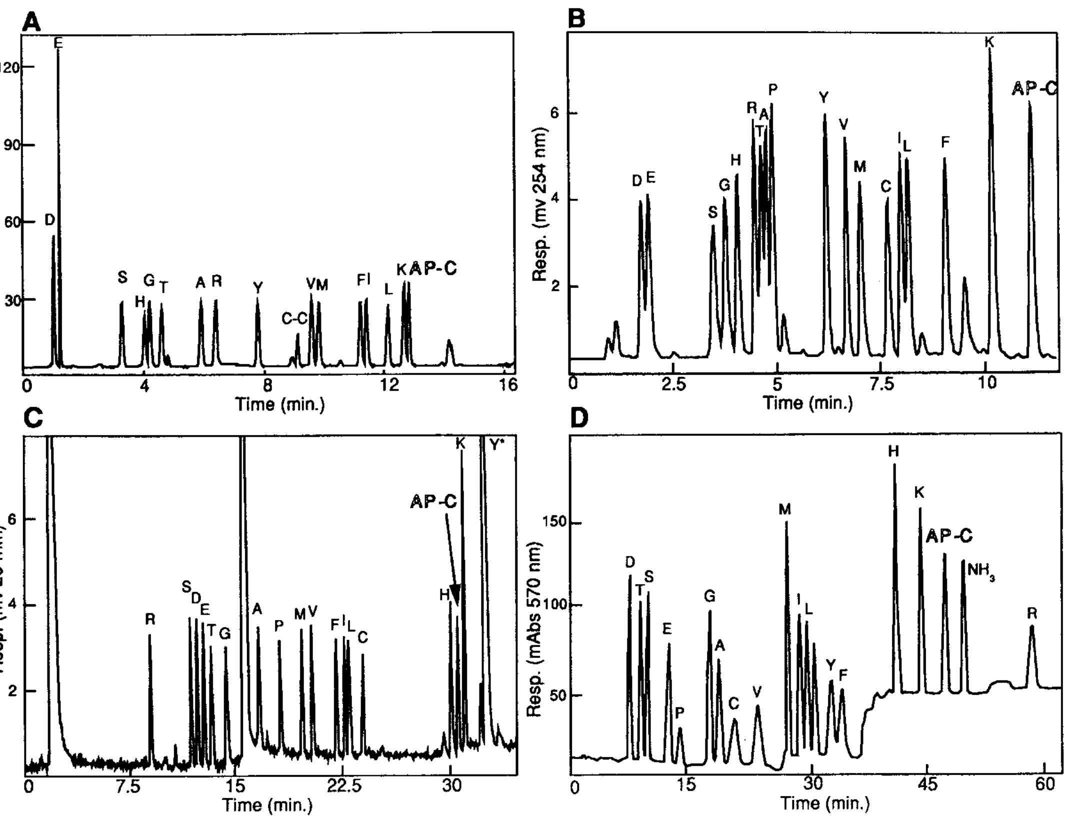

Fig. 4 Elution position of *S*-3-aminopropylcysteine (AP-C) in four different amino acid analysis systems. Purified *S*-3-aminopropylcysteine was spiked into amino acid standards and subjected to amino acid analysis. Standards run on (A) an AminoQuant (OPA) system, (B) a Waters PICO-TAG (PITC) chemistry system, (C) a Varian Amino Tag (FMOC) chemistry system, Y* = tyrosine and FMOC-Cl, (D) a Beckman System 6300 (ninhydrin) amino acid analyzer. (From Ref. 90. Copyright 1994 Academic Press, Inc.)

There are two strategies for circumventing this problem. The first is to employ an enzymatic digestion. There does not appear to be many applications of this strategy. The primary problem encountered appears to be incomplete digestion (94,95). An additional problem common to many types of enzymatic digestions is spurious contributions due to autodigestion. Also complexity and time consumption of procedures is an issue (96).

The second strategy is chemically to convert the side chain of these amino acids into a moiety that is inert to acid hydrolysis. An example of this is reaction with bis(trifluoroacetoxy)iodobenzene (BTI). This reagent converts asparagine and glutamine into diaminopropanoic acid and diaminobutanoic acid, respectively (via the Hofmann degradation). Early employment of this reagent involved the indirect measurement of asparagine and glutamine (97). This is because ion-exchange amino acid analyzers of that time period could not adequately resolve these conversion products from lysine (diaminohexanoic acid). Glutamic and aspartic acid levels are measured after acid hydrolysis, with and without prior reaction with BTI. The measured difference between these two procedures is assumed to be due to the inherent levels of glutamine and asparagine.

Fouques and Landry (98) report the direct measurement of these conversion products by reversed-phase HPLC of their phenylthiocarbamyl derivatives. Additionally, they report that (in a study using pure proteins of known composition) conversion of glutamine and asparagine by BTI is incomplete. Conversion rates to their respective diaminocarboxylic acids were reported between 50% and 83%. However, losses of glutamine (followed by measuring decreases in total glx) were typically 95%, in spite of the fact that recoveries of the corresponding diaminobutanoic acid were far less than 95% (the disappearance of asparagine is reported to be variable and as low as 65%). The disappearance of glutamine ought to track (inversely) the appearance of diaminobutanoic acid. This discrepancy is ascribed to the conversion of glutamine to unknown intermediate products. The authors conclude that these results indicate the use of the indirect method for measuring glutamine (although not asparagine) is advisable.

Since then, Kuhn et al. (99) have optimized the reaction with BTI and have successfully analyzed various pure proteins. They report average recoveries of glutamine (by direct detection of dansylated diaminobutanoic acid) at 93 ± 4%. This study also concludes that analysis of asparagine by this method is inappropriate.

Kuhn et al. have also claimed successful analysis of various protein and peptide-based enteral products (100) using the BTI method. Unfortunately, the sample preparation procedures were lengthy and involved the removal of fats and carbohydrates before reaction with BTI. The removal of fats and carbohydrates involved extraction/precipitation with isopropanol/chloroform. After centrifugation, the pellets were resuspended, dialyzed, and then reacted with BTI. The authors admit that as much as 30% of the protein was lost during this sample preparation (measured as protein nitrogen). The authors then make the assumption that the loss of 30% of the protein did not skew the results for the relative abundance of glutamine content measured in the protein that remained in the pellet. This seems a dangerous assumption for the partially hydrolyzed protein products. The efficiency of extraction/precipitation of peptides can be highly dependent on the molecular weight (size) and composition of the peptide. Furthermore, there is an implicit assumption of a statistically random distribution of glutamine residues throughout the peptide population.

d. Lysine

Lysine is an essential amino acid. Since lysine is a fairly acid-stable amino acid, its analysis as total lysine by the traditional hydrochloric acid hydrolysis is straightforward. Fairly recent examples for the successful determination of total lysine employing either ion-exchange (82) or reversed-phase (101) HPLC are available.

While the determination of total lysine is rather straightforward, the determination of "free" or "available" lysine is more problematic. In this situation, the term "free" is meant to imply that the ϵ-amine of the lysine side chain has not reacted with various components of the sample matrix (most commonly carbohydrates via Maillard browning). This is important because reaction of the ϵ-amine can render lysine nutritionally unavailable and the nutritive value for that protein is then diminished if lysine is the limiting amino acid (which is often the situation with soy proteins). While enzymatic digestion in the human gut may not release the modified lysine in a nutritionally available state, often these lysine adducts are labile to the standard acid hydrolysis in 6N HCl at 110°C. This results in total lysine values that overestimate the amount of nutritionally available lysine.

Traditionally, most chemical methods for the direct determination of available lysine depended on the reaction of the ϵ-amine with a chromophoric derivatizing reagent and then spectrophotometric measurement. The most commonly employed chromophore is 1-fluoro-2,4-dinitrobenzene (FDNB), which was originally employed by Carpenter (102). Since then, numerous articles (103–106) have reported the direct (or indirect by difference) HPLC measurement of the FDNB-derivatized lysine. However, these methods can suffer from the problem that the reaction product of the FDNB-derivatized lysine (dinitrophenyllysine) is not entirely stable during acid hydrolysis (especially in the presence of carbohydrates) and correction factors are recommended (107).

Reaction with FDNB is not the only option for determining lysine with unreacted ϵ-amine moieties. Comparative studies (108,109) discuss the merits of derivatizing with trinitrobenzenesulfonic acid, *o*-phthalaldehyde, and azo dyes. A slightly different strategy mentioned in these articles is to employ the reducing agent sodium borohydride. This is intended to "fix" the lysine-carbohydrate adduct, making it stable to acid hydrolysis. Thus, total lysine values will not be inflated (compared to nutritionally available values), because none of the modified lysine is converted to free lysine during the acid digestion.

C. Internal Standards

Brief mention needs to be made regarding the employment of internal standards. While it is desirable to employ an internal standard for procedures that involve significant sample preparation procedures, there is no ideal choice for an internal standard for amino acid analysis. This fact is due to the wide spectrum of chemistries exhibited by the various amino acids. If one is analyzing for a single amino acid (or class, e.g., the hydrophobic amino acids), it is possible to choose an internal standard that mimics the chemistry of that particular amino acid very well. However, for the overall amino acid profile, an internal standard will do nothing more than allow the analyst to make nonvolumetric solution transfers and correct for variability of the injection volume by the HPLC injector. Unfortunately, the employment of an internal standard can actually skew the apparent recoveries for the overall amino acid profile.

Since no single internal standard can possibly mimic the chemistry of all the amino acids (for overall profile), the choice of internal standard has been based primarily on two criteria. The first is chemical stability. The internal standard must not be labile under the conditions employed. The second is that the internal standard must offer chromatographic resolution. This is not easy, since the overall profile produces a chromatogram that is already very cluttered. A review of the literature reveals that three internal standards are the overwhelming popular choices: norleucine, norvaline, and α-amino-*n*-butanoic acid (AABA). It should be noted that norleucine and norvaline are very hydrophobic amino acids, whereas AABA is relatively hydrophilic. How these standards might behave during sample preparation steps (e.g., filtration) as a function of their hydrophobicity should be taken into consideration.

III. SEPARATION TECHNIQUES

A. Ion Exchange

Early efforts at developing automated methods for amino acid analysis were based primarily on ion-exchange chromatography (IEC) (110–112). Almost without exception, these chromatographic methods now employ some type of cation-exchange resin. Most commonly this has been a sulfonated polystyrene. Development of smaller particle size and pellicular packings has greatly improved resolving power and separation run times (although still relatively slow compared to reversed-phase HPLC).

The amino acids are separated in their free form, usually by stepwise elution. True gradient elution is possible but seldom employed. Solvent systems are typically a series of sodium- or lithium-based buffers. For acid hydrolysates, the resulting 16 amino acids can adequately be separated employing the stepwise application of three sodium buffers affording a typical run time of 1.5 hours. For free amino acid profiles from serum samples (or other applications involving additional and/or nontraditional amino acids), it may be necessary to use a series of four lithium buffers and a longer run-time up to 4 hours. Grunau and Swiader (113) reported the separation of 99 amine-containing (actually, ninhydrin-reactive) compounds in only 2 hours. This was accomplished using lithium buffers and a true gradient elution scheme.

As an interesting side note, sometimes alternative ion-exchange resins are employed. An example (114) is the separation of various amino acids with ion-exchange systems based on poly(hydroxyethyl methacrylate) stationary phases modified with various ionizing groups. Bonded phases that were examined included carboxymethyl (a weak cation exchanger), sulfobutyl (strong cation exchanger), and diethylaminoethyl (weak anion exchanger). While these are interesting possibilities, they have not seen widespread use for determination of amino acids in foodstuffs.

Detection of amino acids is typically by UV absorption after postcolumn reaction with ninhydrin. Precolumn derivatization with ninhydrin is not possible, because the amino acids do not actually form an adduct with the ninhydrin. Rather, the reaction of all primary amino acids results in the formation of a chromophoric compound named Ruhemann's purple. This chromophore has an absorption maximum at 570 nm. The secondary amino acid, proline, is not able to react in the same fashion and results in an intermediate reaction product with an absorption maximum at 440 nm. See Fig. 5. Detection limits afforded by postcolumn reaction with ninhydrin are typically in the range of over 100 picomoles injected. Lower detection limits can be realized with postcolumn reaction with fluorescamine (115) or *o*-phthalaldehyde (OPA) (116). Detection limits down to ~5 picomoles are possible. However, the detection limits afforded by ninhydrin are sufficient for the overwhelming majority of applications in food analysis.

Although there will be slight differences amongst different manufacturers' columns, buffer systems, and instrumentation, Fig. 6 provides a representative chromatogram for the ion-exchange separation of amino acids. Some general trends in selectivity can be deduced. The acidic amino acids (e.g., aspartic acid and glutamic acid) elute early, and the basic amino acids (e.g., lysine) elute late. This mode of separation is a simple electrostatic repulsion/attraction due to the negative charge on the sulfonated resins. Similarly, taurine and cysteic acid will elute very early (with cysteic acid almost eluting in the column void volume). Clearly, hydrophobic interaction also factors into the separation (more hydrophobic eluting later). This is evidenced by alanine's eluting before valine, which, in turn, elutes before leucine.

Automated amino acid analyzers are available from a number of manufacturers (e.g., Beckman, Biotronik, Dionex, LKB, Pickering). These are integrated systems in which the column resin, buffer system, and various instrumentation parameters have been co-optimized for that particular system. All are reported in the literature to perform very well. The advantage to purchas-

Fig. 5 Reaction of amino acids with ninhydrin.

ing one of these prepackaged analyzers is the ease of use and reliability. The disadvantage to all these systems is the expensive equipment (and columns) and the relatively high cost of maintenance. In spite of the cost of these integrated ion-exchange systems, they remain popular. As recently as 1989, a survey (117) of the field found that ion-exchange separation was the predominant method of choice (by far). This, of course, is changing as reversed-phase HPLC for the determination of amino acids becomes increasingly popular.

B. Reversed Phase

The explosive growth in reversed-phase HPLC determinations of amino acids is inextricably linked to the proliferation of precolumn derivatizing agents (these will be detailed in a subsequent section of this chapter). Some of the more common derivatizing agents are phenylisothiocyanate (PITC) (118), *o*-phthalaldehyde (OPA) (119), 9-fluorenylmethylchloroformate (FMOC) (120), and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (121). All of these chemistries are now commercially available as reaction kits for automated amino acid analysis. The development of these derivatizing agents was essential for two reasons. First, they allow for the spectroscopic detection of the amino acids (e.g., UV absorption and/or fluorescence). Second, they impart a higher degree of hydrophobicity to the amino acids. Most underivatized amino acids

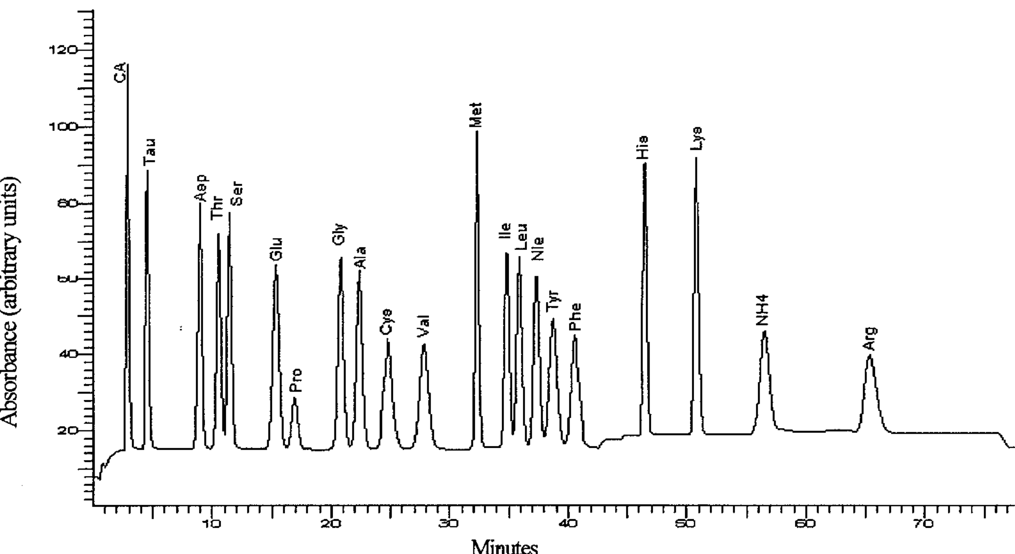

Fig. 6 Typical ion-exchange separation of amino acids followed by postcolumn reaction with ninhydrin. Separation was achieved employing Beckman 6300 amino acid analyzer with cation-exchange column. Three sodium buffers were used with a stepwise elution scheme as supplied/recommended by the manufacturer. Detection was the sum of 440-nm and 570-nm absorbance. Standard three-letter abbreviations for amino acids are used; also, CA = cysteic acid, tau = taurine, and nle = norleucine. Data was supplied by Stephen D. Smith, Ross Products Division of Abbott Laboratories, Columbus, OH.

do not exhibit much hydrophobicity and are not retained sufficiently on typical reversed-phase packings.

With very few exceptions, the column packing of choice is an alkyl-bonded silica particle. The bonded phase is either an octyl- or, more commonly, an octadecylsilane. The most commonly employed particle size is 5 μm, while 3 μm is growing in popularity. In general, the C_{18} columns from different commercial manufacturers offer similar performance. Perhaps the most noticeable difference amongst columns is the density of bonded-phase coverage on the silica particle and the degree to which residual, uncapped silanol groups are exposed to the particle surface. An abundance of uncapped silanol groups on the stationary phase can cause unwanted tailing of peaks (especially for the basic amino acids, e.g. lysine, histidine). This can be obviated, to a large degree, by the addition to the mobile phase of an organic cation like triethylamine.

Solvent systems encompass a dizzying array of permutations of organic solvents, buffers, and other mobile-phase additives. However, the most commonly employed solvent systems involve acetonitrile, methanol, and/or tetrahydrofuran. Buffers are typically acetate (pK_a 4.8) or phosphate (pK_a 1.3 and 6.7) at approximately 100 mM. For the analysis of a small number of free amino acids, isocratic elution is often possible. For the determination of an overall amino acid profile from a hydrolysate sample, complicated ternary gradients are often necessary.

Although the separations afforded by the different derivatizing agents exhibit small differences in selectivity, the general elution order for the amino acids tend to be the same. This is not surprising, since the source for differences in retention are based primarily on the side chains of the amino acids. Perhaps the most noticeable differences amongst various derivatizing agents is the overall run time necessary and the presence of different critical peak pairs in the chromatogram. Even the run times don't vary much, because one can modify other parameters. For a more hydrophobic tag like FMOC, an increased organic content of the mobile phase can be

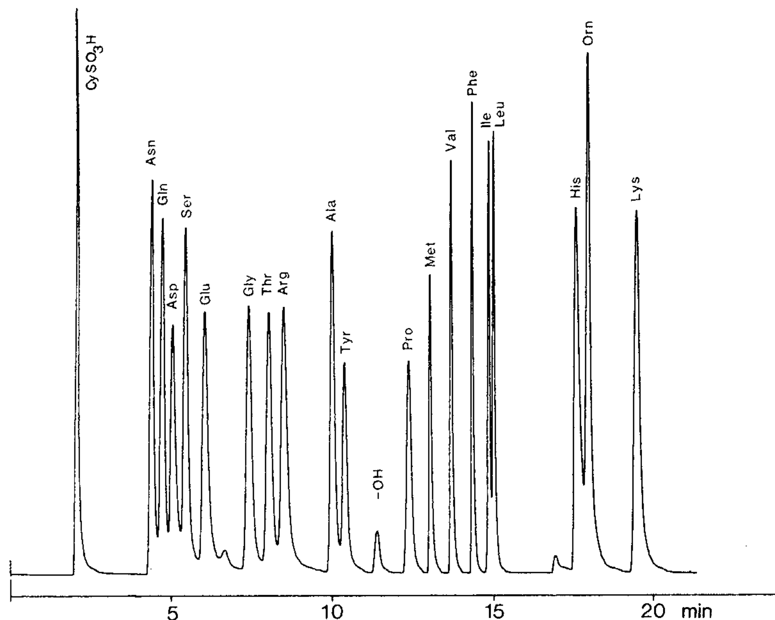

Fig. 7 Typical reversed-phase separation of amino acids. Precolumn derivatization of a standard amino acid mixture was achieved employing FMOC. Resolution was achieved by gradient elution with acetonitrile, methanol, and acetate buffer (pH 4.2) on a C_{18} column. Standard three-letter abbreviations for amino acids are used; also, $CySO_3H$ = cysteic acid. (From Ref. 164. Copyright 1983 Elsevier Science.)

employed or a C_8 bonded phase can be used instead of the more typical C_{18}, both of which will shorten the run time. A representative chromatogram from a reversed-phase separation is shown in Fig. 7.

C. Ion Pair

Only brief mention of this separation technique will be made. Ion-pair chromatography has not gained general acceptance as a means for amino acid determination. Perhaps this is because this method exhibits a complexity that seems to exceed that of both ion-exchange and reversed-phase separations. As much as anything, ion-pair separation for amino acid analysis seems to be a victim of historical timing. It was developed (at least partly) in response to dissatisfaction with the long run times of ion-exchange separations of that period but was soon eclipsed by the explosion of precolumn derivatization chemistries that became available for reversed-phase separations. Regardless, a few references (122–125) are included because ion-pair chromatography does present the opportunity to create highly tailored separation modes/selectivities for unusual/demanding separations that may not be accessible by traditional ion-exchange and reversed-phase modes of separation.

D. Chiral

The enantiomeric separation of the D- from the L-stereoisomers of amino acids is an area of growing interest. It is generally recognized that heat- and alkali-treatment of proteins can result in the racemization of L-isomers of amino acid residues to the D-analogs. Almost without exception, humans cannot utilize the D-isomers of amino acids, and some are thought to be toxic (although

this is still debated). At the very least, it is agreed that the presence of even a small number of D-enantiomer residues in a protein can drastically lower that protein's digestibility.

It may also be surprising how easily this racemization may occur. Friedman and Liardon (126) studied the racemization kinetics for various amino acid residues in alkali-treated soybean proteins. They report that the racemization of serine, when exposed to 0.1M NaOH at 75°C, is nearly complete after just 60 minutes. However, caution must be used when examining apparent racemization rates for protein-bound amino acids. Liardon et al. (127) have also reported that the classic acid hydrolysis, employed to liberate constituent amino acids, causes amino acids to racemize to various degrees. This will necessarily result in D-isomer determinations that are biased high. Widely applicable correction factors are not possible since the racemization behavior of free amino acids is different from that of amino acid residues in proteins (which can be further affected by sequence). Of course, this is not a problem for free amino acid isomer determinations since the acid hydrolysis is unnecessary. Liardon et al. also describe an isotopic labeling/mass spectrometric method for determining true racemization rates unbiased by the acid hydrolysis. For an extensive and excellent review of the nutritional implications of the racemization of amino acids in foods, the reader is directed to a review article written by Man and Bada (128).

There are two major approaches for achieving enantiomeric separation of D- and L-amino acids. The first involves precolumn derivatization with a chiral reagent followed by separation of the resulting diastereomers on regular, reversed-phase HPLC columns. The second approach is the direct separation of underivatized enantiomers. This can be accomplished by employing a column with a chiral bonded phase (e.g., Pirkle column). This can also be accomplished by making a regular C_{18} column behave as if it were a chiral packing. This is analogous to a stereochemical version of ion-pair chromatography. For example, a chiral crown ether can be added to the mobile phase. This also is essentially the concept behind a mode of separation named "ligand-exchange HPLC." This typically involves the addition of an optically active amine and a metal ion to the mobile phase. The amine–metal chelate acts as a chiral mobile-phase modifier. More recently, it has become common to see the chiral amine incorporated as part of the bonded phase, and then the column is loaded with a metal ion. A few recent examples of these various types of enantiomeric separations are tabulated in Table 4.

IV. DETECTION SYSTEMS

A. Nonderivatizing

1. Native UV and Fluorescence

Most underivatized amino acids (except those with aromatic side chains) do not possess suitable chromophores for spectroscopic detection except at very short wavelengths (~210 nm). This is quite inconvenient, because spectroscopic selectivity at that short wavelength is poor (i.e., there are many types of organic compounds that absorb at that wavelength). This can give rise to cluttered chromatograms due to the presence of many interference peaks. Also, this limits the choices of solvents and buffers to those that are transparent to detection at that wavelength. This is especially severe if gradient elution is to be employed. Because of these limitations, spectroscopic detection of amino acids in their native form is seldom attempted. However, spectroscopic detection of amino acids that have been derivatized with chromophores is a valuable technique and will be discussed later in this chapter.

As mentioned, the predominant exception to the aforementioned problem is the detection of tryptophan. Tryptophan (also tyrosine and phenylalanine) exhibits reasonably strong UV absorbance at longer wavelengths. Better yet, tryptophan exhibits strong native fluorescence (λ_{ex} = 295 nm, λ_{em} = 345 nm) that has often been employed for facile detection of free tryptophan.

Table 4 Enantiomeric Separation Schemes

Strategy	Ref.	Comments
Diastereomeric derivatization	129	Reaction with OPA and chiral thiols, *N*-isobutyryl-L,D-cysteine; separation on C_{18} column; application to many food types
	130	Reaction with OPA and chiral thiol, D-3-mercapto-2-methylpropionic acid
	131	Reaction with fluorescent chiral Edman-type reagent
	132	Reaction with OPA and *N*-acetyl-L-cysteine; separation on C_{18} column with gradient elution
Chiral mobile-phase modifiers	133	Separation of dansylated amino acids using β-cyclodextrin in mobile phase and reversed-phase column
	134	Various *N*-alkyl-L-proline complexes of Cu(II) used as additive; postcolumn OPA reaction/detection
	135	α-Aminoalkylphosphonates and Cu(II) as additive
	136	Phenylalanineamide/Cu(II) complex as mobile-phase additive compared to covalent attachment of phenylalaninamide as true bonded phase
	137	Alkylpenicillamine/Cu(II) complex dynamically (?) adsorbed to reversed-phase stationary phase
Chiral stationary phase	138	Covalently attached β-cyclodextrin bonded phase; exploratory separations of PITC-, dansyl-, dabsyl-, and AQC-derivatized amino acids
	139	Separation of NBD-F derivatized amino acids with (*S*)-1-napthylglycyl-3,5-dinitrophenylamide bonded phase on silica
	140	Allyl-terguride as covalent bonded phase; various chromophoric derivatizing agents employed
	141	L-Hydroxyproline incorporated as bonded phase for ligand-exchange mode separation

2. *Refractive Index*

Refractive-index detection is seldom used for many of the same reasons just mentioned for the spectroscopic detection of amino acids in their native forms. In fact, these problems are even more severe. Refractive-index detection has almost no selectivity whatsoever. Nearly every sample component passing the detector will register a signal. Also, this makes refractive index entirely incompatible with gradient elution. Even for isocratic separation, and detection of only a select few amino acids, refractive index can be very troublesome because of the detector's tendency to drift due to temperature changes in the laboratory (perhaps newer models have fixed this problem?). Finally, detection limits tend to be very poor for refractive-index detection.

3. *Electrochemical*

The direct detection of amino acids with typical electrochemical detection conditions (e.g., glassy carbon electrode with constant potential) is generally acknowledged to be limited to amino acids possessing aromatic or sulfur-containing side chains. The other amino acids simply do not possess functional groups that are sufficiently electrochemically active. Because of this, derivatization procedures are often employed (precolumn or postcolumn) to attach electrochemically active moieties to the amino acids. This situation appears to mirror that for the spectroscopic detection of amino acids. Derivatization reactions can be chemical or photochemical in nature and can target either the amine or the carboxylic acid functional groups.

Another approach to dealing with the nonelectrochemically active nature of most amino acids is to generate, in situ, chemical reactions at the electrode surfaces to produce electrochemically active products for detection. Related to this concept, is the online use of immobilized enzymes (142) to react with amino acids. A by-product of this reaction is hydrogen peroxide, which is then quantified by amperometric detection.

Relative to the UV-spectroscopic and/or fluorometric determination of derivatized amino acids, electrochemical detection of amino acids seems to be more complex and problematic. Furthermore, electrochemical detection has not gained sufficient popularity to warrant an extensive examination here. For those readers specifically interested in electrochemical detection of amino acids, an excellent review article has been written by Dou et al. (143).

4. *Chemiluminescent Nitrogen*

In recent years, several new methods for detection of amino acids (with HPLC) have been published that don't fit neatly into any general category of detection. One of these newer techniques, reported by Fujinari and Manes (144), is dubbed chemiluminescent nitrogen detection (CLND). Detection is effected in the following manner: any nitrogen-containing compound that elutes from the column undergoes high-temperature oxidation (1000–1100°C). All chemically bound nitrogen is released in the form of nitric oxide. The nitric oxide is then reacted with ozone to produce excited-state nitric oxide. Subsequently, the excited nitric oxide will emit a photon that is measured by a photomultiplier tube. The author touts this method for its inherent transparency to nonnitrogenous compounds, a specificity that could be useful. The problem is that this detection method excludes the use of acetonitrile as a mobile-phase solvent. Whether or not the apparent cost and complexity of this instrument prevents it from gaining widespread acceptance remains to be seen.

5. *Evaporative Light Scattering*

Another technique (145) that is fairly new to the application of amino acid analysis employs evaporative light-scattering detection. The detection scheme is self explanatory and acts as a mass-sensitive detector. A linear log-log relationship exists between peak area and mass of analyte passing the detector. Detection limits are approximately 20 nanograms. The major problem with this detection method is that all solvents and buffers must be highly volatile. This severely limits the chromatographic selectivities available to the analyst. To circumvent this limitation, the authors gain resolution capability by employing column switching between two very different columns (C_8 and cation exchange). Again, it will be interesting to see whether the public decides that this represents an improvement over more popular detection schemes.

B. Derivatization (Amine vs. Carboxyl)

Almost without exception, derivatization is achieved by electrophilic reaction with the α-amine group. Nucleophilic substitution at the α-carboxyl group is a rarity. This is likely due to the poor reactivity afforded and also a lack of specificity on the part of the derivatization reagents available. Reaction can be effected either precolumn or postcolumn. There are advantages and disadvantages to both schemes. Most of the derivatizing reagents can be (and have been) employed both ways, the exception being ninhydrin, which can be used only as a postcolumn derivatizing reagent.

1. *Postcolumn*

The earliest approach to amino acid analysis involved postcolumn reaction. This scheme offers several advantages compared to precolumn reaction. First, it simplifies the sample preparation necessary. Often, precolumn derivatizations require sample cleanup steps to eliminate sample

components that may interfere with the derivatization reaction. Overall, postcolumn detection schemes offer separations that are less matrix sensitive.

Second, it takes full advantage of the chromatographic differences among the various amino acids in their native form. To illustrate, let's view this from the other strategic vantage, precolumn derivatization. Once the amino acids have become adducts with a large, polycyclic aromatic hydrocarbon like the fluorene backbone from FMOC, these molecules all look pretty much the same to a column's stationary phase (a little anthropomorphism). They are all large, very hydrophobic molecules with only slight differences in their overall chemical makeup due to their original side chains. No wonder that precolumn derivatizations with reversed-phase separations often exhibit very tightly resolved critical peak pairs.

The disadvantages to postcolumn reaction are well known. The first disadvantage is simply the cost and maintenance problems associated with the extra equipment (reagent pump, mixing manifold, reaction bed/coil, oven). Postseparation band broadening can be another problem. Finally, the variety of reaction chemistries available for postcolumn use is limited to those reagents exhibiting sufficiently fast reaction kinetics so that reaction coils need not become excessively lengthy.

2. *Precolumn*

An advantage to precolumn derivatization is that there are no limitations with respect to reaction kinetics. Of course, rapid reaction is always desirable, but need not be the principal factor when choosing a derivatizing reagent. This means that a wide variety of chemistries are available for derivatization. However, it is important that very stable derivatives be formed. The limited stability of OPA derivatives is the reason why OPA is most commonly employed for postcolumn reaction. If it is to be used in the precolumn fashion, very well-controlled and automated (mechanized) sample/reagent handling immediately prior to injection must be employed.

Another advantage is that the reversed-phase separations of precolumn derivatized amino acids tend to be much faster than those for ion-exchange separations. Reversed-phase separations of the 16 hydrolysate amino acids can be achieved in as little as 15 minutes (with reasonable resolution). Much of this credit is given to the technical improvements that accompanied the development of alkyl-bonded silica-based packings with very small particle size. While the development of these high-efficiency chromatographic packings has been important, one should not underestimate the contribution of the precolumn derivatization scheme to shortening separation times (i.e., the amino acids, as chromophoric adducts, are now all much more similar molecules that exhibit a narrowed range of retention times).

3. *Derivatizing Reagents*

A wide array of derivatizing reagents has been developed, and this list keeps growing with each passing year. However, not all the reagents will be discussed here. Only those that have found more widespread acceptance will be detailed. Many of the more popular derivatizing chemistries are also available as commercial kits. This is more expensive (for materials), but also much more convenient. Additionally, any extra cost in materials will likely be offset by cost savings in method development (and in aspirin for headaches).

For the majority of derivatizations listed here, the pH of the reaction solution is very important and should typically be buffered in the region of pH 8.5 to pH 10. Additionally, it is typical that a 10-fold excess of reagent be used to ensure complete derivatization of analytes.

Employing a large excess of reagent forces the analyst to consider whether or not the native molecule (and/or its hydrolysis product) is fluorescent. For many of the reagents, this results in a huge peak eluting in the middle of a chromatogram and preventing the quantitation of neighboring analyte peaks. Often, this is addressed by employing some sort of liquid–liquid extraction

(e.g., pentane) to remove excess reagent after the reaction is completed. Most investigators seem to assume that, while effectively removing unreacted reagent, the derivatized amino acids are not significantly extracted (lost) into the organic phase. This can be a dangerous assumption, especially for the more hydrophobic amino acids and also for lysine, which is typically di-derivatized, rendering the adduct very hydrophobic. A list of the popular reagents, and a thumbnail sketch of their more important attributes, follows.

AQC (6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate) λ_{ex} = 250 nm, λ_{em} = 395 nm. Derivatives are very stable (days) at room temperature and can be formed from both primary and secondary amino acids. A principal advantage of this reagent is that its reaction with amino acids is not highly matrix sensitive. Presence of salts, detergents, lipids, and many other sample components do not interfere with reaction. This greatly simplifies sample preparation. Fluorimetric detection limits are typically in the low picomole range. Unreacted AQC is not fluorescent, and the hydrolysis product, AMQ, is only weakly fluorescent. Thus, extraction of excess reagent is not required. Reaction time is fast at 1 minute. However, the reaction initially creates both mono- and di-derivatized tyrosine. Heating the reaction solution for 10 minutes at 50°C drives all tyrosine to the mono-derivatized product. Fluorescence of tryptophan adduct is very poor. This is perhaps the newest chemistry to be made commercially available in a prepackaged kit. It is referred to as "AccQ Tag" by Waters Corporation. Representative references include 146–150. Also see Fig. 8 for a typical separation.

Dabsyl Chloride (4-dimethyl-aminoazobenzene-4-sulfonyl chloride) λ_{abs} = 420 nm. Derivatives are very stable (days) and can be formed from both primary and secondary amino acids. Detection is by absorption only. Detection limits are in the high picomole range. Reaction time is typically around 10 minutes at 70°C. Completeness of reaction can be adversely affected by the presence of high levels of various salts. Because reaction efficiency is highly matrix dependent and variable for different amino acids, standard amino acid solution should be derivatized under similar conditions/matrix for accurate calibration. Commercial systems available uti-

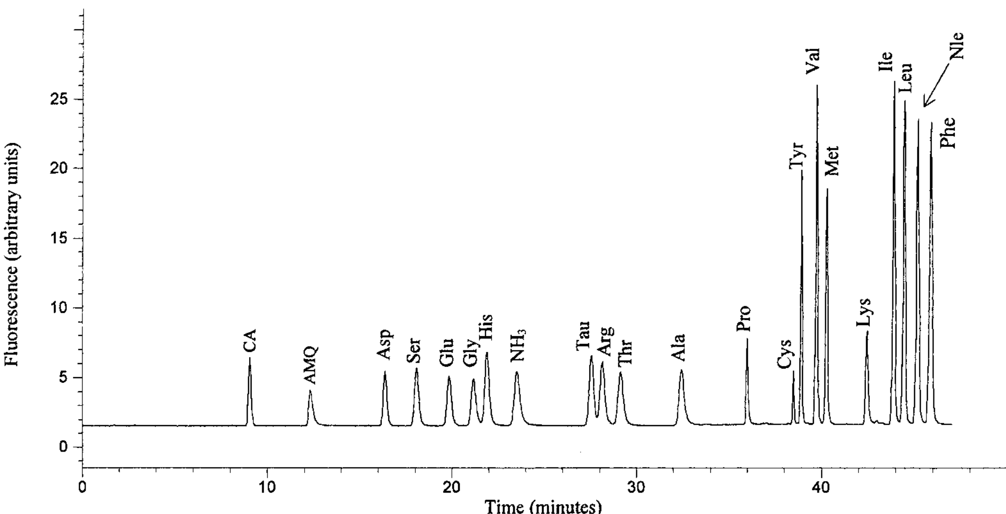

Fig. 8 Separation of standard mixture employing precolumn derivatization with AQC. Gradient elution with acetonitrile and acetate buffer (pH 5.0) was employed with a C_{18} column. Standard three-letter abbreviations for amino acids were used; also, CA = cysteic acid, AMQ = hydrolyzed excess reagent, and nle = norleucine. Data was supplied by Stephen D. Smith, Ross Products Division of Abbott Laboratories, Columbus, OH.

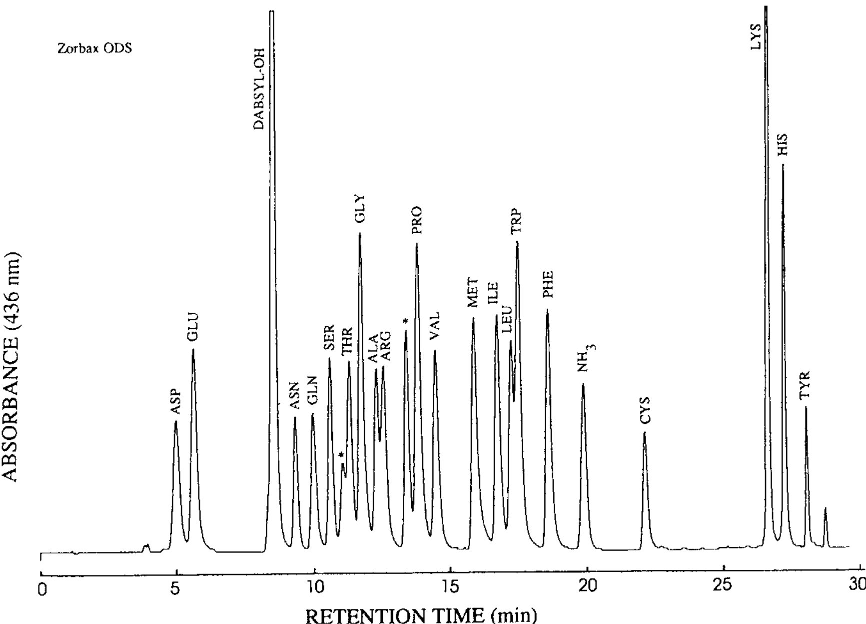

Fig. 9 Chromatogram of the separation of the dabsyl derivatives of all 20 naturally occurring amino acids on a C_{18} column. Gradient elution employed acetonitrile, methanol, and acetate buffer (pH 6.5). Note the presence of a large peak due to the hydrolysis product of excess derivatizing reagent. Peaks marked with asterisks have not been identified. (From Ref. 154. Copyright 1991 Elsevier Science.)

lizing this chemistry include the "System Gold/Dabsylation Kit" (Beckman Instruments, Inc.). See Fig. 9 for a typical separation. Representative references include 151–155.

Dansyl Chloride (5-*N*,*N*-dimethylamino-napthalene-1-sulfonyl chloride) λ_{ex} = 350 nm, λ_{em} = 510 nm. Derivatives are stable (days, if protected from light) and can be formed from both primary and secondary amino acids. Detection is preferably fluorimetric but also absorbs very strongly at 250 nm. Fluorescence measurement affords detection limits in the low picomole range. Hydrolysis product of excess reagent is highly fluorescent and can interfere with separation. Reaction time is relatively very slow at ~1 hour (60°C). This will commonly form multiple derivatives/peaks with histidine. Under certain pH conditions, dansyl chloride will also react with phenolic groups (tyrosine). Representative references include 156–160.

FMOC (9-fluorenylmethyl chloroformate) λ_{ex} = 265 nm, λ_{em} = 315 nm. Derivatives are stable (days) and can be formed from both primary and secondary amino acids. Fluorimetric detection limits in the high femtomole range are typical. Tryptophan adducts do not fluoresce due to quenching by the indole ring. Histidine and cyst(e)ine adducts fluoresce weakly. Reagent is highly fluorescent in its native form (also hydrolysate, FMOH). Excess reagent must be extracted or converted into noninterfering adduct prior to injection. Conversion of excess reagent into adduct with very hydrophobic amine, 1-amino-adamantane (ADAM), results in late elution, which does not interfere with analyte peaks (161,162). Reaction time with FMOC is very fast at approximately 90 seconds at room temperature. Representative references include 163–167.

NBD-F (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) λ_{ex} = 470 nm, λ_{em} = 530 nm. Derivatives are very stable (days) and can be formed from both primary and secondary amino acids. Fluorimetric detection limits in the low picomole range can be expected. Tryptophan adducts do not fluoresce. Reacts with other phenols and thiols to give nonfluorescent adducts. Reaction time

is relatively fast, 1 minute at 60°C. Reactivity of NBD-F is 100 times that for the corresponding chloride, NBD-Cl. This reagent has been used much more commonly for the determination of biogenic amines in foods than in amino acids. Representative references include 168–171.

Ninhydrin (2,2-dihydroxy-1,3-indandione) λ_{max} = 570 nm, λ_{max} = 440 nm. This reagent has already been described earlier in this chapter (Sec. III.A. Ion Exchange). Additionally, it should be noted that ninhydrin reagent solutions are susceptible to photodegradation and air oxidation. Older solutions will yield progressively poorer response as the ninhydrin oxidizes. It is an arbitrary decision when to swap a new solution for the old. Representative references include 172–174.

OPA (ortho-phthalaldehyde) λ_{ex} = 340 nm, λ_{em} = 455 nm. Derivatives are not stable (minutes). OPA is best used as a postcolumn derivatizing reagent. Precolumn use necessitates well-controlled and automated (mechanized) handling of the sample/reagents to ensure uniformity of time elapsed from reaction to injection. It reacts with primary amines only. Proline can be detected only if first converted to primary amine by oxidative reagents like chloramine T or sodium hypochlorite. Conversely, the concomitant reaction with OPA followed by FMOC obviates this problem and is the basis of the "AminoQuant" system marketed by Hewlett-Packard (175–177).

The reaction of OPA with amino acids (see Fig. 10) requires a mercaptan cofactor that is incorporated as part of the final derivative product. The choice of mercaptan can affect derivative stability and chromatographic selectivity (178). Mercaptoethanol is the most commonly used coreagent. Cysteine is not well detected, because this amino acid can react at the α-amine group or it can react via the side chain thiol. Thus, cysteine is determined only after conversion of the thiol group by oxidation or alkylation. Reaction time with OPA is very fast, 1 minute at room temperature. Detection limits are typically in the low picomole range. Representative references include 179–183.

PITC (phenylisothiocyanate) λ_{abs} = 254 nm. Phenylthiocarbamyl amino acid derivatives are moderately stable at room temperature (1 day). PITC reacts well with both primary and secondary amino acids. Reaction time is approximately 5 minutes at room temperature. Excess reagent must subsequently be removed under vacuum. Also, for hydrolyzed samples, hydrochloric acid must be completely removed prior to derivatization. As a result, even though the actual reaction time is reasonably fast, the total time for various sample manipulations can add up to 2 hours. This is partially compensated by the extremely fast separation possible (12 minutes). Detection is by UV absorption only. Detection limits are typically in the high picomole range. Short column life can result due to unreacted PITC getting into the column. Unlike some of the other reagents, PITC quantifies tyrosine and histidine very well. PITC analysis is available as a commercially prepackaged system dubbed "Pico-Tag" by Waters Corporation. Representative references include 184–188. See Fig. 11 for a typical separation.

Fig. 10 Reaction of amino acids with OPA.

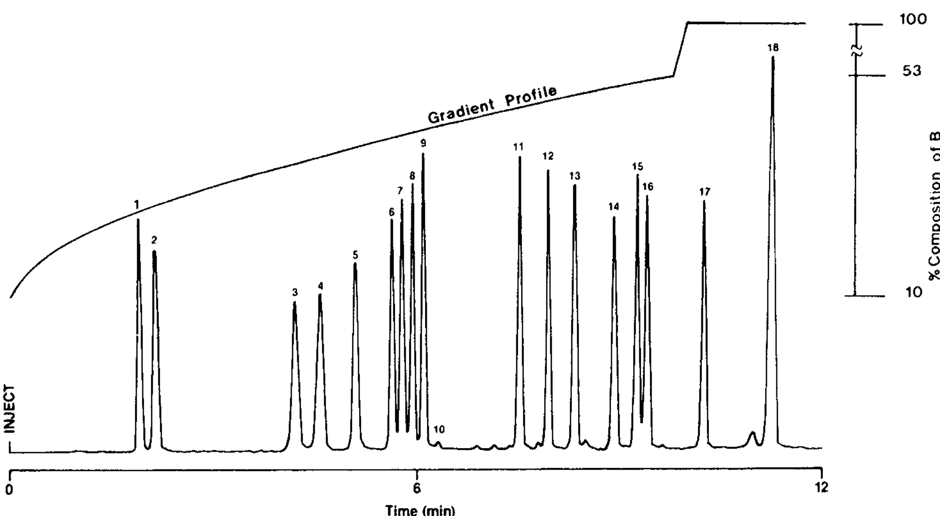

Fig. 11 Separation of amino acid standards derivatized with PITC. Eluent A: sodium acetate/triethylamine buffer (pH 6.4); eluent B: 60/40 acetonitrile/water. Pico-Tag column. Peak identification: 1 Asp, 2 Glu, 3 Ser, 4 Gly, 5 His, 6 Arg, 7 Thr, 8 Ala, 9 Pro, 10 ammonia, 11 Tyr, 12 Val, 13 Met, 14 Cys, 15 Ile, 16 Leu, 17 Phe, 18 Lys. (From Ref. 184. Copyright 1984 Elsevier Science.)

V. APPLICATIONS IN FOOD

This final section of the chapter will be composed primarily of a tabulated literature survey categorizing references based on the type of sample analyzed and the chemistries employed for separation/detection. However, because of its unique nature (and because this topic didn't fit well into any other section of the chapter), special attention will be paid to the analysis for taurine and its application in nutritional products.

A. Taurine

2-Aminoethanesulfonic acid is a β-amino acid that is not incorporated into proteins (see Fig. 12). It is even more untraditional, since it does not contain a carboxylic acid moiety. Taurine is implicated in a number of important biochemical processes, a principal one being the conjugation with bile acids to form bile salts essential for fat adsorption. Additionally, taurine appears to be involved in regulation of the central nervous system and is crucial for proper brain and retinal development (189). There is an increasing recognition that taurine is a conditionally essential nutrient (190,191). Neonates and infants exhibit a limited ability to synthesize taurine due to the incomplete development of their enzyme systems. As such, the demand for taurine by infants often exceeds de novo metabolic supply (192). This recognition has resulted in the supplementation of infant formulas (and parenteral nutritional products) with free taurine. It should also be noted that taurine is recognized as essential for cats and is typically added to cat foods.

Naturally, a variety of methods have been developed to address the increasing need to determine taurine content in these nutritional products (also to catalog levels in naturally occurring foods). A smattering of the more recent studies will now be mentioned. Many of the chro-

$$H_3\overset{+}{N}-CH_2-CH_2-SO_3^-$$

Fig. 12 Chemical structure of taurine.

mophoric derivatizing reagents, mentioned earlier, can be found in the literature. Early attempts to quantify taurine were accomplished using the classic ion-exchange amino acid analyzer followed by postcolumn derivatization with either ninhydrin or OPA (193–196).

Since then, there have been a number of reversed-phase separations employing precolumn derivatization. Interestingly, fluorescamine (not frequently employed for RP-HPLC of amino acids with precolumn reaction) has been reported for taurine analysis in milk (197) and human plasma (198). Precolumn derivatization with OPA/2-mercaptoethanol has been reported for the analysis of infant formula and human breast milk (199). Although not the principal focus of the study, Carratu et al. (200) report taurine values in parenteral solutions as determined by FMOC. In an excellent article, Woollard and Indyk (201) report the dansylation of taurine for its determination in a wide variety of dairy-related products. Subsequently, the same authors report the results of a large collaborative study (202) for the determination of taurine (again, by dansylation) in milk and infant formula. This study afforded an overall interlaboratory RSD of 7.0% and established a lower limit for determination at 5 mg taurine per 100 g of product.

B. Tabulated Survey

Tables 5 and 6 simply categorize additional references (plus a few already cited) based primarily on the type of food (sample matrix) that has been analyzed. This survey is not comprehensive and

Table 5 Total Amino Acid Analysis in Foods

Sample	Separation and detection chemistry	Refs.
Soybeans and eggs	RP-HPLC, butylisothiocyanate (BITC)	203
Animal feeds	RP-HPLC, PITC	5,204,205
	RP-HPLC, AQC	147,206,207
	RP-HPLC, OPA/ethanethiol	208,209
	RP-HPLC, FMOC	210
	Ion exchange, ninhydrin	25,211
Peas, lentils, beef, etc.	RP-HPLC, PITC	212
Infant formula	RP-HPLC, PITC	186,213,214
	RP-HPLC, PITC (cysteine and methionine only)	215
	RP-HPLC, PITC (tryptophan only)	216
Infant formula (microwave heated)	RP-HPLC with L-phenylalaninamide/Cu(II) in mobile phase, dansylated D,L-amino acids	217
Human milk	RP-HPLC, precolumn OPA	218
Chestnuts	Ion exchange, ninhydrin	219
Kelp	RP-HPLC, PITC	220
Green beans	RP-HPLC, PITC	221
Honey	RP-HPLC, detection not specified	222
Beer	Ion exchange, ninhydrin	223
African oil beans	RP-HPLC, PITC	224
Collagen	RP-HPLC, AQC	225
Milk powder	Ion exchange, ninhydrin	65
Cheese	RP-HPLC, OPA/FMOC vs. ion exchange	119
Assorted variety	Ion exchange, ninhydrin	24,27,34
Soy	RP-HPLC, AQC	149
	Ion exchange, OPA	116
Tuna, soy	RP-HPLC, dansyl chloride	226

Table 6 Free Amino Acid Analysis in Foods

Sample	Separation and detection chemistry	Refs.
Beer, soy sauce	RP-HPLC, PITC	227
Sake, soy sauce	RP-HPLC, dansyl chloride	228
Fruits and vegetables	C_{18} with ion pairing, EC detection of native cyst(e)ine	229
	RP-HPLC, precolumn DTNB rxn with cysteine	230
Soup, oyster sauce, baby food, pistachios	RP-HPLC? (cyano column), conductometric determination of native glutamic acid	231
Wine	RP-HPLC, FMOC	232
	RP-HPLC, PITC	233,234
	RP-HPLC, OPA/*N*-isobutyryl-L-cysteine (IBC)	235
	RP-HPLC, dansyl chloride	157
Shrimp	RP-HPLC, OPA/NBD-Cl	236
Crustaceans (L,D-isomers)	RP with ion pair, D-amino acids by precolumn rxn with chiral fluorophore	237
Peanuts	Ion exchange, ninhydrin	238
Cheese (L,D-isomers)	RP-HPLC, OPA/IBC	239
Green tea	RP-HPLC, PITC	240
Bread(s)	RP-HPLC, dansyl chloride	241
Yogurt	RP-HPLC, intrinsic tryptophan fluorescence	242
Glutamic acid content of various foods	RP-HPLC, PITC	243
Potatoes, cat food, etc.	RP-HPLC, PITC	244
Fruit juice	RP-HPLC vs. IEC vs. GC method comparison	245
	Ion exchange, ninhydrin	246
	RP-HPLC, precolumn OPA	247
Wine, cheese, fish	RP-HPLC, FMOC	165
Potatoes	RP-HPLC, FMOC/OPA	182
Cheese, ham	RP-HPLC, dabsyl chloride	155
Beer	RP-HPLC, FMOC/OPA	175
	RP-HPLC, OPA with electrochemical detection	248
	RP-HPLC, precolumn OPA	249,250

is meant only to illustrate the many permutations of sample matrices, chromatographic modes, and detection schemes that can be found in the literature.

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3
HPLC of Peptides

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I. INTRODUCTION

Peptides are present naturally in foods, arising mainly from the partial degradation of protein polypeptide chains. Some peptides, like the dipeptides carnosine, anserine, and balenine in vertebrate muscle or glutathione in fruits, are not proteinic in origin. In other cases, peptides are present in foods because they are used as additives (sweeteners, flavor enhancers, bulking agents in light drinks, etc.).

Enzymatic hydrolysis of food proteins yields peptides that are of great interest to the food industry and are utilized for various purposes, e.g., improving the functional properties of foods, parenteral feeding (casein hydrolyzates), or milk protein substitutes in cases of intolerance.

The multiple functions of peptides in foods (antioxidants, antimicrobial agents, surfactants) and their role in the development of characteristic flavors (sweetness, bitterness), as well as the information they can provide about the genuineness of foods, make peptide analysis a necessity. Producers as well as government laboratories have considerable interest in the study of peptides, both for research purposes and for the control of raw materials and manufactured foods. For this reason, substantial attention is now being focused on the development of analytical techniques designed to separate, characterize, and quantify peptides.

II. HPLC VERSUS OTHER ANALYTICAL TECHNIQUES

Techniques such as mono- and bidimensional thin-layer chromatography and high-voltage electrophoresis have been used in peptide separations for many years. However, the versatility, short analysis times, and high resolution of high-performance liquid chromatography (HPLC), coupled with the possibility of automating the analysis process, make this analysis technique the method of choice in peptide analyses at the present time (1–6a).

Practically all known mechanisms have been employed in the separation of peptides by chromatography, i.e., separation based on molecule size (gel permeation or size-exclusion chromatography), charge (ion-exchange chromatography), hydrophobicity (reversed-phase and interaction chromatography), and even combinations of these mechanisms. However, reversed-phase (RP) chromatography is the most frequently employed. It affords the possibility of changing the

stationary phase, the pH, the ionic strength of the aqueous buffer, or the type of organic modifier, of using different gradient shapes, and of working at a variety of temperatures, making this method suitable for analyzing highly different peptides. Moreover, the option of using microcolumns (7,7a) makes it possible to detect quantities on the order of picomoles and has been an important step toward HPLC–mass spectrometry coupling. Electrophoresis may be more useful in analyzing hydrophobic peptides, because the insoluble peptides can be solubilized with the aid of detergents (8).

Another technique that may be considered a complement or alternative to HPLC in peptide analysis is capillary electrophoresis (CE). This method has a high resolving power (enabling the separation of peptides that differ in only a single amino acid), is simple and fast, uses small sample sizes, and permits quantitative analysis. Several commercial instruments have been brought out, which points to the great strides made by this method as a complement to HPLC. Peptide separation by CE is not yet a routine technique in food analysis, but several applications have been published (9,9a). Moreover, the application of two analytical techniques (HPLC-CE, SEC-CE) and coupling MS to HPLC have acquired great importance for characterization of complex peptide samples (9b–d). Several papers have been published about automated liquid chromatography in which RP-HPLC were used after others types of columns (inmunoaffinitty, immobilized enzyme, perfusion, size exclusion) and coupled with mass spectrometry for targeted component analysis (9e,f).

III. THEORY

A. Physicochemical Properties

Peptides are a very heterogeneous group of compounds that share the common characteristic of being made up of chains of amino acids joined by an amide bond, also known as a peptide bond. The large number of different amino acids that exists in nature and the large variations in the number of amino acids in a given peptide mean that peptides can be highly diverse.

The dividing line between peptides and proteins has not been sharply defined, and it is assumed by convention that a peptide has fewer than 100 residues (molecular weight < 10 kilodaltons). The authors have also made this assumption in the present chapter. Peptides with a low molecular weight (MW) of fewer than 10 residues are known as oligopeptides. Also by convention, the amino acid with the free amino group is placed at the left of the chain and is known as the N terminus; the amino acid with the free carboxyl group is placed at the right of the chain and is known as the C terminal.

In order to simplify peptide names, the amino acids that make up the chain are represented by three letters, which usually correspond to the first three letters of their English names, or by one-letter symbols.

The acid–base behavior of peptides (10) is determined by the free α-amino group on the N-terminal residue, by the free α-carboxyl group on the C-terminal residue, and by the ionizable R groups located at intermediate positions. The pK values of the terminal α-carboxyl groups are somewhat higher and those of the α-amino groups somewhat lower than those of the corresponding free amino acids (Table 1) (11).

The terminal amino and carboxyl groups react in the same way as the corresponding amino acids (acylation, amination, esterification, etc.). Some of the reactions of the amino groups (with ninhydrin, orthophthaldehyde, fluorescamine, etc.) are used for detection purposes, as will be discussed later. Peptides also react in ways that free amino acids do not, like the classic biuret reaction, which consists of the formation of a colored complex with a transition metal (Cu, Ni, etc.)

Table 1 Dissociation Constants and Isoelectric Points of Various Peptides (25°C)

Peptide	pK_1	pK_2	pK_3	pK_4	pK_5	pI
Gly-GLy	3.12	8.17				5.65
Gly-Gly-Gly	3.26	7.91				5.59
Ala-Ala	3.30	8.14				5.72
Gly-Asp	2.81	4.45	8.60			3.63
Asp-Gly	2.10	4.53	9.07			3.31
Asp-Asp	2.70	3.40	4.70	8.26		3.04
Lys-Ala	3.22	7.62	10.70			9.16
Ala-Lys-Ala	3.15	7.65	10.30			8.98
Lys-Lys	3.01	7.53	10.05	11.01		10.53
Lys-Lys-Lys	3.08	7.34	9.80	10.54	11.32	10.93
Lys-Glu	2.93	4.47	7.75	10.50		6.10
His-His	2.25	5.60	6.80	7.80		7.30

Source: Ref. 11.

in an alkaline medium and which can be measured quantitatively by spectrophotometry. This reaction is used in peptide quantification.

B. Sensory Properties

Peptides have a wide range of flavors, from bitterness to more desirable savory flavors, and their importance in the sensory perception of foods has been recognized. Table 2 shows an example of peptides with sensory properties.

Bitter peptides have been identified in hydrolyzates of casein (12,13), cheese (13a,b), and soy bean (14,15,15a). The bitter taste has been related to the hydrophobic amino acid content (16–20) and to chain length. Ney and Retzlaff (21) established a formula relating the bitterness of peptides to their amino acid composition and chain length. Too large a proportion of hydrophobic amino acids gives rise to bitterness; yet above a certain molecular weight, bitterness is not perceptible even when there are hydrophobic amino acids (21). Peptides that were responsible for bitterness in Cheddar cheese were rich in Pro, which occurred predominantly in the penultimate position (21a).

Otagiri et al. (22) used model peptides composed of arginine, proline, and phenylalanine to ascertain the relationship between bitter flavor and chemical structure. They reported that the presence of the hydrophobic amino acid at the C terminus and the basic amino acid at the N terminus brought about an increase in the bitterness of di- and tripeptides. They further noted a strong bitter taste when arginine was located next to proline and a synergistic effect in the peptides $(Arg)_l$-$(Pro)_m$-$(Phe)_n$ ($l = 1, 2$; $m, n = 1, 3$) as the number of amino acids increased. Birch and Kemp (23) related the apparent specific volume of amino acids to taste.

Some peptides enjoy the property of masking the bitter taste of foods. Ohyama et al. (24) conducted sensory analyses using synthetic peptides and found that neutralized peptides consisting of aspartic acid and glutamic acid had a taste similar to that of monosodium glutamate. They termed this "umami taste" or "relish."

Other peptides, such as L-aspartyl-L-phenylalanine methyl ester (aspartame), have a sweet taste. Several studies have been carried out to relate the structure and taste of analogs of this dipeptide (25). Tsang et al. (26) reported that the analogs at the lower end of the L-aspartyl-α-aminocycloalkanecarboxylic acid methyl ester series were sweet, the dipeptides containing α-

Table 2 Examples of Peptides with Sensory Properties

Peptide	Structure	Activity
Aspartame	Asp-PheOMe	Sweet
Alitame	L-Asp-D-AlaNH_2	Sweet
Sweet lysine dipeptides	*N*-Ac-Phe-Lys *N*-Ac-Gly-Lys	Sweet
Aminomalonate dipeptides	D-Ama-L-PheOMe D-Ama-L-PheOEt	Sweet
Soy protein peptides	Gly-Leu, Phe-Leu, Leu-Phe, Leu-Lys, Arg-Leu, Arg-Leu-Leu	Bitter
Casein tryptic peptides	Gly-Pro-Phe-Pro-Val-lle	Bitter
Cheese peptides	Lys-Pro, Phe-Pro, Val-Pro, Leu-Pro	Bitter
Gamma-glutamyl peptides	Glu-γ-Gly, Glu-γ-Ala, Glu-γ-Glu	Sour/astringent
Fish protein hydrolysates	Glu-Glu, Ser-Glu-Glu, Glu-Ser, Thr-Glu, Glu-Asp, Asp-Glu-Ser	*Umami*
Acidic peptides	Gly-Asp, Gly-Asp-Gly, Ala-Glu, Ala-Glu-Ala, Val-Glu-Val, Glu-Leu	*Umami*
Basic peptides	Orn-Tau.HCl, Lys-Tau.HCl, Orn-Gly.HCl, Lys-Gly.HCl	Salty/*Umami*
Buffering peptides	Gly-Leu, Pro-Glu, Val-Glu, β-Ala-His	Flavor enhancers
Glutamate oligomers	Glu, Glu, Glu-Glu-Glu	Bitterness-masking agents

Source: From Ref. 11a, with permission.

aminocyclohexanecarboxylic acid methyl ester and α-aminocycloheptanecarboxylic acid methyl ester were bitter, and analogs containing α-aminocyclooctanecarboxylic acid methyl ester were tasteless. Rodriguez et al. (27) also found that a series of L-aspartyl-D-alanyl tripeptides went from sweet to bitter to tasteless as the size of the ring of the C-terminal amino acid increased. There is, thus, a close relationship between size and taste in these peptides. Peptides belonging to L-aspartyl-D-alanyl amides are strong sweeteners and may be used as good sugar substitutes (27a).

Low-molecular-weight peptides play an important role in the flavor intensity of meat and beef broth (27b). A beefy meat peptide isolated of beef imparts desirable sensory properties and has potential as a flavor enhancer in heat-processed foods (27c,d). Peptides released in dry-cured ham during processing were evaluated by HPLC and related to the ham flavor formation (27e,f).

C. Functional Properties

The functional properties of some proteins are improved in certain enzymatic hydrolyzates of those proteins, and these hydrolyzates can be used as additives to enhance food quality. Resulting peptides have lower molecular weight and fewer secondary structures, as well as a higher number of ionizable groups and exposure of hydrophobic groups, than native proteins. These facts imply that solubility, surface activity, foaming, and emulsifying properties may be different from those of the intact protein (28). Because size control and hydrophobicity are important factors in preparing functional peptides, HPLC is an essential technique for characterization of functional food peptides (28a,b).

Certain protein hydrolyzates can be used for their antioxidant properties. Thus, a hydrolyzate of egg white slowed oxidation when added to a sample of cookies containing lard (28c). The rise in stability was proportional to the amount of protein hydrolyzate added. Dipeptides were

also found to have a greater inhibiting effect on the autoxidation of linolenic acid than did mixtures of the corresponding free amino acids (28c). Antioxidative activity of peptide fragments from soybean protein and carnosine were also established (28d–f). Antioxidative peptides from soybean showed sinergistic effects with nonpeptidic antioxidants.

Adler-Nissen and Eriksen (29) established that the enzymatic hydrolyzates of soybean proteins conferred interesting functional properties on beverages. They pointed out that medium-size peptides had a marked bulking effect, while large peptides acted as foaming agents. Other workers have also reported that polypeptides and proteins have an effect on foam stability (28,30). The separation of these peptides by HPLC, in order to determine their foaming activity, may be of great interest in relation to certain beverages, such as sparkling wines and beer, in which foam formation plays an important role.

Increases in the emulsifying capacity of various proteins have also been brought about by enzymatic hydrolysis. Shimizu et al. (31) studied the emulsifying properties of such peptides by isolating a peptide that contained 23 residues from the peptic hydrolyzate using of α_{s1}-casein. This peptide fraction displayed an emulsifying capacity similar to that of α_{s1}-casein at a neutral pH and concentrations greater than 2%. Subsequently, using HPLC, Shimizu et al. (32) reported that this peptide fraction contained small quantities of other peptides that interfered with emulsifying activity. The emulsifying activity of these peptides would seem to be synergistic, since the purified peptide exhibited a lower emulsifying capacity. Such a sinergistic effect was also mentioned by Caessens et al (28). Hydrolysates of hydrophobic proteins from gluten (zeins, gliadins) have been prepared with a view to increasing solubility and diversifying their functional properties (28b,32a). Functional properties of caseinmacropeptide and peptides from milk protein hydrolysates have been widely studied (32b–d).

IV. METHODS OF SAMPLE PREPARATION FOR HPLC ANALYSIS

All systems for purifying samples to be used in the HPLC analysis of peptides from foods require two prior steps, extraction and deproteinization, and a series of stages that differ according to the degree of polymerization of the peptides to be analyzed. When analyzing low-molecular-weight peptides, the anionic compounds are usually separated first, after which the amino acids are separated from the peptides. This stage is not necessary in the case of high-molecular-weight peptides, which do not elute together with the amino acids. After the peptide fraction has been isolated, but prior to chromatographic analysis, derivatization of the peptides is sometimes carried out to improve detection. In any event, samples are always filtered through membranes with a pore size of less than or equal to 0.50 μm.

Extraction is an essential step when analyzing solid samples. In some cases homogenization with a solvent suffices, but in others the sample must first be comminuted. Water, solutions of acetic acid or sodium chloride, or more complex saline solutions are used as solvents. Mixtures of water and methanol or water and ethanol are also employed. The choice of solvent depends on the degree of selectivity desired in the extraction and whether the extraction yield is intended for quantitative analysis. Optimization of the extraction procedure is required in all cases, to fit the nature of the sample to be analyzed and the range of molecular weights of the peptides to be separated. For example, water has been used as the extraction solvent for cheese (33) and legumes (34). Saline solutions have been utilized to extract peptides from meat (35–38) and flour (39,40). Benedito de Barber et al. (41) examined differences in the extractability of amino acids and short peptides in various solvents (1M acetic acid, 70% ethanol, and distilled water); they concluded that extraction with 1M acetic acid yielded the maximum amino acid and peptide contents.

The extraction of hydrophobic peptides is normally performed using organic solvents. Several authors have extracted bitter peptides from cheese using a mixture of chloroform and methanol (2:1) (42–44).

To separate the peptides from the proteins, proteins have been precipitated by means of solutions of ethanol, methanol, acetone, or acids [trichloroacetic (TCA), sulfosalicyllic (SSA), phosphotungstic (PTA), picric], followed by centrifugation or filtration.

Yvon et al. (45) studied the solubility of 75 peptides from hydrolysates of casein in solutions of 1, 4, 8, and 12% trichloroacetic acid. They found 36 completely soluble peptides and 6 completely insoluble peptides at all concentrations of TCA and 30 peptides whose solubility fell as the TCA concentration rose. They attempted to relate solubility to size, hydrophobicity, and charge at a given pH and reported that solubility decreased with size in 2% TCA but was unrelated to molecular weight in 4, 6, and 12% TCA.

When 5% phosphotungstic acid was used as the precipitant agent in cheeses, the soluble fraction consisted only of amino acids and low-molecular-weight peptides (46,47).

Ultrafiltration is a simple method of fractionating peptides and removing proteins (28, 28a,47a). A wide variety of membranes of different materials, filtration surfaces, and exclusion sizes is commercially available, enabling samples ranging in size from several microliters to liters to be fractionated. The procedure can be aided by using peristaltic or centrifugal pumps, depending on the volumes involved. A preparative procedure including water extraction, membrane ultrafiltration, and RP-HPLC separation to isolate bitter peptides in cheddar cheese was developed by Lee and Warthesen (47b).

A. Separation from Peptides of Sugars, Organic Acids, Salts, Etc.

When peptides with an MW of less than 1000 are to be studied, it is advisable to separate them from sugar, organic acid, salts, etc. This step is not necessary for all food samples, though it is normal for flour and bread dough (39–41,48) and is also used for vegetables (49,50). A first step is usually passage through an anion-exchange resin, in which the amino acids and peptides are not retained, followed by passage through a cation-exchange resin, in which any anions that may have passed through with the eluate from the first column are not retained, and the amino acids and peptides are retained and elute later.

Bio-Rex membranes (AG1, AG50), which are crosslinked anion- and cation-exchange resins enmeshed in polytetrafluoroethylene, are an alternative to ion-exchange resins. These membranes achieve the same effectiveness as open ion-exchange columns, but analysis times are shorter (51).

When precipitation of the proteins has been carried out using salts, these should preferably be removed. Dialysis is one of the simplest methods for removing the salts.

B. Separation of Amino Acids and Peptides

When low-molecular-weight peptides are to be analyzed, it is advisable to separate them from the amino acids, since these elute together with the peptides, complicating the chromatograms. Ion-exchange chromatography on DEAE-cellulose and DEAE-sephadex with Cu^{2+} complexes or ligand-exchange chromatography on Cu^{2+}-modified stationary phases can be used in such separations (48). Chelex resins with chelating capacity or Chelex ion-exchange membranes are used to remove the trace metals (Ca^{2+}, Cu^{2+}, Mg^{2+}, etc.) from the peptides.

González de Llano et al. (47) separated amino acids from low-molecular-weight peptides by means of size-exclusion chromatography on Sephadex G-10, with water as the solvent, as a preparatory step before RP-HPLC analysis of peptides from blue cheeses soluble in 5% PTA (Fig. 1). This technique has also been used (51a) to eliminate the amino acids from the ethanol-

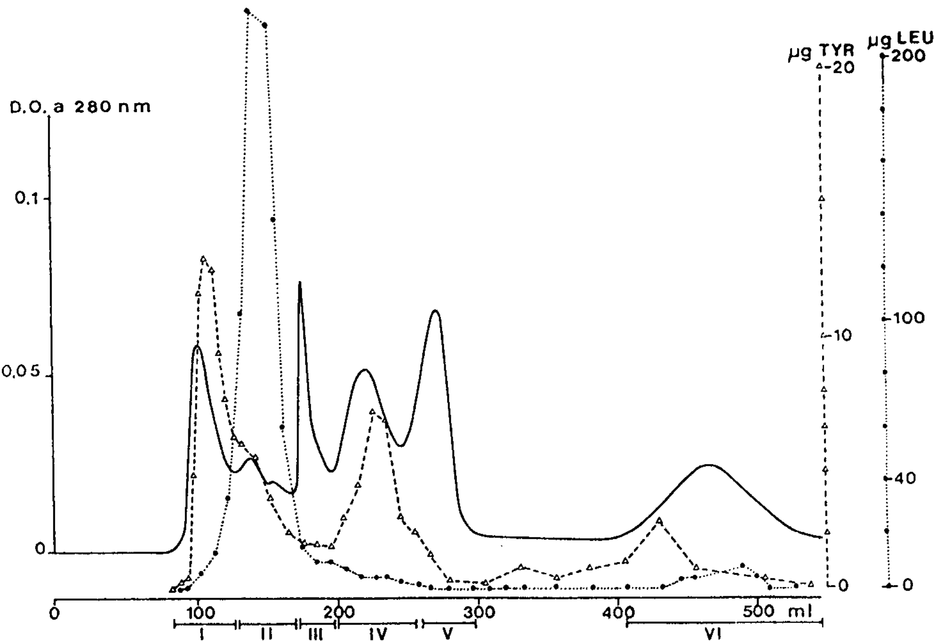

Fig. 1 Elution profile of the PTA-soluble nitrogen of Cabrales cheese on Sephadex G-10 using Milli Q water as eluent.

soluble fraction from white wines and to fractionate the peptides in two groups with molecular mass higher and lower than 700 Da, respectively, before the HPLC analysis.

Passage through Sep-Pak C_{18} cartridges has been used as a final step prior to analysis by means of liquid chromatography (34,52). Solid-phase extraction procedures for peptide isolation and fractionation based on polar and ionic interactions were evaluated by Herraiz and Casal (52a). Higa and Desiderio (53) made a thorough study of the recovery of peptides from a Sep-Pak C_{18} cartridge, examining both the migration time of the sample through the cartridge and the elution time. They found that recovery of the peptides was constant at all flow rates using nanomoles of sample but greater at lower flow rates when femtomoles of sample were used, and they concluded that experimental conditions had to be optimized for each peptide.

V. SEPARATION MECHANISMS USED IN HPLC ANALYSIS OF PEPTIDES

A. Reversed-Phase (RP) Chromatography

The use of reversed-phase liquid chromatography is growing in applications for separating mixtures of peptides. In this type of chromatography, the stationary phase is nonpolar, whereas the mobile phase is polar. The stationary phase is normally porous silica with bonded *n*-alkyl chains, mainly octadecyl but also octyl, hexyl, butyl, and propyl chains.

Although the silica-based columns are the most widely used in RP-HPLC separations of peptides, the use of polymeric carriers (polystyrene divinylbenzene) and composite materials (silica particles with a polymeric coating), which are more chemically stable in that they do not break down at pH values higher than 8 as silica does, is gaining currency (54,55). The mobile phase usually consists of a mixture of water and an organic solvent, generally acetonitrile, methanol, or

isopropanol, but other organic solvents, such as methoxyethanol, ethanol, butanol, and tetrahydrofuran, are also used. The RP-HPLC analysis of peptides normally requires an elution gradient (32,43,47,56–62a), though isocratic separations have also been performed.

Because of their high polarity, peptides do not interact sufficiently with the hydrophobic chains of the stationary phase in reversed-phase chromatography. On the other hand, they do interact with free silanol groups present in the stationary phase. In order to block these silanol groups, salts or strong acids must be added to the mobile phase. To reduce the polar nature of the peptides, the chromatographic procedure should be carried out at a pH lower than 3, so that the carboxyl groups of the amino acids (aspartic acid and glutamic acid) are not present in dissociated form. However, at these pH values the basic amino acids histidine, lysine, and arginine are charged, and they must therefore be blocked through the formation of ion pairs. The selectivity of chromatographic systems can be modified according to the type of ion-pair-forming reagent employed and the pH (63).

Since its introduction by Bennet et al. (64) as the ion-pair-forming reagent for preparatory purposes, trifluroacetic acid (TFA) has been widely used in peptide separations. The advantages over other commonly used acids and buffers afforded by TFA for the elution of peptides (65) is that it is transparent to ultraviolet light, an excellent solvent for large peptides (thereby facilitating their recovery during preparatory chromatography), a strong ion-pair former, and volatile (therefore easily removed by lyophilization), and it does not block the terminal amino groups, so derivatization of the peptides is possible. It is also miscible with most organic modifiers. Concentrations of TFA of around 0.1% are normally employed, although concentrations of 1–2% have sometimes been used (66). Acids (phosphoric, formic, hydrochloric, heptafluorobutyric) and quaternary ammonium salts are also used as ion-pair formers (6a,63). Food-grade HPLC mobile phases suitable for subsequent tasting of eluted components were developed by Lee and Warthesen (66a). Acceptable resolution of peptides was observed when acetonitrile was substituted with absolute ethanol and TFA was replaced with food-grade HCl.

The retention times of peptides with fewer than 20 residues in reversed-phase chromatography can be predicted with a high degree of accuracy based on their amino acid composition and the characteristics of their N-terminal and C-terminal amino acids. A number of researchers (66–75) have studied the role of amino acids in peptide retention and have established retention coefficients for the different amino acids. The retention coefficient value of each amino acid is normally calculated by regression analysis of the retention times for peptides of known composition.

Table 3 (73) compares the retention coefficients for synthetic peptides from various sources. To ensure comparability, the data has been standardized with respect to lysine and assigned a value of 100. The table shows that there are discrepancies between the results obtained using different chromatographic systems. Predictions of retention times should therefore be made using chromatographic systems similar to those used to calculate the retention coefficients for the amino acids. Casal et al. (75a) have made a comparative study of the prediction of the retention behavior of small peptides in several columns by using partial least squares and multiple linear regression analysis.

The retention time predicted for a given peptide is equal to the sum of the retention coefficients for each residue and the terminal group, plus t_o (the elution time for an unretained compound) and t_s (the difference between the observed t_s and the calculated t_r for a standard peptide). The retention times predicted for peptides with more than 20 residues are poorly correlated with experimental data, because the retention coefficients do not take steric factors into account. Moreover, the peptides may be denatured through the action of the solvents.

Reversed-phase columns with a pore size of between 60 and 100 Å are highly appropriate for separating small peptides. For separating large peptides (MW > 4000), columns with pore

Table 3 Comparison of Predicted Retention Coefficients of Amino Acid Residues

	Retention coefficient at pH 2 relative to Leu taken as 100				
Amino acid	I	II	III	IV	V
Trp	109	136	82	−9	157
Leu	100	100	100	100	100
Phe	100	119	96	80	131
Ile	91	104	33	184	73
Met	68	55	28	113	42
Val	62	28	18	34	48
Tyr	56	80	30	28	70
Cys	32	—	−46	−40	48
Pro	25	30	26	−13	32
Ala	25	9	37	−4	10
Glu	14	10	−36	−24	11
Thr	7	28	4	−25	−6
Asp	2	0	−15	61	−5
Gln	0	12	−2	14	−21
Gly	−2	15	−6	−26	2
Ser	−2	4	−21	21	−29
Arg	−7	0	−18	−43	−20
Asn	−7	−43	−29	−56	−31
Lys	−26	−12	−19	−17	−30
His	−26	33	−11	−85	−23
α-Amino	−85, −37[a]	—	21	49	9
α-COOH	−10	—	12	49	17

[a] The charged α-amino group had a smaller effect in an N-terminal Arg residue than in an N-terminal residue with an uncharged side chain.

Conditions: (I) Synchropak RP-P C_{18} column (250 × 4.1 mm i.d.), gradient (A = 0.1% aq. TFA. B = 0.1% TFA in acetonitrile) at 1% B min flow rate, 1 ml/min, 26°C; (II) Waters μBondapak C_{18} column (300 × 4.0 mm i.d.), gradient (A = 0.1% aq. TFA, B = 0.07% TFA in acetonitrile) at 1% B min flow rate, 2 ml/min; (III) Waters μBondapak C_{18} column gradient (A = 0.1% aq. TFA, B = 0.1% TFA in acetonitrile) at 0.33% B min flow rate, 1.5 ml/min; (IV) Waters μBondapak C_{18} column gradient [A = aq. 50 mM NaH_2PO_4, B = A-acetonitrile (1:1)] at 0.83% B min flow rate, 1 ml/min 18°C; (V) Bio-Rad C_{18} column (250 × 4.0 mm i.d.) gradient (A = aq. 0.1 M NaH_2PO_4-0.2% H_3PO_4, B = 0.1% H_3PO_4 in acetonitrile) at 0.75% B min flow rate, 1 ml/min, room temperature.

Source: Ref. 73.

sizes of between 300 and 500 Å yield better results, because the molecules may pass freely through the pores, the alkyl chains thus remain more accessible, and column efficiency and loading capacity are enhanced. The literature dealing with high-molecular-weight peptides in foods includes instances of the use of such columns (56,58,62).

B. Ion-Exchange Chromatography (IEC)

The separation of peptides by means of this technique makes use of two types of ion exchangers: cation exchangers to separate neutral and basic peptides (76,76a,b) and anion exchangers to separate neutral and acid peptides (77). Some workers have separated peptides in polymeric cation-exchange resins (polystyrene divinylbenzene) (78), and automatic peptide analyzers similar to those used in analyses of amino acids have even been designed, with postcolumn detection using

ninhydrin. The possibility of using volatile solvents facilitates the separation of peptides for subsequent study.

The polystyrene divinylbenzene (PS-DVB) columns used to separate polypeptides have to have a low percentage of crosslinking and cannot handle high flow rates. Consequently, these columns have been relegated to analyses of low-molecular-weight peptides and amino acids, which require a higher degree of crosslinking, and this limitation on the flow rate does not exist. With the introduction of silica-based ion-exchange columns, the deformation effects encountered using PS-DVB resins have been obviated.

C. Size-Exclusion Chromatography (SEC)

This chromatographic method separates substances based on their size: Large molecules emerge from the column first; smaller molecules are retained for longer times.

The material used as the stationary phase may be inorganic, e.g., silica or alumina. The active surfaces of these materials have the drawback of adsorbing charged molecules, and hence they must be deactivated, for instance, with organosilanes. Organic materials such as methacrylate-glycerol, sulfonated styrene divinylbenzene, and polyesters with hydroxyl groups have also been used. Although this chromatographic technique has been applied mostly in the separation of proteins, it has been employed in the separation of peptides. For example, Vijayalakshmi et al. (79) separated peptides with molecular weights of between 2000 and 3000 using a hydroxylated polyester column (TSK-2000) with a 50-mM phosphate buffer containing 0.1% TFA and 35% methanol as the solvent. Subsequently, Lemieux and Amiot (61) used this chromatographic system as a method for obtaining peptide fractions from phosphorylated and dephosphorylated casein hydrolysates, which were then separated by means of reversed-phase chromatography.

The different methods of separation (RP-HPLC with small-pore or large-pore packing, IEC, and SEC) are complementary, and better separations are sometimes obtained using combinations of the various techniques.

VI. DETECTION SYSTEMS

A. Ultraviolet Detection

Peptides are usually detected by absorbance at between 200 and 220 nm at concentrations of between 100 and 1000 ng (80). Since many solvents and even other components in the samples absorb at these wavelengths, samples must be carefully purified, and the solvents used must be transparent to ultraviolet light. Detection of peptides with aromatic amino acids (Phe, Tyr, Trp) can be carried out at 254 nm; if tyrosine or tryptophan is present, detection at 280 nm is feasible. In some cases detection has been carried out at 230 nm (59).

The introduction of rapid-scanning UV-VIS detectors based on diode-array technology has been of great assistance in confirming the identity of peptides that contain aromatic residues (Phe, Tyr, and Trp). The advantage of these detectors lies in their ability to store spectra for subsequent use.

Second-order derivatives of the spectrum of Phe, Tyr, and Trp present characteristic absorption minima at 257, 280, and 290 nm. In addition, each aromatic amino acid has other minima of lower intensity: 250 and 264 nm for Phe, 272 nm for Tyr, and 268 and 278 nm for Trp (81). In peptides composed only of Tyr and Phe, the spectral contribution of each aromatic amino acid to the derivative of the peptide spectrum can be distinguished quickly by the Phe (257 nm) and Tyr (278 nm) absorption minima. However, identification of Tyr in the presence of Trp is unclear,

because of spectral interference by the Trp minimum (81). The ratio between absorbance at the two wavelengths 255:270 nm or 255:265 nm is also a useful aid in identifying aromatic amino acids or combinations thereof (82). Wavelengths of the spectrum maxima, the convexity interval, and wavelengths of the second-derivative spectrum maxima—all over the 190–340-nm range—allow identification of the aromatic amino acids that form the peptides as well as identification of HPLC coeluted compounds, such as the cinnamic derivatives detected in the peptide fractions of wines (82a).

In order to avoid the difficulties associated with ultraviolet detection in the region of 200–220 nm, derivatives that can be detected at higher wavelengths, and that are therefore more specific for given chromophore groups, are often formed in peptide analysis using derivatizing agents. The major amine derivatizating agents that have been applied to peptide analysis have been recently reviewed by de Anthonis and Brown (82b). Table 4 summarizes the major peptide derivatizing agents, the characteristic of each reagent, and the different detection systems used for peptide analysis.

Dansyl chloride and phenylisothiocyanate (PITC) are the derivatizating agents most used in UV detection. Dansyl chloride reacts with the primary and secondary amino groups of peptides in a basic medium (pH 9.5), forming dansylated derivatives that are very stable to hydrolysis but are photosensitive. The derivatives are detectable in UV at 254 nm and by fluorescence. Dansyl sulfonic acid is formed as a by-product of the reaction, and excess reagent reacts with the dansyl derivatives to form dansyl amide; the conditions of derivatization must therefore be optimized in order to avoid the formation of such by-products to the extent possible. The conditions of the reaction with dansyl chloride and of the separation of the derivatives thus formed have been thoroughly studied (83,84). Martin et al. (85) carried out derivatization using an excess concentration of dansyl chloride of 5–10-fold in a basic medium (lithium carbonate, pH 9.5) in darkness for 1 h.

Dansyl chloride has been widely utilized in peptide analyses to determine the N-terminal amino acid (60,86). Méndez et al. (87) suggested that derivatization of the peptides with dansyl

Table 4 Summary of the Peptide Derivatizing Agents

	Detection mode[a]	Sensibility	Derivative stability	Reaction kinetics	1°/2° amines	Single peak/pep[b]
OPA	F, A, E	fmol	Poor	Rapid	1°	Yes
PITC	A	pmol	Good	Moderate	1°/2°	Yes
FMOC	F, A	fmol	Excellent	Rapid	1°/2°	No
DANSYL	F, A	pmol	Good	Slow	1°/2°	No
DABS	A	pmol	Excellent	Slow	1°/2°	Yes
NIN	A	pmol	N.A.[c]	Rapid	1°/2°	N.A.
FC	F, A	fmol	Poor	Rapid	1°/2°[d]	Yes
AQC	F, A	fmol	Excellent	Rapid	1°/2°	Yes
CBQCA	F, A	amol	Good	Slow	1°	No
NDA	F, A, E, C	amol	Excellent	Slow	1°	Yes

[a] F = fluorescence; A = absorbance; E = electrochemical; C = chemiluminescence.
[b] Yes = derivatizing agent reaction forms single derivatives for each target analyte; No = multiple peaks are formed for some analytes.
[c] N.A. = not applicable.
[d] 2° amines do not form fluorescent derivatives.
Source: Ref. 82b, with permission.

chloride could be used in peptide mapping, because the resolution of the dansylated peptides was satisfactory and the detection range was picomoles. One of the disadvantages of the use of dansyl chloride in peptide analysis is that it also reacts with alcoholic and phenolic hydroxyl groups.

Phenyl isothiocyanate reacts with both primary and secondary amino groups. The derivatives thus formed are stable and absorb over a broad region of the spectrum, with an absorption peak at 254 nm. It is employed basically to determine the peptide sequence by Edman degradation. The terminal NH_2 groups react with the PITC, forming a phenylthiocarbamylic derivative. When treated with acid in an organic solvent, cyclization takes place and phenylthiohydantoinamino acid is formed; this can be separated from the rest of the chain, which remains intact. The process can then be repeated.

Phenyl isothiocyanate has also been utilized to analyze small quantities of peptides or short-chain peptides (88) by carrying out sequential degradation with detection by dansylation. Using dansyl–Edman degradation, Leadbeater and Bruce-Ward (60) identified 60 tryptic peptides of β-casein that had previously been separated by high-voltage paper electrophoresis.

Phenyl isothiocyanate is also used for peptide sequencing in combination with colored Edman's reagent 4-*N*-,*N'*-dimethylaminoazobenzene-4'-isothiocyanate (DABITC). Prieto (48) used this DABITC-PITC procedure to determine the partial terminal N–NH_2 sequence of the low-molecular-weight peptide fraction from bread dough and bread.

This reagent has been used as a precolumn derivatizing reagent in the analysis of phenylisothiocarbamoyl derivatives of amino acids (89). The Waters Chromatography Division of Millipore (90) developed an automatic method (PICO-TAG) permitting analysis of the phenylthiocarbamoyl derivatives of amino acids in under 12 min, with a detection limit of picomoles.

Various applications of PITC in analyses of acid hydrolysates of purified proteins and peptides (91) and of hydrolysates of feeds (92) have been described. Bidlingmeyer et al. (93) showed reversed-phase chromatographic analysis of free amino acids and hydrolysates of foods (soybean flour, mozzarella cheese, beer, and soy sauce) with formation of PITC derivatives to be a fast, reproducible method that presented a very good correlation with the results obtained by ion-exchange chromatography.

B. Detection by Fluorescence

Peptides may also be detected by means of a fluorescence detector using the natural fluorescence of certain amino acids or by forming fluorescent derivatives. Tyrosine and tryptophan are fluorescent, and this property can be used to detect peptides that contain these amino acids. The excitation wavelength for these amino acids has been established to be between 220 and 280 nm, with the greatest sensitivity at 220 nm. In alkaline solutions the emission band is 365 nm for tryptophan and 310 nm for tyrosine. Peptides containing both these amino acids have been detected at an emission wavelength of 330 nm (94).

Some fluorescent derivatives are specific to certain functional groups. For example, peptides containing tyrosine may be detected by adding a —CHO group to the tyrosine of the peptide by formylation with chloroform in an alkaline medium, followed by reaction with 1,2-diamino-4,5-dimethoxybenzene. The derivatives thus formed are fluorescent and can be detected at an excitation wavelength of 350 nm and an emission wavelength of 425 nm (95).

Fluorescamine reacts instantaneously with the primary amino groups of peptides, yielding a fluorescent product with an excitation peak at 390 nm and an emission band at 475 nm. It is insoluble in water and is usually prepared in acetone. Neither the reagent nor the degradation products of the excess reagent in an aqueous medium are fluorescent, which is a great advantage, particularly when postcolumn derivatization is used.

The secondary amines also react with fluroescamine, but the reaction products are not fluorescent. The intensity of the fluorescence is unaffected at pH values of between 4 and 10, but it is affected by the composition of the solvent (96).

o-Phthaldehyde (OPA) is another reagent for primary amines. This water-soluble reagent reacts with the primary amines at an alkaline pH 9–11 in the presence of a thiol like 2-mercaptoethanol, 3-mercapto-1-propanol, or ethanothiol. The isoindole derivative thus formed is unstable but becomes stabilized in an acid medium. Neither the reagent nor its breakdown products are fluorescent, which again is an advantage, as in the case of fluorescamine. In order to prevent the appearance of spurious peaks in the chromatogram, the proportion of derivatizing reagent should be 50–200 times greater than the amino acid nitrogen content (97). The reaction between OPA and the —NH_2 groups takes place in 1 min, which makes this reagent extremely suitable for both precolumn and postcolumn online derivatization. Since the reaction products break down quickly and small differences in reaction time may result in large differences in the intensity of fluorescence, the reaction should be carried out automatically to prevent operator error. Frister et al. (98) used *N,N*-dimethyl-2-mercaptoethylammonium chloride as the thiol in the detection of amino acids, peptides, and proteins and concluded that the derivatives were more stable.

o-Phthaldehyde does not react with secondary amino groups and therefore does not react with peptides that have proline as the N-terminal amino acid. It reacts weakly with lysine, probably because of the formation of two fluorescent isoindole structures that interact and bring about internal quenching of the fluorescence. On the other hand, when lysine occupies an intermediate location on the peptide chain, the ϵ-NH_2 group forms a derivative with a fluorescent intensity nearly 50 times greater than that of non-lysine-containing peptides (94).

The reaction of amines and amino acids with orthophthaldehyde has been widely used in postcolumn and precolumn derivatization in analyses of foods (99–104) and in analyses of peptides from biological samples. Figure 2 (87) presents a chromatogram for OPA derivatives of tryptic peptides from two proteins. The sensitivity of the method was on the order of picomoles. The authors have themselves performed postcolumn OPA derivatization of low-molecular-weight peptides from blue cheeses separated by reversed-phase chromatography (86).

Online photodiode array detection and OPA-derivatization have been used to corroborate the peptidic nature of the peaks obtained by RP-HPLC and to identify the aromatic amino acid residues contained in wine peptides (104a). Figure 3 shows the flowchart proposed by the authors for the interpretation of both spectral data and OPA-fluorescence response.

Other reagents used lately for fluorescent derivatization of peptides are naphthalene-2,3-dicarboxaldehyde (NDA), 3-(4-carboxy-benzoyl) 2-quinolinecarboxaldehyde (CBQCA), and 6-aminoquinoyl-*N*-hydroxysuccimidylcarbamate (AQC) (6a,82b). Peptides from dry-cured Parma ham were detected using precolumn derivatization with AQC (27f). This reagent reacts with primary or secondary amino groups and is advantageous compared to OPA or PITC because the excess reagent cannot be seen in the chromatogram.

C. Mass Spectrometry

Mass spectrometry (MS) is an analytical technique of great interest, one that provides structural information and quantitative data not easily obtained by other techniques. In view of these advantages, mass spectrometers have been widely used as detectors in gas chromatography; however, adapting them for use with HPLC systems has been more difficult, because the sample is not in the gaseous phase and the solvent must be removed prior to ionization. These difficulties have been overcome by the development of a number of sample-introduction and ionization tech-

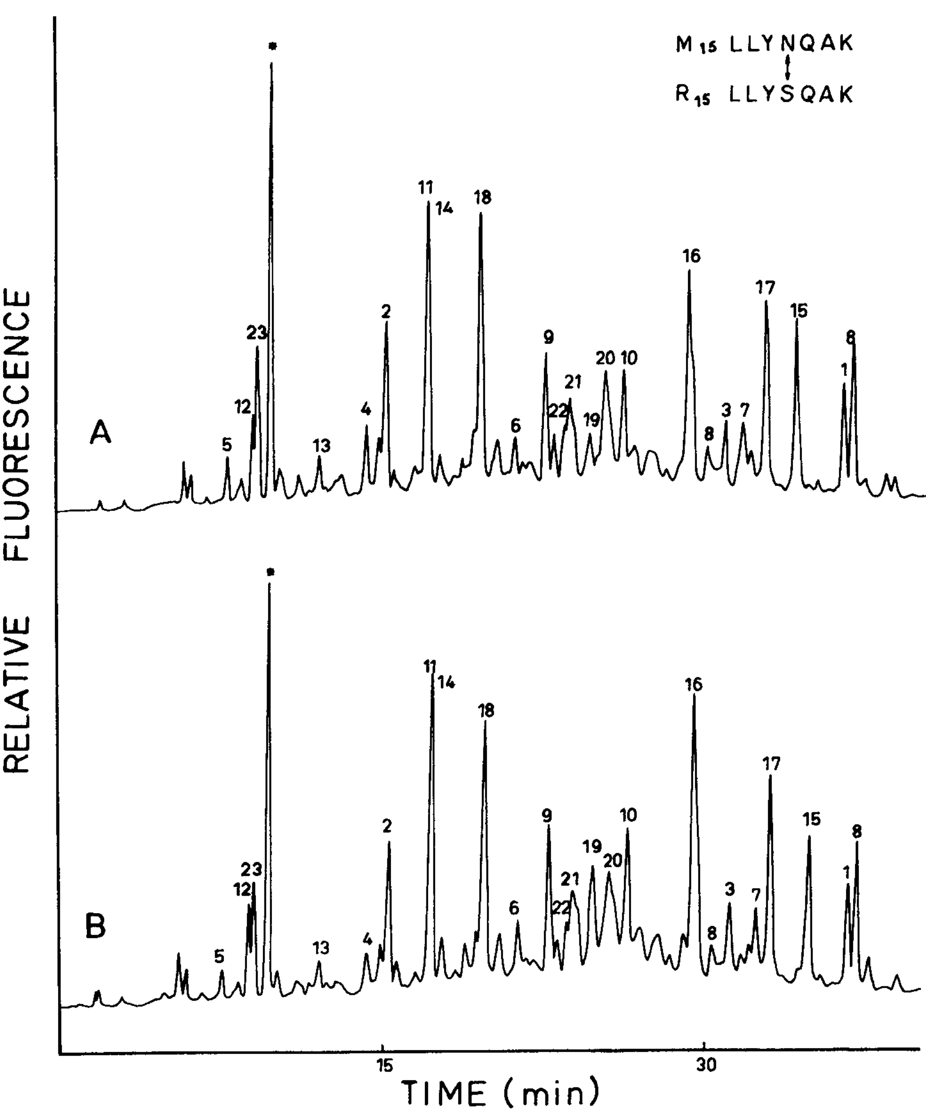

Fig. 2 Distributions of OPA-derivatized amino acids and peptides chromatographed by the automatic on-line OPA/2-mercaptoethanol system. A and B: 50 pmol of tryptic peptide digest of proteins M and R, respectively. The peak marked with an asterisk is due to the derivatizing reagents. Column: 5-μm Resolve C_{18} (15 cm × 3.9 mm). Emission at 425 nm and excitation at 338 nm. A comparison between the sequences of peptides M_{15} and R_{15} is also shown.

niques, e.g., thermospray (TSP), direct liquid introduction (DLI), moving-belt transport devices, continuous-flow (or dynamic) fast atom bombardment (FAB), atmospheric pressure ionization systems, plasmaspray, and electrospray ionization (ESI). The advantage of ESI/MS in the analysis of high-MW biopolymers lies in the formation of multiple-charged molecular ions and the compatibility with liquid phases. The coupling of electrospray ionization spectrometric detection online with HPLC is a method referred to as LC-MS. Presently, electrospray ionization and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, soft

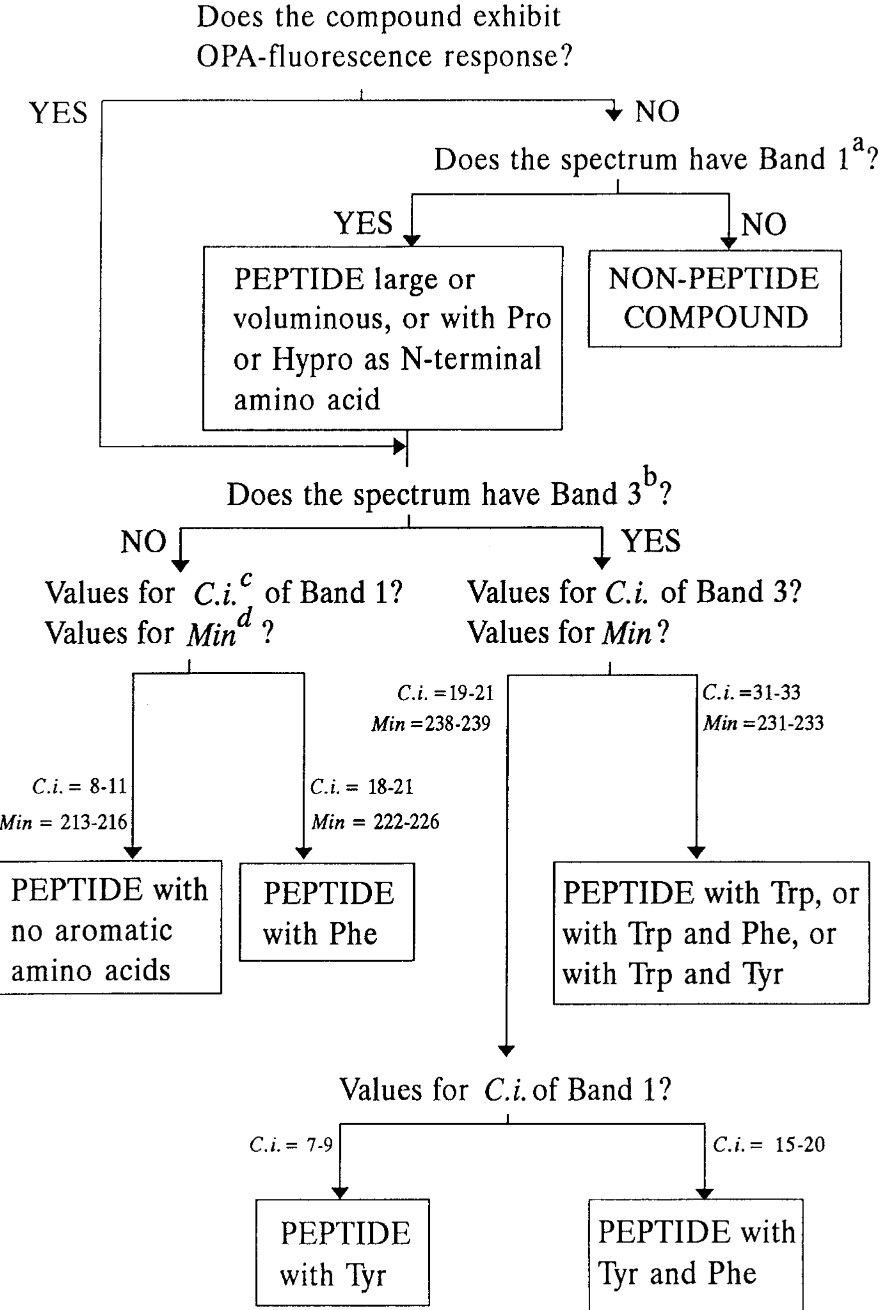

Fig. 3 Flowchart for the interpretation of both spectral data and OPA-fluorescence response. [a]Band 1: spectral band (201–207 nm) due to both the peptidic bonds and the carboxy-terminal group of the peptide molecule. [b]Band 3: spectral band (276–280 nm) due to the respective hydroxyphenyl and indol groups of the tyrosine and tryptophan residues. [c]*C.i.*: convexity interval (distance between the inflection points before and after the maximum in the original spectrum). [d]*Min*: minimum in the original spectrum. (From Ref. 104a, with permission.)

ionization methods, provide suitable means to determine accurate mass on peptides and proteins with high sensitivity (subpicomole range) (9b,104b). Furthermore, both methods are suitable for the creation of peptide ions for analysis using tandem mass spectrometry and collision-induced dissociation (CID) methods (104b).

Mass spectrometry, in conjunction with database searching, plays an increasingly important role in the characterization of peptides, and many reviews dealing with advances in mass spectrometry and the coupling of MS to HPLC have been published (9d,104c). Although MS has been applied mostly to the characterization of peptides of great biological significance (105,106), it is growing in food peptide characterization. The first structural characterization of peptides were made by the ionization methods of FAB and plasma desorption (PD), which are still used but which lack in terms of achievable mass range and sensitivity when compared to newer methods. Casein phosphopeptides in grana Padano cheese were analyzed by HPLC and characterized by FAB-MS (107,108). However, Gagnaire et al. (109) characterized tryptic peptides (phosphopeptides) of bovine caseins micelles by means of RP-HPLC online electrospray ion source–MS analysis. Both techniques, ESI-MS and FAB-MS, were used to identify peptides of the water-soluble nitrogen fraction from starter-free cheese (110). Peptides from β-lactoglobulin hydrolysates prepared by incubation with different enzymes were identified by MALDI-TOF and Edman degradation (111).

Reversed-phase HPLC is widely utilized to generate a peptide map from digested protein, and the MS online method provides rapid identification of the molecular mass of peptides. The HPLC-MS-FAB online system is a sensitive and precise method for low-MW peptides (<3000 Da); even picomol quantities can be detected. However, as the MW of the analytes increases, the ionization of peptides becomes more difficult and decreases the sensibility of the FAB-MS (112). Electrospray ionization (ESI-MS) was found to be an efficient method for the determination of molecular masses up to 200,000 Da of labile biomolecules, with a precision of better than 0.1%. Molecular weights of peptide standards and an extensive hydrolysate of whey protein were determined by the HPLC-MS-FAB online system and supported by MALDI-TOF (112). Furthermore, HPLC-MS-FAB results were compared with those of Fast Performance Liquid Chromotography (FPLC) analysis. Mass spectrometry coupled with multidimensional automated chromatography for peptide mapping has also been developed (9f,112a).

Mass spectrometry has also become a powerful tool for the sequencing of small amounts of peptides and proteins (112b,c). Tandem mass spectrometry and ion-source collision-induced dissociation (CID) produce information specific to the amino acid sequence and the specific covalently modified amino acid (104b,112d). Chait et al. (112e) developed a method, referred to as "protein ladder sequencing," for the N-terminal sequencing of peptides that utilizes multiple steps of partial Edman degradation chemistry prior to the analysis of the reaction mixture by MALDI-TOF MS. Characterization of 16 peptides isolated by RP-HPLC from a water-soluble nitrogen fraction of cheddar cheese was performed using MALDI-TOF MS, by determining their masses and applying N-terminal sequencing (112f).

The greatest advance in the analysis of peptides has been the coupling with spectrometric techniques, both for identification and for characterization. Their high accuracy, sensitivity, and, in some instances, tolerance to solvents make mass spectrometers ideal detectors for analyses of HPLC-separated peptides.

D. Other Detection Systems

Differential refractometer detectors are used in preparative separations of peptides, though not in the analytical separations themselves, because of their low sensitivity.

Electrochemical detectors can also be used in the detection of peptides. The $-NH_2$ group of amino acids, and hence the free NH_2 group of peptides, is electroactive, but the phenol, indole, and thiol substitutes of tyrosine, tryptophan, and cysteine, respectively, are even more so (113). Saetre and Rabenstein (114) and Mills et al. (114a) used an electrochemical detector to determine the cysteine and glutation in fruit juices. Pulsed electrochemical detection was also used to detect caseinomacropeptide (114b). In specific cases, conventional liquid detectors lack selectivity, particularly at low analyte concentrations, and in the presence of interference substances and biospecific detectors may have a great potential. Bioreactors and biosensors with enzymes or antibody/antigen-immobilized systems have rapidly advanced. A review of biospecific detection in column liquid chromatography has been published by Emnéus and Marko-Varga (114c). In the future, increased use of biospecific detection in food peptides can be expected.

VII. SEPARATION OF STEREOISOMERS

One of the problems attaching to the HPLC separation of peptides is the analysis of stereoisomers (enantiomers and diastereoisomers), that is, of peptides that differ only in the configuration of their amino acid residues.

Natural peptides should be made up only of L-amino acids, for only these are coded for by genes. However, some peptides contain D-amino acids, because they are synthesized by microbiological or enzymatic processes. Synthetic peptides may contain D-amino acids as a result of racemization or deviation during synthesis.

Since diastereoisomers have differing physical and chemical properties, they can be separated by HPLC without undue difficulty on any of its conventional phases (reversed-phase or ion-exchange HPLC). However, enantiomers share the same physical properties in all achiral media, so HPLC analysis of enantiomeric peptides is therefore more complicated. The separation of enantiomers is of great interest in a number of fields (biology, medicine, pharmacology, etc.). It is extremely important in the field of food science, since organoleptic receptors distinguish between different stereochemical compounds. It has been established that some small, optically active peptides exhibit biological and sweetening activities (25,26).

The separation of enantiomers can be effected either by transforming them into diastereoisomers using a chiral reagent and separating them on conventional phases or by separating the enantiomers on chiral phases. The utilization of chiral phases has not yet become routine, but studies of enantiomeric dipeptides have been carried out (115,116). Pirkle et al. (117) and Hyun et al. (118) separated enantiomeric di- and tripeptides (methyl esters of *N*-3-5-dinitrobenzoyl derivatives) on chiral stationary phases (CSPs) derived from (*R*)-*a*-arylalkylamines, (*S*)-*N*-(2-naphthyl) valine, or (*S*)-1-(6,7-dimethyl-1-naphthyl) isobutylamine. These workers were able to separate four peaks for each dipeptide derivative, corresponding to the two enantiomeric pairs (R,R)/(S,S) and (R,S)/(S,R). Cyclodextrin-bonded stationary phases and chiral stationary immobilized α-chymotrypsin phases were used to separate enantiomeric peptides (118a,b).

Separation of diastereoisomeric peptides by HPLC is more common. Since each diastereoisomer has different physicochemical and biological properties, this is of great interest. Separations of diastereoiosomeric di- and tripeptides have usually been performed on reversed-phase columns. Cahill et al. (119) separated diastereoisomeric amino acids and derivatized dipeptides using esters of the *N*-hydroxysuccinamide of *t*-butyl carbonyl-L-amino acid on C_{18} and C_8 columns. Linder et al. (120) separated amino acid and peptide derivatives on an RP-C_8 column, adding a metal chelate. Mixtures of DL and LD-dipeptides can be separated by RP-HPLC into two peaks, one containing LL- and DD-isomers, the other containing LD and DL-isomers. Sep-

aration of such dipeptides by weak-anion-exchange HPLC also gives rise to two peaks, but the order of elution is the opposite of that in RP-HPLC (77).

Numerous HPLC analyses have been carried out on biologically active diastereoisomeric peptides (encephalins, endorphins, hormones) in order to isolate them so that their activity can be studied; however, the separation of stereoisomers is not yet common in the field of food science.

VIII. APPLICATIONS

From the foregoing it can be concluded that the various versions of HPLC make up a tool of great use in resolving complex mixtures of peptides because of its versatility, short analysis times, and high resolution. The HPLC analysis of peptides from foods has been approached either from the basic standpoint of characterizing the bioactive peptides in protein hydrolysates that may have important nutritional and physiological properties or with the practical purpose of characterizing meats, fish, or milks of different species in order to be able to detect possible adulterations. It has also been employed to isolate and characterize peptides, which play an important role in food technology. Peptides can be used in the food industry as artificial sweeteners, flavor enhancers, and bulking agents. A few reviews on the applications, methodologies for isolation, and RP-HPLC analysis of food-derivatized peptides have been published (121,121a). Table 5 lists several examples of research topics dealing with food-derivatized peptides.

A. Peptide Mapping

In peptide mapping, a protein is enzimatically or chemically cleaved into small peptide fragments, and the resultant mixture is separated by HPLC to generate a peptide map, or fingerprint,

Table 5 Research Topics in Food-Derived Peptides

Research topic	Fraction analyzed	Sample
Food characterization	Peptide pattern	Meat, soya, and others
Proteolysis and ripening	Free peptides, phosphopeptides, tryptic digests	Cheese, β-casein
Foaming properties	Free peptides	Beer
Bitter peptides	Casein hydrolysate	Cheese, casein
Aroma precursors	Proteolysate	Cocoa
Flavor characterization	Meaty octapeptide and other peptides	Meat
Genetic variants and protein characterization	Enzymatic digest	α-S1-casein, β-lactoglobulin, thionins, gliadins, and glutenins
Proteinases of microbial and animal origin	Proteolysate	Milk, β-casein, k-casein, α-S1-casein, and α-S2-casein
Microbiological degradation	Proteolysate	Milk
Adulteration detection	Caseionomacropeptide	Milk
Surface-active peptides	Enzymatic digest	β-lactoglobulin
Bioactive peptides	Enzymatic hydrolysate	Milk, casein
Peptides associated with coeliac disease	Gliadin digests	Wheat
Antioxidant peptides	β-conglycinin hydrolysate	Soybean
Antimicrobial peptides	Enzymatic digest or free peptides	Several sources

Source: Ref. 121a, with permission.

that can be used to identify the protein. Peptide mapping is a topic of great interest, since it reveals small differences between individual proteins and is capable of detecting posttranslational modifications of amino acids, such as glycosilation sites or disulfide linkages. It can also be applied in studies of the action of extracellular proteases or in identification probes for proteins obtained by genetic engineering. Automated methods for peptide mapping have been developed (112a,121b). Immobilized enzyme columns coupled in tandem with analytical RP-HPLC generated the protein digests and achieved reproducible separation of the peptides resultant. Besides the great development in the coupling of electrospray ionization, spectrometric detection online with HPLC makes this technique ideal for characterizing and identifying peptides.

Of the different types of foods, the proteins from dairy products have been most thoroughly studied. Carles and Ribadeu-Dumas (122) set out the elution gradient conditions for separating mixtures of peptides hydrolyzed from β-casein using trypsin (β-*CN*-tryptic digest I). Leadbeater and Bruce-Ward (60) described an RP-HPLC method for rapidly separating the proteolytic fragments of bovine β-CN split off by trypsin using a gradient of 0–50% acetonitrile in 0.1% TFA. Lemieux and Amiot (61) separated 213 peptides from hydrolysates of dephosphorylated casein and 187 peptides from hydrolysates of phosphorylated casein using a C_{18} column, TFA in the mobile phase, and acetonitrile as modifier in an elution gradient system.

Cobb and Novotny (7) obtained improved separations using C_{18} microcolumns as a method for separating quantities on the order of 4 picomoles of tryptic peptides of phosphorylated and dephosphorylated β-casein. Figure 4 shows two peaks with different retention times, corresponding to the phosphorylated and dephosphorylated forms of the same peptide. The rest of the peptide map is similar. Using this microcolumn, phosphorylation of a single amino acid on a protein can be detected. The method is reproducible with standard deviations smaller than 2%. Characterization of bovine β-lg tryptic peptides by RP-HPLC on a Nucleosil C18 column was also reported (123).

B. Characterization of Foods

The specific peptide composition can be used to characterize foods. Abe (124) separated the peptides carnosine, anserine, and balenine from the white and red muscle of nine species of marine fishes. Carnegie et al. (37,38) developed an HPLC method using a Partisil-10SCX column with 0.2 M lithium formate at a pH of 2.9 and a temperature of 40°C under isocratic conditions with postcolumn derivatization using OPA to separate the dipeptides of histidine, anserine, carnosine, and balenine from the muscles of various species (pork, chicken, beef, lamb, and mutton) in order to identify the origin of the meat used in meat products. The concentration of balenine and the balenine:anserine ratio were higher in pork than in the other meats, and these relationships were useful in determining the presence of pork in mixtures with other meats.

Medina and Phillips (36) analyzed the peptide pattern obtained by the enzymatic hydrolysis of proteins isolated from beef, pork, chicken, and soybean sequentially, using TLC and HPLC to identify the proteins. The analysis was performed using RP-HPLC on a μBondapak C_8 column under isocratic conditions using triethylamine phosphate buffer (0.0833 M, pH 3) as solvent. Figure 5 depicts the peptide pattern of fraction IV isolated from beef, soybean, chicken, and pork by TLC. Identification of the different foods was accomplished by applying discriminant analysis to the peptide pattern.

The detection of cow's milk in ewe's or goat's milk and cheese is yet another application of the HPLC analysis of peptides. Tobler et al. (125) used HPLC to examine the differences between the caseins in the milks of various species. Goat's- and cow's-milk cheese caseins were hydrolyzed with trypsin, and the peptides thus obtained were separated by reversed-phase HPLC. The chromatograms for the caseins of each species were reproducible and distinct. Subsequently,

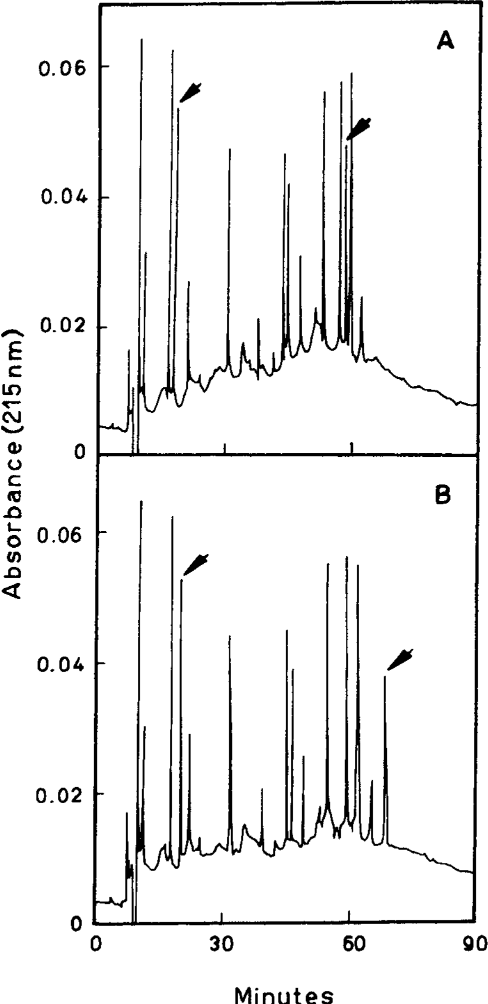

Fig. 4 Comparison of tryptic digests from (A) phosphorylated and (B) dephosphorylated forms of β-casein, separated by microcolumn HPLC. Arrows point to the two peaks that exhibit different retention times between the two forms. Each chromatogram was obtained from a 300-ng protein sample.

Kaiser and Krause (57) used HPLC to separate the tryptic peptides in cow's-milk and goat's-milk cheeses and cheeses made from mixtures of these milks. These authors reported that the quantitative detection limit could be as low as 1% cow's milk in goat's-milk cheese. Mayer et al. (125a) have developed a procedure for the separation of bovine, ovine, and caprine para κ-casein using cation-exchange HPLC.

Liquid chromatography has proved effective in detecting the adulteration of milk powder with cheese whey through the detection of caseinmacropeptide (CMP), the hydrophilic fragment of κ-casein (106–109) released by chymosin during milk clotting. The quantitative determination of CMP was achieved by SE-HPLC (126) or RP-HPLC, either with UV (62) or pulsed electrochemical detection (114b). Nevertheless, during prolonged cold storage of milk or buttermilk, psychrotrophic bacteria can proliferate and bring about enzymatic proteolysis of the caseins,

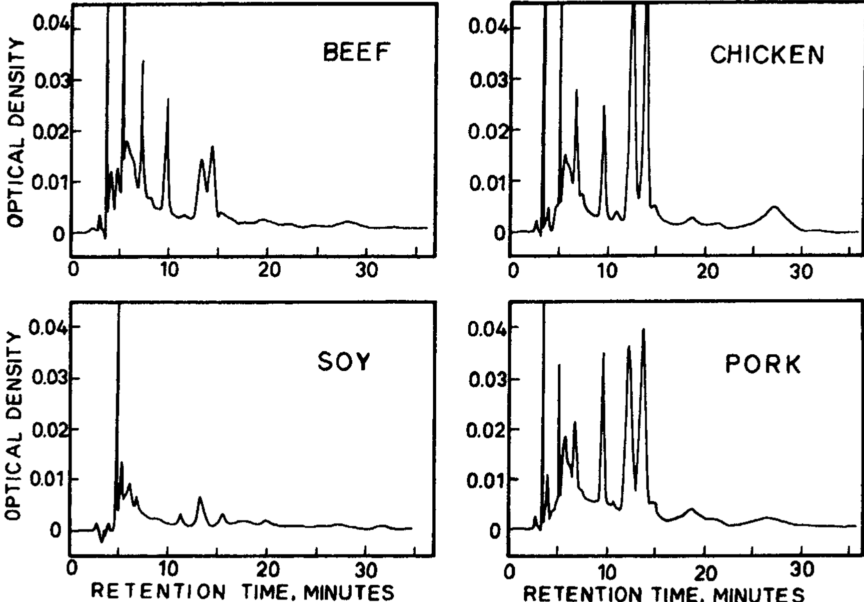

Fig. 5 HPLC peptide patterns of fraction IV beef, soy, chicken, and pork.

leading to false-positive results. Lopez Fandiño et al. (126a) and Recio et al. (126b) have showed that CMP and pseudo-CMP produced by *Pseudomonas* proteinases are formed during storage of raw and Ultra High Temperature (UHT) milk.

Quantification of the degradation products of raw-milk proteins by HPLC may furnish information on the shelf life of subsequently prepared UHT milk. Based on two earlier methods, Mottar et al. (127) applied HPLC to determine the specific proteolytic components that provide information concerning the presence and activity of gram-negative psychrotrophic bacteria.

Reversed-phase HPLC has been widely used for peptide analysis in different types of cheeses (21a,62a,127a), and it may provide valuable information about the proteolysis and ripening time of cheeses.

C. Characterization of Bitter Peptides

The hydrolysates of certain proteins, such as those of soybeans or caseins, give rise to bitter peptides. There seems to be a relationship between hydrophobicity and bitterness, and therefore RP-HPLC could be a useful method for separating bitter from nonbitter peptides. Champion and Stanley (43) used RP-HPLC on a C_{18} column to separate extracts of bitter peptides from cheddar cheese coagulated with pepsin. A total of 71 substances, some of them bitter, was found, and the bitter fraction was reported to exhibit slightly higher mean hydrophobicity values and to present higher valine and leucine contents than the nonbitter fraction. Lee et al. (21a) evaluated separations of bitter peptides in cheeses by means of RP-HPLC, with alternative mobile phases to allow subsequent sensory evaluation of the eluents. More recently, Gomez et al. (13b) studied, by means of RP-HPLC, the relationship between the level of hydrophobic peptides and bitterness in cheese made from pasteurized and raw milk.

Because of the bitterness observed in cheeses in which ripening had been accelerated by the addition of enzymes, a reversed-phase method was developed to study the peptide profile in

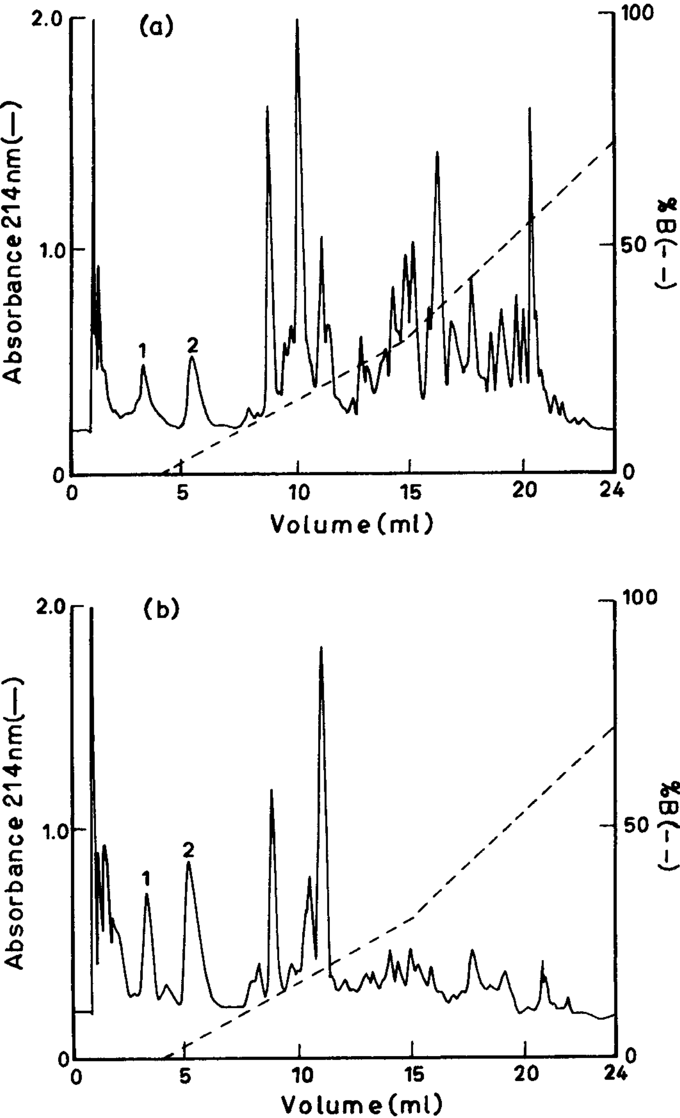

Fig. 6 Reversed-phase chromatography of peptide fractions from curd slurries on a C_{18} (50 × 5 mm) column. Neutrase added (a) followed by starter peptidase (b). Elution was with the following solvent systems: solvent A, 0.1% v/v TFA in H_2O; solvent B, 0.1% v/v TFA in methanol.

such cheeses and to monitor the removal of bitter flavors achieved by adding intracellular peptidases from *Lactococcus lactis* (which reduce the bitter taste by hydrolyzing the bitter peptides) (16). The chromatograms in Figure 6 show a reduction in the number of peaks with long retention time, which correspond to hydrophobic and bitter peptides, and an increase in the bands with short elution times, which may possibly be the breakdown products of the bitter peptides.

High-performance liquid chromatography has become an important technique for the analysis of peptides and has contributed to the identification and characterization of various classes of biologically active and flavor-enhancing peptides that have been isolated during the last few years.

ACKNOWLEDGMENTS

This work was supported by the Spanish Interministerial Commission of Science and Technology (Project ALI97-0630).

ABBREVIATIONS

AQC	6-aminoquinoyl-*N*-hydroxysuccinidylcarbamate
CBQCA	3-(4-carboxy-benzoyl)2-quinilinecarboxaldehyde
CE	capillary electrophoresis
CID	collision-induced dissociation
DABITC	4-*N,N'*-dimethylaminoazobenzene-4'-isothiocyanate
DABS	4-dimethyl-aminoazobenzene-4-sulfonyl chloride
DANSYL	5-N,N-dimethyl aminonaphthalene-1-sulfonyl chloride
DLI	direct liquid introduction
ESI	electrospray ionization
FAB	fast atom bombardment
FC	fluorescamine
FMOC	9-fluorenylmethyl chloroformate
FPLC	Fast Protein Liquid Chromatography
GPC	gel-permeation chromatography
HPLC	high-performance liquid chromatography
MALDI-TOF	matrix-assisted laser desortion/ionization time of flight
MS	mass spectrometry
NDA	naphthalene-2,3-dicarboxaldehyde
NIN	ninhydrin
OPA	*o*-phthaldehyde
PITC	phenyl isothiocyanate
PTA	phosphotungstic acid
RP	reversed phase
SEC	size-exclusion chromatography
SSA	sulfosalicylic acid
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TSP	thermospray

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4

HPLC of Food Proteins

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I. INTRODUCTION

The qualitative and quantitative analysis of proteins or protein digests by different high-performance liquid chromatography (HPLC) modes has become routine in most laboratories dealing with protein research and food analysis. There is no doubt that the present high-resolution chromatographic techniques offer many advantages for the separation and analysis of proteins. However, it has been recognized that the chromatography of proteins is in many respects different from that of small, more "rigid" biomolecules such as saccharides and organic acids. Modern laboratory practice requires not only a complete resolution of all compounds in a mixture in a short time but also, especially on a preparative scale, the highest possible recovery of bioactivity. Furthermore, foodstuffs are seldomly one-compound systems, and, despite the high sensitivity and resolution of modern HPLC systems, isolation of the proteins of interest from the food matrix is still required in most cases, introducing a possible additional source of errors. Since protein structure can be important from the technological and functional points of view, detection techniques that allow the determination of the intergrity of a protein are particularly useful.

II. FOOD PROTEINS

Proteins are defined as complex polymers composed of different monomers, i.e., amino acids, that are incorporated in the protein molecule in a fixed sequence given by the information encoded in the deoxyribonucleic acid (DNA) string. Proteins constitute the primary material of living organisms (1). The use of different protein sources can vary widely from one country to another, depending on local habits and traditions. In general, protein sources can be divided into two major groups: plant proteins and animal proteins. A major portion of the world protein supply is derived from plant proteins such as cereals, legumes, and oilseed proteins (2). The soybean has traditionally been used as a major protein source for food, but there has been an increasing interest for other legume seeds, such as pea, fababean, and lupin, as a protein source (3,4). Also, completely new sources of proteins are reported, e.g., the so-called "myco-protein," or fungal protein, with a protein content of 12.1% (5). The most important animal proteins, from the economic and quantitative points of view, are muscle tissues, eggs, and milk (6). Table 1 shows different plant

Table 1 Average Protein Content of Various Classes of Foodstuffs

Food class	Protein content (g/100 g)	Food class	Protein content (g/100 g)
Cereals	10.4	Fish	18.8
Roots and tubers	1.8	Crustaceans	16.0
Legumes	24.8	Mollusks	10.0
Nuts and seeds	14.6	Cow's milk	3.5
Meats	17.0	Cheeses	18.0

Source: Ref. 7.

and animal protein sources and their average protein content. In general, animal proteins are of higher quality than plant proteins, due to the fact that they are 90% digested and absorbed in the human body, whereas for plant proteins this may be only 60–70% (8).

Proteins are important from the nutritional and technological points of view. Proteins affect every property that characterizes a living organism, and they play different roles in the human body. Proteins are also very important in food technology and are responsible for many food properties. The physical properties of proteins and their interactions with other components contribute significantly to the functional behavior and quality of several food products, such as cheese, bread, and meat products (9). An overview of the functional roles of proteins in different food systems is presented in Table 2. Food preferences by human beings are based not on nutritional quality but on sensory attributes to the food, such as appearance, color, flavor, texture, and

Table 2 Functional Roles of Food Proteins in Food Systems

Function	Mechanism	Food system	Protein source
Solubility	Hydrophilicity	Beverages	Whey proteins
Viscosity	Water binding, hydrodynamic size, shape	Soups, gravies, salad dressings	
Water binding	H-bonding, ion hydration	Meat sausages, cakes, breads	Muscle proteins, egg proteins
Gelation	Water entrapment and immobilization, network formation	Meats, gels, cakes, baked goods, cheeses	Muscle proteins, egg and milk proteins
Cohesion, adhesion	Hydrophobic, ionic, H-bonding	Meats, sausages, pasta, baked goods	Muscle proteins, egg proteins, whey proteins
Elasticity	Hydrophobic bonding, disulfide crosslinks	Meats, baked goods	Muscle proteins
Emulsification	Adsorption at interfaces, film formation	Sausages, bologna, soups, cakes, dressings	Muscle proteins, egg proteins, milk proteins
Foaming	Interfacial adsorption, film formation	Whipped toppings, ice cream, cakes, desserts	Egg proteins, milk proteins
Fat and flavor binding	Hydrophobic bonding, entrapment	Simulated meats, baked goods, doughnuts	Milk proteins, egg proteins

Source: Ref. 10.

mouth feel, which are all manifestations of different complex interactions among constituents. Food proteins play a major role in these attributes (2). The importance of proteins in foods and the discovery or development of new protein sources require the analysis of the protein fraction via techniques that are able to identify the amount of protein present. In most cases, the protein fraction is constituted of different proteins, and often there is a relationship between, e.g., the functional properties and the proteins present in the protein fraction of a food. This is the case for wheat flour, where the glutenin fraction is related to the flour bread-making quality (11,12), and milk proteins, where the caseins are related to the properties of cheeses (13). Thus, information about the composition of the protein fraction is a prerequisite for product quality assessment and product development.

In the previous century, protein analysis was done by determining the nitrogen content of a food product after complete oxidation of the product and the subsequent conversion to protein content using conversion factors. In the beginning of this century, colorimetric protein determination methods became available and have since been further developed for the analysis of proteins in foods and food products. Examples of such colorimetric methods are the Biuret procedure and the Folin–Ciocalteau method, which was further optimized to what is now known as the Lowry method. Later on, near-infrared reflectance spectrophotometry was introduced for the determination of the protein content in dry products. In 1930, free or moving boundary electrophoresis was introduced, which has evolved into electrophoresis in porous gel media, which are nowadays frequently applied in food protein analysis. Most currently used methods to detect and/or quantify specific protein components in foods can be cataloged in the field of spectrometry, chromatography, electrophoresis, or immunology or a combination of these (14). The main advantages of the HPLC analysis of food proteins lay in its high-resolution power and its versatility. Both the composition of the protein fraction and the amount of proteins in the different subfractions can be obtained in a single run without either the requirement of additional staining procedures or a specified incubation time, and analyses can be automated. However, equipment is more costly and rather complex, and variability in solvent purity, column stability and batch-to-batch reproducibility, gradient formation, and technical operation may introduce some variability. It is also important to realize that separation by column chromatography is of a sequential nature in contrast to, e.g., electrophoretic techniques, the latter facilitating simultaneous separation of several samples. Besides, equipment for electrophoresis is fairly simple and inexpensive, but results may vary with reagent purity, polymerization conditions, temperature factors, nonuniform electric fields, and staining and destaining methods. On the other hand, samples and standards can be run at the same time.

III. SAMPLE PREPARATION

Proteins can be separated and purified from physiological fluids, cell or tissue homogenates, and food matrices. To separate proteins by HPLC techniques, the removal of cell debris, lipids, and nucleic acids is usually required, as is solubilization of the protein fraction. A homogenization step can be used to disrupt tissues and cell membranes, followed by filtration to remove the unwanted homogenized tissue or cell debris. Particulate matter and tissue fractions can also be removed by centrifugation, which can also be used to remove precipitates. In addition, ultracentrifugation can be used to separate different classes of proteins. Separation of the different classes of protein in a sample can be achieved by selective precipitation. Usually this involves the use of salts, organic solvents, or heat. Solubilization of unsoluble proteins might be accomplished via the addition of reagents for reduction (e.g., β-mercaptoethanol or dithiothreitol), destruction of H-bonding (e.g., urea), or the destabilization of hydrophobic interactions [e.g., sodium dodecyl-

sufate (SDS)]. However, the use of these reagents might alter the chromatographic properties of the proteins of interest, something that has to be taken into account when selecting a separation scheme.

IV. SEPARATION TECHNIQUES

The central part of a chromatographic system is the column and, more specifically, the packing material, consisting of a base support (core or carrier) supporting the stationary phase. The stationary phase might be the support itself or an interfacial immobilized layer. Since proteins are rather complex biomolecules, more than one interaction mechanism between the protein molecules and the stationary phase may exist. On the other hand, this complexity enabled the development of a wide range of available stationary phases, ranging from the classical, commercially available silica gels to tailor-made phases, prepared by the user himself by coupling an appropriate ligand to, e.g., glass, silica gel, or an organic phase.

A. Apparatus

Modern HPLC apparatuses used today are reliable and, in most cases, suitable for protein analysis. Pumps deliver solvents at fairly constant flow rates and give good run-to-run reproducibility, isocratically as well as with gradient elution. Gradients can be generated either by using two pumps having outputs controlled by a gradient programmer or by using one pump that delivers a gradient generated before the pump inlet. With respect to protein chromatography, Pharmacia Biotech developed a technique for the separation of proteins in their native state, called *fast protein liquid chromatography* (FPLC). Since FPLC needs only a relatively low backpressure, compared to classical HPLC, to drive the high rates of flow at which the separations are performed, the risk of denaturation caused by shearing forces diminishes. The mechanical components are designed to be resistant to corrosive buffers (e.g., the use of glass, fluoropolymers, and titanium) and in such a way that there is no contamination or inactivation of the compounds of interest. Corrosive buffers might result in contamination of the protein sample by heavy metals due to corrosion of high-grade stainless steel, traditionally used in the construction of HPLC. However, nowadays there is no longer a clear distinction between FPLC and HPLC, since the introduction of new, inert materials for HPLC resulted in good resistance to corrosive buffers and a reduced interaction with the sample preparation.

B. Base Support

The application of HPLC to the analysis of proteins has only become possible due to developments in packing materials. Traditionally, only nonrigid and semirigid gels with relatively low mechanical strength could be used for the chromatographic separation of macromolecules like proteins. This limited the chromatography of such molecules to low flow rates and large particle sizes, thus resulting in analyses with poor resolution and long separation times. This favored the use of alternative techniques such as electrophoresis (15). In 1979, Waters (Millipore, USA) introduced the first commercially available HPLC column for the separation of proteins, the Protein Pak I 125 for gel filtration chromatography (16). Since then, continuing efforts have been made to improve the separation of proteins by the introduction of new packing materials. Although chromatographic parameters, such as mobile-phase composition, stationary-phase design, and column geometry, must be optimized for each protein separation, some common criteria dictate the selection of chromatographic media. First, they should be chemically and physically

stable and should possess good mechanical strength to allow high flow rates. In addition, they should not contain groups that bind proteins nonspecifically, but should be easily derivatizable to allow the introduction of functional groups for interactive chromatography applications. It is also important that they withstand regeneration and cleaning procedures commonly applied in liquid chromatography. Finally, they should be produced with controllable size and pore size distributions and should be reproducible from batch to batch (17). The base support plays a determining role in the mechanical, chemical, and thermal stability of packing materials. According to their chemical composition, base materials may be divided into inorganic polymers and organic polymers. Developments in base support materials have been based on improvements in the production processes of these polymers or have been directed toward the use of composite materials. The primary approach in the research on composite design consists of associating the rigidity of a carrier with the biocompatibility and chemical stability of another component, acting as stationary phase. Composite packings may be divided into three main categories, (a) surface-bonded stationary phases, (b) pore–matrix composites, and (c) mixed carriers. An excellent review of recent developments in the field of composite materials can be found in Ref. 17.

1. Inorganic Polymers

Undoubtedly, silica is the most widely used chromatographic material, being available in a wide range of particle sizes and porosities. The most common porous silica particles are obtained by polycondensation of silicic acid followed by thermal treatments. In general, amorphous silica is used for chromatographic applications. Silica is very stable under high pressure and easily derivatizable. Unmodified silica has some major drawbacks that preclude its use for the chromatography of proteins. Silica is unstable at mild alkaline pH values and dissolves drastically above pH 8. Since the pK_a value of the silanol groups is 6–8, deprotonation occurs above pH 4 and nonspecific interactions might take place between these groups and the basic part of the protein molecule. This drawback can be limited by using high-purity silica with a high population of internally bonded or associated silanol groups. An alternative method is a chemical modification of the silanol groups with monomeric or polymeric silanes. However, the siloxane bonds tend to hydrolyze at low pH, causing changes in the chromatographic performance. Due to the chemical instability of silica, the synthesis of new base supports, based on other metal oxides such as alumina (Al_2O_3), titania (TiO_2), and zirconia (ZrO_2), have gained increasing interest. Since these oxides are capable of interacting with the carboxyl groups of proteins, procedures to suppress the reactive sites are necessary. Another well-known inorganic chromatographic support for protein separations is calcium phosphate hydroxyapatite [$(Ca_5(PO_4)_3OH)_2$]. It has unique separation properties: at neutral pH, basic proteins (isoelectric point, pI, >7) are mainly adsorbed via electrostatic interactions with negatively charged phosphate ions, while the carboxylic groups of acidic proteins interact with the calcium sites (18). However, hydroxyapatite has a relatively low mechanical strength, which limits its use for high-flow-rate HPLC applications, and it is stable over only a limited pH range (5–10) (17).

2. Organic Polymers

The organic polymers used for chromatographic purposes are natural polysaccharides and synthetic polymers. Examples of polysaccharides used are agarose, cellulose, dextran, and, to a lesser extent, amylose and starch. They can be produced with a suitable porosity and are stable over a wide pH range. Their high hydroxyl group content makes them suitable for derivatization, and they generally do not interact with proteins under normal chromatographic conditions. The main disadvantage of polysaccharides is their poor mechanical strength and their swelling ability. Recently, however, macroporous particles made of agarose or cellulose that could sustain rel-

atively high flow rates were developed. The organic polymers have an increased stability compared to silica, but they have a lower pressure tolerance, they swell in the presence of certain organic solvents, and they have a broader pore size distribution and a decreased efficiency (17). Some examples of synthetic polymers are polyacrylamides, polyacrylates, and polyvinyl polymers, with the last the most commonly used for chromatographic purposes. Most polyvinyl polymers are based on polystyrene-divinylbenzene (PS-DVB) copolymers. Developments in the polymerization process made it possible to obtain rigid beads with a high degree of crosslinking that are capable of withstanding high pressures. Compared to silica gels, PS-DVB matrices are stable over the whole pH range and are ion free. However, they have a hydrophobic character that limits their use as such for the separation of proteins, except in reversed-phase mode. An increase of the polarity of the PS-DVB matrix can be obtained by surface modifications.

3. New Approaches in Base Support Design

Two new approaches can be distinguished in the research for the optimization of base support materials for HPLC of macromolecules, i.e., the development (or rediscovery) of micropellicular, or nonporous, supports and the gigaporous supports (19). Micropellicular stationary phases consist of nonporous sorbents with a fluid impervious core. They have the advantage that diffusion effects due to the pore structure of conventional supports can be eliminated. This is particularly useful for the rapid analysis of proteins with high efficiency and resolution (20). However, the capacity of the initial supports, developed in the late 1960s, was very low due to the small surface area. In the '80s, the particle size was decreased to 1.5–7 μm in order to increase the loading capacity. The nonporous particles are mainly silica- or PS-DVB-based, although other polymers, such as polymethacrylate, are also used. Columns packed with nonporous particles, with a diameter ranging from 2 to 10 μm, for analytical purposes are now commercially available (Table 3). They are usually shorter than the conventional columns, since a higher pressure generally arises from the use of small particles. Nonporous supports are particularly useful for quality control, online monitoring, and purity check of biomolecules due to the short analysis times (21). The introduction of supports having pore diameters greater than 1 one-hundredth of the particle diameter is a more recent development. Two types of such gigaporous supports are well described in the literature. One type is derived from a crosslinked PS-DVB matrix, with appropriate surface modifications for chromatography of biopolymers. They have a unique pore structure that allows intraparticle convection in the throughpores ($d_{pore} > 500$ nm), the so-called perfusion, combined with a network of smaller pores (30 nm $< d_{pore} <$ 70 nm) branching from the throughpores. At low mobile-phase velocities they appear to be monodisperse, whereas at higher mobile-phase velocities they show more bidisperse properties (22,23). Columns packed with these gigaporous particles are designed for fast separations, which do not require a high efficiency. A second class of gigaporous supports are the so-called "gel-in-a-shell" configurations. In these

Table 3 Commercially Available Prepacked Columns with Nonporous Sorbents

Support	Base support	Particle size (μm)	Manufacturer
Hytach C18	Silica	2	Glycotech
Micra NPS RP-18	Silica	1.5	Micra Scientific
Develosil NP ODS series	Silica	2, 5	Nomura
Hydrocell NP10 series	PS-DVB	10	BioChrom Labs
Hamilton RP-∝	PS-DVB	4	Hamilton
Bio-Gel MA7 series	Polymethacrylate	7	Bio-Rad
TSKgel NPR series	Polymer	2.5	TosoHaas

Source: Adapted from Ref. 21.

composite materials, the pores of rigid macroporous material (pore size 200–600 nm) are filled with a retentive soft and porous polymer network, a hydrogel. The hydrogel is permeable, at least in part, to the biopolymers to be separated. Columns with these type of stationary phases may have significantly higher loading capacities than those packed with the open gigaporous sorbents, but their efficiency is likely to diminish much faster upon increasing the mobile-phase flow rate (19).

C. Operation Modes

A wide variety of chromatographic operation modes exists for the separation of proteins, due to the variation in their behavior. The diverse biological and biochemical functions of proteins originate largely from differences in the amino acid side chains that convey different properties onto the protein. This provides the protein analysts with a guide to select the possible operation modes. Possible techniques are:

Hydrophobic interaction (HI) and reversed-phase (RP) chromatography, based on differences in the polarity of amino acid side chains

Size-exclusion chromatography (SEC), exploiting differences in the size of the protein molecules due to differences in structure

Ion-exchange chromatography (IEC), on the basis of differences in the pKa values of the functional groups of the side chains resulting in changes in the ionization function of pH

Metal chelate and bioaffinity chromatography: based on the affinity of the protein for a specific molecule that can be incorporated in the stationary phase (not commonly used for food analysis)

Hydroxyapatite chromatography, separating molecules by differential surface binding to phosphate and calcium sites on a microcrystalline matrix of hydroxyapatite ($[Ca_5(PO_4)_3OH]_2$)

Reversed-phase chromatography was introduced in the early 1950s (24) and has become a widely applied method of HPLC because of its wide scope of applications. Reversed-phase chromatography is generally achieved on an inert column packing, typically covalently bonded with a high density of hydrophobic functional groups, such as linear hydrocarbons 4, 8, or 18 residues in length, or the relatively more polar phenyl group. Interaction takes place between a nonpolar surface of the protein and the stationary phase, thereby shielding the stationary phase from the polar (aqueous) mobile phase. Differential desorption is achieved by applying a gradient with increasing concentration of an organic solvent. This favors the formation of a solvent shell around the protein by lowering the surface tension of the mobile phase and also favors desorption due to direct competition between the protein and the organic molecules for the stationary phase. Despite the fact that a lot of theoretical models are already developed for the description of the complex kinetics and hydrodynamic phenomena involved in the interaction between mobile phase, stationary phase, and sample molecules, the mechanisms involved in RP-HPLC are not yet fully understood. Today, an almost frightening choice of stationary phases for RP-HPLC, in which silica gel derivatives still predominate, exists. A list of some commercially available prepacked columns for the RP-HPLC of proteins is given in Table 4. Separation selectivity can be altered by changes in column source or type, changes in the organic solvent, the use of ion-pairing conditions, and variation of pH or ionic strength. The mobile-phase composition is the most readily changed variable in a RP-HPLC separation. In normal usage, the mobile phase consists of a mixture of water, a miscible organic solvent, and dissolved buffers and salts. The organic solvents commonly used for the RP-HPLC of proteins are acetonitrile, propan-1-ol, and propan-2-ol. The propanols are much more viscous, thus giving higher backpressures, and the

Table 4 Survey of Commercially Available Reversed-Phase Prepacked Columns for HPLC of Proteins[a]

Base support	Support	Particle size (μm)	Pore size (nm)	pH range	Manufacturer
Divinylbenzene	Jordi NPR-DVB	4	np[b]	—	Jordi
Polymer	Hamilton PRP-3	10	30	1–13	Hamilton
	Hamilton PRP-Infinity	4	np[b]	1–13	
	TSKgel Phenyl-5PW	10	100	2–12	TosoHaas
	TSKgel-Octadecyl-4PW	7	50	2–12	
Polyvinyl alcohol copolymer	Astec C4, C8, C18	5	30	2–13	Astec
Silica	Adsorbosphere XL C4, C8, C18	3	30	—	Alltech
	Macrosphere C4, C8, C18	5–7	30	2–8	
	Hi-Pore RP304 (C4), RP318 (C18)	5–15	30	2–7	Bio-Rad
	Kromasil C4, C8, C18	5–10	10	<9	EKA Nobel
	Sephasil Protein C4	5 or 12	30	2–8	Pharmacia Biotech
	Selectosil C4 300 A	5–10	30	—	Phenomenex
	Hypersil C4, C8	5	30	2–8	Shandon Scientific
	LC-304 (C4), 308 (C8), 318 (C18)	5	30	2–7	Supelco
	LC-8-DB (C8), 18-DB (C18)	3–5	12, 30	2–7	
	TSKgel Super-ODS	2	11	2–7.5	TosoHaas
	TSKgel TMS-250 (C1)	10	25	2–7.5	
	Zorbax SB C8	5	30	—	Rockland Technologies
Not specified	Bakerbond WP C4, C8, C18	5	30	—	J.T. Baker
	Bakerbond WP Diphenyl	5	30	—	

[a] Data obtained from the respective manufacturer's catalogues. The mention of a manufacturer does not imply any preference of the authors toward that manufacturer's products.
[b] np: nonporous

acetonitrile concentration necessary to elute a protein is considerably higher than the equivalent propanol concentration. In most cases, minor components such as trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), tetrabutylammonium phosphate, and triethylamine are added to the mobile phase (25). These reagents avoid secondary equilibrium effects due to partial ionization of amine or carboxylic groups present in proteins through ion suppression (26). Furthermore, these compounds give hydrophobicity to the protein through ion pairing between the carbon chain and the protein, thereby increasing retention time (16). Most reversed-phase separations of protein samples are carried out using a "standard" combination of conditions, i.e., a short-chain alkyl-silica column (e.g., C3 or C4) and gradient elution with an acetonitrile-water mobile-phase system containing ca. 0.1% TFA (25). The most important advantage of RP-HPLC over the other HPLC techniques available for proteins is its high resolution power and suitability for protein analysis at low ionic strengths. The main disadvantages are the risk of protein denaturation and the loss of biological activity and interferences of hydrophobic contaminants. Besides these, the separation process still remains empirical, and some of the solvents used are toxic. Reversed-phase HPLC requires high-quality equipment, since the control of the gradient elution is very critical, and the reproducibility of isocratic separations remains difficult (14). It is advisable that, prior to protein analysis, the RP-HPLC column be washed with a solvent more hydrophobic than

that used in the gradient (e.g., 100% acetonitrile) to remove any tightly bound proteinaceous material. Decreased resolution, as judged by separations of a reference sample, or increased operating pressure may indicate tightly bound or insoluble material on the column. Regeneration with solutions such as 2-propanol, urea, dimethylsulfoxide, or chloroform usually restores column performance.

A further extension to RP-HPLC is called hydrophobic interaction HPLC (HI-HPLC). Hydrophobic interaction HPLC is also based on the hydrophobicity of the biomolecules. However, milder conditions are used in contrast to RP-HPLC, i.e., using less hydrophobic supports and no organic solvents, since elution can be achieved by changes in the ionic strength of the mobile phase. Solutions of neutral salts with a high salting-out effect are preferred for hydrophilic (weakly binding) proteins, whereas salts with a low salting-out effect are used for hydrophobic (strong binding) proteins. This technique has a high resolving power with less risk of protein denaturation due to the milder conditions. Proteins can be analyzed at high ionic strengths, and no toxic solvents are used. However, the use of high salt concentrations has some corrosive effects on the HPLC equipment (14). Substantial changes in retention and selectivity are achieved by several factors, such as type of salt, the gradient, use of various ligand chains attached to the support matrix, addition of detergents or surfactants to the mobile phase, and optimization of pH and temperature (16). The hydrophobic interaction is strong in the presence of high concentrations of ions, depending on the type and concentration of the salt. Retention is higher for salts exerting a larger salting-out effect on proteins. The strength of the hydrophobic interaction increases with increasing temperature. On the other hand, retention of the protein is reduced upon the addition of low amounts of organic solvents or chaotropic reagents (e.g., urea, guanidinium chloride). Theoretically, the bonding ought to be the strongest at the isoelectric point of the protein, where it exhibits a net zero charge. In practice, however, the pH of the mobile phase can affect the retention behavior of different proteins to a large extent depending on the type of matrix. This is probably attributable to the effect of charges on the stationary phase. Differential elution of proteins is achieved by applying changes in the mobile phase, i.e., gradient elution. Besides the type of stationary phase, temperature, and type of salt, factors that can be manipulated to improve resolution are the use of longer columns and lower flow rates and the addition of chaotropic agents or organic solvents (27). Some commercially available columns for HI-HPLC of proteins are given in Table 5.

Size-exclusion chromatography separates the sample molecules on the basis of their size, or, more precisely, their hydrodynamic volume, and has become very popular in the field of protein analysis since the introduction of Sephadex (Pharmacia Biotech, Sweden) in 1959 (28). Size-exclusion HPLC became operational in 1980, after the development of uniform rigid particles whose uniform pores were sufficiently large to be entered by protein molecules. Larger molecules do not enter the pores of the column particles and are excluded, so they are eluted in the void volume of the column. The pores of a column particle are differentially accessible to smaller particles, depending on their size. Commercially available columns for SE-HPLC are listed in Table 6. In contrast to other chromatographic modes, in SE-HPLC one buffer is normally used to elute proteins over the whole gradient volume. In ideal SE-HPLC, there is a linear relationship between the logarithm of the molecular weight and the elution volume of a protein. Therefore, SE-HPLC can be used not only to analyze a protein fraction but also to determine the molecular weight of the proteins. Fractionation limits are related directly to the pore size of the column particles, but other parameters are also important in the separation process. At higher flow rates, the resolution will decrease, especially for higher-molecular-weight proteins, because of slower mass transfer. Other parameters of influence are the viscosity of the sample and sample volume, which should not exceed 1–2% of the column bed volume. Two basically different mobile phase systems can be used in SE-HPLC: physiological buffers of near-neutral pH, sometimes with addition of mild nondenaturing detergents, and SDS-containing buffers and denaturing conditions for

Table 5 Survey of Commercially Available Prepacked Columns for Hydrophobic Interaction HPLC of Proteins[a]

Base support	Support	Surface	Particle size (μm)	Pore size (nm)	pH range	Manufacturer
Polymer	Bio-Gel Phenyl-5-PW	phenyl	10–15	100	2–12	Bio-Rad
	CQH3ps	phenyl	—	—	—	Mitsubishi Kasei
	CQH3es	ester	—	—	—	
	CQH3bs	butyl	—	—	—	
Hydroxylated polyether	TSKgel Ether-5PW	ether	10	100	2–12	Toya Soda
	TSKgel Phenyl-5PW	phenyl	10	100	2–12	
Crosslinked agarose	Phenyl Sepharose CL 4B	—	—	—	2–13	Pharmacia Biotech
	Phenyl Sepharose FF	—	—	—	2–13	
Silica	Spherogel	—	—	—	—	Beckman
	CAA-HIC	methyl polyether	5	30	—	
	Bakerbond WP HI-Propyl	propyl	5–15	30	2–10	J.T. Baker
	LC-HINT	diol	5	10	2–7.5	Supelco

[a] Data obtained from the respective manufacturer's catalogues. The mention of a manufacturer does not imply any preference of the authors toward that manufacturer's products.

sample treatment. In many cases, salt is added to the buffer system at a concentration above 0.1 M to minimize ionic interactions between the protein and the stationary phase, especially in the case of modified silica-based supports, which can still contain free negatively charged silanol groups. However, high salt concentrations should be avoided, because they will favor hydrophobic interactions. Most common buffers for SE-HPLC are sodium phosphate buffers containing NaCl or tris-HCl buffers containing SDS, both with a pH around 7 (29). Due to the mild separation conditions used in SE-HPLC, no protein denaturation occurs due to the chromatographic matrix itself. Disadvantages are nonspecific hydrophobic or electrostatic interference of the column packing and erroneous behavior of nonglobular and associated proteins (14).

A fourth technique widely used for protein analysis, is ion-exchange HPLC. Ion-exchange chromatography (IEC) is based on the ionic interactions between charged molecules on the stationary phase and in the mobile phase and the ionic sample species. Because a protein can exist either as an anion or as a cation, IEC provides a custom-designed system for the separation of proteins. The type of ionic sites on the protein will vary with pH, and their number will vary with the protein type and its tertiary structure. Elution of proteins can be achieved by a gradient of increasing ionic strength, a pH gradient, or a combination of both, although this is less common. Two types of IEC can be distinguished, based on the type of stationary phase. Anion-exchange HPLC systems contain positively charged groups, which bind reversibly to anionic biomolecules; cation-exchange HPLC operates on the reverse mode. The functional groups that determine the variation of surface charge with pH can be used to define the various classes of ionic exchangers as strong or weak (30). Both silica- and polymeric-based columns are regularly used for ion-exchange chromatography, and a wide choice of prepacked columns is commercially available (Table 7). A pore size of 30 nm is usually recommended for proteins; for macromolecules with a high molecular weight, i.e., >150,000, however, a pore size of 100 nm is better (31).

Table 6 Survey of Commercially Available Prepacked Columns for the Size-Exclusion HPLC of Proteins[a]

Base support	Support	Particle size (μm)	Pore size (nm)	Molecular weight range	pH range	Manufacturer
Polyetheretherketone	SigmaChrom GFC 100, GFC 1300	12–15		–<100–<1,300	3–12	Sigma
Polymer	Bio-Gel SEC 10	10	<10	<2	2–12	Bio-Rad
	Bio-Gel SEC XL series	6–13	<20–>100	<8–200,000	2–12	
	Superdex HR series	13		–0.1–7 thru 10–600	3–12	Pharmacia Biotech
	Superose HR series	10–13		–1–300 thru 5–5,000	3–12	
	PolySep-GFC-P series	8–12		–<2–<10,000	3–12	Phenomenex
	Poly-Sep-GFC-Linear	8–12		–<100,000	3–12	
Silica	Macrosphere GPC series	7	6–400	28–25,000	—	Alltech
	Bio-Sil SEC series	5–17	12.5–40	5–100 thru 20–1,000	2–7	Bio-Rad
	Aquapore OH series	10	10–100	<90–<20,000	—	Brownlee
	BioSep-SEC-S series		5 14.5–50	1–300 thru 15–2,000	2.5–7.5	Phenomenex
	Zorbax GF series	4–6	15–30	4–400 thru 10–1,000	2.5–8.5	Rockland Technologies
	LC-Diol, LC-3Diol	5	12–30	—	—	Supelco
	TSKgel G SW series	10–13	12.5–45	5–100 thru 20–7,000	2–7.5	TosoHaas
	TSKgel G SWxl series	5–8	12.5–45	5–150 thru 20–10,000	2–7.5	
	QC-PAK series	5	12.5–25	5–150 thru 10–500	2–7.5	
Not specified	Ultraspherogel SEC series	5–7	14.5–34.5	—	—	Beckman

[a] Data obtained from the respective manufacturer's catalogues. The mention of a manufacturer does not imply any preference of the authors toward that manufacturer's products.

Table 7 Survey of Commercially Available Prepacked Columns for the Ion-Exchange HPLC of Proteins[a]

Base support	Support	Type	Surface	Particle size (μm)	Pore size (nm)	pH range	Manufacturer
Hydroxylated polyether	Bio-Gel SP-5-PW	Strong cation	Sulfopropyl	10	100	2–12	Bio-Rad
	Bio-Gel DEAE-5-PW	Strong anion	Diethylaminoethyl	10	100	2–12	
Polymer	Bio-Gel MA7P	Weak anion	Polyethyleneimine	7	np[b]	2–12	
	Bio-Gel MA7C	Weak cation	Carboxylic acid	7	np[b]	2–12	
	Bio-Gel MA7S	Strong cation	Sulfopropyl	7	np[b]	2–12	
	Bio-Gel MA7Q	Strong anion	Quaternary amine	7	np[b]	2–12	
	PRP-X500	Anion	—	7	—	1–13	Hamilton
	PRP-X600	Weak anion	—	7	—	—	
	Mono P HR5/5	Weak anion	Tertiary amine	10	—	2–12	Pharmacia Biotech
	Mono Q HR5/5	Strong anion	Quaternary amine	10	—	2–12	
	Mono S HR5/5	Strong cation	Sulfonic acid	10	—	2–12	
	BioSep-DEAE-P	Weak anion	Diethylaminoethyl	7	—	2–12	Phenomenex
	BioSep-Express-PEI	Weak anion	Polyethyleneimine	4	—	2–12	
	TSKgel DEAE-5PW	Weak anion	Diethylaminoethyl	10	100	2–12	TosoHaas
	TSKgel CM-5PW	Weak cation	Sulfopropyl	10	100	2–12	
	TSKgel SP-5PW	Strong cation	Carboxymethyl	10	100	2–12	
Polymethacrylate	ProtEx-DEAE	Strong anion	Diethylaminoethyl	5	100	2–11	Mitsubishi Kasei
	ProtEx-SP	Strong cation	Sulfopropyl	5	100	2–11	
Silica	Adsorbosphere XL HAAX	Anion		3–7	30	—	Alltech
	Macrosphere 300 WAX	Weak anion	Diethylaminoethyl	7	30	—	
	Macrosphere 300 WCX	Weak cation	Carboxylic acid	7	30	—	
	Macrosphere 300 SAX	Strong anion	Quaternary ammonium	7	30	—	
	Macrosphere 300 SCX	Strong cation	Sulfonic acid	7	30	—	
	Aquapore AX-300	Weak anion	Diethylaminoethyl	7	30	—	Brownlee
	Aquapore CX-300	Weak cation	Carboxymethyl	7	30	—	
	Bakerbond WP PEI	Weak anion	Polyethyleneimine	5–15	30	—	J.T. Baker
	Bakerbond WP CBX	Weak cation	Carboxyethyl	5	30	—	

[a] Data obtained from the respective manufacturer's catalogues. The mention of a manufacturer does not imply any preference of the authors toward that manufacturer's products.

[b] np: nonporous

V. DETECTION SYSTEMS

An integral part of an HPLC system is the detector, since, in addition to the column, the quality of the separation achieved as measured from the chromatogram depends upon the detector response time, the analyte response signal to the detector relative to the detector noise signal, and the flow-cell design. A wide range of possible detectors exists (16,31), and the choice of a particular detector for a specific analysis depends on the properties or characteristics of the solute, the sensitivity of the detector, its selectivity specifically in complex matrices, and its convenience and versatility of operation. In general, ultraviolet (UV) and fluorometric detectors are the more widely used detectors in liquid chromatography of proteins. The peptide bond absorbs at λ = 210–230 nm, the absorbance being independent of the amino acid composition. The sensitivity is 10- to 100-fold higher than at λ = 250–280 nm, where only the aromatic amino acids absorb. However, working at lower wavelengths restricts the choice of solvent, places greater demands on its purity (since many nonprotein materials also absorb at 210 nm), and requires the effective exclusion of oxygen (27). Ultraviolet detectors may be divided into three types: simple fixed-wavelength, dispersion-type variable-wavelength double-beam UV spectrophotometers, and diode-array UV photometers. Fixed-wavelength detectors usually employ a low-pressure mercury vapor lamp with a maximum emission at λ = 253.7 nm or a medium-pressure mercury lamp in conjunction with suitable filters to have a wider choice of fixed wavelengths. The disperion-type variable-wavelength detectors have a broad range of possible wavelengths (usually within the range 260–850 nm) and use a deuterium discharge lamp (UV) or a quartz halogen lamp (visible light, VIS). With a diode-array variable-wavelength detector, the whole of the absorption spectrum of the UV-absorbing analyte is monitored simultaneously on a millisecond time scale. Thus, the UV/VIS spectrum of each chromatographic peak may be measured without disturbing the eluate flow. However, this type of UV detectors usually has a reduced sensitivity and a smaller linear working range and is more expensive than the traditional UV detector (31). Overall, UV detection is selective, i.e., responds to some property of the solute, but the response varies widely for different solutes. Fluorescence detection provides a highly selective monitoring but is limited to proteins with a high content of aromatic amino acids. Proteins containing tryptophan and tyrosine residues show emission spectra between 330 and 370 nm when excited at 220 nm or 280 nm. Excitation at 220 nm provides greater sensitivity (32). Both detectors can be used to quantify the amount of a certain protein present in a sample, since the absorbance is linearly related to the concentration of the solute, according to the law of Lambert-Beer. A disadvantage of both type of detectors is the fact that they provide little or no biopolymer identification or characterization. This is because most biopolymers have very similar absorbance and emission spectra in UV and fluorescence spectrometry (33). Possible ways to overcome this are the further characterization of the fractions obtained after HPLC separation, e.g., with electrophoretic techniques (34–37), and the use of techniques that enable the online characterization of the effluent, e.g., laser light scattering (33,38) and mass spectrometry (39,40). Both techniques still have to be optimized for use as a detector system for HPLC, but they both offer great opportunities.

VI. FOOD APPLICATIONS

Since the introduction of HPLC in the field of protein analysis, this technique has become very popular because of its versatility. The development of improved or new packing materials has often resulted in a decrease in the analysis time. However, with regard to the analysis of food proteins, the application of HPLC has remained limited mainly to wheat proteins and milk proteins. A quick survey of recent literature confirms this observation, and most publications about the

HPLC analysis of food proteins deal with either wheat or milk proteins. Nevertheless, although applications to other food proteins seems to be lagging behind, the following paragraphs describe the application of various HPLC techniques to food protein analysis. Attention will also be paid to the issue of food authentication. In many countries, food legislation has become progressively more onerous, leaving little latitude for a fraudulent food-processing operation. Usually, food authenticity issues do not pose a threat to public health and in most cases are related to the composition of the commodity concerned. Although various types of authenticity fraud can be perpetrated, the objective in all types of fraud is usually to sell a cheap product as a more expensive type. Typical areas involved in food authentication are the identification of species or variety, the region of origin, the use of a specific process, industry fraud, and originality of a certain brand (41).

A. Animal Proteins

The sources of animal proteins can be subdivided into proteins present in edible tissues, e.g., muscles, and those present in edible fluids, e.g., milk and eggs.

1. Milk Proteins

Milk proteins are subdivided into random coiled caseins, which can be precipitated by acidification of raw skim milk to pH 4.6 at 20°C, and into more globular whey proteins, which remain in the serum after precipitation of the caseins (42). In Table 8, an overview is given of the molecular structure and basic properties of the major protein fractions present in milk. Some specific properties that might be of importance for their determination in foods and food products are also listed. For the young of mammals, including humans, milk is the first and, for most, the only food ingested for a considerable period of time. With the domestication of animals, it became possible to include milk in the diet of adult humans as well. For much of the world, particularly in the West, milk from cattle (*Bos taurus*) accounts for nearly all the milk processed for human consumption (43).

An overview of some applications of HPLC for milk protein analysis is given in Table 9. Unless whole milk is to be analyzed, sample preparation usually includes the separation of the casein fraction from the whey fraction. This can be achieved during processing of the food, e.g., during the production of caseinates, whey protein isolates (WPIs), or whey protein concentrates (WPCs), or it has to be done prior to analysis. Possible means are centrifugation of the skim milk in the presence of Ca^{2+} to precipitate the casein fraction (35), renneting of the milk to obtain sweet whey (36,44), acidification of the milk to pH 4.6, with acid (45–56) or lactic acid bacteria, to obtain acid whey (36,37). Acidification to pH 4.6 can also be used to remove denatured whey proteins (57,58). Other sample pretreatments can include the removal of low-molecular-weight compounds, such as salts and lactose, by dialysis (35,37,59), ultracentrifugation (60), and the addition of salts, e.g., $(NH_4)_2SO_4$ (60), to remove aggregated, denatured proteins. When cheese samples have to be analyzed, the protein isolation usually includes solubilization of the proteins, applying reducing solvents like β-mercaptoethanol (61,62), and a defatting, e.g., by ultracentrifugation (61,62). In almost all cases, the protein sample is filtered over a 0.22- or 0.45-μm filter membrane prior to injection on the column.

The use of HPLC for the analysis of bovine milk proteins was introduced by Diosady et al. (59), who compared SE-HPLC on two SynChropac GPC-100 columns in series and RP-HPLC on a 10-μm-particle-size RP-8 column, both with UV detection, for the separation of dialyzed freeze-dried whey proteins. Column temperature was 40°C and 47°C, respectively. Samples were eluted with Tris-buffer pH 6 for SE-HPLC and a linear gradient of two solvents, i.e., 98% 0.5 M KH_2PO_4, pH 2, and 98% isopropanol, each with 2% 2-methoxyethanol for RP-HPLC. They

Table 8 Molecular Structure and Properties of the Major Milk Proteins

Food protein	Protein component	% of total protein	Molecular weight	Isoelectric point	Molecular structure
Caseins	α_{s1}-Caseins	32–40	22,068–23,724	44.4–4.76	Primarily random coiled Rich in Pro and hydrophobic amino acids Strong tendency for association by hydrophobic interactions
	α_{s2}-Caseins	8–10	25,230	4.20–4.60	Primarily random coiled Most hydrophilic of major caseins
	β-Caseins	24–29	23,944–24,092	4.83–5.07	Either highly asymmetrical or random coiled Most hydrophobic of major caseins Strong tendency for association by hydrophobic interactions
	κ-Caseins	5–10	19,007–19,039	5.30–5.80	Random coiled, containing distinct hydrophobic (para-κ-casein) and hydrophilic (macropeptide) regions Tendency for association by disulfide bonds and hydrophobic interactions
Whey proteins	β-Lactoglobulin	8–17	18,205–18,363	5.35–5.49	Globular protein, containing one cysteine and two cystine residues Formation of dimers in pH range 5.2–7.5; octamerization may occur at pH 3.5–5.2
	α-Lactalbumin	2–7	14,147–14,175	4.2–4.5	Compact globular protein, containing four cystine residues High stability between pH 5.4 and pH 9.0
	Bovine serum albumin	2	66,267	4.7–4.9	Rod-shaped protein, containing one cysteine and 17 cystine residues Partial unfolding at low (<4) and high (>8) pH values
	Immunoglobulins	2–4	150,000–1,000,000	5.5–8.3	Monomers or polymers of four-chain molecular containing two light (MW 20,000) and two heavy (MW 50,000–70,000) chains linked by disulfide bonds

Source: Adapted from Ref. 14.

Table 9 Overview of the Application of Different HPLC Methods to the Analysis of Milk Proteins

Motivation for protein determination	Applications in food analysis	Method of HPLC	Refs.
Process evaluation and optimization	Changes in solubility and aggregation of a whey protein isolate during heating in slightly alcaline media	Size-exclusion FPLC	60
		Anion–exchange FPLC	60
		Hydrophobic-interaction FPLC	60
	Effect of heating on milk protein denaturation	Size-exclusion	53, 57
		Reversed-phase	58, 63
	Classification of heat-treated milk powder	Size-exclusion	50
		Reversed-phase	64
	Enzymic milk coagulation	Size-exclusion	65
		Reversed-phase	65
	Effect of high-pressure treatment on protein aggregation	Size-exclusion	66
	Cheese maturation	Anion-exchange FPLC	62
		Cation-exchange FPLC	62
Evaluation of storage conditions	Effect on protein interaction and complex formation	Reversed-phase	64
	Effect of elevated temperature storage on whey protein denaturation	Size-exclusion	57, 67
Determination of food composition	Protein composition determination of whey and/or casein fraction	Size-exclusion	67
	Individual animal variation in milk protein composition	Reversed-phase	68
	Characterization of protein concentrates and/or isolates	Size-exclusion	69, 70
		Reversed-phase	71
	Characterization of caseinates	Size-exclusion	72
Product authentication	Separation of soy proteins from whey proteins	Reversed-phase	73
	Phenotyping of milk	Reversed-phase	74
	Detection of mixtures from different animal species	Reversed-phase	45, 46, 54, 75
		Anion-exchange FPLC	56, 76
		Cation-exchange	61
	Detecting renneted whey solids in skim milk powder	Size-exclusion	77
		Reversed-phase	78
Optimization of protein fractionation and determination methods	Evaluation of HPLC for fractionation of milk proteins	Size exclusion	35, 44, 59, 79, 80
		Size-exclusion FPLC	55, 81
		Reversed-phase	46, 49, 59, 68, 79, 82
		Reversed-phase FPLC	55, 81
		Anion-exchange FPLC	36, 37, 44, 55, 82
		Cation-exchange FPLC	48, 51, 83
		Anion-exchange	84
		Hydrophobic interaction	47, 85
		Hydroxyapatite	82
		Perfusion chromatography	48

found that both HPLC techniques were effective in quick separations, i.e., 15 min for SE-HPLC and 20 min for RP-HPLC, and determinations of the protein distribution in whey. Bican and Blanc (35) succeeded in separating dialyzed whey proteins and native casein fractions, obtained from fresh raw milk, with SE-HPLC using only one column at 22°C (MicroPak TSK 3000 SW, Varian, USA), 40 mM Tris-Cl, 50 mM NaCl, pH 7.6 as elution buffer, and peak detection at 220 nm. The resolution obtained, however, was too low for a clear quantification of the different fractions. The use of different chromatographic techniques to separate caseins was evaluated by Barrefors et al. (55) and Visser et al. (82). Reversed-phase chromatography gave the highest resolution for the separation of caseins, with an analysis time of about 1 hour. Faster separations could be obtained using hydroxyapatite HPLC (HA-HPLC) (82) or IE-FPLC (55) but with a lower resolution. A good separation and quantification of the major casein fractions, i.e., α_{s1}-, α_{s2}-, β-, and κ-casein, was obtained by Hollar et al. (83) using anion-exchange FPLC on a Mono Q HR 5/5 and Mono S HR 5/5 column (Pharmacia Biotech, Sweden). Performance of both columns was comparable, and results were obtained within 45 min. Ion-exchange HPLC was also very effective in the quantitative analysis of caseins during the ripening of cheese in order to evaluate maturation (62). However, both anion-exchange and cation-exchange chromatography were necessary to quantify all caseins, since peptides, formed during the ripening, interfered. In a study to characterize the functional properties of different WPCs and WPIs, de Wit et al. (69) used SE-HPLC on two TSKgel 2000 SW columns (TosoHaas, USA) in series to evaluate the purity. They used a 0.1 M potassium phosphate buffer containing 0.15 M Na_2SO_4 pH 6, and UV detection at 205 nm. The α-lactalbumin (α-la) and β-lactoglobulin (β-lg) isolates showed a high purity, with only one fraction detected, whereas the bovine serum albumin (BSA) and immunoglobulin (Ig) isolates included two fractions. This result was expected for the Ig isolates, which are known to contain different proteins, but no explanation was found for the BSA isolate. A separation method of whey proteins by anion-exchange HPLC was developed by Irvine and co-workers (36,44) on a Mono Q HR 5/5 column. Gradient elution was done with water and a buffer containing 0.7 M sodium acetate, pH 6.3, and peak detection was at 280 nm. A separation (40 min, including cleaning time) and quantification of α-la and the β-lg genetic variants A and B, which primary structures differ only at two positions of their 162 amino acid sequence (86), in whey was achieved, while the BSA fraction was heavily contaminated and could not be reliably quantified. Girardet et al. (37) evaluated the parameters influencing the chromatographic behavior of whey proteins on the Mono Q HR 5/5 column and concluded that fractions with different degrees of purity can be obtained by changing the elution buffer or the gradient, i.e., a nonlinear-type gradient. Typical elution profiles obtained with the Pharmacia Mono Q column, using different buffer systems, is shown in Fig. 4.1. Girardet and co-workers also showed that previous physicochemical treatments of whey proteins can have an influence on the separation profile; e.g., the chromatographic behavior of Ig toward ion-exchange material can be altered by the sample preparation, resulting in coelution with other whey proteins. The behavior of whey proteins in RP-HPLC and HI-HPLC was studied by de Frutos et al. (49). They applied circular dichroism spectroscopy to evaluate the influence of reversed-phase and hydrophobic-interaction chromatographic systems on the confirmation of proteins. Hydrophobic-interaction HPLC led to a low degree of whey protein denaturation, which is an advantage when used with a preparative aim, but a higher resolution was achieved with RP-HPLC (e.g., the A and B variants of β-lg could be separated, but accompanied by severe denaturation of the molecules). The same research group optimized an RP-HPLC method for the separation of whey proteins, including the β-lg A/β-lg B pair, by using a two-segmented linear gradient of 10% acetonitrile in water and 90% acetonitrile in water, both containing 0.1% TFA and 0.1% morpholine. They were able to reduce the analysis time to less than 10 min on a Vydac 214 TPB 10 column (The Separations Group, USA). A breakthrough in the rapid analysis of whey proteins came with the introduction of gigaporous supports, or the

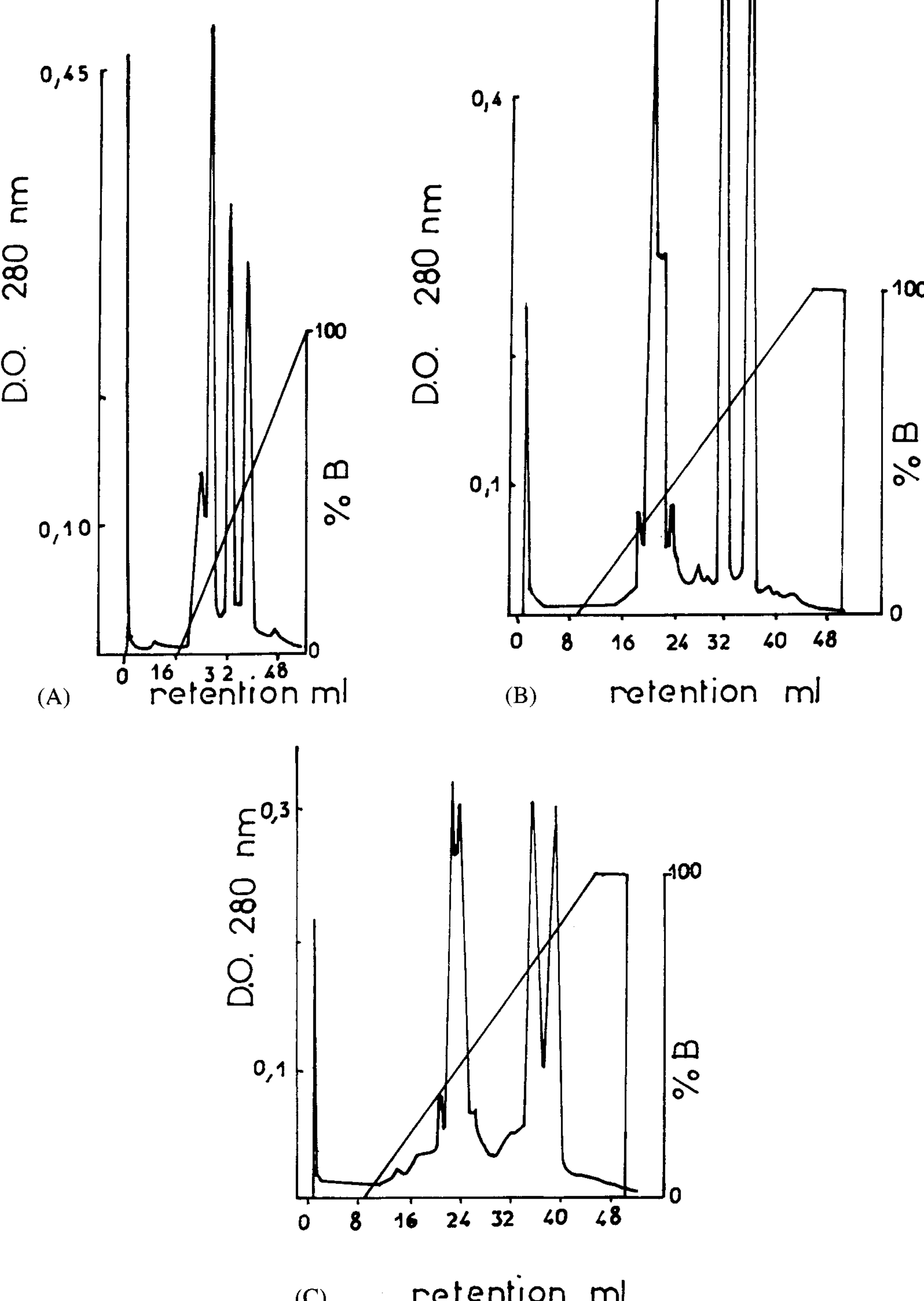

Fig. 1 Ion-exchange chromatography of whey proteins on a Mono Q column using different buffer systems: (A) a 100-μl sample of total whey protein dissolved in 0.05 M sodium acetate buffer, pH 6.3, was injected into the column and eluted with a 0.05–0.7 M sodium acetate linear ionic-strength gradient; for (B) and (C) the sample was dissolved in 0.02 M Tris-HCl, pH 7 (B) or pH 8 (C), and a 0–0.35 M NaCl linear gradient was applied. *Key*: 1: immunoglobulins, 2: α-lactalbumin, 3: bovine serum albumin, 4: β-lactoglobulin B, 5: β-lactoglobulin A. (From Ref. 37.)

so-called perfusion chromatography. Torre et al. (48) were able to separate the main whey proteins (α-la, β-lg A, β-lg B, and BSA) in about 1.5 min, with a standard deviation (S.D.) $\leq$3.75 mg per 100 ml in the quantitative determination of α-la and the β-lgs. Chromatographic conditions were a POROS 1 10R (PerSeptive BioSystems, USA) packed column held at 50°C, linear binary gradient of 5% acetonitrile, 20% formic acid and 93% acetonitrile, and UV detection at 280 nm. The load capacity of the perfusion column was about 10 times smaller than of silica-based stationary phases, with overload having an adverse effect on column resolution. This requires a higher sensitivity of the detection system, which might be a disadvantage of this technique.

The evolution in separation methods described in the preceding paragraph is focused completely on the separation of bovine milk proteins. Much less information is available on the use of HPLC techniques to separate nonbovine milk proteins. Law and co-workers applied cation-exchange FPLC to the quantitative separation of ovine casein (51) and caprine casein (52) on a Mono S HR 5/5 column, using a 20 mM acetate, 6 M urea, pH 5, and 20 mM acetate, 6 M urea, 1 M NaCl, pH 5 elution buffer system. They observed that ovine and caprine casein contains much less α_s- and much more β-casein compared to bovine casein. However, caprine casein contains a higher κ-casein content compared to bovine or ovine casein (52).

Skim milk is used in the food industry for the manufacture of baked goods, dairy products, and processed foods. During processing of skim milk and milk products, various heat treatments are used that can denature whey proteins. Pre–heat treatment of skim milk before spray drying is used widely as a means of improving water absorption and functional properties. Since high temperatures might have a pronounced effect on the functional properties of whey proteins by inducing protein unfolding (87), a large number of studies have been carried out to establish the possibilities for HPLC to determine the degree and kinetics of whey protein denaturation since its introduction to protein research. The classification of low-heat and extra-low-heat skim milk powder was possible via the determination of the peak area of undenatured whey proteins after separation on a Zorbax Bio Series GF-250 SE-HPLC column (Rockland Technologies, USA) (50). Results were comparable to these obtained according to the American Dry Milk Institute method. Dumay and Cheftel (60) compared SE-FPLC, HI-FPLC, and anion-exchange FPLC to determine the thermal denaturation (0–10 min at 65–82°C) of α-la and β-lg in a β-lg concentrate at slightly alkaline pHs (7.7–9.2). They found that HI-FPLC on a Phenyl Superose HR 5/5 (Pharmacia Biotech, Sweden), with binary gradient buffer 50 mM Tris-HCl, 1.27 M $(NH_4)_2SO_4$, pH 7.5, and 50 mM Tris HCl, 35% acetonitrile, pH 7.5, and peak detection at 280 nm, gave the best results for quantifying the residual native or "quasi-native" α-la and β-lg A and B after precipitation of the aggregated denatured proteins with $(NH_4)_2SO_4$. Patel et al. (70) found a positive correlation between the thermal properties of six cheddar-cheese-type whey protein concentrates and the amount of β-lg present as determined by SE-HPLC on a Bio Sil TSK 250 column (Bio-Rad), using a dissociating phosphate buffer with 0.1% dithioerythritol and 5% SDS and peak detection at 280 nm. This indicated that the thermal properties of the WPCs were determined to a large extent by the β-lg fraction. Determination of whey protein denaturation based on the peak area of the individual peaks for the different whey proteins, separated with RP-HPLC, was found to be a good way to determine the thermal damage of whey proteins during (low) thermal processes used in the dairy industry (58,63,64). Variations in the quantification were smaller compared to the classical Kjeldahl protein determination method after removal of caseins and denatured whey proteins by isoelectric precipitation (58,63). Similar results were obtained by Law et al. (53), who used SE-FPLC and IE-FPLC (Mono Q HR 5/5 and Mono S HR 5/5) to investigate the effect of different time–temperature profiles on the alteration of milk proteins. The extent of denaturation of the whey proteins increased with the severity of heating, and most of the Ig, BSA, α-la, and β-lg were denatured when milks were heated for 5 min at temperatures equal to or greater than 80°C. Caseins were less sensitive to heating, and changes in the elution profile were only observed on heating milk for 5 min at 120°C or for 1 min or more at 140°C, due to

changes in the charges of the negatively and positively charged amino acid residues on heating. Only a few studies have focused on changes in the functional properties of caseins during the production of commercial caseinates. Lynch et al. (72) showed different levels of modifications in the negative and positive charge distribution, the percentage of high-molecular-weight proteins, and the amount of free amino acids and SH groups for nine commercial sodium caseinates upon heating to high temperatures. They used SE-FPLC [Superose 12 HR 10/30 column (Pharmacia Biotech, Sweden) with 2.5% SDS, 7.63 mM Tris, 0.76 mM EDTA, pH 8.2 as eluting buffer] to show the formation of covalently bonded high-molecular-weight aggregates and IE-FPLC (Mono Q HR 5/5 and Mono S HR 5/5) to prove alterations in the charge distribution, occurring during processing of the caseinates.

In the dairy industry it is common practice for cheese whey, which is not to be processed immediately, to be either cooled or pasteurized. Morr (57) used SE-HPLC on a TSK 3000 SW Spherogel column to prove that prolonged storage ($\geq$10 h) of cheese whey at temperatures used for pasteurization results in losses of Ig (30–35%) and BSA (13–21%) in sweet and acid whey and up to 30% for α-la in sweet whey. β-Lactoglobulin was considerably more stable, showing no loss. The effect of heat treatment, water activity, and storage temperature on the formation of protein polymerization products during the storage of whole milk powder was proven by Stapelfeldt et al. (67), applying SE-HPLC (TSKgel G3000 SW-XL (TosoHaas, USA) with 50 mM phosphate buffer, pH 6.8, as mobile phase).

Milk is a natural product and thereby subjected to seasonal variations linked with feeding (supplementary or not) and calving. These seasonal variations associate with a similar variation in the composition of milk as well as the functional properties of the resultant whey protein concentrations (71). Furthermore, seasonal variations have been demonstrated to influence cheese-making properties (88) and to affect the heat stability of milk powders (89). Regester and Smithers (71) studied WPC prepared from cheddar cheese whey at weekly intervals over consecutive lactation cycles between August 1986 and April 1987 and between October 1987 and May 1988. They used RP-HPLC (Spherisorb S5 C6 resin) to quantify α-la, β-lg, and glycomacropeptide after resolubilization of 0.1% WPC powder in a 150 mM NaCl:HCl solution, pH 2.1. Seasonal changes generally included a reduction in the α-la content of WPC manufactured during the final three months of the lactation, concomitant with a rise in the level of β-lg. However, Groen et al. (68) found a higher whey protein content in early lactation than in mid-lactation in their case study on individual animal variation in milk protein composition as determined by RP-HPLC (HI-Pore RP-318; 10% acetonitrile and 90% acetonitrile in water, both containing 0.1% TFA, in a multisegment linear gradient, column temperature 30°C, peak detection at 220 nm). Since they do not report on individual whey proteins, these results are not necessarily in contradiction. These researchers also observed a significant effect of the stage of lactation on α_{s1}-casein, the mass fraction being higher in early lactation compared to the rest of the lactation. Another parameter influencing the composition of milk and some milk-processing parameters is the occurrence of certain milk protein genetic variants (90). Visser et al. (74) were able to identify simultaneously various casein variants and the whey proteins α-la, β-lg A, and β-lg B in a single run in an RP-HPLC system consisting of a Hi-Pore RP-318 column with a multisegmented gradient of 10% acetonitrile, 1% TFA in water, and 90% acetonitrile, 0.7% TFA in water, column temperature 30°C, and peak detection at 220 nm.

Milk and dairy products have been important trading goods since historical times. Thus, they may well have been one of the earliest foodstuffs to be subjected to adulteration. There are several ways in which the authenticity of milk may be challenged, and, since milk is used as the raw material for other products, such as cheese, there is also the potential to use waste from the milk-processing industry (e.g., whey) as an extender. Another possibility is the use of milk of other species of animal. There may be safety issues attached to this type of adulteration, since many people are allergic to cows' milk and therefore choose milk, cheese, and yogurt from other

animals, such as sheep and goats. Analytical methods to detect adulterations in the dairy industry are based on differences in fat and protein composition. Differences in the protein fraction can be detected by electrophoretic techniques, which is time consuming, and immunological methods, which are limited by the availability of specific antisera. Applications of HPLC in this field have been reported by a number of research groups. A method to detect bovine milk in mixture with ovine milk using anion-exchange FPLC (Mono Q HR 5/5) was described by Laezza et al. (56). They found a good correlation between the amount of bovine milk present in the mixture (up to 60%) and the peak area of bovine α-la B. The research group of de Frutos (45,46) used RP-HPLC on a column packed with Vydac 214 TPB 10 to enable the separation of whey proteins from bovine, ovine, and caprine species and the detection of milk mixtures from different species. Using gradient elution with mobile phases A and B, comprised of 33% acetonitrile and 43% acetonitrile, each containing 0.1% TFA, they succeeded in identifying cows' milk in ewes' and goats' milk, detecting ewes' milk in milk mixtures, and incorporating bovine milk (>10%) during the production of cheese made from ovine milk by assigning peaks to whey proteins of the different species. Separation time was about 8 min. In order to obtain reproducible results for the quantification of whey proteins on an unused column, de Frutos et al. (46) found that a preconditioning of the column, i.e., a saturation of the active sites still present on the support, was necessary. This could be done by injecting 10–20 μg of protein (α-la) for a 5-cm × 4.6-mm internal-diameter column. Following this procedure, linear calibration curves (protein concentration versus peak area) for the main whey proteins were obtained ($r \geq 0.997$). Very similar results with RP-HPLC were obtained by Romero et al. (54), Pellegrino et al. (91), and De Noni et al. (75). In contrast to de Frutos et al. (46), Romero et al. (54) could not distinguish ewes' from goats' milk, and the analysis time was about 25 min. This could be due to differences in packing material (PLRP-ES column, Polymer Laboratories, UK) and solvent gradient used. A further reduction in analysis time to less than 2 min was achieved by Torre et al. (48) using a POROS 1 10R perfusion chromatography column. They could detect the presence of ovine milk in bovine milk or vice versa (0.1 in 1), the addition of ewes' milk to goats' milk (0.1 in 1), and the addition of bovine milk to caprine milk (9 in 1). The use of casein fractions to detect cows', ewes', and goats' milk occurs to a lesser extent. Haasnoot et al. (76) evaluated the use of Mono Q HR 5/5 with a 0.02 M Tris-HCL, 6 M urea, pH 8.5, and 0.02 M Tris-HCl, 6 M urea, 1 M NaCl, pH 8.5 buffer gradient system for the separation of caseins of different species. Adulterations of goats' and ewes' milk can be detected quantitatively with bovine α_{s1}-caseine as a marker and a detection limit of about 2% and 4%, respectively. The presence of cow casein in sheep and goat milk cheeses can be estimated with the cow milk α_{s1}-caseine degradation product α_{s1}-I-caseine as an indicator. Analysis time was about 30 min. Mayer et al. (61) reported the determination of the percentages of cows', ewes', and goats' milk in cheeses, based on the quantification of para-κ-casein from the different species within 45 min. Chromatographic conditions were a Shodex IEC CM-825 column (Waters Corp., USA); multisegment linear gradient using 10 mM malonic acid, 5 M urea, and 10 mM malonic acid, 5 M urea, 0.5 M NaCl, both pH 6; peak detection at 280 nm. The inclusion of rennet whey and buttermilk in skim milk powder is financially attractive, considering the lower price of these products. However, according to EU regulations (Regulations 2188/81 and 1725/79), skim milk powder may not contain solids from whey or buttermilk. A sensitive SE-HPLC method to detect the adulteration of skim milk powder is presented by Olieman and van den Bedem (77) and is included in EU Regulation 625/78. Addition of more than 0.8% of rennet whey total solids to skim milk powder and sweet buttermilk powder was accurately detected, based on the glycomacropeptide peak, after separation on two TSK 2000 SW columns in series at 35°C with a phosphate buffer containing Na_2SO_4 as mobile phase and detection at 205 nm. Excessive growth of psychrotrophic bacteria in milk and the presence of components, eluting after the GMP peak, might cause false positives, however. Six years later, Olieman and van Riel (78) presented a detection method for GMP on RP-HPLC that is able to detect more than 0.2% addi-

tion of rennet whey solids but requires an analysis time of about 50–60 min, which is about five times longer than the application of the previously described method (77) on a Zorbax GF-250 column.

2. *Egg Proteins*

Proteins present in whole egg, yolk, and albumen (egg white) are excellent sources of nutrients, and they possess valuable functional properties. Shell eggs consist of 8–11% shell, 56–61% albumen, and 27–32% yolk. The solid content of albumen is about 11–13%, depending on the strain and on the age of the hens, and the solid content of yolk is about 52–53.5%. Albumen solids contain mainly protein, whereas lipids are the major constituents of egg yolk (92). Yolk can be separated by centrifugation into sedimented granules and a supernatant, plasma. The granules contain the major part of the yolk proteins. The main proteins in albumen and yolk are ovalbumin, ovotransferin, lysozyme, ovomucoid, ovomucin, and immunoglobulin Y (93).

Very few publications report on the use of HPLC of egg proteins for analytical purposes. A possible reason may be that food regulations concerning eggs usually deal with physical properties rather than with functionality or composition. Until recently, there has been less emphasis on ensuring egg quality from a regulatory perspective (94). Another possible reason may be the complexity of the protein fraction, especially the lipoprotein fraction present in yolk, compared to milk proteins. Sajdok et al. (95) used a combination of HPLC and immunochemical techniques to prove that the determination of egg content in food products, based on immunochemical assay, is reliable only when limited thermal treatment is involved in processing. Size-exclusion FPLC on a Superose 12 column was used to study aggregate formation in egg white at acid and alkaline pH values. At acid pH, high-molecular-weight aggregates (>500,000 Da) were formed in the early stage of denaturation (10 min at 70°C), whereas no aggregation was detected at alkaline pH. Heat denaturation of hen egg white was also accompanied by protein splitting, resulting in lower-molecular-weight compounds (ranging from 23,000 to 30,000). Takeuchi et al. (96) described a rapid method for the analysis of egg white by RP-HPLC. Using a Bondasphere C4-300 (Waters Corp., USA), they were able to separate and identify the major proteins present in egg white, within 40 min. A limitation to the method is the coprecipitation of lysozyme with ovomucin upon dilution of the sample, especially at low ionic strength.

3. *Meat Proteins*

Meat can be considered as the product resulting from muscle tissue after chemical modifications occurring after death and exsanguination of an animal. In this regard, the term *meat* is used to indicate the contractive tissues from all species of animals used for food, including beef, pork, lamb, turkey, chicken, fish, and crustaceans. This is a rather narrow definition compared to, e.g., the UK Meat Products and Spreadable Fish Products Regulations (1984), which define meat as "the flesh, including fat, and the skin, rind, gristle and sinew in amounts naturally associated with the flesh used, of any animal or bird which is normally used for human consumption." Many of the proteins in meat can be easily extracted using salt solutions, but extractability depends strongly on salt concentration, pH, and species. The water-soluble fraction contains the muscle pigment myoglobin and enzymes, the salt-soluble fraction is composed mainly of contractile proteins, and the remaining unsoluble fraction consists of connective tissue proteins, membrane proteins, and also some unextracted contractile proteins. Main contractile proteins are myosin, paramyosin (in invertebrates), actin, titin (connectin), actinin, actomyosin, tropomyosin, troponin, desmin, myomesin, and nebulin. The main fraction of connective tissue is collagen (97).

Heating of meat during processing or cooking results in both chemical and physical changes that can be noticed as protein denaturation and solubility loss. Davis and Anderson (98) used SE-HPLC (Spherogel TSK-3000 SW) to evaluate heat-induced changes in the molecular-

weight-distribution profiles of water-soluble compounds extracted from bovine and porcine tissue. Twelve protein fractions were separated, of which six were below the effective size-exclusion capabilities of the column. The authors attributed this to adsorption effects. The six major proteins appeared to be very heat sensitive, this fraction representing 41.5% and 37.5% of the water-extractable proteins of porcine and bovine, respectively. Slight differences in the molecular weight of myoglobin for both species were obtained. The color of meat is considered an important aspect of the quality and is determined primarily by the concentrations and chemical states of myoglobin and hemoglobin pigments. Myoglobin is the primary pigment, whereas hemoglobin, the pigment of blood, is of secondary importance, since most is removed when animals are slaughtered and bled. Hemoglobin consists of four myoglobins linked together as a tetramer (99). Oellingrath et al. (100) were able to separate myoglobin and hemoglobin completely in minced beef samples using HI-HPLC after selective precipitation in mild salt solutions. A quantitative separation of myoglobin and hemoglobin in beef and chicken muscles was developed by Han et al. (101). Extraction of pigments from minced samples was carried out using a phosphate buffer (0.01 M, pH 6.3). Myoglobin and hemoglobin coeluted on an SE-HPLC column (Spherogel TSK 2000SW from Beckman, USA), indicating dissociation of the hemoglobin quaternary structure. Hydrophobic-interaction HPLC (Hydrophase HP-butyl from Interaction Chem., USA) resulted in the separation of myoglobin and hemoglobin from bovine samples but not from chicken samples. The best quantification of pigments was obtained based on peak areas on the SE-HPLC chromatogram and indicated differences in total pigments among cattle or among chickens and interactions between animal and type of muscle. The authors concluded that SE-HPLC was the best-suited method to determine myoglobin in meat, especially from species with low myoglobin content.

Adulteration of meat and meat products may involve the addition of substances of lower value and/or quality above the amounts required for normal or accepted processing or product formulation, e.g., the replacement of meat from high-value species with meat from lower-value species, the addition of an excess amount of fat or rind, or the addition of water, nonmeat proteins, or other substances. Most of the detection methods are based on electrophoresis and immunological methods (102,103). However, the identification of meat with liquid chromatography is also possible (103–107). Ashoor et al. (104) described an RP-HPLC method [Hi-Pore RP-304 column (Bio-Rad, USA); binary linear gradient using water and 95% acetonitrile in water, both containing 0.1% TFA, detection of peaks at 280 nm] for the identification of raw beef, pork, veal, lamb, chicken, turkey, and duck based on water-soluble proteins. Meat cuts and parts from the same species had similar chromatographic profiles and differed only quantitatively. However, meat cuts or parts from different species resulted in different chromatographic profiles, which enabled the detection and identification of meat blends. However, the method is applicable only to fresh and frozen meats, since the heat treatment of meat resulted in less distinctive chromatograms. Also, the effects of age, sex of the animal, different breeds, geographical location, and seasonal variation were not evaluated, and these factors might have an influence. The use of an external standard further complicates the method, and the interference of other (nonmeat) proteins was not examined. The same procedure was applied to determine the quantity of chicken and turkey in unheated chicken–turkey mixtures (105). Mixtures of 5–100% of raw chicken in turkey could be detected. Reversed-phase HPLC (Hi-Pore RP-304) of water-extractable proteins from fish white muscle tissue was used for the identification of seven fish species (108). The use of bovine serum albumin (BSA) was necessary for the qualitative identification of fish species, since only the expression of retention times, relative to BSA, gave good results. Bovine serum albumin was also used as an internal standard for quantification. However, response factors of the different peaks were not known, which makes quantification of unknown samples difficult. Qualitative and quantitative differences were noticed in the chromatograms of the seven species studied (salmon, cod, monk, trout, rockfish, catfish, and shark). The analysis time for optimum resolution

was about 90 min. A major drawback of HPLC methods for species identification are changes in peak retention times, widths, and resolution due to even slight variations in the chromatographic parameters, such as mobile-phase composition, gradient generation, and temperatures. The long analysis time (50–60 min) magnifies these effects in consecutive chromatograms, and special attention is necessary to overcome these problems. In another study (107), Armstrong et al. reported a method to overcome these problems. One peak was taken as the reference peak, and relative retention times of all other peaks were calculated. Star-symbol plots, with the radials representing the possible peaks and their lengths proportional to the corresponding percentage area, generated unique plots for each species. Species identification of unknown samples was correct, even after irradiation or drying of the samples, but was not applicable to cooked samples. According to the authors, the presented method is universally applicable, and results obtained by different laboratories would easily be comparable and interchangeable. The star-symbol plot was shown to be little affected by seasonal changes or intraspecies variability. The effect of capture or storage conditions, however, was not studied. It is known that these factors might have an effect on the sarcoplasmic fraction, due mainly to the existence of proteases acting during the postmortem period (97), and freezing may also induce changes due to denaturation of sarcoplasmic proteins (109). Piñeiro et al. (106) used RP-HPLC (Hi-Pore RP-304) to identify gadoid fish species. Although phylogenetically related fish species were analyzed, the chromatograms obtained were different, which enabled species identification based on the presence or absence of one or more peaks. The methodology described seemed to be independent of any protein damage occurring during storage at low temperatures, e.g., chilling and freezing temperatures. Another possible means of adulteration is, as already mentioned, the addition of an (excess) amount of nonmeat proteins. Since the addition of nonmeat proteins may be the subject of government regulations—for instance, concerning the maximum allowable amount added to meats—simple and reliable analytical methods for the determination of nonmeat proteins in meat products should be available. Reversed-phase HPLC (Hi-Pore RP-304) was proven to be a possible technique for determining the presence of nonmeat proteins in unheated meats (110). The presence of soy protein, caseinate, and whey protein in unheated beef, pork, chicken, and turkey was possible, as was the identification of the nonmeat protein added. However, quantitative determination was possible only in the case of soy protein and caseinate, since in the case of whey proteins the major peaks overlapped with peaks of meat proteins. The application of the presented RP-HPLC method to sterilized meat products was not tested.

To conclude, it can be said that HPLC gives a rapid and sensitive method for species identification and is simple enough to be employed for routine analysis. Although immunological detection is very specific and sensitive, special antisera are required and may not yield quantitative responses. Unlike electrophoresis, staining and destaining procedures are not required, and direct quantification of the separated proteins is possible, using an external or, preferably, an internal standard. Furthermore, electrophoretic methods lack the reproducibility needed for quantitative results and involve many preparation steps. Nevertheless, in the case of heated or cooked meat, electrophoresis seems to be the only reliable analysis method and is in all cases the only method recognized by the AOAC for fish species inspection.

B. Plant Proteins

Worldwide, about 70% of the protein consumed by humans is derived from plant tissues. Although thousands of crops are available as possible food source, only 100–200 species are of major importance in the world trade. The major cultivated species produced in the world include sugar cane, wheat, maize, rice, potato, sugar beet, barley, cassava, sweet potato, and soybean. The protein content varies greatly among the different species, although it generally represents only a small percentage of the fresh weight (111). However, grains from cereals and seeds from legumes

may have high protein contents. Hence, most applications of HPLC for protein analysis deal with the storage proteins in cereals and beans.

1. Cereal Proteins

Cereal grains can be defined as the fruits of cultivated grasses that are processed in order to make them into palatable foods or drinks (112). Cereal crops include wheat (*Triticum aestivum* L.), corn, rice, oat, and barley and are, worldwide, the direct or indirect source of about 75% of our dietary protein. Wheat proteins can be divided into two major classes, the monomeric and polymeric proteins, depending on whether they consist of single- or multiple-chain polypeptides. The monomeric proteins can be subdivided into two groups: the gliadins (zeins for maize, hordeins for barley, secalins for rye, and avenins for oats), with a molecular weight ranging from 30,000 to 80,000, and monomeric albumins and globulins, with a molecular weight of about 20,000–30,000 (137). Gliadins are soluble in 70–80% alcohol, whereas albumins are soluble in water and globulins in salt solutions but not in water (111). Three main groups of proteins constitute the multichain or polymeric proteins: glutenins, made up of the high-molecular-weight (80,000–120,000) and low-molecular-weight (30,000–55,000) glutenin subunits, the high-molecular-weight albumins, which are mainly β-amylases, and triticins, which are globulin-type proteins (113). Glutenins are insoluble in neutral aqueous solutions, saline solutions, or alcohol. However, according to Huebner and Bietz (114), low-molecular-weight glutenins are soluble in 70% ethanol and are coextracted with the low-molecular-weight monomeric gliadins, and are therefore referred to as ethanol-soluble glutenin. A summary of the major proteins in wheat and some relevant properties are listed in Table 10. In wheat, the principal storage proteins are the gliadin

Table 10 Molecular Structure and Properties of the Major Wheat Proteins

Protein components	% of total proteins	General characteristics
Albumins and globulins	12–20	Extractable by water of dilute salt solutions (e.g., 0.1–0.5 M NaCl) Contains enzymes (amylases, proteinases, lipoxygenases) Rich in Lys, Asp, Ala, Cys, Leu, and Arg; low in Met content
Gliadins	35	Soluble in aqueous solutions of alcohol (e.g., 70% ethanol or 55% isopropanol) Primarily globular proteins (MW 30,000–40,000) stabilized by intramolecular disulfide bonds Rich in Glu, Pro, and hydrophobic amino acids, and low content of basic amino acids; Glu and Asp in primarily amide form; low net charge
Soluble glutenins	15–35	Extractable in dilute acid or alkali (e.g., 0.05 N acetic acid) High-MW (100,000–3,000,000) complexes containing low-MW (40,000–10,000) subunits linked by disulfide bonds; extra stabilization by hydrogen and hydrophobic interactions Rich in Glu, Pro, and hydrophobic amino acids, and low content of basic amino acids; Glu and Asp in primarily amide form; low net charge
Insoluble glutenins ("residue protein")	10–40	Not extractable by dilute acid or alkali Composed primarily of high-MW aggregated glutenin molecules containing several intermolecular disulfide bonds

Source: Adapted from Ref. 14.

and glutenin fractions. These fractions represent about 80–85% of the endosperm proteins (111). Extraction procedures for proteins from cereals are usually based on stirring the milled cereals for a certain amount of time in a buffer solution under reducing or nonreducing circumstances. Complete solubilization of all the wheat flour proteins is possible in dilute NaOH solutions (115) or by introducing reducing agents like β-mercaptoethanol, but this results in loss of information about how the polypeptides interact. In most cases, however, only part of the proteins is extracted, depending on the composition of the extraction buffer. Extraction with phosphate buffer containing SDS usually gives high extraction efficiencies (between 55 and 90%), depending on the wheat genotype (116). Application of mechanical shear, e.g., sonication, instead of simple stirring increases the extraction efficiency (117) but is not complete (118). An overview of some applications of different types of HPLC to the analysis of cereals is given in Table 11.

a. Wheat

Liquid chromatography is the second major method used to isolate and characterize wheat proteins, the most important method still being electrophoresis (136). High-performance liquid chromatography has proven its usefulness in the field of varietal identification. The processing quality of grain depends on its physical and chemical characteristics, which are at least partially genetic in origin, and a wide range of qualities within varieties of each species exists (112). The selection of the appropriate cultivar is an important decision for a farmer, since it largely influences the return he receives on his investment. The use of HPLC in the field of cereal analysis was introduced by Bietz (137) in 1983. Reversed-phase HPLC on a SynChropak RP-P C18 column with a linear binary gradient of 15% acetonitrile and 80% acetonitrile, both containing 0.1% TFA, and peak detection at 210 nm was able to differentiate between the various protein fractions present in wheat and showed a good resolution of the gliadin fraction, comparable to or exceeding that obtained by electrophoresis. This is very important, since the gliadin fraction, a heterogeneous group of wheat endosperm storage proteins, is a sensitive genotype indicator (138) because its composition varies among wheat genotypes (122). According to Bietz et al. (138), the validity of RP-HPLC for gliadin separation is dependent on proper techniques for protein ex-

Table 11 Reason for Determination of Cereal Proteins in Foods and Food Products

Reason for protein determination	Applications in food analysis	Method of HPLC	Refs.
Control of overall performance of food products	Prediction of quality of baked goods via protein content of cereal grains	Size-exclusion	116, 119, 120
	Relationship between flour protein composition and dough quality	Size-exclusion	118
Improvement of yield and quality of foods	Cultivar identification and discrimination of cereal proteins	Size-exclusion Reversed-phase	116, 121 122–124
Food authentication	Wheat varietal identification	Reversed-phase	125–128
	Detection of mixtures from different wheat cultivars	Reversed-phase	129, 130
Optimization of protein fractionation and determination methods	Separation of cereal proteins	Reversed-phase Size-exclusion Cation-exchange FPLC	114, 123, 127, 131–133 114, 117, 134 135

traction. These researchers recommend a simple extraction procedure, based on the extraction of nondefatted samples with 70% ethanol for about 30 min, for routine RP-HPLC analysis of gliadins. The usefulness of this procedure was reported in a subsequent paper (122) to identify hard red winter, hard red spring, soft red winter, white, and durum wheats; only varieties that have very similar pedigrees, for which variability among gliadins is slight or nonexistent, gave similar or identical chromatograms. The same method was also used to evaluate the effect of various fertilizers on the gliadin fraction of wheats, grown on sulfur-deficient soils (139). The intensity of the major peaks in the late-eluting gliadin fraction was greater when sulfur fertilization was used on sulfur-deficient soils. Further improvements were made to the HPLC method of Bietz (137) with regard to gradient composition, flow rate, temperature, and the use of standards (123). Sufficient resolution of the gliadin fraction for varietal identification within less than 15 min was obtained on an RP-HPLC column (SynChropak, RP-P C18, Brownlee Aquapore RP-300 C8 or Beckman Ultrapore RPSC) by using a binary solvent system comprised of a 1% TFA in water solution and CH_3CN + 0.1% TFA. Column temperature was raised from 30°C to 70°C, which probably enhances protein dissociation, and alkylphenones (C2–C10) were included as internal standards to eliminate intra- and interlaboratory variability. The identification of proteins is then based on a hydrophobicity index rather than elution time. A computerized library search procedure to perform wheat varietal identification based on RP-HPLC of gliadins was developed by Scanlon et al. (125). Normalized peak retention times and relative peak heights comprise the chromatogram fingerprint and were used in the library as primary and secondary discrimination parameters, respectively. The computer scores peak as being matched if both retention time and relative peak heights of compared components are within prescribed difference thresholds. The use of a weighted matching procedure in the search minimizes the effect of mismatching small peaks, whose retention times may be altered by noise and peak slope changes. The effects of long-term use of the column on variety identification was evaluated in a subsequent paper (126). It was shown that chromatographic resolution was relatively constant over time, but prolonged column use retarded peak retention times significantly, especially for early eluting components. Appropriate normalization of peak retention times and peak height is thus necessary in order to have sufficient long-term precision in cultivar identification. Due to the development of new, highly stable columns, however, the long-term stability of RP-HPLC columns will become less critical (133). Variations in the RP-HPLC of the gliadin fraction also originate from qualitative and quantitative differences in this fraction among single kernels of one wheat variety (140). Electrophoretic analysis of individual gliadin peaks showed component multiplicity, but photodiode-array detection of coeluting components did not further improve the automatic discrimination of wheat varieties by RP-HPLC, indicating that the coeluting proteins were very closely related (127). Wieser et al. (141) examined the amino acid composition of single components of the gliadin fraction, obtained from 16 wheat varieties and separated on a SynChropak C18 column, and found the following elution order: ω-gliadins eluted first, followed by α-gliadins; the last compounds to be eluted were the γ-gliadins. Differences between the three groups were very small and caused by exchange, insertion, or deletion of single amino acids in the protein chain. Gliadin patterns differed significantly, quantitatively and qualitatively, among the varieties studied, proving the possibility of varietal identifications based on the gliadin fraction. Although RP-HPLC is the method most widely used for the high-resolution separation of cereal proteins, the use of IE-HPLC is also reported (135,142,143). Anion-exchange FPLC on a Mono-Q column (linear gradient of 1 M urea, 0.01 M 3-(cyclohexylamino)-propanesulphonic acid as starting solution and 0.5 M acetic acid in starting solution, both pH 10.4) was able to differentiate between 13 wheat genotypes within 30 min, including column regeneration, but a relatively low number of peaks are obtained compared with RP-HPLC, indicating a lower resolution. Extraction of the wheat flours was done with the starting buffer for 30 min at room temperature (142). Cation-exchange FPLC of the gliadin fraction of wheat on a Mono-S column gave a higher resolution,

with more peaks resolved, than anion-exchange FPLC (135,143). Electrophoretic analysis of individual fractions showed a relatively high purity of subsequent fractions, indicating a possible use for preparative work.

According to Italian law, the manufacture and marketing of pasta products are subject to certain rules, stating that pasta must be prepared only with durum (*Triticum durum* Desf). National legislation of other member states of the European Union does not allow the sale of pasta products made from common wheat (*Triticum aestivum* L) or a mixture of common and durum wheat. However, owing to the price differences between types of wheats, it is most likely that a lot of pasta products are prepared from mixtures of common and durum wheat. So analysis methods to detect the wheat varieties used for pasta production are necessary to enforce legislation or to prove authenticity. Possible methods are electrophoretic separation of proteins (144,145), identification of fatty acid sterol esters (146), and immunochemical methods (147). According to McCarthy et al. (129), RP-HPLC is able to differentiate between common wheat and durum wheat based on the gliadin fraction. Elution of aqueous 2-chloroethanol (3:1) extracts of milled wheat samples on a Delta Pak C18 column (Waters Corp., USA) with a linear gradient of 15% acetonitrile and 80% acetonitrile, both containing 0.1% TFA, showed a doublet peak in common wheat that was consistently found to be absent in durum wheat and which might be used to detect adulteration of durum wheat by common wheat. Possible ways to quantify adulteration were proposed, based on ratios of specific peaks, which changed upon mixing common and durum wheat. Another detection method (130) is also based on RP-HPLC (PLRP-S column, Polymer Lab., UK) but of water-soluble proteins (albumins). Less than 3% of common wheat flour can be detected in durum wheat semolina; however, the method is applicable only to low-heat-dried pasta, since high-heat-dried pasta did not produce well-resolved patterns.

With respect to the processing of wheat, important properties are grain hardness, e.g., in milling processes, and dough rheological properties that related to the processing of different breads. Although considerable progress has been made toward understanding the molecular basis of the functionality of wheat proteins in foods, there is still no detailed understanding of the contributions of individual components of wheat to the functionality. A number of publications deal with the application of HPLC to the prediction or evaluation of the functional behavior of wheat varieties, based on the relationship of some specific peaks or specific regions of the chromatogram to quality characteristics. Huebner and Bietz (148,149) applied RP-HPLC to detect quality differences among wheats and showed that peak 1 in the chromatogram of unreduced wheat flour extracts is related directly to the mixing time, indicating a possible use for breeding programs. However, the effects of year and growing location, which might have an influence on baking quality (116), and the long-term stability of the column were not included. The effect of this last was studied by Scanlon et al. (120). They showed that the long-term use of an RP-HPLC column (SynChropak C18) and change of the column did not diminish the predictive capacity of their method, based on the gliadin fraction, when only a selection of peaks was used. Since they studied only one particular protein fraction and one quality parameter, i.e., the extensibility, this conclusion cannot be generalized without further research. Differences in the gliadin fraction of wheat lines with different baking quality were also found by Lookhart and Albers (140). An ω-gliadin was found to be present only in the good-quality lines, whereas two β-gliadins were present only in the poor-quality lines. Data obtained by RP-HPLC (SynChrom C8 column, SynChrom Inc., USA) and classical polyacrylamide gelelectrophoresis showed correlation. Differences in the gliadin fraction of flours with different baking properties were also observed by Menkovska et al. (150) in a combined electrophoresis/RP-HPLC study. Changes in the gliadin fraction during the flour–bread crumb transformation were more pronounced in good breadmaking flours than in poor breadmaking flours. Differences in the breadmaking potential of wheat flours seemed to be at least partially related to the heat-labile α-, β-, and γ-gliadins. In an attempt

to find a relationship between RP-HPLC patterns and baking quality, Seilmeier et al. (151) studied the reduced glutenin fraction of 19 wheat varieties having different baking properties. An extract of defatted wheat flour, obtained with salt and 70% aqueous ethanol solution, was reduced in a 0.05 M Tris HCl solution, containing 6 M urea and 0.05 M dithioerythritol and injected on a SynChropak C8 column (linear gradient of 20% acetonitrile and 50% acetonitrile, each containing 1% TFA and 5 M urea, pH 2.2; column temperature 65°C, and peak detection at 222 nm). The resulting glutenin fraction consisted of about 20 fractions, varying significantly for the different varieties, and three protein groups could be distinguished, corresponding to subunits with middle, high, and low molecular weights. A distinct correlation existed between the baking quality of the varieties and the HPLC pattern of the high-molecular-weight glutenin group. Weiser et al. (152) further examined the glutenin fraction of four wheat varieties. The ethanol-soluble reduced glutenin fraction, separated on two SynChropak RP-8 columns in series at 60°C, was composed mainly of middle-molecular-weight glutenins and the major portion of the low-molecular-weight glutenins, whereas the ethanol-insoluble reduced glutenin fraction, separated on a SynChropak RP-8 column at 60°C, contained the high-molecular-weight subunits and the minor protein of low-molecular-weight glutenins. The RP-HPLC pattern of the former fraction was unique for each variety, the pattern of the latter being related to the baking quality. Besides RP-HPLC, SE-HPLC is also applied to separate wheat proteins. Dachkevitch and Autran (116) analyzed unreduced extracts of wheat flour, obtained from bread wheat lines grown at different locations and from different genotypes in 1985, 1986, and 1987, by SE-HPLC. Extraction was done using a 0.1 M sodium phosphate buffer containing 2% SDS, pH 6.9. The chromatographic conditions were a TSK 4000-SW column, elution buffer 0.1 M sodium phosphate, 0.1% SDS, pH 6.9, and detection of peaks at 214 nm. Four distinct areas of material absorbing at 214 nm were observed and the best assessment of baking quality was obtained using %F1 or F1/F2 ratio, F1 and F2 being the excluded peak and the peak associated with intermediate aggregates, respectively, whereas loaf volume in French baking technology seemed to be more associated with the percentage of the F2 fraction. The same extraction procedure was applied by Ciaffi et al. (118). These researchers found a strong correlation between the insoluble polymeric proteins (obtained after a second extraction under reducing conditions) and parameters like dough strength and tenacity, indicating that large, insoluble gluten aggregates are important for the assessment of baking quality. Size-exclusion HPLC (Protein Pak 300, Waters Corp.) of unreduced proteins from flours, obtained by sonication in a 0.05 M sodium phosphate buffer containing 2% SDS, resulted in three fractions of different molecular size range. The quantity of glutenin (peak 1) was highly correlated with dough quality parameters like loaf volume, dough resistance, extensibility, and others (119,121). Size-exclusion HPLC has some advantages over RP-HPLC for the assessment of baking quality. Size-exclusion HPLC equipment is simple, since no gradient controller is necessary, purity of reagents seems to be less critical, chromatograms are much simpler to interpret (e.g., only four major fractions obtained by SE-HPLC compared to 20–30 in RP-HPLC), and it has the potential to work with large aggregates, which makes unnecessary a total reduction or dissociation of native protein aggregates, with concomitant loss of information concerning structure and interactions. However, fingerprinting of genotypes is not possible with SE-HPLC and requires a technique like SDS-PAGE or RP-HPLC (116). An example of RP-HPLC chromatograms of the gliadin and glutenin fraction of wheat, obtained with different columns, is shown in Fig. 2.

b. Nonwheat Cereals

Most publications dealing with HPLC of cereals report on wheat. However, the results obtained for wheat are not necessarily valid for other cereals. As with wheat, research on nonwheat cereals usually deals with identification of varieties or cultivars and the evaluation of processing quality of the different varieties.

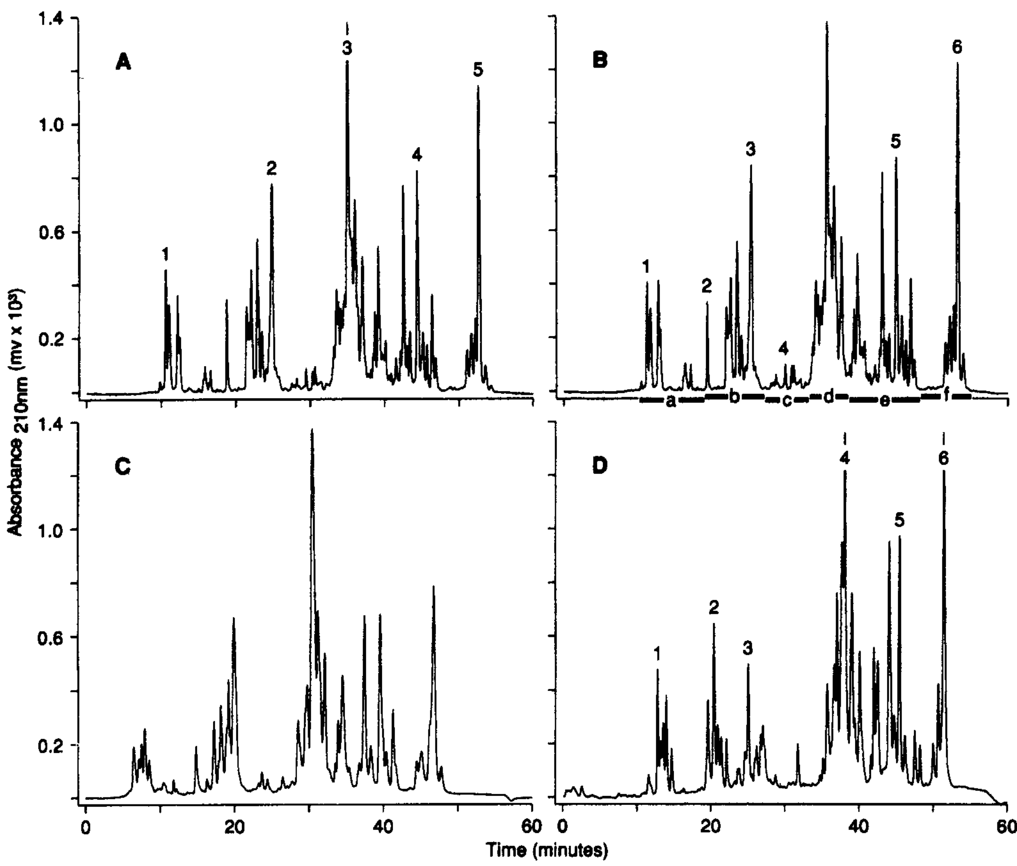

Fig. 2 Comparison of reversed-phase high-performance liquid chromatograms (obtained using 60-min gradients) of gliadins and glutenins extracted under reducing conditions from the wheat variety Neepawa using the following columns: A, Zorbax Rx-300-C8 (first analysis); Zorbax Rx 300-C8 (450th analysis); C, Supelcosil LC-308 (first analysis); D, Zorbax Rx-300-CN (first analysis). 1,000 mv = 1 AU. (From Ref. 133.)

Identification of oat (*Avena*) cultivars by HPLC was first reported by Lookhart and coworkers (153–155) in combined electrophoresis/HPLC experiments. The HPLC technique used was a modification of the procedure described by Bietz (137) for wheat. Generally, the prolamin fraction, i.e., the alcohol-soluble fraction, of oat species generates complex polyacrylamide gel-electrophoresis (PAGE) and RP-HPLC patterns, with increasing complexity as ploidy of the selections increased. Readily (visible) identification of the cultivars was possible only when PAGE and RP-HPLC results were combined. An HPLC procedure for the characterization of the major oat protein fractions was developed by Lapveteläinen et al. (156). Salt-soluble, alcohol-soluble, and alkali-soluble protein fractions were extracted with 0.1 M NaCl, 52% ethanol, and 1% SDS in 0.05 M borate (pH 10), respectively. For the five cultivars examined, RP-HPLC separations of salt- and alkali-soluble proteins were very similar, whereas the prolamin fraction enabled cultivar differentiation, except for very closely related cultivars.

The hordein fraction in barley (*Hordeum vulgare* L.), categorized into B, C, and D hordeins based on migration during SDS-PAGE, affects the malting process and therefore plays an integral role in determining quality and profitability of barley cultivars (157). Marchylo and Kruger

(158) adapted the method of Bietz (137) to study the potential of RP-HPLC for identifying Canadian barley cultivars. Elution profiles of hordein showed a significantly larger number of components as compared with electrophoretic techniques. Analysis of 12 Canadian cultivars showed that 10 could be distinguished qualitatively on the basis of elution profiles, whereas the two remaining cultivars showed reproducible quantitative differences in the proportions of C and B hordeins. No significant influence of year and location of growth was found on the distinguishability of cultivars or differences in the hordein fraction RP-HPLC. The differences in the hordein RP-HPLC pattern could be related to the malting quality (157). The hordeins of two barley cultivars with contrasting malting quality showed marked differences. For instance, the rate of decrease of total hordein during malting differed among varieties, and the rate of decrease of the individual groups of hordein also differed with the D hordein being degraded most rapidly. However, significant differences in the relative extractability characteristics exist among the B, C, and D hordeins within and between barley cultivars. Since malting and brewing quality consists of a group of different characteristics, a wide range of barley cultivars with different quality should be examined. Eight widely varying barley cultivars were analyzed by SE-HPLC on a TSK4000-SW column in order to evaluate the effect of nitrogen fertilization on the hordein fraction (159). The insoluble-protein fraction and total protein content were significantly influenced by the fertilization. Some fractions of the SE-HPLC pattern were cultivar-dependent; others, particularly the hordein C–rich fraction, were fertilizer dependent. The latter fraction was also related to the malting quality of the barley cultivars, a higher proportion of this fraction being detrimental to the quality.

The application of HPLC to rice (*Oryza sativa* L) proteins focuses mainly on cultivar identification (160–162). A SynChropak RP-P C-18 column at 45°C with a binary gradient of acetonitrile and water, each with 0.1% TFA, and peak detection at 210, was used by Lookhart et al. (160) to differentiate among 29 rice cultivars, including all major long-, medium-, and short-grain types produced in the United States, based on their prolamins obtained with extraction with 70% ethanol. The RP-HPLC patterns were different for the 19 cultivars examined, and the patterns for the subgroups of each grain type correlated well with their respective ancestry. Since the prolamin fraction represents only a minor fraction in rice, 1.7–7.6% in milled rice (163), identification methods based on this fraction might be less useful. A method based on the RP-HPLC analysis of alcohol-soluble (prolamins), salt-soluble, and glutelins was reported by Huebner et al. (162). They confirmed the abilities of RP-HPLC for varietal identification, but stated that glutelin analysis is preferable to that of prolamins or total protein, since the glutelins, the rice's major storage protein, differed most among varieties. However, the poor solubility of the glutelin fraction might impair the full extension of this method, and the addition of specific reagents (e.g., SDS) is necessary. A strongly dissociating solvent composed of 0.1 M acetic acid, 3 M urea, and 0.01 M cetyltrimethylammonium bromide (AUC) was used in an SE-HPLC experiment to determine the molecular mass distribution of proteins in six rice varieties (164). The number of peaks, i.e., ten, and peak resolution was much lower than compared with RP-HPLC, and no clear differences among the varieties examined was found; however, varietal differences were noticed when an analysis of variance was conducted for individual peaks. Results obtained in this study are difficult to compare with other research, since no direct relation exists with other, classical, extraction procedures.

2. *Noncereals*

High-performance liquid chromatography of plant proteins is limited mainly to cereal grains, since they have a relatively high protein content compared to other plants, like vegetables and fruits. The proteins in cereals also play an important role in processing. With regard to noncereal

plants, HPLC analysis of proteins is limited to the analysis of the storage bodies, i.e., beans or nuts.

The domestic soybean [*Glycine max* (L.) Merrill] is a legume that originated in eastern Asia and that consists of roughly 42% protein, 35% carbohydrate, 20% oil, and 5% ash. The major proteins are globulins, mainly glycinin (11S) and β- and γ-conglycinin (7S), which are to a large extent soluble in water at near-neutral pH. The soy proteins have the highest nutritional value of all plant proteins for human food, but the relatively high content of phytic acid may bind some minerals, which may limit the bioavailability of these minerals. Soy proteins are widely used in cereal grain foods, dairy-type foods, snack foods, soups, beverages, and meat, poultry and fish products (165). As with cereal proteins, RP-HPLC of protein extracts from different cultivars gives qualitatively unique chromatograms, unless closely related cultivars are compared, indicating that major differences of protein classes exist among cultivars. For closely related cultivars, comparison of quantitative RP-HPLC protein profiles permits the separation of genetically diverse genotypes (166,167). A good chromatographic separation (W-Porex 5 C4, Phenomenex Inc., US, and detection at 280 nm) within 40 min can be achieved using native nondefatted flours, extracted with a 35 mM Tris-HCl buffer (pH 7.6) in the presence of 10 mM β-mercaptoethanol and 0.4 M NaCl (166). The most hydrophobic proteins account for the largest peak area percentages, but few peaks contain homogenous proteins, owing to the heterogeneity of the protein fraction. In the same study, the use of SE-HPLC (TSK 4000-SW) for the separation of soybean proteins is described. Nine peaks eluted from the column, corresponding to the different S-fractions, with one peak, showing a high variability for the relative peak area, as a possible differentiator among different cultivars. Since soy proteins are often used in food products, a rapid assessment of soy protein products (e.g., textured soybean, soybean flour, soybean milk, and soybean infant formulae) is necessary. A rapid RP-HPLC technique was developed by García et al. (168). A linear binary gradient of acetonitrile and water, each with 0.1% TFA and column temperature of 50°C, resulted in the separation of the globulins (five peaks) in 9 min, with a differentiation among different commercial soybean products based on peak areas. The use of soybean proteins as supplement in bovine milk is forbidden in many countries, whereas in some countries there is a regulation concerning the maximum allowance level of soybean products in meat products. On the other hand, detecting the presence of milk proteins in soy products may be necessary because of possible allergy in some individuals. The determination of soy proteins in unheated meat (110) was already described in a previous paragraph; their detection in dairy products is possible by SE-HPLC after treatment of the sample with a tetraborate-EDTA buffer (169). Since soy proteins are insoluble in this buffer, they can be selectively separated from the milk protein fraction. Analysis of treated cheese samples on Zorbax GF-250 columns resulted in the detection of added soy protein in the total protein of melted cheese as low as 6 g/kg. However, the response of different types of soy products and the interference of residual milk proteins limits the application to unknown samples. The presence of animal whey proteins in commercial vegetable milks and of soybean proteins in animal milk can be achieved by RP-HPLC within 20 min (170). Quantification of animal whey proteins at low concentrations in vegetable milks required an acidic precipitation prior to injection of the sample into the chromatographic system.

Peanuts (*Arachis hypogaea* L.) are native to South America and represent an important food crop in many of the tropical and subtropical areas of the world. In the United States, peanuts are used primarily as peanut butter or as a snack food, i.e., whole roasted peanuts. Throughout the rest of the world, peanuts are defatted to produce a cooking oil and a feed meal (171). Peanut seed proteins can be classified into arachin, con-arachin (globulins), and albumins. The indeterminate fruiting characteristics of peanuts result in seeds of varying maturity on the plant as harvest time approaches. The proper time to harvest is when the greatest weight of sound, mature kernels is available (172). Differences, qualitatively and quantitatively, in peanut seed protein composition

were detected by SE-HPLC (Protein Pak 300 SW, Waters Corp., USA) and contributed to genetic differences, processing conditions, and seed maturity. The molecular weight of the different fractions ranged from 80,000 to 480,000 (173). In a subsequent paper, the use of the SE-HPLC pattern as an indicator of seed maturity was further evaluated (172). It was found that the relative area of one particular peak decreased with increasing maturity and remained unchanged toward later stages of seed maturity. The peak was present in all cultivars examined, all showing a "mature seed protein profile" with respect to the particular peak, which was therefore called Maturin.

The application of HPLC to nonstorage plant proteins is, as already mentioned, very limited, and very few research papers are published on this topic. The application of HI-HPLC to soluble grape proteins was reported by González-Lara and González (174) for classification of musts according to grape variety. Must, obtained by lightly pressing grapes, was dialyzed against tap water, concentrated by weak-anion solid-phase extraction, and chromatographed on a Nucleosil C4 column, with peak detection at 220 nm and a decreasing gradient of $(NH_4)_2SO_4$ in phosphate buffer. At pH 3.44, where the must proteins are positively charged, eight protein fractions were separated, probably in their native form. Since these proteins are most likely enzymes, the recovery in their native state may be useful for preparative work.

VII. CONCLUSION

Since its introduction as an analysis technique, HPLC has evolved into a widely applied, accurate, and precise technique for the analysis of food proteins. The development of rigid, wide-pore supports was without doubt a milestone for the chromatographic separations of proteins. Compared to other techniques, HPLC can offer many advantages, for instance, its versatility, high resolution, and excellent reproducibility. Further improvements in column technology led to the development of highly stable columns with a wide working range with regard to pH and a long-term stability, the latter being a prerequisite for the identification of varieties of certain species based on databases or the comparison of results over a long period. However, in some cases HPLC is still not applied as a routine analytical technique because of its sequential nature combined with relatively long analysis times for each run. Improvements in the column support, e.g., the development of gigaporous supports, reduced the analysis time, but long-term stability and loading capacity still have to be optimized. Nevertheless, HPLC has already proven its suitability for building a link between food composition and food functionality, since its versatility enables the researchers to evaluate different properties of the proteins by using different column supports. However, since food functionality is seldom based on a single food component but usually results from complex interactions among the different components, a lot of research still has to be done. Moreover, the protein fraction in a lot of foodstuffs is not yet fully characterized. With regard to varietal identification and food adulteration, it has been proven that HPLC is a very valuable technique, on its own or in combination with, for instance, electrophoretic techniques, and it is able to differentiate between closely related species. The use of HPLC in this field, however, is not yet fully implemented, and its use should be combined with advanced statistics to benefit fully from its possibilities.

ACKNOWLEDGMENTS

The first author is a research assistant of the Fund for Scientific Research—Flanders (Belgium). The authors wish to express their thanks to Monique Jooris for the supply and copying of docu-

ments. Luis R. Bracho (Universidad Central de Venezuela/Mavesa S.A., Valencia, Venezuela) is kindly acknowledged for his contribution to the Introduction section.

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5
HPLC of Lipids

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I. INTRODUCTION

Fats and oils are made up predominantly of triesters of glycerol with fatty acids and commonly are called *triglycerides* (TGs). The designations *fats* and *oils* are used merely for convenience, in that customarily at ambient temperatures fats are solids and oils are liquids. Both classes of compounds are triglycerides, differing only in melting point.

Fats are distributed widely in nature. They are derived from vegetable, animal, and marine sources and often are by-products in the production of vegetable proteins or fibers and animal and marine proteins. Fats of all types have been used throughout the ages as foods, fuels, lubricants, and starting materials for other compounds. This wide utility results from the unique chemical structures and physical properties of fats. The chemical structures of fats are very complex, owing to the combinations and permutations of fatty acids that can be esterified at the three (enzymatically nonequivalent) hydroxyl groups of glycerol.

Many naturally occurring fats are made up of fatty acids with chain length greater than 12 carbon atoms; the vast majority of vegetable and animal fats are made up of fatty acid molecules of more than 16 carbon atoms. Marine fats (and some *Cruciferae* fats) are characterized by their content of longer-chain (up to C_{24}) fatty acids. Thus, because the fatty acid portions of the TGs make up the larger proportion (ca. 90% fatty acids to 10% glycerol) of the fat molecules, most of the chemical and physical properties result from the effects of the various fatty acids esterified with glycerol.

Naturally occurring fats contain small amounts of soluble minor consituents: pigments (carotenoids, chlorophyll, etc.), sterols (phytosterols in plant fats, cholesterol in animal fats), vitamin A (from carotenes), vitamin D (calciferol), waxes (esters of long-chain alcohols and fatty acids), ethers, and degradation products of fatty acids, proteins, and carbohydrates. Most of these minor compounds are removed in processing, and some are valuable by-products.

A. Glycerides

The number of TGs in a given natural fat is a function of the number of fatty acids present and the specificity of the enzyme systems involved in the particular fat synthesis reactions. Many plant seed fats have the potential to provide 125–1,000 different TGs, animal fats contain potentially 1,000–64,000 TG species, and butterfat could generate 2,863,288 TGs from 142 different fatty acids (1).

In many instances, TGs exist in polymorphic forms. Crystal structure is very important to the properties of margarines, shortenings, and specialty fats. The very unstable α form is readily transformed to the more stable β' form, which in some TGs has a higher melting point (more stable) than the β form. In single-acid TGs, the order is $\alpha \rightarrow \beta \rightarrow \alpha'$, but some mixed TGs show a lower-melting (less stable) β form. This is further complicated by the existence of multiple β' and β forms, depending upon the detailed TG structures at hand.

B. Fatty Acids

Most of the fatty acids in fats are esterified with glycerol to form glycerides. However, in some fats, particularly where abuse of the raw material has occurred leading to enzymatic activity, considerable (>5%) free fatty acid (FFA) is found. Hydrolysis occurs in the presence of moisture. This reaction is catalyzed by some enzymes, acids, bases, and heat. Most producers of fats attempt to prevent the formation of free fatty acid because certain penalties are assessed if they are present in the trading of crude and refined fats.

C. Phospholipids

Phospholipids occur in most natural fats, with differing amounts and compositions depending on the source of the fat. Owing to their complexity, these fat-soluble, biologically important compounds also have presented some intriguing analytical problems to chemists and biochemists.

From a technical standpoint, phospholipids (e.g., from soybean) are composed mainly of lecithin, cephalin, or phosphatidylinositol. These complex mixtures (2–3% in soybean oil) are hydrated during the degumming step, removed, and dried. These products are sold as commercial lecithin used in margarines, confections, and shortenings where a fat-soluble emulsifier is required.

D. Antioxidants

The most commonly occurring natural antioxidants in vegetable fats are the tocopherols (vitamin E active). These derivatives of 6-chromanol are not synthesized by mammals and occur in their fats only through ingestion of plant materials and vegetable fats. Antioxidants tend to protect fats by inhibiting autooxidation and subsequent rancidity. A certain percentage of the tocopherols are removed in some refining steps and are recovered as by-products of vegetable oil processing.

E. Pigments

The major pigments of fats are the carotenoids. Palm oil, usually bright reddish orange, contains as much as 0.2% β-carotene. Many seed oils, particularly if processed from immature seeds, also contain significant levels of chlorophyll pigments that lend a greenish tinge to the fats. Cottonseed oil is heavily colored by gossipol-type (phenolic) pigments. Most of these pigments are removed in the alkali refining and bleaching steps. A few pigments (fixed) are difficult to remove in processing and may result from heat or oxidative abuse of the fat-containing raw materials or the crude fats themselves. The carotenoid pigments are decolorized mainly by heat, light, or oxidative treatment, but the quinones generated by oxidation of the tocopherols generally cause the darkening of fats.

F. Vitamins

The principal components in vegetable fats with vitamin activity are the tocopherols. Vitamin A is found in butterfat and in fish oils. The carotenes (provitamin A) are found at significant levels in palm oil and butterfat and as traces in other fats. Vitamin D is found primarily in some fish oils.

G. Sterols

Most of the unsaponifiables in vegetable and animal fats are sterols. The animal fats contain predominantly cholesterol, and most vegetable fats contain only traces of this sterol. Plant sterols, collectively called *phytosterols*, are made up mainly of sitosterols and stigmasterol, but some individual vegetable fats contain additional phytosterols. The pattern of typical sterols has been suggested as useful in detecting the adulteration of one oil with another (2).

H. Minor Constituents

In addition to the materials just listed, waxes, hydrocarbons, ketones, aldehydes, and mono- and diglycerides are found in fats and oils at varying but low levels. The waxes in some seed oils (e.g., corn, sunflower, and safflower) are troublesome and are removed in processing to prevent haze formation in the finished products. The ketones and aldehydes probably arise from oxidative damage and can produce flavors and odors in fats. The mono- and diglycerides result from hydrolytic reactions either in the raw materials or during processing but do not pose particular problems in end products. The hydrocarbons are mainly analytical curiosities and are of no technological consequence.

II. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Since a TG molecule is built up of three fatty acids, combinations of just a few kinds of fatty acid can give rise to several different TGs. The complexity of TG analysis is therefore obvious; in most cases it is carried out by transesterification of the TGs into methyl esters followed by gas chromatography (GC) analysis of these methyl esters.

If the analysis of fatty acid methyl esters gives a large amount of information on the composition of fats, there remain some unsolved problems, related to the two major flaws of the approach. The transesterification of TGs derived from polyunsatured acids is not always quantitative. A more important and general problem is that the method does not provide any information regarding the actual composition of the TGs.

Common triglycerides may be separated by GC at high temperature (350°C) by total fatty acid chain length (e.g., tripalmitin and tristearin have total fatty acid chain lengths of 48 carbons and 54 carbons, respectively).

HPLC has a number of advantages over other chromatographic techniques for lipids; namely, (a) it can be quantitated more easily than TLC, (b) lipids that cannot be separated by GC because they would be decomposed by the high temperatures required or by the catalytic activity of the column may be analyzed using HPLC, and (c) the separated components can be collected and subjected to further analysis, e.g., by spectroscopic techniques. Examination of the literature reveals a number of points of agreement on the difficulties and present limitations of the method.

In a partially hydrogenated fat, the number of fatty acid isomers is dramatically increased by the appearance of geometrical isomers (i.e., cis and trans configurations) and various positional isomers (i.e., different positions of double bonds within the fatty acid chain). Thus the in-

creased number of fatty acid isomers will result in a drastic increase in the number of possible TGs. For example, OOO (three cis double bonds) may convert to geometrical isomers OOEl (two cis, one trans), OElEl (one cis, two trans), and ElElEl (three trans double bonds).

The importance of being able to determine the geometrical isomers of TGs in partially hydrogenated fats can be ascribed to their effect on the physical behavior of the fats, such as their polymorphic behavior and melting properties.

A. Detectors

As early as 1972, Perry et al. (3) described the necessary properties of a high-performance liquid chromatography detector system, requirements that still serve as a basis for detector evolution. The detector characteristics should be such that it (a) responds to all compounds (except those comprising the mobile phases) or possesses a predictable specificity; (b) gives a highly sensitive and predictable response; (c) gives a linear response; (d) is insensitive to change in the mobile-phase composition, flow rate, and temperature; (e) does not contribute to band spreading; (f) is nondestructive; (g) gives some information to assist compound identification; (h) is easy to use and capable of continuous separation; and (i) has a low initial cost as well as low running costs. Most current HPLC detectors are a compromise of these criteria, and, despite recent developments, many compounds pose difficulties in detection, particularly if the molecules do not possess an ultraviolet (UV) chromophore or a fluorophore, are not electrochemically active, or do not give a good response with other commercial detectors. Lipids are a group of important compounds for which HPLC could provide valuable information if the problems encountered in their analysis could be overcome.

1. *Refractive Index Detector (RID)*

Refractive index detectors (RIDs) are relatively universally applicable but are relatively insensitive. The choice of RID is made necessary by the fact that lipids have only the weak ester chromophore with a small absorbance in the far-UV range (180–200 nm) and do not absorb light above 220 nm. The use of an RID is not compatible with gradient elution, however. The difference between the refractive indexes of two solvents (typically ca. 0.05–0.2) is huge compared to the modest change in the refractive index associated with the elution of a compound that has a small concentration in the sample. Thus, no component detection would be possible on the enormous slope of the drifting baseline during a gradient elution analysis.

The analysis of oils and fats was formerly done by isocratic liquid chromatography using an RID. The composition of the solvent mixture was chosen as a compromise between the opposite requirements of a significant retention of the low-molecular-weight esters and a reasonable analysis time. The analysis of samples having triglycerides with a broad molecular weight distribution is difficult. It was not possible to analyze properly the triglycerides having more than about 46 alkyl carbon atoms, and quantitation was difficult.

With RIDs, the analysis is generally carried out in isocratic conditions, using acetonitrile-tetrahydrofuran, acetonitrile-acetone, propionitrile, or acetone-tetrahydrofuran-*n*-hexane mixtures.

The isocratic elution of short- and medium-chain-length triglycerides using the solvents just described is relatively easy. Some authors, like Ken Jie (4), found that it is difficult to elute triglycerides with more than 46 carbon atoms in their alkyl chains (tripalmitin, tristearin, and tribehenin have, respectively, 46, 51, and 63 carbon atoms in their alkyl chains) with the strongest solvents he could use, acetone-acetonitrile (2:1). Higher-molecular-weight triglycerides tend to crystallize out in the column (5).

Tristearin and triglycerides of higher molecular weight have a very low solubility in the mobile phase used, and either precipitate at injection (in the valve loop or in the connecting tube between the injection valve and the column) when the sample solution is diluted in a rather poor solvent or are so strongly retained that they are eluted only after a prohibitively long time, giving broad peaks barely discernible from baseline oscillations.

This is confirmed by the results achieved by Podlaha and Torregard (6), who could not elute tristearin at ambient temperature with propionitrile on a 50-cm RP-18 column but could do it at 30°C in about 105 min (flow rate 0.5 ml/min, column capacity factor around 13). It is known that the solubility of TGs increases very rapidly with increasing temperature.

2. *Ultraviolet (UV) Detector*

Low-wavelength UV detection (200–210 nm) is more sensitive and permits the use of gradients but precludes the use of certain common lipid solvents, such as chloroform and acetone, which are opaque in the UV region of interest. With low-wavelength UV detection, the response will also be somewhat dependent on fatty acid composition. For these reasons the mobile phases used in lipid analysis by HPLC may seem rather strange to workers familiar with the Thin Layer Chromatography (TLC) or open column separations.

3. *Infrared Detector*

A detection system based on spectroscopy in the infrared region of the spectrum has been reported by Hamilton et al. (7) to develop a system that would enable the UV-visible detector to be used for lipid analysis, utilizing a specific functional group present in the molecule (e.g., the carbonyl group). This system has the advantage that it can be used as a universal detector, monitoring the C—H frequency at 3.40 μm, or by choosing a wavelength corresponding to a particular atomic grouping, e.g., the C=O frequency at 5.7 μm.

The two major problems associated with the infrared detector are:

The lack of "spectral windows" in the spectrum of the mobile phase, i.e., regions in the spectrum where the transmission is greater than 30%. This limits not only the choice of mobile phase but also the path length of the detector cell; e.g., acetonitrile in a 1-mm cell has a spectral window of 6–8 μm and 8.5–11 μm, but with a 3-mm cell, both of these regions are spectrally opaque. Thus, the choice of path length is a compromise between spectral transmission and sensitivity.

The change in absorbance of the mobile phase during gradient elution. This leads to baseline shifts, the size of such shifts depending on the extent of compositional changes and on the absorbance characteristics of the mobile phase, the optical path length, and the detector sensitivity. This problem can be overcome by absorbance matching, i.e., the blending of solvents to give mobile-phase pairs that have a similar absorbance but different solvent strengths for use in gradient elution.

4. *Laser-Light-Scattering Detector*

This detector was first described by Charlesworth (8). Nebulization of the column effluent in a stream of tepid (30–35°C) gas, followed by total vaporization of the solvent in a warm (40–45°C) drift tube, leaves a cloud of particles made of the nonvolatile material contained in the eluent. These particles are carried by the gas stream across a laser beam and then vented.

Laser light scattered by the particles is collected by the tip of a glass rod, ensuring an efficient light collection, in a rather wide spatial angle. The direct laser light is absorbed in a

Raleigh horn. The collected scattered light is transmitted by an optical fiber to a photomultiplier. The photomultiplier current is amplified and recorded.

A first important property of the detector is that a very low background signal is observed, since there is no light scattered from the laser beam by the solvent vapors. This requires the use of clean solvents, with only trace amounts of nonvolatile impurities and careful filtration of the column eluent, using a tight metal frit to eliminate the suspended particles, such as the fine dust contained in the column-packing material. If there is no background signal with the pure solvents, there will be no baseline drift in gradient elution.

The detector can be used with any solvent or mixture of solvents, as long as they are volatile or pure enough. The reproducibility of the detector is very good. Another advantage of the detector is that the response is practically independent of the nature of the compounds eluted, as long as they condense as liquids. Finally, the detector makes a very small contribution to band broadening, because of the rapid transit of the solutes and a very small time constant. This permits its effective use with fast, efficient columns as well as with 1- and 2-mm-ID columns.

One of the rare drawbacks of the detector is its total lack of response for any volatile material. Accordingly, the classical method of the determination of the void volume by elution of an isotopic analog of one of the eluent components cannot be used.

5. *Chemical Ionization Mass Spectrometry (CIMS) Detector*

The inherent difficulties of combining HPLC with conventional mass spectrometry have been elaborated by McFadden et al. (9,10).

Erdahl and Privett (11) developed a simple interface for the purpose of combining HPLC with CIMS for the analysis of lipids based on their continuous conversion to volatile products by reduction with hydrogen prior to their introduction into the mass spectrometer. Hydrogen was introduced into the reactor in this system to convert the acyl groups of the lipids to hydrocarbons. However, some of the double bonds in the acyl groups were hydrogenated, voiding identification of the fatty acid constituents. Hence they turned to the use of an inert gas in place of hydrogen.

With an inert carrier gas in this system, the acyl groups of the lipid classes are split from the backbone structures and identified as the $[RCOOH + 1]^+$ and $[RCO]^+$ ions. Since the products of the backbone structures of the lipid classes are produced by thermal degradation, their formation does not appear to be affected by the change to an inert gas.

B. Columns and Mobile Phases

Most HPLC is based on the use of so-called normal-phase columns (useful for class separations), reverse-phase columns (useful for homolog separations), and polar columns (used in either the normal- or reverse-phase mode). Since reverse-phase HPLC columns are generally easier to work with, almost all authors use high-performance reverse-phase liquid chromatography with octadecyl chemically bonded silica as the stationary phase and nonaqueous solvents as mobile phases (so-called NARP, or nonaqueous reverse-phase chromatography).

The importance of the amount of carbon load on the column, which varies widely between columns from different manufacturers, has been discussed for a long time. The differences in chromatographic retention and selectivity are a result of the utilization of different silica materials as supports and a variety of reagents and procedures to produce the bonded phases. Several studies have shown that the capacity factor, k', generally increases with increasing carbon content (12). However, sometimes the results show that k' values are not always correlated with the differences in carbon content. This may be explained, as Unger (13) illustrates, by the fact that the carbon content alone is often misleading in the comparison of columns because of differences

in the surface area of the original silica, which results in different surface coverage of the bonded alkyl groups, and also differences in the packing density of the stationary phase.

III. FREE FATTY ACIDS

The development of an analytical method for the routine simultaneous identification and quantification of a variety of fatty acids is desirable for use in various fields. Although gas chromatography (GC) methods are traditionally used for fatty acid analyses, they are accompanied by some disadvantages, particularly with respect to heat-labile or short-chain fatty acids, and, moreover, the separation of cis-trans isomers is possible only with capillary columns.

In order to overcome some of these shortcomings, a number of HPLC methods have been introduced. These methods usually offer good resolution of the most important fatty acids, but the detection of underivatized fatty acids is neither sensitive nor selective, because these compounds generally do not contain suitable chromophores. Absorption of underivatized fatty acids near 200 nm cannot be recommended, because it is adversely affected by the properties of solvents and frequent impurities in organic solvents, which is specifically undesirable in gradient elution.

Separations are usually carried out on reverse-phase columns using isocratic or gradient elution with methanol, acetonitrile, and water. The order of elution is governed by the length of the fatty acid carbon chain and the number of double bonds in it (14). The retention time increases with increasing chain length and decreasing number of double bonds. These opposing tendencies lead to the occurrence of several pairs of fatty acids that are difficult to separate and the separation of which may be considered a criterion of the resolution efficiency of an analytical procedure. Examples of such critical pairs are linolenic (18:3) and myristic (14:0), palmitoleic (16:1) and arachidonic (20:4), and palmitic (16:0) and oleic (18:1) fatty acids, although difficulties with the separation of some other fatty acid pairs may also be expected. The trans isomers are generally eluted after the corresponding cis isomers. Positioning of the double bond in the proximity of the carboxy group of an acid usually leads to a decrease in retention, compared with an isomer of a fatty acid, with the double bond shifted in the direction of the methyl end of the carbon chain.

Free fatty acids are separable by GC by the inclusion of phosphoric acid in the packing; so, for HPLC analysis, the phosphoric acid or other equivalent strong acid is included in the mobile phase. On a SUPELCOSIL LC 18 column, a model mixture of free fatty acids was separated with a mobile phase containing tetrahydrofuran, acetonitrile, water, and phosphoric acid (6:64:30:0.1) at pH 2 (Fig. 1) (15). Oleic and elaidic acids, palmitoleic and palmitelaidic acids, and linoleic and linoelaidic acids were well separated, but margarine fatty acids presented a difficult problem. Ultraviolet detection of 220 nm was used to prepare this chromatogram.

In order to increase the sensitivity and selectivity of detection, a number of UV-absorbing or fluorescent derivatives have been prepared. Several reasons can be given for the use of derivatization in chromatography: to enhance solute volatility (GC), to enhance separation, and to enhance detectability. The selection of a derivative can often become much more difficult than the actual derivatization itself. In all areas of chromatography these effects have been succesfully applied to difficult or otherwise impossible separation.

In liquid chromatography, derivatization for detection enhancement is frequently needed, since no universal, sensitive, and simple-to-operate detector exists (the preparation of UV-absorbing derivatives is essential to obtain the sensitivity required for samples in the nanogram range). Since most fatty acids do not absorb UV radiation (at least not in the wavelength ranges of most commercial UV monitors), detection of quantities in the 1-ng range can be difficult. Tag-

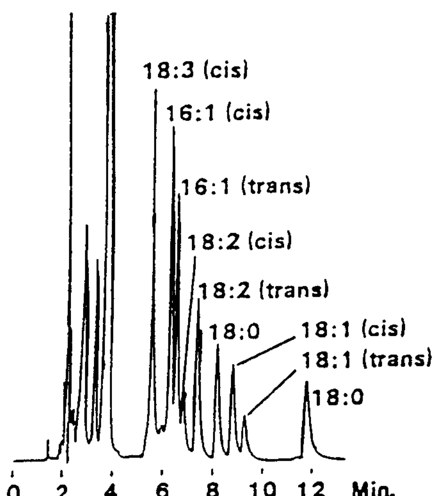


Fig. 1 HPLC of free fatty acids. Column: SUPELCOSIL LC 18. 25 cm × 4.6-mm ID. (5μ): mobile phase: tetrahydrofuran/acetonitrile/0.1% phosphoric acid, pH 2.2 (21.6:50.4:28.0); flow rate: 1.5 ml/min; temperature: 35°C; detection at 220 nm; sample concentration 1–2 mg/ml per component. 16:1 (cis) = *cis*-9-hexadecenoic acid (cis-palmitoleic acid); 16:1 (trans) = *trans*-9-hexadecenoic acid (trans-palmitoleic acid); 18:0 = octadecanoic acid (stearic acid); 18:1 (cis) = *cis*-9-octadecenoic acid (oleic acid); 18:1 (trans) = *trans*-9-octadecenoic acid (elaidic acid); 18:2 (cis) = *cis*-9-*cis*-12-ctadecadienoic acid (linoleic acid); 18:2 (trans) = *trans*-9-*trans*-12-octadecadienoic acid (linolelaidic acid); 18:3 (cis) = *cis*-9-*cis*-2-*cis*-15-octadecatrienoic acid (linolenic acid).

ging the acids with a strongly UV-absorbing species, by the deformation of suitable derivatives, is essential.

A. Derivatization of Fatty Acids

The derivatization of fatty acids, and of acidic substances in general, has been a problem in analytical-organic chemistry for many years. The classically used derivatives have been amides and esters. The main problem in using methyl esters of fatty acid in HPLC is that one cannot then use the UV detector, because of inadequate absorption. Thus a search for an alternate method of forming UV-sensitive derivatives has been initiated in several laboratories.

Derivatization can be performed precolumn as well as postcolumn (16,17). Postcolumn derivatization is most convenient for detection. The reaction does not have to be complete, provided it is reproducible; however, in most cases additional equipment is needed. Reagent must be added, with extra pumps, and long reaction times require the use of reaction coils and phase separators. Precolumn reactions, on the other hand, should be complete. While the derivatives have to be stable until the analysis, generally no extra equipment is required.

Politzer et al. (18) used 1-benzyl-3-*p*-tolutriazine as a diazo-like precursor that will react with free fatty acids to form benzyl esters. Other authors (19) have taken the same basic approach, except that the derivatizing agent was 1-*p*-nitrobenzyl-3-tolutriazine to give *p*-nitrobenzyl esters of the fatty acids. These possess stronger UV absorption than the benzyl esters, and detection limits are reported to be in the 1- to 10-ng range. Disadvantages of this technique are the necessity to vent the nitrogen evolving during the reaction and the fact that the derivatizing reagent is quite expansive and must be used in large quantities. The reaction also produces by-products that may interfere with the subsequent chromatographic analysis.

Cooper and Anders (20) reported the HPLC analysis of unsaturated C_{18} and C_{20} fatty acids. Since the methylene-interrupted polyunsaturated acids show no specific UV absorption, the 2-naphtacylesters were prepared for UV detection at 254 nm [the column was a 3-ft × 0.07-in.-ID stainless steel tube packed with CORASIL-C_{18}, methanol/water (85:15) served as the eluent, and a flow rate of 12 ml/h was obtained at a pressure of 300 psig]. The lower detection limit was 4–90 ng of ester.

Phenacyl esters were easily prepared by Borch (21) for the HPLC analysis of C_{12}–C_{24} fatty acids (90 × 0.64-cm μ-BONDAPAK C-18 column, acetonitrile/water eluent programmed from 67:33 to 97:3 or from 80:20 to 100:0 in composition, flow rate set at 2.0 ml/min) (Figs. 2 and 3). Several characteristics of this separation should be noted. First, separation by chain length alone is dramatic, with the retention volume increasing with increasing chain length. For example, in Fig. 3 see the separation of the myristic (14:0), pentadecanoic (15:0), palmitic (16:0), margaric (17:0), and stearic (18:0) acids represented by peaks 4, 9, 12, 17, and 18, respectively. Second, increasing unsaturation decreases the retention volume of the phenacyl esters. Thus, the C_{20} fatty acids 20:0 (arachic acid), 20:1 (gadoleic acid), 20:2 (eicosadienoic acid), 20:3 (eicosatrienoic acid), and 20:4 (arachidonic acid) are represented in Fig. 2 by peaks 21, 19, 16, 11, and 6, respectively. Third, the trans unsaturated fatty acid derivatives are intermediate in retention volume between the saturated and the cis unsaturated derivatives, as expected on the basis of conformational changes induced by the introduction of the respective double bonds. Each

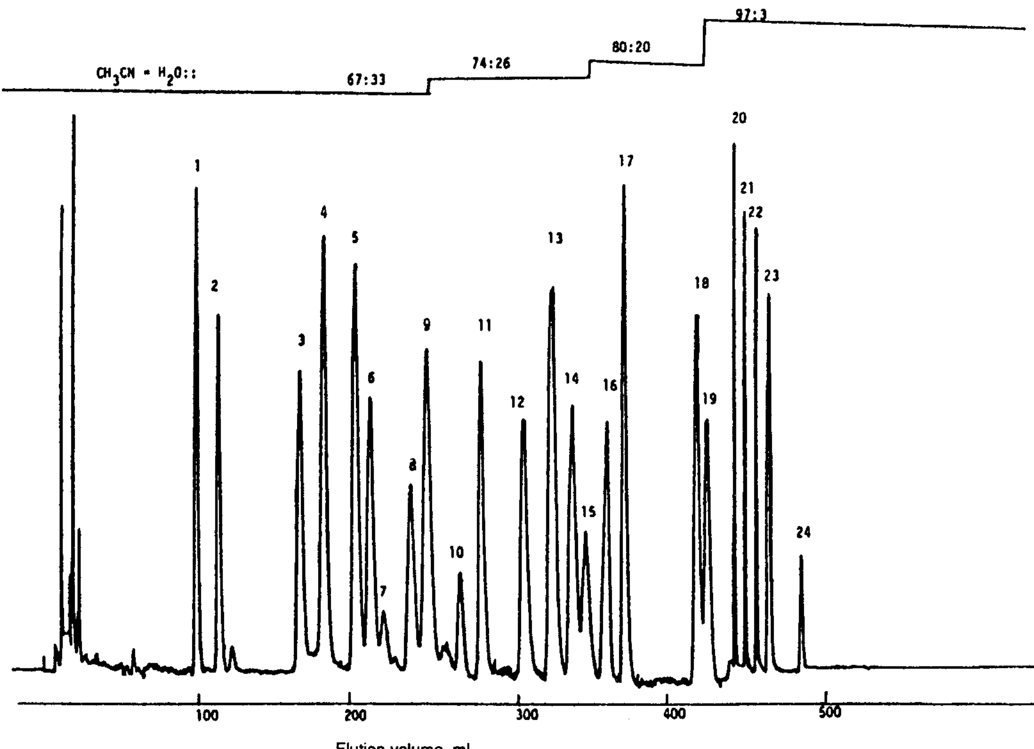


Fig. 2 HPLC of fatty acid phenacyl esters. Peaks: 1. C12:0; 2. C14:1; 3. α- and γ-C18:3; 4. C14:0; 5. C16:1; 6. C20:4; 7. trans-C16:1; 8. C18:2; 9. C15:0; 10. trans-C18:2; 11. C20:3; 12. C16:0; 13. C18:1, Δ^9 and C18:1, Δ^{11}; 14. C18:1, Δ^6; 15. trans-C18:1; 16. C20:2,11,14; 17. C17.0; 18. C18:0; 19. C20:1; 20. C19:0; 21. C20:0, and C22:1; 22. C21:0; 23. C22:0 and C24:1; 24. C24:0. Column 90 × 0.64-cm μ-Bondapak C_{18}; eluent acetonitrile/water; flow rate 2.0 ml/min.

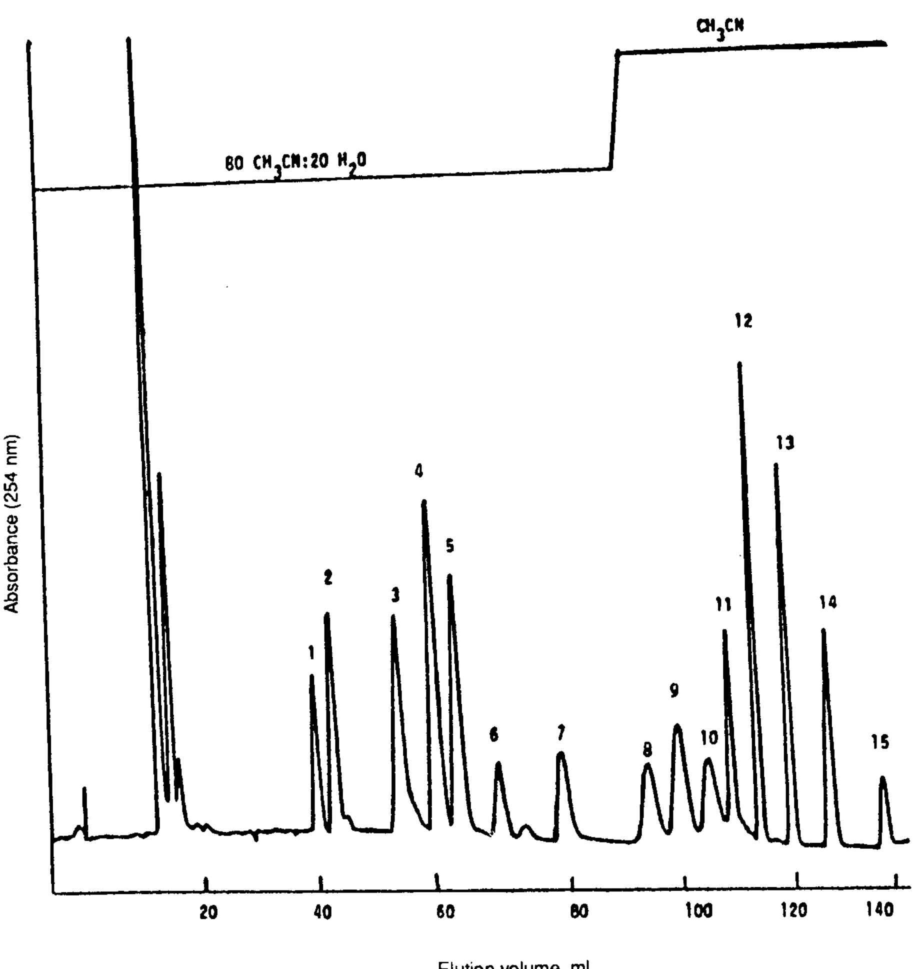


Fig. 3 HPLC of phenacyl esters. Peak: 1. C12:0; 2. C14:1; 3. C18:3; 4. C14:0; 5. C20:4; 6. C18:2; 7. C20:3; 8. C16:0; 9. C18:1, Δ^9; 10. C18:1, Δ^6; 11. C20:1; 12. C18:0; 13. C20:0; 14. C22:0; 15. C24:0. Column 90 × 0.64-cm μ-Bondapak C_{18}; eluent acetonitrile/water; flow rate 2.0 ml/min.

of peaks 4, 13, 21, and 23 represents a mixture of two fatty acid derivatives. Peak 23 can be resolved by omitting the solvent change to 97:3 acetonitrile/water and completing the separation using 80:20 acetonitrile/water.

The order of elution is governed by the length of the fatty acid carbon chain and the number of double bonds in it. These opposing tendencies lead to the occurrence of several pairs of fatty acids that are difficult to separate and the separation of which may be considered a criterion of the resolution efficiency of an analytical procedure.

The key elements in such an area analysis are palmitoleic and arachidonic acids (peaks 5 and 6, Fig. 2). Complete resolution of these two acids precludes the use of a starting solvent system with acetonitrile composition of over 67%. If only one of these two acids is present, however, a rapid analysis is feasible. A separation of this type is illustrated in Fig. 3. The time required for this analysis is 70 min, compared to 4 h for the separation shown in Fig. 2.

To enhance the UV detection, Durst et al. (22) report the formation of phenacyl esters using *crown ether catalysts*. Since the time of their discovery, crown ethers have been shown to have

a tremendous ability to complex metal salts, especially those of potassium, and aid, by solvation of the cation, the dissolution of these salts in nonpolar, aprotic solvents. Anions of these salts in solution have been shown by several groups to be unusually reactive, especially the carboxylate anions. The ability of the crown ether to aid in the dissolution or extraction of the fatty acid into an aprotic solvent, coupled with the unusually reactivity of the carboxylate ion under these conditions, forms the basis of the derivatization reaction. The column used was a nonyl group (C_9) bonded to CORASIL II (temperature 40°C), with detection at 254 nm and a mobile phase of methanol/water (75:25).

Because the resolution of various adjacent peaks of fatty acid phenacyl esters on RP columns is further significantly affected by the column temperature and the proportions of methanol and acetonitrile in the mobile phase, Hanis et al. (23) compared various separation conditions in order to achieve an optimal resolution of the most important fatty acids (Table 1).

Analyses of phenacyl esters of fatty acids were performed on 250 × 4-mm-ID columns packed with 5-μm SEPARON SGX C18 octadecyl-bonded spherical silica and coupled with an octadecyl 7-μm guard column (50 × 4-mm ID). Samples of derivatized fatty acids dissolved in methanol (1–10 μg/ml each) were injected into the chromatograph, with detection at 242 nm, maintaining the column temperature at 40°C.

Elution was performed using a concentration gradient of a methanol–acetonitrile–water ternary mixture. The initial proportions of the components at the beginning of the run were 40:40.5:18.5. The concentration of acetonitrile was then decreased linearly so that it reached 0% at 25 min while its concentration in the mobile phase was replaced with methanol at the same gradient rate. Elution was completed with a linear gradient of the methanol–water mixture so that the mobile phase usually contained 90% of methanol at 60–70 min and was 100% methanol at 90 min. The elution of phenacyl esters of 6:0–22:1 fatty acids was completed within 80 min at a flow rate of 1 ml/min (the detailed composition of the mobile phase is described in Table 1, elution mode E) (Fig. 4).

The mobile phases usually used for HPLC separation of fatty acid derivatives are acetonitrile–water, methanol–water, and acetonitrile–methanol–water. Elution with methanol–water mobile phase only failed to resolve linolenic (18:3) and myristic (14:0) acids.

Table 1 Elution Conditions Used to Separate Fatty Acid Phenacyl Derivatives

Elution mode[a]	Mobile-phase components present: CH_3OH	CH_3CN	H_2O	Concentration gradient, CH_3OH-CH_3CN-H_2O (%, v/v) (min in parentheses)[b]
A	x		x	83:17(0–35).90:10(70),100:0(90)
B		x	x	70:30(0–40),90:10(60),100:0(80)
C	x	x	x	40:40.5:19.5(0),80.5:0:19.5(30),90:0:10(70),100:0:0(90)
D	x	x	x	40.5:41:18.5(0).81.5:0:18.5(28–30),90:0:10(70), 100:0:0(90)
E	x	x	x	40:40.5:19.5(0),81.5:0:18.5(25–27),90:0:10(70), 100:0:0(90)
F	x	x	x	40:40.5:19.5(0).81.5:0:18.5(30),90:0:10(70),100:0:0(90)
G	x	x	x	40:40.5:19.5(0),81.5:0:18.5(28–30),90:0:10(70), 100:0:0(90)

[a] Column, 250 × 4-mm ID, Separon SGX C_{18}, 5 μm; guard column, 50 × 4-mm ID, Separon SGX C_{18}, 7 μm; temperature, 40°C; flow rate, 1.0 ml/min.

[b] Time to reach the composition cited using a linear gradient.

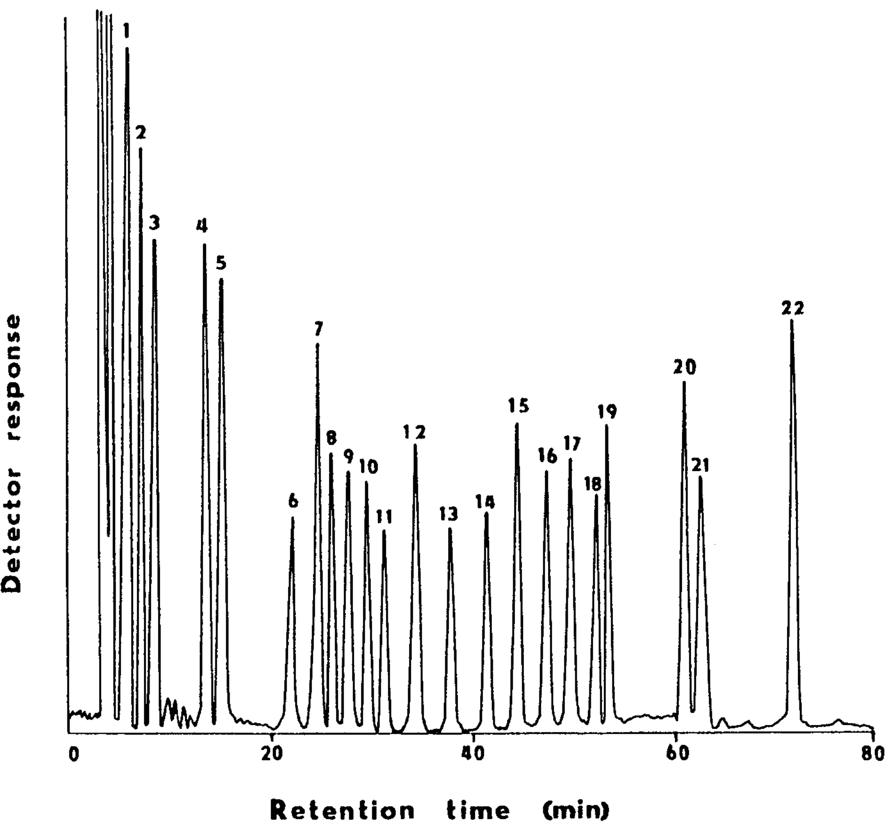


Fig. 4 HPLC of a standard mixture of fatty acid phenacyl esters (40 ng of each). Elution conditions as in Table 1, elution mode E. Peaks: 1. C6:0; 2. C8:0; 3. C10:0; 4. C12:0; 5. C14:1; 6. C18:3; 7. C14:0; 8. C22:6; 9. C16:1; 10. C20:4; 11. C18:2 cis,cis; 12. C15:0; 13. C18:2 trans,trans; 14. C20:3; 15. C16:0; 16. C18:1 cis; 17. C18:1 trans; 18. C20:2; 19. C17:0; 20. C18:0; 21. C20:1; 22. C22:1.

Korte et al. (24) in addition could not resolve the phenacyl esters of oleic and elaidic acids (cis and trans isomers of 18:1, *n*-9 acids), stearic (18:0) and *cis*-11-eicosenoic (20:1) acids, and all-*cis*-4,7,10,13,16,19-docosahexaenoic (22:6) and palmitoleic (16:1) acids.

An acetonitrile–water mobile phase could not resolve adjacent peaks of palmitoleic (16:1) and arachidonic (20:4) acids. Partial separation could be achieved with a decreased ratio of acetonitrile to water in the mobile phase, as reported by Borch (25), but this was inconvenient, owing to the substantial prolongation of the elution time (up to 4 h at a flow rate of 2 ml/min) and the specific column requirements (90 × 0.64-cm ID). These results demonstrate the importance of the carbon chain length and the number of double bonds with respect to the solubility of phenacyl derivatives in these two solvents. The retention time of phenacyl esters increases with increasing chain length and decreasing number of double bonds.

Whereas in acetonitrile the number of double bonds seems to be more important for solubility, in methanol the chain length seems more important. Utilization of the different properties of these two solvents offers some possibility for the separation of fatty acids whose differences in chain length and degree of unsaturation may make them difficult to separate with the use of either acetonitrile or methanol alone.

Isocratic elution with any mixture of these solvents cannot benefit from all the potential advantages of the system. Acetonitrile improves the resolution of fatty acids eluted at the beginning

of the run (18:3 and 14:0), but significantly reduces the resolution of fatty acids eluted later (16:1, 20:4, 16:0, and 18:1). For these reasons, a ternary concentration gradient elution with acetonitrile, methanol, and water seems to be the best way to optimize the separation of the phenacyl derivatives of fatty acids by RP-HPLC.

This method is more efficient in the resolution of cis and trans conformational isomers of fatty acids than the usually used GC methods with packed columns, and there are no problems with derivatization of short-chain fatty acids or heat-labile polyunsaturates. Another advantage over GC methods is that the separated fatty acids are not destroyed during their detection, which enables further analysis to be performed. The trans isomers are generally eluted after the corresponding cis isomers. Positioning of the double bonds in the proximity of the carboxy group of an acid usually leads to a shift in the direction of the methyl end of the carbon chain.

Miwa et al. (26) have demonstrated that both short- and long-chain fatty acids can also be converted into their 2-nitrophenylhydrazides and separated bv RP-HPLC with acetonitrile–water as the eluent. They have described a method for the direct derivatization without an extraction step and the simultaneous microanalysis of 14 kinds of $C_{10:0}$–$C_{22:6}$ fatty acid hydrazides in a reverse-phase HPLC system (27).

Analyses were carried out with an ODS guard column (30 × 4.6-mm ID) and a reverse-phase column of YMC-C8 (250 × 4.6-mm ID, particle size 5 μm) maintained at 30°C; the absorbance was monitored at 230 nm. All analyses were performed isocratically using acetonitrile–water as the solvent system at a flow rate of 1.2 ml/min. The pH of the solvents was maintained at 4.5 by adding 0.1 M HCl to acetonitrile.

The simultaneous separation of a mixture of 15 kinds of fatty acid hydrazides with mobile phases comprising various mixtures of methanol and water was not possible because of the long retention times. Figure 5 shows that with methanol–water (86:14) the separation of linoleic and eicosapentenoic acid hydrazides, and of linoleic, arachidonic, and docosahexenoic acid hydrazides, were difficult.

The elution volumes of the fatty acid derivatives are affected principally by the number of carbon atoms and the number of unsaturated bonds in the fatty acid chains. In acetonitrile–water, unlike methanol–water, the number of unsaturated bonds seemed to be of greater importance. Also, the effect of the column temperature was important. A value of 30°C was used to shorten the analysis time, with good resolution. Thus, satisfactory resolution and favorable retention times (within 15 min) of the $C_{10:0}$–$C_{22:6}$ fatty acid hydrazides were obtained in RP-HPLC with isocratic elution using acetonitrile–water (85:15), as shown in Fig. 5b.

Miwa and Yamamoto (31) described a simple and rapid method with high accuracy and reliability for the determination of $C_{8:0}$–$C_{22:6}$ fatty acids, which occur in esterified forms in dietary fats and oils and in living cells [the biological effects of routinely consumed fats and oils are of wide interest because of their impact on human health and nutrition (28,29), in particular, the ratio of ω-3 polyunsaturated fatty acid to ω-6 polyunsaturated fatty acids (ω-3/ω-6) seems to be associated with atherosclerosis and breast and colon cancers (30)]. They report improved separation of 29 saturated and mono- and polyunsaturated fatty acids (C_8–C_{22}), including cis-trans isomers and double-bond positional isomers, as hydrazides formed by direct derivatization with 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) of saponified samples without extraction. The column consisted of a J'sphere ODS-M 80 column (particle size 4 μm, 250 × 4.6-mm ID), packed closely with spherical silica encapsulated to reach a carbon content of about 14% with end-capped octadecyl-bonded-spherical silica (ODS), maintained at 50°C. The solvent system was acetonitrile–water (86:14, v/v) maintained at pH 4–5 by adding 0.1 M hydrochloric acid with a flow rate of 2.0 ml/min. Separation was performed within only 22 min by a simple isocratic elution (Fig. 6). The resolution of double-bond positional isomers, such as γ-linolenic (ω-6) and α-linolenic acid (ω-3) hydrazides and ω-9, ω-12, and ω-15 eicosenoic acid hydrazides was achieved by use of this column.

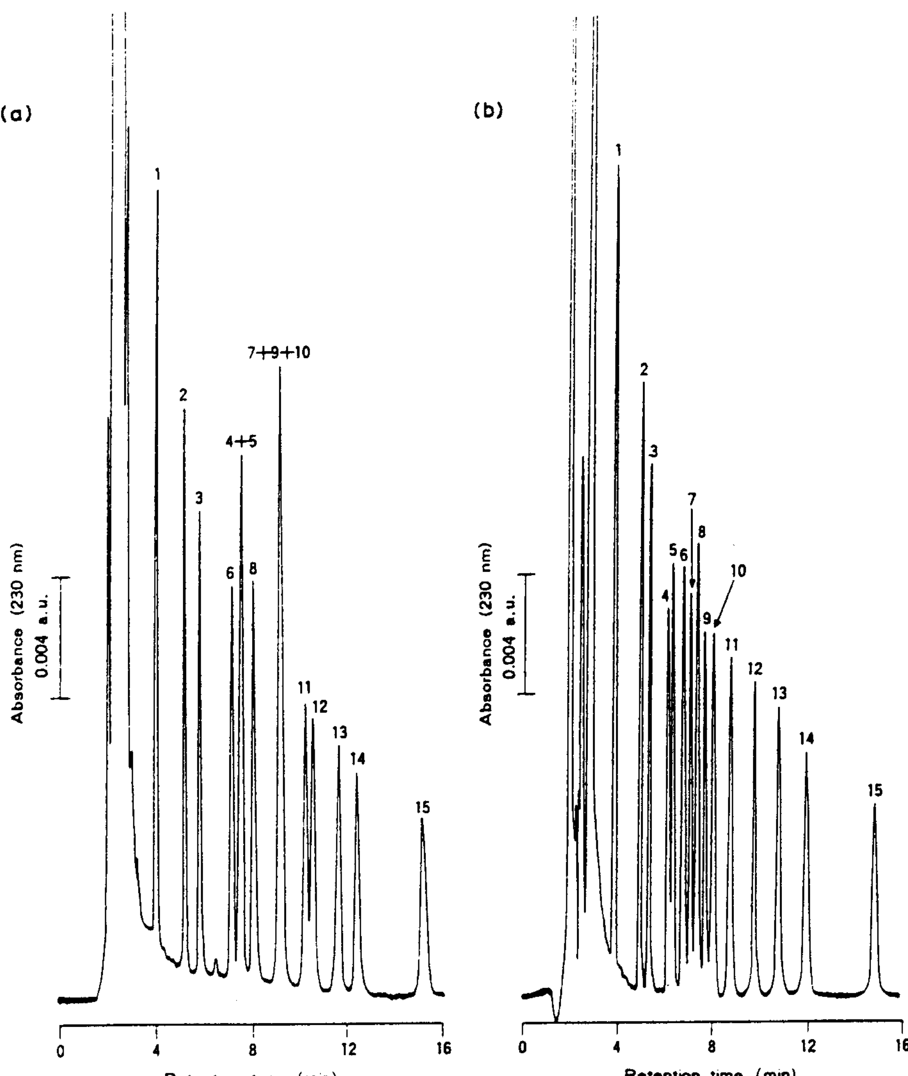


Fig. 5 Chromatograms of the 2-nitrophenylhydrazides of a mixture of saturated and unsaturated long-chain fatty acids obtained with UV detection. Flow rate: 1.2 ml/min. Eluent and column temperatures: (a) methanol/water (86:14 v/v) and 50°C; (b) acetonitrile/water (85:15 v/v) and 30°C. Peaks: 1. capric; 2. Lauric; 3. myristoleic; 4. eicosapentenoic; 5. linolenic; 6. myristic; 7. docosahexenoic; 8. palmitoleic; 9. arachidonic; 10. linoleic; 11. eicosatrienoic; 12. palmitic; 13. oleic; 14. margaric (internal standard); 15. stearic acid hydrazide. Each peak corresponds to 150 pmol.

By increasing the proportion of acetonitrile in the mobile phase, γ-linolenic (ω-6) and myristic acid hydrazides were resolved, as were docosahexaenoic (ω-3) and palmitoleic acid (ω-7) hydrazides. This change, however, also resulted in decreased resolutions of myristoleic (ω-5) and octadecatetraenoic acid (ω-3) hydrazides and of palmitic and docosatetraenoic acid (ω-6) hydrazides and of oleic (ω-9) and elaidic acid (ω-9) hydrazides.

Column temperature also has a significant effect on resolution. Increasing the column temperature from 30° to 50°C leads to inversion in selectivity. Therefore, increasing column temper-

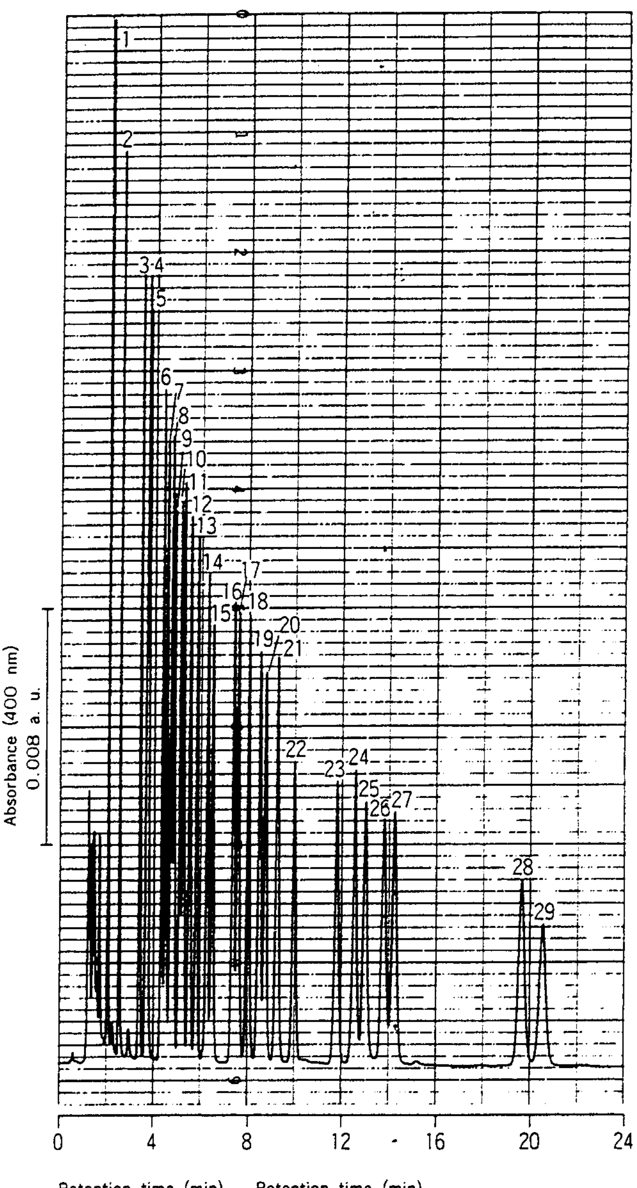


Fig. 6 Chromatogram of 2-nitrophenylhydrazides of a standard mixture of 29 fatty acids obtained with visible detection. Peaks: 1 = caprylic (C8:0); 2 = capric (C10:0); 3 = lauric (C12:0); 4 = myristoleic (C14:1, ω-5); 5 = ostadecatetraenoic (C18:4); 6 = eicosapentaenoic (C20:5, ω-3); 7 = α-linolenic (C18:3, ω-3); 8 = γ-linolenic (C18:3, ω-6); 9 = myristic (C14:0); 10 = docosahexaenoic (C22:6, ω-3); 11 = palmitoleic (C16:1, ω-7); 12 = arachidonic (C20:4, ω-6); 13 = linoleic (C18:2,cis,cis, ω-6); 14 = linoelaidic (C18:2,trans,trans, ω-6); 15 = eicosatrienoic (C20:3, ω-3) and dihomo-γ-linolenic (C20:3, ω-6); 16 = palmitic (C16:0); 17 = docosatetraenoic (C22:4, ω-6); 18 = oleic (C18:1,cis, ω-9); 19 = elaidic (C18:1,trans, ω-9); 20 = eicosadienoic (C20:2, ω-6); 21 = margaric (C17:0) (IS); 22 = docosatrienoic (C22:3, ω-3); 23 = stearic (C18:0); 24 = eicosenoic (C20:1, ω-9); 25 = eicosenoic (C20:1, ω-12); 26 = docosadienoic (C22:2, ω-6); 27 = eicosenoic (C20:1, ω-15); 28 = arachidic (C20:0); 29 = erucic (C22:1, ω-9) acid hydrazide. Each peak corresponds to 150 pmol.

ature caused greater resolutions of the latter three critical pairs but loss of resolutions of the former two critical pairs. For this study, column temperature was set at 50°C to shorten analysis time and yet achieve good resolution. However, resolution of eicosatrienoic (ω-3) and dihomo-γ-linolenic acid (ω-6) hydrazides could not be achieved.

Table 2 compares the major fatty acids of vegetable and fish oils and animal fats. Vegetable oils differ among themselves in the percentage of C_{16}–C_{18} fatty acids, with the exception of coconut oil. In animal fats, fatty acid chain lengths extend from C_{10} to C_{20}.

Sardine oil has similar fatty acids to other edible fats and oils and also contains longer-chain ω-3 polyunsaturated fatty acids, such as eicosapentaenoic acid and docosahexanoic acid. Table 2 indicates that the principal polyunsaturated fatty acids of sardine oil are in the ω-3 family.

The picolinyl esters (32) permit the location of double bonds, since these give distinctive fragmentations that are characteristic of the double-bond positions. They are easily prepared and are not too polar for separation by gas-liquid chromatography (GLC). It has been confirmed that the picolinyl esters are the most useful, since they permit unequivocal identifications even with polyunsatured components. It was also demonstrated that derivatives of this type, prepared from natural mixtures, give satisfactory resolutions when subjected to GLC on capillary columns of fused silica coated with a nonpolar methylsilicone phase, for identification by mass spectrometry (MS).

Table 2 Fatty Acid Composition of Fats and Oils

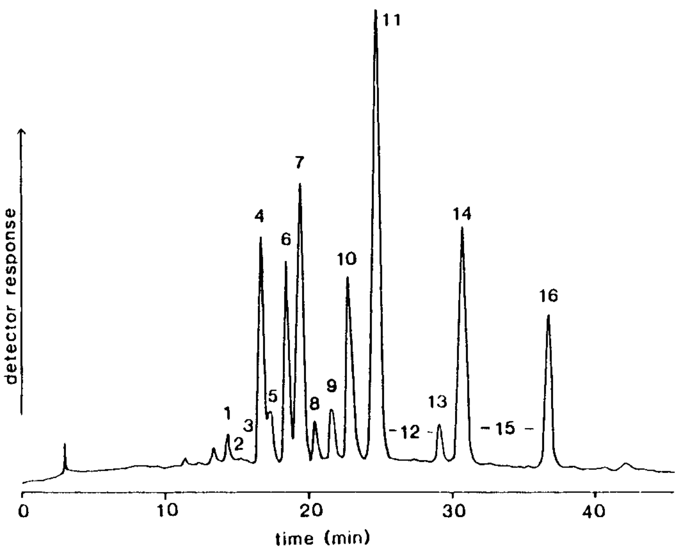
	Fatty acid, mol %								
	Vegetable						Animal		
Fatty acid	Coconut	Olive	Soybean	Corn	Safflower	Margarine	Beef tallow	Lard	Sardine
$C_{8:0}$	6.30	—	—	—	—	—	—	—	—
$C_{10:0}$	5.28	—	—	—	—	—	0.08	0.18	—
$C_{12:0}$	50.06	—	—	—	—	0.74	0.13	0.16	1.03
$C_{14:0}$	20.51	—	—	—	—	2.83	2.82	2.89	11.57
$C_{14:1}$ (ω-5)	—	—	—	—	—	—	0.35	0.27	3.92
$C_{16:0}$	7.93	11.85	8.91	12.52	10.20	35.39	31.77	28.96	19.17
$C_{16:1}$ (ω-7)	—	0.63	—	—	—	—	1.71	3.35	10.21
$C_{18:0}$	2.68	4.28	3.72	1.80	2.25	7.29	27.79	13.88	2.70
$C_{18:1\,cis}$ (ω-9)	5.31	75.58	23.73	25.30	11.69	39.61	29.92	37.14	11.64
$C_{18:1\,trans}$ (ω-9)	—	—	—	—	—	—	0.86	0.76	—
$C_{18:2\,cis,\,cis}$ (ω-6)	1.93	7.01	54.24	58.72	74.12	13.42	3.04	10.85	3.48
$C_{18:3}$ (ω-3)	—	0.65	7.71	1.66	1.74	0.08	0.54	0.30	0.90
$C_{18:3}$ (ω-6)	—	—	1.69	—	—	—	—	—	—
$C_{18:4}$ (ω-3)	—	—	—	—	—	—	—	—	0.12
$C_{20:1}$ (ω-9)	—	—	—	—	—	0.64	—	0.57	2.34
$C_{20:2}$ (ω-6)	—	—	—	—	—	—	0.42	0.42	0.30
$C_{20:3}$ (ω-3 and ω-6)	—	—	—	—	—	—	0.57	0.45	—
$C_{20:4}$ (ω-6)	—	—	—	—	—	—	—	—	0.82
$C_{20:5}$ (ω-3)	—	—	—	—	—	—	—	—	16.17
$C_{22:1}$ (ω-9)	—	—	—	—	—	—	—	—	4.11
$C_{22:2}$ (ω-6)	—	—	—	—	—	—	—	—	0.28
$C_{22:6}$ (ω-3)	—	—	—	—	—	—	—	—	11.24
Total, mmol/g	5.897	2.281	2.462	2.482	2.637	2.006	2.682	2.770	2.495

Data is means ($n = 3$).

The free fatty acids were reacted with 3-(hydroxymethyl)-pyridine (a tenfold excess) and 4-dimethylaminopyridine (1.2 molar proportion) in dichloromethane for 3 h at room temperature. The products were taken up in hexane–diethyl ether (1 : 1) by volume and washed with 2 M HCl and then twice with water.

For the optimum HPLC separation, the liquid chromatograph was connected to a mass detector. A column (250 × 5-mm ID) of SPHERISORB C8 (5-μm particles) was eluted with a solvent gradient, with reservoir A containing methanol and reservoir B containing water–pyridine–acetic acid (98.5 : 1.5 : 0.025). The mobile phase was A:B = 92:8, and this composition was held for 10 min. The flow rate was 0.75 ml/min.

The separation obtained with the picolinyl derivatives (of the fatty acids of cod liver oil) is shown in Fig. 7. As with reverse-phase separations with other fatty acid derivatives, the retention volumes for each component increased with chain length and decreased with the number of double bonds.



Peak	Major components	Minor components
1	14:1 (*n*-5)	14:1 (*n*-7)
2		18:4 (*n*-3), 13:0
3		16:2 (*n*-4)
4, 5	14:0, 20:5 (*n*-3)	18:3 (*n*-3) , 18:3 (*n*-6) , 20:5 (*n*-6)
6	16:1 (*n*-7)	16:1 (*n*-5), 16:1 (*n*-9)
7	22:6 (*n*-3)	20:4 (*n*-6) , 20:4 (*n*-3)
8	18:2 (*n*-6)	18:2 (*n*-4)
9	22:5 (*n*-3)	17:1 (*n*-9), 7-methyl-16:1 (*n*-7)
10	16:0	20:3 (*n*-6) , 20:3 (*n*-3) , 22:5 (*n*-6)
11	18:1 (*n*-9), 18:1 (*n*-7)	18:1 (*n*-5)
12		15-methyl-16:0, 5-methyl-16:0[ab] 19:1, 20:2 (*n*-6)
13	18:0	
14	20:1 (*n*-9), 20:1 (*n*-11)	20:1 (*n*-7)
15		phytanic acid
16	22:1 (*n*-9), 22:1 (*n*-11)	

Fig. 7 Separation of picolinyl ester derivatives of the fatty acids of cod liver oil by HPLC in the reverse-phase mode.

$$R\text{-}COOH + HO\text{-}CH_2\text{-}(\text{anthracene}) \xrightarrow{BMP} R\text{-}C(=O)\text{-}O\text{-}CH_2\text{-}(\text{anthracene}) + H_2O$$

Chemical Structure 1

To improve the separation of the derivatives of fatty acids with the same effective carbon number, e.g., palmitoleic (C16:1), linoleic (18:2), and myristic (C14:0), Baty et al. (33) reported the preparation of the anthrylmethyl esters derivatives of several fatty acids (with 9-hydroxymethylanthracene and the catalyst 2-bromo-1-methylpyridinium iodide (BMPI)) with a view to analysis by HPLC and LC-MS (with gradient elution on a ZORBAK 5-μm C_{18} column) (see Chemical Structure 1). The excess reagents were evaporated under nitrogen at 50°C, and the derivatized acids were taken up in 1 ml of mobile phase prior to chromatography. This method did not allow the resolution of the C16:1, C18:2, and C14:0 esters, although HPLC data obtained for the other acids correlated well with that obtained by capillary gas-liquid chromatography.

The authors have evaluated different C_{18} and C_8 columns, studying the variation in capacity factors with the proportion of water in the mobile phase for all six acid derivatives. The detector was a fluorescence detector equipped with a 360-nm excitation and a 420-nm emission filter. They were unable to resolve derivatives of the six acids on three different C_{18}, 5-μm columns, despite using a wide range of solvent compositions. Table 3 shows capacity factor values for three critical acid derivatives on a number of columns. When a C_8, 5-μm column was used with an isocratic solvent system [acetonitrile/water (98:2)], they observed a marked improvement in the resolution of the six derivatives. However, it was not possible to achieve complete resolution of the six compounds under isocratic conditions, despite numerous experiments with a range of organic solvent mixtures consisting of acetonitrile, methanol, tetrahydrofuran, and water.

Modification of the isocratic solvent composition shown in Fig. 8 by increasing the percentage of water did not improve matter, since this caused the $C_{20:4}$ acid derivative to be eluted with the $C_{14:0}$ derivative. This led to an investigation of the variation in capacity factors of all six derivatives with different acetonitrile/water mlxtures.

At water compositions of 10–14%, the arachidonic acid derivative and the $C_{14:0}$ com-

Table 3 Capacity Factors for the C12:0, C18:3, and C20:4 Derivatives on Columns with Differing Percentages of Carbon Load

Mobile phase	Column	Load (%)	Surface area (m^2/g)	k′ $C_{12:0}$	k′ $C_{18:3}$	k′ $C_{20:4}$
Acetonitrile–water (85:15)	Spherisorb C_8	6	220	6.77	8.66	10.08
	Apex-MF_8	7–8	170	6.61	9.33	11.48
	LiChrosorb C_8	12	250	4.41	5.32	5.97
Acetonitrile–water (92:8)	Spherisorb ODS	8	220	6.01	6.65	7.23
	Nucleosil ODS	16	300	18.12	22.71	26.80
	LiChrosorb ODS	22	150	11.51	13.65	15.84

Spherisorb columns from Phase Separations (Queensferry, U.K.); LiChrosorb columns from Merck (Darmstadt, F.R.G.); Nucleosil columns from Macherey-Nagel (Duren, F.R.G.); APEX-MF columns from Jones Chromatography (Llanbradach, U.K.).

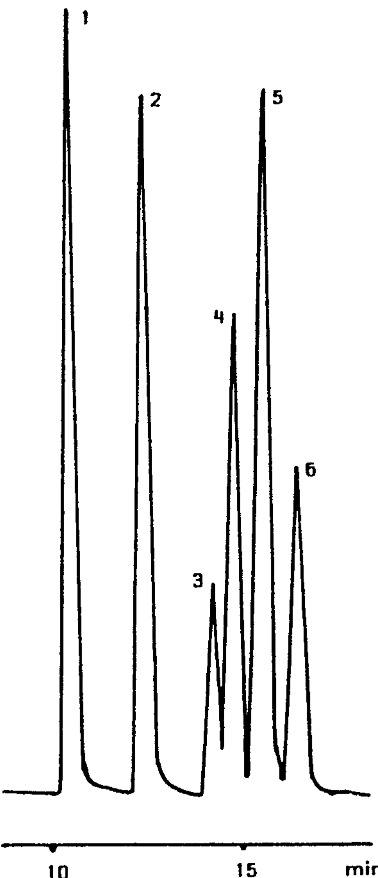


Fig. 8 Separation of the "critical" set of acid derivatives. Column APEX MF8, 250 × 4.6-mm ID (5 μm): flow rate: 1 ml/min; mobile phase: acetonitrile/water (92:8 v/v); detection: fluorescence (excitation: 360 nm; emission: 420 nm). Peaks: 1. C12:0; 2. C18:3; 3. C20:4; 4. C14:0; 5. C16:1; 6. C18:1.

pounds were unresolved; at percentage water compositions below 3%, the $C_{14:0}$ was eluted together with the $C_{16:1}$ and $C_{18:2}$ derivatives. It was found necessary to employ a gradient that started with 7% water and then to introduce more water for a short time (using a concave elution program) to allow adequate separation of the six derivatives. Following the separation of the six "critical" compounds, the elution of the remaining derivatives was straightforward and was accomplished by using 100% acetonitrile (Fig. 9).

In their study on the automation of the derivatization reaction of carboxylic acids, Wolf and Korf (34) chose bromomethylmethoxycoumarin (Br-MMC) as a label because of its commercial availability. The automation of this reaction is problematic, because elevated temperatures are required in the manual procedures and the reaction had to take place in an aprotic environment. The addition of a solid salt also complicates the procedure. They describe a solution to this problem using a suspension of potassium carbonate and appropriate reagent concentrations. The samples were detected by a fluorescence detector equipped with a 5-μl cell, using an excitation wavelength of 325 nm and a cutoff filter of 398 nm.

The labeled fatty acids were separated on a CHROMPHER C_{18} column (200 × 3-mm ID) at ambient temperature. The chromatograms were obtained either under isocratic conditions in acetonitrile–water (90:10) or in a gradient of acetonitrile–water (80:20) to 100% acetonitrile in 45 min. The elution was performed at 0.5 ml/min. The reaction of Br-MMC with fatty acids

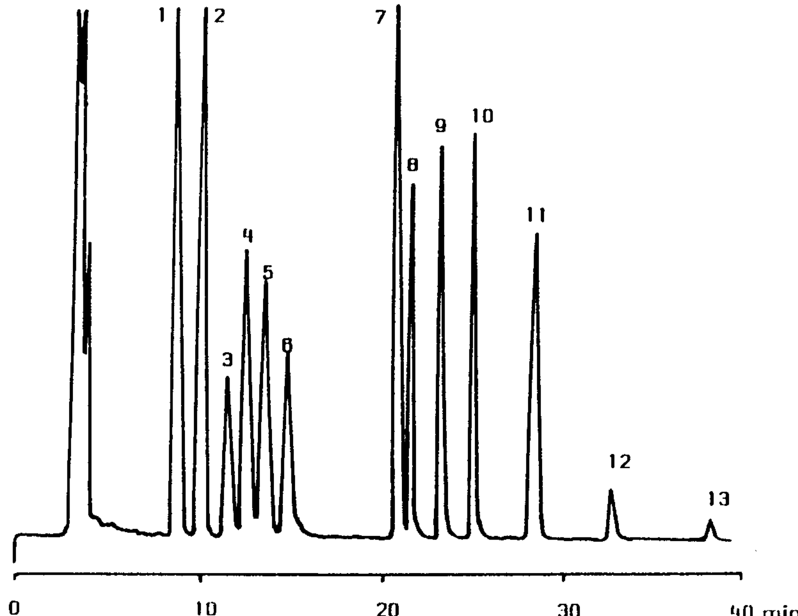


Fig. 9 Separation of a standard mixture of saturated and unsaturated anthrylmethyl derivatives. Column SPHERISORB C8 250 × 4.5-mm ID (3 μm); flow rate: 1 ml/min; gradient elution, mobile phase: acetonitrile/water (93:7 v/v) for 12 min: (86:14 v/v) for 5 min; (100:0 v/v) for 23 min; detection: fluorescence (excitation 360 nm; emission 420 nm). Peaks: 1. C12.0; 2. C18:3; 3. C20:4; 4. C14:0; 5. C16:1; 6. C18:2; 7. C16:0; 8. C18:1; 9. C17:0; 10. C18:0; 11. C20:0; 12. C22:0; 13. C24:0.

in an aprotic, nonaqueous environment catalyzed by solid potassium carbonate or other bases is completed at 60°C within 15 min.

The methylmethoxycoumarin ethers of fatty acids are generally separated on a C_8 or C_{18} column with acetonitrile or methanol mixtures as eluent (Fig. 10). The authors chose acetonitrile because of its compatibility with the reaction medium. The addition of water and different gradients did not result in a better separation. The higher the modifier percentage, the lower the quantum yield and thus the sensitivity.

The comparison of three coumarin compounds, 4-(bromomethyl)-7-methoxy-coumarin (Br-MMC), 7-(diethylamino)coumarin-3-carbohydrazide (DCCH), and 7-(diethylamino)-3-[(4-(iodoacetyl)amino)phenyl]-4-methylcoumarin(DCIA) as potential chemiluminescence with HPLC was reported by Grayeshi and Vasto (35) (see Chemical Structure 2).

The detection of fluorophors by peroxyoxalate chemiluminescence (CL) was first demonstrated by Curtis and Seitz (36). This approach was later adapted to HPLC detection by Kobayashi and Imai (37).

Bis-(2,4,6-tricholophenyl)oxalate (TCPO) reacts with hydrogen peroxide to form a proposed energetic intermediate, 1,2-dioxethanedione, which transfers its energy to a fluorophor via a proposed electron transfer and excites it to the first excited singlet state followed by the emission of a photon. The reason for the interest in CL detection is that chemical excitation, as opposed to conventional photoexcitation, is lower, because of the lack of problems from scattering and fluctuations of the source.

For chemiluminescence measurements, a postcolumn reactor with a pulse-dampening filter was added to the HPLC apparatus. A 3-cm piece of narrow-bore tubing joined the pulse dampener to a Valco 1-μl "T" chemiluminescent reagent with the chromatographic eluent. A C_8 ECONOSPHERE (250 × 4.6-mm ID) column was used. Modifications were made with the

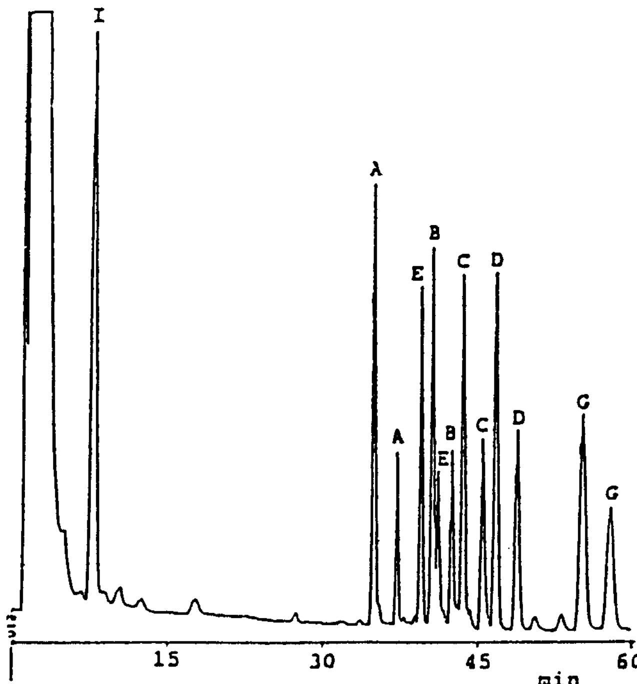


Fig. 10 Separation of fatty acids as their methylmethoxycoumarin esters; flow rate, 0.5 ml/min; room temperature; fluorescence detection (excitation 325 nm, cutoff filter, 398 nm); eluent: acetonitrile/water (80:20 v/v) to 100% acetonitrile in 45 min. Peaks: A. C14.0 + C16:1; B. C16:0; C. C17:0; D. C18:0; E. C18:1; G. C20:4; I. unknown.

CH_2BR
CH_3O

4-Bromomethyl-7-methoxycoumarin (Br-Mmc)

$CNHNH_2$
ET—N
ET

7-Diethylaminocoumarin-3-carbohydrizide (DCCH)

$NHCCH_2I$
CH_3
ET—N
ET

7-Diethylamino-3-(-4-iodoacetylaminophenyl)4-methylcoumarin (DCIA)

Chemical Structure 2

HPLC apparatus to reduce postcolumn pump pulsation and improve CL detection levels of the DCIA derivatives. The CL solution concentrations and flow rate remained the same as those previously described. A 10-μm C_8 column (250 × 4.6-mm ID) plus a guard column (30 × 4.6-mm ID) packed with 30-μm C_{18} material was used in replacement of the ECONOSPHERE column.

Solutions of 1.0 M hydrogen peroxide in acetonitrile and 4.45 mM trichlorophenol (TCP) in ethyl acetate were used as postcolumn reagents, with the flow rates optimized at 1.0 ml/min each. The chromatographic eluent ranged from 70 to 80% acetonitrile–4 mM sodium phosphate buffer at apparent pH 7.5. The absorbance and fluorescence wavelengths for coumarin tags were as follows:

Derivatizing agent	λ_{max}, nm	
	Absorption	Emission
Br-MMC	325	390
DCCH	415	480
DCIA	375	470

The products obtained by derivatizing with Br-MMC were readily detected by fluorescence, but no chemiluminescence was observed for these derivatives.

The derivatization of DCCH to a carboxylic acid required conversion of the acid to acid chloride using thionyl chloride before reaction with the carbohydrazide group. To evaluate the chemiluminescent characteristics of this coumarin compound, the amide derivative of DCCH was isolated by preparative TLC. Although CL was observed from this product, detection limits were not determined because of the difficulty in quantitative transfer from TLC plates.

In the case of DCIA, the iodoacetyl-containing aminocoumarin was reacted with the same carboxylic acids as Br-MMC, and the chromatograms were monitored by chemiluminescence (Fig. 11).

The improvement in fluorescence efficiency afforded by the electron-donating amino group in DCIA improves its fluorescence detectability by more than an order of magnitude when compared to Br-MMC. A fivefold increase in detectability was also observed when comparing chemiluminescence with fluorescence detection of the DCIA derivatives.

Aryldiazoalkanes have therefore been developed because they react readily with carboxylic acids at room temperature without catalysts. These are generally unstable against heating and light and cannot therefore be well purified by recrystallization.

9-Anthryldiazomethane (ADAM) has been widely used for HPLC of various biologically important carboxylic acids.

The proportion and content of FFA in four species of causative phytoplankton of red tides, *Chatonella* antiqua, *Heterosigma* akashiwo, *Skeletonema* costatum, and *Chaetoceros* didymum, cultured in axenic conditions were determined by fluorometric HPLC after derivatization with ADAM by Suzuki and Matsuyama (38).

In experimental assay systems, free fatty acids (FFAs) destroy red blood cells and show hemolytic activity (39). Free fatty acids, especially the highly unsaturated fatty acids (HUFAs) contained in the raphidophyte flagellates, have been implicated as one of the causative substances that damage the epithelial tissues of the fish gills.

The concentrates were suspended in 1 ml of chloroform/methanol (2:1, vol/vol) and sonicated (3 × 5 min). The combined extracts were evaporated to dryness under reduced pressure and dissolved in 2 ml of 80% methanol. Free fatty acids were extracted three times with 2 ml of *n*-

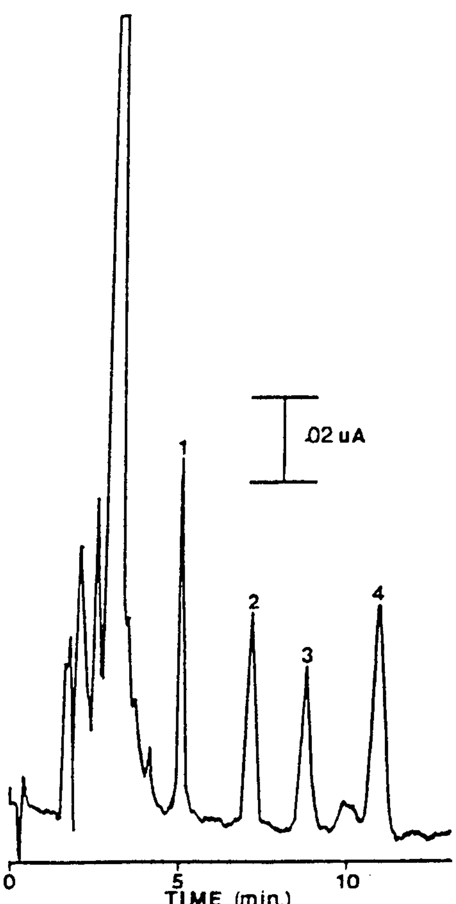


Fig. 11 DCIA chemiluminescence detection: detector source off; PMT voltage 1400 V; 0.2 μA full scale. 1. C18:3; 2. C20:4; 3. C16:1; 4. C18:1.

hexane. The combined extracts were dissolved in 3 ml of *n*-hexane after evaporation. An aliquot (300 μl for S. costatum, C. antiqua, and H. akashiwo and 75 μl for C. didymum) of 3 ml *n*-hexane solution was put in a colored vial and dried under nitrogen. The residue was esterified in 100 μl of 0.1% ADAM (9-anthryldiazomethane)/methanol solution for 1 h in the dark at ambient temperature, and 10 μl of the solution was injected directly into the HPLC.

The HPLC separation was carried out with a Develosil ODS-5 column (250 × 4.6-mm ID) with a gradient of solvent A (acetonitrile/methanol/water, 8:1:1, vol/vol/vol) and solvent B (methanol) at ambient temperature and a flow rate of 1.1 ml/min. The following linear gradient was used: segment 1, 100% A for 20 min; segment 2, initial conditions changed to 100% B over 30 min; and segment 3, 100% B for 50 min. The peaks of fluorescent derivatives were monitored at the excitation and emission wavelengths set at 365 and 412 nm, respectively. Figure 12 shows the chromatogram of FFAs as ADAM derivatives obtained from H. akashiwo.

Major FFAs in C. antiqua were 20:5, 18:4, and 16:0. The FFA composition of C. antiqua was characterized by a high proportion of 18:4 in comparison with that of other species. On the other hand, major FFAs present in H. akashiwo were 20:5 and 16:0. The very high percentage of 20:5 in H. akashiwo is noteworthy. The sum of the HUFA (18:4, 20:4, 20:5, and 22:6) in C. antiqua and H. akashiwo accounted for a 60.3% and 67.5% (wt%) in identified FFAs, respectively.

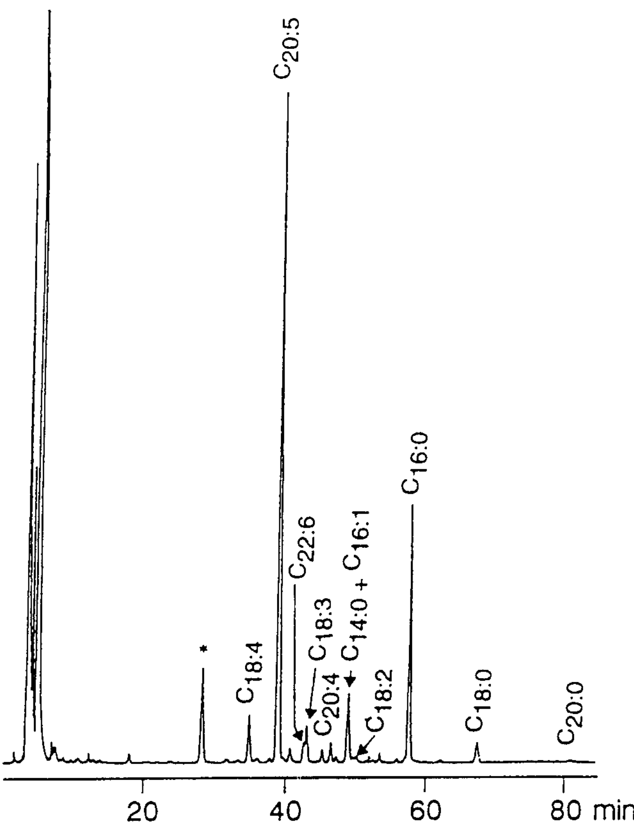


Fig. 12 High-performance liquid chromatographic resolution of the 9-anthrylmethyl esters of free fatty acids obtained from *Heterosigma akashiwo* cultured in axenic conditions. An aliquot (1/100 of 31 mg of phytoplankton) of the extract was injected. The peak of 20:5 corresponds to 746 ng. *Unidentified peak with 0.42 in relative retention time with respect to 18:0. See text for chromatographic conditions.

A more stable aryldiazoalkane, 1-pyranyldiazomethane (PDAM), has been prepared as a new fluorescent labeling agent for carboxylic acids by Nimura et al. (40). The PDAM readily reacts with carboxylic acids at room temperature without a catalyst to give an intensely fluorescent ester.

For detection the excitation wavelength at 340 nm was employed, which gives greater fluorescence intensity. The column was stainless steel (150 × 4-mm ID) packed with a octadecylsilyl silica gel (TSK-GEL-120A) (particle size, 5 μm). The mobile phase was a gradient elution of water with acetonitrile (acetonitrile conc.: 0–30 min, 85%; 30–60 min, 85–100%). The flow rate was 1.0 ml/min. Figure 13 shows a chromatogram of PDAM esters of saturated and unsaturated long-chain fatty acids. Each 100 fmol of the acids was clearly separated and detected. The esters of unsaturated fatty acids were stable, as were those of saturated fatty acids.

B. Cis and Trans Isomers of Unsaturated Fatty Acids: Silver Ion Chromatography

A number of methods have been devised for the separation and/or determination of cis and trans isomers of unsaturated fatty acids, but none of these is entirely satisfactory. Infrared spectrometry

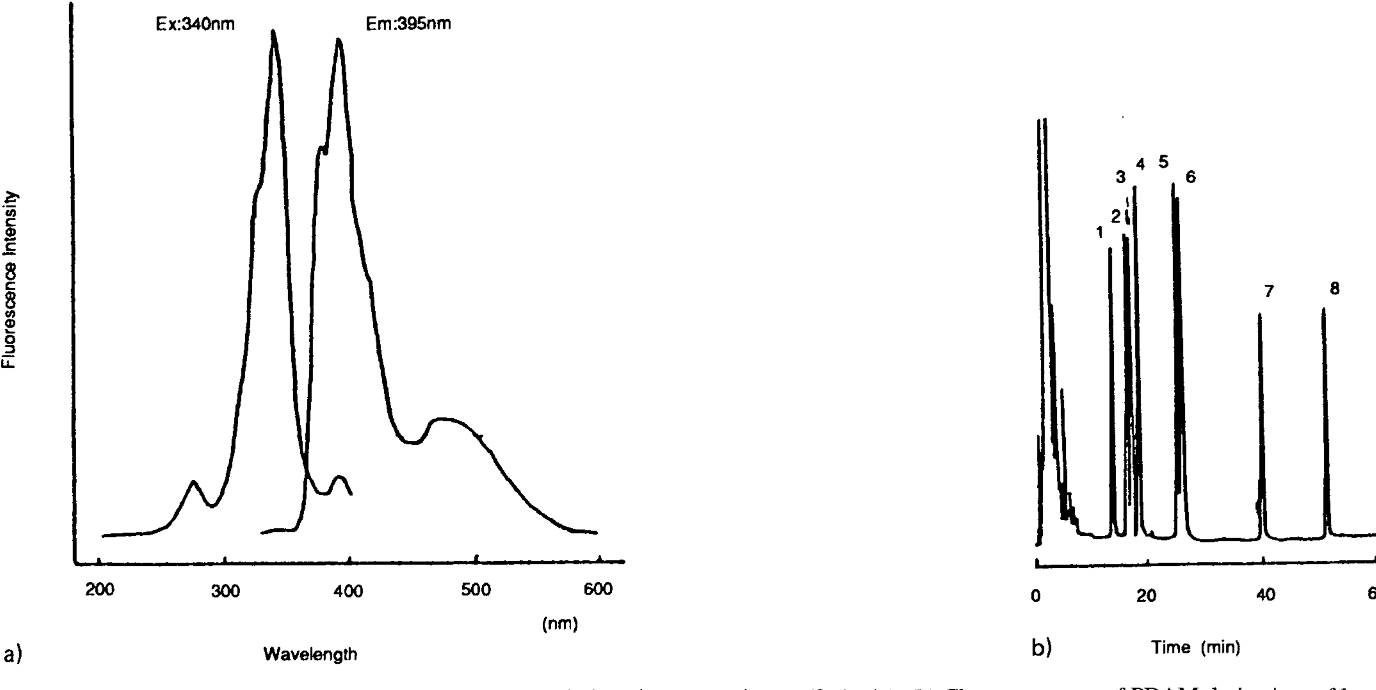


Fig. 13 (a) Fluorescence spectra of 1-pyrenyl methyl palmitate in acetone/water (9:1 v/v). (b) Chromatograms of PDAM derivatives of long-chain fatty acids with fluorometric detection. Mobile phase, gradient elution of water with acetonitrile; acetonitrile conc. 0–30 min, 85%; 30–60 min, 85–100%; flow rate: 1.0 ml/min. Each peak corresponds to about 5 pmol of fatty acid. Peaks: 1. C18:3; 2. C16:1; 3. C14:0; 4. C18:2; 5. C16:0; 6. C18:1; 7. C18:0; 8. C20:0.

has been much used for this purpose, but GC on long packed columns or capillary columns of fused silica with highly polar liquid stationary phases appears to be generally preferred.

Silver ion chromatography is a useful technique for separating geometrical isomers of fatty acids (as the methyl ester derivatives) for subsequent analysis by GC. On the other hand, a stable ion-exchange column loaded with silver ions has been developed for HPLC that has proved of value in the simplification of complex mixtures of fatty acids (FAs) of natural origin for subsequent identification by GC-MS and for separating molecular species of triacylglycerols (41).

Christie and Breckenridge (42) describe the application of this column to the isolation and determination of FAs containing trans double bonds in samples of natural and industrial origin. A column (250 × 4.6-mm ID) of NUCLEOSIL 5SA was flushed with 1% ammonium nitrate solution at a flow rate of 0.5 ml/min for 1 h, then with distilled water at 1 ml/min for 1 h. Silver nitrate (0.2 g) in water (1 ml) was injected onto column via the Rheodyne valve in 50-μl aliquots at 1-min intervals; silver began to elute from the column after about 10 min, and 20 min after the last injection the column was washed with methanol for 1 h, then with 1,2-dichloroethane-dichloromethane (1:1 v/v) for 1 h. For most of the analytical work, the column temperature was maintained at 30°C in a thermostatted oven. 1,2-Dichloroethane-dichloromethane (1:1) (mixture A) at a flow rate of 1.5 ml/min was the mobile phase (detector operated at 242 nm) for the separation of isomeric monoenes, and the same solvents with the addition of 0.5% acetonitrile (mixture B) at a flow rate of 0.75 ml/min were employed for isomeric dienes and trienes.

With samples containing a wide range of components, such as hydrogenated fats, the column was eluted with solvent A for 13 min, then changed in one step to A–B (75:25), with a gradient to 100% B over 20 min. The free fatty acids were converted into the phenacyl derivatives and, prior to HPLC analysis, were purified by elution from a BOND ELUT NH_2 column with hexane–diethyl ether (9:1).

The separation achieved with the isomeric dienes derived from oleic acid is shown in Fig. 14. Three peaks are apparent, the last of which is the natural 9-cis,12-cis isomer; the first must be 9-trans,12-trans octadecadienoate, and the second peak is presumably a mixture of the 9-cis,12-trans and 9-trans,12-cis compounds. With the geometric isomers of linolenic acid six

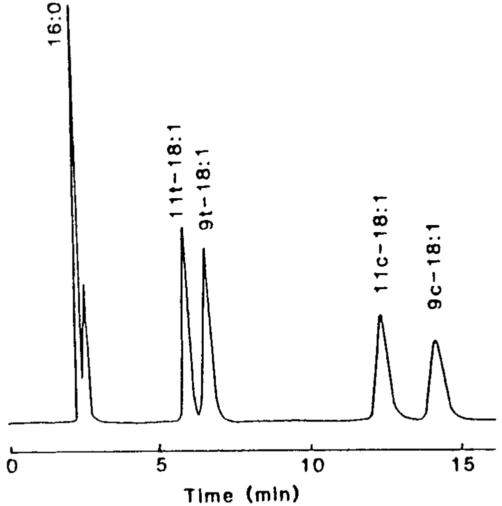


Fig. 14 Separation of the phenacyl derivatives of C16:0: 11t-C18:1, 9t-C18:1, 11c-C18:1, and 9c-C18:1 by HPLC in the silver ion mode. The column temperature was 38°C and the mobile phase was 1,2-dichloroethane/dichloromethane (1:1 v/v) at a flow rate of 1.5 ml/min, with detection at 242 nm.

peaks emerge, the first of which is presumably the all-trans isomer and the last the all-cis isomer (Fig. 15).

Although all-trans-octadecatrienoate tended to elute with the cis-dienes, this was not a problem in practice with samples of hydrogenated fats containing dienoic and trienoic fatty acids. A gradient elution scheme was adapted from the isocratic mobile phase used for monoenes and polyenes for the separation of such mixtures.

While silver ion liquid chromatography has been utilized to separate fatty acid methyl esters (FAMEs) by number of double bonds and by the configuration (cis/trans) of the double bonds [1–3], the lack of commercial HPLC silver ion columns has limited the impact of this technology.

A commercially available column containing silver ions has recently been developed by Chrompack (ChromSpher Lipids HPLC column). A solvent system (43) composed of dichloromethane, dichloroethane, and small amounts (0.01–0.025%) of acetonitrile (ACN) is used with both the Nucleosil and Chrompack HPLC columns. Because chlorinated solvents are opaque at the wavelengths (200–210 nm) used for FAME analyses, the use of UV detectors is precluded unless the phenacyl derivatives of the fatty acids are first prepared.

Adlof (44) developed a UV-compatible solvent system (acetonitrile in hexane) for the separation of polyunsaturated cis- and trans-FAMEs.

The ChromSpher Lipids column (250 mm × 4.6-mm ID stainless steel; 5 μm) was purchased from Chrompack (Middelburg, Netherlands) and used as received. Solvent flow was standardized at 1.0 ml/min and run temperatures at 22–23°C. A small cooling fan was used to minimize temperature fluctuations and bubble formation at the solvent pump mixing solenoid.

Acetonitrile, the cosolvent of choice for increasing the eluting capability of solvents in silver ion chromatography (45), is soluble in hexane to ca. 1.5% (v/v) at 23°C. A binary system composed of hexane and 0.5% ACN in hexane was found to provide a wide range of solvent strengths and excellent baseline stability. However, elution times for methyl linoleate differed when a binary system was used [A–B (50:50), where A = hexane and B = 1.0% ACN in hexane] vs. a single solvent reservoir containing 0.5% ACN in hexane. Whether this was due to the HPLC pump, the solvent reservoirs, or the solvent mixing solenoid was not investigated. Thus, an isocratic system with a single reservoir containing the appropriate percentage of ACN in hexane mixture was more suitable for column characterization and reproducible FAME separations.

The separation of safflower oil (SFO)–linseed oil (LSO) methyl esters is shown in Fig. 16. Free fatty acid methyl ester elution reproducibility, resolution, and baseline stability were maintained at sample sizes of 17–170 μg, although capacity factors (κ) decreased approximately 25% between the 17- and 170-μg sample sizes. The trend of longer retention times with smaller sample sizes was consistent throughout their studies. Peak distortion, such as observed when gas chromatographic columns are overloaded, was not observed in their system. Perhaps larger FAME samples compete for silver ion sites the same way the ACN cosolvent competes for those sites. Excellent peak shapes were obtained, even with sample elution times of 1.5–2.0 h.

Separation of the cis/trans isomers of methyl 18:3 (8 isomers) is illustrated in Figs. 16 and 17. The order of elution for the methyl linoleate isomers (Fig. 16) was trans-9,trans-12-, trans-9,cis-12-, cis-9,trans-12-, and cis-9,cis-12-18:2. The observed elution order differs from that obtained with capillary GC (SP 2330, SP 2340, or SP 2560 stationary phases) in which cis-9,trans-12-18:2 elutes before trans-9,cis-12 18:2.

The separation of all eight cis/trans isomers of methyl 18:3 was similar to, but with better resolution than, the separation obtained with a 50-m CP Sil 88 capillary GC column (46) or by packed capillary supercritical fluid chromatography (47]. The FAMEs eluted in four peaks or sets of peaks corresponding to the total number of cis and trans double bonds. These are marked as A (three trans), B (two trans, one cis), C (one trans, two cis), and D (three cis).

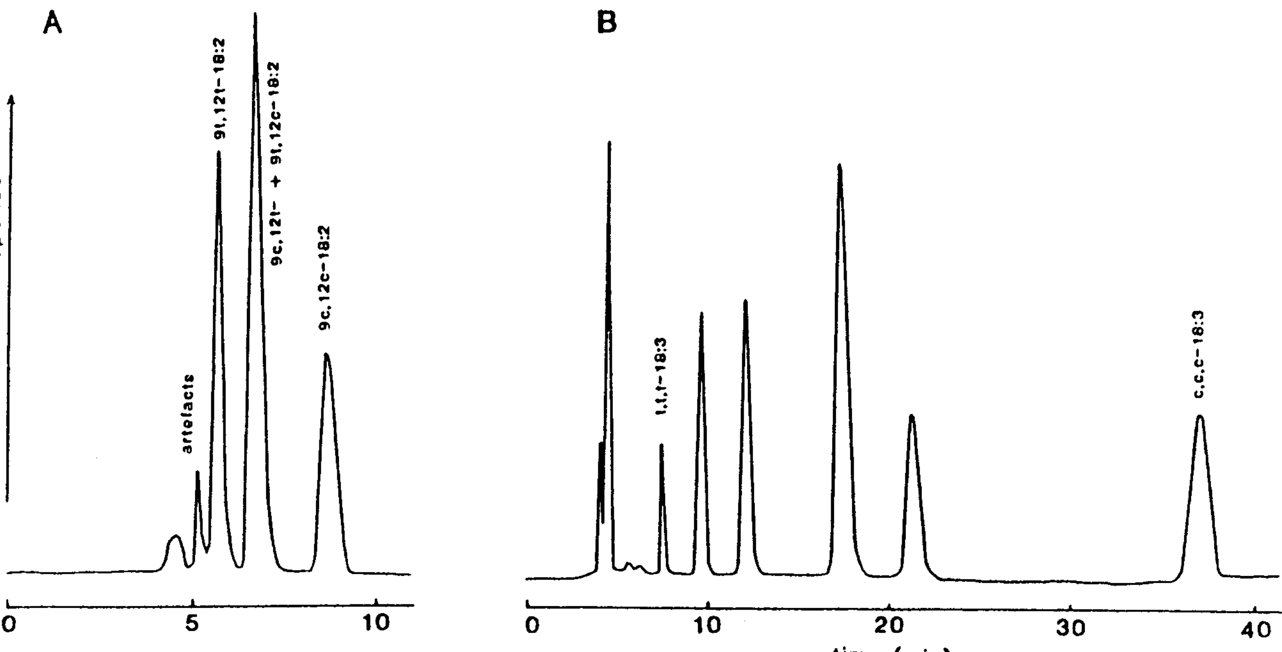


Fig. 15 Separation of the phenacyl derivatives of the geometrical isomers of (A) linoleic and (B) linolenic acids by HPLC in the silver ion mode. The column temperature was 38°C, and the mobile phase was 1,2-dichloroethane/dichloromethane/acetonitrile (49.75:49.75:0.5 v/v/v) at a flow rate of 0.75 ml/min, with detection at 242 nm. Note the change of scale on the time axis.

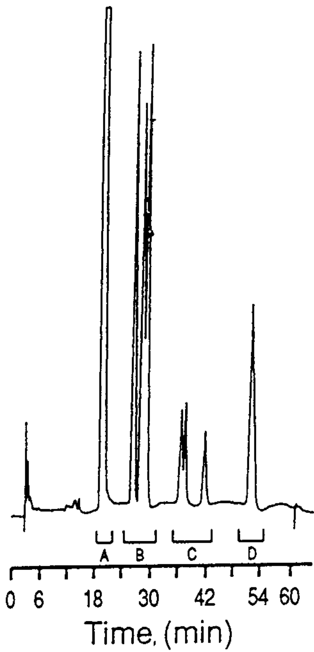


Fig. 16 Separation of isomerized methyl linolenate. Sample size: 20 μg. Flow rate: 1.0 ml/min 0.125% ACN in hexane. UV detection at 210 nm. Groups: A = three trans; B = two trans, one cis; C = one trans, two cis; D = three cis.

The resolution of 15 of the 16 possible cis/trans isomers of methyl 20:4 far exceeded the capabilities of current GC, other HPLC, or other analytical methodology. Again, the eluted isomers are grouped by total cis and trans double bonds, with an elution pattern of A (four trans), B (three trans, one cis), C (two trans, two cis), D (one trans, three cis), and E (four cis). At 0.15% ACN in hexane, the 20:4 isomers could be separated within 25 min; with this solvent system, all four (three trans, one cis) isomers can be separated, but one (two trans, two cis?) of the other isomers is unresolved.

Another point of interest was the time required to equilibrate the system after changes were made in solvent composition. While the ChromSpher Lipids column had a column volume of ca. 3 ml, an increase in ACN concentration was not noted until the introduction of 7–8 ml of solvent (determined with refractive index detector). The problem of ACN–silver ion interaction and subsequent ACN retention is not new and may be noted in all forms of chromatography employing silver ions in the stationary phase. In the isocratic system, the column was equilibrated with the appropriate solvent mix for at least 0.5 h before sample injection. Since ACN dissolves very slowly into hexane, the ACN–hexane solvent mix was thoroughly stirred for 5 min before use. To obtain reproducible retention times, thorough mixing of the ACN and hexane is essential.

C. ω-3-Polyunsaturated Fatty Acids

Interest in ω-3-polyunsaturated fatty acids initially was prompted by observations that Greenland Eskimos experienced a significantly lower incidence of death from ischemic heart disease than Western populations. Results of subsequent studies indicated that this alleged or potential health benefit and other physiological effects were due to a diet high in marine oils containing esterifed ω-3-polyunsaturated fatty acids (PUFAs).

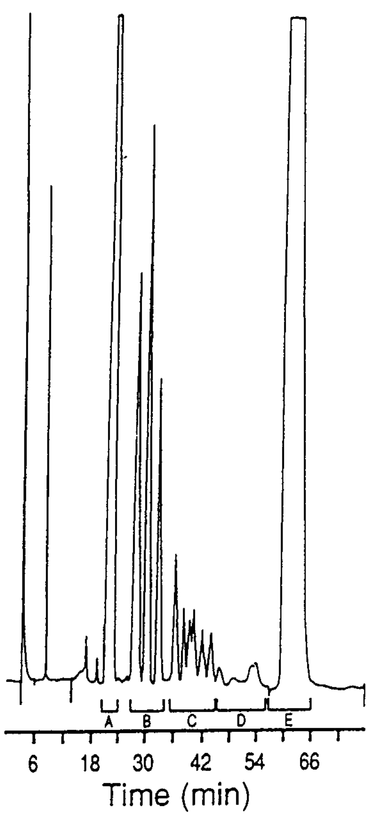


Fig. 17 Separation of isomerized methyl arachidonate. Sample size: 100 μg. Flow rate: 1.0 ml/min 0.125% ACN in hexane. UV detection at 210 nm. Groups: A = four trans; B = three trans, one cis; C = two trans, two cis; D = one trans, three cis; E = four cis.

Either native winterized or concentrated whole-body fish oils or fish liver oils have been utilized in most studies as dietary source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The physiological effects and possible health benefits of administered fish oils generally have been attributed to either EPA or DHA alone or to a synergistic effect between the two. As a result, there has been controversy over the contribution of individual fish oil constituents to particular pharmacological actions and the optimal dosages required for achieving established and/or suspected beneficial effects. The predominant marine triglyceride-derived ω-3-fatty acids are all-*cis*-5,8,11,14,17-eicosapentaenoic acid ($C_{20:5,3}$, EPA) and all-*cis*-4,7,10,13,16,19-docosahexaenoic acid ($C_{22:6,3}$, DHA).

Normally, the method of choice for the analysis of complex mixtures of polyenoic fatty acids such as those derived from fish oils is capillary gas chromatography with prechromatographic derivatization and mass spectrometric detection. However, GC is impractical for the purification of the large amounts of polyenoic fatty acids required for biological and clinical studies. Moreover, the temperatures required in GC may cause degradation of oxidized long-chain polyunsaturated fatty acids that are present as minor components of the mixture.

In the past 20 years, complex mixtures of these polyunsaturated long-chain fatty acids or their *n*-alkyl esters contained in natural and modified products, such as margarines and vegetable oils, have been successfully separated by RP-HPLC. Under the relatively mild conditions of RP-

HPLC, little degradation of polyenoics occurs, and methods can be easily scaled up for semi-preparative or preparative use.

Because methylene-interrupted polyunsaturates do not have strongly absorbing chromophores in the UV region, detection by refractive index or far-UV detection (205–214 nm) has been utilized in RP-HPLC separations of free fatty acids and their aliphatic esters. Refractive index detection is less sensitive than UV detection. However, with far-UV detection, solvents absorbing UV below 210 cannot be used. The RP-HPLC separations reported to date have generally involved derivatization designed to incorporate aromatic chromophores allowing detection by fluorescence or UV detection.

The separation and purification of ethyl esters of EPA, DHA, and the heretofore-minor unreported polyunsaturate octadecatetraenoic acid ($C_{18:4,3}$, OTA) on a preparative scale by modification of an analytical RP-HPLC procedure has been described by Beebe et al. (48). They used a liquid chromatograph equipped with a differential refractometer as detector operated at room temperature and an ST Macrobore column (350 × 4.6-mm ID) of C18 reverse-phase material, 25-μm particle size.

The esters were eluted with acetonitrile/THF/water (466:233:300) at a flow rate of 2.0 ml/min. Following the transesterification and urea crystallization steps, the concentrated ethyl esters were dissolved (1 mg/μl total fatty acid ethyl esters) in peroxide-free THF containing 0.005% BHT as an antioxidant and injected into the chromatograph.

A preparative chromatograph fitted with an ST/2000-1 guard column (1 × 2-in. ID) and an ST/2000B preparative column (25 × 2-in. ID), each packed with ST MACROBORE C18 (25-μm particle size), was employed for separation of esters. The system was equipped with a differential refractometer with a flowing reference (as opposed to an air-filled reference) in order to minimize baseline drift.

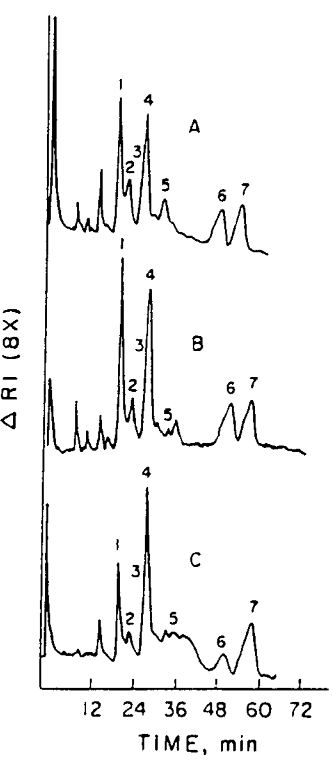
Samples of 1 or 2 ml (equivalent to 0.8–1.6 g) of total esters were introduced into the system via a 10-ml syringe immediately followed by a 1-ml eluent fresh. The esters were eluted with acetonitrile/THF/water (466:233:300) at a flow rate of 220 ml/min. As the esters were eluted, typically 24 fractions of approximately 175 ml each were collected; the fractions were stored in tightly closed containers in a freezer at 60°C until analyzed.

The results of method development experiments were monitored by analytical RP-HPLC of appropriate collected fractions. Prior to chromatography, the esters in each fraction were extracted from the eluent into hexane (2 ml) and the layers were separated. Next, the fractions were brought to dryness under a stream of nitrogen and reconstituted in 200 μl of peroxide-free THF containing 0.005% BHT.

An RP-HPLC procedure for the analysis of ω-3 fatty acid esters that could eventually be adapted to the preparative isolation of quantities of EPA and DHA was also developed by Beebe et al. (48). The separations were performed at room temperature on an ODS-3 RAC II column (100 × 4.6-mm ID, 5-μm particle size) with a guard column (30 × 4.6-mm ID dry-packed with SUPELCOSIL LC-18 40-μm material). The esters were eluted with a mobile phase of acetonitrile/tetrahydrofuran/water (9:5:11) at a flow rate of 2.0 ml/min (detector differential refractometer).

The chromatographic profiles representing saturated mono-, di-, and polyunsaturated long-chain fatty acid esters from marine triacylglycerols were very complex. In general, polyunsaturates are eluted before saturates of equal chain length and shorter-chain before longer-chain fatty acid esters.

Representative chromatograms of fatty acid methyl esters, derived from the three species most frequently used in dietary supplements, are shown in Fig. 18. In the chromatograms, the number and types of fatty acid methyl esters appear to be the same. However, ratios of the



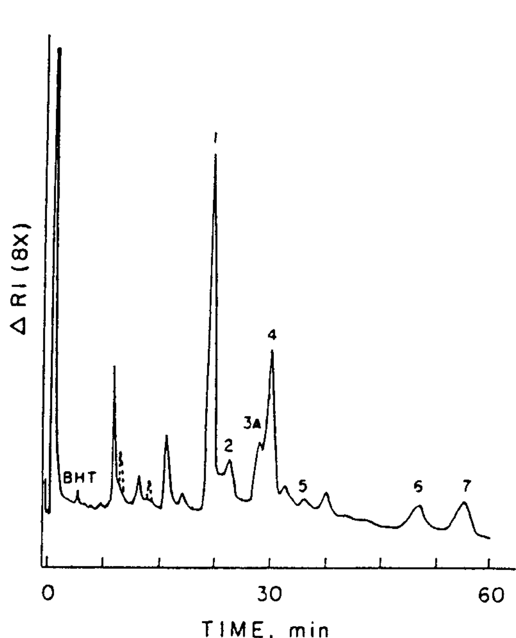


Fig. 18 RP-HPLC of methylesters derived from three different fish oil sources: sardine (A), menhaden (B), and cod liver (C). Identified fatty acid methyl esters in order of their elution: 1. C20:5ω3; 2. C14:0; 3. C16:1ω9; 4. C22:6ω3; 5. C18:2ω6; 6. C16:0; 7. C18:1ω9. BHT is eluted right after the void volume; remaining peaks have not been positively identified. Mobile phase: acetonitrile/THF/water (9:5:11) at 2.0 ml/min; stationary phase WHATMAN ODS-3 RAC II (100 × 4.6-mm ID); detection, refractive index at ambient temperature.

amounts of methyl esters of EPA to DHA differ according to the species of fish. The elution order of the identified FAMEs is: $C_{20:5\ \omega3} > C_{14:0} > C_{16:1\ \omega7} > C_{22:6\ \omega3} > C_{18:2\ \omega6} > C_{16:0} > C_{18:1\ \omega9}$.

Figures 19, 20, and 21 show the ethyl esters, *n*-butyl esters, and benzyl esters. The deterioration in peak shape with increased retention may be due to the decreased solubility of the esters in the mobile phase as well as to the band broadening that occurs with increased retention time.

D. Monohydroxy Fatty Acids

Complete resolution and quantitation by HPLC of mixtures of monohydroxy fatty acids containing positional isomers of various chain lengths is a technical problem, because their separation occurs according to both chain length and the position of the hydroxyl group on both reverse-phase and silica adsorption HPLC columns.

Bandi and Ansari (50) reported the use of *O*-(*p*-nitrobenzyl)-*N*,*N*′-(diisopropyl)-isourea (PNBDI) for the formation of PNB esters of monohydroxy fatty acids and the HPLC analysis of the derivatives on both reverse-phase and adsorption HPLC columns. They found that separation of positional isomers of PNB esters of monohydroxy fatty acids could be carried out successfully

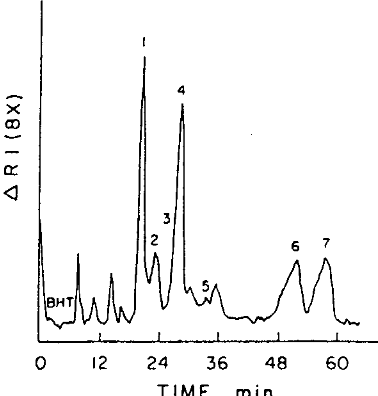


Fig. 19 RP-HPLC of ethyl esters of long-chain fatty acids derived from sardine oil. Identified fatty acid ethyl esters in order of their elution: 1. C20:5ω3; 2. C14:0; 3. C16:1ω7; 4. C22:6ω3; 5. C18:2ω6; 6. C16:0; 7. C18:1ω9.

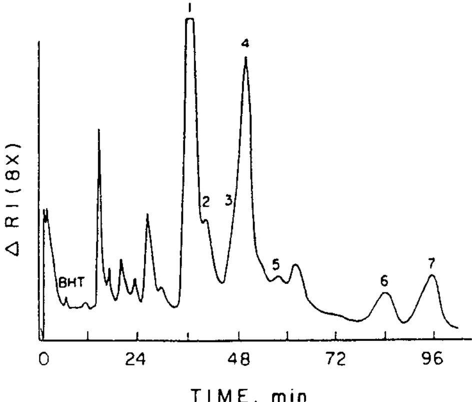


Fig. 20 RP-HPLC of *n*-butyl esters of long-chain fatty acids derived from sardine oil. Identified fatty acid *n*-butyl esters in order of their elution: 1. C20:5ω3; 2. C14:0; 3. C16:1ω7; 4. C22:6ω3; 5. C18:2ω6; 6. C16:0; 7. C18:1ω9.

on adsorption (silica) HPLC columns, but resolution according to chain length by RP-HPLC was only partially achieved, because separation of positional isomers occurred in addition to the separation according to chain length. In addition, UV-absorbing components of the PNBDI reagents interfered with adsorption chromatography, because the polarities of the hydroxy fatty acid PNB esters were similar to those of the PNBDI components.

To improve the purification by adsorption TLC and to reduce the separation of positional isomers by RP-HPLC, Bandi and Ansari (51) reduced the polarity of the hydroxyl groups forming *tert*-butyldimethylsiloxy derivatives of PNB esters of monohydroxy fatty acids (PNB-TBDMS-OHFA) (with *tert*-butyldimethylimidazole in dimethylformamide).

They chose the TBDMS derivatives for reducing the polarity of hydroxyl groups of hydroxy fatty acid PNB esters because the TBDMS derivatives were resistant to hydrolysis, stable in common organic solvents, and changed the adsorption TLC and RP-HPLC retention of PNB hydroxy fatty acids more than smaller siloxy groups and because the TBDMS groups could easily be removed by fluoride ions (sodium fluoride or tetra-*n*-butyl ammonium fluoride) to free

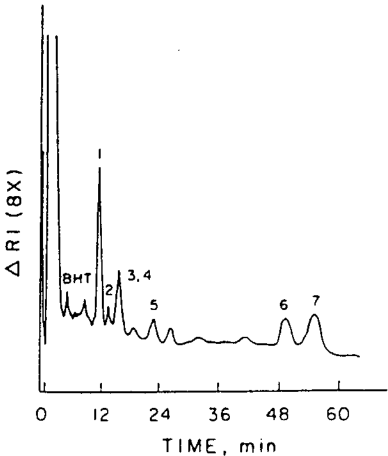


Fig. 21 RP-HPLC of benzyl esters of long-chain fatty acids derived from sardine oil. Identified fatty acid benzyl esters in order of their elution: 1. C20:5ω3; 2. C14:0; 3. C16:1ω7; 4. C22:6ω3; 5. C18:2ω6; 6. C16:0; 7. C18:1ω9.

```
              CH3
               |
        CH3 — C — CH3
               |
        CH3 — Si — CH3
               |
               O                O
               |                ||
CH3 — (CH2)5 — CH — (CH2)10 — C — O — CH2 —⟨benzene ring⟩— NO2
```

p-Nitrobenzyl 12-*tert*.-butyldimethylsiloxyoctadecanoate (12h-C_{18} PNB-TBDMS-PHFA).

Chemical Structure 3

the hydroxyl groups for separation of positional isomers by adsorption HPLC (see Chemical Structure 3).

The reverse-phase analysis was carried out on a SUPELCOSIL LC-18, 3-μm particle size, 150 × 4.6-mm ID column (solvent system: A, acetonitrile; B, acetonitrile-tetrahydrofuran-chloroform (50:27.5:22.5); linear gradient from 30% to 100% of B in 70 min, flow rate: 0.5 ml/min) (Fig. 22). The upper part of Fig. 22 shows that various chain lengths (C_{12} to C_{24} with one-carbon increment) of PNB-TBDMS-OHFA separated well enough in 30 min for effective recovery of the components by an absorbance slope–detecting fraction collector-detector combination. The separation of the positional isomers present in the used mixture was only minor, and it did not interfere with the fractionation according to chain length.

E. Dimer and Trimer Acids

While the analysis of the composition products from the thermal and oxidative treatment of fats and oils has been widely studied, systematic studies concerning separation, determination, and elucidation of the chemical structure of the higher-molecular-weight materials such as dimers and

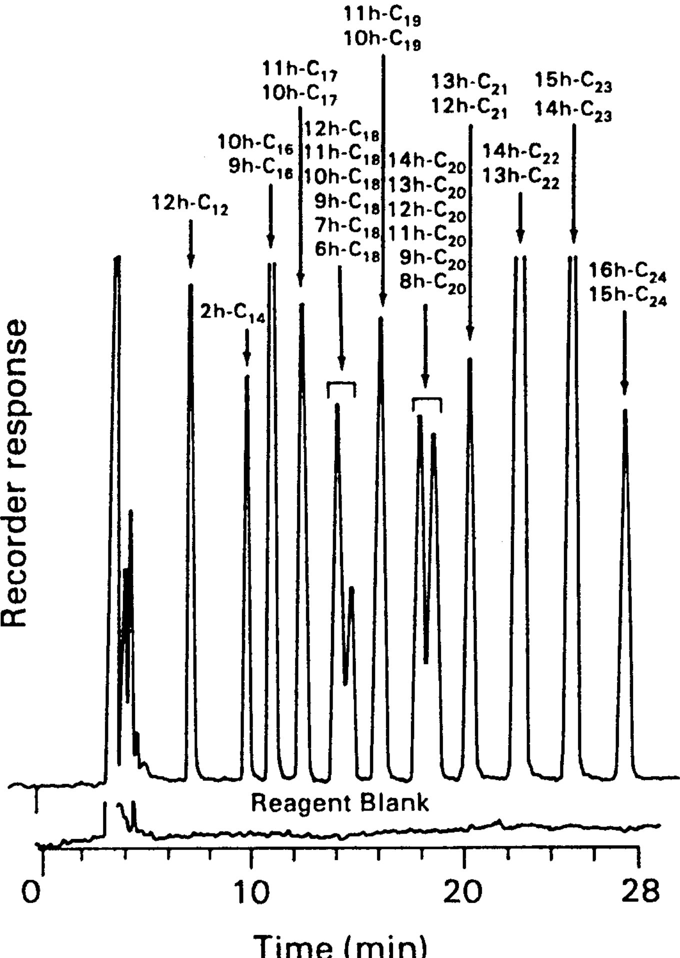


Fig. 22 RP-HPLC of PNB-TBDMS-OHFA. A SUPELCOSIL LC 18 (3 μm), 15 cm × 4.6-mm ID column was used. Solvent system: A. acetonitrile; B. acetonitrile/tetrahydrofuran/chloroform (50:27.5:22.5). A linear gradient from 30% to 100% of B in 70 min was used. Flow rate was 0.5 ml/min at ambient temperature. Detector was set at 265 nm.

higher polymers formed during thermal oxidation reactions are far from complete. Exposition of fats and oils to high temperatures may lead to significant changes in their chemical composition, especially when the fat is rich in polyunsaturated fatty acids. The generated compounds include polymerization products possessing the ability to cause adverse physiological effects such as appetite and growth depression, diarrhea, oily and mattered coats, lower basal metabolism, lower body temperatures, and higher mortality.

The analysis of polymerized fatty acids (also termed dimer acid) for neutrals, monomers dimer, and polymer has been approached by means of many different techniques, but a truly satisfactory solution remained elusive until 2 or 3 years ago. Many early analyses were done by distillation of the methyl esters, but the integrity of the sample is compromised when subjected to severe distillation conditions. Chromatographic methods have the greatest promise for this analysis, and some success has been realized in applying these techniques.

The isolation and structure elucidation of dimers formed as the result of thermal oxidation of fats during deep frying has been of great interest. However, the compositional determination of this fraction from used or heated fats and oils has been neglected because of the difficulties encountered during the isolation of dimers from heated fats and the complexity of the structures present within this fraction.

The presence of polar dimers in thermally oxidized corn oil at 200°C was reported by Perkins and Kummerow (52). Dimers and higher polymers isolated had a molecular weight range of 692–1,600 daltons; they were noncyclic and of high oxygen content, and they contained unsaturation difficult to remove by hydrogenation. The oxygen present in the fractions was shown to be in the form of hydroxyl and carbonyl groups. Similarly, Sahasrabudhe and Farn (53) presented results of the effect of heating corn oil in air at 200°C. Dimeric and polymeric products were isolated from the heated oil; and evidence was presented for the presence of hydroxy acids in the saponified polymeric fractions.

Firestone et al. (54) heated cottonseed oil at 205° and 225°C in the presence of air. The dimers and higher polymers that were isolated contained moderate amounts of carbonyl and hydroxyl groups and unsaturation difficult to remove by hydrogenation. However, the dimers after bromination-dehydrobromination and oxidation absorbed in the UV region at 250–260 nm and 270–280 nm, indicating the presence of cyclic structures. In addition, Barrett and Henry (55) presented chromatographic and spectral evidence for the presence of dimers in cottonseed oil that had been used for frying.

Ohfuji and Kaneda (56) oxidized soybean oil at 295°C for 12 h in the presence of nitrogen or air. The dimeric fraction had a molecular weight of 586 daltons and contained several functional groups, such as carbonyl, hydroxyl, and epoxide groups. Treatment with sodium borohydride and hydroiodic acid revealed no peroxidic or ether linkages. The content of conjugated dienes was low, but the strong UV absorption at 223 nm suggested ketone conjugates with double bonds. Furthermore, this dimeric fraction was proven to be very toxic to mice.

Zeman and Scharmann (57) reported the presence of 0.3% nonpolar dimers composed of diunsaturated bicyclic and tetraunsaturated acyclic structures in peanut oil subjected to thermal and oxidative action. Perrin et al. (58) analyzed samples from two different peanut and sunflower oils oxidized by deep fat frying to a stable foam formation. They reported the presence of dimers at levels between 12.1% and 12.9% of the oxidized mixtures. Peanut oil oxidized by deep fat frying before and after stable foam formation yielded 7.8% and 14% dimers, respectively. Gere et al. (59) reported nonpolar and polar dimeric triglycerides in sunflower oil used in deep fat frying.

Kupranycz et al. (60) reported the presence of dimeric triglycerides in various butterfat samples oxidized at 185°C in the presence of air for 8 and 16 h. Nonpolar and polar dimeric TGs were found in lard samples used in deep fat frying (61).

The results of dimer formation in 10 different oils oxidized to the level of stable foam formation were presented by Perrin et al. (58). The dimer content was found to be between 1.6 and 17.5% of the nonvolatile decomposition products, with 1.6% for the beef tallow and 17.5% for the soybean oil sample, respectively.

Thermal as well as oxidative dimers have also been isolated and characterized from pure fatty acid and TGs oxidized under simulated deep fat frying conditions. These model systems have been employed in order to simplify and control the various parameters affecting the thermal-oxidative reactions and to facilitate the structure elucidation of the decomposition products.

Michael et al. (62) heated methyl linoleate diluted with an equal weight of methyl laurate at 200°C for 200 h in the presence of air. The dimer was isolated from the reaction mixture and further separated into nonpolar and polar fractions. Analytical data for the nonpolar dimers was consistent with the cyclic DIELS-ALDER structure, whereas the polar dimer material was non-

cyclic, containing hydroxyl, peroxy, and/or carbonyl groups. Perkins and Wantland (63) subjected pure 1-linoleyl-2,3-distearin to thermal oxidation at 200°C and presented evidence for the presence of cyclic and noncyclic dimeric species in the reaction mixture.

Chang et al. (64) presented studies on the nonvolatile decomposition products from pure trilinolein, triolein, and tristearin produced under simulated deep fat frying conditions at 185°C for 74 h. Chromatographic, chemical, and spectrometric analysis indicated the presence of dimers in all three oxidized TG mixtures. A cyclic carbon-to-carbon linked dimer and a noncyclic dimer that was formed through carbon-to-carbon linkage and contained two hydroxyl groups per molecule were identified from the oxidized trilinolein mixture. The cyclic and noncyclic dimers presented 4.9% and 2.8% of the treated trilinolein, respectively. Triolein also yielded two different noncyclic dimers joined by carbon-to-carbon linkages. Each dimer constituted 1.36% of the treated triolein: one was determined as the dimer of methyl oleate and the other as the dimer of methyl oleate with one carbonyl group per molecule. Finally, tristearin yielded noncyclic dimers joined by carbon-to-carbon linkages and constituted 0.7% of the oxidized tristearin.

Size-exclusion chromatography (SEC) has been used by several workers to separate monomer, dimer, and trimer. Bartosiewics (65) reported the determination of monomer and dimer acids with cross-linked polystyrene beads but did not attempt quantitative analysis.

Chang (64) used a porous styrene/divinylbenzene gel to determine small amounts of fatty acid dimers in tall oil. Hase and Harva (66) separated the monomeracid methyl esters from dimers and higher oligomers using a modified dextran gel SEPHADEX LH-20.

Inoue et al. (67) accomplished a GPC resolution of the methyl esters up to and including tetramer acids in 24 h and obtained evidence of the presence of pentamer and higher oligomers. Using LH-20 and BIO-BEADS SX-1, Perkins et al. (68) separated both fatty acid and TG polymers from heated corn oil.

Aitzetmuller (69,70) indicated that SEC can be used as a measure and indication of the extent óf heating and polymerization of heated fats and oil. Harris et al. (71) achieved SEC separation of monomer, dimer, and trimer acids within 3 h, and quantitation was possible with the use of heptanoic acid as intemal standard.

El-Hamdy (72), using BIO-BEADS SX-1 and SX-2, fractionated thermally oxidized olive oil into six fractions, with molecular weights ranging from 300 to more than 10,300 daltons. This technique separates the oligomers by size but does not give information about the neutrals content or functionality of the oligomer. Paper chromatography and TLC yield separation of dimer acid, but again quantitation is difficult. Column chromatography of the free acids is slow and imprecise.

Gas chromatography of dimer methyl esters at high temperature on a short column also has been reported (73). The separation of trimer from dimer was incomplete in this method, and it is likely that some sample degradation occurs during the analysis because of the high temperatures involved. More promising approaches have been developed with the application of HPLC to this problem (74,75).

Veazey (76) utilized a reverse-phase separation employing infrared detection of the carbonyl species. This separation is similar to the SEC methods, in that monomer, dimer, and polymer are eluted in order of size. This method does have the advantage of separating neutral species. There is also some partial separation of the dimer-sized species.

A technique utilizing normal-phase separation was reported that yields good separation and quantitative measurement of the dimer acid species (77). This method incorporates a preparative separation using a UV detector as an indicator for the elution of neutral, monobasic, dibasic, and polybasic fractions. The fractions are collected manually, the solvent is removed, and the residue weighed to yield weight percent data. The normal-phase separation employed is advanta-

geous, because in contrast to SEC, the separation is on the basis of functionality and not size (Figs. 23, 24). Thus the monobasic fraction contains monomer-sized molecules as well as mono-decarboxylated dimer-sized molecules (Fig. 25). This is useful information when the dimer is to be used in polymerization reactions where the monobasic species act as chain stoppers.

The accuracy and utility of the normal-phase separation is evident, but the gravimetric method of quantitation has several major drawbacks:

The gravimetric method specifies using preparative chromatographic columns, which must be prepared rather than obtained commercially.

During the separation, the analyst must observe the UV response for clues to the proper time to make gradient and fraction changes. The judgment of the proper time to make these changes may vary from analysis to analysis and from analyst to analyst.

The time invested in each analysis is considerable; typically only three or four samples per day can be analyzed using this technique.

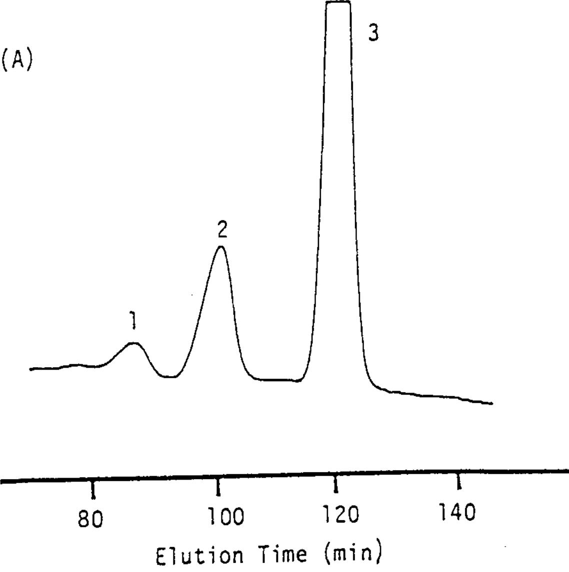
Veazey (76) has investigated a novel quantitation alternative utilizing a commercially available analytical-scale HPLC column with a flame ionization detector (FID). Using this detector and a normal-phase HPLC separation, he has obtained quantitative results for analyses of crude dimer and final dimer products. The samples were separated on a 5-μm SUPELCOSIL LC-Si column 250 × 4.6-mm ID, thermostatted at 30°C (protected by a 50-mm guard column packed with 5-μm silica). The mobile phases consisted of 99.3% cyclohexane/0.5% isopropyl alcohol/0.2% glacial acetic acid (solvent A) and 89.8% cyclohexane/10.0% IPA/0.2% glacial acetic acid (solvent B). The flow rate was 1 ml/min. The elution program was a multistep gradient as follows: 2% B at time zero to 5% B in 6 min, hold 3 min, to 48% B at time 15 min, then to 59% B at time 22 min. Reverse to 2% B in 2 min and allow baseline to settle before next injection (3 min).

The FID uses a continuous quartz braid to transport the column effluent (sprayed onto the braid through a 0.1-mm orifice) through a solvent removal zone and into the analytical and cleaning flames. The FID flows were as follows: 140 ml/min H_2 and 400 ml/min air for the analytical flame; 300 ml/min H_2 and 150 ml/min O_2 for the cleaning flame. The oven temperature control was set to slightly less than mid-range, which yields a block temperature of approximately 140°C.

The chromatographic conditions specified produced a chromatogram in which, for Unidyme-18-dimer acid, there are four broad peaks corresponding to neutrals, monobasic, dibasic, and polybasic components. Each peak represents the elution of many isomers; for example, the dibasic peak is composed of linear, cyclic, and aromatic dimer, each in various geometric, structural, and conformational isomeric forms (the peaks are very broad as a result of the presence of these isomers).

A systematic study has been developed by Christopoulou and Perkins (78). They employed three different types of detectors (differential refractometer, variable wavelength detector set at 205 or 232 nm, and infrared detector at 5.72 μm) and three commercially packed columns (I, LC-Si, 250 × 4.6-mm ID, 5-μm particle size; II, LC-18, 250 × 4.6-mm ID, 5-μm particle size; III, LC-18, 150 × 4.6-mm ID, 5-μm particle size). The various mobile phases used were: system I, 1.5% isopropyl alcohol (IPA) in hexane; system II, acetonitrile/acetone (1:1); system III, acetonitrile (spectro); system IV, acetonitrile/methylene chloride (3:1). Columns I and II were used with solvent systems I and II, respectively, and refractometry was the mode of detection. Column III was used with solvent system III and UV detection at 205 or 232 nm, as well as with solvent system IV and infrared detection at 5.72 μm.

Attempts were first made in this study to separate dimers by normal-phase liquid chromatography. Silica gel absorption chromatography was first used as a separation method. Hexane, as the mobile phase, gave very long retention times for the various dimers, and no separation was



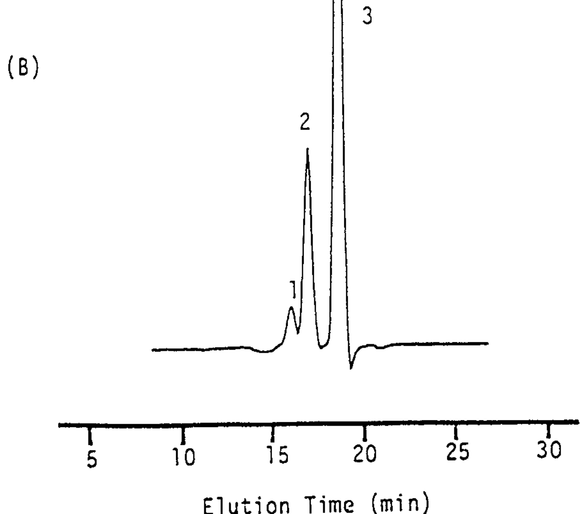


Fig. 23 HPSEC separation of standard mixtures. GPC (A) and HPSEC (B) separation of standard mixtures. For GPC (A) separation: Columns: two glass 109 cm × 12.5 mm ID packed with Bio-Beads SX2; Eluent: toluene at a flow rate of 1 ml/min. Detector: Refractive index; Injection volume: 100 μl of 50–100 mg solute/ml toluene. Ambient temperature for HPSEC (B) separation. Columns: LiChrogel Ps4 + LiChrogel Ps1 (each 25 cm × 0.7 cm ID); Eluent: toluene at a flow rate of 0.5 ml/min. Detection: refractive index; Injection volume: 20 μl. Sample conc.: 1.5 ml/min: Temperature: ambient: Peaks: 1, trimer (Empol 1014); 2, dimer (Empol 1010); and 3, monomer (methyl stearate) as the methyl ester. (From Ref. 77.)

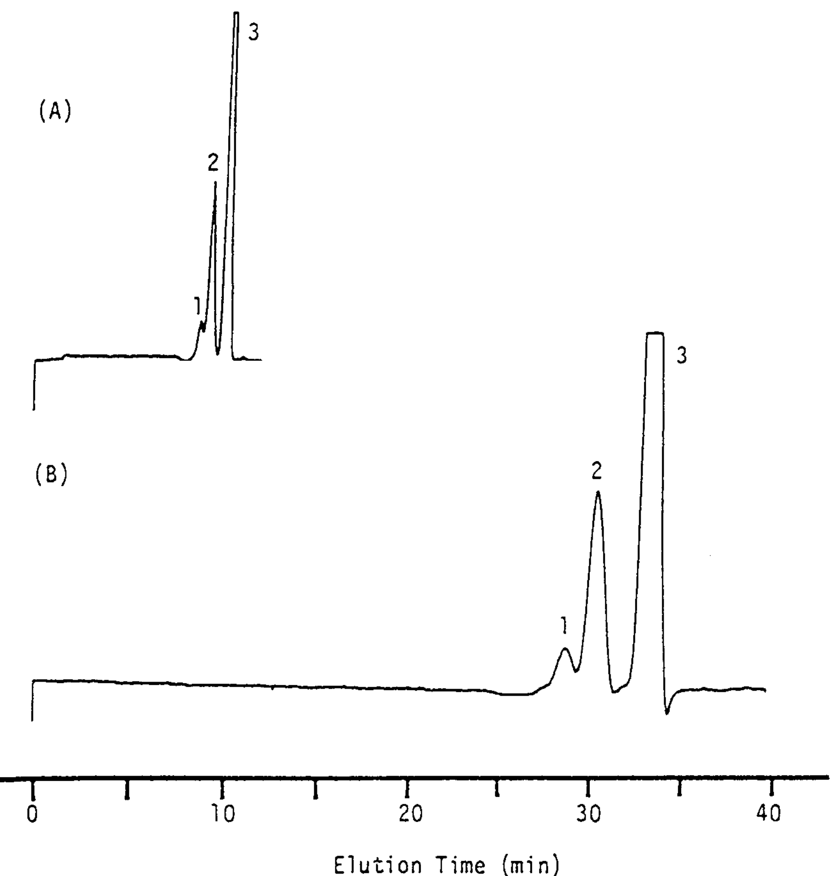


Fig. 24 Effect of solvent flow rate on the HPSEC separation of standard mixture. A, 1 ml/min and B, 0.3 ml/min. Chromatographic conditions and peak identification as in Fig. 23. (From Ref. 77.)

observed within either the polar or the nonpolar dimers. The addition of 1–3% IPA as a polarity modifier to hexane resulted in a considerable reduction of the retention times of all dimers, complete separation of polar and nonpolar dimers, and partial separation within each dimer group, with the best results obtained when the concentration of IPA was 1.5%.

More satisfactory separations were obtained when reverse-phase liquid chromatography was used. The separation of the standard dimeric mixture was carried out on an LC-18 column with refractometry as the mode of detection and acetonitrile/acetone (1 : 1) as the mobile phase (system II). Separation proceeded according to the polarity of the various dimers, and complete separation of all dimers, except those of the thermal dimer of methyl linoleate and the dehydrodimer of methyl oleate, were obtained at a flow rate of 0.5 ml/min. The resolution of the two unresolved peaks would be increased by using another LC-18 column in series, but a sacrifice in the analysis time would have to be made.

An increase in the polarity of the mobile phase produced by increasing the amount of acetone in the mobile phase resulted in a decrease in resolution within the polar and nonpolar dimers. On the other hand, a decrease in the polarity of the mobile phase by increasing the amount of acetonitrile resulted in the insolubility of the samples at concentrations required for refractive index detection (20–40 mg/ml), thus limiting the use of the mobile phase of polarity less than that of system II.

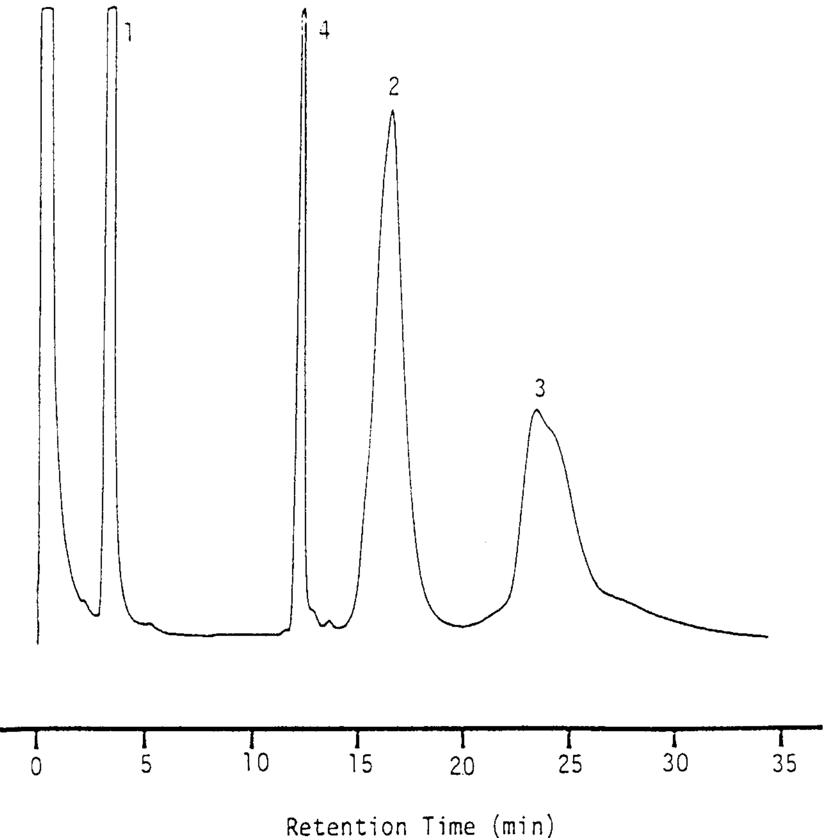


Fig. 25 GLC separation of standard mixture. Peaks: 1, monomer (Methyl stearate); 2, dimer (Empol 1010); 3, trimer (E,pol 1014) and 4, dotriacontane (internal standard). (From Ref. 77.)

Acetonitrile was used in the mobile phase when ultraviolet detection of the carbonyl group at 205 nm was used for the various dimers. Separation of the thermal dimer of methyl linoleate and the dehydrodimer of methyl oleate was again incomplete. The sample at concentrations as low as those required for UV detection (1–5 mg/ml) was sparingly soluble in the mobile phase, resulting in increased retention times. Finally, incomplete separation was obtained when various systems of acetonitrile/methyl chloride were used as the mobile phase in HPLC with infrared detection of the carbonyl group at 5.72 μm.

IV. TRIGLYCERIDES

Separation of TGs has been a general problem for many years. For several years such separations were carried out more or less by GC procedures. However, the GC method gives insufficient information to provide a complete TG composition of a complex mixture, even when combined with the total fatty acid composition or distribution.

These analyses are important in the natural oil industry for process and product quality control purposes. Also, at the research/development level, detailed triglyceride data might facilitate the understanding of TG biosynthesis and deposition in plant and animal cells.

Common triglycerides contain a series of even-chain-length saturated fatty acids and several corresponding series of unsaturated fatty acids. Such TGs may be separated by GC at high temperatures (350°C) by total fatty acid chain length (e.g., tripalmatin and tristearin have total fatty acid chain lengths of 48 and 54 carbons, respectively). Unsaturation has relatively little effect on such separations. In older liquid chromatographic methods, including TLC, it has been found that the introduction of one double bond is approximately chromatographically equivalent to shortening the alkyl chain by two carbon number atoms (e.g., tripalmitin and triolein have an equivalent carbon number of 48). The addition of silver ions to the chromatographic media (*argentation chromatography*) produces separations that are dependent on the number of double bonds but relatively independent of fatty acid chain lengths.

Reverse-phase liquid chromatography is now virtually the only method used in the analysis of the TG mixtures. The first paper on TG-HPLC analysis was published in 1975 by Pei et al. (81). Triglycerides were separated on a VYDAC reverse-phase (35–44 μm) column and eluted with methanol–water (9:1). Since Pei et al. first applied RP-HPLC to the separation of triacylglycerols, a number of reverse-phase systems have been developed as rapid and efficient resolution of complex triacylglycerol mixtures can be achieved.

Earlier work in the HPLC analysis of TGs used a differential refractometer as the detector; a number of papers have detailed isocratic systems combined with refractive index (RI) detectors, often with acetonitrile/acetone mobile phases. Although aqueous mobile phases were generally used with alkyl-bonded phase columns, due to the lipophilicity of TGs, water could not be used in the mobile phase for this particular application; therefore the mobile phases generally employed consisted of mixtures of acetone and acetonitrile and occasionally tetrahydrofuran, methylene chloride, or hexane (the conspicuous absence of water in the mobile phase prompted the term *nonaqueous reverse phase*, or NARP, to describe these systems).

Plattner (82) briefly examined the effects of solvent composition upon TG separations. Mixtures of acetone/methanol and acetone/acetonitrile were examined. In this work, acetone/acetonitrile mobile phases were reported to give less complex chromatograms of TG mixtures than acetone/methanol mixtures.

Pauls (83) examined the effect of the composition of binary solvent mixtures upon the selectivity and resolution of olive oil TG components. Separation factors (α values) and resolution were calculated for the linoleyldiolein (LOO)-linoleyl-palmitylolein (LPO) and triolein (OOO)-palmityldiolein (POO) pairs in olive oil. Five strong solvents (isopropanol, dichloromethane, chloroform, tetrahydrofuran, and acetone) as well as two weak solvents (methanol and acetonitrile) were employed.

The technique of time normalization was employed in order to compare the effect of varying solvent composition upon TG resolution under conditions of a fixed analysis time. In time normalization, two variables are varied simultaneously so that the retention time of the last eluting component remains fixed. Thus all comparisons are carried out within the same analysis time. Under these conditions, changes in resolution could be related directly to changes in solvent properties. This approach allowed the selection of the optimal mobile phase yielding the greatest resolution. The two variables modified were the components comprising the binary mobile phase and the percentage of strong solvent in the binary mixture. Retention of the last component of interest, POO, was mantained at a k' of 7.5 by adjusting the amount of strong solvent. Plots of log k' against the percentage of strong solvent for all seven solvent pairs were found to be linear, and this allowed the prediction of the solvent composition yielding a k' of 7.5.

For studies on the relationship between log k' and the percentage of strong solvent, olive oil was dissolved in the appropriate strong solvent at a concentration of 50 mg/ml. For time-normalization studies, olive oil was dissolved in the mobile-phase mixture at this same concentration level whenever possible. In cases where olive oil was not soluble in the mobile phase, it was dissolved in the strong solvent. The column void time was determined by measuring the av-

erage retention time of THF, isopropanol, and acetonitrile. A mobile phase of THF/acetonitrile (35:65) at a flow rate of 2.0 ml/min was employed for this measurement. An average void time of 75.6 ± 1.1 s was obtained. Capacity factors, k', and separation factors, α, were calculated by standard procedures. Resolution, R, was calculated by:

$$R = \frac{2(t_2 - t_1)}{w_1 + iw_2}$$

where t_1 and t_2 are the retention times of the two components and w_1 and w_2 are the peak widths at the baseline. In cases where the peaks were too badly overlapped to allow measurement of the total peak width, the width of the back half of the peak was doubled.

A more fundamental expression for resolution is given by the following equation:

$$R = \frac{(N)^{1/2}}{4[(\alpha - 1)/\alpha][(k'/(k' + 1)]}$$

where N is the number of theoretical plates and k' is the capacity factor of the second peak.

Such solvent systems continued to be used even though the lack of solubility of triacylglycerols with carbon numbers greater than 46 in this mobile phase has been noted. The solvent gradients that would be required for optimum separations of complex triacylglycerol mixtures are not compatible with RI detection. Therefore, ultraviolet detectors have also been used, but the range of mobile phases is limited, since TGs absorb only in the far-UV range.

Singleton and Pattee (84) investigated the various parameters necessary to optimize conditions for the analysis of TGs using a UV detector at 210 nm. The parameters tested were (a) the effect of sample solvent on TG analysis; (b) the effect of mobile-phase composition on the sorption behavior of TGs; (c) the effect of sample load level on TG analysis; and (d) the temperature effects on TG analysis.

The sample diluent affects the solute dispersion. If we consider the effects of three different diluents (hexane, chloroform, and acetone) on the chromatographic behavior of a TG mixture on RP columns using, for example, acetonitrile and ethanol as the mobile phase, we can see that the TGs dissolved in hexane provided only a minute chromatographic trace, whereas dissolution in chloroform yielded excellent detection and resolution. These results can best be explained by invoking the solvophobic theory of Horvath and Melander (85).

According to this theory, a solute molecule can be brought into solution by making an appropriate hole in the solvent followed by a reduction in free volume caused by van der Waals forces and electrostatic interactions. The energy required to make such a cavity in the solvent is calculated from the surface area of the solute molecule and the surface tension of the solvent. The physical properties of chloroform (solute diluent) lowers the energy level required to make a cavity in the mobile phase, which allows the solute molecules to interact with the mobile phase. On the other hand, hexane, because of its physical properties, cannot lower the energy enough to bring about dissolution of the solute molecules into the mobile phase. Acetone, the most polar of the TG diluents, results in reduced detection and resolution compared with the chloroform-dissolved TGs. The high dielectric constant of acetone apparently resulted in less reduction of the solute cavity size and therefore less solvent–solute interactions.

To determine the effect of mobile-phase composition on the sorption behavior of TGs on reverse-phase columns, two mixtures were employed: acetonitrile/ethanol (80:20) and acetonitrile/methanol (80:20). A very rapid analysis resulted, with excellent peak shape and adequate resolution, when ethanol was used as the secondary solvent. Substituting an equal amount of methanol for ethanol resulted in increased solute retention, poor detector response, and asymmetrical peaks. Methanol forms a monomolecular layer on octadecyl-derived silica, which may explain the increase in solute retention caused by methanol. Also, the use of methanol would

increase the hydrophobicity of the mobile phase, thus decreasing solute–stationary phase interactions.

When acetonitrile/methanol was used as the mobile phase, the concentration of methanol had to be increased to 50% before the TGs could be eluted in a reasonable length of time; when ethanol was used as the polar organic modifier with ACN, resolution and detection were improved and only 20% ethanol was required.

Temperature is another parameter that can be used for optimal analysis on RP columns. The overall resolution of a particular solute matrix can be improved and analysis time can be reduced because temperature affects every term in the resolution equation:

$$R = \frac{[k'/(1 + k')](\alpha - 1)N^{1/2}}{4}$$

where k' is the capacity factor, α is the column selectivity, and N is the number of theoretical plates.

Increasing the temperature from ambient to 60°C reduced the capacity factor and dramatically increased the number of theoretical available plates. Also, the viscosity of the mobile phase and the solute matrix was reduced, thus permitting more interaction of the solute molecules with the bonded phase.

Also, Jensen (86) studied the influence of column temperature upon the retention of TGs; retention was found to increase as column temperature decreased, and linear plots between log k' and $1/T$ were obtained.

The advances in column and instrument technology have significantly enhanced HPLC performance in recent years. Results comparing the effects of various column packings on TG separation by RP-HPLC were presented by El-Hamdy and Perkins (87). Six commercially packed columns produced by different manufacturers were used: PARTISIL ODS-1 and ODS-2 octadecyl-bonded silica of 10-μm partical size, ZORBAK-ODS octadecyl-silica of 6–7-μm diameter (250 × 4.6-mm ID), 5-μm octyl-bonded spherical silica LC-8, 5-μm methyl-bonded spherical silica LC-1, and a 5-μm octadecyl-bonded spherical silica LC-18 (150 × 4.6-mm ID). The mobile phase employed consisted of mixtures of methanol/acetone/isopropanol/acetonitrile ranging from 1:0:3:4 to 1:6:3:4. Triglycerides were solubilized in either THF or acetone at 100 mg/ml for each compound.

Improved separation of natural oil TGs using short columns packed with 3-μm alkyl bonded-phase particles was reported by Dong and DiCesare (88). The HPLC columns used were HS-3 high-speed columns packed with 3-μm C_{18} bonded-phase particle (100 × 4.6-mm ID) with a column void volume of ca. 0.8 ml and efficiencies in the range of 13,000–15,000 theoretical plates (measured under optimized conditions) and HS5 C_{18} columns (125 × 4.6-mm ID packed with 5-μm particles). Two detectors were used: a modified refractive index detector having an 8-μl flow cell and 0.007-in. ID inlet tubing and a variable-wavelength UV/visible detector.

Figure 26 shows the separation of a synthetic mixture of TG standards and an olive oil sample using two 3-μm C_{18} columns connected in series with NARP conditions. The mobile phase used was 7:3 acetone/acetonitrile at a flow rate of 2.5 ml/min. The analysis time was ca. 8 min. Attempts to duplicate this separation on several 5-μm C_{18} columns packed with different packing materials were not successful.

Figure 27 shows the separation of olive oil TGs using three 3-μm columns connected in series. The efficiency of this column system exceeds 40,000 theoretical plates. The mobile phase was unchanged, and the flow rate was reduced to 1.8 ml/min (the analysis time was increased by a factor of 2).

Figure 28 shows the separation of the same sample of olive oil using a different mobile phase (3:7, THF–acetonitrile) with UV detection at 220 nm. Compared to the separation in

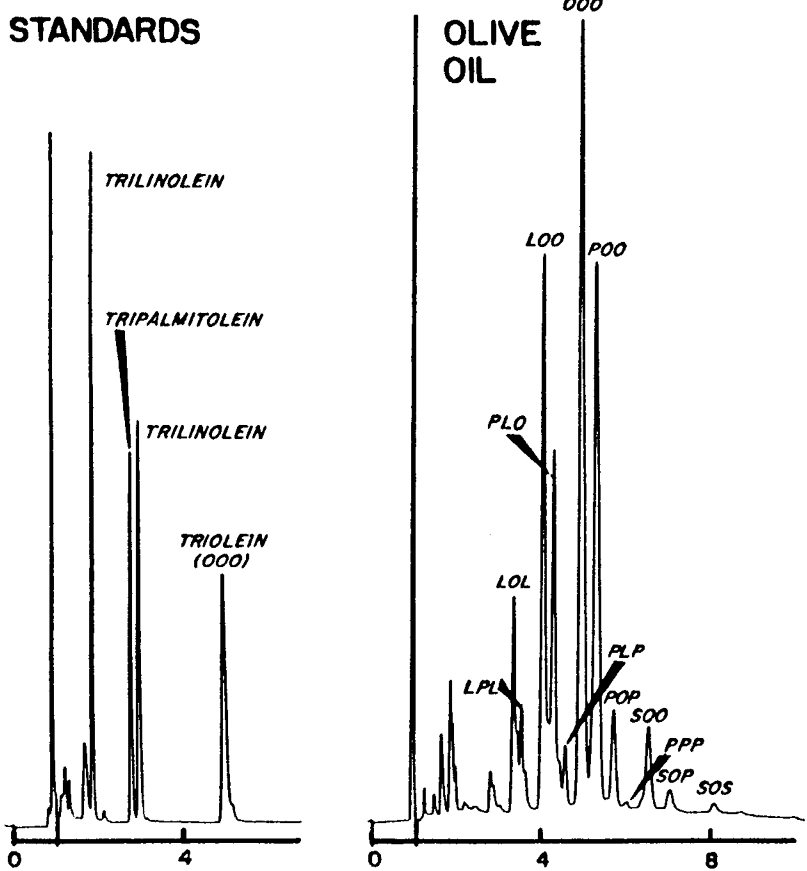


Fig. 26 HPLC separation of olive oil TGs using three 3-μm columns HS-3 (100 × 4.6-mm ID) connected in series. Mobile phase: acetone/acetonitrile (7:3 v/v) at a flow rate of 2.5 ml/min. LOL = linoleyloleyllinolenin; LPL = linoleylpalmityllinolenin; LOO = linoleyldiolein; PLO = palmityllinoleylolein; PLP = palmityllinoleylpalmitin; POP = palmityloleylpalmitin; PPP = tripalmitin; SOO = stearyldiolein; SOP = stearyloleylpalmitin; SOS = stearyloleylstearin.

Fig. 27, using an acetone/acetonitrile mobile phase and refractive index detection, the chromatogram in Fig. 28 appears remarkably similar for TGs of high retention times. However, peaks corresponding to the lower retention times appear to be enhanced, presumably due to the higher molar absorptivity of these species.

A. Influence of Silver Ions

Silver ion chromatography has, for several decades, been utilized for the separation of geometrical isomers of lipids, such as fatty acids and TGs (89–95). This type of separation is possible due to the formation of π-complexes between double bonds and silver ions.

Trans olefins form weaker π-complexes with silver ions than do cis olefins; hence cis-trans isomers can be separated using silver ion chromatography. Initially silver ions were used in conjunction with TLC, with silver nitrate being incorporated in the silica gel layer. In recent years the technique has been adapted to HPLC.

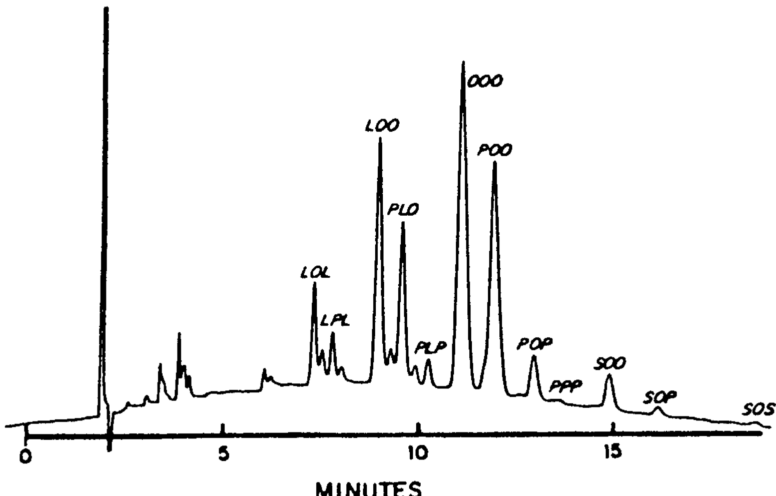


Fig. 27 High-resolution separation of TGs in olive oil. Column and mobile phase were as described in the text, except three columns were connected in series. Mobile-phase flow rate and inlet pressure, 1.8 ml/min, 28 MPa (4100 psi). Refractive index detector.

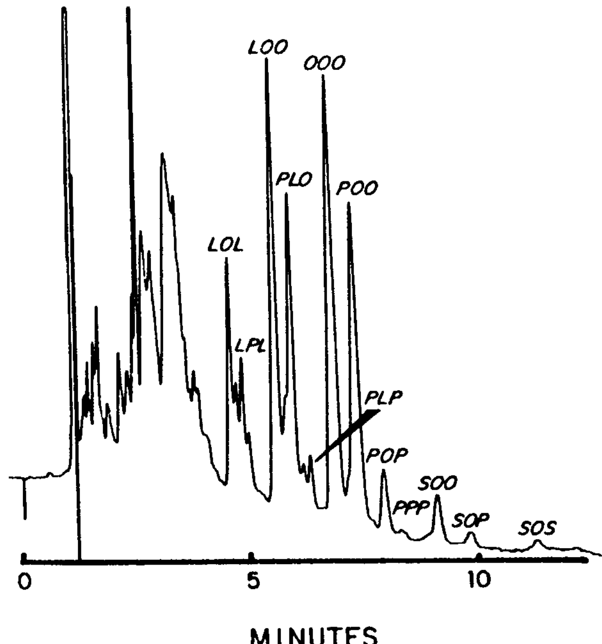


Fig. 28 Separation of TGs in olive oil using UV detection. Column as in the text. Mobile phase: tetrahydrofuran/acetonitrile (3:7 v/v) at 2.0 ml/min; 22-MPa (3200-psi) inlet pressure. UV detector at 220 nm.

Christie (96) has demonstrated such a separation of some geometrical isomers of TGs into the groups SSEl, SSM, SElM, and SMM (S = saturated fatty acid, M = monounsaturated fatty acid) using an ion-exchange column impregnated with silver ions.

Hammond and Irwin (97) have demonstrated a similar separation of StElSt and StOSt (St = stearic acid) on a silica column impregnated with silver ions. It is also worth mentioning in

conjunction with silver ion chromatography that many workers have shown the separation of symmetric and asymmetric isomers of mixed saturated and unsaturated fatty acids, e.g., SOS and SSO (98).

An HPLC chromatograph was used together with a mass detector (when required, a stream splitter (app. 10 : 1) was inserted between the column and the detector). A column (250 × 4.6-mm ID) of NUCLEOSIL 5SA was flushed with 1% aqueous ammonium nitrate solution at a flow rate of 0.5 ml/min for 1 h, then with distilled water at 1 ml/min for 1 h. Silver nitrate (0.2 g) in water (1 ml) was injected onto the column in 50-μl aliquots at 1-min intervals; silver began to elute from the column after about 10 min. Twenty minutes after the last injection, the column was washed with methanol for 1 h, then with 1,2-dichloroethane-dichloromethane (1 : 1) for another hour. The three solvent reservoirs contained the following: (A) 1,2-dichloroethane-dichloromethane (1 : 1); (B) acetone; and (C) acetone-acetonitrile (9 : 1). For linoleic acid–rich seed oils, gradients of A were employed to 50% A–50% B over 15 min, then to 50% B–50% C over a further 25 min and held there for 5 min. For linolenic acid–rich seed oils, C was changed to acetone–acetonitrile (4 : 1), and the flow rate was increased to 1 ml/min; gradients of A were utilized to 50% A–50% B over 10 min, then to 70% B–30% C over 20 more min, and finally to 100% C over another 30 min.

Figure 29 shows the separation of triacylglycerols from sunflower seed oil. In the analysis of linoleic acid–rich seed oils, well-shaped peaks are obtained, and excellent resolution of all the main fractions is achieved, with species containing linoleic acid being predominant.

In some commercial samples of these vegetable oils, there appeared to be some isomerized linoleic acid, and additional small peaks containing these components eluted in front of the corresponding main peaks on silver ion chromatography.

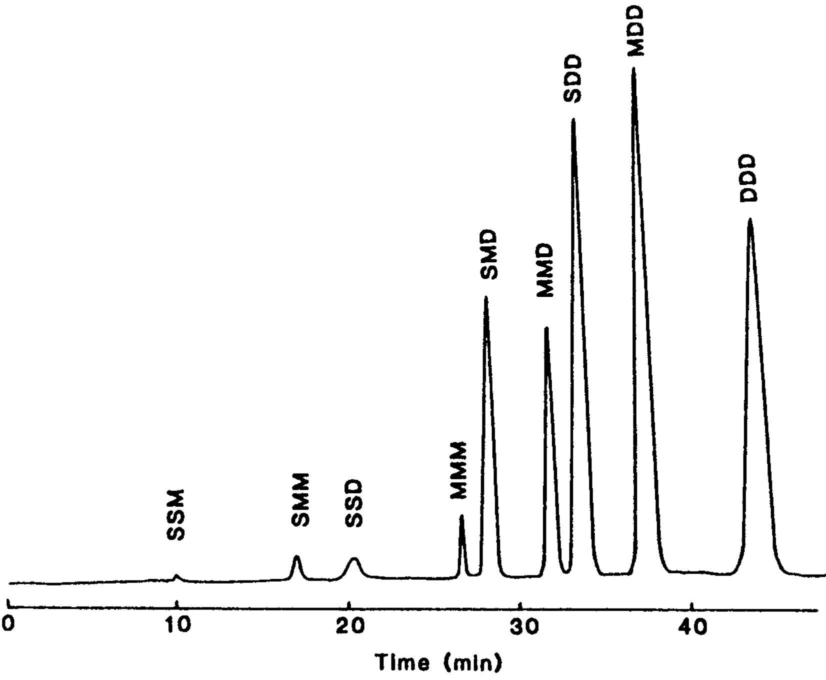


Fig. 29 Separation of triacylglycerols from sunflower seed oil by HPLC with a silver ion column and mass detection. For conditions see text. S = saturated fatty acid; M = monounsaturated fatty acid; D = diunsaturated fatty acid.

Neff et al. (99) applied Ag-HPLC with an FID to the quantitative detection of triacylglycerols (TAGs) of *Crepis alpina* oil (CrAO). The Ag-HPLC column was a Chromsphere Lipids (4.6-mm ID × 250 mm; 5 micron). All TAGs were eluted in 120 min by an isocratic mobile phase of 0.5% acetonitrile in hexane at a flow rate of 1.0 ml/min. The FID block temperature was set at 110°C, the cleaning flame hydrogen at 300 ml/min, and the oxygen flow at 175 ml/min.

Eleven TAG fractions were isolated. The TAG composition of these fractions was determined by transmethylation, by identification and quantitation of the resulting methyl esters by GC-FID and identified as S_3, CrS_2, CrSO, CrSL, CrOL, CrL_2, Cr_2S, Cr_2O, Cr_2L, CrL_2, and Cr_3 [S = saturated FA (palmitic, P, and stearic, St); O = monoene FA (oleic); L = dienoic FA (linoleic); and Cr = FA with both alkene and alkyne bonds (crepenynic)] (Fig. 30). The major TAGs are Cr_3 (34.5%), Cr_2L (33.8%, two isomers), and CrL_2 glycerols (16.3%), which together account for 85% of the TAGs. Further, the Ag-HPLC-FID system allowed 95% of all the TAGs to be absolutely identified, as opposed to the RP-HPLC-FID system, in which only 70% of the TAGs were identified.

Petersson et al. (100) described the separation of the major geometrical isomers of TGs in partially hydrogenated vegetable fats. It includes the separation of geometrical isomers, such as all combinations of oleic acid and elaidic acid. The proposed method was applied to partially hydrogenated soya bean oil and palm oil (both soya bean oil and palm oil were hydrogenated to a melting point of 41°C and referred to as Soya 41 and Palm 41, respectively). Samples were first separated by preparative HPLC into partition numbers (PNs). The PN fractions yielded were collected and evaporated to dryness at 60°C under a stream of nitrogen. Finally, the fractions were dissolved in benzene to give a concentration of ca. 20 mg/ml.

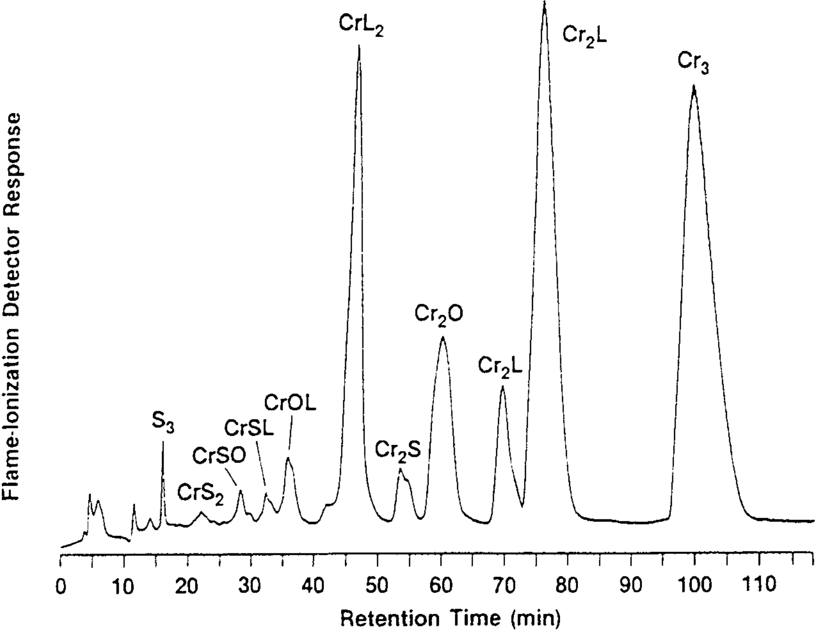


Fig. 30 Silver ion high-performance liquid chromatography (Ag-HPLC-FID) with flame ionization detector (FID) analysis of the triacylglycerols of chromatographed *Crepis alpina* seed oil. Ag-HPLC-FID conditions: 0.5-mg sample; 5-micron Chromspher Lipids column (Chrompack International, Middelburg, The Netherlands) (4.6 × 250 mm); mobile phase 0.5% acetonitrile in hexane (v/v); flow rate 1.0 ml/min; FID. Chromatogram peak triacylglycerol fatty acid abbreviations: S, saturated (palmitic and stearic); O, oleic; L, linoleic; and Cr, crepenynoic fatty acids.

The HPLC separation into geometrical isomers was performed on a 5-μm particle size Hibar RP C_{18} LiChrosorb column (250 mm × 4-mm ID). The mobile phase was methanol-2-propanol (3:1; v/v), with $AgNO_3$ in a concentration of 0.085 M, at a flow rate of ca. 1 ml/min. The major PN fractions, at a concentration of 20 mg/ml benzene, were injected in volumes of 5–10 μl.

The aim of the preparative fractionation was to divide the TGs into well-defined groups, each containing a moderate number of TGs. Three procedures were considered for this purpose: separation according to PN, separation according to the number of double bonds (NDB), and separation according to carbon number (CN). However, since CN analysis is performed by GC, this procedure is not suitable for fractionation. Separation according to NDB can be performed by separation of brominated TGs. However, the bromination reaction produces irreversibly chemically altered compounds that become identical for both cis and trans configurations, so this procedure was therefore also excluded. The third procedure, separation according to PN, proved to be the best solution. It follows the following simple relation:

$$PN = CN - 2 \cdot NDB$$

By way of example, PN separation of Soya 41 is illustrated in Fig. 31A. Since the PN48 fraction from Soya 41 includes the geometrical isomers of at least nine different TGs, the PN peak is broadened. Among others, it includes the four geometrical isomers OOO, OOEl, OElEl, and ElElEl, which are partly separated as pure standards (Fig. 31D). For purposes of comparison, Fig. 31C shows the resolution of OOO and ElElEl only. The triunsaturated OOO and the saturated PPP (Figure 31B) are nearly separated at baseline, but, since Soya 41 includes several TGs with retention times between those of OOO and PPP, the PN48 peak looks like the peak of a single component. This is also due to the relative proportions of the different TGs within the peak. If the relative proportions are altered, the peak may become asymmetrically shaped.

Because the retention times of the different TGs within a PN differ, it is important to collect the whole peak at fractionation. One total fractionation, with an elution time of ca. 1.5 h, will provide enough material for the subsequent separation procedure based on argentation-HPLC (utilizing silver ions in the mobile phase), in order to separate geometrical isomers.

Several points had to be taken into consideration when choosing the mobile phase. First, the mobile phase had to dissolve the silver nitrate properly while at the same time being sufficiently nonpolar for the elution of saturated TGs. Second, the mobile phase had to be inert to silver ions so that no reaction would take place. Third, the refractive index of the mobile phase had to be different from that of the TGs, since TGs were to be detected using a refractive index detector. For these reasons, mobile phases such as propionitrile, acetonitrile, methanol, and 2-propanol were tested. The mobile phase that gave the best separation results was methanol-2-propanol (3:1; v/v) with dissolved silver nitrate.

As we said before, the silver ions act in two ways. They act as complexing agents with double bonds (π-complexes), and they also increase the polarity of the mobile phase. From Fig. 32 it can be seen that a saturated TG (PPP) has a longer retention time when the silver concentration is increased. The silver ions cannot form π-complexes with a saturated compound, and this longer retention time must be attributable to the increased polarity of the mobile phase. Unsaturated TGs are, of course, also affected by the polarity of the mobile phase. The formation of π-complexes is also illustrated in Fig. 32, from which it can be seen that PPP-StElEl and ElElEl-StOO are eluted in inverted order when the silver concentration is increased. When the elution order of PPP and StElEl is inverted in this way, the trans double bonds must have formed a complex with silver ions, for otherwise, with only a change in the polarity of the mobile phase taking place, no inversion would have resulted. The inversion in the order in which ElElEl and StOO are eluted demonstrates that there is a difference in the strength of silver complexes with cis versus trans double bonds. StOO with only two cis double bonds is so strongly affected by the increase

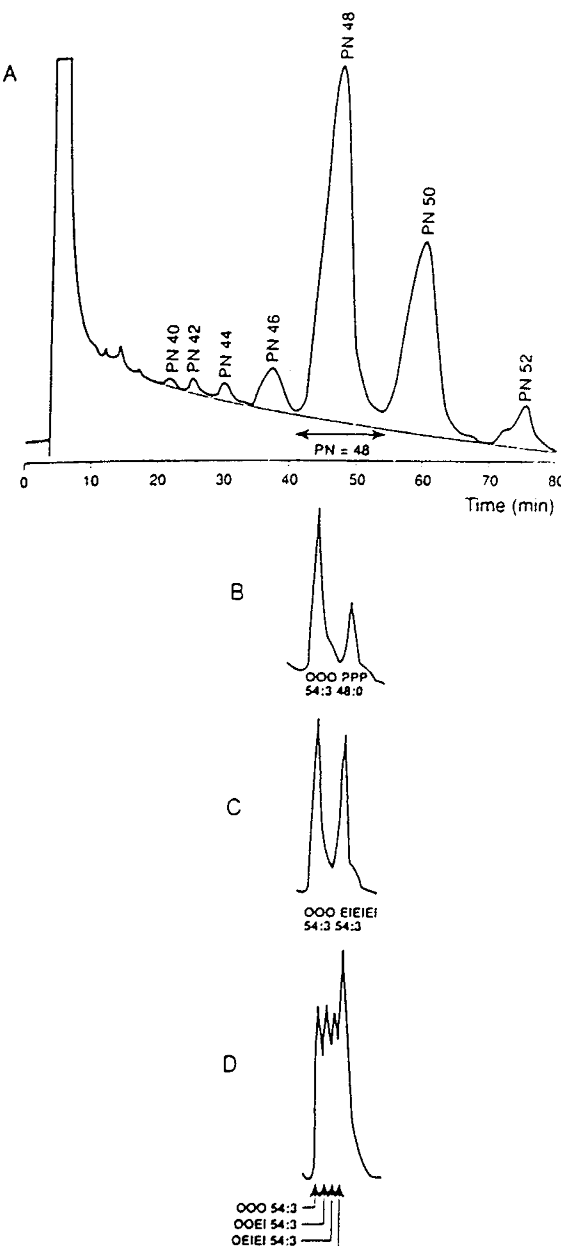
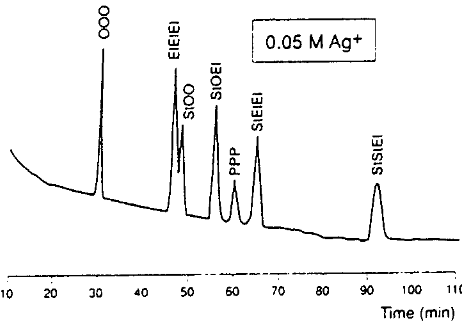


Fig. 31 PN separation of Soya 41 and illustration of the separation of different types of TG isomers included in the PN48 peak. Conditions: column Hibar RP C_{18} (250 mm × 4-mm ID; mobile phase methanol–acetone (3:2 v/v). Ambient temperature, flow rate 1 ml/min.

in the silver concentration that it elutes before ElElEl, in which there are *three* trans double bonds. Since, due to the increased polarity of the mobile phase, the stearic acid in StOO contributes to an increase in retention time, it is clear that the silver complexes with cis double bonds are stronger than those with trans double bonds. This is probably due to a sterical hindrance from the trans double bonds.



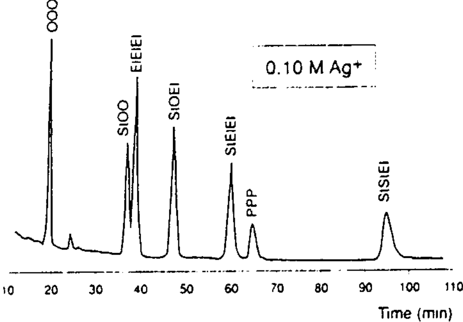


Fig. 32 Influence of silver nitrate concentration on retention times of different TGs, including geometrical isomers. Conditions: Column Hibar RP C_{18} LiChrosorb (250 mm × 4-mm ID); mobile phase methanol-2-propanol (3:1 v/v); system temperature 20°C; flow rate 1 ml/min.

The resolution of unsaturated and saturated TGs with the same PN is strongly increased in the subsequent separation using silver in the mobile phase, compared with the initial fractionation. For example, the separation of OOO and PPP increased dramatically. Consequently, an extended space between the first and the last eluted component, within a PN, becomes available for the geometrical isomers of that particular PN.

Particle number, CN, and NDB remain constant within a series, and only the geometrical configurations vary. A transition from oleic acid with a cis configuration to elaidic acid with a trans configuration contributes to a major change in retention time, due to the effect of the silver ions in the mobile phase. As a result, resolution between isomers that differ in their geometrical configuration by just one double bond is excellent. Also, for TGs containing palmitic acid (P), the resolution between isomers that differ in their geometrical configuration by just one double bond is excellent.

The change from one oleic acid moiety in OOLc,c to one elaidic acid moiety (OElLc,c) affects retention time to the same extent as a corresponding change from linoleic acid with both double bonds in cis configuration in OOLc,c to linoleic acid with both double bonds in trans configuration (OOLt,t).

A nonaqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) with refractive index (RI) detection was described and used for palm olein and its fractions obtained at 12.5°C for 12–24 h by Swe et al. (101). The objective of their research was to find the optimum separation for analysis of palm olein triglycerides by NARP-HPLC, and to find a correction factor to be used in calculating CN and fatty acid composition (FAC). The NARP-HPLC method used to determine the triglyceride composition was modified from the method of Dong & DiCesare (88). Palm olein was melted completely at 70°C in an oven for 30 min prior to crystal-

lization. An oil sample weighing 50 ± 0.005 g was placed in each bottle and crystallized in a water bath that was placed in a cold room with the temperature set at 12.5°C. The crystals that formed were collected between 12 and 24 h at 3-h intervals. The crystals were separated from the liquid oil by centrifugation at 3000 rpm for 3 min at 12.5°C. Triglycerides were separated on a single commercially packed (250 × 4.0-mm ID) RP-18 (Merck, Darmstadt, Germany) column with a particle size of 5 μm. The mobile phase was a mixture of acetone–acetonitrile (63.5 : 36.5), and the flow rate was set at 1 ml/min. The column temperature was set at 35°C. The injection volume was 10 μl of 5% (w/v) oil in acetone.

A typical triglyceride chromatogram for palm olein is shown in Fig. 33. Based on the NARP-HPLC chromatogram of palm olein, it can be estimated that there are altogether 17 identifiable triglycerides found in palm olein. They are lauric-lauric-lauric [LaLaLa (0.08%)], lauric-lauric-myristic [LaLaM (0.43%)], myristic-myristic-lauric [MMLa (0.16%)], myristic-myristic-myristic [MMM (0.59%)], myristic-palmitic-oleic [MPO (0.69%)], myristic-palmitic-linoleic [MPL (3.01%)], palmitic-palmitic-oleic [PPO (27.95%)], palmitic-palmitic-linoleic [PPL (10.65%)], linoleic-linoleic-linoleic [LLL (0.007%)], palmitic-oleic-stearic [POS (5.12%)], palmitic-oleic-oleic [POO (27.49%)], palmitic-linoleic-oleic [PLO (12.64%)], oleic-oleic-stearic [OOS (3.82%)], stearic-oleic-stearic [SOS (0.58%)], stearic-linoleic-stearic [SLS (0.20%), oleic-oleic-oleic [OOO (4.57%)], and oleic-oleic-linoleic [OOL (2.19%)].

The triglyceride chromatograms for palm olein solid fractions collected after 12, 15, 18, 21, and 24 h at low-temperature (12.5°C) storage were very similar. The unidentified peaks that appeared in the early region of the chromatograms were expected to be of partial glycerides. Evidence of a stable and straight baseline and the sharp and symmetrically defined peaks show the

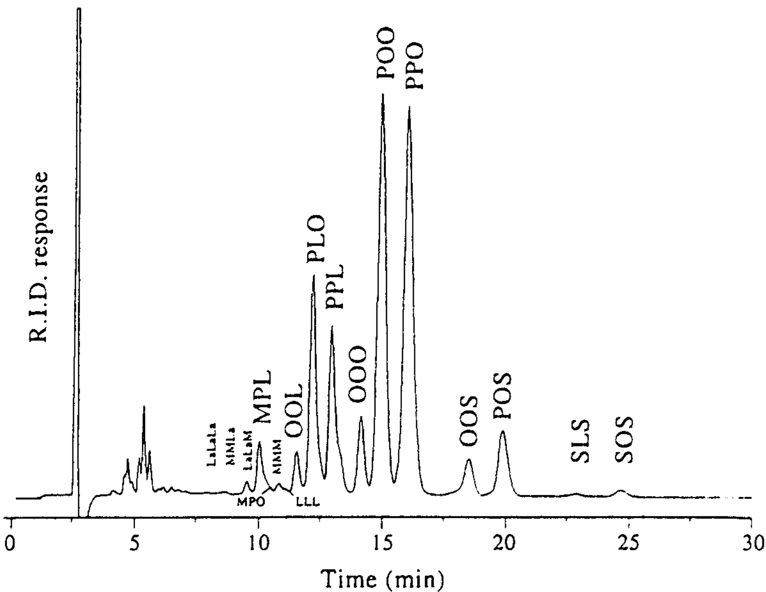


Fig. 33 NARP-HPLC chromatogram of typical palm olein at room temperature (about 27°C): LaLaLa, lauric-lauric-lauric: LaLaM, lauric-lauric-myristic; MMLa, myristic-myristic-lauric; MMM, myristic-myristic-myristic; MPO, myristic-palmitic-oleic; MPL, myristic-palmitic-linoleic; PPO, palmitic-palmitic-oleic; PPL, palmitic-palmitic-linoleic; LLL, linoleic-linoleic-linoleic; POS, palmitic-oleic-stearic; POO, palmitic-oleic-oleic; PLO, palmitic-linoleic-oleic; OOS, oleic-oleic-stearic; SOS, stearic-oleic-stearic; SLS, stearic-linoleic-stearic; OOO, oleic-oleic-oleic; and OOL, oleic-oleic-linoleic. R.I.D., refractive index detector.

quality of separation using the improved NARP-HPLC method. According to Frede (102), the baseline could be stabilized by indirect thermostating of the detector. The increase of selectivity with decreasing temperature and the increasing sharpness of peaks at higher temperature were also observed. A high temperature was indispensible for saturated long-chain compounds, which tend to crystallize on the column. To fulfill this requirement, and to avoid loss of selectivity, temperature programming was investigated. At higher temperatures and additional axial temperatures, gradients up to 0.05K/cm were applied to enhance peak symmetry. However, in the case of palm olein, the preset temperature of 35°C was found to be sufficient. The analysis time for each injection was found to be 30 min at a flow rate of 1.0 ml/min. Table 4 tabulates the results of triglyceride composition and content. An increase was observed in PPO and POS, and a decline was found in the composition of POO, PLO, OOO, OOL, and MPL during the first 18 h of crystallization. The rest of the components did not show obvious changes.

It is possible to use the NARP-HPLC results to determine triglycerides according to the unsaturation degree, in that it allows the calculation of CN as well as FAC to be made according to the following formula:

$$\text{Calculated composition of CN} = \Sigma \text{ individual triglycerides with same CN}$$

For example:

$$C_{52} = \text{POO} + \text{POS} + \text{PLO}$$

$$\text{Calculated FAC} = \Sigma \text{ individual fatty acid found in total glycerides}$$

For example:

$$C_{14} = \frac{\text{MPO}}{3} + \frac{\text{MPL}}{3}$$

The CFs (correlation factors) can be calculated by using the following equation:

$$\text{CF} = \frac{\text{Actual result}}{\text{Calculated result}}$$

The CFs for C48, C50, C52, C16:0, C18:0, and C18:1 were found to be ~1.0, showing a good agreement between calculated and actual analysis results. Using the following equations, derived by incorporating the CF, the composition of CN and FAMEs can be calculated accurately from the NARP-HPLC results:

$$\text{Actual CN composition} = \text{CF of CN} \times \Sigma \text{ individual triglycerides of CN with same CN}$$

For example:

$$C_{52} = \text{CF of } C_{52} \times (\text{POO} + \text{POS} + \text{PLO})$$

$$\text{Actual FAC} = \text{CF of fatty acid} \times \Sigma \text{ individual fatty acids found in total glycerides}$$

For example:

$$C_{14} = \text{CF of } C_{14} \times \left(\frac{\text{MPO}}{3} + \frac{\text{MPL}}{3}\right)$$

By applying these equations to NARP-HPLC results, one can determine the composition of CN and FAMEs.

Table 4 Triglyceride Composition Based on HPLC Analysis During Low-Temperature Storage (wt%)

	Triglyceride*								
Hours	MPO	MPL	PPO	PPL	POS	POO	POL	OOS	SOS
Control	0.69^{abc}	3.01^{a}	27.95^{c}	10.65^{d}	5.12^{c}	27.49^{a}	12.64^{a}	3.28^{ab}	0.58^{b}
12	0.66^{d}	2.96^{a}	29.32^{b}	10.72^{cd}	5.42^{b}	26.42^{b}	12.31^{a}	3.36^{a}	0.62^{ab}
15	0.66^{cd}	2.72^{b}	32.92^{a}	10.86^{bc}	6.04^{a}	24.05^{d}	11.17^{b}	2.69^{c}	0.67^{a}
18	0.68^{bcd}	2.69^{b}	33.53^{a}	10.95^{b}	6.04^{a}	23.98^{d}	11.13^{b}	3.04^{b}	0.67^{a}
21	0.72^{a}	3.14^{a}	29.82^{b}	11.15^{a}	5.29^{bc}	25.54^{c}	12.83^{a}	2.74^{c}	0.57^{b}
24	0.70^{ab}	2.96^{a}	30.10^{b}	10.96^{b}	5.48^{b}	26.32^{b}	12.22^{a}	3.17^{ab}	0.62^{ab}
$LSD_{0.05}$	2.96×10^{-2}	0.19	1.21	0.15	0.29	0.16	0.72	0.24	5.3×10^{-2}

La, lauric; M, myristic; P, palmitic; S, stearic; O, oleic; and L, linoleic acid.
* Means of four readings.
[a–d] Mean in a column followed by different letters are different ($P < 0.05$).

B. Identification of Individual Triglycerides

1. Equivalent Carbon Number (ECN)

The first problem in the HPLC analysis of TGs is the identification of the individual TGs. Plattner et al. (90) observed that under isocratic conditions the logarithm of the elution volume of a triacylglycerol is directly proportional to the total number of carbon atoms (CN) and inversely proportional to the total number of double bonds (X) in the three fatty acyl chains. This elution behavior is controlled by the equivalent carbon number (ECN) of a triacylglycerol, which may be defined as

$$ECN = CN - X \cdot n$$

where n is the factor for double bond contribution, normally close to 2. No distinction is made here between triacylglycerols that are positional isomers.

The straight lines corresponding to the different unsaturation characteristics were shown to be parallel. Tracing of these parallel lines from a few known TGs theoretically permits the identification of unknown TGs on the chromatogram. On the other hand, Goiffon et al. (91) have established several linear relationships between the number of double bonds of TGs and the logarithm of the retention time with respect to that of trioctadecenoin (triolein OOO), expressed as log α. In particular, this relationship was observed with the series of TGs obtained by substituting a given fatty acid successively for the three component fatty acids of a simple TG. They concluded that log α for a given TG was the sum of log α for the three constituent fatty acids, the latter values being equal to 1/3 log α of the corresponding simple TG. The retention time of any TG or the identification of a TG whose retention time is known can thus be either determined graphically or calculated from log α for each fatty acid.

During the analysis and separation of TGs, difficulties in obtaining superior resolution arise as a result of the complex mixtures of varying TG structure caused by varied acyl components. One of these difficulties is the formation of "critical pairs," which has made it more difficult to separate certain methyl esters from each other as well as their TGs on RP-HPLC using 5-μm octyl or 10-μm octadecyl bonded phases. Critical pairs have been found to have close behavior on RP-chromatography, in spite of the difference in the chain lengths, the number of double bonds, and the geometrical configuration.

Critical pairs have been defined as those structures with an equivalent carbon number (ECN). This includes critical pairs that contain one or more critical acyl groups on the glycerol,

Triglyceride*							
SLS	OOO	LaLaLa	LaLaM	LLL	MMLa	MMM	OOL
0.20[c]	4.57	0.08[a]	0.43[b]	0.007[ab]	0.16[b]	0.59[b]	2.19[ab]
0.31[a]	4.41	0.11[a]	0.50[a]	0.04[ab]	0.17[ab]	0.56[b]	2.08[bc]
0.20[c]	4.07	0.06[ab]	0.10[c]	0.03[b]	0.167[b]	0.52[b]	1.9[c]
0.28[b]	3.98	0.08[a]	0.10[c]	0.009[a]	0.195[a]	0.52[b]	1.89[c]
0.16[d]	4.61	0.0[b]	0.0[d]	0.04[ab]	0.16[b]	0.70[a]	2.39[bc]
0.17[d]	4.36	0.0[b]	0.07[c]	0.04[ab]	0.165[b]	0.57[b]	0.08[bc]
1.16×10^{-2}	0.31	6.11×10^{-2}	3.70×10^{-2}	5.54×10^{-2}	2.64×10^{-2}	0.102	0.28

such as triolein (54:3), palmityldiolein (52:2), oleyldipalmitin (50:1), and tripalmitin (48:0), all having the same ECN of 48. Positional isomers of TGs also are critical pairs, because they have the same acyl groups but differ in the position of these acyl groups. Triglycerides containing geometrical isomers also form critical pairs; they differ only in the configuration of the double bond, whether cis or trans. The separation of TG critical pairs requires the optimization of the mobile-phase composition and highly efficient stationary phases that have enough theoretical plates to resolve such critical pairs into their individual components.

The IUPAC Commission on Oils, Fats and Derivatives undertook the development of a method and collaborative study for the determination of triglycerides in vegetable oils by liquid chromatography. Three collaborative studies were conducted from 1985 to 1987. Refinements were made in the method after the first collaborative study, and the second and third collaborative studies demonstrated that the method produces acceptable results. Materials studied were soybean oil, almond oil, sunflower oil, olive oil, rapeseed oil, and blends of palm and sunflower oils and almond and sunflower oils. Six test samples were analyzed by 18 laboratories from 11 countries in the second study; 4 test samples were analyzed by 16 laboratories from 12 countries in the third study. The method for the determination of triglycerides (by partition numbers) in vegetable oils by liquid chromatography was the first action adopted by AOAC INTERNATIONAL as an IUPAC-AOC-AOAC method (103).

Triglycerides in vegetable oils are separated according to their equivalent carbon number by reversed-phase liquid chromatography and detected by differential refractometry. Quantitation is by area normalization. Elution order is determined by calculating the equivalent carbon numbers, ECNs, often defined (as we said before) as CN − $2n$, where CN is the carbon number and n is the number of double bonds. To calculate ECNs more accurately, the origin of the double bond is taken into account:

$$\text{ECN} = \text{CN} - d_o n_o - d_l n_l - d_{ln} n_{ln}$$

where n_o, n_l, and n_{ln}, are the number of double bonds of oleic, linoleic, and linolenic acids, respectively, and the coefficients d_o, d_l, and d_{ln} are calculated from reference triglycerides. Under the conditions in this method, ECN approximates as

$$\text{ECN} = \text{CN} - (2.60 \times n_o) - (2.35 \times n_l) - (2.17 \times n_{ln})$$

With several reference triglycerides, resolution (α) can be calculated with respect to triolein:

$$\alpha = \frac{RT'_i}{RT'_{\text{triolein}}}$$

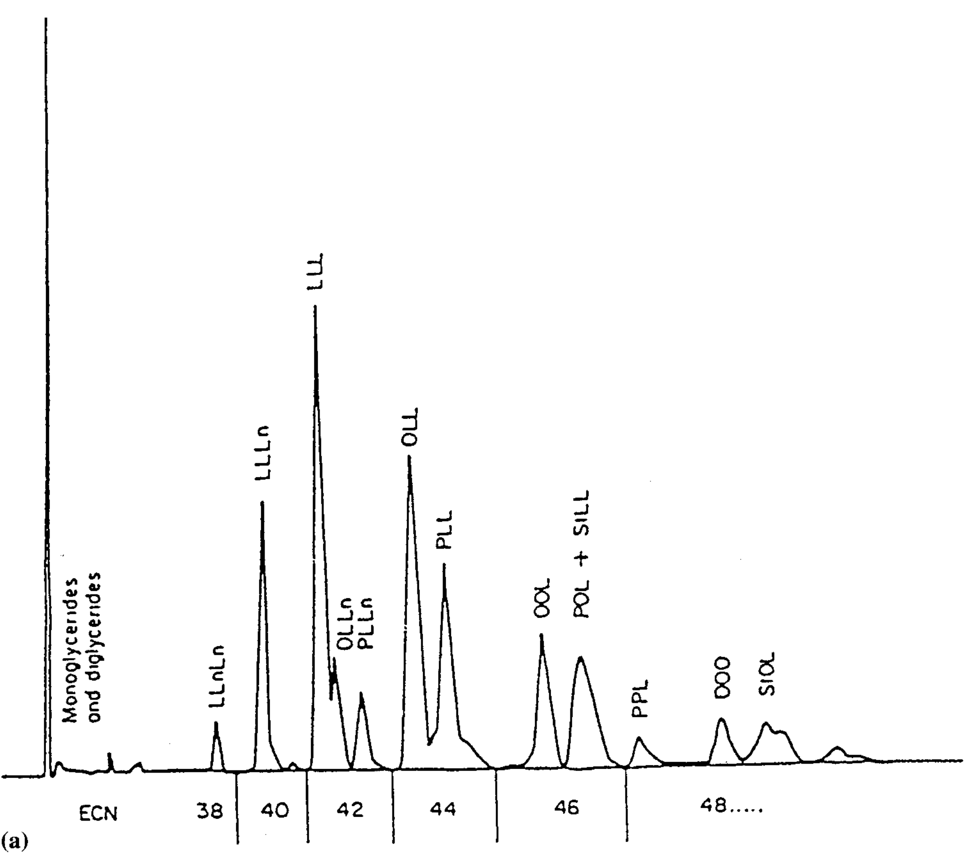


Fig. 34 (a) Typical LC chromatogram for soybean oil as reference material for the identification of triglycerides: O, oleic acid; P, palmitic acid; L, linoleic acid, St, stearic acid; Ln, linolenic acid. Thus, OOO represents triolein. (b) Graph of log $\alpha = f(n)$, where α is the resolution of the reference triglyceride with

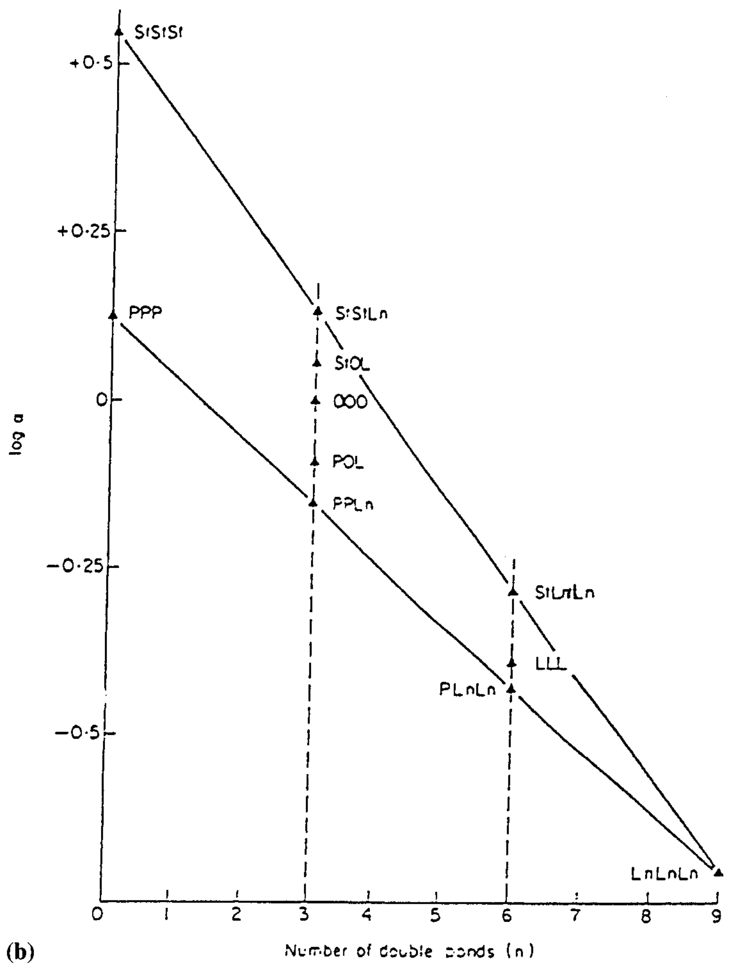
using reduced retention time $RT'_i = RT_i \times RT_{solvent}$. One can determine retention values for all triglycerides of fatty acids contained in the reference triglycerides (Fig. 34) by graphing log α vs. n (number of double bonds).

C. The Problem of TG Detection

The major difficulty in the HPLC of triacylglycerols is that of detection. Traditional modes of detection, such as refractive index and ultraviolet absorbance, offer only low sensitivity toward these triacylglycerols.

The detection by means of UV absorbance at low wavelengths (190–237 nm) is caused by the ester carbonyl group and isolated double bonds. Baseline drift may occur during the operation of gradients, and solvents that absorb strongly at these wavelengths cannot be used in the mobile phase. Otherwise, the refractive index mode of detection is much less sensitive to TGs than is UV detection, and it is incompatible with the use of gradient elution.

The complexity of natural TG mixtures would, however, require gradient elution to utilize fully the resolution power of liquid–liquid chromatographic systems. This is not possible to achieve in combination with IR detectors, and is difficult with UV detectors in this case due to the short wavelengths that have to be used.



(b)

Fig. 34 Continued respect to triolein (α_i = RT$'_i$/RT$'_{\text{olein}}$) and n is the number of double bonds in the triglyceride. The graph determines retention values for all triglycerides of fatty acids contained in the reference triglycerides.

The introduction of the commercially available mass detector meant an expansion of the possible conditions for TG separations, including that of gradient elution. The mass detector showed very narrow and symmetrical peaks, which indicated its usefulness for this application. The goal was to find a system that could be used to separate any natural TG mixture regardless of its carbon number distribution. The basic principle of this detector, i.e., the light scattering, originally was presented by Charlesworth (104).

Herslof and Kindmark (105) report the use of the mass detector (light-scattering detector) in this sense. The column used (250 × 4.6-mm ID) was packed with LICHROSPHER RP 100 and thermostated at 22°C. The mass detector oven temperature was 40°C, and the inlet gas pressure was 15 psi. The mobile phase consisted of mixtures of acetonitrile, ethanol, and hexane. Gradients from 0 to 100 of ternary premixtures of the three solvents were used at a flow rate of 1 ml/min. The sample was dissolved in hexane–isopropanol (1 : 1), and 12 μl were injected (approx. 150 μg total sample). The chromatogram of the standard mixture of TGs is shown in Fig. 35. The separations of soybean oil and a mixture of soybean and coconut oils illustrate the resolution of vegetable oils into TG species (Fig. 36).

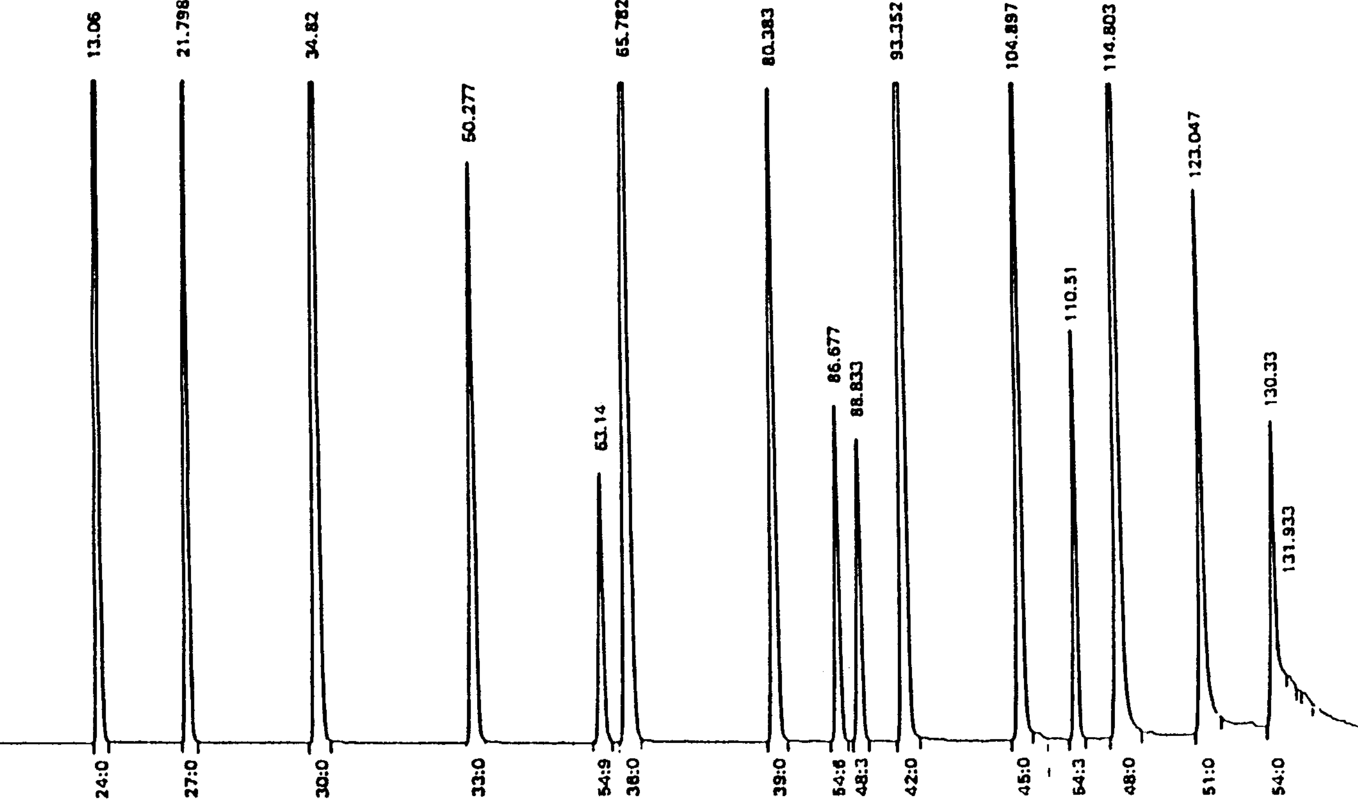


Fig. 35 Separation of a standard TGs mixture (G No. 1, Nu-Chek Prep.). Linear gradient from 100% A to 100% B in 120 min. Column 250 × 4.6-mm ID. LICHROSPHERE 100 RP18; solvent A: acetonitrile; solvent B: acetonitrile/ethanol/hexane (40:40:20 w/w/w); flow rate: 1.0 ml/min.

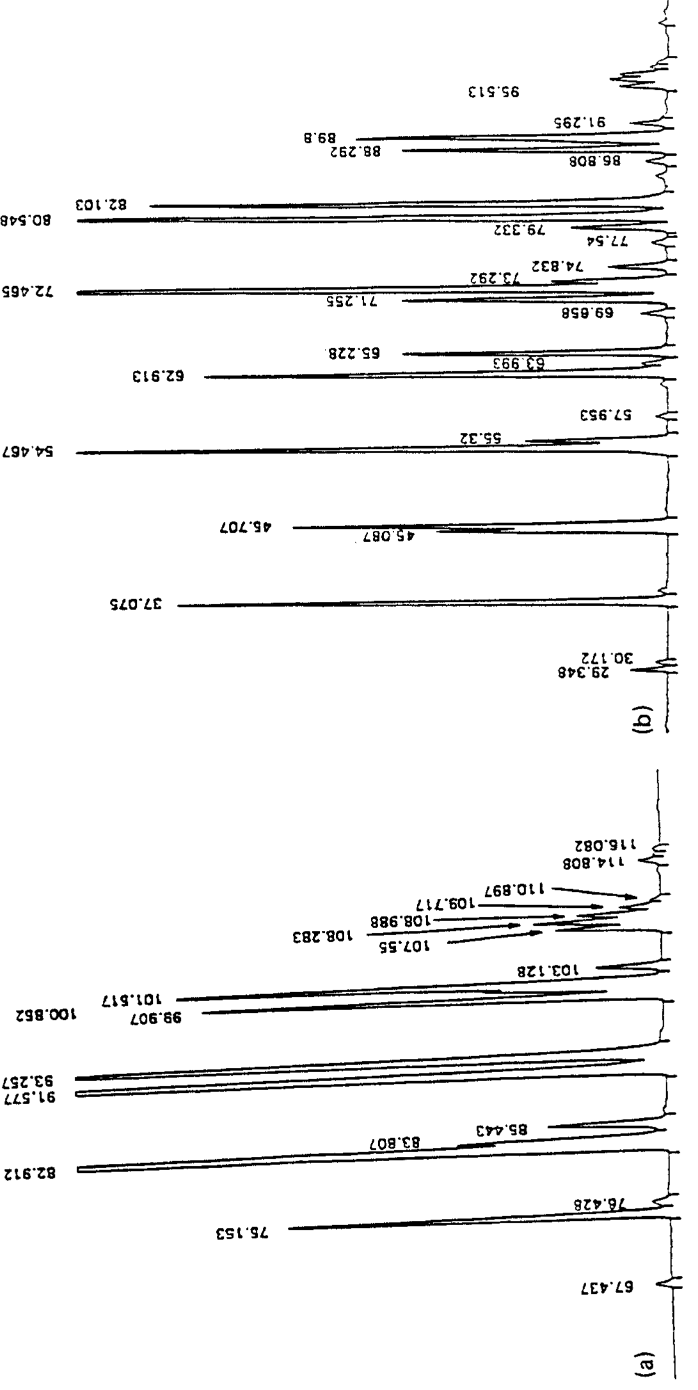


Fig. 36 (a) Separation of soybean oil TGs. Chromatographic conditions as in Fig. 35. (b) Separation of a mixture of equal amounts of soybean oil and coconut oil. Chromatographic conditions as in Fig. 35.

Stolyhwo et al. (106) described a different analytical methodology, one that circumvents most of the problems encountered so far and permits the analysis of TGs up to and including tribehenin and the quantitative analysis of these complex mixtures with few calibrations, using a laser-light-scattering detector. For the achievement of most separations, a 250 × 4.0-mm ID HIBAR LICHROSPHER 1000 CH-18/3 (5-μm particles) was used. For the achievement of some difficult separations, a second, similar column was connected online to the first one.

If acetonitrile appears to be the best weak solvent for the analysis of TGs, there seems to be a wide choice for the strong solvent. Most previous analysts have reported the use of acetone. Indeed, TGs up to tripalmitin can be analyzed properly with acetone/acetonitrile mixtures. Higher-molecular-weight TGs, above PPP, have a solubility in these mixtures, which decreases dramatically with increasing molecular weight. They used acetone, benzene, $CHCl_3$, CH_2Cl_2, and 1,2-dichloroethane mixed with acetonitrile in different concentrations. Similar results have been achieved with $CHCl_3$/acetonitrile (40:60) and with acetone/acetonitrile (70:30). The best results have been consistently obtained with $CHCl_3$, however. It seems also that because of the rapid increase of the solubility of TGs with increasing temperature, the use of programmed-temperature LC could be another solution to the problem.

Fig. 37A shows the chromatogram obtained in isocratic conditions for a mixture of homogeneous TG standards. The mobile phase is a mixture of $CHCl_3$ and acetonitrile (49:51). Tribehenin (BBB) is eluted easily in 18 min, while tristearin (SSS) could not be eluted without serious difficulties in previous studies where acetone/acetonitrile mixtures were used. This is certainly due to the use of a strong eluent, containing about half $CHCl_3$, a solvent in which fats are known to be very soluble.

The use of the laser-light-scattering detector permits the record of excellent chromatograms in gradient elution. An analysis of the test mixture of standards is shown in Fig. 37B for comparison with the same analysis obtained in isocratic conditions. The analysis lasts about 30 min before the elution of BBB instead of 18 min in isocratic conditions, but the first part of the chromatogram is considerably improved, and a number of impurities in the standards can be resolved.

Both of these systems produced efficient separations of complex triacylglycerol mixtures, but retention times were long. One gradient system that can be used to separate all known natural triacylglycerol mixtures, regardless of their carbon number distribution, was investigated by Palmer and Palmer (107). They described a rapid separation of TGs using a single gradient system that was sought to enable the method to be adapted for routine analytical work for identification of oils and possible detection of adulteration. Their work was carried out using a 3-μm, 150 × 4.6-mm ID, reverse-phase column and light-scattering detection. Solutions of 0.05% of each standard TG and 1% oil and fat samples in dichloromethane–acetonitrile (1:1) were filtered through a 0.45-μm filter and injected onto the HPLC system.

Resolution of the standard TGs was achieved with a linear gradient of 30–70% dichloromethane in acetonitrile with a flow rate of 0.6 ml/min, but optimum resolution of the complex TG mixture present in natural oils and fats requires a step in this gradient. Using the stepped gradient, the resolution and retention times of these standards were found to be reproducible.

The method was then applied to a range of edible oils and fats (trinonadecanoin was used at a level of 0.01% as an internal standard). Figure 38 shows TG profiles obtained by this method. Triglycerides elute from this system in order of increasing carbon number. The presence of double bonds reduces the retention time of the TG.

The TG profiles for soybean oil and olive oil are significantly different. Olive oil contains no triacylglycerols eluting before LLO, whereas soybean oil contains a large proportion of triacylglycerols eluting before LLO. This difference could be used to detect the addition of low levels of soybean in olive oil (Figs. 39 and 40). Olive oil is the main oil used in many countries in food preparation, cooking, and frying, and large volumes are imported every year. El-Hamdy and

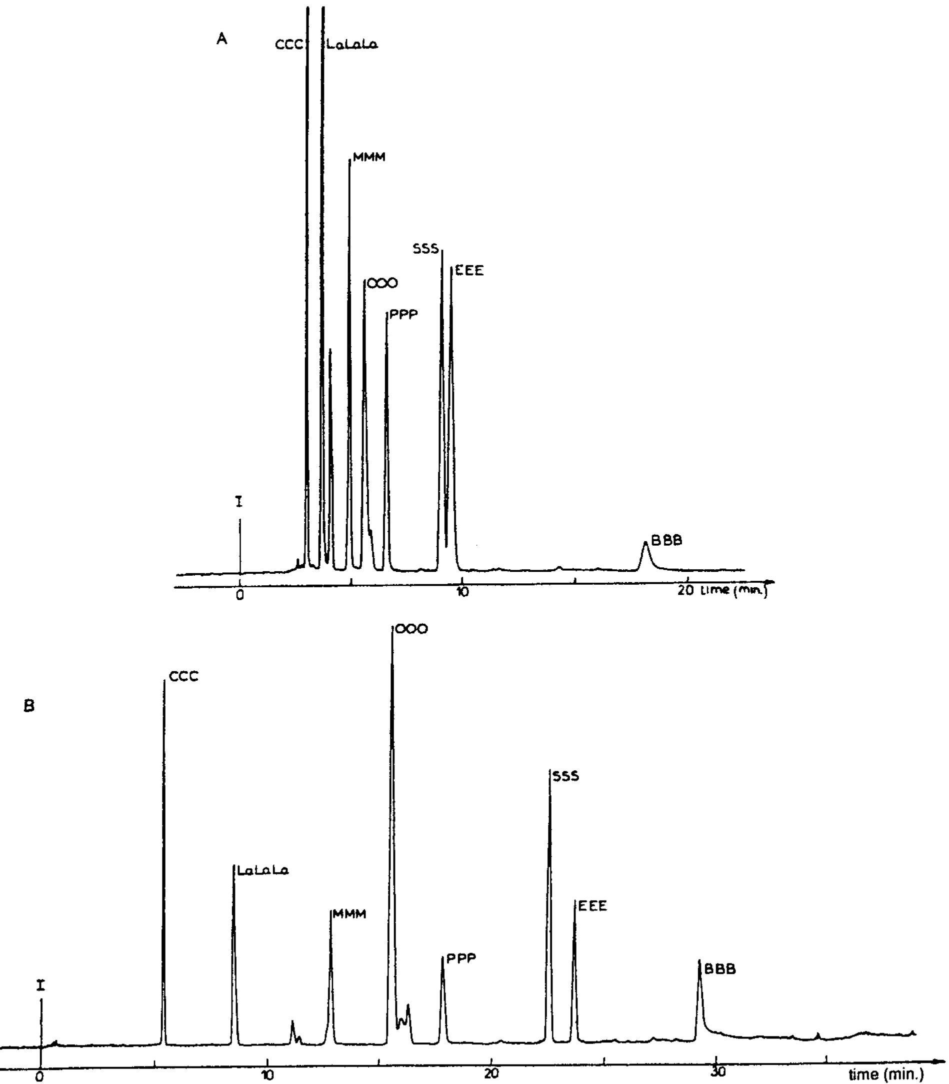


Fig. 37 Chromatograms of a test mixture of homogeneous tryglyceride standards. Columns: 250 × 4-mm ID LICHROSPHERE RP18 (5 μm); constant flow rate: 1.0 ml/min. A, isocratic conditions: solvent: chloroform/acetonitrile (49:51 v/v) (one column). B, gradient elution: chloroform in acetonitrile. Starting conditions: 30:70, program rate: 1%/min for 25 min to 55:45 and then 3%/min for 15 min to 100:0 and hold at 100% chloroform for 8 min (two columns in series). Some impurities are contained in the standard compounds.

El-Fizga (108) developed a simple, rapid method for the detection of oils high in linoleic acid in olive oil by RP-HPLC and a simple authenticity factor and a derived equation to determine the extent of adulteration with a one short chromatographic step, completed in less than 15 min. They used two 150 × 4.5-mm ID stainless steel columns packed with an octyl-bonded silica stationary phase (Supelcosil-LC 8) (Supelco. Bellefonte, PA, USA) and a differential refractometric detector. The isocratic mobile phase was acetone–acetonitrile (70:30, v/v) (Table 5).

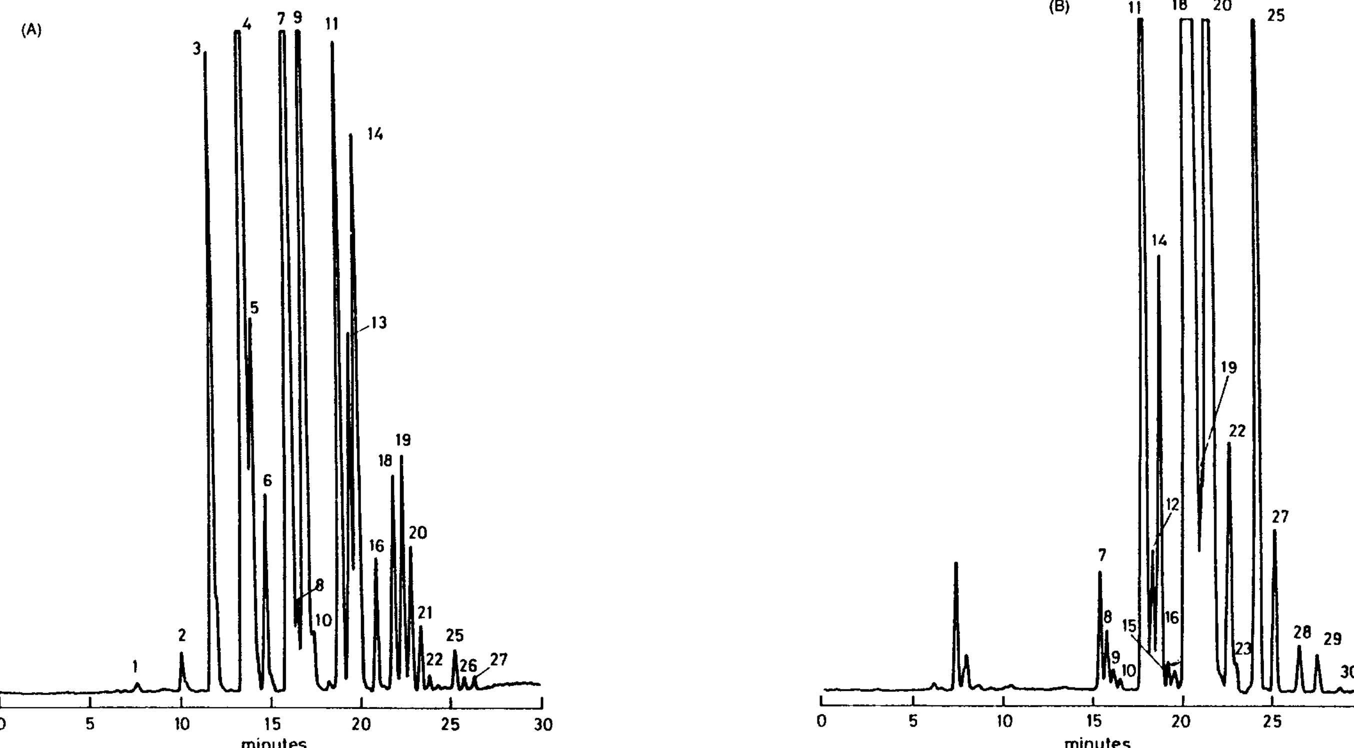


Fig. 38 (A) Triacylglycerol profile of soybean oil. For peak identification see Table 5. (B) Triacylglycerol profile of olive oil. For peak identification see Table 5.

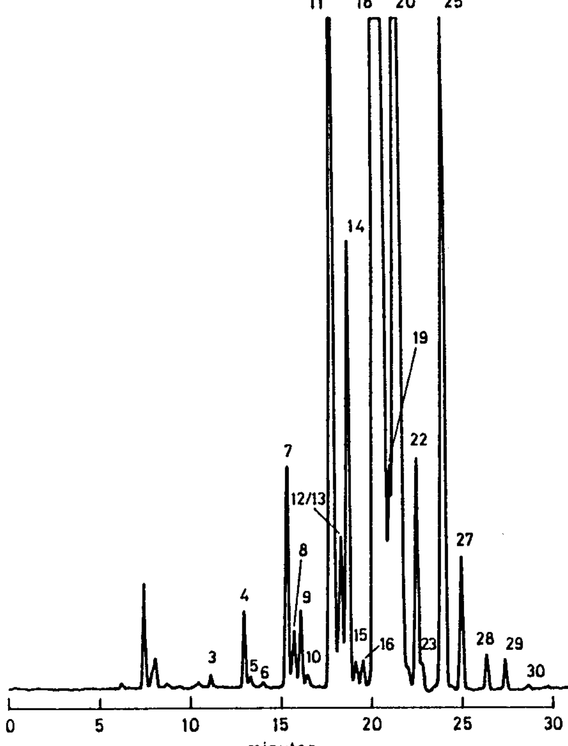


Fig. 39 Triacylglycerol profile of 10% soybean oil in olive oil.

Figure 41 shows typical chromatograms for olive, corn, soybean, and sunflower oil triacylglycerols separated according to their equivalent carbon number (ECN). Soybean oil contains 1.2% of triacylglycerols, with ECN 38 and 7.0% of ECN 40 triacylglycerols. The ECN 42 triacylglycerol contents in corn, sunflower, and soybean are 24.2 ± 0.04, 22.4 ± 0.10, and 24.9 ± 0.11%, respectively, while that in olive oil is 1.0 ± 0.02%. The ECN 42 triaclyglycerol group was used as an indicator of adulteration because it shows the greatest difference in triacylglycerol content between olive oil and the high linoleic acid oils.

The presence of vegetable oils of high linoleic acid content in olive oil can be detected by measuring its authenticity factor (Au) as follows:

$$Au = \frac{100 - ECN \times 42\,(\%)}{ECN \times 42\,(\%)}$$

Virgin olive oil separated hy RP-HPLC has Au = 98.2 ± 3.86. The authenticity factors for corn, sunflower, and soybean oils are 3.2 ± 0.02, 3.5 ± 0.06, and 3.2 ± 0.19, respectively. Figure 41 shows that the addition of as little as 1% of corn, sunflower, and soybean oils decreased the olive oil Au to 81.6 ± 2.5, 80.3 ± 4.05, and 79.0 ± 3.54, respectively, and the addition of 5% of these oils decreased Au to 46.2 ± 1.38, 48.3 ± 1.26, and 46.4 ± 1.70, respectively.

Plotting the percentage of added high-linoleic-acid oil versus the percentage of ECN 42

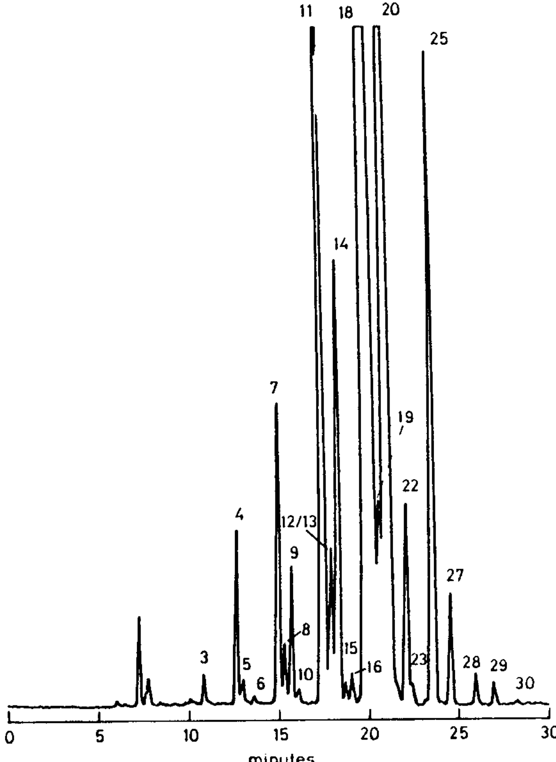


Fig. 40 Triacylglycerol profile of 5% soybean oil in olive oil.

Table 5 Identification of Triacylglycerol Peaks

Peak no.	Triacylglycerol	Peak no.	Triacylglycerol
1	LnLnLn	18	OOO
2	LLnLn	19	OLS
3	LLnL	20	OOP
4	LLL	21	PLS
5	LLnO	22	POP
6	LLnP	23	PPaS
7	LLO	24	PPP
8	OLnO	25	OOS
9	LLP	26	SLS
10	OLnP	27	POS
11	OLO	28	PPS
12	OPaO	29	SOS
13	LLS	30	SPS
14	OLP	31	SOA
15	OPaP	32	SSS
16	PLP	33	SSA
17	SLnP		

P = palmitic acid, Pa = palmitoleic acid, S = stearic acid, O = oleic acid, L = linoleic acid, Ln = linolenic acid, A = arachidic acid.

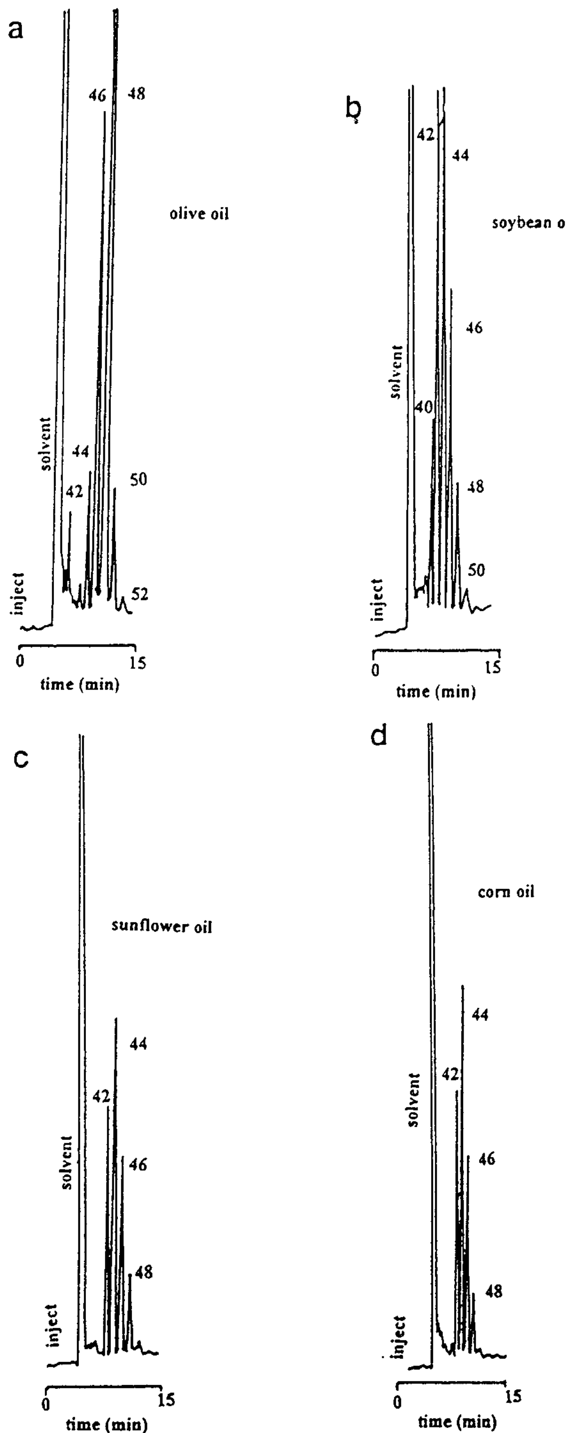


Fig. 41 Separation of triacylglycerols on Supelcosil-LC 8 with acetone–acetonitrile (70:30, v/v) as the mobile phase and refractive index detection. Flow rate, 1.0 ml/min. (a) Olive oil, (b) soybean oil, (c) sunflower oil, (d) corn oil.

triacylglycerol group showed the possibility of measuring the extent of olive oil adulteration by the following simple equation:

$$\text{Added oil (\%)} = \frac{\text{ECN 42 (\%)} - b}{a}$$

where a and b are constants that differ slightly according to the oil added. Thus corn, sunflower, and soybean oils added to olive oil can be calculated using the following equations:

$$\text{Corn oil (\%)} = \frac{\text{ECN 42 (\%)} - 0.9820}{0.2326}$$

$$\text{Sunflower oil (\%)} = \frac{\text{ECN 42 (\%)} - 0.9954}{0.2142}$$

$$\text{Soybean oil (\%)} = \frac{\text{ECN 42 (\%)} - 0.9801}{0.2388}$$

The overall equation for oils added to olive oil is

$$\text{Added oil (\%)} = \frac{\text{ECN 42 (\%)} - 0.9850}{0.2285}$$

V. MILK FAT

Butterfat is a mixture of more than 100,000 different triacylglycerols (TAGs) with a melting range from −40 to +40°C (109). About 400 different fatty acids (FAs) are present in butterfat, 25% of which were short-chained and 45% were long-chained saturates (110). Such variety in composition is responsible for butterfat's unique physical properties.

Butter has a limited plastic range. At refrigerator temperature (~1–0°C), butter behaves essentially as a solid and lacks spreadability, whereas at room temperature (21–25°C), it oils off and exhibits moisture exudation.

Nutritionally, butterfat contains a high percentage of hypercholesterolemic fatty acids (mid-length saturated FA) located predominantly at the sn-2 position of TAG (111). Conversely, it contains only a small percentage of trans fatty acids.

Milk fat has presented a particular challenge to analysis in terms of the identification and separation of TGs due to their complex variety of molecular species. The most complex mixtures of natural triacylglycerols require HPLC with gradient elution.

In the beginning, the refractive index detector was the most used detection system, although it has two important drawbacks: (1) solvent gradients cannot be used, and (2) it has low sensitivity and different responses to saturated and highly unsaturated TGs (112). Moreover, use of the ultraviolet (UV) detector is difficult, because the most adequate solvents also absorb in the same range and therefore cause an important baseline drift with gradient elution systems (106).

Other authors have analyzed milk fat TG with a flame ionization detector (FID) and with nonlinear gradient elution (113). They found that differtent TG responses were variable, although the variation was much less than with UV detection.

Introduction of the light-scattering detector has made the development of more efficient methods for the separation of natural complex mixtures of TG possible, including those of gradient elution. The evaporator temperature in the mass detector oven may influence the response of thermolabile compounds or compounds with low boiling points. Consequently, low tempera-

tures would be desirable, provided that the evaporation of mobile-phase solvents is guaranteed. However, temperatures of 10 or 15°C above ambient are required for the detector to work correctly.

Usually, mobile phases of acetonitrile and acetone have been used in the analysis of TG from milk fat, most often in isocratic elution (114,115) and in gradient eiution, and they provide a resolution of 50 chromatographic peaks (Numela). One of the main difficulties in the analysis of TG is the identification of the chromatographic peaks, because of the small number of mixed TGs in a pure state. Bornaz et al. (115) and Dotson et al. (114) identified butterfat chromatographic peaks from the relationship between the retention time and the theoretical carbon number according to the model proposed by El-Hamdy and Perkins (87). An alternative method is the fractionation of total TG in milk fat by reversed-phase HPLC and analysis of the fatty acids in each fraction (116,117).

The presence of high proportions of the short-chain (C4–C8) fatty acids in butterfat has long been known (118), as has been their overall positional distribution and molecular association. Early studies by high-temperature gas chromatography GC) and silver ion thin-layer chromatography ($AgNO_3$-TLC) (119) showed that the bulk of these acids occurred with the frequency of one short-chain fatty acid per triacylglycerol molecule. Furthermore, evidence was obtained that the short-chain fatty acids were confined largely to the sn-3 position (120,121).

More recently, butterfat composition has been analyzed by capillary GC on polarizable liquid phases (122,123), reversed-phase high-performance liquid chromatography (124,125), and GC and HPLC with mass spectrometry (MS) (126), as well as by MS-MS (127). These studies have led to an extensive resolution of butterfat triacylglycerols, but the possible presence of small amounts of positional and reverse isomers as well as of species containing two or three short-chain acids per molecule has not been addressed.

As an aid in the identification of such triacylglycerols in butterfat, Marai et al. (128) have investigated the reversed-phase LC-MS behavior of randomized butterfat, which contains the various isomeric triacylglycerols in known and sufficient amounts for analysis. The results show that conventional C_{18} reversed-phase columns would not resolve the positional and reverse isomers of mixed acid triacylglycerols, but that the resolution of isologous triacylglycerols is retained also when two or three short-chain fatty acids occur per molecule.

The purpose of their investigation was to determine the relative order of elution of the molecular species of rearranged butterfat triacylglycerols for which the exact quantitative composition can be calculated by statistical considerations. The initial part of the study was concerned with verifying that a random distribution of the fatty acids had indeed been obtained, which was established by detailed GC and HPLC resolution and MS identification of the species.

The triacylglycerols were resolved by reversed-phase HPLC with a Supelcosil LC-18 reversed-phase column (5 μm, 25 cm × 0.46-cm ID) using a linear gradient of 0–90% propionitrile in acetonitrile or 10–90% propionitrile in acetonitrile (2.2 ml/min). The column was installed in a liquid chromatograph connected via a direct liquid inlet interface to a mass spectrometer. Alternatively, these analyses were performed with a liquid chromatograph equipped with the reversed-phase HPLC column coupled to a light-scattering detector. This column was operated to a flow rate of 1 ml/min (90 min) using a linear gradient of 10–90% isopropanol in acetonitrile.

The rearranged butter oil was segregated on basis of degree of unsaturation by TLC on silica gel G impregnated with 15% $AgNO_3$ (20 × 20 cm, 250-μm-thick layer) using chloroform containing 0.75% ethanol as the developing solvent. The resolved triacylglycerols were located spraying the plate with a 0.05% solution of 2,7-dichlorofluorescein in methanol–water (50:50) and viewing the plates under UV light. A total of five fractions were collected by eluting with chloroform–methanol (2:1) the silica gel scraped from areas corresponding to the following R_f

values: 0.70–0.85 (band 1), 0.55–0.70 (band 2), 0.35–0.55 (band 3), 0.25–0.37 (band 4), and 0.06–0.25 (band 5).

The individual triacylglycerols in the various HPLC peaks were identified by online LC-MS: single-ion profiles for the diacylglycerol type of fragments were systematically matched against those recorded for the total ion current, and the partition number and elution order were established for all triacylglycerols by reference to the fatty acid moiety lost during fragmentation. The identification of individual molecular species was confirmed by examining the full mass spectra taken from the ascending and descending limbs or the heart section of each peak.

Figure 42 gives the LC-MS profile for the saturated diacylglycerol moieties of the triacyl-

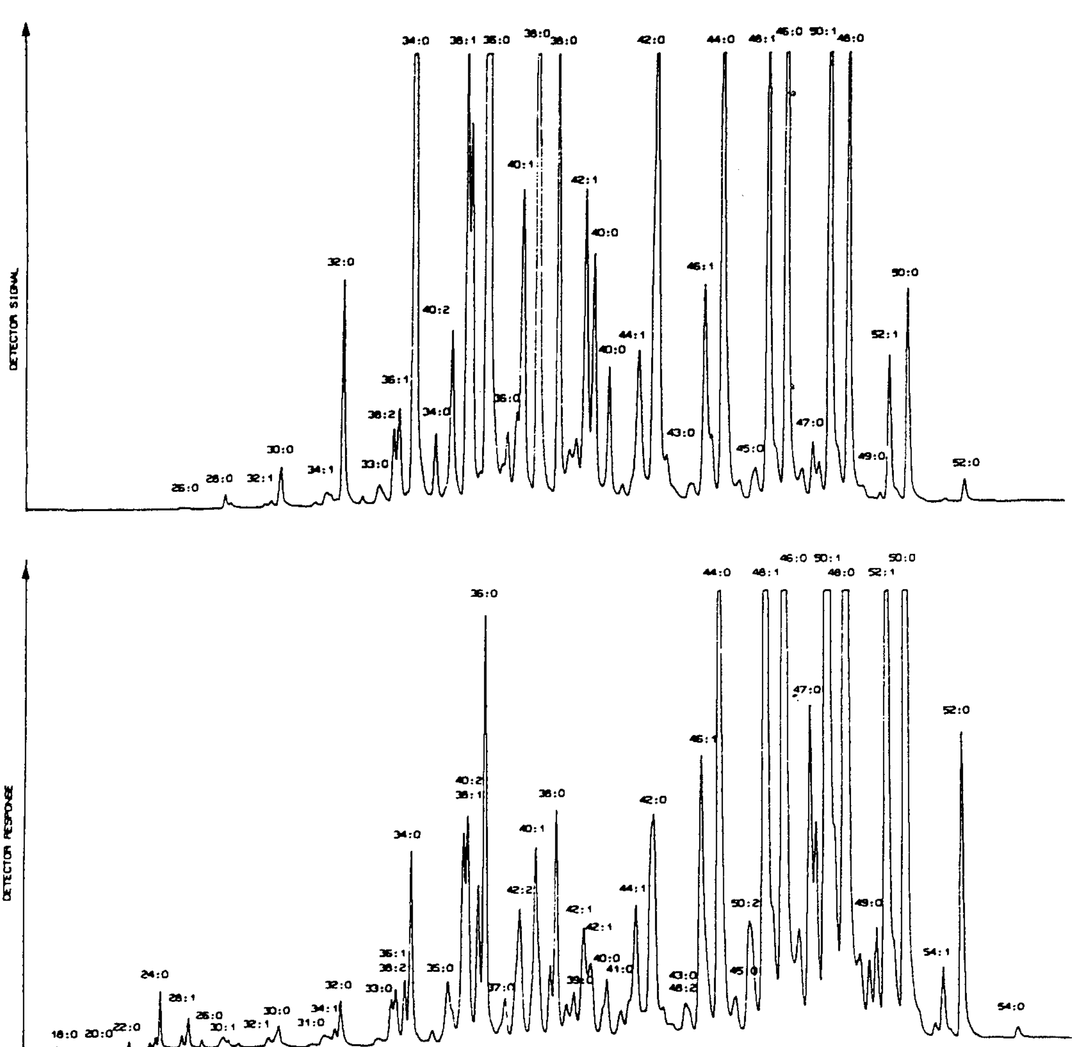


Fig. 42 Reversed-phase HPLC profiles of natural (top) and rearranged (bottom) butterfat triacylglycerols as obtained with the light-scattering detector. HPLC conditions: Hewlett-Packard Model 1050 liquid chromatograph equipped with a Supelcosil LC-18 column (25 cm × 0.46-cm ID) coupled to a Varex ELSD II light-scattering detector. Solvent: linear gradient of 10–90% propanol in acetonitrile at 25°C over a period of 90 min (1 ml/min); recording stopped at 70 min. Peak identification by carbon and double-bond numbers of triacylglycerols.

glycerol peaks in the randomized butter oil. The major saturated even-carbon-number diacylglycerols are determined as the $[MH-RCOOH]^+$ range from 8:0 to 36:0 and are combined randomly with all the fatty acids present in the rearranged oil. The relative proportions of the various diacylglycerols are indicated by the ion counts given in the left-hand corner of each ion plot, while the nature of the fatty acid lost during fragmentation is indicated by its carbon and double-bond numbers attached to each diacylglycerol peak. The splittings in the partition number of the triacylglycerols correspond to the to the splitting in the peaks for the diacylglycerol fragment ion and are due to differences in elution time of the species containing fatty acids of different chain length within isologous triacylglycerols. There is a clear indication of the separation of various critical pairs of species, e.g., the palmitates from oleates and the myristates from palmitoleates. Among the triacylglycerols containing short-chain fatty acids, e.g., butyrates, caproates, and caprylates, there is a clear-cut resolution triplet, with the butyrates being preceded by caproates, which are preceded by caprilates and higher chain lengths within each isologous series of triacylglycerols. Thus, the triacylglycerols yielding a diacylglycerol (DG) fragment $DG_{22:0}$ were made up of a triplet of peaks within the partition numbers 26–40. This was due to the presence of diacylglycerols containing such combinations of saturated fatty acids as 18:0–4:0, 16:06:0, 14:0–8:0, and 12:0–10:0, which are well resolved when combined with 4:0–18:0 saturated fatty acids in the original triacylglycerols. Likewise, there is resolution among isologous triacylglycerols containing two short-chain fatty acids. Thus, $DG_{8:0}$, mainly 4:0–4:0, when combined with 18:0, is retained longer than $DG_{10:0}$, mainly 6:0–4:0, when combined with 16:0, which is retained slightly longer than $DG_{12:0}$, mainly 6:0–6:0 and 8:0–4:0, when combined with 14:0. The shouldering is due to the earlier elution of 14:0–6:0–6:0 when compared to that of the isologous 14:0–8:0–4:0. Similar orders of elution can be recognized among other short-chain triacylglycerols.

The split in the partition number 50 is due to the presence of two major triacylglycerols, 18:0–18:1–16:0 and 18:0–16:0–16:0, with the 18:1-containing species being eluted ahead of the 16:0-containing species. In addition, the front part of this triacylglycerol peak also contained 18:0–18:0–16:1 and the rear part 18:0–14:0–18:0 as major species. The composition of the diacyl–glycerol moieties derived from the rear peak of partition number 48 revealed the presence of 18:0–16:0, 14:0–16:0, and 14:0–18:0 diacylglycerol species, in nearly equal proportions, corresponding to 14:0–16:0–18:0. The front of partion peak 48 yielded diacylglycerol ions for 16:1–18:1, 18:1–18:1, 16:0–18:1, 18:0–18:1, and 18:0–16:1, corresponding to triacylglycerols 16:1–18:0–18:1 and 16:0–18:1–18:1. The triacylglycerols eluted between the even-partition-number peaks were due to odd-carbon-number species.

Another problem is quantitative analysis of a multicomponent mixture, because we have to take into account the fact that only a small fraction of the components will appear as isolated peaks, in spite of using extremely efficient columns. For this reason, different deconvolution techniques have been elaborated.

Ruiz-Sala et al. (129) described a reversed-phase HPLC method with a light-scattering detector for the analysis of TGs in milk fat. The identification of TGs was carried out by a combination of HPLC and gas-liquid chromatography (GLC), and was based on the equivalent carbon numbers and retention times of different standard TGs. Finally, quantitation of peak areas from HPLC chromatograms was carried out after applying a deconvolution program to the parts of chromatograms with poor resolution.

The samples were raw ewe's milk, raw cow's milk and raw goat's milk. Cream was separated from milk by centrifugation, and TGs were extracted with *n*-hexane. For the HPLC analysis, two stainless steel columns, 25 cm and 15 cm × 4.6-mm ID with 3 μm Spherisorb ODS-2 (Phase Separations, Queensferry, United Kingdom; Symta, Madrid, Spain) were connected in series. The mass detector (ACS 750/14; The Arsenal, Macclesfield, United Kingdom) drift tube temperature was 45°C, and the inlet gas pressure was 1.38 bar. The mobile phase consisted of a

gradient elution from 0 to 70% (v/v) acetone in acetonitrile, in two stages—a linear increase of 0.7%/min in acetone for the first 50 min, an isocratic elution for 20 min, a second linear increase of 0.7%/min in acetone for another 50 min, and, finally, an isocratic elution for 42 min until the end of the analysis. The flow rate was 0.9 ml/min, and the pressure was 172 bar.

They studied the effect of the mass detectors' drift tube temperature on the low-molecular-mass TGs. Solutions of 10 mg/ml of tributyrin, tricaproin, tricaprylin, tricaprin, and trilaurin were injected twice at each of the following drift tube temperatures: 20, 25, 30, 45, and 60°C. Five replications of the HPLC analysis were performed for one sample of ewe's milk fat to determine the reproducibility of the HPLC method. The TG composition was estimated in accordance with the method based on the calculation of the equivalent carbon numbers (ECNs) of the HPLC chromatographic peaks and in the molar composition in fatty acids, analyzed by GLC, collected at the HPLC chromatograph outlet. The HPLC fractions were collected every 40 s at the outlet of the column after 14 min; there were no peaks before that time.

The ECN was calculated according to the formula given by Herslof et al. (89):

$$ECN = CN - (a' \cdot NDB)$$

where CN is the total carbon number of the three fatty acids and NDB is the number of double bonds in the TG molecules. The value of the constant a′ was calculated by multiple linear regression analysis of the experimental values of the dependent variable, log k', and the independent variables CN and NDB for the TGs available in pure form having been injected seven times:

$$\log k' = a' + b' \cdot CN + c' \cdot NDB$$

where a' is quotient of the coefficients c' and b'. Finally, a simple linear regression was applied to relate ECN to log k'.

The percentage in which possible TGs can be found in every fraction is estimated by the molar percentage of every TG. These molar percentages are calculated from the fatty acids in every fraction percentage, taking into account that the three positions in the glycerol are equivalent. This fact is justified because HPLC analysis cannot separate position isomers.

Figure 43 shows the HPLC chromatogram for TGs of cow's, ewe's, and goat's milk fat. The three species (ewe, cow, and goat) show chromatograms with 111 peaks. The estimate of ECN was carried out with two different groups of equations because of the complicated HPLC gradient method used: one for the first gradient and isocratic period, for 70 min, and the other for the second gradient and isocratic period until the end of the analysis. At first, the authors tried to calculate the ECN with one group of equations, but the problem was that most of the TGs eluted in ECN ascending order, as expected. However, certain series of critical pairs eluted in ECN descending order.

Standards with retention times shorter than 70 min (tributyrin, tricaproin, tricaprylin, tripelargonin, tricaprin, trilinolenin, trimyristolein, and trilaurin) were used to calculate the first a' coefficient. The resulting equations were as follows:

$$\log k' = -0.81130 + 0.06025 \cdot CN - 0.1280 \cdot NDB$$

$$ECN = CN - 2.12 \cdot NDB$$

$$\log k' = -0.81143 + 0.06025 \cdot ECN \qquad (SE = 0.0289)$$

The second a′ was calculated from the rest of the TG standards retention times (1,2-dilauroyl-3-myristoyl glycerol, tritridecanoin, 1,2-dimyristoyl-3-lauroylglycerol, trilinolein,tripalmitolein, trimyristin, 1,2-dilinoleoyl-3-oleoyl glycerol, 1,2-dimyristoyl-3-oleoyl glycerol, 1,2-dimyristoyl-3-palmitoyl glycerol, tripentadecanoin, 1,2-dipalmitoyl-3-myristoylglycerol, triolein, 1,2-dioleoyl-3-palmitoyl glycerol, 1,2-dipalmitoyl-3-oleoyl glycerol, tripalmitin, 1,2-dioleoyl-3-stearoyl glycerol, 1-oleoyl-2-palmitoyl-3-stearoyl glycerol, 1,2-distearoyl-3-myristoyl glycerol,

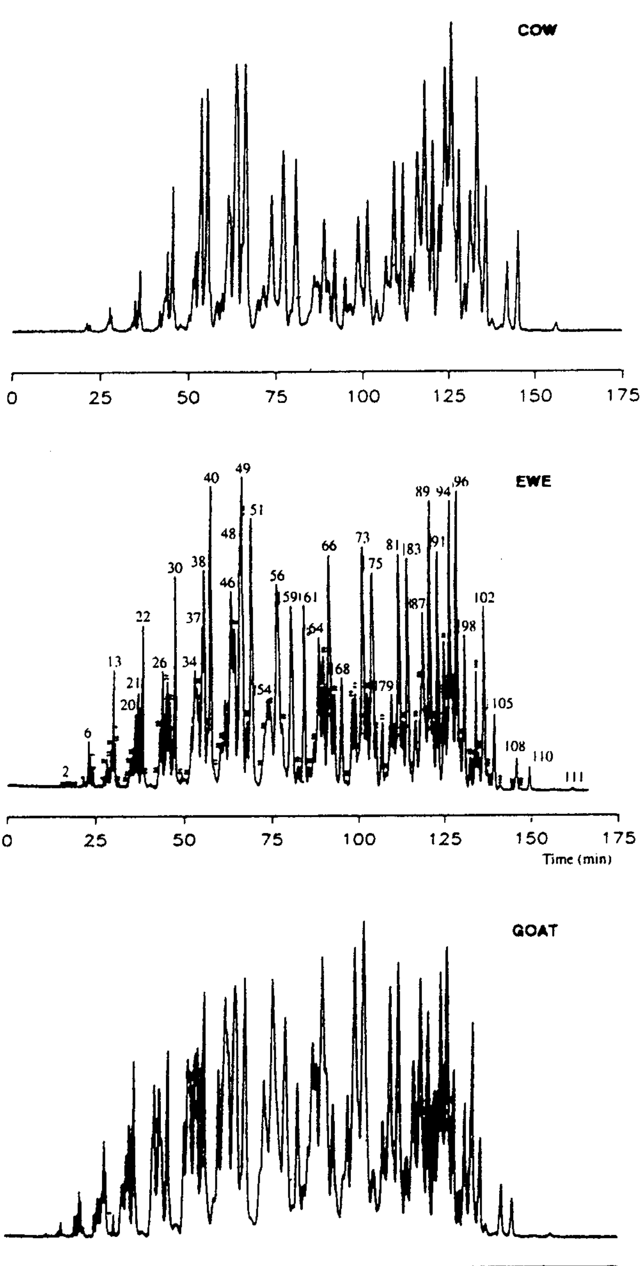


Fig. 43 HPLC analysis of cow's, ewe's, and goat's milk triglycerides. The numbers of the peaks correspond to Table 6.

trimargarin, 1,2-distearoyl-3-oleoyl glycerol, 1,2-distearoyl-3-palmitoyl glycerol and tristearin). The results obtained were:

$$\log k' = 0.72097 + 0.01919 \cdot \mathrm{CN} - 0.04121 \cdot \mathrm{NDB}$$

$$\mathrm{ECN} = \mathrm{CN} - 2.15 \cdot \mathrm{NDB}$$

$$\log k' = 0.72093 + 0.01919 \cdot \mathrm{ECN} \qquad (\mathrm{SE} = 0.0151)$$

Application of these two equations reduced the number of possible TGs to those containing adequate molecular parameters (CN, NDB) for each peak in their retention time.

One hundred and eighty-one molecular species of TGs have been identified: 79 of them were saturated, 44 monounsaturated, and 58 polyunsaturated. The majority of the unsaturated TGs (61) contained only one unsaturated fatty acid, 41 contained two, and 5 had all three fatty acids unsaturated. Furthermore, ten TGs that contained linear or branched odd-carbon-number fatty acids have been identified. In Table 6, identified species are mentioned with retention times and peak numbers corresponding to the chromatogram in Fig. 43.

One hundred and fifty-one molecular species of TGs have been described previously in milk fat (125,130); the other 30 are described in Ruiz-Sala's (129) analysis for the first time. Due to the fact that this identification of TGs is based not only on the estimation of ECN, but also on a fatty acids analysis of HPLC fractions of the TG, some of the species described previously by other authors have not been found in this study. Bornaz et al. (125) show 26 molecular species that contained linolenic acid. However, their study shows only 9, and they contained other fatty acids in higher amounts. The rest of them are not shown because they were found at less than 0.01%.

Their study agrees with the Myher et al. (130) estimation in the identification of TGs with odd-carbon-number fatty acids (CN = 15 and 17), either branched or linear. However, they have been able to differentiate between iso and anteiso isomers not only in the GLC analysis of fatty acids but also in TG estimation. Tridecanoic and nonadecanoic acids were identified by GLC but were not included in TG estimation due to their low amounts in whole milk fat content.

In agreement with Barron et al. (117) *trans*-vaccenic acid was taken into account, because it was found at 1.9% in the fatty acids analysis of the total TG fraction. The aforementioned study detected 116 TG molecular species instead of their 181, because they only considered 14 fatty acids for the calculation of the composition due to the lower sensitivity of their GLC analysis.

VI. OXIDIZED FATS

It is important to prevent the oxidation of edible fats and oils and of foods that contain oils to maintain their quality and safety. Oxidation of fats and oils can be initiated by heat, light, and metals in the fats and oils. The oxidation products from oils, which include hydroperoxides and cyclic peroxides, decompose to produce a variety of volatile compounds, which result in undesirable flavors and odors in oils (131–134). Oils damaged by oxidation also have been reported to cause biological problems, such as diarrhea, growth depression, and tissue damage in living organisms.

The oxidative stability of an oil depends on the fatty acid (FA) composition and triacylglycerol (TAG) structure, as well as on non-TAG components, such as tocopherols, carotenoids, ascorbic acid, citric acid, free fatty acids, and sterols, which may either prevent or promote oxidation. Several investigations have reported correlations of FA composition, TAG structure, and oxidative stability (135–140). For example, the oxidative stability of purified TAG from soybean oil (SBO) in air in the dark at 60°C is correlated positively with a greater concentration of oleic acid (O) and lower concentrations of linoleic (L) and linolenic (Ln) acids of SBO TAG.

Hydrogenation has been a useful process to increase the oxidative stability of edible oils, because it changes polyunsaturated FA to monounsaturated and saturated FA (141–143).

Hydrogenated oils can be suitable basestocks for margarine, shortening, and frying oil with improved stability and altered physical properties. But hydrogenation has not completely solved oxidative-stability problems, and there is increasing concern about the nutritional safety of partially hydrogenated oils.

Interesterification of edible oils is an important process for the modification of physical and functional properties, as are hydrogenation and fractionation.

Table 6 Identified TGs in Ewe's Milk Fat

Peak number	Retention time (min)	TG	Peak number	Retention time (min)	Peak TG	Peak number	Retention time (min)	TG
1	15.658	BuBuP	39	55.908	CoCaS			CaPL
2	16.602	CoCoM	40	56.421	BuMP	63	85.931	LaLaO
3	17.592	BuCop			BuLaS	64	87.193	CaMO
4	19.017	BuBuS	41	57.425	Bu*ai*PdP			CaMV
5	21.282	BuCaL			BuPdP	70	96.852	LaMO
		BuCyO			BuPdP			CoSO
6	22.614	BuCaM			CaCaPd	66	90.191	LaLaP
7	23.045	BuCyP	42	59.550	CyOLn			LaMM
8	23.927	BuCoS			CoOL			CaMP
9	26.805	CoCyO	43	59.746	CaMLn			CaLaS
10	27.632	BuCaO	45	60.817	BuOV			CyMS
11	28.112	CoCyP	51	67.890	LaLaLa			CyPP
12	29.017	BuLaM	46	62.248	CoPL	67	91.679	CoPS
13	29.329	BuCaP			CoMO			BuSS
14	29.908	BuCyS	47	63.140	BuPO			CaPd
15	31.033	BuLaPd			BuPV	68	93.732	CaOO
16	33.300	CoMyL	48	64.758	CoMP			LaOL
17	34.008	BuML			CaCaP	69	95.348	LaPL
18	34.258	CoCaO	49	65.084	BuPP			MML
19	34.782	BuLaO			BuMS			CaSL
		BuMyP	50	66.858	CoPdO			PaOL
20	35.393	CyCaM			Bu*ai*MaO	71	97.712	CaPO
		CoLaM			Bu*i*MaO	72	98.925	CySO
21	36.213	CoCaP			BuMaO			MMyP
22	37.427	BuMM	58	77.800	LaLaM	73	99.827	MMM
		BuLaP			CaLaM			LaMP
23	38.242	BuCaS[a]			CyLaP	74	101.043	CaPP
24	41.992	CaLnLn			CoLaS			LaLaS
25	42.344	CoOLn	52	71.308	CaOLn	75	102.663	CyPS
		BuOL	53	71.958	CyOL			CaMS
		CyMyPa	54	72.470	CoOO	76	103.922	CoSS
26	42.922	CaCaL	55	73.279	CoSL	77	105.654	OLL
		CyLaL			CaML			OOLn
27	44.349	CoML			CyPL			SLLn
		BuPL	56	73.253	CoPO	84	114.098	CySS
28	45.006	CyCaO			BuSO			PLL
29	45.983	CoLaO	57	76.827	CyMO	85	115.205	LOO
30	46.495	BuMO			CyLaS			SLL
		BuPPa			CaMPa			POL
31	48.725	BuMyS	65	88.462	CyPO	86	116.833	PSLn
32	49.650	N.I.			CaMM	87	117.232	MOO
33	51.119	CaLaLa	59	79.345	CyMP	88	118.050	PPaO
34	51.962	CaCaM			CoPP			MOV
35	52.596	CyLaM			BuPS	89	119.204	PPL
36	53.191	CyCyS			CaLaP			MSL
37	54.027	CyCaP	60	81.592	CaPaO			MPO
38	54.619	CoLaP	61	83.021	CyOO			MPV
		CoMM	62	84.705	LaML	90	120.220	LaSO

Table 6 Continued

Peak number	Retention time (min)	TG	Peak number	Retention time (min)	TG	Peak number	Retention time (min)	TG
91	121.571	MPP	97	127.975	MSO	104	137.041	N.I.
		MMS			PPV	105	138.014	PPS
		LaPS	98	129.358	PPP	106	139.829	MSS
		CaSS			MPS	107	143.005	PdSS
92	123.374	OOO	99	131.123	LaSS	108	144.391	SSO
93	123.883	LOS	100	132.587	SOO	109	145.142	SSV
94	125.013	POO	101	133.421	SOV	110	148.111	PSS
95	125.831	SLP	102	134.924	PSO	111	160.683	SSS
96	127.079	PPO	103	135.978	PSV			

[a] Bu: butyric; Co: Caproic; Cy: caprylic; Ca: capric; La: lauric; M: myristic; My: myristoleic; *ai*Pd: *anteiso*-pentadecanoic; Pd: pentadecanoic; P: palmitic; Pa: palmitoleic; *ai*Ma: *anteiso*-margaric; *i*Ma: *iso*-margaric; Ma: margarie; S: stearic; O: oleic; V: *trans*-vaccenic; L: linoleic, Ln: linolenic.

The oxidative stability of the product or margarine basestock obtained from SBO and methyl stearate by chemical interesterification with regioselectivity was evaluated and compared with that of the basestock from which FAs were randomized by H. Konishi et al. (144).

The degree of unsaturation or average number of double bonds and oxidizability is calculated from the FA composition as

$$\frac{0.02 \times [\mathrm{O}\ \%] + [\mathrm{L}\ \%] + 2 \times [\mathrm{Ln}\ \%]}{100}$$

Reverse-phase HPLC analysis indicated 26 triacylglycerol molecular species (TAGMS) in the products and the original SBO. The RP-HPLC chromatogram of the regioselective product is given in Fig. 44. In the regioselective product, LLS, LOS, SLP, SLS, and SOP contents were increased greatly compared with those in the original SBO. Also, the contents of LLL, LLO, LOP, OOO, and POO were decreased. Therefore, stearic acid was incorporated mostly in the soybean TAG that previously contained linoleic and oleic acids in the original SBO. This data, combined with the FA positional distribution of the original SBO and the regioselective product, indicated that stearic acid was located mostly on the 1(3) glycerol carbons of TAG.

As a result of the regioselective reaction, LOS, SLP, LLS, SOO, SLS, and SOP contents were increased moderately, and the LLL, LLP, and LnLL contents were decreased. The changes in TAGMS composition were the result of the random rearrangement of FAs concentrated on the 1(3) or 2 carbons of regioselective product TAG.

Reversed-phase HPLC has been used to analyze the oxidation products of triacylglycerols in edible oils. The detection is often based on monitoring the conjugated dienes with an ultraviolet detector (234–235 nm). However, the UV detector provides no information about oxidation products without a conjugated diene structure, e.g., products of oleic acid. Information about these compounds is important when oils with a high oleic acid content are studied. The most common universal detector types—refractive index and flame ionization detectors—are not sensitive enough to detect small amounts of oxidation products.

Viinanen and Hopia (145) described an evaporative light-scattering detector (ELSD) that can be used to detect autoxidation products of TAG standards [trilinolenin (TLn), trilinolein (TL), and triolein (TO)] and of a natural mixture of rapeseed oil (RSO) TAGs. The samples were oxidized at 40°C in the dark in open 10-ml test tubes. Sample aliquots of 500 mg were taken for

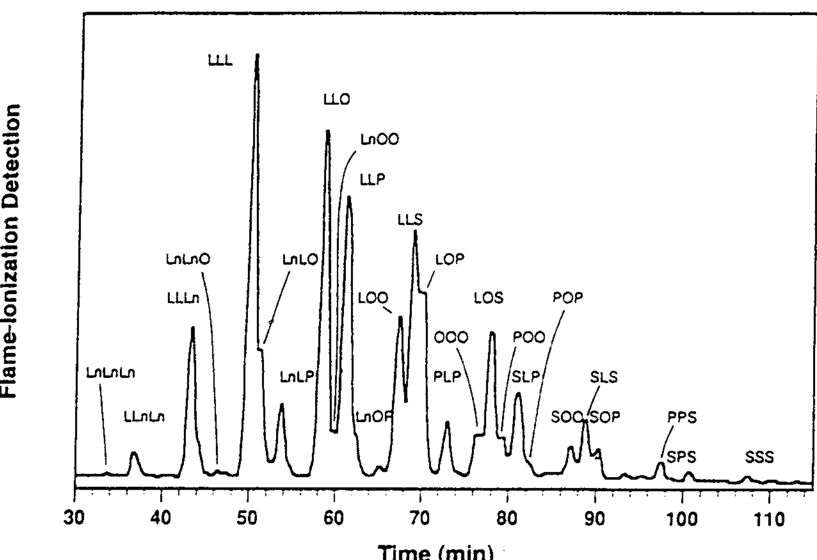


Fig. 44 Reverse-phase HPLC analysis of purified regioselective product triacylglycerols. Sample size: 0.5–1.0 mg; 5 μ C-18 column (0.49 × 50 cm); 120-min solvent gradient acetonitrile/methylene chloride (70:30 to 40:60, v/v); flow rate, 0.8 ml/min; flame ionization detector. Column cleaned with methylene chloride after analysis. L, linoleic; Ln, linolenic; O, oleic; P, palmitic; S, stearic.

analyses at regular intervals. Peroxide values (PVs) were measured by the colorimetric ferric thiocyanate method. Autoxidized samples were separated into polar and nonpolar components through solid-phase extraction (NH_2 stationary phase, Bond Elut 500 mg). The sample size for solid-phase extraction was 100–150 mg. After separation of the polar and nonpolar components, the polar fractions were concentrated into 1-ml dichloromethane and analyzed by RP-HPLC with a Nova-Pak C18 cartridge column (3.9 × 150 mm, 60 Å, 4 μm, Waters, Milford, MA).

The mobile phase was acetonitrile/dichloromethane/methanol (80:10:10, v/v/v), and the flow rate was 1.5 ml/min. The injection volume was 10–100 μl, representing 1–10 mg of original sample. Autoxidation products were detected with a Cunow DDL21 (Cunow Department DMS; Gergy St. Christophe, France) ELSD and a Waters 486 Tunable Absorbance Detector (UV, 235 nm). The gas used in the ELSD was compressed air filtered before the detector through a 0.45-μm filter. The gas pressure was 1.0 bar, giving a flow rate of 7 L/min. Makeup gas was off. The detector temperature was 87°C, and the photomultiplier sensitivity was 650 mV (gain area 400–800 mV). The RP-HPLC chromatograms of the autoxidized TO and RSO-TAG obtained from the UV and ELS detectors are shown in Figures 45–48.

Comparison of the two different types of detector, UV and ELSD, showed that the chromatographic profiles of the autoxidized samples are similar except for TO. Compounds without conjugated dienes in their structure cannot be detected with a UV detector at 235 nm; thus, the autoxidation products of TO are not detected. The detection limit of ELSD was 150 ng, quantified by 1,3-diolein and 2-monolinolein, and its sensitivity in detecting autoxidation products of TLn and TL approached that of the UV detector used in the study. The two detectors could therefore be used in series.

Each of the standard TAG chromatograms exhibits one main peak, which grows during the autoxidation process of the samples. These main peaks do not characterize one pure compound, because shoulders can be seen in them. Present knowledge about the oxidation of TAGs and the

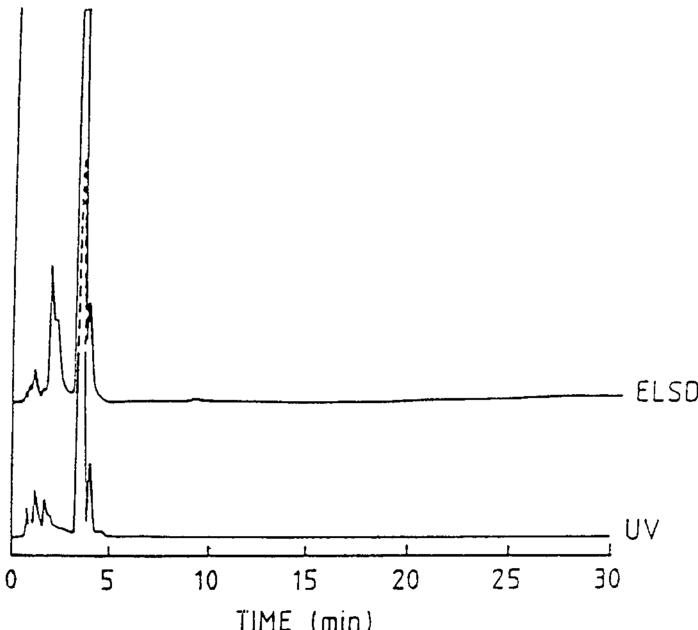


Fig. 45 Reversed-phase HPLC of autoxidized trilinolenin (peroxide value = 236.4 meq/kg). Nova-Pak C18 cartridge column (Waters, Milford, MA) (3.9 × 150 mm, 60 Å, 4 μm), mobile phase acetonitrile/dichloromethane/methanol (80 : 10 : 10). Ultraviolet (UV) detector (235 nm) and evaporative light-scattering detector (ELSD). Primary oxidation products, double peak at 3.6 min: secondary oxidation products elute before primary oxidation products.

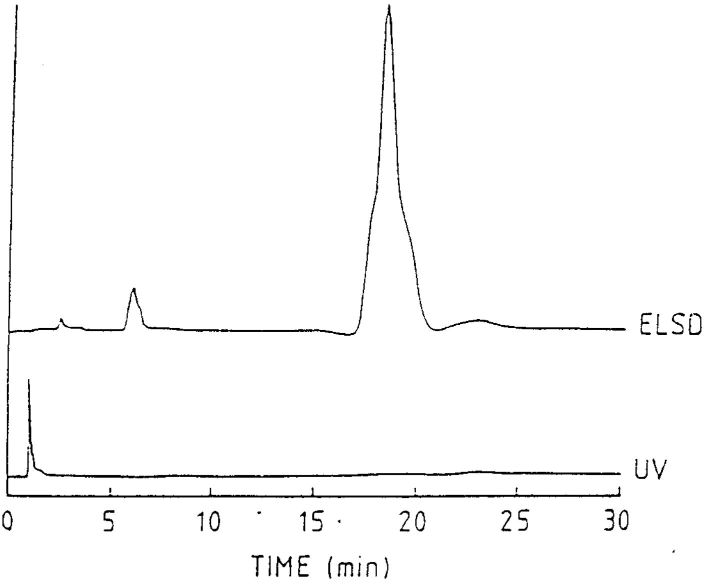


Fig. 46 Reversed-phase HPLC of autoxidized triolein (peroxide value = 149.7 meq/kg). See Fig. 45 for abbreviations and chromatographic conditions. Primary oxidation products, peak at 18.6 min, secondary oxidation products elute before primary oxidation products.

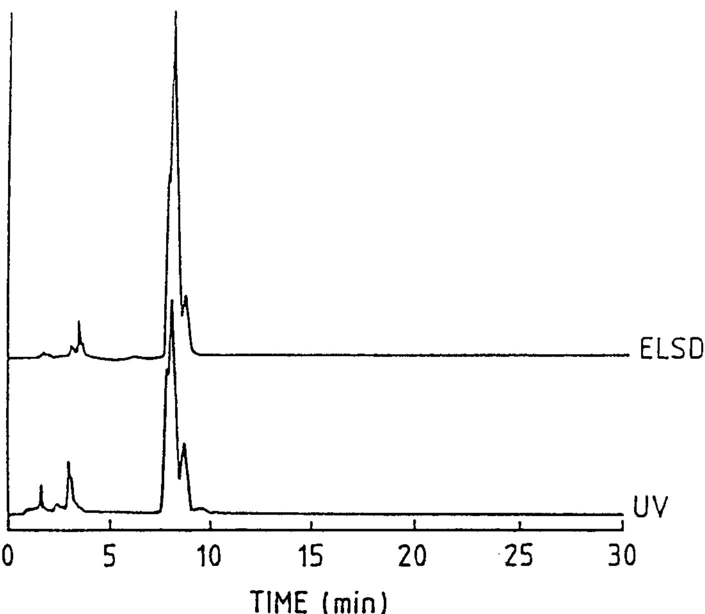


Fig. 47 Reversed-phase HPLC of autoxidized trilinolein (peroxide value = 198.2 meq/kg). See Fig. 45 for chromatographic conditions and abbreviations. Primary oxidation products, peaks at 7.4–9.1 min, secondary oxidation products elute before primary oxidation products.

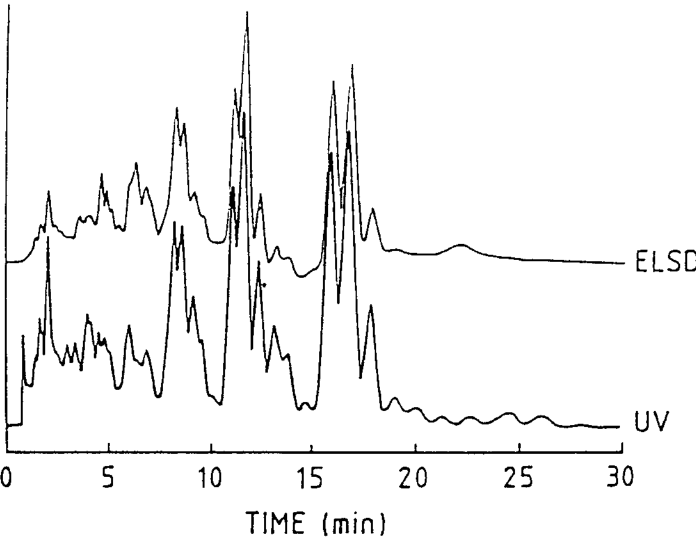


Fig. 48 Reversed-phase HPLC of autoxidized rapeseed oil triacylglycerols (peroxide value = 393.8 meq/kg). See Fig. 45 for abbreviations and chromatographic conditions. Peaks correspond to both primary and secondary oxidation products of rapeseed oil triacylglycerols.

capabilities of RP-HPLC methods to separate different compounds suggest that these main peaks are mixtures of different geometric isomers of monohydroperoxides (146).

The peaks in the chromatograms most likely represent not only primary but also secondary oxidation products of TAGs. According to Neff and co-workers (140), the minor peaks eluting before the main peaks of standard compounds illustrate secondary oxidation products, such as hydroperoxy epidioxides or *bis*- or *tris*-hydroperoxides.

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6
Phosphopholipid Analysis by HPLC

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I. INTRODUCTION

Phospholipids are lipids containing a glycerol backbone with two fatty acyl chains. The third hydroxyl group is linked to the phosphate, to which an organic group may be esterified. Both the nature of the polar headgroup and the esterified fatty acids influence the physicochemical properties of these natural surfactants (1–8). Phospholipids are the main components of all cell membranes, so they occur in all living organisms, plants as well as animals, and hence in most food products derived from them. Besides being a natural component of various food products, phospholipids are also added as a functional ingredient (2–8). Hence, they are used as emulsifiers in foods such as margarines, mayonnaises, chocolates, and ice cream, as baking improvers and antistaling agents in bread and bakery products, as wetting agents in instant products, and as antioxidants in vegetable oils.

In commercial formulations, phospholipids are not available as pure products. Mostly they are obtained as a by-product of the process of refining vegetable oils, during the so-called degumming step (3,4), from which a liquid-to-pasty product is obtained that is referred to as *lecithin*. Lecithin contains about 65% phospholipids plus about 30% residual neutral lipids and minor amounts of glycolipids. For historical reasons, most commercially available lecithins are derived from soybean oil, but lecithins of other oils could be used as well. From this discussion it follows that the determination and quantification of phospholipids is of importance both to control how efficiently the phospholipids have been removed from vegetable oils and to control the quality of the lecithin. For this purpose it is important to know not only the total amount of phospholipids but also the amount of the different types of phospholipids present, because it is well known that the functional properties of the various phospholipids differ widely (2–8).

Historically, thin-layer chromatography (TLC) was widely used to separate phospholipid classes, for it was a simple and rather inexpensive method (9,10). Especially using two-dimensional TLC, very good separations were achieved. Unfortunately, quantitative detection was a large problem. Initially, the quantification was accomplished mostly by determining the phosphorus content of the spots that were scraped from the plates (9). Nowadays, a much faster method is to scan the TLC plates after staining or charring (10). However, the use of these sophisticated, computer-controlled in situ scanners is responsible for the fact that modern TLC can no longer be selected as a cheaper alternative to HPLC.

Another alternative solution to circumvent the detection and quantitation problems of classical TLC involves the use of silica-coated quartz rods, so-called *chromarods*. This has been pro-

posed because the separated compounds could be detected using a flame ionization detector (10,11). Although the latter should theoretically perform as a mass-sensitive detector, nevertheless large differences in response were observed for different phospholipid classes and even for different molecular species within the same class. Besides, the response deviates from linearity, and rod-to-rod variability badly affects the reproducibility of both retention times and peak areas.

Nowadays, ^{31}P-NMR (nuclear magnetic resonance) is sometimes proposed as a better alternative, because this technique requires only a minimum amount of sample pretreatment (12–14). In our opinion, its high investment cost and need for highly qualified operators will cause P-NMR to remain a research technique rather than a quality-control method. However, being an absolute technique, P-NMR seems ideally suited to certify the composition of standard solutions whose quality largely determines the accuracy of both TLC and HPLC results (15,16).

The separation of phospholipids by micellar electrokinetic capillary electrophoresis (MEKC) has been described (17–19). In this technique, solutes are separated based on their distribution between a mobile (usually aqueous) and a pseudostationary (micellar) phase. Szucs et al. found that the major soybean phospholipids were fully resolved in only 7 minutes using deoxycholic acid for micelle formation in combination with 30% *n*-propanol at 50°C (18). However, quantification of the separated compounds remains troublesome. This is due first of all to the fact that only UV detection can be used, thus making the response highly dependent on the degree of unsaturation of the phospholipids. Besides, the comparison of peak areas in MEKC is more complicated than in HPLC, because all compounds are moving with different velocities.

Supercritical fluid chromatography (SFC) has also been used in phospholipid analysis. According to Lafosse et al., phospholipid classes can be separated by SFC using a simple isocratic solvent consisting of 78.4/21.6 (w/w) mixture of carbon dioxide and a mixture of methanol, water, and triethylamine (95/4.95/0.05) in combination with a Zorbax Sil stationary phase; detection was performed by evaporative light-scattering (20).

Up to now, however, HPLC has remained the method of choice. This is due mainly to the fact that this technique is much easier to automate as compared to TLC. Besides, a wide variety of stationary and mobile phases are available, so the technique is highly flexible. Besides, the investment cost is much lower as compared to P-NMR, whereas quantitation is more straightforward than in MEKC. Hence, the more recent official methods for the analysis of phospholipids, as proposed by the American Oil Chemists' Society (AOCS), by the International Union for Pure and Applied Chemistry (IUPAC), and by the International Lecithin and Phospholipid Society (ILPS), all use HPLC. In this chapter, a review is presented of HPLC procedures that have been described during the past 10 years; the older literature was discussed in a previous edition of this handbook (21).

II. THE CHEMISTRY OF PHOSPHOLIPIDS

Glycerophospholipids contain a glycerol skeleton to which two fatty acids are esterified; saturated fatty acids occupy mostly sn-position 1, whereas unsaturated fatty acids are mainly present on sn-position 2. The third hydroxyl is linked to a phosphate group to which an organic base is mostly esterified (Fig. 1). The most important components of soybean lecithin are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). Phosphatidic acid (PA) may become important due to the presence of phospholipase D: this enzyme slowly converts PC into PA in vegetable lecithins. Phosphatidylserine (PS), phosphatidylglycerol (PG), and lysophosphatidylcholine (LPC) are known as minor components; lysophospholipids contain only one acyl group per molecule. Besides, ether phospholipids occur in which one or both fatty acyl

With : -X =

$-CH_2CH_2N^+(CH_3)_3$ *Phosphatidyl choline [PC]*

$-CH_2CH_2N^+H_3$ *Phosphatidyl ethanolamine [PE]*

(inositol ring: OH OH OH OH OH) *Phosphatidyl inositol [PI]*

$-H$ *Phosphatidic acid [PA]*

$-CH_2CH(COO^-)N^+H_3$ *Phosphatidyl serine [PS]*

$-CH_2CHOHCH_2OH$ *Phosphatidyl glycerol [PG]*

$CH_3(CH_2)_{12}CH{=}CHCHOHCH(NH{-}C(=O){-}R)CH_2{-}O{-}P(=O)(O^-){-}O{-}CH_2CH_2N^+(CH_3)_3$

Sphingomyelin [SPH]

Fig. 1 Chemical structures of the main phospholipids.

groups is replaced by an aliphatic residue linked either by an ether or a vinyl ether bond; in the latter case plasmalogens originate.

Sphingomyelin (SPH) is the major representative of the sphingophospholipids. This phospholipid consists of a ceramide unit, containing a fatty acid linked by an amide bond to the amine group of a long-chain base called *sphingosine*, linked at position 1 to phosphorylcholine (Fig. 1). It is a major component of animal complex lipids, but is not present in plants or microorganisms.

From the structure of the phospholipids, presented in Fig. 1, it is obvious that these compounds have a pronounced amphiphilic character: besides the strongly lipophilic fatty acid chains, they contain a hydrophilic part consisting of the phosphate group and the esterified organic base. Hence, if a separation of different phospholipid classes is desired, the stationary phase should interact with their polar part, so normal-phase HPLC is preferred. On the other hand, a separation

of the different molecular species within one phospholipid class is accomplished by reversed-phase HPLC, causing the phospholipids to elute according to their fatty acid composition.

III. SAMPLE PREPARATION AND DERIVATIZATION TECHNIQUES

A. Extraction

Prior to phospholipid analysis, it is imperative to extract the lipids from their matrix and free them of any nonlipid contaminants. Phospholipids are generally contained within the lipid fraction, which may be recovered by the traditional Bligh and Dyer or Folch extraction procedure (9,22). In any phospholipid extraction method it is recommended to include a rather polar solvent in addition to a solvent with high solubility for lipids. The former is needed to break down lipid–protein complexes that prevent the extraction of the lipids in the organic phase. Traditionally, mixtures of chloroform and methanol (especially 2:1, v/v) have been recommended. These are washed with water or aqueous saline to remove nonlipid contaminants. Comparing the recovery of phospholipids, Shaikh found that the neutral phospholipids PC, PE, SPH as well as DPG were nearly quantitatively extracted by all solvent systems studied (Table 1), although Bligh and Dyer, in which the lower phase was removed only once, was somewhat worse (23).

However, Shaikh demonstrated that the aforementioned traditional methods are inappropriate to recover completely lysophospholipids as well as acidic phospholipids: classical Folch gave 85–90% recovery of LPC and LPE, whereas Bligh and Dyer yielded only 75–80% recovery. Extraction with a mixture of chloroform and methanol, on the other hand, provided nearly complete recovery of acidic and lysophospholipids, but up to 15% losses were observed during subsequent washing, according to Folch. These losses could be circumvented by purification of the crude extract on Sephadex G-25, but this column chromatographic procedure is quite time-consuming.

As far as the acidic phospholipids are concerned, the recovery of PI, PS, PA, and PG was limited to only 30–50% by Bligh and Dyer extraction, whereas Folch extraction yielded about 85% recovery of PI and PS but only 20% and 35% recovery of PA and PG, respectively (Table 1). The poor recovery of acidic PL is thought to be due to adsorption of PL to proteins in a neutral

Table 1 Recoveries (in %) of Individual Phospholipids (PLs) by Different Extraction Techniques

	Extraction technique				
PL class	Folch/column	Classical Folch	Bligh & Dyer	Acid butanol	Modified Folch
PC	100%	94%	85%	98%	100%
PE	99	92	83	99	99
SPH	100	99	98	100	99
DPG	95	88	84	97	97
PI	97	87	40	97	99
PS	98	84	43	99	100
PA	86	22	23	97	98
PG	93	35	32	97	98

Source: From Ref. 23 with permission of *Analytical Biochemistry*.
Phospholipid standards representing 6.0-μg P each were added to the respective solvent mixtures; following extraction, the phosphate content was measured.

biphasic system. Besides, the presence of metal ions badly affects the recovery of acidic PL, which become tightly bound to proteins, as well as of FFA and lysophospholipids, which form water-soluble complexes. A solvent containing dilute acid is required to break ionic complexes and lipid–protein associations, thereby minimizing losses during extraction. Hence, Shaikh proposed a modified Folch extraction procedure, that allowed highly reproducible quantitative analyses of all lipid fractions, including lysoPL, FFA, and acidic phospholipids. The last involved extraction of the tissue with a C/M/saline biphasic system. The upper phase was re-extracted twice with pure Folch lower phase and once with lower phase containing HCl. The last extract was neutralized with NH_3 vapor. A detailed study of the recovery after each individual step of this procedure revealed that up to 78% of lyso-PL and 90% of neutral PL can be recovered with a single neutral solvent (Fig. 2). Two additional extractions with neutral lower phase produced nearly complete recovery of PE and PC, but up to 8% of lyso-PL still remained in the upper phase. These were recovered by acidic lower phases. In addition, both the Bligh and Dyer and the Folch procedures are troublesome for products with a low lipid content and a high protein content, such as whey protein concentrate (WPC), not only because of the presence of stable lipid–protein complexes, but also because the protein present prevents phase separation by forming a stable emulsion. In addition, the Roese–Gottlieb and Mojonnier methods, used extensively for extraction of lipids in dairy products, are also not appropriate for PL extraction, because the applied heat treatment and the addition of acid or base may induce oxidation and/or hydrolysis of the phospholipids (24).

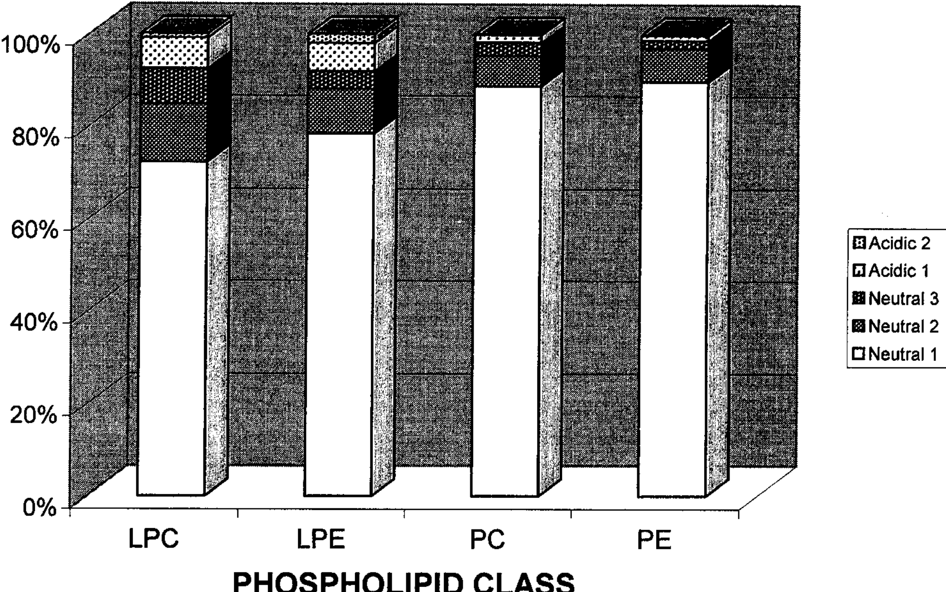

Fig. 2 Successive distribution of pure isolated, radiolabeled phospholipids mixed with bovine serum albumin in C/M/NaCl and C/M/HCl biphasic mixtures. Pure ^{32}P-labeled phospholipids bound to defatted bovine serum albumin were extracted by a modified Folch technique, as described in the text, and radioactivity was determined in successive lower phases. "Neutral 1-3" and "Acidic 1-2" represent successive lower phases that were withdrawn from neutral and subsequent acidic biphases, respectively. (Adapted from Ref. 23.)

In order to prevent the formation of a stable emulsion at any stage of the extraction procedure, the water content of the hydrated WPC has to be controlled so as not to obtain a biphasic solvent system during extraction with mixtures of chloroform and methanol. Besides, nonlipid contaminants are removed from the extract by gel filtration on nonlipophilic Sephadex G-25 instead of traditional aqueous washing: total lipids were eluted with a 19/1 (v/v) mixture of chloroform/methanol, saturated with water, whereas a 1/1 (v/v) mixture of water and methanol eluted nonlipid contaminants. The method yields a similar total lipid content to the Folch method, but it is about four times faster (24).

Given that chloroform is believed to give rise to birth defects and may also be carcinogenic, Heitman et al. tried five alternative solvent combinations in order to replace the traditional C/M/W combination (25). Six different solvent mixtures were selected, so the total elution power of C/M/W (4/8/3) calculated from the eluotrope order–taking silica gel as the reference adsorbent was nearly the same. The solvent mixtures 1-propanol/water (4/1), MIBK (methylisobutylketone)/M/W (40/80/30) and petroleum ether/2-P/W (40/112/38) were claimed to be excellent alternatives for C/M/W (40/80/30) in order to extract both phospholipids and glycosphingolipids from animal tissues. Nevertheless, the mass of the total lipid extracts from mouse hybridoma cells for all three alternatives was only 85% of the mass extracted with C/M/W. The latter was confirmed by Vaghela and Kilara: they found an at-least 15% higher recovery using chloroform and methanol as compared to alternative extraction procedures that use hexane and 2-propanol (24). According to Vaghela and Kilara this may be due to the inability of hexane and 2-propanol to break the tertiary structure of whey proteins, which is essential for breaking the lipid–protein interactions. Hence, these less toxic solvents seem inappropriate to extract total lipids from high-protein products.

Extraction of fat by supercritical carbon dioxide was investigated as an important option for minimizing the expanded use of frequently flammable and carcinogenic solvents in food analysis. Unfortunately, the presence of moisture in foods has an adverse effect on the quantitative extraction of fat by supercritical fluid extraction (SFE). Hence, samples have to be lyophilized first. The total fat content of freeze-dried meat and oilseed samples was found to be comparable to values derived from Soxhlet-extracted samples (26). Besides, only small amounts of residual lipids could be recovered by an additional extraction of the SFE-extracted matrix by the Bligh and Dyer solvent extraction procedure. As far as the minor constituents are concerned, it was found that the extraction recovery ranged from 99% for PC to 88% for PA. Hence, Snyder et al. concluded that SFE can be used as a rapid, automated method to obtain total fat, including total phospholipids, from foods (26).

Horne and Holt-Larkin introduced an alternative approach to extract PL from very dilute aqueous media using styrene-divinylbenzene (SDB) Empore disks (27). The latter have small SDB particles (10 μm) enmeshed in a PTFE backbone and combine the adsorbing qualities of the SDB macroreticular resin in a convenient disk form. The PL present in the aqueous dispersion adsorb to the disk, which is subsequently washed with water. In the elution step, 8 ml of methanol is used, followed by 8 ml of a mixture of C/M/NH_4OH (80/19.5/0.5). The recovery of samples spiked with 5 μg of five different phospholipids was 92% on the average for most phospholipids, but only 65% of PS was obtained. Nevertheless, the recovery of the neutral phospholipids was at least comparable to that obtained for liquid–liquid extraction and was clearly superior for the acidic PL. In addition, the use of solvent and time required for sample preparation was reduced by roughly 80% relative to liquid–liquid extraction. And solvent impurities become less problematic during the subsequent concentration of the extracts. The capacity of the 47-mm disks for total phospholipids was found to exceed 0.3 mg. The SDB filtration method is flexible, since sample size is easily varied from 1 ml up to 1 L with no change in the operating procedure.

B. Fractionation and Preconcentration

Because the PL concentration in the total lipid extract is generally rather low, a preconcentration step is generally required prior to HPLC analysis; crude vegetable oils e.g., contain only 0.5–2.0% of phospholipids.

Traditionally, fractionation of the lipid extract was accomplished using open glass columns containing rather coarse and irregular silicic acid particles (9). Neutral lipids were eluted by chloroform, glycolipids were recovered using acetone, and methanol was used to obtain the phospholipids. Thus, Mounts et al. isolated PL from 5 g of oil on a 10-g column of silica gel (60–200 mesh) by sequential elution with 200 ml of chloroform, 100 ml of acetone, 100 ml of methanol, and 100 ml of 0.1% phosphoric acid in methanol (28). The last two fractions were combined to recover the phospholipid fraction.

Recently, however, prepacked solid-phase extraction (SPE) columns have been preferred. In this case, too, the phospholipid fraction is often recovered by elution with methanol. Thus, Nash and Frankel developed a method to recover the phospholipids from 1-g portions of crude oils using silica gel Sep-Pak cartridges (29). The oil was applied as a 10% solution in a 95/5 (v/v) mixture of petroleum ether and diethyl ether, and neutral lipids (about 94–97%) were eluted with 10 ml of additional solvent; the so-called unsaponifiables (2–3%) were recovered using 20 ml of diethyl ether, and the phospholipid fraction was obtained using 10 ml of methanol.

A similar procedure, originally developed by Kaluzny et al. (30), was used by Caboni et al. (31) and Vaghela and Kilara (24). An amino phase was preferred to purify the PL fraction by SPE. Caboni et al. applied 250 mg of lipids, dissolved in 250 μl of chloroform, to a 500-mg column, whereas Vaghela and Kilara used only 80 mg of total lipids at the most, dissolved in less than 2 ml of chloroform, with Mega Bond Elut columns containing 2 g of amino propyl packing. After preconditioning with hexane, neutral lipids, free fatty acids, and phospholipids are sequentially eluted with chloroform/2-propanol (2/1), 2% acetic acid in diethyl ether, and methanol (Table 2). Vaghela and Kilara determined gravimetrically that the recovery of the total lipids after SPE was more than 99%, and Caboni et al. found that 99% of the PL fraction of ground meat was recovered.

However, in a more recent paper, Caboni et al. described that the aforementioned method was not applicable for all types of food products (32). Hence, only 30% and 53% of the PL of a total lipid extract of cheese and dried egg powder, respectively, were recovered. High-performance liquid chromatography analysis revealed that the acidic phospholipids PG and PI were not

Table 2 Recoveries of Various Lipid Classes (in %) After SPE Using Bond Elut-Aminopropyl Disposable Cartridge Column

	Lipid class							
Fraction	Ch	FFA	PC	PE	SPH	PI	PS	PA
A	102.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.7%
B	0.6	102.4	0.0	0.0	0.0	0.0	0.0	0.0
C	0.0	0.0	100.2	97.4	97.2	0.0	0.0	0.0
D	0.0	0.0	0.0	3.8	0.0	95.6	107.0	101.7

Source: From Ref. 33 with permission of *Journal of Lipid Research*.

Fraction A was obtained by a 2/1 mixture of chloroform and 2-propanol, fraction B was recovered using 2% acetic acid in ether, fraction C was eluted by methanol, and fraction D resulted from elution by a 420/350/100/50/0.5 mixture of hexane, 2-propanol, ethanol, 0.1 M aqueous ammonium acetate and formic acid.

recovered in the PL fraction (Table 2). A similar observation was also described by Kim and Salem (33). Different approaches have been proposed to overcome the limited recovery of the phospholipid fraction. As far as the mobile phase is concerned, both the inclusion of water as a more polar solvent and the use of a pH modifier such as ammonia or phosphoric acid have been suggested.

Kim and Salem (33) found that the acidic PL were eluted from an aminopropyl bonded phase with over 95% recovery with 4 ml of a mixture of H/2-P/Ethanol/0.1 M aqueous NH_4-acetate/formic acid (420/350/100/50/0.5) containing 5% phosphoric acid (Table 2). Neither the solvent mixture without phosphoric acid nor methanol containing 5% phosphoric acid was able to elute the acidic PL. Actually, Table 2 indicates that a fractionation of both neutral and acidic PL is enabled by the sequential elution with methanol and the aforementioned solvent mixture. In order to remove the phosphoric acid from the acidic PL fraction, it is first dried under N_2 for 10 min to remove the hexane and then extracted three times with 1 ml of chloroform after the addition of 1 ml of water.

Carelli et al., on the other hand, elaborated a basic (rather than an acidic) fractionation scheme to collect the PL present in a chloroform solution of 50–150 mg of sunflower oil: most triglycerides were recovered from a 500 mg of bonded diol SPE cartridge using 2.5 ml of chloroform, whereas PL were obtained by elution with 7 ml of methanol containing 0.5 ml/100 ml of a 25% ammonia solution (34). Using this procedure, the recovery of more than 98% pure soybean PE, PA, PI, and PC standards ranged from 94% to 107% at concentrations of 0.1, 0.6, and 1.0% in purified sunflower oil; the coefficient of variation was typically of the order of magnitude of 5%.

Instead of modifying the mobile-phase composition, Caboni et al. suggest changing the solid-phase chemistry (32). Using reverse-phase C_8 cartridges, phospholipid recoveries ranging from 93.0% to 99.7% (Table 3) were obtained for various foods, such as egg powder, chicken meat, ripened cheese, and salami. In the method proposed, 50–200 mg of total lipids in 0.5 ml of chloroform/methanol (2/1) is applied on a 500-mg C_8 cartridge that is preconditioned with 3 ml of methanol. Elution is carried out by adding 4 ml of methanol, 5 ml of chloroform/methanol (3/2), and 5 ml of chloroform. Phospholipids elute in the first fraction. Although this method seems to be ideally suited to preconcentrate phospholipids in the total lipid extract, it is important to realize that it is not applicable to quantifying the total PL fraction gravimetrically, because the percentage of non-PL, such as MG, DG, and FFA, in the SPE fraction ranged from 22% to 43%.

For the sake of completeness, it must be mentioned that two additional solid phases were also included in this study (Table 3). However, only 43% and 23% of the PL present in the lipid extract of egg powder were recovered from a C_{18} and a silica column, respectively (32). Table 3 reveals that the PL recovered from C_{18} were depleted in the neutral phospholipids PE, PC, and

Table 3 Average Concentrations (in mg/g) of Single Phospholipids in Egg Powder, Obtained via Different SPE Purification Methods

	Phospholipid							
Phase	PG	PE	PI	PC	SPH	LPC	Total	Recovery (%)
C8	0.6	17.0	1.3	79.0	9.4	2.9	110.2	98.0
C18	0.6	8.3	1.4	34.0	1.8	1.8	47.9	42.6
Silica	0.2	12.0	1.3	9.6	1.1	1.3	25.5	22.7
NH_2	0.0	15.6	0.0	20.1	1.4	1.3	38.4	34.1

Source: From Ref. 32 with permission of the American Oil Chemists' Society.

SPH, whereas the silica-phase fraction showed a particularly low content of PC. For the C_8 phase, no selectivity was observed, resulting in nearly complete recovery.

Singleton further automated the preconcentration step of PL from crude vegetable oil by including it in the HPLC procedure (35). Repeated injections of 4 mg of crude oil were made on a 2-cm silica (40 μm) column every 12 s until the sample was sufficiently enriched. Hexane was used as the eluting solvent during this enrichment process. The neutral lipids were eluted to the waste flask. The HPLC system was then flushed with the starting solvent containing 2-propanol and hexane in a 4-to-3 ratio. The precolumn was attached to the 250 × 4.6-mm 5-μm silica column, and a gradient was started with a final composition of 2-propanol/hexane/water (8/6/1.5). Using this procedure, the solvent removal of the PL fraction eluted from the preconcentration column was avoided, thus providing a 40% reduction in analysis time and less solvent usage and reducing the risk of lipid deterioration during the transfer between the concentration and analysis steps. In a more recent paper, this whole procedure was completely automated using an HPLC system consisting of two pumps, a 50 × 4-mm concentrator column (40 μm silica) and a 100 × 8-mm analytical column (6 μm silica) and two sixway valves (36).

Actually, solid-phase extraction is used not only as a rough preliminary fractionation procedure. Prieto et al. described the complete fractionation of the total lipids from wheat into eight neutral lipid, two glycolipid, and four phospholipid classes: in addition to PC and LPC, *N*-acyl PE and *N*-acyl LPE were detected (37). However, two separate stationary phases (silica and aminopropyl) as well as seven different mobile phases were needed. Moreover, 14% cross-contamination of PC and LPC was observed, and the recovery of the phospholipids was limited to about 85%. Hence, SPE is a rapid and efficient technique for preliminary fractionation, but loses its advantages if more complex separations are tried.

C. Derivatization

Derivatization may be used either to facilitate separation or to improve detection. In the former case the derivatization has to be included during the sample pretreatment. Improved detector sensitivity, on the other hand, may be accomplished by either pre- or postcolumn derivatization.

Derivatization is never used in the analysis of the different phospholipid classes present, but is very widely used in the analysis of molecular species. The main reason for (mostly precolumn) derivatization in this case is to reduce significantly the detection limits. To this end, both UV-absorbing and fluorescent derivatives are frequently used, as described in more detail in Secs. IV.B and V.3.

D. Storage of Phospholipids

According to Caboni, it is important to carry out HPLC analyses of the purified PL fractions without frozen storage, because the latter operation leads to selective insolubilization of some components, especially PE (32).

IV. SEPARATION TECHNIQUES

A. Phospholipid Classes

Because HPLC methods were originally elaborated to replace TLC separations, the stationary- and mobile-phase selection was largely inspired by previous experience with TLC. Hence, normal silica was used mostly as the stationary phase, although both aminopropyl- (43–46) and diol-modified silica (47,48) have become increasingly popular. Besides, smaller column diameters are

now frequently preferred: instead of the traditional 4.6-mm-ID column, columns with inner diameter of only 1–4 mm are widely used (32,54,55,57,77).

As far as the mobile phase was concerned, however, the traditional TLC solvent systems, which were based mainly on chloroform, methanol, and water, could not be used because of their incompatibility with UV detection. Two main alternatives have been proposed during recent decades: besides combinations of acetonitrile, methanol, and water (Table 4), mixtures of hexane, 2-propanol, and water (Table 5) are frequently applied (21). In order to reduce retention time variability, different types of modifiers have been suggested to be added to the aqueous component. Modifications of these two traditional solvent systems compatible with UV detection are still widely used, even though UV detection has been largely replaced by evaporative light-scattering detection (ELSD). However, a third group of mobile phases has originated from the use of ELSD: because solvents do not need to be UV transparent anymore, mixtures of chloroform, methanol, and an aqueous component are becoming increasingly popular in HPLC analyses too (Table 6).

1. Acetonitrile-Based Mobile Phases (Table 4)

The main advantage of acetonitrile-based mobile phases is their ability to resolve (nearly) all phospholipid classes using a single isocratic mobile phase. Besides eliminating the need for expensive gradient hardware, this also produces a very stable baseline in UV detection. In addition, column re-equilibration in between subsequent injections can be omitted, resulting in faster

Table 4 Recent HPLC Phospholipid Class Separation Methods Using an Acetonitrile-Based Mobile Phase

Stationary phase	Mobile phase	Detection	(Phospho)lipids	Ref.
Plain silica				
Porasil, 10 μm	A/M/H_3PO_4 (130/5/1.5)	UV (205 nm)	PE, PC, LPC	38
Ultrasphere Si, 5 μm	A/M/85% H_3PO_4 (100/10/1.8)	UV (203 nm)	PS, PE, PC, SPH	42
Nucleosil-100, 7 μm	A/H/M/H_3PO_4 (918/30/30/17.5)	UV (206 nm)	PI, PS, PE, PC, SPH	40
Ultrasphere Si	A/M/H_2SO_4 (100/3.5/0.05)	UV (202 nm)	PI, PS, PE, PC, LPC, SPH	41
LiChrocard Si-60, 5 μm	A/M/0.2% H_3PO_4 (gradient)	UV (205 nm)	DPG, PI, PS, PE, LPE, PC, SPH	39
Diol-bonded silica				
LiChrospher Si100, 5 μm + LiChrospher diol, 5 μm	A A/M/H_3PO_4 (93/5/1.5)	UV (205 nm)	GL, PI, PS, PE, PC, LPC, SPH, LPE	47
LiChrospher 100 diol, 5 μm	A; A/W (80/20); A/HAc (99.5/0.5)	UV (200 nm) ELSD	PI, PE, PC, SPH	48
Amino-bonded silica				
Nucleosil 5 NH_2, 5 μm (50 × 4.6 + 175 × 4.6 mm)	A/M/W/MPA (1460/500/30/0.3)	UV (205 nm) + fluorescence	PC, SPH, LPC, PG, PE, PI, PS	43
Zorbax NH_2, 5 μm	A/M/10 mM $NH_4H_2PO_4$ (pH 4.8) (64/28/8)	UV (206 nm) RI	PC, PG, LPC, LPG	44
Daisopak-SP-120-5-APS (aminopropyl)	A/M/10 mM $NH_4H_2PO_4$ (pH 5.8) (61.5/26.4/15)	UV (205 nm)	PC, SPH, LPC, PE, PI, PS	45
Spherisorb S5 NH_2 or: Zorbax NH_2, 5 μm	A/M/0.1 M NH_4Ac (pH 4.8) (52/32/16)	ELSD	Ch, PC, LPC, PG, LPG	46

The phospholipids are mentioned in the order that they were eluted from the column.

Table 5 Recent HPLC Phospholipid Class Separation Methods Using a Hexane/2-Propanol–Based Mobile Phase

Stationary phase	Mobile phase	Detection	Phospholipids	Refs.
Plain silica				
LiChrosorb Si-60, 10 μm	H/2-P/W (gradient)	UV	PE, (PI, PA), PS, PC	49, 50
Silica gel, 5 μm	H/2-P/W (6/8/0–1.5)	UV	PA, PG, PE, PI, PC	51
Spherisorb S3W, 3 μm	H/2-P/W (58/39/3.2) & (55/44/5)	ELSD	PE, PI, PA, PC	52
Spherisorb 80A, 3 μm	H/2-P/W (40/52–58/2–8)	ELSD	Ch, FFA, PE, PS, PC, SPH	53, 54
LiChrosorb Si-60, 5 μm	H/2-P/W (40/52–58/2–8)	ELSD	Ch, FFA, PE, PS, PC, SPH	53, 54
LiChrosorb 7 μm Si-60	i-C8/THF (99/1): 40–100%; 2-P; W: 0–9%	FID	FFA, DPG, PE, PG, PI, PA + PS, PC, LPC	55
LiChrosorb Si-60, 7 μm	H/THF (99/1): 40–100%; 2-P; W: 0–9%	ELSD	TG, FFA, GL, NAPE, PE, PG, LPE, PC, LPC	56
LiChrosorb Si-60, 5 μm	H (40–100%); 2-P; 0.04% triethylamine (0–9%)	FID	FFA, DPG, PE, PG, PI, PA + PS, PC, LPC	57
LiChrosorb Si-60, 5 μm	H/2-P/Ac (pH 4.2) (8/8/1)	UV (206 nm)	PE, PI, PA, PC	34, 38, 18, 58
LiChrosorb Si-60, 5 μm	(A) isooctane/THF (99/1); (B) 2-P/C (4/1); (C) 2-P/W (1/1)	ELSD	PE, PI, PS, LPE, PC, SPH, LPC	61
Spherisorb S3W, 3 μm	(A) isooctane/THF (99/1); (B) 2-P/C (4/1); (C) 2-P/W (1/1)	ELSD	DPG, PE, PI, PS, PC, SPH, LPC	62
Spherisorb S3W, 3 μm	(A) isooctane/THF (99/1); (B) 2-P/C (4/1); (C) 2-P/W (1/1)	ELSD	PE, PI, PA, PC, LPC	63
Ultrasphere Si, 5 μm	(A) isooctane/THF (99/1); (B) 2-P/C (4/1); (C) 2-P/W (1/1)	ELSD	CE, TG, Ch, CL, PE, PI, PS, PC, SPH, LPC	64
LiChrosorb Si	(A) isooctane/THF (99/1); (B) 2-P/C (4/1); (C) 2-P/W (1/1)	ELSD	CE, TG, Ch, PE, PS, PI, PC, SPH	65
Zorbax, 7 μm	i-C8/THF/2-P/C/W (415/5/446/104/30) (216/4/546/154/80)	ELSD	PE, PI + PA, PS, LPE, PC, SPH, LPC	66
Diol-bonded silica				
Lichrospher 100 Diol, 5 μm	H/2-P/HAc/triethylamine (gradient) (75/25/7.5/0.05) & (21/78/1.5/0.05)	MS	PA, PG, PE, PC, PI	60
Lichospher 100 Diol, 5 μm	H/1-P/W/formic acid/triethylamine (gradient) (79/20/0/0.6/0.08) & (0/88/10/0.6/0.08)	MS	PG, PE, PC, PS, PI	109
Nucleosil 100-7 Diol	H/2-P/BuOH/THF/i-C8/W (64.5/17.5/7/5/5/1) (0/73/7/5/5/10) + 180 mg NH_4Ac/L	ELSD	PE, PI, PA, PC	69
LiChrosphere 100 Diol, 5 μm	H/2-P/BuOH/THF/i-C8/W (64/20/6/4.5/4.5/1)(0/75/6/4.5/4.5/10) + 180 mg NH_4Ac/L	ELSD	DGDG, PE, PA, PI, PS, PC, SPH, LPC	70

Table 6 Recent HPLC Phospholipid Class Separation Methods Using a Chloroform/Methanol-Based Mobile Phase

Stationary phase	Mobile phase	Detection	Phospholipids	Refs.
Ultrasphere Si, 5 μm	(A) C (B) M/28.7% NH_4OH (86/14)	FID	TG, *N*-acyl PE, FFA, PE, PC, PI, LPC	72
LiChrospher Si60, 5 μm	C/M/W/30% NH_4OH: (A) 80/19.5/0/0.5 (B) 60/34/5.5/0.5	ELSD	CER, PE, PI, PS, PC, PA, SPH, LPC	73, 26
Encapharm 100, 5 μm	C/M/W/30% NH_4OH: (A) 80/19.5/0/0.5 (B) 60/34/5.5/0.5	ELSD	PG, DPG, PE, PI, PS, PC, PA, SPH, LPC	74
LiChrosorb Si60, 10 μm	C/M/W/30% NH_4OH: (A) 80/19.5/0/0.5 (B) 60/34/5.5/0.5	ELSD	PG, PE, PI, PC, SPH	31, 32
Rosil Silica-D	C/M/W/30% NH_4OH: (A) 80/19.5/0/0.5 (B) 60/34/5.5/0.5	ELSD	PE, PI, PC	75
Spherisorb Si60, 3 μm	C/M/W/30% NH_4OH: (A) 80/19.5/0/0.5 (B) 60/34/5.5/0.5	ELSD	PE, PI, PS, PC, SPH	27
Ultrasphere Si, 5 μm	(A) 84/15.5/0/0.5 (B) 60/34/5.5/0.5	ELSD	FFA, PC, LPC	76
Inertsil, 5 μm (250 × 2.1 mm)	(A) 80/20/0/0 (B) 60/34/6/0.25	ELSD	CER, PI, PE, PS, PC, SPH, LPC	77
LiChrospher Si100, 5 μm	(A) C (B) M/W/NH_4OH/C (92/5/2/1)	ELSD	MG, DG, FFA, PE, PI, PS, PC, SPH	78
LiChrosorb Si-60, 10 μm	(A) C/THF (1/1) (B) M/NH_4OH/C (92/7/1)	ELSD	PE, PI, PA, PC	79
LiChrospher Si60/II, 3 μm (250 × 4 mm)	(A) C/tBME (75/15) (B) M/NH_4OH/C (92/7/1)	ELSD	PE, PI, PA, PC	80, 28

analyses and requiring less solvent per analysis. Hence, this type of mobile phase continues to be used in routine analyses because of its simplicity, low cost, and low solvent consumption. Actually, UV detection has to be used with these solvent systems because they include mostly phosphoric or sulphuric acid that would damage the ELSD upon evaporation of the volatile organic solvents. Typical examples have been described by Balazs et al. (38), Seewald and Eichinger (39), Arduini et al. (40), Engen and Clark (41), and Rehman (42). In all these cases, silica gel was used as the stationary phase.

Bernhard et al., on the other hand, selected 5-μm spherical Nucleosil-NH_2 as the stationary phase (43). In order to improve the resolution of the early-eluting components and to reduce the overall time needed per analysis, they set up an isocratic HPLC system, including two separate columns. By means of a switch valve, the second 175-mm column could be excluded after the elution of PC, SPH, LPC, *N*-methyl PE, PG, and PE. This enabled the rapid elution of the acidic phospholipids PI and PS from the 50-mm column and hence significantly decreased the total run time (Fig. 3). The mobile phase contained 1460 ml A, 500 ml M, 30 ml W, and 600 μl

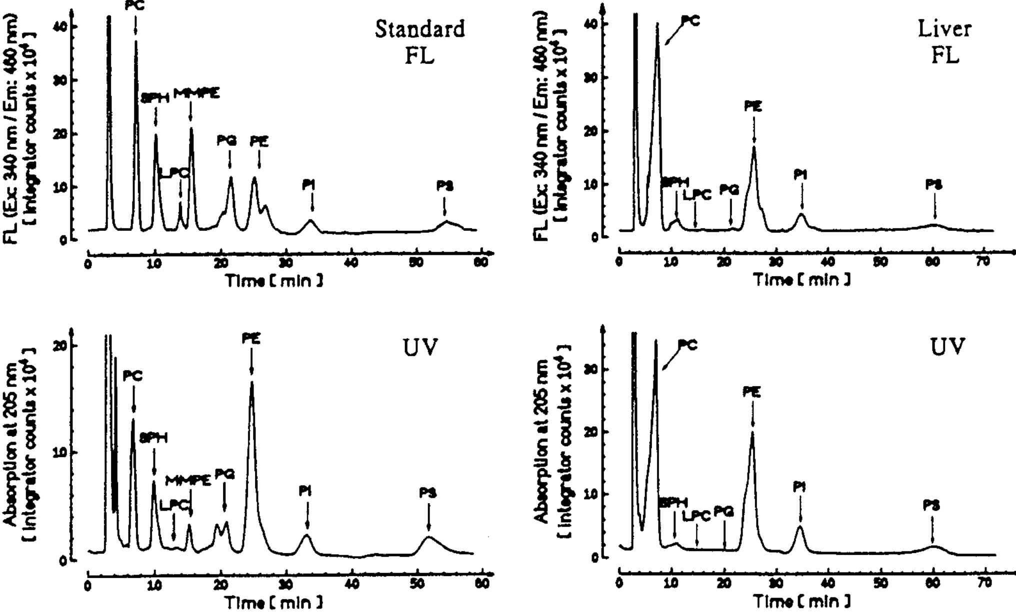

Fig. 3 Separation of standard and liver phospholipids by HPLC on a 5-μm Nucleosil 5 NH_2 stationary phase with an isocratic mobile phase consisting of acetonitrile, methanol, water, and methyl phosphonic acid and subsequent UV and fluorescence detection. The second column was cut off from the eluent stream by valve switching after about 30 min. (Reprinted from Ref. 43 with the kind permission of *Analytical Biochemistry*.)

methylphosphonic acid (MPA; 50% in W); the pH was adjusted to 6.3 by titration with 25% NH_4OH in water. In this method, the column was maintained at 50°C. This did not only reduce the viscosity of the mobile phase and hence the column backpressure, but also seemed to be essential for the separation of SPH, LPC, *N*-methyl PE, and PG into four fully resolved peaks. Table 4 reveals that the elution order of the phospholipids was widely different for unmodified and aminopropyl-silica: the former stationary phase strongly retained the quaternary ammonium phospholipids PC, LPC, and SPH, whereas the latter phase seemed to be characterized by a high affinity for the acidic phospholipids PI and PS.

Soudant et al. also observed that the elution order of the different phospholipid classes depends upon the stationary phase used: with a 5-μm LiChrosorb diol (250 × 4-mm) column it was GL, PI, PC, PS, PE, LPC, SPH, and LPE, whereas with a 5-μm LiChrospher Si100 (250 × 4-mm) silica gel column it was GL, PI, PS, PE, LPE, PC, LPC, and SPH (47). However, each of the columns did not provide adequate resolution of all PL classes of a molluscan lipid extract when used separately: the diol column did not provide a separation of PI and PC or of PS and PE, whereas the silica gel column did not have enough resolution for the two major fractions, PC and LPE, present abundantly in molluscs. According to the authors, this poor resolution was due mainly to the great variety of fatty acids in molluscan lipids, resulting in wider peaks and hence lower resolution during normal-phase chromatography. Nearly perfect resolution was obtained by using both columns in series. When the GL, PI, PS, PE, and PC classes have moved out of the diol column, it is disconnected and the former PL are separated with very good resolution in the silica gel column. Once these PL have been detected, the silica gel column is disconnected and the diol column is connected again. Hence, the most polar PLs are recovered within a relatively

short time. As far as retention times are concerned, the diol column is remarkable for its stability for all PL classes, including the most polar ones. The silica gel column shows an appreciable and steady reduction in retention time during the course of the day and must be regenerated by storing under acetonitrile overnight.

In this case, a binary gradient is used, which is meant mainly to yield a better resolution of glycolipid classes. When the lipid extracts do not contain glycolipids, this phase of the separation can be omitted, and hence an isocratic mobile phase can be used. Also, for extracts poor in molecular species, the silica gel column may be omitted, for the diol column on its own provides resolution of the major phospholipid classes.

In order to enable the use of the generally preferred ELSD, Jaaskelainen and Urtti tried to find an alternative modifier to replace phosphoric acid (46). In this regard, a systematic study of the effect of ammonium acetate (pH 4.8) on the retention of PL on an amino-modified silica column was performed. It was found that increasing the volume from 8 to 16% of a 0.1 M solution or increasing the concentration from 0.01 to 0.1 M in using 8% buffer largely reduced the retention times; this was especially true for the acidic PLs. Hence, the authors proposed to use a 52/32/16 combination of acetonitrile, methanol, and 0.1 M ammonium acetate (pH 4.8). Despite the rather high buffer content, a stable baseline was obtained during ELSD.

Bruch et al. also used ELSD in combination with an acetonitrile-based mobile phase (48). Evaporative light-scattering detection was enabled by using acetic acid rather than phosphoric acid as the aqueous phase modifier. According to the authors, the limit of detection is only 10 ng of PL.

2. *Hexane/2-Propanol–based Mobile Phases (Table 5)*

Traditionally, mixtures of hexane, 2-propanol, and water were used both in isocratic and in gradient mode. The former procedure was applied mainly if UV detection was used, whereas the latter became especially popular following the introduction of the ELSD. Mounts and Nash used a gradient of H/2-P and W; although PS was clearly resolved, PI and PA were only partly separated (49). Letter used a ternary mobile phase of hexane, 2-P, and water (ranging from 2 to 8%) to separate Ch, FFA, PE, PS, PC, and SPH in less than 15 min (53). The Varex ELSD, model IIA, gave a stable baseline, with detection limits for most compounds in the low nanogram range. Using nonbuffered mobile phases, however, the elution order of the PL classes could vary for normal-phase silica gel of different manufacturers: Singleton and Stikeleather (51), e.g., found that PA was obtained before PE and PS, whereas several other authors (52, 57, 58, 63) observed that PA emerged after PE and PI. In order to prevent this variability, pH adjustment, either by acids or by bases, is now preferred. As an example, both the IUPAC method (58) and the AOCS method Ja 7b-91 (59) are based upon the use of an 8/8/1 mixture of H, 2-P, and 0.2 M acetate buffer (pH 4.2) as the mobile phase, with silica gel as the stationary phase. In these isocratic methods, UV detection at 206 nm is recommended. This is due to the fact that ELSD detectors were not yet widely used at the time these methods were elaborated. Besides, the IUPAC and AOCS methods were intended primarily for the quantification of the phospholipid classes of soybean lecithins only, so that fatty acid variability is largely excluded, thus justifying UV detection. Using this simple isocratic system, the main components of vegetable lecithins, i.e., PE, PI, PA, and PC, are well resolved within a short time (Fig. 4), whereas *N*-acyl PE may be found as a shoulder on the PE peak (58). Both methods completely ignore the presence of PS and lysophospholipids, most probably because they are indeed only minor components in soybean lecithin. By spiking with PS, Szucs et al. found this phospholipid to elute between PI and PA, but it could not be resolved from them (18).

Moreau used a ternary gradient including 0.04% triethylamine in water; 23 different classes of both nonpolar and polar lipids, including glycolipids and phospholipids, were resolved within 1 hour (57). Besides NL, FFA, DPG, PE, PG, PI, PS, and PC, three major hopanoid classes were

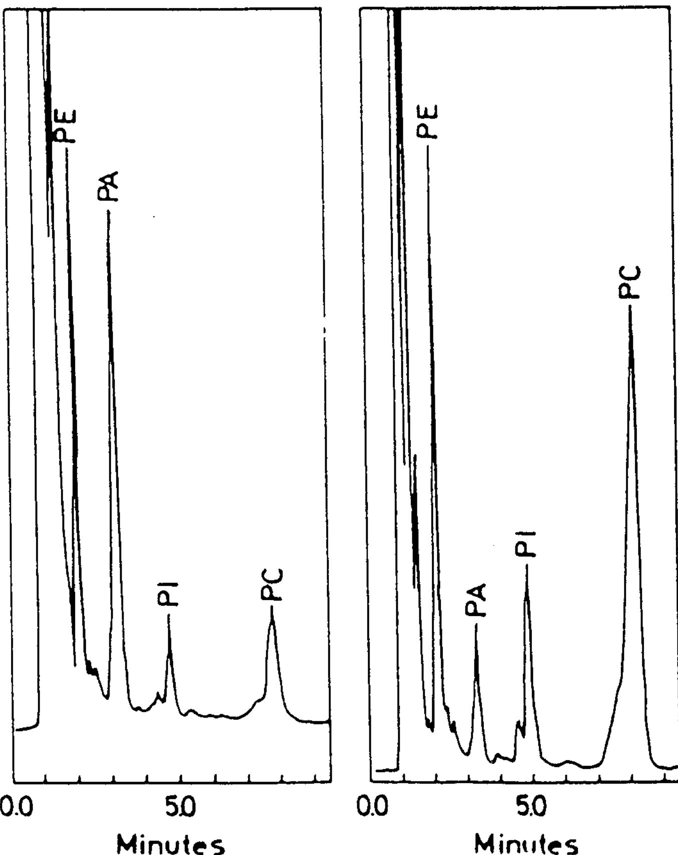

Fig. 4 HPLC separation of water-degummed and crude sunflower oil phospholipids on a 250 × 4-mm column packed with Lichrosorb Si-60 according to the AOCS method. The mobile phase consisted of hexane, 2-propanol, and acetate buffer of pH 4.2 in a volumetric ratio of 8/8/1 at 2 ml/min. Detection was accomplished by UV absorption at 206 nm. (Reproduced from Ref. 34 with permission of the American Oil Chemists' Society.)

separated: pentacyclic triterpene lipids found in many species of bacteria and cyanobacteria, a few species of fungi, and a few species of higher plants.

Very detailed separations have been obtained by numerous authors (61–66) based upon the method originally developed by Christie (67). This method is based mainly on iso-octane (similar to hexane), 2-P, water containing 500 μM serine adjusted to pH 7.5 with ethylamine, and trace amounts of tetrahydrofuran (THF) as a mobile-phase modifier. Lutzke and Braughler modified slightly the mobile-phase system proposed by Christie by including a flow rate gradient to maintain low column backpressure (62). According to the authors, this positively affected detector response to PLs. Markello et al. used the procedure described by Christie, albeit without the addition of serine or ethylamine (65). Melton proposed the use of two solvent mixtures only, but they included exactly the same solvents as proposed by Christie (66). However, PI and PA were not resolved.

The International Lecithin and Phospholipid Society proposed a method including a gradient of two mixtures of H/2-P/W/acetic acid/triethylamine with a composition of 81.5/17/0/1.5/0.08 and 0/84.5/14/1.5/0.08, respectively (68). In it a 5-μm LiChroCart 100 diol-bonded silica stationary phase was selected to give more stable retention times. Quantification is performed us-

ing ELSD calibration curves that were obtained using a standard sample whose phospholipid class composition was first determined by ^{31}P-NMR.

Olsson et al. (69), as well as Arnoldsson and Kaufmann (70), further improved the mobile-phase composition. Experimental design revealed that besides THF, *n*-butanol as well as ammonium acetate were appropriate modifiers too. The contribution of THF and *n*-butanol was kept constant throughout the whole solvent program and was about 5% for each solvent; each mobile-phase mixture also contained 180 mg of ammonium acetate per liter. As a typical example, the chromatogram of the ILPS-proposed mixed soybean phospholipids standard, which is also referred to as the Spectralipid SN standard mixture, is shown in Fig. 5.

3. *Chloroform-Based Mobile Phases (Table 6)*

The mobile-phase compositions including chloroform, methanol, and an aqueous component often refer to the method of Becart et al. (73). However, this methanolic ammonium hydroxide gradient in chloroform was originally developed by Erdahl et al. back in 1973 (71); it was not taken into consideration for over 15 years because it was not compatible with UV detection. A gradient elution with pH modifier was required for proper resolution and peak symmetry of the acidic phospholipids PS and PI. This solvent's main advantage is that it is less viscous and contains solvents better as compared to acetonitrile, hexane, and 2-propanol. Using this mobile phase, Becart et al. obtained an excellent separation of CER (cerebrosides), PE, PI, PS, PC, PA, SPH, and LPC within 20 min on a 125-mm Lichrocart cartridge filled with 5-μm Lichrospher Si60 at a flow rate of only 1 ml/min, as illustrated by Fig. 6 (73). The detection limit, which was defined as twice the noise level, for PE and PC was only 20 ng. For the sake of completeness, it has to be men-

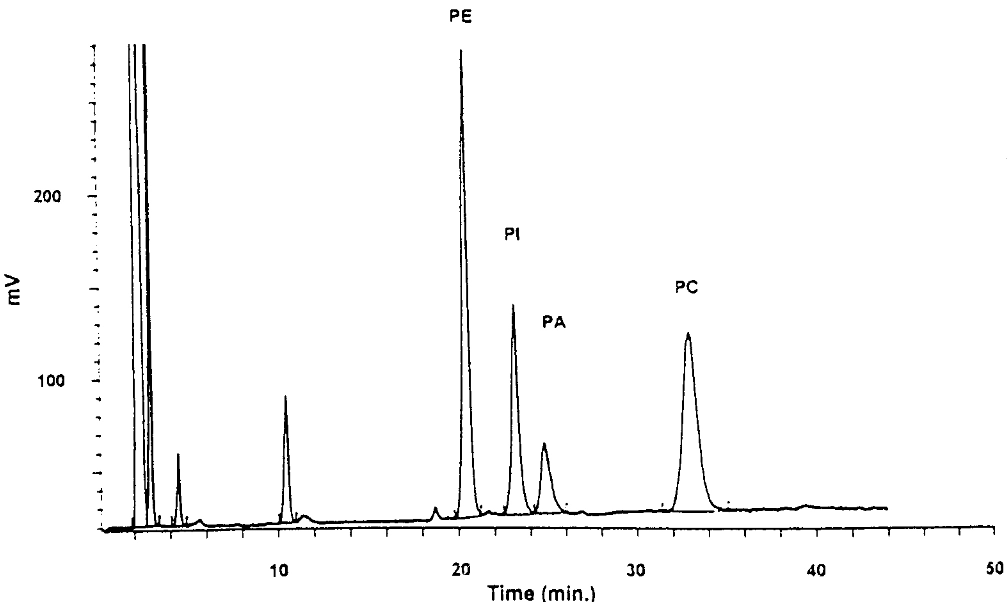

Fig. 5 HPLC profile of the Spectralipid SN standard mixture monitored by ELSD after HPLC separation of the phospholipid components on a Nucleosil 100-7 Diol stationary phase. The mobile phase included hexane, 2-propanol, butanol, tetrahydrofuran, iso-octane, and water in a binary gradient. (Reprinted from Ref. 69 with the kind permission of the authors and of Elsevier Science Publishers.)

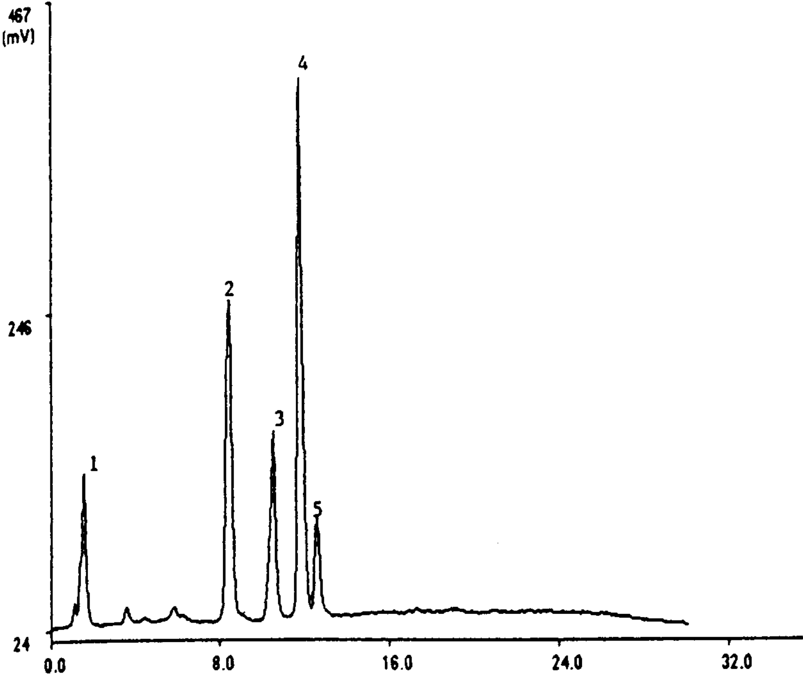

Fig. 6 ELSD chromatogram of a commercial soy lecithin following HPLC on a 5-μm Ultrasphere Si stationary phase with a gradient of chloroform, methanol, and 28.7% NH_4OH. 1 = nonpolar lipids, 2 = PE, 3 = PI, 4 = PC, 5 = PA. (Reproduced from Ref. 73 with the permission of the *Journal of High-Resolution Chromatography.*)

tioned that a similar mobile phase had been proposed one year earlier by Grieser and Geske (72), but this method used FID, so it has been most neglected since then.

According to Vaghela and Kilara, ammonium hydroxide, though volatile, at high concentrations dissolves the silica packing, thus reducing column lifetime (77). Hence, the Becart system was modified, with the main aim being to reduce the ammonium hydroxide content as much as possible. Using a 2-mm-ID narrow-bore column, the solvent consumption was reduced by 80% and the detection limit was improved more than tenfold. In addition, Vaghela and Kilara observed that the chloroform used should not be stabilized by pentene, for it seems to promote silica dissolution and hence unstable baselines with numerous ghost peaks and short lifetime (77). The use of chloroform stabilized with ethanol solved the problem.

Mounts et al. proposed a binary gradient of chloroform/THF (1/1) on the one hand and methanol/NH_4OH/C (92/7/1) on the other hand (79). According to Abidi et al., incorporation of pure THF to a mobile phase consisting of C/M and ammonium hydroxide enhances the separation of PC from PA (80). However, impurities were often found in THF, causing severe ghost peak interference with PL analytes. The use of tertiary butyl methyl ether (tBME) instead of THF eliminated these problems.

4. *Miscellaneous Methods*

Demopoulos et al. demonstrated that PL separation can be accomplished by using a cation-exchange column with an isocratic elution system of A/M/W (300/150/35). Thus, most PLs, such

as PI, LPE, PC, SPH, and LPC, were clearly resolved; PE and PS coeluted between PI and LPE (81).

Abidi et al. reported the separation of the major soybean PLs within 25 min at a flow rate of 1 ml/min on a beta-cyclodextrin–bonded silica using isocratic elution with mobile phases containing hexane, 2-propanol, ethanol, and 5 mM tetramethyl ammonium phosphate (TMAP) in water (pH 6.3) at a ratio of 35/32.7/26.8/5.5 (82). The latter seemed to be critical to improve resolution and enhance peak symmetry. The elution order appeared to follow the order of increasing phospholipid polarity with increasing retention times: PE $<$ PC $<$ PI $<$ PA. However, UV detection at 208 nm was required due to the presence of TMAP. Hence, the method is only qualitative.

B. Molecular Species

In general it has to be stated that molecular species analysis of phospholipids is not frequently applied in food analysis; most of the studies involving molecular species are instead found in the fields of biochemistry and nutrition. Thus, in the recent reviews by Bell and by Olsson and Salem, special emphasis has been given to the characterization of biological tissue samples (83,84). However, the molecular species composition has been shown to affect the accuracy of the quantification of phospholipid classes and hence is important in food analysis too (47,52). In the vast majority of published methods, isocratic elution has been used. In our opinion, this should be ascribed mainly to the fact that the traditional UV detector remains. Keeping account of the inherent problems associated with UV detection of underivatized phospholipids, it is astonishing that ELSD has hardly been exploited in this subdomain. As far as the stationary phase is concerned, nearly all methods prefer octadecyl-coated stationary phases.

1. Ultraviolet-Absorbing Derivatives

Most molecular species methods involve enzymatic conversion by phospholipase C of the phospholipids to diacylglycerols, which are subsequently converted either to UV-absorbing or fluorescent derivatives. This procedure has two main advantages. First of all, the same HPLC method can be used for all phospholipid classes, because the polar head group is removed during the sample preparation. In addition, quantification is highly simplified and becomes much more sensitive.

Overall, UV-absorbing derivatives, such as benzoate derivatives, have been preferred because of the wide availability of UV detectors. Diacyl glycerobenzoate molecular species may be resolved by either a gradient of 2-propanol in acetonitrile (85) or an isocratic mobile phase consisting of methanol, acetonitrile, ethanol, and water in a 29/21/47/2.8 volumetric ratio (86); they are detected at 230 nm. Cantafora and Masella suggest including an internal standard to correct for incomplete hydrolysis and derivatization (86). Others have used dinitrobenzoyl-diacylglycerol (DNB-DAG) derivatives; they are characterized by a strong absorption at 254 nm (87–89). It was established that an excellent correlation exists between the carbon and double-bond numbers in the aliphatic residues and the relative retention time of the dinitrobenzoyl derivatives. To this end, an equivalent carbon number that takes into account the number of cis and trans double bonds per molecular species was defined as

$$ECN^* = CN - 2n_{cis} - n_{trans}$$

where CN is the number of carbon atoms in the aliphatic residues. The logarithm of the retention time increases linearly as a function of ECN* (Fig. 7). Applying isocratic elution with A/2-P (80/20), all compounds were eluted from the HPLC column within 20 min only. The detection limit of DNB-DAG (18:0;16:0) is about 6 pmol.

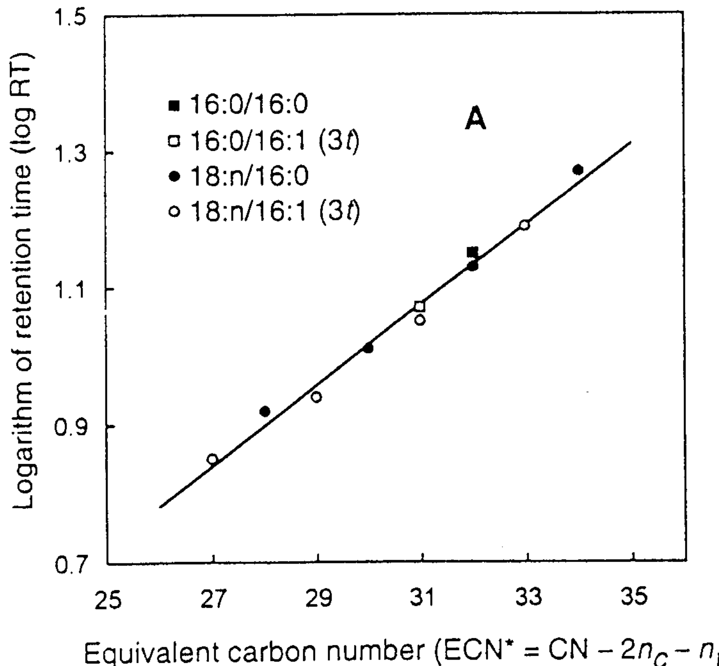

Fig. 7 Correlation between the logarithm of the retention time of the 3,5-dinitrobenzoyl derivatives of PG molecular species expressed in min (log RT) and the corrected equivalent carbon number ECN*. (Reprinted from Ref. 89 with the permission of the American Oil Chemists' Society.)

2. *Fluorescent Derivatives*

The main advantage of fluorescent derivatives is that they may be quantified down to the femtomole level. Takamura and Kito separated anthroyl-diacylglycerol derivatives of PL molecular species by A/2-P (70/30) (90).

McKeone et al. proposed to use 7-methoxy coumarin-3-carbonyl azide (7-MCCA) to prepare fluorescent derivatives to be detected with absorption at 340 nm and emission at 370 nm (91). Reversed-phase HPLC was carried out with A/2-P/W (64/25/11); some typical chromatograms are shown in Fig. 8.

The preliminary enzymatic hydrolysis step can be omitted in the case of lysoPL. The latter may be derivatized at the *sn*-2 position of the glycerol group by 7-diethylaminocoumarin-3-carbonylazide (94). According to McKeone et al., however, 7-MCCA proved to be better suited than 7-diethylaminocoumarin-3-carbonyl azide, even though the latter had a higher molar extinction coefficient (91).

For PE and PS, the amino groups may be derivatized. Abidi et al. compared various fluorescent labels for PE and PS, such as pyrene, dansyl, and fluorescein (92,93). They observed that the HPLC separations were drastically influenced by the nature of the fluorophore attached. The fluorescent molecular species were separated using isocratic elution with A/M/W (70/28/2) to which either NH_4Ac or a tetra-alkyl ammonium phosphate (TAAP) was added. In the absence of mobile-phase electrolyte, the polar lipids, each having a negative charge at the phosphoryl moiety, exhibited little retention on a reversed-phase column. The main advantage of NH_4Ac buffers is that they are vaporizable, making them useful in LC-MS. The detection limit at a signal-to-noise ratio of 4/1 was found to be about 4 ng.

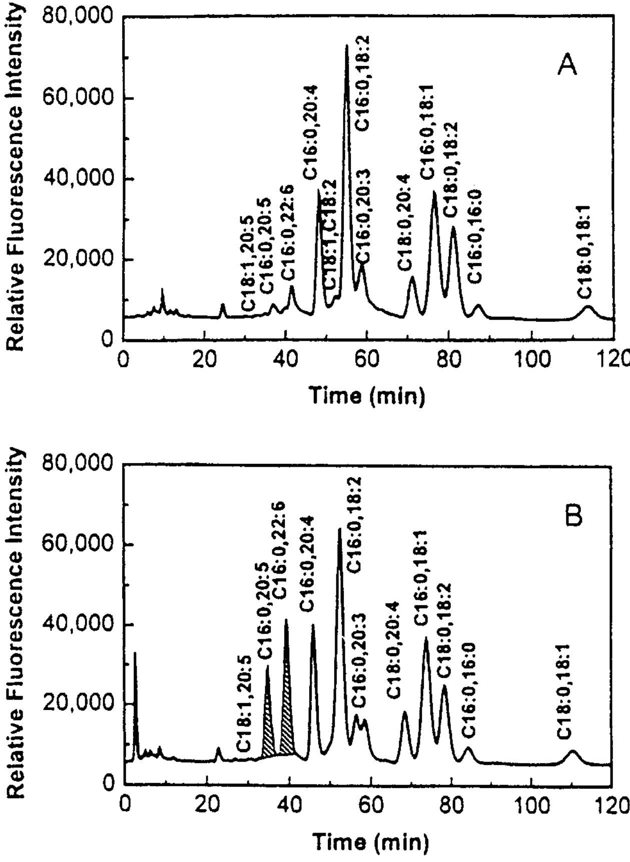

Fig. 8 Serum PC molecular species distribution after 6 weeks of treatment for both the placebo control group (A) and the drug-treated group (B). The molecular species were derivatized with 7-methoxycoumarin-3-carbonyl azide prior to analysis and detected by fluorescence with excitation at 340 nm and emission at 370 nm. (Reprinted from Ref. 91 with the permission of the *Journal of Lipid Research.*)

3. *Underivatized Phospholipids*

Using underivatized PLs makes it easier to preserve polyunsaturated fatty acids and avoids time-consuming enzymatic and chemical procedures. In addition, it eliminates errors due to incomplete degradation and derivatization. In this case, UV detection at about 205 nm is generally used. Initially, methods were focused mainly on the separation of neutral phospholipids, such as PE and PC. Glass as well as Therond et al. used an isocratic mobile phase containing 5% aqueous NH_4Ac and 95% methanol to separate the molecular species of these phospholipids (95,96). Therond et al. proposed 10 mM NH_4Ac (pH 5), whereas Glass preferred 0.1 M NH_4Ac (pH 7.4). Singleton and Stikeleather, on the other hand, did not include any salts; the molecular species of PC were separated isocratically with a 91/3/6 mixture of methanol, acetonitrile, and water (51). Similarly, Guillot-Salomon et al. used a 90.5/2.5/7 mixture of the same solvents to separate the molecular species of both PC and PE (97).

Abidi and Mounts separated the molecular species of the neutral phospholipids PC and PE using A/M/W (70/22/8) containing tetra-alkyl ammonium phosphates (TAAPs). The competitive interaction of TAAPs and analyte solutes with the reversed-phase HPLC column resulted in

reduced retention of PC and PE with concomitant increase in detection sensitivity. While PC or PE components eluted at longer retention times with a larger size of TAAP, an increase in the TAAP concentration invariably caused a decrease in retention time (98). Hence, to obtain narrow peaks, the lowest member of the TAAPs was selected; 25–100 mM tetramethyl ammonium phosphate was proposed.

As far as the acidic phospholipids are concerned, Abidi and Mounts could not obtain any resolution of either PG or DPG molecular species, nor any noticeable degree of solute retention, using an A/M/W mobile phase in the absence of mobile-phase additives. Upon addition of 1.25–5 mM of a quaternary ammonium phosphate (QAP) to the mobile phase, however, adequate separations of the lipid components were obtained (99). Ion pairing of the negative charge at the acidic PL head group with alkyl groups in a QAP enhances the hydrophobic interactions of the molecular species with the C18 stationary phase and improves column selectivity. Capacity factors increased with increasing chain length as well as with increasing concentration of QAP. The concentration effect is similar for all negatively charged PLs, but opposite to what is observed for neutral PLs (98). In order to reduce the retention time of the molecular species within a reasonable range for rapid analyses, the authors advise the use of a high member of QAP at a low concentration. Because the ion-pairing mobile-phase system is not compatible with ELSD, UV detection has to be used. As a further consequence, the separation yields only qualitative results.

A similar method was described by Rezanka and Podojil (100) and by Wiley et al. (101), including choline chloride as the source of the quaternary ammonium counterion. The molecular species of PE, PC, and PI were separated by a mixture of methanol, 286 mM aqueous choline chloride, and acetonitrile in a 90.5/7/2.5 ratio. Graphs of the logarithm of the relative retention time versus the equivalent chain length of the fatty acid found in the 1-position yields a straight line for a given fatty acid in the 2-position. For different fatty acids in the 2-position, a series of parallel straight lines is obtained.

Only recently, Kaufman and Olsson demonstrated the use of ELSD in combination with the separation of the molecular species of intact PC and PE (102). As a matter of fact, the use of a different detection system also had implications for the mobile-phase composition. Hence, nonvolatile additives had to be omitted, whereas gradient elution was enabled: a gradient of 1-propanol, water, and iso-octane, changing from 52/47/1 to 58/33/9, was used (Fig. 9). Mounts et al., on the other hand, used isocratic elution with mixtures of methanol, water, and chloroform to resolve the molecular species of PC and PE on a C18 reversed phase in combination with ELSD; the volumetric ratio was 20/1/1 in the case of PE, whereas for PC a 30/1/1 ratio was preferred (28).

In an attempt to avoid interactions with residual silanol groups, Abidi and Mounts investigated the separation of the molecular species of PC, PE, and SPH on polymeric C18 columns by RP-HPLC (103). Of the three polymer columns evaluated, the best HPLC results were obtained with an octadecanoyl polyvinyl alcohol (ODPVA) stationary phase. High-performance LC on ODPVA with an A/M/W mobile phase provided significantly faster analysis and greater detection sensitivity than assays with C18 silica columns.

V. DETECTION SYSTEMS

A. Evaporative Light-Scattering Detection (ELSD)

Evaporative light-scattering detectors have become by far the most widely used detectors in the analysis of phospholipid classes. Generally, this is due not only to their compatibility with a very wide range of eluents but also to the fact that the responses obtained are much more uniform (20).

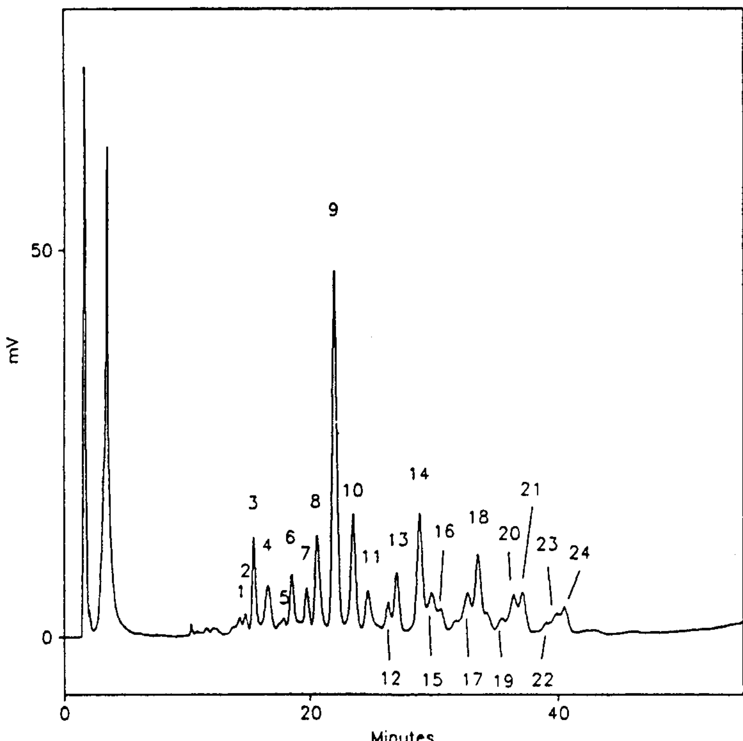

Fig. 9 ELSD chromatogram of the molecular species of bovine milk PC. Underivatized molecular species were separated on a 250 × 4.6-mm column packed with Superspher RP-100 at 75°C. The mobile phase contained 1-propanol, water, iso-octane, and ammonium acetate in a complex gradient. (Reprinted from Ref. 102 with the permission of *Chromatographia*.)

Although several authors have claimed that the calibration curves obtained are linear, the major drawback of ELSD is that the response is not linearly related to the amount of sample applied. Although at first sight, this may sound contradictory, it is not: both experimentally and theoretically, it has been shown that the calibration curves are always sigmoidal in shape (104). However, if only a limited range is considered, the calibration curve can be fitted to a simpler form. In the very low-concentration range, the sigmoidal curve can be described by a quadratic or an exponential function (31,64,73); Caboni et al. (31) found exponential calibration curves within the range from 0.25 to 4 μg, according to the equation $y = (a + bx)^2$. Using the method of Caboni et al., the minimum quantifiable amount of PL was about 100 ng. As a further consequence of the nearly exponential response at very small sample loads, it follows that ELSD is rather insensitive toward minor components. Hence, the purity of the fractions obtained during preparative HPLC may be overestimated by ELSD (63).

Buenger and Pison elaborated calibration curves for nine different phospholipid classes in the range from 1 to 25 μg (74). Curve fitting according to the exponential function $Y = A \times X^b$ revealed that the value of the exponent b ranged from 1.63 to 1.83, whereas the value of A varied from 42 to 67. The detection limits, defined as twice the noise level, of natural PL ranged from 30 ng for PA to 100 ng for PG. According to Vaghela and Kilara a detection limit of only 10 ng can be obtained with ELSD (77).

For intermediate sample loads, the sigmoidal calibration curve may be substituted by a linear function, but this statement can surely not be generalized. Balazs et al. assume the ELSD

response is linear; however, a closer look at their experimental data suggests an exponential response instead (38). Similarly, Vaghela and Kilara used linear regression to describe experimentally determined calibration curves in the range from 0.25 up to 3 μg; although high determination coefficients were obtained, the residuals were nevertheless not randomly distributed, showing that a linear model was not appropriate (77).

Melton observed that ELSD calibration curves become linear from 10-μg sample loads onward (66). It is essential, though, to keep in mind that the response is described by a linear function only within a limited range, but it is not at all linear. Hence, one-point calibration, as proposed by Klein and Dudenhausen, surely yields erroneous results (105). Also, linear calibration models may be applied only within the linear range, which should be clearly determined and specified. This is illustrated by the decidedly negative values of the intercept observed in all so-called linear calibration curves; according to the linear model, very small peak areas would refer to negative concentrations (66). This behavior also implies that successive injections may be required for a reliable quantitative analysis; the major components may be quantified using a small sample load, whereas for minor components, such as lysophospholipids, a higher load is required to operate within the linear part of the calibration curve (73).

The ELS detector was previously also referred to as a mass detector, pointing to the fact that the response is (mainly) determined by the mass of the sample rather than by its chemical structure. Van der Meeren et al., though, demonstrated that the ELSD calibration curves of phospholipid classes were also dependent on the fatty acid composition (52). The dependence on the fatty acid composition is, however, completely different in nature and much less pronounced than for UV detection. The reason for this behavior is to be found in the partial resolution of molecular species, even during normal-phase chromatography. Thus, the peak shape depends not only on the chromatographic system but also on the fatty acid composition and molecular species distribution of the PL sample (47). Because it was shown before, based on both theoretical considerations and practical experiments, that the ELS detector response is generally inversely proportional to peak width (62,104), it follows that the molecular species distribution of the PL standards used should be similar to the sample components to be quantified. It was shown that up to 20% error may be induced if an inappropriate standard is used (52).

Arnoldsson and Kaufman optimized the response of the ELSD with a full factorial design evaluating the influence of evaporator temperature and air inlet pressure (70). As indicated in Fig. 10, the optimum conditions for both peak height and low baseline were achieved at high drift tube temperature and low-to-moderate nebulizer air pressure. The conditions chosen were 96°C and 1.6 bar.

Despite the widespread use of ELS detection in the quantification of phospholipid classes, it is striking that it is hardly used in molecular species analysis. This is due mainly to the rather high detection limit in ELSD: according to Abidi and Mounts, ELSD is at least 10 times less sensitive than UV detection of underivatized phospholipids, which in turn is roughly 10–1000 times less sensitive than UV or fluorescence detection of derivatized phospholipids. Given that the different phospholipid classes first have to be separated by normal-phase chromatography, and hence that the amount of sample available is usually very small, the sensitivity of the molecular species analysis method is of utmost importance. In addition, it has to be mentioned that ELSD is not compatible with ion-pairing reagents, such as quaternary ammonium phosphates, which are known largely to improve molecular species analysis (98,99).

B. Light Absorption

Traditionally, UV detection was by far the most widely used detection method, both in phospholipid class analysis and in molecular species analysis. Even up to now, this detection system re-

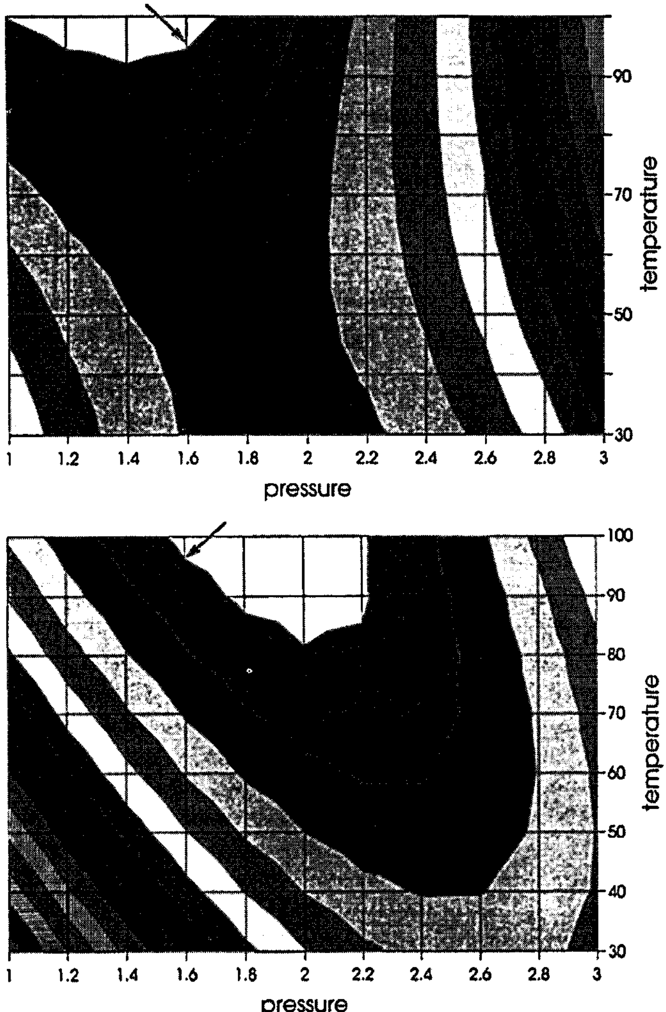

Fig. 10 Dependance of ELSD response on air inlet pressure and drift tube temperature during HPLC of phospholipid classes. *Top*: peak height; *bottom*: rise of baseline. Optimal conditions are shown by the arrow. (Reprinted from Ref. 70 with the permission of *Chromatographia*.)

mains popular in phospholipid class analysis, especially for qualitative analyses, because it is a simple and cheap technique. Besides, it is not destructive and hence is the detector of choice in preparative applications. As far as molecular species analysis is concerned, light absorption remains the most important detection principle. In this case, it may be used both for underivatized phospholipid molecular species and for UV-absorbing derivatives.

A systematic investigation of the relationship between the chemical structure and the UV detector response was performed by McHowat et al. (106). In this study, the response factors of 37 underivatized phosphatidylcholine molecular species were determined in the 2–100-nmol range, integrating the UV absorbance at 203 nm, which is the absorbance maximum. The absorption of UV energy in the 190–210-nm wavelength range is due mainly to the π–π^* electronic transition arising in molecules containing nonconjugated C=C double bonds, so it follows that the detector response is determined primarily by the degree of unsaturation of the phospho-

Table 7 Relative Sensitivity of Standard Phospholipids for Fluorescence and Ultraviolet Detection

Phospholipid	Fluorescence	UV absorption
Egg PC	1.00	1.00
Dipalmitoyl PC	1.14	0.03
Dioleoyl PC	0.95	0.38
SPH	0.96	0.82
LPC	0.11	0.02
PG	0.90	0.85
PE	1.07	3.11
PI	0.60	2.02
PS	0.86	1.16

Source: From Ref. 43 with the permission of *Analytical Biochemistry*.
Data calculated from the peak areas of diphenylhexatriene fluorescence (340/460 nm) and UV absorption (205 nm) following HPLC separation of the phospholipids on a 5-μm Nucleosil 5 NH_2 stationary phase. Egg PC was set as 1.

lipids, as illustrated by the relative UV absorption response of egg PC, dipalmitoyl PC, and dioleoyl PC (Table 7). As a further consequence, it follows that the PL to be detected must contain at least one C=C group at the *sn*-1 or *sn*-2 position in order to generate a detectable UV absorbance response. In addition, the calibration curve must be constructed with a PL standard with the same fatty acid composition as the corresponding PL in the sample to be analyzed. Given that the fatty acid composition, and in particular the amount of unsaturated fatty acids of a PL class, may change with the developmental stage or disease of an organism or with nutritional conditions and differs among organs as well as species, it becomes obvious that perfectly matching PL standards for UV detection of phospholipid classes do not exist. Hence, quantification is the major problem in UV detection of phospholipid classes.

C. Fluorescence Detection

Whereas fluorescence detectors are used only rarely in phospholipid class analysis, they are very often preferred in molecular species analysis using fluorescent derivatives. First of all, fluorescence detectors are very sensitive; detection of anthroyl-diacyl glycerol derivatives, e.g., is about 140 times more sensitive than UV detection of dinitrobenzoyl-diradylglycerol derivatives. Besides, the response is linear within a very broad range: it extends from 17 fmol to 850 nmol in the case of anthroyl-diacyl glycerol derivatives (90). In addition, the peak areas are not affected by differences in the structures of the molecular species, as exemplified by Fig. 11. Bernhard et al. (43) demonstrated that fluorescence detection may also be used in normal-phase chromatography of phospholipid classes, as exemplified by Fig. 3. In this case, fluorescence is achieved by post-column formation of mixed micelles with 1,6-diphenyl-1,3,5-hexatriene. The derivatizing reagent consisted of double-distilled water to which 250 μl of Brij 35, 50 mg of NaN_3, and 150 μl of diphenylhexatriene solution (3 mmol/liter THF) were added per liter. It was mixed with the eluent (at a flow of 1 ml/min) at a flow of 4.5 ml/min. Formation of the fluorescent micelles was achieved in 2000 × 0.8-mm teflon tubing kept at 50°C. Using this technique, the sensitivity seemed to be comparable for all phospholipids except PI and LPC (Table 7). The lower limit of detection was 0.5 nmol for all PLs, except PI (1 nmol), LPC, and PC (2 nmol). The fluorescence

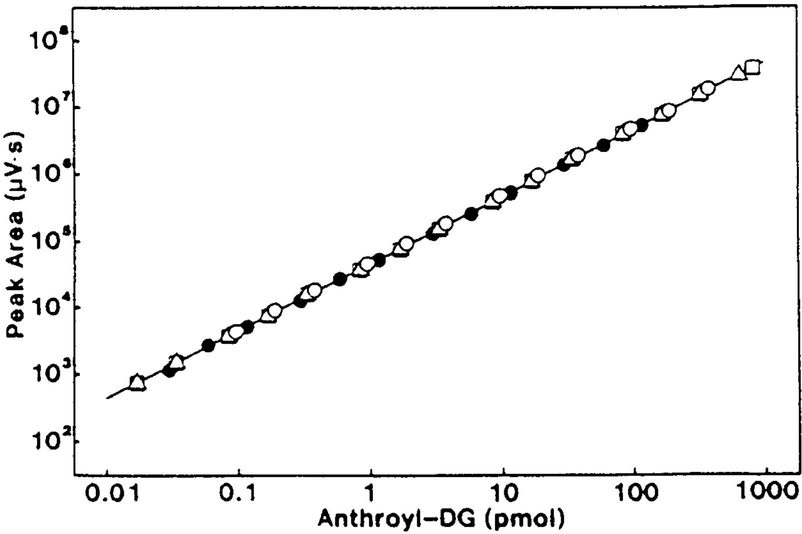

Fig. 11 Proportionality between the amount of the anthroyl-DG derivative and the peak area obtained by fluorescence detection, with excitation at 360 nm and emission at 460 nm following RP-HPLC with acetonitrile/2-propanol (70/30) as the mobile phase on a 5-μm Ultrasphere ODS stationary phase at 25°C. Open circles represent C16:0,C16:0 (diacyl), open squares C16:0/C18:1 (diacyl), triangles 16:0/20:4 (diacyl), and filled circles 16:0/16:0 (alkylacyl). (Reprinted from Ref. 90 with the permission of the *Journal of Biochemistry*.)

response was linear up to at least 60 nmol of PL. The authors also found that the UV/fluorescence peak area for both PC and PE increased linearly with the average number of double bonds per molecule. Hence, this ratio might be used as an indicator of changes in the unsaturated fatty acid content. According to the authors this detection technique is preferable to ELSD, because the latter limits the choice of mobile-phase solvents and phase modifiers to volatile solvents. Besides, the ELSD response is not linear and the sample is destroyed.

D. Refractive Index Detection

Refractive index (RI) detection used to be a (rather insensitive) alternative for UV detection. It is not compatible with gradient elution, so it is generally restricted to samples containing a limited number of components. Mustranta et al. used RI detection in the separation and quantitation of oleoyl myristoyl PC formed during the enzymatic transesterification of DMPC (dimyristoyl phosphatidylcholine) in the presence of oleic acid on a Nova-Pak C18 (8 $\times$ 100-mm, 4 μm) using 100 mM tetramethylammonium chloride in methanol (107). Grit et al. also preferred to use RI detection in order to quantify phospholipid hydrolysis in aqueous media (44). According to these authors, the molar RI detector response within a phospholipid class is not significantly affected by either the degree of unsaturation or the length of the fatty acyl chains.

E. Flame Ionization Detection (FID)

Flame ionization detection has been proposed in the past as a universal detection system that is compatible with gradient elution. According to Moreau et al., the limit of detection of this detector is about 1 μg of each lipid component, which is comparable to or slightly worse than ELSD

(55). Two additional features are also shared by both FID and ELSD: the calibration curves are nonlinear and the detector is destructive. Moreau et al. conclude that similar results are obtained with either FID or ELSD detection (57). However, because FID limits the flow rate to less than 1 ml/min, the total analysis time is quite large. Hence, FID is used only rarely and should preferably be replaced by ELSD.

F. Mass Spectrometry (MS) Detection

The mass spectra of phospholipids produced from filament-on thermospray ionization give simple and characteristic fragment ions, such as protonated molecular ions (MH^+), diacylglycerol fragment ions (DG^+), monoacylglycerol fragment ions (MG^+), and some low-mass ions originating from the head groups, which are useful to identify the head group and the constituent fatty acids. Ma and Kim observed that the intensities of the monoacylglycerol fragment ions and MH^+ increase linearly with increasing sample size in the range studied (i.e., from 0.05 to 10 μg), but the diacylglycerol fragment ion intensities increase in a second-order polynomial way (108). Hence, the relative intensities of the various fragments observed in the mass spectra of the PL change with the sample size: at the submicrogram-to-microgram range, diacylglycerol fragment ions are the main peak, whereas at smaller sample size, monoacylglycerol fragment ions become dominant in the spectra. Ma and Kim developed an online RP-HPLC/MS method to determine rapidly the major PL molecular species without prior isolation of each PL class. With this method, both the classes and the molecular species of the phospholipids can be identified in a single run using a mobile phase containing 0.5% (v/v) NH_4OH in M/H/W (108). The detection limit is in the 20–50-ng range using selected ion monitoring (SIM) detection.

Valeur et al. used online plasmaspray (PSP) ionization mass spectrometry to detect the separated phospholipid classes and to derive the fatty acid composition from the abundance of diacylglycerol and monoacylglycerol fragments (60); according to the authors, the PSP ionization technique provides significant advantages over "filament-on" thermospray (TSP) with regard to sensitivity and the range of HPLC mobile phases available. In a more recent publication of the same research group, atmospheric pressure chemical ionization (APCI) was compared with PSP (109): full scan sensitivity in APCI was found to be the same as with PSP but was about two orders of magnitude less than with electrospray (ES). In the full-scan mode under the conditions described, the detection limit was in the range of 1 to 5 pmol, depending on the phospholipid. In the SIM mode the sensitivity was found to be 70–100 fmol at a signal-to-noise ratio of 3 (109). Because the response factor depends on the degree of unsaturation, accurate quantitation of individual species is difficult (108,109). Hence, MS detection is an identification, rather than a quantification, technique.

VI. APPLICATIONS IN FOODS

Although phospholipids are natural components of nearly all food products, the analysis of the phospholipid composition is of importance mainly in the certification and quality control of lecithins. According to the European Analytical Subgroup of the International Lecithin and Phospholipid Society (ILPS), there is an urgent need for a standard method for the determination of the PL composition, for this would allow a better characterization of lecithin and PL products (15,16). Besides, the nonavailability of good calibration standards is a major problem when comparing analytical results between companies. In order to try to solve the latter problem, the ILPS proposes a calibration standard whose composition is certified by ^{31}P-NMR as an absolute tech-

nique. This so-called Spectralipid standard is commercially available from Spectral Services in Germany (Fig. 5).

Balazs et al. compared the AOCS phospholipid class analysis method to a so-called "simplified" method and to the ILPS recommended method using both fluid and deoiled lecithin (38). The simplified method was developed basically to detect PE, PC, and LPC on a 10-μm Porasil stationary phase using an isocratic acetonitrile-based mobile phase and UV detection at 205 nm. The best chromatographic performance resulted from the ILPS method. And this method showed the highest precision for both within- and between-day experiments, which might be ascribed to column stability as a consequence of the use of a diol-bonded stationary phase. Moreover, this study clearly demonstrated that the selection of the reference standards was an important issue in defining the results. Thus, a significant difference in the lipid class composition was observed depending on the use of either individual pure PL standards or a mixed PL standard solution: the calculated PC content was about 8% higher and the PE content even 18% higher using individual standards rather than the certified lecithin standard. Comparing results of the ILPS method and the AOCS method, the former yielded a higher PE and a lower PI content for both fluid and deoiled lecithin. For PC, no clear trend could be observed.

Recently, Mounts et al. studied the effect of genetic modification on the content and composition of tocopherols, sterols, and phospholipids in soybean oil (28). Although, in general, there was little impact on the phospholipid classes, a higher PA content was found in some crude oil samples. Compositional variations in molecular species indicated that genetic modification of soybeans affected the phospholipids at the molecular level.

Analysis of the phospholipid fractions can also provide useful information on the quality of oilseeds as affected by stress due to environmental conditions in the field and during storage. Mounts and Nash found that the FFA content increased and the overall phospholipid content decreased as stress was applied (49). As far as individual PL classes were concerned, the content of PC and PI decreased significantly, while the PA and PE content increased, thus giving rise to an increased nonhydratable phospholipid (NHP) content in the crude oil. The NHPs refer to the phosphatides that, during degumming of crude oils with water, do not hydrate, swell, form gel, or precipitate and hence are not removed by centrifugation.

Similarly, Singleton and Stikeleather developed an HPLC method to investigate the influence of different stress situations on the total PL content, on the PL class distribution, and on the molecular species distribution of peanut oil prior to refining (51). In many cases, the crude oil of stressed beans is unusable for edible purposes and becomes increasingly difficult to refine. High-performance LC analysis revealed that the concentrations of PA, PE, and PC were higher in immature than in mature seed. Freeze damage resulted in a highly increasing PA and a decreasing PC concentration, which is assumed to be due to phospholipase-D activity. Postharvest treatments also affected the molecular species distribution. Immature PC was more unsaturated, whereas heat-damaged PC was more saturated.

Zhang et al. studied the effects of the expander process on the degumming efficiency of the crude oil and on the phospholipid composition in the crude soybean oil and the lecithin obtained (110). The expander is a simple form of an extruder. The high temperature caused by steam injection and the shearing action convert soy flakes into porous collets due to destruction of the cell structure, protein denaturation, and the sudden release of the steam from the product at the exit. The results revealed that expander-processed soybean oil contained more phospholipids. However, the degumming efficiency was much higher (i.e., 93% as compared to 79% for conventional oil), so a lower phospholipid content was obtained in the degummed oil. Moreover, the expander-processed lecithin contained more PC and less PE as compared to the traditional lecithin, which is generally accepted as being superior in functional properties, such as O/W emulsifier. According to the authors, the effects could be due to heat deactivation of lipoxygenase (110). This

enzyme can oxidize unsaturated fatty acids of PC; the resulting oxidized PL form complexes with soy protein, thus reducing degumming yield. This is true particularly because the presence of PC also enhances the degumming efficiency of the other phospholipids. Besides, the extractability of PL from the meal may be improved due to a more porous structure of the collets.

Carelli et al. used the IUPAC method to compare the PL composition of water-degummed and crude sunflower oil (Fig. 4): crude sunflower oil contained about 1.0% of PL, 46% being PC, and 16% PA, whereas degummed sunflower oil contained only 0.1% of PL, with only 9% of PC and 64% of PA (34). Moreover, Carelli et al. found that the oil obtained by hexane extraction had a higher PL concentration than that obtained by hot-pressing. However, no significant differences in the PL profiles were observed. The average efficiency of water degumming was about 83%, independent of the method of oil extraction.

Weete used HPLC to evaluate the effect of thermalization, i.e., heating at 125–200°C for 30–120 min, on the phospholipid composition of soybean lecithins (111). It was found that phospholipids were broken down in the following order: PE > PI > PA > PC. Diglycerides were the principal products formed. The noncholine PL were selectively degraded, leaving PC as the sole phosphatide, so thermalization greatly enhanced the properties of lecithin as an emulsifier. The methanol soluble fraction of the acetone insolubles of thermalized lecithin contained 96% PC and 4% monoglycerides. According to the authors this process can be achieved in a controlled continuous-flow process and can be adapted to scale up production of the thermalized material for emulsifying purposes or as a source of PC.

Phospholipid analysis has also been used to investigate in detail the functional properties of food components. Vaghela and Kilara studied the phospholipid composition in whey protein concentrate, because the residual lipids are known to be detrimental to both the functionality and the flavor of whey protein products (24,112). Three major phospholipids in whey protein concentrates were sphingomyelin, PC, and PE, followed by PI and PS, in proportions that were quite similar to those of PLs in milk fat globule membrane rather than in milk as such. This result suggests that the majority of PLs in whey came from the fat globule membrane. Conforti et al. described a method for separating the lipid components of wheat flour: the starch lipids contained about 77% LPC and 10% LPE, thus demonstrating the huge importance of lysophospholipids in the functionality of wheat flour (56).

In the framework of an investigation of the mechanisms of flavor formation, Caboni et al. observed that cooking did not have a significant effect on the PL composition and the fatty acid content of the single PL in ground beef (31). However, SPH decreased uniformly in all cooked samples.

Finally, several researchers investigated the influence of food uptake on the phospholipid composition and molecular species composition of important tissues. Olsson et al. investigated the effects of chronic alcohol consumption on the membrane lipid class composition of human brains: no significant differences in the lipid class composition were observed between a test group and a control group (69). McKeone et al. studied alterations in serum PC fatty acyl species by eicosapentaenoic and docosahexaenoic ethyl esters in patients with severe hypertriglyceridemia (91). The drug-treated group received 4 g/day of an 85% concentrate of the ethyl esters of the n-3 fatty acids for 6 weeks, whereas control patients received 4 g/day of ethyl esters of corn oil fatty acids. Figure 8 demonstrates that the most substantial increases were seen in the relative percentages of $C_{16:0,20:5}$ and $C_{16:0,22:6}$ species. Hence, the n-3 fatty acids were taken up and incorporated into plasma PCs. It follows that the biomembrane composition may be altered due to nutritional, environmental, or xenobiotic exposure. Fatty acyl distributions appear to be most easily affected, whereas lipid class composition is less amenable to alteration.

Several studies have also focused on the phospholipid requirements in larval fish and crustaceans. In particular, the effects of diets supplemented with different mixtures of phospholipids

were compared in order to optimize feed composition (113,114). Although phospholipids seem to be an essential nutrient, up to now their role in promoting the growth of fish larvae has not been clear.

ABBREVIATIONS

A	acetonitrile
Ac	acetate
BuOH	butanol
C	chloroform
Ch	cholesterol
CE	cholesterol esters
DG	diglycerides
DPG	diphosphatidylglycerol
ELSD	evaporative light-scattering detection
FFA	free fatty acids
FID	flame ionization detection
GL	glycolipids
H	hexane
HAc	acetic acid
i-C8	iso-octane
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
M	methanol
MG	monoglycerides
NAPE	*N*-acyl phosphatidylethanolamine
2-P	2-propanol
O	oil
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL	phospholipid
PS	phosphatidylserine
RI	refractive index
SPE	solid-phase extraction
SPH	sphingomyelin
tBME	tertiary butyl methyl ether
TG	triglycerides
THF	tetrahydrofuran
UV	ultraviolet
W	water

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7
HPLC Determination of Carbohydrates in Foods

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I. THEORY

The analysis of food carbohydrates by high-performance liquid chromatography (HPLC) has been dealt with and reviewed by several authors, either as books or as chapters of books or as scientific papers, most notably in the current decade (1–6).

Table 1 summarizes the most important food carbohydrates, along with their structure. It also briefly describes where they are usually found. Carbohydrates, among the cheapest of food constituents, have traditionally been classified by food researchers into sugars and polysaccharides, although mixtures of them, such as glucose syrups, are also used, taking advantage of their respective characteristics. Sugars are utilized for their sweetening power, preservative action (osmotic pressure), and crystallinity in foodstuffs; polysaccharides provide foodstuffs with texture, body, and colloidal properties.

Nowadays, the assay of carbohydrates is a very important criterion for the quality control of drinks and foodstuffs, especially in dietary products. Other reasons for their determination include:

Monitoring of food-labeling claims
Analysis of sweeteners, bulking agents, and fat substitutes
Establishing authenticity
Fermentation monitoring in the production of alcoholic beverages

In this sense, HPLC is an analytical method that cannot be put aside in the determination of carbohydrates, since the wide variety of these species and their inherent polydispersity and heterogeneity require separation techniques of high resolving power and high selectivity (7).

A. Properties

Although all sugars have similar densities in the dry state, they differ significantly in other physicochemical properties. For instance, viscosity increases with increasing molecular weight, whereas osmotic pressure logically decreases. As for the crystallinity of sugars, it governs their textural characteristics as well as the sweetness-impact response, since hydrogen bonding is responsible for the packing of sugar molecules in the crystal cell and subsequently for their solu-

Table 1 Important Food Carbohydrates

Name	Structure[a]	Remarks
Glucose	Monosaccharide	Naturally very widespread in foods; also present in glucose syrups and invert sugars
Fructose	Monosaccharide	Found in most foods in small amounts; higher concentrations in honey and fruit
Galactose	Monosaccharide	Scarcely found in the free state in foods
Sorbitol	Monosaccharide	Reduction product of glucose; used mainly in foods for diabetics
Sucrose	Disaccharide (*Glu* + *Fru*)	Widely used in foods
Lactose	Disaccharide (*Glu* + *Gal*)	Found naturally only in milk and dairy products; used as a dusting powder for some baked goods
Maltose	Disaccharide (*Glu* + *Glu*)	Found mainly in glucose syrups and malt extracts
Raffinose	Trisaccharide (*Glu* + *Fru* + *Gal*)	Found naturally
Starch	Polysaccharide (*n* times *Glu*)	Used in foods as a filler and thickening agent; naturally present in many foods

[a] *Glu* = glucose; *Fru* = fructose; *Gal* = galactose.

bility, availability for taste receptors, and notably low vapor pressures. This last property determines the high melting points of these substances. Nevertheless, their crystalline lattices are so strong that they often decompose without melting, thus originating an array of pyrolisis products, such as those giving rise to sweet, syrupy, and caramel-type odors.

The dissolution of sugars in water is determined by the way the sugar molecules disturb the solvent structure. This depends mainly on the hydration shell surrounding each molecule, which probably affects its taste characteristics.

Sweetness appears to be associated with the sugars, although their sweetening power is really very low compared to most of the intense sweeteners. This characteristic depends on molecular structure and is determined specifically by the configuration at a single chiral center in the sugar molecule. Therefore only one change in configuration at one carbon atom may make a sweet molecule tasteless or even bitter. Additionally, sweetness is modified by the presence of other compounds; for example, when a sugar is mixed with a bitter substance (e.g., quinine sulfate), the bitterness depresses the sweetness. In a similar way, the sweetness of sucrose is depressed by the sourness of citric acid.

B. HPLC versus Other Analytical Techniques

The reader is directed to Ref. 5, which makes an interesting comparison between HPLC and other analytical methodologies for the determination of carbohydrates in foods. Additionally, notable progress has been made in the application of high-performance capillary electrophoresis (HPCE) in this field (8–11). However, given the scope of this chapter, we will focus on the advantages and drawbacks of other chromatographic techniques versus HPLC.

The wide range of currently available chromatographic techniques have all been applied, to varying extents, to the determination of sugars in foodstuffs. Earlier methods, such as open-column chromatography, paper chromatography (PC), and thin-layer chromatography (TLC), have now largely been replaced by HPLC and gas-liquid chromatography (GLC). Instrumental

techniques, in general, provide more rapid analyses with greater specificity and precision. Nevertheless, for multiple samples, where only qualitative or semiquantitative data is required, PC or TLC may still be the most appropriate alternative. Gas-liquid chromatography has become established as an important method in carbohydrate determinations since the early 1960s (12), and many diverse applications have been reported since then (6). In more recent years, HPLC has probably replaced GLC as the most commonly used technique, at least as witnessed by the relative volume of publications in this area. However, although in general HPLC does offer considerable advantages over GLC for carbohydrate analysis, in many ways both techniques are far from ideal. A comparison between them for this purpose is shown on Table 2. The earliest applications of column chromatography to carbohydrate analysis made use of either ion exchange or gel filtration, the former involving complex formation (e.g., borates) or use of the exchanger in a partition mode. Although both types of chromatography are still utilized under high-pressure conditions (with microparticulate stationary phases), partition chromatography has now become the more important, especially for low-molecular-weight carbohydrates.

Whether eluted from columns or from thin-layer plates, the quantitative determination of sugars was traditionally based on colorimetric reactions involving the use of chemical reagents, e.g., anthrone. These detection methods have been largely replaced in modern HPLC by the refractive index detector, although ultraviolet detectors are also employed. Recently we have also seen the introduction of other types of detector (e.g., the mass detector), as will be discussed later.

A flow diagram corresponding to a typical scheme—covering all the stages—for the HPLC analysis of a food material is shown in Fig. 1. However, the major difficulty found in the HPLC analysis of sugars in foodstuffs is not the chromatographic determination itself but rather in obtaining extracts of suitable concentration containing an acceptably low level of interferents. In some foods this is relatively simple to achieve, whereas in more complex processed foods the cleanup procedure may be considerably more tedious than the separation stage. Surprisingly, this is not always taken into account as it should be, since an inefficient sample preparation is liable to introduce errors. Only recently has much attention been paid to new manual methods of sample treatment as well as to automated procedures (faster and increasingly efficient). Due to their importance, these will be dealt with in the following section.

Table 2 Comparison Between HPLC and GLC for Sugar Analysis

HPLC	GLC
More suitable for the determination of medium- and high-molecular-weight polysaccharides	Far more sensitive
Shorter (50%) analysis times	Allows separation of α and β anomers
Higher recoveries with greater accuracy	Preferable for monosaccharides
Directly applicable to sugar samples	Derivatization required

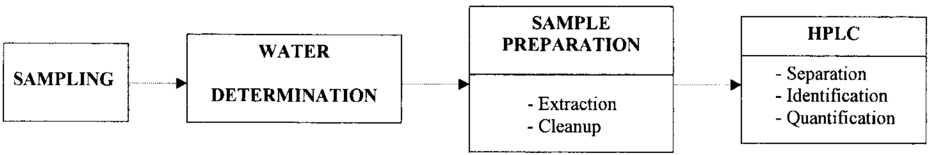

Fig. 1 Flow diagram for HPLC sugar analysis.

II. SAMPLE PREPARATION

Samples to be analyzed can be of very complex nature (dairy products, fruit juice, etc.). Therefore, and provided that the preliminary steps—namely, sampling and moisture determination—have been properly attended to, the first important stage is sample preparation, which comprises extraction and cleanup. Once a sufficiently clean extract has been obtained, the carbohydrates therein may be separated prior to their detection and quantitation.

A. Extraction

Liquid–liquid extractions (13) permit the elimination of slightly polar molecules (phospholipids, fatty acids, etc) that may interfere in the HPLC determination of carbohydrates. Hence, for solid foodstuffs, some form of extraction will be required prior to the chromatographic procedure, and even in liquid food samples it may be necessary to modify the solvent composition of the liquid phase to make it compatible with the HPLC eluent. The major role of the extracting solvent is to obtain all of the carbohydrate present in the food sample dissolved in a liquid phase, be it for direct injection into the chromatograph or for subsequent cleanup stages prior to HPLC.

If the extraction is to be efficient, then all of the carbohydrates of interest must be readily soluble in the chosen solvent; hence, water would theoretically be ideal. Unfortunately, water will also extract an important amount of other polar components, most notably amino acids or proteins, which can interfere in the chromatographic process. The extraction solvent must therefore be chosen to have a suitable solubilizing effect on the components of interest (carbohydrates) but not on the others. Bearing that in mind, it is hardly surprising that ethanol–water mixtures containing 75–80% alcohol are the most frequently used (14). It also ensures a simultaneously acceptable selectivity and efficiency. For larger carbohydrates the extraction may be enhanced by replacing ethanol with methanol or, alternatively, by decreasing the proportion of ethanol in the mixture (15,16), although both procedures may ultimately require an extra cleanup stage.

Regardless of the solvent utilized, the extraction may take place at a high temperature (reflux) or at room temperature. In order to ensure complete extraction, the process is repeated four or five times until a colorimetric test confirms that no sugars remain in the final extract. Table 3 summarizes some of the most important problems encountered in the extraction step.

The second half of the 1990s saw an increase in the use of dialysis (as a liquid–liquid extraction procedure). Its main advantage is the possibility of operating in an automatic mode by coupling a dialysis unit with an automatic injector, as demonstrated not only in HPLC analysis (17) but also in flow-injection determinations of reducing sugars in wines (18) and alcoholic fermentation broths (19).

Table 3 Major Problems Found in the Extraction of Carbohydrates from Food Samples

Effect	Cause	Solution
Degradation of labile carbohydrates	High temperature	Work at room temperature
Inversion of sucrose	High temperature and low pH of the extract	Work at room temperature, and addition of $NaHCO_3$
Contamination of the extract	Presence of yeasts	Add sodium azide as a preservative, or use EtOH/water
Low selectivity	Use of water alone as solvent	Try EtOH/water

B. Cleanup

Some factors will condition the choice of the cleanup procedure, namely: (a) the extraction solvent used in the previous stage, (b) the concentration of the carbohydrates present in relation to potential interfering compounds, and (c) the complexity of the analyzed food. Additionally, the nature of these interferents and the need to minimize the loss of analyte during the cleanup will become key aspects to be considered. In any case, and regardless of the final method chosen, it is essential that the percentage recovery of the cleanup procedure be determined. The most frequently utilized procedures will be commented next.

1. *Clarifying Agents*

The main purpose of a clarifying agent is to precipitate proteins and/or fatty acids, which are particularly troublesome in sugar analysis by HPLC. However, unlike in traditional methods of sugar analysis (such as the titration with the Soxhlet reagent), where the utilization of clarifying (clearing) agents is a common practice, in HPLC analyses this is not so widespread, due to the fact that a nonselective detector is often used. Consequently, any excess of clarifying agent must be carefully removed to avoid undesirable chromatographic interferences. $Pb(CH_3COO)_2$, a widely used agent in traditional sugar analysis, has found some application, its excess being eliminated by ion-exchange chromatography (20). Another example is the use of Carrez reagents (zinc acetate + zinc ferrocyanide) (21), which does not appear to produce spurious peaks or distortion of the chromatograms. Unfortunately, in this case amino acids and salts are not removed, with the subsequent difficulties.

2. *Solvent Precipitation*

This procedure is often used when the extraction solvent chosen has been water alone, since in this case polar compounds such as salts and amino acids may contaminate the extract. Precipitation with an appropriate solvent, e.g., a mixture of acetate buffer/acetonitrile (22), is then required. Water-soluble macromolecules also interfere and may be removed to a considerable extent by simple precipitation with an organic solvent, for example, ethanol. Nevertheless this may be troublesome, since a proportion of the carbohydrates of interest may become absorbed to the precipitated material, which will be subsequently lost. On the other hand, the sugars must be sufficiently soluble in the solvent used to avoid its precipitation from the solution.

Acetonitrile precipitation has been the subject of some papers dealing with the analysis of mono- and disaccharides in milk and dairy products, oligosaccharides in soybeans, and general methods for sugars in foods (23). Aqueous ethanol has also been frequently utilized in the extraction of lipids as undesirable components along with the carbohydrates. A further treatment with chloroform will free the hydroalcoholic extract from the lipids.

3. *Ion Exchange*

As already mentioned, the presence of some high- and low-molecular-weight ionic species in the food material (and hence in its aqueous alcoholic extract) may seriously interfere in the chromatographic process. For example, the risk of overlapping peaks increases when salts and/or amino acids occur in the sample, since some of them under certain chromatographic conditions may elute very near to a carbohydrate of interest (24). On the other hand, proteins can become irreversibly bound to the columns, resulting in a very rapid deterioration of performance. In most of these cases the use of a mixed-bed ion-exchange resin permits the complete removal of all these interfering species, with no loss of carbohydrates. Nonetheless, it has been observed that some ion exchangers tend to adsorb sugars, thus yielding low recoveries and decreasing the

efficiency of this process. Care must then be taken if they are to be used. Ion-exchange cleanup methods have been used most commonly with fruit-based products, such as wines and juices, where not only proteins or amino acids but also organic acids are removed. However, particular samples, e.g., fermentation juices (25), need a more complex preparation because of the presence of many degradation products.

4. Sep-Pak Cartridges

Apart from ion exchange, the use of a chemically modified silica (e.g., C_{18}) has also become a common chromatographic procedure for extract cleanup, either in an open column or—as usually preferred—in the form of a Sep-Pak cartridge attached to the end of a syringe. In this last, once the aqueous carbohydrate extract is forced through the cartridge, any nonhydrophilic molecules (including proteins) will be retained and the carbohydrates will pass straight through. No decrease in their concentration will then take place in the extract. This method has been advantageously employed when complex extracts are to be obtained from direct extraction, as is the case with cereal products. Despite its relatively high cost, this technique is straightforward, with a considerable simplicity and excellent yields concerning carbohydrate recovery. Unfortunately, these cartridges have a limited useful life, since even their washing with hydrophobic solvents cannot—in the long run—avoid the irreversible binding of macromolecules.

5. Guard Columns

Provided that the eluent utilized is compatible with both the cleanup and the chromatographic separation, ion exchange can be employed in a guard column placed inline between the injector and the analytical column. This type of column should remove all interfering compounds (which may give rise to overlapping peaks in the chromatogram) without decreasing the final resolution. For this purpose, relatively long (4–6 cm) and narrow-bore (2–2.5 mm) guard columns packed with pellicular phases or other coarse material are usually employed. It must be noted that the packing material filling the guard column may be different than the one used in the analytical column. This leads to a selective retention of compounds, depending on the more or less hydrophobic character of the guard column. Nevertheless, in those cases in which it serves only as a replaceable "column top," a common analytical practice consists of packing it with the same chromatographic material. Anyway, the use of guard columns is especially recommended when extracts from complex foods are to be analyzed; some interesting examples are found in the literature (3,6).

6. Column Switching

The technique of column switching is perhaps the most recently developed method of chromatographic cleanup. It may be considered a multidimensional chromatography, according to which the eluate from the HPLC column (containing the analytes) is switched to a second column, where further separation takes place. In this method, different column types are used, with their selectivities also being different. One typical example would involve the utilization of a column with a separation based on gel filtration (different molecular sizes) followed by the elution of the remaining low-molecular-weight carbohydrates by means of a partition column. Nonetheless, this case is far from being ideal, for mainly two reasons: (a) dilution of the carbohydrate solution resulting from the first elution turns out to be unavoidable, which will obviously affect both the resolution and the final sensitivity, (b) contamination of the first column (gel filtration) as a consequence of adsorption. Fortunately, the automation of this procedure that has been developed so far guarantees—to a certain extent—its success (6).

III. SEPARATION TECHNIQUES

The chronology of the development of the different separation techniques employed for carbohydrate analysis in foodstuffs may be summarized as follows:

Paper chromatography → Gel filtration → Ion exchange → Partition (bonded phases)

These modes of chromatography will be discussed in detail, with the exception of paper chromatography, which has now largely been replaced by column chromatography and which therefore is of interest in sugar analysis only from a historical point of view. Table 4 summarizes the main developments in this field, including some recent contributions (26).

A. Gel Filtration

This was the first successful column separation technique, although time-consuming analyses and poor resolution have always been two major drawbacks, especially taking into account that the earliest gels (usually Sephadex or Bio-Gel) applied to carbohydrate analysis were nonrigid and thus could not be used under high-resolution conditions. Nevertheless the situation changes significantly when simple or very heterogeneous (in terms of molecular size) carbohydrate mixtures are to be chromatographed; in this case satisfactory results can be obtained. On the other hand, long analysis times can be shortened by utilizing a wide range of commercially available rigid gel-filtration media, which permit higher flow rates and pressures. Advantage has been taken of this procedure, most notably in the characterization of polysaccharides (see Sec. V).

B. Ion-Exchange Chromatography

Both anion- and cation-exchange resins have traditionally been utilized in carbohydrate analysis. However, this technique has recently been superseded by the use of partition systems, mainly because the former involves long analysis times as well as the need to operate the column at high temperatures, whereas the latter improves the separation of higher-molecular-weight oligosaccharides.

Typical applications of ion exchange in sugar analysis include: (a) complexation of borates, which accentuates ionic interactions with the exchanger (27), (b) use of hydroorganic eluents, especially acetonitrile/water, with rigid, fine-particulate anion columns (28), (c) use of basic eluents, since most carbohydrates are weak acids with pK_a of 12–13 (29), (d) complexation with cations, Pb^{2+}, Ca^{2+} and Ag^{+} being the most frequently employed, (e) the use of cation exchangers in a heavy metal form, e.g., Aminex HPX-85.

Recent advances in the development of ion-exchange resins have made possible their use at higher flow rates, with a consequent reduction in analysis time. On the other hand, ion-exchange systems can readily separate glucose, mannose, and galactose, which is a problem area

Table 4 Major Types of Chromatographic Separations in Sugar Analysis (According to the Stationary Phase)

Bare silica gel	Octadecyl-bonded phases
In situ amine-impregnated silica gel	Copper silicate gel
Amine- or amide-bonded phases	Cyclodextrin-bonded phases
Diol- and polyol-bonded silica gel	Hydroxyapatite-covered silica
Anion exchangers	Graphitized carbon
Cation exchangers	

with partition systems. Finally, another important advantage of "ion exchange" over partition systems is that the former normally uses water as the eluent, which avoids all the potential toxicity problems associated with the use of acetonitrile in the latter.

C. Partition Chromatography

Partition systems employing polar bonded phases are now the cornerstone of HPLC analysis of carbohydrates in foodstuffs. In this case, the emergence of stationary phases with low-dispersion silica microparticles gave a spectacular start to the use of this separation procedure. Amine-bonded silica gels are the most frequently utilized systems for mono- and oligosaccharide separations, with silica usually being bonded by a primary amine and, to a lesser extent, by secondary and tertiary amines (30). However, care must be taken to avoid or minimize the formation of Schiff bases between reducing sugars and the amino moieties (31). Additionally, the lifetime of these amino-bonded phases remains quite short because of a certain degree of phase hydrolysis (32), although this problem may be largely overcome by making use of trifunctional-silane (e.g., 3-aminopropyltriethoxysilane) bonded phases or, alternatively, an eluent recycling system to extend the column life (33). In addition to facilitating good steric accessibility to the amino group, the propyl spacer arm may provide the phase with some hydrophobic character, which, under certain eluent conditions, can give rise to unexpected elution sequences. This behavior is, however, rarely found with carbohydrates, unless they are derivatized to increase their hydrophobicity.

Theoretically, a wide range of polar mobile phases could be used with these polar amino-bonded phases, with water being a major component to ensure adequate solubility of the sugars. Nonetheless, the eluent is usually an acetonitrile/water mixture, the relative proportions of which can alter the retention times (the less complex the carbohydrate molecule, the greater the percentage of acetonitrile required). This is especially surprising if we consider the following: (a) in terms of polarity, solutions of aqueous methanol can be prepared of equivalent eluting power but providing higher solubility for monosaccharides; (b) aqueous solutions of acetonitrile are toxic and in confined spaces can produce explosive vapors (no vessel containing acetonitrile should be left open to the atmosphere, including those for waste solvent). Efforts have been made to find a suitable alternative eluent, including ethyl acetate/ethanol/water and dichloromethane/methanol (34), but none of them has shown any improvement over aqueous acetonitrile.

Several new phases applied to maltosaccharide analysis that seem to be promising have been described in the literature, for instance, a silica-phase covered with polymeric polyamine resin, and an entirely polymeric resin containing an amide function (35). In any case, partition chromatography is restricted mainly to the utilization of polar-bonded phases, as already described. Nevertheless, there have been a certain number of applications of reversed-phase chromatography that permit relatively simple separations to be achieved. Octadecyl-bonded silica phases are the most widely used, although few applications involve carbohydrate analysis. Their interest lies rather in the analysis of derivated sugars, where the selectivity increases (36).

D. Adsorption Chromatography

In spite of the fact that this chromatographic technique is not generally associated with carbohydrate analysis, some applications are found in the literature where silica gel has been employed either directly or indirectly (after modification of the phase or the analytes). The use of silica gel for this purpose involves a polar eluent such as ethylformate/EtOH/H_2O (6:2:1, v/v/v) and has been applied to the chromatographic separation of some mono- and disaccharides.

Precolumn derivatization has been a widely used resource in adsorption chromatography of carbohydrates, although it alters their related properties and in some cases their detectability.

The derivatization allows the use of scarcely polar eluents, e.g., hexane/ethyl acetate (2:1, v/v), since it greatly reduces the polarity of the carbohydrates. Another important advantage is that these derivatives are straightforward, with a high absorption in the ultraviolet region, and thus a higher sensitivity is achieved with respect to the use of refractive index detectors. On the negative side, one of the most outstanding features of HPLC in carbohydrate analysis when compared to GLC, the possibility of avoiding derivatization, disappears.

Finally, other proposals include the introduction of an amine into the eluent so that, unlike in bonded phases, preparation of the amino phase takes place in situ. This competes advantageously in terms of degradation of the column, since with this procedure columns may be immediately repaired by recoating and therefore losses in performance are minimized and even avoided.

IV. DETECTION SYSTEMS

Detection has always been troublesome in the HPLC analysis of carbohydrates. Until recently, only the refractive index detector could be used without significant problems, since other alternatives, such as fluorescence and UV-V detectors, required a previous derivatization of the sugars (they do not fluoresce and absorb only at low wavelengths). Derivatization techniques, either pre- or postcolumn, were thus nearly a must in this field. Fortunately, new detection systems have been proposed in the last two decades that have partially solved the difficulties, although detection still remains far from ideal. In this section this somewhat problematical stage of sugar analysis will be covered, with special attention being paid to selectivity, detection limits, and compatibility with elution gradients.

A. Derivatization Techniques

Colorimetric and ultraviolet detection in sugar analysis need to resort to derivatization techniques, due to the fact that these compounds are colorless and do not have easily exploitable UV chromophore groups. Postcolumn reactions are then required to produce derivatives for detection in the visible and UV regions. Different colorimetric methods have been utilized, many of which are based on the reaction of orcinol in concentrated sulfuric acid (37). Another alternative is the use of tetrazolium blue as a reagent for detecting reducing sugars (38), although sorbitol, sucrose, or raffinose (nonreducing properties) would demand a preliminary hydrolysis. This growing trend toward the use of derivatization procedures can be partly explained by the increased demands for a sensitive and selective detector for carbohydrates (given the low sensitivity of the refractometric detection), and there is no doubt that it may continue in the near future. Figure 2 shows a scheme of the configuration used for postcolumn derivatization detection.

An alternative approach, which additionally favors the separation of sugars from interfering compounds, is the precolumn formation of the derivatives; in this case trace sugar detection is sometimes possible, as nmol or pmol orders are approached. Examples include the use of dansylhydrazine (with either fluorimetric or colorimetric detection) (39) and 1-phenyl-3-methyl-5-pyrazolone (UV absorption or electrochemical detection) (40).

B. Refractive Index Detection

This is still the most widely used detection method for carbohydrates, in spite of its lack of sensitivity. It has the advantage of being universal, and some of its drawbacks can be avoided by suitable sample preparation. This low sensitivity does not present a big problem in most foodstuffs,

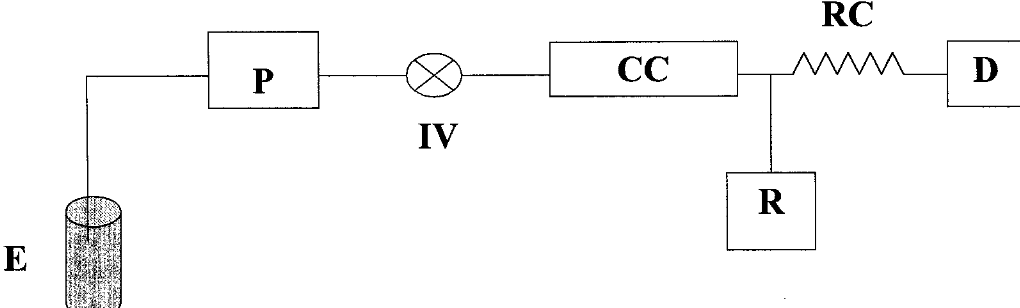

Fig. 2 Configuration used for postcolumn derivatization detection in HPLC. E = eluent; P = HPLC pump; IV = injection valve; CC = chromatographic column; R = pump reagent; RC = reaction coil; D = detector.

since carbohydrates often occur as major components and levels of individual sugars down to 0–5% may be quantified. Nonetheless, the situation changes dramatically when trace amounts are present, due to the fact that the refractometric detector is nonspecific and its response may be altered by (a) changes in environmental conditions, such as temperature, pressure, composition, and even levels of dissolved air, (b) any dissolved solute (e.g., salt) that will produce a peak irrespective of its nature, which may appear near a carbohydrate of interest, giving rise to confusing peak assignments. Furthermore, its principle of operation, with a reference cell for the mobile phase, rules out any possibility of making use of gradient elution. On the other hand, the response factor depends on the nature of the solute (41) as well as on the flow rate of the eluent.

As a consequence, some steps must be taken when using the refractive index detector: (a) solvent delivery must be degassed and pulse free; (b) the composition of the mobile phase should remain constant during a single run (isocratic elution); (c) the detector must be carefully thermostated; and (d) the temperature of the column must also be kept constant, since a variation may affect the equilibrium of the solvent components between both phases and therefore the composition of the eluate from the column.

Some firms are now introducing more sensitive refractometric detectors, most of them based on interference effects, with an increase in sensitivity. They offer low detection limits (42), and some of them have even been adapted for gradient elution (43), which was a major disadvantage of this type of detector, especially in the case of complex mixtures or for mixtures containing carbohydrates with a wide range of molecular weights, as in glucose syrups. Nevertheless, they are still very susceptible to environmental changes.

C. Ultraviolet Detection

It is commonly accepted among chromatographists that this detection system does not offer significant advantages over the refractometric detector. One of the main problems is that at the monitoring wavelength at which carbohydrates show an acceptable absorbance (190–210 nm), most other organic components encountered in foodstuffs also absorb, as do the usual eluents. Therefore, on one hand the sample extract must be completely purified, since the slightest interference can render chromatograms unusable; on the other hand, only aqueous acetonitrile of a suitable grade can meet the transparency requirements in this region of the spectrum. Obviously, because of this high absorbance of organic compounds, it is difficult or even impossible to use elution gradients if a certain sensitivity is required, since a considerable shift in the baseline would be unavoidable. It should also be noted that, in the case of direct UV detection, molar re-

sponse factors depend on the nature of the eluted carbohydrates, with the limit of detection of simple sugars varying from 1 to 12 μg (31).

D. Evaporative Light-Scattering Detection

Also known as the mass detector, this is an evaporative analyzer in which the mobile phase is removed by nebulization and evaporation prior to the determination of nonvolatile carbohydrates by light scattering (44). Unlike the refractive index detector, it allows gradient elution (eluent is removed before detection) and is more sensitive. The detection limit can go up to a few tens of nanograms injected.

Another important advantage over the previously discussed detection systems is that the detector response varies very little with the nature of the carbohydrate. Additionally, it is straightforward, with a great flexibility of use, given the absence of baseline drift and its rapid equilibration. And last but not least, it is not sensitive to temperature changes or to variations in the mobile-phase flow. The former may be of great importance in preventing the possibility of thermal degradation of the sugars. In this sense, evaporation temperatures as low as 35°C have successfully been tested. Still, this promising, easy-to-use detector of universal nature is still little utilized, although some interesting papers have appeared concerning its use and applications (45,46).

E. Electrochemical Detection

Electrochemical detectors in HPLC have encountered many applications in the analysis of foodstuffs. Nonetheless, although single potential instruments provide excellent signal-to-noise ratios and selectivity, they are not recommendable for sugar detection, since electrochemical reaction products contaminate the surface of the electrode by depositing on it. Fortunately, the use of a pulsed amperometric detector (PAD) overcomes this trouble and allows specific electrochemical detection without derivatization, by direct oxidation on a gold electrode in a highly alkaline medium (47). It is based on a three-step potential cycle lasting a few fractions of a second; the —OH groups can be oxidized at the first potential (≈200 mV), the gold electrode surface is then oxidized to remove reaction products (≈600 mV) and then finally reduced to its initial state by the application of the third voltage (≈−800 mV).

This detection system is coupled with anion-exchange chromatography (48,49), which has the same alkaline pH requirements. The sensitivity of PAD is considerably higher than that yielded using the refractometric detector, with detection limit being of the order of 10 pmol (29). On the other hand, its response also depends heavily on the nature of the carbohydrate. Finally, other alternatives described in the literature include the use of nickel-based or copper-based electrodes and catalytic oxidation (50).

F. Other Detection Systems

Polarimetry takes advantage of the optical activity of carbohydrates. The high selectivity of this procedure makes it especially suitable in the case of complex food extracts, where other components would interfere with ultraviolet or refractive index detection. However, a major disadvantage is its lower sensitivity. The use of immobilized enzymes (51) with detection by fluorescence or electrochemistry has also been applied in fermentation juices (52) and other particular cases.

Indirect conductimetry with a mobile phase containing polyol-borate complexes allows a detection limit of 10^{-5} mol of carbohydrate (53). A lower sensitivity is reached with the moving-

wire detector (transport by wire and detection by flame ionization), although it allows gradient elution. Detection by radioactivity (54) has also been described in very specific applications.

V. APPLICATIONS IN FOODS

High-performance liquid chromatography remains the most widely used technique in the determination of sugars in foodstuffs. In fact, all those foods in which free carbohydrates occur significantly have been analyzed by means of this technique.

There is no ideal HPLC system for the analysis of carbohydrates in foods, but of the techniques offering different and complementary selectivities, each has its own advantages and drawbacks. The characteristics of the principal systems that, at present, are most widely used are summarized in Table 5. Additionally, the reader is directed to the recent review by Churms (4), in which the performance of several HPLC systems is compared with respect to their capacity factors for sugars and polyols.

A representative selection of applications is shown in Table 6, which illustrates the wide choice of techniques that have been used, with attention being paid to the type of sugar and matrix, eluent, and stationary phase, as well as the appropriate detection method. An example of a chromatogram is presented in Fig. 3.

Some particular applications can also be mentioned because of their interest—for instance, the determination of oligo- and polysaccharides derived from hydrolyzed glucose syrup, as well as the analysis of sugar alcohols (especially in dietetic hard candy and chewing gum), for which nutritional labeling requirements must be met in some countries. High-performance LC is also in daily use in the sugar industry to analyze nutritive sweeteners, be they natural or substitutes (e.g., sucralose or kestose). This is of growing importance because of the increasing public concern

Table 5 Major HPLC Systems for Carbohydrate Analysis

Mobile phase	Stationary phase	Retention mechanism	Detection system	Major advantages	Major drawbacks
NaOH & H_2O	Anion-exchange resin	Ion exchange	Pulsed amperometry	High selectivity for mono-, di-, and trisaccharides; high sensitivity	Need for specific instrumentation; instability of some products at basic pHs
CH_3CN/H_2O	Amino-bonded silica	Partition, hydrogen bonding	Refractometry & ELSD[a,b]	High selectivity for mono- and disaccharides	Formation of Schiff bases; chemical instability of the column
H_2O	Octadecyl-bonded phase	Molecular sieve	Refractometry ELSD[b]	Stability of the column	Separation of anomers of oligosaccharides; poor separation of monosaccharides
H_2O	Cation-exchange resin	Partition, exclusion, and others	Refractometry ELSD[b]	High selectivity for monosaccharides	Need for periodical regeneration; column fragility

[a] ELSD = evaporative light-scattering detector.
[b] An alternative is detection after derivatization.

Table 6 Examples of Applications of HPLC to Carbohydrate Analysis

Sugars	Sample	Mobile phase	Stationary phase	Detector
1, 2, 3	Tonic water	CH_3CN/H_2O (75:25)	LiChrosorb NH_2	UVS (190 nm)
1, 2, 3	Fruit juice	CH_3CN/H_2O (85:15)	Brownlee amine column	UVS (190 nm)
1, 2, 3,4	Cereals (sweetened)	CH_3CN/H_2O (80:20)	μ-Bondapak carbohydrate	RID
1, 2, 3	Sorghum	H_2O (25°C)	Aminex HPX-87	RID
2, 5, 6	Milk	H_2O (80°C)	Aminex HPX-87	RID
1, 2, 3, 4, 6	Cream, cereals, beverages	CH_3CN/H_2O (80:20)	μ-Bondapak carbohydrate	RID
3, 7, 8	Winged beans	H_2O	Aminex 50W X-4	UVS (254 nm)
1, 2	Melon	H_2O	Dextropak	RID
1, 2, 5, 9, 10	Coffee	CH_3CN/H_2O (84:16)	Spherisorb-NH_2	ELSD
1, 2, 3, 4, 6, 11, 12, 13	Molasses, mango, chicory	CH_3CN/H_2O (75:25) + 0.01% amine modifier	LiChrosorb Si60	Postcolumn reaction
1, 2, 3, 4	Vegetables	CH_3CN/H_2O (75:25)	μ-Bondapak carbohydrate	RID
2, 3, 7, 8	Soyabean, yoghourt	CH_3CN/H_2O (64:36)	Spherisorb NH_2	RID
1, 2	Honey	CH_3CN/H_2O (80:20) + 0.02% amine modifier	LiChrosorb Si60	RID

1 = fructose; 2 = glucose; 3 = sucrose; 4 = maltose; 5 = galactose; 6 = lactose; 7 = raffinose; 8 = stachyose; 9 = arabinose; 10 = mannose; 11 = ribose; 12 = xylose; 13 = melibiose. RID = refractive index detector; UVS = ultraviolet spectrophotometer; ELSD = evaporative light-scattering detector.

over health and nutrition. Special emphasis must be put on the determination of levels of fermentable and nonfermentable sugars at every stage of beer and wine production, since the former is related to the final alcohol content, whereas the latter contributes to the flavor and "body" of the resulting product. Therefore, HPLC plays an important role in the monitoring of these fermentation processes.

The most recent contributions in this field show that high-performance anion-exchange chromatography at alkaline pH, coupled with pulsed amperometric detection (HPAE-PAD), is emerging as perhaps the best approach (55). Sugars, sugar alcohols, oligo-, and polysaccharides can be separated with very high resolution in a single run without derivatization, and can be quantified down to 10^{-12} mole levels. The technique is being applied to a wide variety of routine monitoring and research applications, and official methods have been approved by the International Standards Organization and other official regulatory agencies.

Finally, this section would remain incomplete without a few comments on the applications of HPLC to a particular group of carbohydrates—polysaccharides—whose determination, both qualitatively and quantitatively, has received much less attention than the rest (56). This may be surprising when the importance of these compounds, in terms of both functional properties and nutrition, is considered, but it is not so surprising when the difficulty of the analyses required is studied. High-performance LC can be used in this field, either to characterize the polysaccharides per se or to study their carbohydrate composition and the nature of bonding after acid or enzymic hydrolysis.

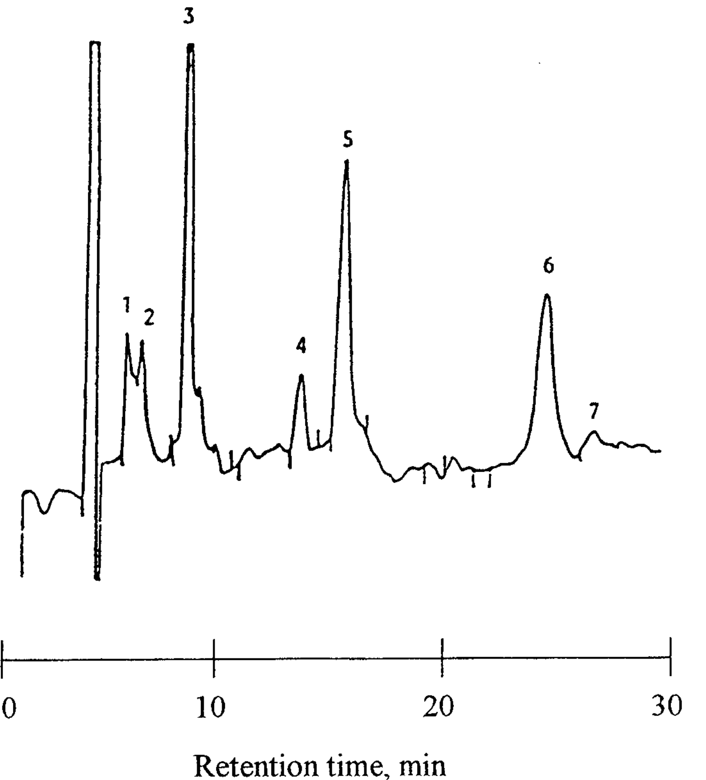

Fig. 3 HPLC separation of carbohydrates in chickpeas. Column (200 × 4.6-mm ID) LiChrosorb NH_2; flow rate 1 ml min^{-1}; mobile phase 20% water in acetonitrile. Refractive index detector. Peak assignment: 1, fructose; 2, glucose; 3, sucrose; 4, raffinose; 5, maninotriose; 6, stachyose; 7, verbascose.

Characterization can be carried out by using HPLC with bonded amino phases to separate polysaccharides with intermediate molecular weights, i.e., up to about 2500, depending on the composition and hence solubility properties. As for larger polysaccharides, gel filtration is undoubtedly the chromatographic technique of choice, as witnessed by the references found in the literature. In this sense, conventional gels, such as Bio-Gel and Sephadex, have been commonly used to characterize polysaccharides according to molecular weight profiles. The combination of rigid gels and high-resolution conditions will enhance the performances of this chromatographic mode, which is still relatively low in terms of resolution and cost. Nevertheless, further development work is required in terms of gel-filtration media with good mass transfer characteristics for macromolecules.

With respect to carbohydrate composition and the nature of bonding, both are currently dominated by GLC, although in some cases HPLC reveals itself as a valuable complementary technique. Determination of the sugar composition of a polysaccharide begins with a preliminary hydrolysis stage by means of dilute acids. The resulting free carbohydrates may then be determined utilizing the usual methods mentioned earlier, with a previous neutralization of excess acid and removal of its salt by ion exchange.

As regards the nature of the glycosidic bonds in a polysaccharide molecule, it may be determined by derivatization (formation of methyl ethers) prior to acid hydrolysis. As a consequence of this process, the —OH groups formerly involved in bonding are transformed into acetyl esters, whereas the free hydroxy groups occur as methyl ethers (analyzed by GLC).

Nonetheless, adsorption or reversed-phase chromatography may also play an important role in this characterization, provided that the correct choice of derivatization procedures is carried out. Some development work is needed, but—as in other areas of food analysis—HPLC has a lot to offer and will probably do in the near future.

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8
HPLC Analysis of Alcohols in Foods and Beverages

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I. INTRODUCTION

The reasons for determining the alcohol content of foods and beverages are many. A brief examination was carried out by Amelio (1), large parts of which are reexamined here.

> The determination of alcohols in foods and beverages is of interest to many sectors, from market classification, to quality control, from research of adulterations, to the understanding of the natural processes which control their formation or the effect of technological processes on products intended for human consumption. The determination of the alcoholic content of wines and distilled spirits is perhaps the field which interested the analytical chemist first of all, given the huge importance taken on by the production and commercialization of these products. Because of this, the producing countries have for a long time had regulations, which control their production and commercialization, often with widely differing provisions. The current tendency leans towards the greatest possible uniformity of regulations, in an attempt to tune them in with ever-wider markets (i.e., European Community). The market classification of alcoholic drinks, given by the alcoholic content, is one of the most important parameters in establishing tax. The importance of the exact determination of alcoholic content, and the risks of adulteration to which these products are exposed, is obvious. However, the interest in the alcoholic content of foods is due also to other reasons which are linked to their properties and which contribute to conferring the characteristics of that food. This is the case, for example, of alcoholic drinks, where ethanol also has the role of emphasizing the aromatic components by increasing their volatility. (Reprinted from Ref. 1, p. 551, by courtesy of Marcel Dekker Inc.)

In general, the alcoholic component, and in particular ethanol, gives wines mellowness, attenuating the components of roughness and the dry sensation given by tannins, and freshness due to acids. Moreover, ethanol generates a pseudowarmth sensation through its vasodilator effect and salivary dehydration. "A similar case is that of the higher alcohols, which, in wines, have an effect on the charateristics of the bouquet" (Reprinted from Ref. 1, p. 551, by courtesy of Marcel Dekker Inc.). In fact, in this case the polyalcohols have the function of contributing to the mellowness and to the velvetyness of the wine.

Of these, probably the most important is

> glycerol, which is formed during the fermentation of musts, with its sweet taste, [and which] contributes to the smoothness and the body of wines. However, in certain circumstances, it can

indicate defects in harvested grapes. In alcoholic fermentation there is the formation of glycerol with yield of 2–3% from the fermented sugar. This content diminishes in acetic fermentation, but the best-quality vinegars contain larger quantities compared with ordinary ones. Very low amounts of glycerol are a signal of possible adulteration. Methanol, given its toxic properties, which require a control of its concentration in drinks, which may contain it, has a different importance. In wines it is naturally present in small quantities, as it originates during the fermentation of pectins in the grapes. In particular, in the preparation of distilled spirits, the methanol content must be kept under control, even though it is concentrated in the "head," which is thrown away. Other alcohols are checked for different reasons: for example, 2-butanol in distilled spirits or in vinegars is indicative of bacterial spoilage in the mashes or wines used for distillation, whereas higher alcohols are indicators of wholesomeness and the botanic origin of the distillates. There are also industrial reasons for determining alcoholic content, such as monitoring vinification processes, and, in general, of fermentation, production of low-alcohol beverages, etc. Since it is thought that the quality of white wines is in inverse relation to the content of higher alcohols (isobutanol, act-amyl and isoamyl alcohols), studies to clarify their origin and conditions of formation have been carried out by LM Klingshirn, JR Liu, JF Gallander (2). In honey, on the other hand, the presence of not small quantities of ethanol is an indication of undesired fermentations which damage the product. (Reprinted from Ref. 1, p. 551, by courtesy of Marcel Dekker Inc.)

Special reference must be made to the study of fermentations, which besides the wines, musts, and beers already mentioned, is of great interest in other alimentary sectors as well. A large number of products undergo fermentations before being put on the market (for example, by-products of milk, vegetables, olives, cocoa, etc.).

Fermentation proceeds via the development of microorganisms that the food industry controls and corrects to obtain the desired results. The main substratum that is transformed is made up of carbohydrates, which may undergo various types of fermentation, with the production of more simple substances that are very important in the determination of the quality of the final product. In some cases, the formation of some substances may indicate undesired processes. There is, therefore, the need to intervene rapidly so that the necessary corrections may be made to the process. Fast analytical methods are required that can in most cases ascertain the content of various sugars, organic acids, glycerol, and alcohols (for example, methanol, ethanol, higher alcohols).

As we will see later, enormous efforts have been made to determine in the best way all the analytes of interest, modifying the operative conditions to each single case. Given the chemical-physical characteristics of these analytes, high-performance liquid chromatography has been shown to be a highly suitable technique in these determinations. This technique gives advantages in terms of rapidity of analysis, often also of precision, and last but not least simplicity in the preparation of samples. The fact that results can be obtained rapidly is certainly an excellent characteristic of this method to be used for "online" checks.

As we have already mentioned, another role is played by alcohols which contribute, through their presence, to the complex volatile fractions which give characteristics of taste and smell to a large number of foods. In milk, for example, these volatile fractions are often used as indicators of quality and their variation in composition is placed in relation to storage time and temperature, to thermal treatments, to exposure to light, etc. A study of these fractions also contributes to clarifying the metabolic mechanisms of their formation, which, in the case of milk, depend on the animal which produces it. Besides, for certain foodstuffs, the aromatic component is decisive in characterizing the product and consents a specific market placing. In other cases (i.e., vegetable oils), the volatile component, to which, as we would like to remind our readers, alcohols belong, has been used to evaluate oxidation during storage. The deter-

mination of alcohols is also used in the sector of adulteration controls, as in the case of olive oils. The determination of the content of fatty alcohols may be useful in recognizing where there are mixtures between pressed olive oils and solvent extracted oils (pomace oil) in samples under examination. As certain technological refinement processes give rise to structural modifications of some triterpenic alcohols in relation to working conditions, their determination may be useful in recognizing fraudulent mixtures of virgin oils (that is, oils obtained only by pressing) with elaborated oils. In the sector of beers, the fraudulent addition of exogenous water (which means not the same water used during the photosynthesis of starch) to the fermentable juice or the type of cereal used may be discovered through suitable analytic determinations (NMR). Other fields of research may be interested in the determination of alcohols, such as, for example, certain sectors of agronomy. It is well known, for example, that some herbivorous insects accept or reject plants according to their lipidic surface composition. The presence of long-chain fatty alcohols can affect the insects. Thus, a knowledge of the composition of these lipids may be useful in the fight against the parasites of certain fruit plants. In a completely different sector, the physiology of nutrition, it has been demonstrated that octacosanol (C28-OH) stimulates the conversion of lipids into energy, and a large number of patents has been issued for the use of these alcohols in the foods and beverages industry. The search for the cheapest sources has incentivated the study of plants which are rich in waxes containing the highest percentages of octacosanol, like sugarcane. (Reprinted from Ref. 1, p. 552, by courtesy of Marcel Dekker Inc.)

II. SEPARATION TECHNIQUES

A. Columns

In most cases the choice of column falls on an ionic-exchange resin of the styrene-divinyl benzene polymer type, to which has been linked a functional group of the sulphonic type in the form H^+ or with another suitable cation, usually lead or calcium. The mechanisms with which these columns act on the various analytes have been examined by Jupille et al. (3) and are considered, as a whole, as ion-moderated partitioning. In normal-phase partition, the analyte shows traces of the effect of intraparticle water and of the slightly less polar mobile phase. With suitable buffers it is possible to regulate its selectivity. Moreover, an ion-exclusion mechanism contributes, thus impeding molecules of like charge from being held back. In reversed-phase partition, the analyte may be considered to be divided between the less polar phase (the resin) and the mobile, more polar phase.

Some chemical-physical characteristics of the resin give the column different separative properties, such as a different rigidity with the increase of resin crosslinkage, which makes it less permeable to higher-molecular-weight substances. In this case a size-exclusion mechanism is applied. Also, these columns are impacted with resin already present in a particular ionic form. This contributes to the selectivity of the column through its charge (ligand exchange mechanism) and, not least important, steric hindrance.

Every analysis performed on columns of this type (for example, carbohydrates, alcohols, organic acids, mixtures of these) can be improved with a column in the most suitable ionic form, even though solutions with compounds of differing types may impose the choice of a compromise. In analytical practice, however, it is not advisable to make an in-column conversion from one form to another, for the resin could shrink or even swell in relation to the type of cation linked. The effect of fixed-cation is made obvious when the compounds under analysis can coordinate with it, as in the case of carbohydrates. For example, the linkage of sugars with three adjacent hydroxyls may be more stable than those that have only two, with obvious effects in the resolution of the analytes. Finally, it is advisable to use a guard column to prolong the life of the

analytical column, which, because more often than not it has to separate analytes of complex matrixes, may easily be contaminated by undesired substances. It may also be added that this type of resin shows good stability to variations both of pressure and of pH in a wide range. Also, experience has shown that for certain separations this type of phase is much more efficient than reversed-phase or ion-pairing techniques, which require very complex mobile phases.

However, papers have been written where, for example, separations of mixtures of alcohols, sugars, glycerol, and hydroxyacids with reversed-phase columns using simple gradient mobile phase of acetonitrile/water are described (4). In the works available in the literature, as a general rule no particular indications are found on how to preserve the columns between one cycle of analysis and another, even though—for example, in Ref. 5—the authors describe a procedure and the method of reconditioning the column for a new analysis.

There are cases where HPLC separation is performed not in order to quantify the alcohols but as a technique for the purification of the analytes to be subjected to further instrumental analysis. This is the case, for example, with the identification and determination of the structure of an abscisic acid in starfruit extract (*Averrhoa carambola L.*). The separation and purification of the analytes was carried out also with HPLC using a mobile phase of diethyl ether, whereas the structure was elucidated by ^{1}H and ^{13}C-NMR (6). In a similar way, to separate the sterols and alkanols from the unsaponifiable matter from olive oils on a silica column, a gradient composed of hexane/diethyl ether was chosen in an offline system (7), whereas an online HPLC-HRGC system uses as its mobile phase hexane/isopropanol (8).

B. Column Length

The choice of length for this type of column depends on how long the analytes of interest are retained by the resin. Since the alcohols to be analyzed are usually present together with other compounds, such as organic acids and carbohydrates (for example, in musts and wines), in most cases the choice falls on columns of 300 × 7.8-mm even though, as in the case of ethanol, columns 100 mm in length can give satisfactory separations in a few minutes. Sometimes, in special cases, columns set up in series may be used, as in the case of the determination of glycerol (and, at the same time, also of ethanol) in wines described by Sanchez Munoz et al. (9), where three 250 × 8-mm columns are set up.

C. Operative Conditions (Mobile Phases, Temperatures, Flows, Run Time)

Various factors influence chromatographic separation, regarding both resolution and retention times. The main ones among these are eluant composition, including any organic modifier, its ionic strength, its pH, the temperature of the column and of the mobile phase, and the flow rate. Here we will not give a lengthy description of the effects that each single factor may have in HPLC separation, but we will simply give a brief summary of the operative conditions that have been used most often in the case of alcohol separation.

In most cases, the separation of alcohols, usually methanol, ethanol, and glycerol, is carried out contemporaneously with the separation of sugars and organic acids, and almost always the desire is to quantify all these analytes. It is seen, therefore, that the mobile phase is often an aqueous acid solution, even though only water may be used (5,9). Sulphuric acid is the one most frequently used, although phosphoric acid is preferred by some, since it is less corrosive on the components of the HPLC system (10). The concentration of sulphuric acid normally varies between 0.004 N and 0.01 N or more. The choice of acid may, however, be dictated by other considerations. This is the case, for example, with the use of a conductivity detector, which requires an appropriate conductivity suppressor system. If such a device is not available for a particular

acid, for example, sulphuric acid, a different acid must be chosen; in some cases it has been substituted with heptafluorobutyric acid (11,12).

The use of organic modifiers is introduced when it is desirable to diminish the adsorption of organic compounds on the matrix. This effect is due to the penetration of the organic solvent into the backbone of the resin, thus reducing the volume available to water. A separation of various alcohols (C2–C3), glycols, glycerol, sugars, and hydroxy acids on a reverse-phase column with an acetonitrile/water mobile phase is described in Ref. 4. Normally special preparations of the mobile phase are not required, apart from the usual filtration at 0.45 μm and degassing with helium, which, however, becomes fundamental with electrochemical detectors, where oxygen must be absent.

Among the other factors to be considered in the choice of separative conditions is temperature. Peak resolution and retention time (RT) depend greatly on this parameter: an increase in temperature diminishes the viscosity of the mobile phase and of the sample, and a reduction of RT is observed in addition to an increase in the efficiency of the column. However, this effect is not always produced, nor for all supports: some sulphonic ion-exchange resins show for analytes such as ethanol, the opposite effect. It is clear that thermostatization of the system must be very thorough so that repeatable results may be obtained. Very commonly used temperatures are those between 25°C and 70°C, values that must be adjusted each time depending on the complex of analytes to be separated.

Finally a reminder is made that the flows of the mobile phase generally are rather modest, about 0.6–0.7 ml/min, and they rarely go over 1 ml/min. In fact, peak broadening may be observed on certain stationary ion-exchange phases, due to the slowness of the mass transfer. This means that very low flows must be maintained. Despite this, most separations may be completed in times that can vary between about 20 min and 40 min. A useful paper to consult, both because of the number of analytes considered, 63, 15 of which are alcohols, and for the choice of operative conditions applied to a cation exchange column is Ref. 13. Table 1 enumerates the alcohols considered; Fig. 1 shows the separation of a standard solution.

Table 1 Peak Identification of the Investigated Compounds

Peak N°	Compound
1	Glycerol
2	1,2,4-Butanetriol
3	1,2-Propanediol
4	1,3-Propanediol
5	2,3-Butanediol
6	Methanol
7	1,3-Butanediol
8	1,4-Butanediol
9	Ethanol
10	2-Propanol
11	*tert.*-Butanol
12	1-Propanol
13	2-Butanol
14	Isobutanol
15	1-Butanol

Source: Reprinted from Ref. 13 with the kind permission of Elsevier Science—NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.

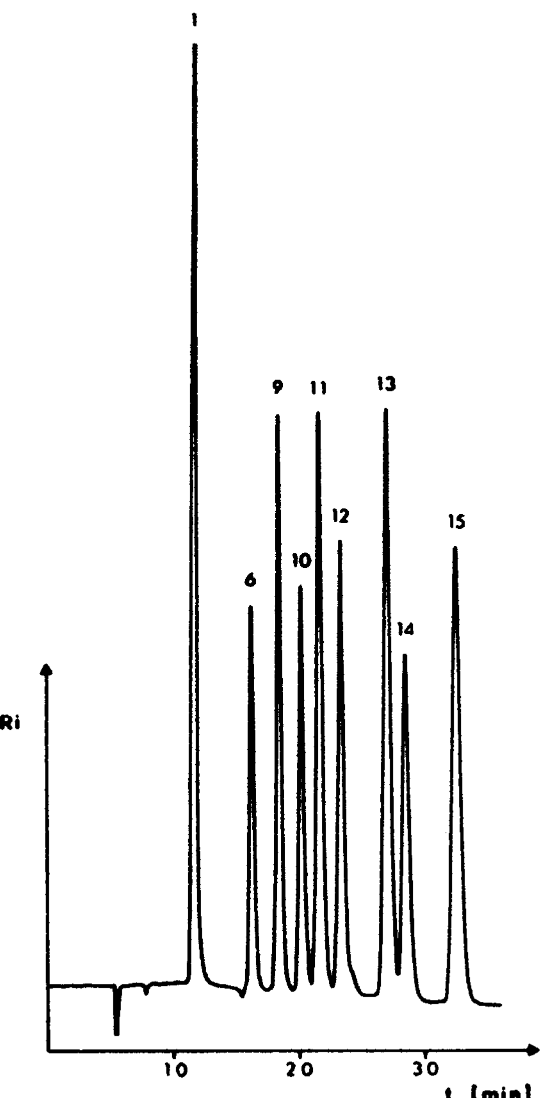

Fig. 1 Optimized separation of alcohols. Column, HPX-87 H (300 × 7.8-mm ID); column temperature, 50°C; mobile phase, 0.01 N sulphuric acid; flow rate, 0.7 ml/min; refractive index detection. For peak identification, see Table 1. (Reprinted from Ref. 13 with the kind permission of Elsevier Science—NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

III. DETECTION AND QUANTITATION

Since, as has already been said, the separation of alcohols is performed very often together with other analytes, such as organic acids and sugars, the choice of the type of detector also takes into account their chemical-physical properties. Usually, the choice is the ultraviolet detector (UV), the refractive index detector (RI), or the electrochemical detector (EC). Of the last, various types exist, which we shall describe briefly.

In some cases, in order to improve the revealability of the analytes, they may be subjected

to a derivatization reaction. The reaction may be performed either before HPLC separation (precolumn derivatization) or after, immediately before the detector (postcolumn derivatization). A thorough examination of this technique is developed in Ref. 14. While precolumn derivatization may be carried out offline before injection of the sample, postcolumn derivatization must take place online using a special device that may differ with the type of detector used. In the case of alcohols, mention is made of derivatizations with phenylisocyanate, both to allow a better response to ultraviolet light (250 nm) and for the use of a fluorescence detector (λ_{ex} = 230 nm, λ_{em} = 315 nm). In some cases, however, the choice may be a postcolumn enzyme reactor followed by an electrochemical detector (15,16). The enzyme (for example, alcohol oxidase), immobilized by covalent bonding to a support such as Sepharose or Eupergit C, reacts with the alcohol, producing H_2O_2, which is revealed by the EC detector. In common practice, given the low response of alcohols to UV, an RI detector is preferred.

The simultaneous presence of analytes that do give good responses to UV has prompted the use of two detectors set up in series, thus allowing the contemporaneous registration of the two chromatograms. It must be remembered, however, that the use of the refractive index detector does not permit elutions in gradient. In recent years the use of electrochemical detectors (ECs) is becoming more common, because they show great sensitivity and a high linear dynamic range. For a description of the theory of its function and the care in its use and upkeep, please see the specialist texts and the handbooks prepared by each company. However, an early survey on ED may be the book by Huber (17).

We wish only to remind readers that there are three main methods of electrochemical revealment: conductivity, direct current (d.c.) amperometry, and integrated amperometry (pulsed amperometry is a form of integrated amperometry). In revealment by conductivity, the analytes, in ionic form, move under the effect of an electric field created inside the cell. The conductivity of the solution is proportional to the mobility of the ions in solution. Since the mobile phase is itself an electrolytical solution, in order to increase the signal/noise ratio and the response of the detector, it is very useful to have access to an ion suppressor before the revealment cell. By means of ionic exchange membranes, the suppressor replaces the counterions respectively with H^+ or OH^-, allowing only an aqueous solution of the analytes under analysis to flow into the detector.

In the forms of amperometric detection, however, currents are produced due to redox reactions, which are then converted to signals of a proportional entity. In a few cases, for example, in the analysis of carbohydrates, oxidation products poison the surface of the electrode (usually made of gold or platinum), thus inhibiting a further oxidation of the analyte. By repeatedly inverting the polarity of the electrodes, the surface may be maintained active and stable; in this way this serious difficulty is overcome. Among the substances that can be usefully revealed with this technique are alcohols, aldehydes, and amines. With this type of detector it is important to avoid the presence of oxygen and/or of air, which could give rise to the formation of bubbles or to redox reactions; for example, the oxygen is easily reduced at fairly negative potentials, −0.4, −0.5 V vs AgCl. To this end it is useful to consider the material with which the HPLC system tubes are made. For example, it may be opportune to substitute PTFE tubing with steel tubing between the mobile-phase reservoirs and the pump and between the column and the detector. Special attention must be paid to the choice of the chemicals, which must be electrochemically inert and free of electroactive contaminants. An interesting study on the optimization of electrochemical detection of alcohols, sugars, and sugar degradation products is in Ref. 4. Useful information on the separation and revealment of alcohols (methanol, ethanol, propanol, and butanol), sugars, and carboxylic acids with a cation-exchange column and conductivity or an amperometric detector may be found, respectively, in Refs. 18 and 19. A more recent work on the use of the pulsed amperometric detector in the analysis of flavor-active alcohols separated using HPLC can be found in Ref. 20.

IV. SAMPLE PREPARATION

Normally samples are subjected to minimal treatment before injection into the separative apparatus. In most cases these are necessary dilutions followed by simple filtrations through filters or membranes of 0.45 μm or 0.2 μm. We report as an example of a more elaborate treatment the procedure set up by McCord et al. (21). The authors, who work on a 1982 Colombard must and on 1982 Chardonnay wine, aim to overcome the disadvantages that derive from the simultaneous elution of various analytes present in musts and wines, as happens in the analyses proposed up until then. Therefore they separate the sample into two fractions: one containing the neutral compounds and the other containing the acid compounds. Separation is obtained by use of a strong base anion-exchange resin (Econocolumns, Bio-Rad Laboratories, 7-mm ID $\times$ 8 cm) on which a sample at pH = 8–9 (regulated with concentrated ammonium hydroxide) is eluted. The resin is then washed with water in order to recover the neutral fraction and then with 10% sulphuric acid to obtain the acid phase. Before injection into HPLC the solutions are filtered (0.45 μm) and passed on to C18 SepPack to remove the phenolic fraction.

V. APPLICATIONS

Regarding glycerol, in most cases it is determined contextually with ethanol. For this reason in the sections dedicated to glycerol reference will be made only to those works that deal mainly with its determination.

A. Wines and Musts

1. Ethanol

Shimazu et al. (22) separate ethanol, polyols, and sugars with a strong cation-exchange resin (Diaion CK-08 S, Ca^{2+} form) and refractive index in about 30 min. The authors verify their method by finding, for wines, a recovery between 91% and 101% and encounter appreciable quantities of glucose, fructose, and glycerol in noble German wines. Frayne (10) intends to separate organic acids, sugars, and alcohols in wine and must using a dual cation-exchange column with samples filtrated only through a 0.45 μm. The column chosen is an Aminex HPX-87H, 300 $\times$ 7.8 mm, with a mobile phase of H_3PO_4 0.065% at 65°C and a flow of 0.7 ml/min. Revealment of the analytes takes place using a UV detector at 214 nm and RI linked together in series.

An example of separation obtained with this method is reported in Fig. 2. Falqué and Fernandez (23) study the influence of time of contact with the skins on the composition of the volatile fraction and of the carboxylic acids in wine produced from Treixadura grapes. Also, these authors quantify glycerol and ethanol, besides carboxylic acids and sugars, through the use of an Aminex HPX-87H (300 $\times$ 7.8 mm) column, but with the mobile phase of H_2SO_4 0.65 mM at 75°C and a flow of 0.7 ml/min. They use a UV detector at 214 nm and an RI in series. The sample requires only a filtration at 0.45 μm, as described in their survey (24).

> Pfeiffer and Radler (25) separated the main constituents of wine with a cation-exchange column, a mobile phase of diluted H_2SO_4 (0.003 N or 0.013 N), and an RI detector. In particular they obtained an excellent separation of ethanol and glycerol from the other components in a single run of 25 min. The authors also compared conventional methods, showing a good agreement in the results. (Reprinted from Ref. 1, p. 565, by courtesy of Marcel Dekker Inc.)

McFeeters (11) separates, in 22 min, numerous analytes of wine, including ethanol, (*R*)- or (*S*)-butane-2,3-diol and the meso-butane-2,3-diol with a Phenomenex Resex organic acid column

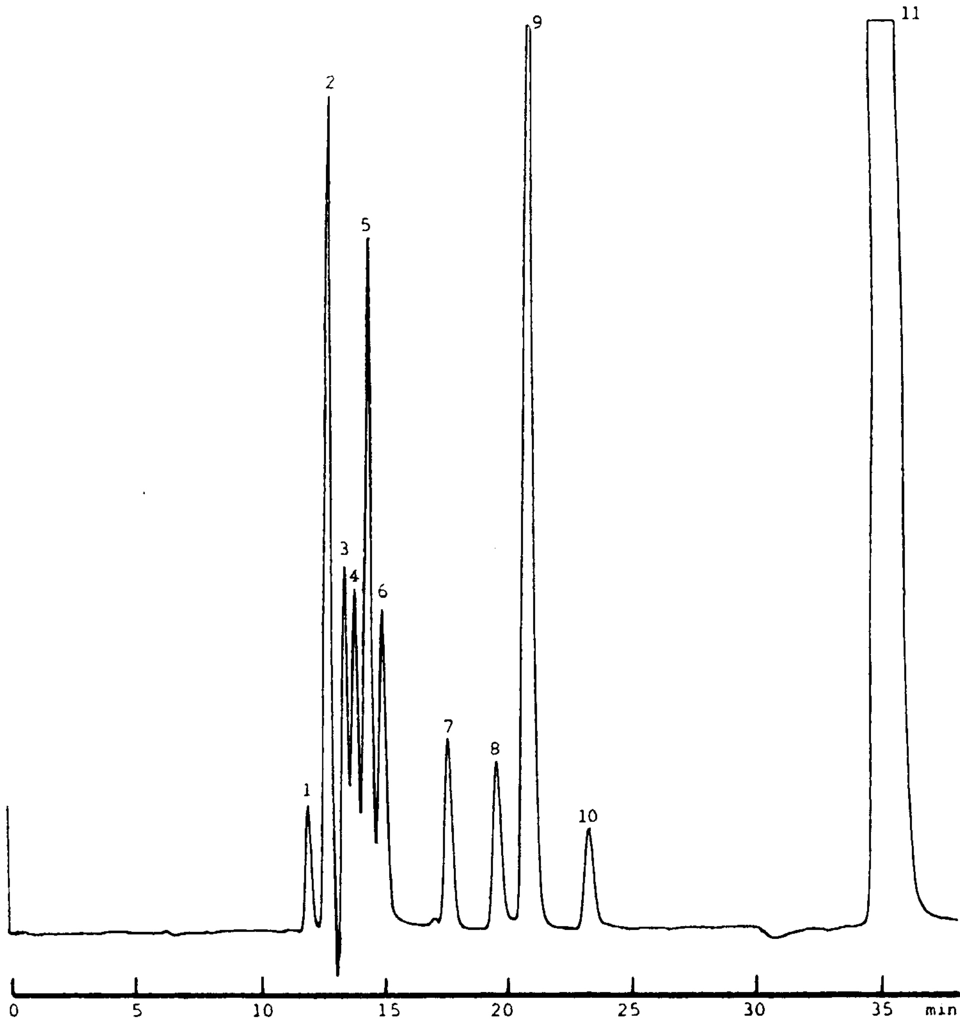

Fig. 2 Separation on two cation-exchange columns connected in series of the dry wine standard using 0.065% H_3PO_4 at a flow rate of 0.7 ml/min and detected by RI. 1—citric acid, 0.5 g/L; 2—tartaric acid, 4.0 g/L; 3—phosphoric acid, 0.065%; 4—glucose, 1.0 g/L; 5—L-malic acid, 2.0 g/L; 6—fructose, 1.0 g/L; 7—succinic acid, 1.0 g/L; 8—L-lactic acid, 1.0 g/L; 9—glycerol, 5.0 g/L; 10—acetic acid, 1.0 g/L; 11—ethanol, 12.0% (v/v). (From: Am J Enol Vitic 37:1986.)

with a mobile phase composed of a solution of heptafluorobutyric acid 3.0 mM, and a conductivity detector (for acids) and a pulsed amperometric detector (for sugars and alcohols) in series. Quantification of the analytes is obtained using an internal standard.

2. *C1–C5 Alcohols*

Pecina et al. (26) used fixed-ion resin columns in hydrogen ion form to separate in a satisfactory way mixtures of low-MW alcohols. At the same time, glycerol is also well resolved from other alcohols present. The authors worked with columns of 300 × 7.8 mm, thermostated at 50°C, using as the eluent a solution of 0.01 N of H_2SO_4 with a flow of 0.7 ml/min and an RI detector. For fast screenings, the use of shorter columns has been suggested (27) with an elu-

> ent that consists of a 0.002 N of H_2SO_4 solution, and greater flows (1.2 ml/min, thermostating at 65°C and an RI detector. (Reprinted from Ref. 1, p. 565, by courtesy of Marcel Dekker Inc.)

3. *Diethylene Glycol*

"With the same type of column used by Pecina et al. (26) (v. C1–C5 alcohols), determinations of DEG in wines were carried out (27). The eluent used is a 0.002 N solution of H_2SO_4 at a flow of 0.6 ml/min, with the column at 65°C. In this case, too, the detector is an RI" (Reprinted from Ref. 1, p. 565, by courtesy of Marcel Dekker Inc.).

4. *Glycerol*

Per AOAC Methods (28), in wine and grape juice (method number 991.46): This is an HPLC method using a strong cation column at 65°C, with an RI detector and a mobile phase of degassed water by boiling or helium sparging. Before starting, the sample must be filtered (0.45 μm), and it is necessary to prepare different standard solutions (external standard) depending on whether one is dealing with wine or grape juices.

In dry wines (method number 920.60): This is a surplus method. In sweet wines (method number 920.61): This is a surplus method.

> Caputi et al. (29) conducted a collaborative study for the determination of glycerol in wine and grape juice. This method uses a strong cation-exchange type of column (H^+ form), degassed water as the mobile phase, and an RI detector. The glycerol was determined on samples simply after filtration (0.45 μm) and with an external standard. For wines, the authors obtained a repeatability of 1.25% and a reproducibility of 2.79% (Reprinted from Ref. 1, p. 566, by courtesy of Marcel Dekker Inc.).

The use of three 250-mm × 8-mm columns in series (Shodex S-801/S; S-802/S; S-802/S) controlled by thermostat at 75°C, using water as mobile phase at 1 ml/min and a refractive index as detector, allowed Sanchez Munoz et al. (30) to quantify, with a single injection, the glycerol in wines after a simple filtration of the sample through a 0.2-μm membrane. Furthermore, the authors were also able to quantify the ethanol and sucrose and conduct, for glycerol, a comparison between the method suggested and an enzymatic one; see Fig. 3.

B. Beers and Malt Beverages

1. *Ethanol*

Castané (31) suggests a method that is an alternative both to the enzymatic one and to gas chromatography. He uses a column, sulfonated styrene divinylbenzene resin in H^+ form (Fast-Fruit Juice), 7 μm, 7.8 × 150 mm and a refractive index detector. The mobile phase, composed of H_2SO_4 0.002 M, is maintained at 60°C. In this way he succeeds in separating and quantifying the ethanol in concentrations between 0 and 1% (v/v). The author also ascertains a reproducibility of 0.04% v/v and a repeatability of 0.07% v/v with a precision value of 0.55% v/v.

> Cieslak and Herwig (32) perfected a method for the quantification of ethanol using a hydrogen-form cation-exchange column. They use a 0.01 N H_2SO_4 water solution as the mobile phase, at 85°C, and an RI detector and obtain the separation of ethanol, glycerol, and glucose in 10 min. Quantification was made with the use of an external standard. The samples to be analyzed are subjected only to ultrasonic degassing. The authors also ascertain an excellent agreement with distillation methods (Reprinted from Ref. 1, p. 573, by courtesy of Marcel Dekker Inc.).

2. *Glycerol*

"See Ethanol, Cieslak and Herwig (32)" (Reprinted from Ref. 1, p. 574, by courtesy of Marcel Dekker Inc.).

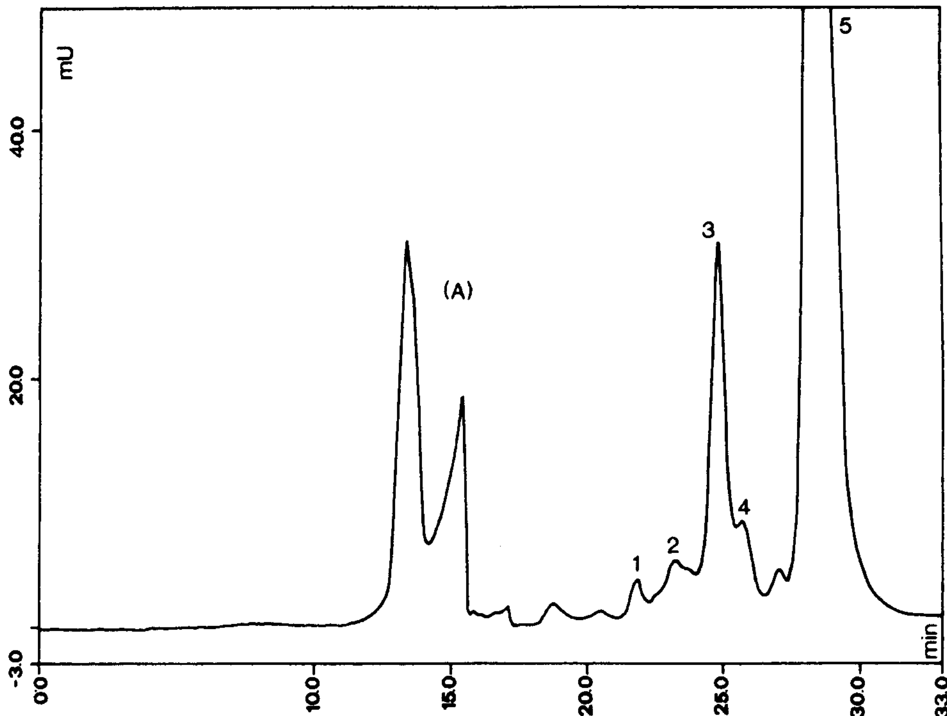

Fig. 3 Chromatogram of a red wine using Shodex S-801/S and S-802/S columns at 75°C with a mobile phase of water at a flow-rate of 1 ml/min and using a refraction index detector. Peaks: A = compounds with the highest molecular mass and with an acid character; 1 = glucose; 2 = fructose; 3 = glycerol; 4 = butan-2,3-diol (= 0.8 g/L added to the initial wine); 5 = ethanol. (Reprinted from Ref. 30 with the kind permission of Elsevier Science—NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

C. Oils and Fats

1. *Fatty Alcohols*

"These may be present either free or combined, mainly with fatty acids in wax esters" (Reprinted from Ref. 1, p. 577, by courtesy of Marcel Dekker Inc.).

Online coupled LC-GC: "The possibility of determining easily the content of free alkanols is offered by an On-line coupled LC-GC system described by Grob (33). A very interesting application for olive oils is illustrated by Grob et al. (34), who make use of a fully automated LC-GC 'Dualchrom' system (Carlo Erba, Milan, Italy).

"The aliphatic alcohols are first heat-derivatized, with pivalic anhydride added directly to the oil. The solution is properly diluted with *n*-hexane and injected for the LC-GC run (Fig. 4).

"Naturally with this method it is possible to determine the total content from the unsaponifiable or, in theory, from the content of wax esters, or by suitable transesterification" (Reprinted from Ref. 1, p. 577, by courtesy of Marcel Dekker Inc.).

2. *Triterpenic Alcohols*

Soulier et al. (35) isolate eight triterpenic alcohols from sal and illipe butters besides other compounds. They separate these compounds after saponification of the fatty matter and fractionation of the unsaponifiable on an aluminium oxide column (hydrated at 5%). Two successive HPLC separations and a TLC-$AgNO_3$ permit the isolation of highly purified fractions. Through the use, among others, of the ^{1}H-NMR and MS techniques, they identify nine

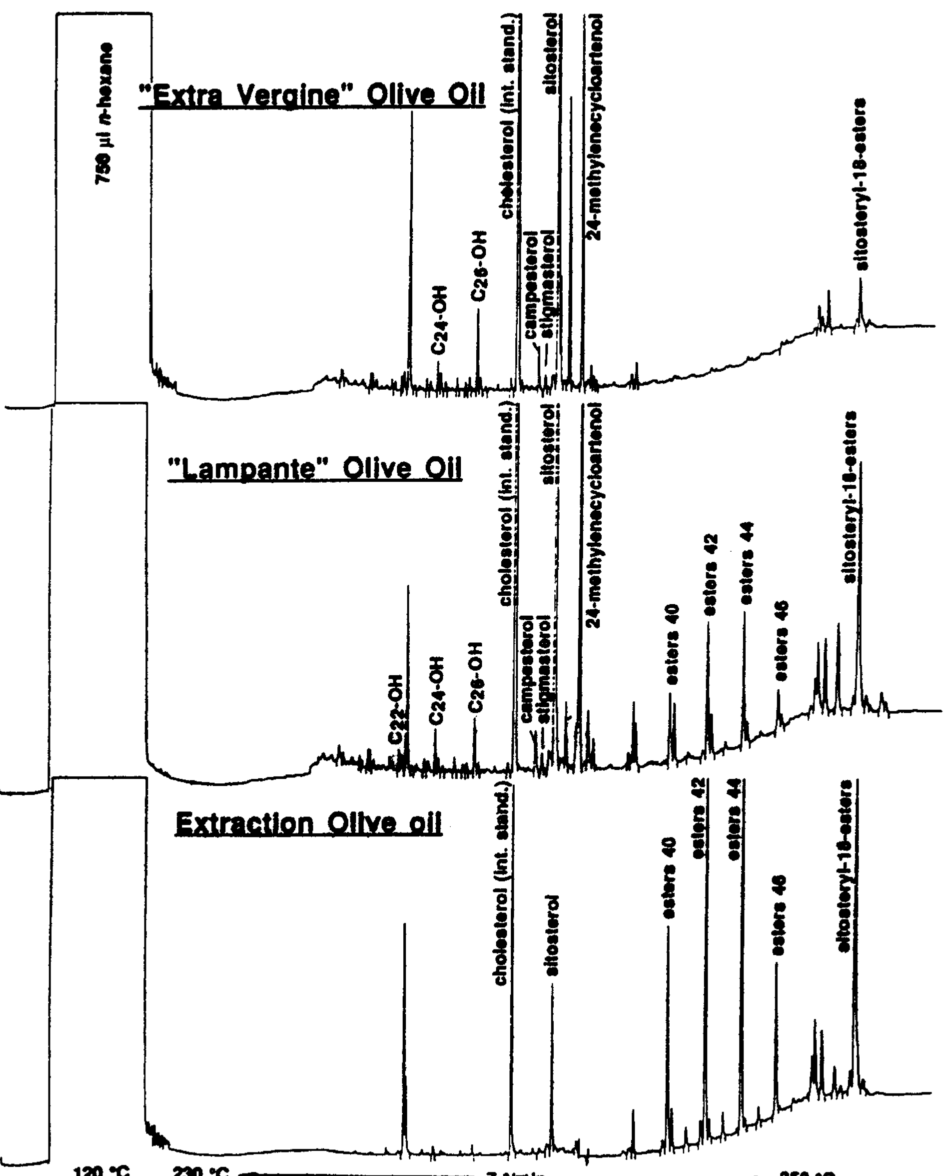

Fig. 4 LC-GC-FID chromatograms for typical olive oils. The nearly complete absence of wax esters (esters 40–esters 46) and very low concentrations of steryl esters indicate a high-quality extravirgin oil. The concentration of free stigmasterol is low. C24–26-OH, fatty alcohols. In lampante oils, more wax esters and steryl esters are found. The concentration of stigmasterol increases more than campesterol if the oil was prepared from olives of low quality. Run at the same sensitivity, chromatograms of solvent-extracted oils are completely overloaded. The refined extraction oil was diluted 1 : 5 before running the chromatogram shown. Wax ester and steryl ester concentrations are very high. (From Ref. 34, p. 626.)

triterpene alcohols (β-amyrin, butyrospermol, cycloartenol, α-amyrin + lupeol, 24-methyleneparkeol, 24-methylenecycloartanol, ψ-taraxasterol, cycloartanol), amongst which 24-methyleneparkeol and ψ-taraxasterol are indicated for the first time in sal and illipe butters. Moreover, the authors suggest an analytic method for recognizing the presence of these fats in foodstuffs. (Reprinted from Ref. 1, p. 580, by courtesy of Marcel Dekker Inc.)

3. *Other Determinations*

Recently Amelio et al. (7) described a method which may find routine applications and which makes use of SPE for the separation and clean-up of the unsaponifiable from olive oil, from which the aliphatic alcohols are separated by means of HPLC (besides sterols and the two triterpenic dialcohols erythrodiol and uvaol). The alkanols are then derivatized and analyzed by means of HRGC. The use of an autosampler and a fraction collector for use with HPLC permits a considerable automatization of the analysis. (Reprinted from Ref. 1, p. 581, by courtesy of Marcel Dekker Inc.)

See Fig. 5.

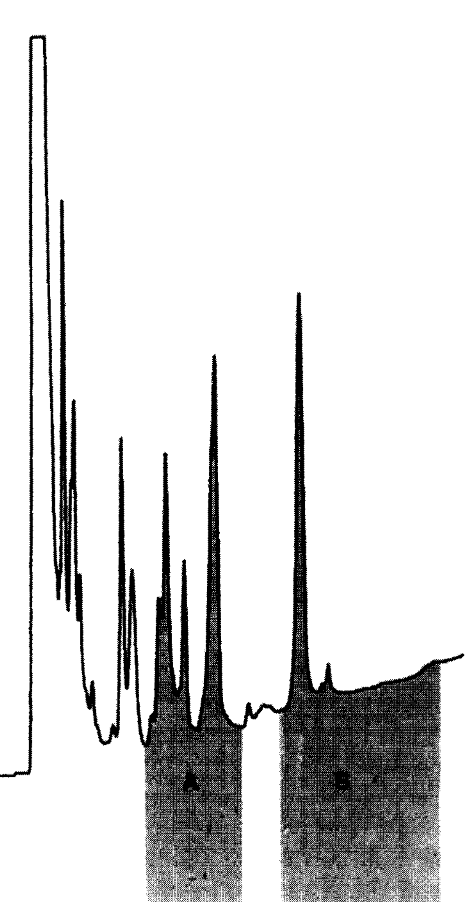

Fig. 5 HPLC of extravirgin olive oil unsaponifiable and collected fractions. A = fraction containing alkanols (6 and 9 min); S = fraction containing sterols (10 and 18 min). (Reprinted from Ref. 7 with the kind permission of Elsevier Science—NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

D. Fruits, Vegetables, and Juices

1. Ethanol

Tomlins et al. (36) analyzed extracts from cocoa beans in order to investigate the qualitative differences of cocoa of varying origins that were subjected to different methods of fermentation. The cocoa beans or cotyledons were weighed and homogenized in benzoic acid solution (0.2%) for 3 min. The homogenized substance (30 ml) was centrifuged for 15 min at 15,000 rpm, then 20 ml of supernatant were preserved at 4°C. High-performance LC separation of the extract, simply filtrated through a 0.45-μm disposable filter, was performed in less than 30 min on an Aminex HPX-87H (300-mm × 7.8-mm) cation-exchange column with a mobile phase of 0.013 N H_2SO_4 at a flow of 0.5–0.8 ml/min and at a temperature of 25°C. Because it was also desirable to determine the sugars and organic acids, a UV (215 nm) detector for the organic acids and an RI for the other analytes were used in series. Figure 6 shows the separation of a standard solution. McFeeters et al. (37) study the composition of fermentation brines of cucumber and suggest for the separation of ethanol, sucrose, and acetic, lactic, and malic acids a Radial-Pak C18 reverse-phase, 5 μm (100 × 8 mm), mobile phase 0.05 M H_3PO_4 (regulated pH at 2.5 with NH_4OH). The results obtained are considered interesting, but they must be taken together with those from a different separation of sugars, conducted separately, in order to evaluate the carbon balance for complex heterolactic acid fermentations so that the course of lactic acid fermentation may be studied in detail. McFeeters (12) quantifies ethanol, glycerol, and 1-propanol in cucumber juices and in fermentation brines. He uses a Phenomenex ROA organic acid column, with a mobile phase of heptafluorobutyric acid 1.6 mM in water, at 65°C and a flow rate of 0.7 ml/min. With the help of a micromembrane suppressor he performs, in less than 30 min, the separation of the analytes, revealed by a pulsed amperometric detector (PAD). The samples under analysis are suitably diluted and centrifuged for 5 min in a microcentrifuge at 15,000 g. See Fig. 7.

2. 1-Propanol

See Ethanol and Ref. 12.

3. Triterpene Alcohols

As an example we mention a study made by Akihisa et al. (38) on nine leguminosae seeds (*Cajanus cajan, Cicer arietinum, Dolichos biflorus, Lathyrus sativus, Lens culinaris, Pisum sativum, Vigna mungo, Glycine max, Vigna cylindrica*). They recognize 25 triterpenic alcohols, including one new compound (24Z-ethylidene-24-dihydroparkeol) by using several TLC,

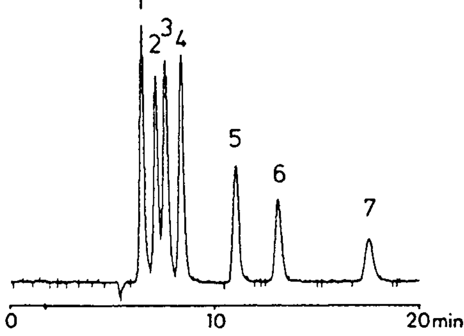

Fig. 6 RI chromatogram of standard solution. 1 = sucrose; 2 = citric acid; 3 = glucose; 4 = fructose; 5 = lactic acid; 6 = acetic acid; 7 = ethanol. (From Ref. 36.)

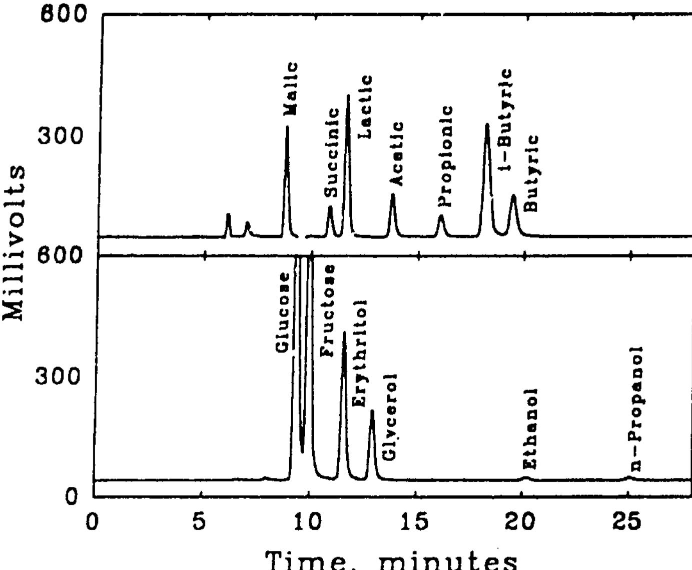

Fig. 7 Chromatograms from a single injection of compounds added to or naturally present in cucumber juice. (Reprinted with permission from Ref. 12. Copyright 1993 American Chemical Society.)

HRGC, and HPLC separations. Identification was also supported by MS and 400-MHz ^{1}H-NMR. (Reprinted from Ref. 1, p. 592, by courtesy of Marcel Dekker Inc.)

4. Bibliographic Update

Since the databases accessible with a personal computer are ever more widespread and accessible, it may be useful to point out some website addresses for Internet users. There are, of course, many other, equally valid alternatives that may prove advantageous. These sites have been suggested not because we believe them to be the best, but simply as examples. Through the STN International Site, Database in Science and Technology, Karlsruhe, Germany (http://www.fiz-karlsruhe.de) it is possible to conduct bibliographic research and request copies of works of interest. Through the CAS site Chemical Abstract Service, Document Detective Service, Columbus, OH (http://www.cas.org) it is possible to check on the availability of required works and to order copies in various different ways. Finally, it is possible to retrieve a bibliography that is updated weekly on the site of the ISI, Institute for Scientific Information, Philadelphia, PA (http://www.isinet.com).

ACKNOWLEDGMENTS

Many thanks to my colleagues: Dr. R. Rizzo and Mr. F. Varazini; Dr. G. Colli, Industrial consultant, Novara, Italy; Mrs. A. Tassara, Centro Servizi Bibliotecari "S. Cannizzaro," Università, Genova, Italy; Dr. L. Maxwell.

ABBREVIATIONS

DEG	diethylene glycol
ED	electrochemical detector

^{1}H-NMR	^{1}H nuclear magnetic resonance
^{13}C-NMR	^{13}C nuclear magnetic resonance
HPLC	high-performance (or pressure) liquid chromatography
HRGC	high-resolution gas chromatography
LC-GC	liquid chromatography–gas chromatography
MS	mass spectrometry
MW	molecular weight
PAD	pulsed amperometric detector
RI	refractive index (detector)
RT	retention time
Surplus method	Such AOAC method is a satisfactory method but is thought not to be in current use for various reasons
TLC	thin-layer chromatography
TLC-$AgNO_3$	argentation thin-layer chromatography
UV	ultraviolet (detector)

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9
The Fat-Soluble Vitamins

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I. INTRODUCTION

The fat-soluble vitamins comprise vitamins A, D, E, and K, whose biological activities are attributed to a number of structurally related compounds known as *vitamers*. Also included are those carotenoids that are precursors of vitamin A. Recommended dietary allowances (RDAs) based on human epidemiological and experimental animal studies have been published in the United States for vitamins A, D, E, and K (1). Other countries and international bodies have compiled similar recommendations. In the United States and Canada, fluid milk is supplemented by law with vitamin D to a level of 400 international units per quart (10 μg/0.95 L) to meet the RDA of 10 μg. Other commodities, such as margarine, milk products, ready-to-eat breakfast cereals, and dietetic foods, are commonly supplemented with vitamins A, D, and E. Except for infant formulas, vitamin K is not added to foods. The addition of vitamins to a particular processed food is intended to provide a specific proportion of the RDA.

Vitamin assays in foods are carried out for a variety of purposes: to check compliance with contract specifications and nutrient labeling regulations; to provide quality assurance for supplemented products; to provide data for food composition tables; to study changes in vitamin content attributable to food processing, packaging, and storage; and to assess the effects of geographical, environmental, and seasonal conditions. The monitoring of foods supplemented with vitamins A and D to ensure that neither inadequate nor excessive quantities are added is particularly important, because, in addition to the serious pathological consequences of deficiency, these two vitamins are toxic at excessive intakes (2,3). The supplementation of animal feeds is another potential route for entry of abnormal quantities of micronutrients into human foods, and hence animal feeds should be monitored too—at least for the highly potent vitamin D.

The nutritional value of a food commodity or diet with respect to a particular vitamin may be expressed in terms of the vitamin's *bioavailability*, which refers to the proportion of the quantity of vitamin ingested that undergoes intestinal absorption and utilization by the body. *Utilization* encompasses transport of the absorbed vitamin to the tissues, cellular uptake, and conversion to a form that can fulfill some biochemical or physiological function, either immediately or after storage.

II. CHEMICAL AND BIOLOGICAL NATURE OF THE FAT-SOLUBLE VITAMINS

A. Vitamin A and the Provitamin A Carotenoids

Vitamin A–active compounds are represented by retinoids (designated as vitamin A) and the carotenoid precursors of vitamin A (provitamin A carotenoids). The retinoids comprise retinol, retinaldehyde, and retinoic acid, together with their naturally occurring and synthetic analogs. Carotenoids are yellow, orange, red, or violet pigments that are responsible for the color of many vegetables and fruits. Certain almost-colorless carotenoids also exist, such as phytofluene, which fluoresces intensely under ultraviolet (UV) irradiation. In nature, the carotenoids are synthesized exclusively by higher plants and photosynthetic microorganisms, in which they play fundamental roles in metabolism. The various retinoids are present in animal tissues as a consequence of the enzymatic cleavage of ingested provitamin A carotenoids to retinaldehyde and subsequent reactions in the absorptive cells of the small intestine.

Vitamin A is a necessary micronutrient in the diet for vision, growth, tissue differentiation, reproduction, and maintenance of the immune system. A deficiency of vitamin A affects reproduction in both male and female experimental animals. In the male, retinol is required for normal spermatogenesis; in the female, the vitamin is necessary for both conception and normal development of the fetus.

Carotenoids, present in fruits and vegetables, are widely believed to protect human health. In particular, some epidemiological studies have correlated the intake of carotenoid-rich fruits and vegetables with protection from some forms of cancer (4,5). This action is probably due to the carotenoids' antioxidant properties rather than to their vitamin A activity.

Dietary vitamin A is stored in the liver and secreted into the bloodstream when needed. The circulating retinol is taken up by target cells and oxidized in part to retinoic acid, which induces the synthesis of proteins through the direct control of gene expression. This type of action—gene activation—establishes vitamin A (in the form of its metabolite, retinoic acid) as a hormone, similar to the steroid hormones and the thyroid hormone.

1. *Chemical Structure and Biopotency*

a. **Retinol**

The parent vitamin A compound, *retinol*, has the empirical formula $C_{20}H_{30}O$ and a molecular weight (MW) of 286.44. The molecule comprises a cyclohexenyl (β-ionone) ring attached at the carbon-6 (C-6) position to a polyene side chain whose four double bonds give rise to *cis-trans* (geometric) isomerism. The predominant isomer, all-*trans*-retinol (Fig. 1), possesses maximal (100%) vitamin A activity and is frequently accompanied in natural foodstuffs by smaller amounts of 13-*cis*-retinol, which exhibits 75% relative activity in the rat (6). Other cis isomers of retinol also occur in nature, but they are of low potency, and their contribution to the total vita-

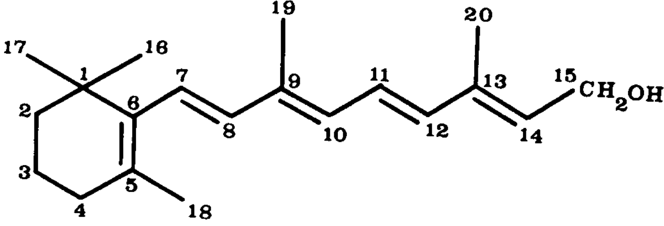


Fig. 1 Structure of all-*trans*-retinol.

min A activity of a foodstuff is generally negligible. In most of the foods that contain vitamin A, the retinol forms esters with long-chain fatty acids, particularly palmitic acid. An exception is egg yolk, in which unesterified retinol (cis and trans forms) represents the major retinoid, accompanied by retinaldehyde and retinyl esters (7).

b. Provitamin A Carotenoids

Carotenoids can be considered chemically as derivatives of *lycopene*—a $C_{40}H_{56}$ polyene composed of eight isoprenoid units (Fig. 2). Using the abbreviation *ip* for the isoprenoid unit, the carotenoids can be represented as *ip-ip-ip-ip-pi-pi-pi-pi*; i.e., the arrangement of the units is reversed at the center of the molecule. Derivatives are formed by a variety of reactions that include cyclization, hydrogenation, dehydrogenation, and insertion of oxygen. Hydrocarbon carotenoids are known as *carotenes*, and the oxygenated derivatives are termed *xanthophylls*. The oxygen functions of xanthophylls are most commonly hydroxy, keto, epoxy, methoxy, and carboxy groups. Some acyclic carotenoids occur widely, e.g., lycopene, but monocyclic and bicyclic compounds are more common. Most carotenoids of plant tissues contain 40 carbon atoms, but shortened molecules known as *apocarotenoids* are also found.

A semisystematic nomenclature for carotenoids has been devised to convey structural information (8). According to this scheme, the carotenoid molecule is considered in two halves, and the nature of the end group of each half is specified. Each carotenoid is considered to be a derivative of a parent carotene, indicated by two Greek letters describing the end groups. The nomenclature recognizes six end groups: β (beta), ϵ (epsilon), κ (kappa), ϕ (phi), χ (chi), and ψ (psi). Three of the more common end groups (β, ϵ, and ψ) are shown in Fig. 2. Changes in hydrogenation level and the presence of substituent groups are indicated by the use of conventional prefixes and suffixes. The numbering system for carotenoids is shown in the structure of β-carotene (Fig. 2).

Many of the naturally occurring carotenoids are chiral, bearing from one to five asymmetric carbon atoms. In most cases a given carotenoid occurs in only one chiral form. The absolute configuration at a chiral center is designated by use of the *R,S* convention. Unless stated otherwise, all double bonds have the trans configuration. *Cis-trans* isomerism is indicated by citing the double bond or bonds with a cis configuration. The *Z,E* terminology for geometric isomerism is seldom used in vitamin A and carotenoid nomenclature. The semisystematic names of some common carotenoids of plant foods are given in Table 1.

From a nutritional viewpoint, the carotenoids are classified as provitamins and inactive carotenoids. To have vitamin A activity, the carotenoid molecule must incorporate a molecule of retinol, i.e., an unsubstituted β-ionone ring with an 11-carbon polyene chain. β-carotene ($C_{40}H_{56}$, MW = 536.88), the most ubiquitous provitamin A carotenoid, is composed of two molecules of retinol joined tail to tail; thus the compound possesses maximal (100%) vitamin A activity. The structures of all other provitamin A carotenoids incorporate one molecule of retinol and hence theoretically contribute 50% of the biological activity of β-carotene. Among the 600 or so carotenoids that exist in nature, only about 50 possess vitamin A activity in varying degrees of potency.

In nature, carotenoids exist primarily in the all-*trans* configuration, but significant amounts of cis isomeric forms have been found in fresh vegetables (10–12). The main cis isomers of β-carotene that have been found in fresh and processed fruits and vegetables are 13-*cis* and 9-*cis*, which have vitamin A activities relative to all-*trans*-β-carotene of 53% and 38%, respectively (13). A third isomer, 15-*cis*-β-carotene, has also been reported to occur in several fruits and vegetables (12). The structures of these three isomers are shown in Fig. 3. With asymmetrical carotenoids, such as α-carotene and β-cryptoxanthin, the number of theoretically possible cis isomers

(a)

(b)

(c)

(d)

(e)

Fig. 2 Structures of lycopene, β-carotene, and three of the six carotenoid end group designations. (a) Lycopene; (b) β-carotene; (c) β (beta); (d) ϵ (epsilon); (e) ψ (psi).

Table 1 Chemical Nomenclature and Provitamin A Activity of Some Common Carotenoids of Plant Foods

Trivial name	Semisystematic name	Type	Vitamin A activity (%)[a]
Lycopene	ψ,ψ-Carotene	Acyclic carotene	Inactive
ζ-Carotene	7,8,7′,8′-Tetrahydro-ψ, ψ-carotene	Acyclic carotene	Inactive
γ-Carotene	β,ψ-Carotene	Monocyclic carotene	42–50
β-Zeacarotene	7′,8′-Dihydro-β,ψ-carotene	Monocyclic carotene	20–40
β-Carotene	β,β-Carotene	Bicyclic carotene	100
α-Carotene	(6′*R*)-β,ε-Carotene	Bicyclic carotene	50–54
β-Cryptoxanthin	(3*R*)-β,β-Caroten-3-ol	Bicyclic, monohydroxy-carotenoid	50–60
Zeinoxanthin	(3*R*,6′*R*)-β,ε-Caroten-3-ol	Bicyclic, monohydroxy-carotenoid	Inactive
Zeaxanthin	(3*R*,3′*R*)-β,β-Carotene-3,3′-diol	Bicyclic, dihydroxy-carotenoid	Inactive
Lutein	(3*R*,3′*R*,6′*R*)-β,ε-Carotene-3,3′-diol	Bicyclic, dihydroxy-carotenoid	Inactive
β-Carotene-5,6-epoxide	5,6-Epoxy-5,6-dihydro-β, β-carotene	Bicyclic, monoepoxy-carotenoid	21
β-Carotene-5,8-epoxide (mutatochrome)	5,8-Epoxy-5,8-dihydro-β, β-carotene	Bicyclic, monoepoxy-carotenoid	50
β-Cryptoxanthin-5,6-epoxide	5,6-Epoxy-5,6-dihydro-β, β-caroten-3-ol	Bicyclic, monoepoxy-, monohydroxycarotenoid	Active (not quoted)
Neoxanthin	(3*S*,5*R*,6*R*,3′*S*,5′*R*,6′*S*)-5′,6′-Epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β, β-carotene-3,5,3′-triol	Bicyclic, monoepoxy-, trihydroxycarotenoid	Inactive
Violaxanthin	(3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*S*)-5,6,5′,6′-Diepoxy-5,6,5′,6′-tetrahydro-β, β-carotene-3,3′-diol	Bicyclic, diepoxy-, dihydroxycarotenoid	Inactive

[a] Activity of all-*trans* forms relative to the activity of β-carotene (9).

is approximately twofold greater than with symmetrical carotenoids, such as β-carotene. In fruits, hydroxycarotenoids (carotenols) exist mainly as mono or bis esters of saturated long-chain fatty acids, such as lauric (C12), myristic (C14), and palmitic (C16) acids (14,15).

In plant and animal tissues the carotenoids are usually found associated with lipid fractions in noncovalent association with membranes and lipoproteins, and they accumulate, together with chlorophylls, in the chloroplasts of green leaves (16). They also occur as very fine dispersions in aqueous systems, such as orange juice.

2. *Occurrence*

a. Vitamin A

The liver of meat animals is a rich source of vitamin A, for this organ is the body's main storage site of the vitamin. Fish liver oils, particularly halibut liver oil, are incredibly rich in the vitamin and are too potent to be consumed as foods. Typical food sources of vitamin A (in μg retinol equivalents/100 g) are sheep and ox liver, 15,000; butter, 830; cheese, 320; eggs, 140; herring and

(a)

15

(b)

13

(c)

9

Fig. 3 Structures of cis isomers of β-carotene that occur in foods. (a) 15-*cis*-β-carotene; (b) 13-*cis*-β-carotene; (c) 9-*cis*-β-carotene.

mackerel, 50 (17). Margarine is fortified with vitamin A to make it nutritionally equivalent to butter.

b. Carotenoids

Vegetables are the major sources of provitamin A carotenoids, followed by fruits. Milk products, egg yolk, shellfish, and crustacea also contain active carotenoids, which are derived from the animal's diet. The carotenoid distribution in green leafy vegetables is generally constant, whereas fruits contain a greater variety of carotenoids in varying concentrations. In ripening fruits, the decrease in chlorophylls is frequently accompanied by an increase in the concentration of carotenoids and an increase in the ratio of carotenes to xanthophylls.

The quantitative distribution of all-*trans* and cis isomers of major provitamins in a selec-

tion of fresh and processed fruits and vegetables is presented in Table 2. In most vegetables and in many fruits, β-carotene constitutes more than 85% of the total provitamin A activity. Notable exceptions are carrots and oranges, which contain both β-carotene and α-carotene (18). β-Cryptoxanthin is the predominant provitamin in orange juice (19) and in some varieties of sweet corn (20). In many fruits and vegetables the concentrations of provitamins are low relative to the concentrations of inactive carotenoids. For example, lutein is the most abundant carotenoid in green leafy vegetables (21), lycopene predominates in tomatoes (22), and capsanthin is the major pigment in red peppers (23). Other inactive carotenoids found in fruits and vegetables include ζ(zeta)-carotene, zeinoxanthin, zeaxanthin, neoxanthin, and violaxanthin.

3. *Supplementation of Foods*

Foods are supplemented with vitamin A in the form of standardized preparations of synthetic fatty acyl esters, nowadays chiefly retinyl palmitate. The preparations are available commercially as either dilutions in high-quality vegetable oils containing added vitamin E as an antioxidant or as dry, stabilized beadlets in which the vitamin A is dispersed in a solid matrix of gelatin and sucrose or gum acacia and sucrose. The oily preparations are used to supplement fat-based foods such as margarines; the dry preparations are used in dried food products such as milk powder, infant formulas, and dietetic foods (24).

Synthetic β-carotene, β-apo-8′-carotenaldehyde (apocarotenal), the ethyl ester of β-apo-8′-carotenoic acid (apocarotenoic ester), and the nonprovitamin carotenoid canthaxanthin are acceptable food color additives (25). Suspensions of micronized crystals of carotenoids in high-quality vegetable oils containing added vitamin E are used to color fat-based foods such as margarines, butter, cheese, and French dressings. Water-dispersible forms of carotenoids prepared as dry, gelatin-coated beadlets have been developed for the coloring of water-based foods such as cake mixes, puddings, and dried and canned soups (24).

4. *Stability*

Retinol is readily oxidized by atmospheric oxygen, resulting in an almost complete loss of biological activity. Fatty acyl esters of retinol are somewhat more stable towards oxidation than the alcohol. Vitamin A is stable towards alkali but is extremely sensitive towards acids, which can cause rearrangements of the double bonds and *cis-trans* isomerization. Solutions of all-*trans*-retinol or retinyl palmitate in hexane undergo slow isomerization to the lower potency cis isomers when exposed to white light. The photoisomerization rate is greatly increased in the presence of chlorinated solvents, but under gold fluorescent light (wavelengths greater than 500 nm) no significant isomerization occurs within 23 h (26). Retinyl palmitate is stable in chlorinated solvents when it is stored in the dark (27).

The carotenoids are stable within their natural plant cell environment, but once isolated they are prone to molecular rearrangement, *cis-trans* isomerization, and degradation by heat, light, oxygen, trace amounts of acids, and active surfaces. Solutions of β-carotene undergo slow isomerization, giving rise to 9-*cis* and 13-*cis* isomers, even when stored in the dark. In general, isomerization is higher in nonpolar solvents than in polar solvents (28). Chlorinated solvents are often contaminated with trace amounts of hydrochloric acid, which can promote stereoisomerization.

Chlorophyll compounds naturally present in extracts of green leafy vegetables have the ability to sensitize the photoisomerization of carotenoids, giving rise to appreciable amounts of cis isomers during even a brief exposure to white light. The reaction rate is slower when vegetable extracts are exposed to gold light (29).

In foods the indigenous retinyl esters and carotenoids are protected from oxidation by

Table 2 Quantitative Distribution of β-Carotene, α-Carotene, and β-Cryptoxanthin Isomers in Fresh and Processed Fruits and Vegetables[a,b]

	β-Carotene						α-Carotene						β-Cryptoxanthin				
Extract	All-*trans*	9-*cis*	13-*cis*	15-*cis*	Other *cis*[c]	Total	All-*trans*	9-*cis*	13-*cis*	13′-*cis*	Other *cis*[d]	Total	All-*trans*	13/13′-*cis*	15-*cis*	Total	Total[e]
Broccoli																	
fresh	29.2	5.0	3.3	1.9	2.0	41.4											41.4
boiled	36.5	6.9	4.2	2.2	2.2	52.0											52.0
Carrot																	
fresh	534.4					534.4	372.7					372.7					907.1
canned	420.4	32.7	90.5	30.4		574.0	290.9	6.1	91.0	55.9	37.1	481.0					1055.0
Collard																	
fresh	205.5	33.4	15.6	8.5	10.9	273.9											273.9
canned	229.5	128.5	18.7	9.2	23.8	409.7											409.7
Orange juice																	
fresh	2.2	tr[f]	0.4	tr		2.6	1.9	tr	0.2	0.1		2.2	2.5	0.2	tr	2.7	7.5
pasteurized	1.5	tr	0.3	tr		1.8	1.3	tr	0.1	0.1		1.5	1.3	0.2	tr	1.5	4.8
Peach																	
fresh	2.2	0.3	0.5	tr		3.0							0.3	0.1	0.1	0.5	3.5
canned	0.9	0.2	0.4	tr		1.5							0.2	0.1	tr	0.3	1.8
Spinach																	
fresh	311.9	38.6	24.5	tr	22.5	397.5											397.5
canned	309.8	96.9	28.6	14.9	22.9	473.1											473.1
Sweet potato																	
fresh	256.5					256.5											256.5
canned	191.0	25.3	76.6	19.4		312.3											312.3
Tomato																	
fresh	71.0	4.8	5.8			81.6											81.6
canned	49.1	5.5	12.0	4.8		71.4											71.4
juice	40.0	4.5	10.1	4.8		59.4											59.4

Source: Reprinted with permission from Ref. 12. Copyright 1997, American Chemical Society.

[a] Data based on an average of two lots, except for orange juice, which had only one lot. Each lot was analyzed by HPLC in duplicate.

[b] Concentrations are in micrograms per gram of dry weight tissue.

[c] Totals the concentration of one unidentified cis isomer of β-carotene.

[d] Totals the concentration of two unidentified cis isomers of α-carotene.

[e] Total provitamin A carotenoid concentration includes all isomers of β-carotene, α-carotene, and β-cryptoxanthin.

[f] tr, trace

vitamin E and other antioxidants that might be present. On depletion of the antioxidants, the retinyl esters and carotenoids become vulnerable to oxidation and subject to attack by highly active peroxides produced during oxidative rancidity. Thus factors that accelerate oxidative rancidity, such as exposure to air, heat, light, and traces of certain metals (notably copper and, to a lesser extent, iron) and storage time, will also result in the destruction of the vitamin A and carotenoids (24). The loss of vitamin A activity during food processing is largely due to thermal *cis-trans* isomerization.

5. *Bioavailability*

Several distinct differences exist between vitamin A and provitamins with regard to their digestion and absorption in the human. When foods containing normal physiological amounts of these micronutrients are ingested, vitamin A is absorbed with an efficiency of 70–90%, compared to 20–50% for the provitamins. The retinyl esters are utilized as efficiently as retinol itself if compared on the basis of their retinol content. The absorption efficiency of vitamin A remains high as the amount ingested increases beyond physiological levels, whereas that of the provitamins falls markedly with increased ingestion to less than 10%. Among the various nutritional factors that influence the bioavailability of carotenoids is the poor digestibility of fibrous plant tissue (30).

6. *Expression of Dietary Value*

The vitamin A value of foods has traditionally been expressed in international units (IU). One IU is defined as the amount of vitamin A activity contained in 0.334 μg of all-*trans*-retinyl acetate, which is equivalent to 0.300 μg of all-*trans*-retinol. In 1965, an expert committee decided to abandon the IU for vitamin A, proposing instead that the vitamin A value be designated in terms of retinol equivalents (RE), expressed in micrograms of retinol. The RE is defined as the amount of retinol present plus the equivalent amount of retinol that can be obtained from the provitamins. It is purely a dietary concept and is not an equivalency in the usual chemical sense:

$$RE = \mu\text{g retinol} + \frac{\mu\text{g } \beta\text{-carotene}}{6} + \frac{\mu\text{g other provitamins}}{12}$$

For comparison with values in the older literature, the IU values can be converted to retinol equivalents. To convert IU into RE on the basis of retinol, 1 RE = 1 μg retinol and 1 IU = 0.3 μg retinol. Therefore, 1 RE = 1/0.3 = 3.33 IU vitamin A activity from retinol.

Since the IU was based on studies that did not take into account the poor absorption and bioavailability of carotenoids in foods, the equivalency of retinol and β-carotene in the IU system differs from that in the RE system. Thus in the RE system, 1 μg retinol ≡ 6 μg β-carotene, whereas in the IU system, 1 μg retinol ≡ 2 μg β-carotene.

To convert IU to RE on the basis of β-carotene, one must first multiply the IU by a factor of 3 (6/2), to make the equivalency the same as that of the RE system, and then multiply by 3.33. Therefore, 1 RE = 3 × 3.33 = 10.0 IU vitamin A activity from β-carotene. RE = (number of IU from retinol)/3.33 + (number of IU from β-carotene)/10. The units for expressing vitamin A activity are presented in Table 3.

For food labeling purposes, which require the actual amounts of vitamin A in the food rather than the nutritional value, data obtained by physicochemical assay are expressed on a weight basis. In plant-derived foods, the appropriate units are β-carotene equivalents expressed in micrograms of β-carotene. By definition, 1 β-carotene equivalent is equal to 1 μg of all-*trans*-β-carotene or 2 μg of other, largely all-*trans*, provitamins in the foods. In animal-derived foods, the units are either micrograms of retinol or retinol equivalents (32).

Table 3 International Units (IU) and Retinol Equivalents (RE) for Expressing Vitamin A Values in Humans

Compounds[a]	μg/IU	IU/μg	μg/RE	RE/μg
Retinol	0.300	3.33	1.000	1.000
Retinyl acetate	0.344	2.91	—	—
Retinyl palmitate	0.55	1.82	—	—
β-Carotene	1.8	0.56	6	0.167
Mixed provitamin carotenoids	3.6	0.28	12	0.083

Source: Reprinted from Ref. 31, p. 1, by courtesy of Marcel Dekker, Inc.
[a] All-*trans* isomers.

B. Vitamin D

Vitamin D is represented by cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), which are structurally similar secosteroids derived from the UV irradiation of provitamin D sterols. In vertebrates, vitamin D_3 is produced in vivo by the action of sunlight on 7-dehydrocholesterol in the skin. Vitamin D_2 is produced in plants, fungi, and yeasts by the irradiation of ergosterol. On irradiation, the provitamins are converted to previtamin D, which undergoes thermal transformation to vitamin D.

Certain human populations depend on dietary sources of vitamin D because of insufficient biosynthesis of the vitamin due to inadequate skin exposure to sunlight. The classic symptoms of vitamin D deficiency are rickets in children and osteomalacia in adults. 25-Hydroxyvitamin D_3 is the major circulating metabolite in the blood, but the hormonally active form of the vitamin is 1,25-dihydroxyvitamin D_3. The latter metabolite stimulates the intestine to absorb calcium and phosphate by two independent mechanisms and acts with parathyroid hormone to mobilize calcium, accompanied by phosphate, from the bone fluid compartment into the bloodstream. 1,25-dihydroxyvitamin D_3 is also involved in the formation of *osteoclasts*—giant cells that are solely responsible for the resorption of bone matrix (33). Resorption is an essential process for the development, growth, maintenance, and repair of bone.

1. *Chemical Structure and Biopotency*

Vitamin D_3 ($C_{27}H_{44}O$, MW = 384.62) and vitamin D_2 ($C_{28}H_{44}O$), MW = 396.63) differ structurally only in the C-17 side chain, which in vitamin D_2 has a double bond and an additional methyl group (Fig. 4). Both compounds occur naturally with 5,6 double bond in the cis configuration. The biological potencies of vitamins D_2 and D_3 in humans are essentially equal.

2. *Occurrence*

As with vitamin A, fish liver oils are exceedingly rich in vitamin D. But natural foods contain only very small amounts, and significant sources are confined to a limited number of animal products. The richest natural food sources (in μg/100 g) are fatty fish, such as herring, 22; sardines and pilchards, 8; and tuna, 6 (17); smaller amounts are found in mammalian liver, eggs, and dairy products. The concentration of vitamin D_3 in milk shows a seasonal variation, which is related to the amount of sunlight available for vitamin D biogenesis in the cow. Milk also contains vitamin D_2, but in smaller concentrations than vitamin D_3. Unlike vitamin D_3, vitamin D_2 is derived by UV irradiation of ergosterol in sun-dried green forage (hay); ergosterol cannot be converted by the animal into vitamin D_2. Eggs normally contain about 1.8 (17), but eggs from hens receiving a vitamin D supplement will have a considerably higher vitamin D content. Except for eggs and fatty fish, a serving of food containing only natural sources of vitamin D would probably supply less than 1 μg of vitamin D (34). It has been estimated that to maintain adequate plasma 25-

Fig. 4 Structures of cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2).

hydroxyvitamin D levels without any input from skin irradiation would require ingestion of at least 12.5 μg of vitamin D per day (35).

The vitamin D activity in animal products is contributed by both vitamin D_3 and 25-hydroxyvitamin D_3. Typical values of the hydroxylated metabolite (μg/100 g) are bovine muscle, 0.2–0.3; bovine liver, 0.3–0.5; bovine kidney, 0.5–1.0 (36); and egg yolk, 1.0 (37). 25-Hydroxyvitamin D_3 is of nutritional significance; it is about five times more potent than vitamin D_3 and occurs in significant amounts. In milk, for example, it accounts for 75% of the total vitamin D activity as estimated by the calcium transport assay (38).

3. *Stability*

In solution, vitamins D_2 and D_3 exhibit reversible thermal isomerization to their corresponding previtamins, forming an equilibrium mixture. Equations and calculations have been published to determine the ratio of previtamin D to vitamin D as a function of temperature and reaction time (39). When equilibrated at 20°C, the ratio of previtamin D to vitamin D is 7:93. The isomerization rates of vitamins D_2 and D_3 are virtually equal (40) and are not affected by solvent, light, or catalysis (41).

The stability of vitamin D in fats and oils corresponds to the stability of the fat itself, as described previously for vitamin A. Vitamin D is, however, more stable than vitamin A under comparable conditions. Once freed from the protection of the food matrix, vitamin D is susceptible to decomposition by oxygen and light. The vitamin is stable towards alkali, but under conditions of even mild acidity the molecule isomerizes to form the 5,6-*trans* and isotachysterol isomers, neither of which possesses any significant antirachitic activity (42).

4. *Bioavailability*

In the only known human study of vitamin D bioavailability from natural sources (43), the average relative bioavailability of vitamin D_2 from meat sources was estimated to be about 60% as compared to a vitamin supplement.

5. Expression of Dietary Value

One IU of vitamin D is the activity of 0.025 μg of crystalline cholecalciferol. An expert committee in 1970 recommended that the intake of vitamin D be expressed as μg of cholecalciferol rather than as IU.

C. Vitamin E

Vitamin E is a generic term that represents four tocopherols and four tocotrienols of varying biological potency. The term *tocopherol* correctly refers to the methyl-substituted derivatives of tocol and is not synonymous with the term *vitamin E.* The tocopherols and tocotrienols may be referred to collectively as *tocochromanols.* Many of the diverse deficiency syndromes observed in animals experimentally deprived of vitamin E can be explained by the vitamin's acting as an antioxidant in stabilizing unsaturated lipids in biological membranes.

1. Chemical Structure and Biopotency

Tocopherols comprise a methyl-substituted chroman-6-ol ring attached at C-2 to a saturated isoprenoid side chain. Tocotrienols are analogous structures whose side chains contain three trans double bonds. The tocopherols and tocotrienols are designated as α, β, γ, and δ, according to the number and position of the methyl substituents in the chromanol ring (Fig. 5).

Tocopherol molecules contain three chiral centers at C-2, C-4′, and C-8′, making possible eight stereoisomers. Tocotrienols possess only the chiral center at C-2. Naturally occurring α-tocopherol (commonly known as *d*-α-tocopherol) should be designated *RRR*-α-tocopherol to

Tocopherol

Tocotrienol

Tocopherol or Tocotrienol	
5,7,8 – Trimethyl	α
5,8 – Dimethyl	β
7,8 – Dimethyl	γ
8 – Methyl	δ

Fig. 5 Structures of the tocochromanols (vitamin E).

describe its stereochemistry. Totally synthetic α-tocopherol (commonly known as *dl*-α-tocopherol) is a mixture of all eight possible stereoisomers in virtually equal proportions and should be designated all-*rac*-α-tocopherol. The most biologically active vitamer is *RRR*-α-tocopherol ($C_{29}H_{50}O_2$, MW = 430.7). The *RRR* forms of the various tocopherols (T) and tocotrienols (T-3) possess the following biological potencies (percent activity) as measured by the fetal resorption assay in the rat: α-T, 100; β-T, 25–40; γ-T, 1–11; δ-T, 1; α-T-3, 27–29; β-T-3, 5 (44).

2. *Occurrence*

The naturally occurring tocopherols and tocotrienols exist in the unesterified forms and are distributed widely in nuts, seeds, fruits, vegetables, and grasses. Major sources of vitamin E in Western diets are the cereal seed oils and the margarine and other products made from them. The indigenous concentrations of vitamin E in fatty foods are much higher than those of vitamins A and D. Typical United States values (in mg of total vitamin E/100 g) are: wheat germ oil, 166–300; soybean and cottonseed oil stick margarine, 45; and mayonnaise, 58. The chief animal sources, in which α-tocopherol predominates, are high-fat products such as eggs, 1.1; milk, 0.1; butter, 1.6; and liver, 0.7 (45).

The acetate ester of α-tocopherol, rather than the free alcohol, is used as a food supplement on account of its greater stability. Both *RRR*-α-tocopheryl acetate and totally synthetic all-*rac*-α-tocopheryl acetate are commercially available, the former having a biological activity of 1.36 IU/mg and the latter 1.00 IU/mg (44). The *RRR*-α-tocopheryl acetate is obtained by extraction from vegetable oils. Since it is not isolated without some chemical processing, it cannot legally be called natural, but it can be described as derived from natural sources.

3. *Stability*

Tocopherols and tocotrienols are destroyed fairly rapidly by sunlight and artificial light containing wavelengths in the UV region. The vitamers are slowly oxidized by atmospheric oxygen to form mainly biologically inactive quinones; the oxidation is accelerated by light, heat, alkalinity, and certain trace metals. The tocotrienols, by virtue of their unsaturated side chains, are more susceptible to destruction than the tocopherols. The vitamers can withstand heating in acid or alkaline solution provided that oxygen and UV radiation are excluded. Because α-tocopheryl acetate lacks the reactive hydroxyl group, air and light have practically no destructive effect.

4. *Bioavailability*

The few reported human studies have shown that vitamin E is absorbed with an efficiency of around 70%, and there is no significant difference in absorption efficiency between α-tocopherol and its acetate ester (30).

5. *Expression of Dietary Value*

One IU of vitamin E is the activity of 1 mg of all-*rac*-α-tocopheryl acetate, but the activity is nowadays expressed as milligrams of α-tocopherol equivalents. When calculating the total vitamin E activity of mixed diets in the United States, the mg of β-T, γ-T, and β-T-3 can be multiplied by factors of 0.5, 0.1, and 0.3, respectively, and added to the mg of α-T to give the total mg of α-tocopherol equivalents (1).

D. Vitamin K

Vitamin K is essential for the activation of specific proteins involved in blood clotting and bone metabolism through its role as a cofactor for γ-glutamylcarboxylase. This enzyme catalyses a

unique conversion of selected glutamate residues into γ-carboxyglutamate (Gla) residues in the proteins, allowing them to bind calcium and thus become activated. Vitamin K is required for the carboxylation of seven protein components of the blood-clotting cascade and for at least two proteins found in bone (46).

Two forms of vitamin K exist in nature. *Phylloquinone* (vitamin K_1) is a specific vitamer synthesized by green plants in the chloroplasts; *menaquinones* (vitamin K_2) are a series of structural analogs that are synthesized by bacteria.

1. Chemical Structure and Biopotency

Phylloquinone ($C_{31}G_{46}O_{21}$, MW = 450.68) comprises a methyl-substituted naphthoquinone nucleus attached at C-3 to a phytyl side chain containing 20 carbon atoms (Fig. 6) and may be designated $K_{1(20)}$ to distinguish it from synthetic structural analogs such as $K_{1(25)}$. Naturally occurring phylloquinone has the C-2′ double bond in the trans configuration. Synthetic phylloquinone usually contains both the trans and cis isomers, but only the trans isomer is essentially responsible for the vitamin's biological activity. The predominant members of the menaquinone family have polyisoprene side chains composed of 6–10 isoprene units and are designated menaquinone-6 (MK-6) to menaquinone-10 (MK-10) accordingly.

2. Occurrence

The highest concentrations of vitamin K (in the form of phylloquinone) are found in green leafy vegetables, with values for cabbage, broccoli, brussels sprouts, spinach, and collards ranging between 98 and 440 μg of phylloquinone/100 g of vegetable (47). Some vegetable oils, including canola (rapeseed), soybean, and olive oils, are rich sources of phylloquinone, whereas peanut oil and corn (maize) oil are not. Those margarines, mayonnaises, and regular-calorie salad dressings that are derived from phylloquinone-rich vegetable oils are second to green leafy vegetables in their phylloquinone content. Livers of ruminant species and some fermented foods, including cheeses, contain significant quantities of menaquinones (48).

3. Stability

Vitamin K vitamers are decomposed by UV radiation, alkali, strong acids, and reducing agents but are reasonably stable to oxidizing conditions and to heat.

4. Bioavailability

Little is known about the bioavailability of vitamin K from foods. It is likely that absorption is least efficient from green leafy vegetables, where the phylloquinone is intimately associated with

Fig. 6 Structure of phylloquinone (vitamin K_1).

the thykaloid membranes of chloroplasts, and most efficient from processed foods, such as oils, margarine, and dairy produce (49).

III. SCOPE OF ANALYTICAL TECHNIQUES

The vitamins were originally isolated and characterized using animal bioassays to monitor their activity. The bioassays were too time consuming and expensive to apply to routine food analysis, and colorimetric methods were later developed for the fat-soluble vitamins. These methods required open-column (gravity-flow) chromatography or thin-layer chromatography to isolate the vitamins from interfering substances. During the 1960s, gas chromatography, using so-called packed columns, was widely applied to the determination of vitamins D and E in foods, but cleanup by open-column or thin-layer chromatographic techniques was still necessary, as was derivatization, to increase the vitamins' thermal stability and volatility (50). The development of fused-silica open tubular capillary columns has revived the interest in gas chromatography, leading to a number of recent applications for the determination of vitamin E (51–54). However, since the mid-1970s and to the present day, the method of choice for determining the fat-soluble vitamins in foods has undoubtedly been HPLC. This is due to the technique's ability to chromatograph the vitamins without the need for derivatization, the nondestructive operation, and the greater separation and detection selectivity. The nondestructive nature of HPLC allows it to be used as a preparative purification method as well as a quantitative technique.

In the latest (1995) edition of *Official Methods of Analysis of AOAC International*, HPLC methods have been introduced for the first time for the determination of vitamin A in milk (55) and vitamins A (56), D_3 (57), E (58), and K_1 (59) in milk-based infant formulas.

At present, there is no universally recognized standard method for determining any of the fat-soluble vitamins that can be applied to all types of food. In this section, selected representative published HPLC methods are tabulated and key practical features discussed. The selected method must always be modified to suit the composition of the sample to be analyzed.

A. Vitamin A and Provitamins

Of the vitamin A commonly found in foods, only all-*trans*-retinol and smaller amounts of 13-*cis*-retinol, both in esterified form, are usually present in significant quantities. For the analysis of vitamin A–fortified foods, HPLC can be applied to determine either the total retinol content or the added retinyl ester (acetate or palmitate), depending on the extraction technique employed.

The vitamin A activity of plant foods is usually based on the HPLC determination of the three most ubiquitous provitamins, namely, α- and β-carotene and β-cryptoxanthin. It is necessary to separate the provitamins from other carotenoids and to quantify them individually. An obvious prerequisite to accurate quantitation is the conclusive identification of the provitamins.

To assess the effects of processing on the nutritional value of a plant food with respect to vitamin A activity, the various isomeric forms of provitamin A carotenoids present in both the fresh and processed states must be accurately measured. In such investigations it must be demonstrated that the analytical procedure does not itself cause trans-cis isomerization of carotenoids.

The methodology depends on the known carotenoid distribution in plant tissues, which can be classified into three main groups: (1) those in which the vitamin A value is due almost exclusively to β-carotene (e.g., green leafy vegetables, peas, broccoli, sweet potatoes, tomatoes, watermelon, mango); (2) those in which primarily α- and β-carotene account for the vitamin A value (e.g., carrots, some varieties of squash); and (3) those in which β-cryptoxanthin and β-carotene are the major contributors (e.g., cashew, apple, peach, persimmon, loquat) (60).

Table 4 Ratios of Vitamins A and E and Cholesterol to Vitamin D in Some Foodstuffs

	Ratio relative to vitamin D on a weight basis			
Foodstuff	D	A	E	Cholesterol
Whole milk	1	1500	5000	600,000
Ox liver	1	6000	1400	300,000
Whole egg	1	75	500	80,000

Source: Ref. 61. By permission of the publisher, Academic Press, London.

B. Vitamin D

The estimation of the very low concentrations of indigenous vitamin D in foodstuffs is difficult, owing to the need to remove interfering substances, such as cholesterol, vitamin A, and vitamin E, which are invariably present in gross excess (Table 4). Most of the published HPLC methods for determining vitamin D in foods are concerned with estimating the vitamin D content in supplemented products, such as milk in various forms, infant formulas, and margarine. In supplemented foods, the amount of naturally occurring vitamin D (if any) is usually considered to be negligible, and it is deemed necessary to determine only the vitamin D that is added. Even so, supplementation levels are very low (e.g., 7.5–12.5 μg/100 g in milk powder) (62), and the determination of vitamin D is by no means a simple task.

A bioassay will account for the activity of previtamin D as well as vitamin D and its various active metabolites. A valid estimate of the vitamin D value of a food should therefore represent "potential vitamin D," i.e., the sum of the vitamin D and previtamin D contents.

When determining naturally occurring vitamin D in animal products for nutritional evaluation purposes, 25-hydroxyvitamin D_3 should be included, because this metabolite contributes significantly to the total biological activity, particularly in milk. 25-Hydroxyvitamin D_3 is present in dairy products, eggs, and meat tissues in sufficient concentration to permit its determination by HPLC using an absorbance detector. In bovine milk the concentration of this metabolite is less than 1 ng/ml (63); hence it is usually determined by a competitive protein-binding assay after fractionation of the extracted sample by HPLC (64).

C. Vitamin E

The nutritional evaluation of the vitamin E–rich vegetable oils and the products made from them using a nonbiological assay necessitates the determination of the individual tocopherols and tocotrienols, for these vitamers vary widely in biological activity. High-performance LC is ideally suited for this purpose, and the overall vitamin E value of such foods can be estimated by applying appropriate factors based on relative biological activities. For the analysis of those animal products known to contain predominantly α-tocopherol, only this vitamer need be determined. In vitamin E–fortified foods it is usually sufficient to determine either the added α-tocopheryl acetate or the total α-tocopherol.

D. Vitamin K

Among the various vitamin K vitamers, only phylloquinone is accounted for in routine food analysis. Milk-based and soy protein–based infant formulas for the full-term infant are supplemented with synthetic preparations of phylloquinone, which invariably contain about 10% of the

biologically inactive cis isomer (65). The analytical method for infant formulas must therefore exclude *cis*-phylloquinone in the measurement.

IV. SAMPLE PREPARATION

Before a food sample can be analyzed, consideration must be given to obtain a representative sample (66,67). The amount of sample material to be taken for analysis depends upon the inherent homogeneity of the material or the homogeneity achieved after comminution.

The lipid fraction of foods containing the fat-soluble vitamins is composed mainly of triglycerides, with much smaller amounts of sterols, carotenoids, phospholipids, and minor lipoidal constituents. All of these substances exhibit solubility properties similar to those of the fat-soluble vitamins, and therefore they constitute a potential source of interference. A proportion of the indigenous fat-soluble vitamin content of a food is bound up with a lipoprotein complex, and hence the fat–protein bonds must be broken in order to release the vitamin. The protective gelatin coating used in certain proprietary vitamin premixes will need to be dissolved before commencing the analysis of supplemented foods.

It is essential for a successful assay that the vitamins be quantitatively extracted from the food matrix in a form that can be accurately measured by the particular HPLC technique to be used. An effective extraction procedure serves to homogenize and concentrate the sample, isolate the vitamin analyte from its association with protein, eliminate as far as possible known interfering substances, and destroy any indigenous enzyme activity. The vitamin-rich fraction thus obtained may require some form of cleanup before the vitamins can be measured, particularly when measuring the trace amounts of naturally occurring vitamins D and K.

The fat-soluble vitamins are photosensitive, and therefore all operations with vitamin solutions and vitamin-containing materials should be carried out in subdued light or in low-actinic amber glassware. Ideally, the laboratory or a dedicated room within the laboratory should have the windows (if any) covered with effective blinds, and artificial lighting should be provided by F40GO "gold" (or equivalent) fluorescent lamps, which exclude radiation wavelengths of less than 500 nm.

A. Extraction Procedures

Factors to consider in selecting a suitable extraction procedure are (a) the analytical information required, (b) the nature of the food matrix, (c) the form in which the vitamin occurs naturally or is added, (d) the nature and relative amounts of potentially interfering substances, (e) the stability of the vitamin in heat and extremes of pH, and (f) the selectivity and specificity of the analytical technique to be used.

Methods of extracting the fat-soluble vitamin from food matrices include alkaline hydrolysis, enzymatic hydrolysis, alcoholysis, direct solvent extraction, and supercritical fluid extraction of the total lipid component.

1. Alkaline Hydrolysis

Alkaline hydrolysis (saponification) effectively removes the preponderance of triglycerides from fatty food samples and is a practical way of extracting a relatively large amount of material. The hydrolysis reaction attacks ester linkages, releasing the fatty acids from glycerides and phospholipids and also from esterified sterols and carotenol esters. The reaction also liberates indigenous vitamins from any combined form in which they may exist (e.g., lipoprotein complex) and breaks down chlorophylls into small, water-soluble fractions. In addition, it dissolves any gelatin that

might have been present in the vitamin premix added to supplemented foods. Saponification can be utilized in assays for vitamins A, D, and E, but it is not expedient for vitamin K vitamers, which are rapidly decomposed in alkaline media.

Prepared samples of many types of food can be saponified directly. High-starch samples, such as breakfast cereals, may be digested with the enzyme takadiastase before saponification so as to avoid the formation of lumps (68).

Saponification is conventionally carried out by refluxing the suitably prepared sample with a mixture of ethanol and 60% w/v aqueous potassium hydroxide (KOH) solution in the presence of pyrogallol as an antioxidant for 30 min. The amount of ethanolic KOH solution required is dependent on the fat content of the sample. A rough guide is to use 5 ml of 60% w/v aqueous KOH and 15 ml of ethanol per 1 g of fat (69). A slow stream of nitrogen is introduced into the saponification flask via a side arm at the start and at the end of the process. A nitrogen flow is not necessary during the actual refluxing because a blanket of alcohol vapor prevents aerial oxidation during boiling. Rapid cooling after saponification is important. The liberation of the unstable retinol and tocopherols from their relatively stable esters demands protective measures against light and oxygen during saponification and throughout the subsequent analytical procedure.

The sterols, carotenoids, fat-soluble vitamins, and so forth, which constitute the unsaponifiable fraction, are extractable from the saponification digest by liquid–liquid extraction using a water-immiscible organic solvent, after adding water to the digest to facilitate the separation of the aqueous and organic phases. The fatty acids, which are precipitated as their potassium salts (soaps), and the glycerol are not extractable under alkaline conditions. Multiple extractions are necessary to ensure a quantitative transference of the vitamin analyte in accordance with partition theory. The combined solvent extracts are evaporated to dryness, and the residue is redissolved in a small volume of a suitable solvent for chromatographic analysis or further purification.

Vitamins A, D, and E, being slightly polar compounds, are extracted more efficiently from the saponification digest using a slightly polar solvent, such as petroleum ether/diethyl ether (1 + 1), than with a nonpolar hydrocarbon solvent, such as petroleum ether or hexane. The washing of diethyl ether–containing extracts with successive portions of distilled water to remove the alkali is troublesome, owing to the solubility of soaps in this solvent. Stable emulsions are produced when soaps, water, and hydrophobic solvents are shaken in the absence of ethanol; thus the washing step must be performed using a gentle swirling motion of the separatory funnel. When using hydrocarbon solvents to extract the unsaponifiable matter, it is important to maintain the optimum proportion of water and ethanol in the extraction system. For the efficient extraction of retinol (70) and the tocopherols (71) using hexane, the ethanol strength must be below 40%. Large amounts of soaps confer hydrophobic properties to the ethanol–water mixture; therefore the minimum number of extractions needed to achieve a quantitative recovery of the vitamins is affected by the amount of fat present in the original sample.

a. Vitamin A

Retinol is stable in alkaline solution and has been reported to survive at least one week while steeping in ethanolic KOH containing pyrogallol (72).

Zahar and Smith (73) developed a rapid saponification method for the extraction of vitamin A from milk and other fluid dairy products, which avoids the need for multiple extractions and washings using separating funnels. Into a series of 50-ml stoppered centrifuge tubes is placed 2 ml of sample, 5 ml of absolute ethanol containing 1% (w/v) pyrogallol, and 2 ml of 50% (w/v) aqueous KOH. The tubes are stoppered, agitated carefully, and placed in a water bath at 80°C for 20 min with periodic agitation. After saponification, the tubes are cooled with running water and

then placed in an ice-water bath before adding 20 ml of diethyl ether/petroleum ether (1 + 1) containing 0.01% (w/v) butylated hydroxytoluene (BHT) as antioxidant. The tubes are again stoppered and vortex-mixed vigorously for 1 min, allowed to stand for 2 min, and again vortexed for 1 min. To each tube is added 15 ml of ice-cold water, and the tubes are inverted at least ten times. After centrifugation, 10 ml of the upper organic layer is accurately pipeted into a tube, and the solvent is evaporated to dryness in a stream of nitrogen or under vacuum at 40°C using a rotary evaporator. The residue is dissolved in 1.0 ml of methanol (for milk samples) to provide a final solution for HPLC.

b. Carotenoids

Saponification causes a significant loss of xanthophylls, even when carried out under relatively mild conditions (ambient temperature for 3 h) (21). In addition, several different saponification procedures have been shown to promote the formation of cis isomers of β-carotene (74). Since saponification prolongs the analysis and is error prone, it should be carried out only when needed, as in high-fat samples or those containing carotenol esters.

Kimura et al. (74) recommended a procedure in which the carotenoids are dissolved in petroleum ether, an equal volume of 10% methanolic KOH is added, and the mixture is left standing overnight (about 16 h) in the dark at room temperature. This treatment caused no loss or isomerization of β-carotene, while completely hydrolyzing β-cryptoxanthin ester. Losses of xanthophylls could be reduced to insignificant levels by using an atmosphere of nitrogen or an antioxidant.

c. Vitamin D

Saponification is obligatory for the determination of vitamin D in fatty foods because of the need to remove the vast excess of triglycerides present. Hot saponification results in the thermal isomerization of vitamin D to previtamin D and the consequent need to determine the potential vitamin D content. Thompson et al. (75) reported that saponification of milk at 83°C in the presence of pyrogallol results in a 10–20% loss of added vitamin D due to thermal isomerization. Several workers have avoided the problem of thermal isomerization by employing cold saponification (i.e., prolonged alkaline digestion at room temperature). Whatever the saponification temperature, it is necessary to perform the reaction in an inert atmosphere. Indyk and Woollard (76) avoided vitamin D losses of 10–20% by flushing the saponification vessel with oxygen-free nitrogen and then sealing the vessel before cold saponification.

A mixture of petroleum ether/diethyl ether (1 + 1) is suitable for extracting vitamin D from the unsaponifiable material and allows vitamins A and D to be coextracted. For the determination of vitamin D alone in fortified milks, margarine, and infant formulas, Thompson et al. (70) extracted the unsaponifiable matter three times with hexane in the presence of a 6:4 ratio of water to ethanol. The combined hexane layers were then washed with 55% aqueous ethanol, after the initial 5% aqueous KOH and water washes, to remove material, including 25-hydroxyvitamin D, that was more polar than vitamin D. This extraction process was based on partition studies that showed that insignificant amounts of vitamin D were extracted from hexane by aqueous ethanol when the ratio of ethanol to water was less than 6:4.

d. Vitamin E

The recovery of α-tocopherol is nearly quantitative after hot saponification in the presence of an antioxidant and exclusion of light, but significant losses of tocotrienols occur (77). The extent of the tocotrienol losses depends upon the concentration of alkali present and upon the temperature

and duration of the digestion. The contribution of the tocotrienols to total vitamin E activity in foods is generally small; thus, for most practical purposes, the losses are of little consequence.

Saponification of meat is essential in order to release the α-tocopherol, which is incorporated into the membranes. Ascorbic acid is often used as an antioxidant to protect the α-tocopherol against peroxidation during saponification. Pfalzgraf et al. (78) reported a rapid saponification method using a single vessel for the extraction of α-tocopherol in pork tissues. Samples of homogenized tissue are weighed into amber 50-ml laboratory bottles followed by the addition of ascorbic acid and methanolic KOH. The bottles are flushed with nitrogen, sealed, and heated at 80°C for 40 min, with occasional shaking. To the cooled digest is added water/ethanol and hexane/toluene (1:1) containing 0.01% BHT. The mixture is vigorously shaken for 10 min and centrifuged. A 20-μl aliquot of the upper layer is injected onto the HPLC column.

In the analysis of foods that contain significant amounts of both naturally occurring tocopherols and supplemental α-tocopheryl acetate, saponification, by hydrolyzing the esterified vitamin E, allows the total α-tocopherol content to be measured as a single peak by HPLC. It should be noted that if totally synthetic all-*rac*-α-tocopheryl acetate is the supplemental form used, its hydrolysis product, all-*rac*-α-tocopherol, is less biologically active than is naturally occurring *RRR*-α-tocopherol, making it impossible to calculate a potency value for the total vitamin E. This problem does not arise if the supplement used is *RRR*-α-tocopheryl acetate.

2. *Alcoholysis*

The lipid content of a food sample can be removed by converting the parent glycerides into their methyl esters by reaction with methanolic KOH solution under conditions that favor alcoholysis rather than saponification (79). Alcoholysis depends upon the KOH and methanol reacting to form potassium methoxide, which, in turn, converts the glycerides into glyceride methyl esters and soaps. The reaction is completed within 2 min at ambient temperature; hence alcoholysis is a very rapid process compared with saponification. Alcoholysis is also a milder process than saponification and does not hydrolyze vitamin A esters; consequently, there is less potential for destruction of vitamin A. Alcoholysis has been utilized in the HPLC determination of vitamin A in fortified nonfat milk and vitamin D_3 in fortified whole milk (80).

3. *Enzymatic Hydrolysis*

Enzymatic hydrolysis is a nondestructive alternative to saponification for removing triglycerides in vitamin K determinations. For the simultaneous determination of vitamins A, D, E, and K in milk- and soy-based infant formulas and dairy products fortified with these vitamins (81), an amount of sample containing approximately 3.5–4.0 g of fat was digested for 1 h with lipase at 37°C and at pH 7.7. This treatment effectively hydrolyzed the glycerides, but only partially converted retinyl palmitate and α-tocopheryl acetate to their alcohol forms; vitamin D and phylloquinone were unaffected. The hydrolysate was made alkaline in order to precipitate the fatty acids as soaps and then diluted with ethanol and extracted with pentane. A final water wash yielded an organic phase containing primarily the fat-soluble vitamins and cholesterol.

4. *Direct Solvent Extraction*

The fat-soluble vitamins can be extracted from the food matrix without chemical change using a solvent system that is capable of effectively penetrating the tissues and breaking lipoprotein bonds. A total lipid extraction is required for the simultaneous determination of vitamers or vitamins with a wide range of polarities, and, for this purpose, a mixture of chloroform and methanol (2 + 1) is highly efficient (82). The Röse–Gottlieb method is particularly suitable for ex-

tracting the total fat from milk products and infant formulas. It entails treatment of the reconstituted milk samples with ammonia solution and alcohol in the cold and extraction with a diethyl ether/petroleum ether mixture. The alcohol precipitates the protein, which dissolves in the ammonia, allowing the fat to be extracted with the mixed ethers. The method is suitable for the extraction of vitamins A and D but not for extracting vitamins E and K, which are labile under alkaline conditions.

Some methods of selectively extracting the lipid fraction from various foods prior to the determination of the fat-soluble vitamins by HPLC are discussed below.

a. Vitamin A and Carotene

In fortified fluid milks, in which the vitamin A ester (palmitate or acetate) in the form of an oily premix is thoroughly dispersed in the bulk product, the total vitamin A content can be extracted directly with hexane. The hexane solution, after removal of the polar material, is then injected into the liquid chromatograph. Thompson et al. (83) developed a method in which sufficient absolute ethanol (5.0 ml) is added to a 2-ml sample of milk in a centrifuge tube so that the milk constituents are suspended in 71% aqueous ethanol; this solvent denatures the proteins and fractures the fat globules. The lipid fraction is then partitioned into hexane, and water is added to induce the aqueous and organic phases to separate. After centrifugation, the upper phase is a hexane solution of the milk lipids containing the vitamin A, and the lower phase is aqueous ethanol in which are dissolved salts, denatured proteins, and polar lipids. The interface contains a mixture of upper and lower phases plus insoluble protein. This extraction technique can, with slight modification, also be applied to the determination of vitamin A and carotene in margarine. It is not recommended for milk powders, since the added vitamin A may be contained within a gelatin matrix, and a quantitative extraction may not be achieved.

b. Carotenoids

When green leafy vegetables are under analysis, the carotenoids are prone to photoisomerization by the sensitizing action of coextracted chlorophylls.

For the determination of carotenoids in fruits and nonleafy vegetables, which contain a large percentage of water, direct solvent extraction using a suitable water-miscible organic solvent is appropriate. Tetrahydrofuran has been found suitable, because it readily solubilizes carotenoids without causing isomerization, and it prevents the formation of emulsions by denaturing the associated proteins (21). However, tetrahydrofuran is known to promote peroxide formation, so it must be stabilized with an antioxidant such as BHT. The extraction may be carried out in the presence of anhydrous sodium sulfate as a drying agent. The addition of magnesium carbonate to the extraction system serves to neutralize traces of organic acids that can cause destruction and structural transformation of carotenoids.

In an extraction procedure described by Khachik and Beecher (84), homogenized vegetables are blended with anhydrous sodium sulfate (200% of the weight of the test portion of vegetable), magnesium carbonate (10% of the weight of the test portion), and tetrahydrofuran. The extract is filtered under vacuum, and the solid materials are re-extracted with tetrahydrofuran until the resulting filtrate is colorless. Most of the solvent is removed on a rotary evaporator at 30°C, and the concentrated filtrate is partitioned between petroleum ether and water to remove the majority of contaminating nonterpenoid lipids. The water layer is washed with petroleum ether several times, and the resulting organic layers are combined, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue is taken up in a small volume of HPLC solvent for analysis.

c. Vitamin D

In a simplified method for screening vitamin D levels in fortified skimmed milk, the milk sample was mixed with water, ethanol, and ammonium hydroxide and then extracted four times with diethyl ether/hexane. The dried residue obtained from the combined organic phase could be analyzed by HPLC without the need for purification (85).

d. Vitamin E

For the determination of vitamin E in seed oils by HPLC, the oils can simply be dissolved in hexane and analyzed directly. Solid-food samples demand a more rigorous method of solvent extraction. In a modified Röse–Gottlieb method to extract vitamin E from infant formulas (86), dipotassium oxalate solution (35% w/v) was substituted for ammonia to avoid alkalizing the medium, and methyl *tert*-butyl ether was substituted for diethyl ether because of its stability against the formation of peroxides.

e. Vitamin K

For the analysis of infant formulas, Ayi and Burgher (87) modified the Röse–Gottlieb procedure by replacing the ammonia/ethanol treatment by acidified ethanol. Shearer (88) extracted phylloquinone from vegetables, fruits, cereals, meats, and fish by grinding in a mortar with fine quartz granules before extracting with acetone. After the addition of water and hexane to the acetone extract, the phylloquinone partitioned entirely in the upper hexane phase, leaving polar impurities in the acetone/water phase.

5. Supercritical Fluid Extraction

Supercritical fluids are compounds that are held above their critical temperature and pressure. Under these conditions they have properties intermediate between those of liquids and gases. By altering the conditions, their density, viscosity, and solubilizing power can be changed. The advantages of this method are that the extraction conditions are milder and can be carried out at lower temperatures than liquid–liquid extraction, thus reducing degradation of the analyte. During the extraction the sample is also in an inert atmosphere and is protected from light. The low viscosity and high diffusion rates of supercritical fluids compared to most liquids speed up the extraction step, and, by adjusting the temperature and pressure, the selectivity of the extraction can be optimized, thereby reducing the need for a complex cleanup stage before analysis (89).

Phylloquinone has been extracted from powdered infant formula using supercritical carbon dioxide at 8000 psi and 60°C for 15 min (65). The extracted material was readily recovered by depressurization of the carbon dioxide across an adsorbent trap and then washed from the trap with a small volume of dichloromethane/acetone (1 + 1) to give a sample suitable for direct HPLC analysis. Trial experiments gave recoveries of 92% of phylloquinone from a Chromosorb W matrix. A similar technique was applied to the extraction of retinyl palmitate from cereal products (90).

B. Cleanup Procedures

The extracts prepared by treatment of the test material may require some form of cleanup before the vitamins can be measured. The requirement for cleanup depends upon the ratio of analyte to interfering substances and also upon the sensitivity and selectivity of the HPLC method employed.

The unsaponifiable fraction of whole milk constitutes 0.3–0.45% by weight of the total fat and is composed largely of cholesterol (91). In vegetable oil types of margarine, the larger part

of the unsaponifiable matter is composed of phytosterols, which are predominantly β-sitosterol, stigmasterol, and campesterol; only trace amounts of cholesterol are generally present (92). Cleanup of the unsaponifiable matter is obligatory in HPLC methods for vitamin D in order to remove the excessive amounts of sterols and other interfering substances, including carotenoids and vitamin E vitamers. Sterols exhibit similar chromatographic properties to vitamin D and, if not removed, would alter the vitamin's retention time. Although sterols exhibit only very weak absorbance at the HPLC detection wavelength used for vitamin D, a vast excess will cause a detector response sufficient to constitute an interference.

Cleanup or fractionation procedures that have been used in the more recent fat-soluble vitamin assays include sterol precipitation, open-column chromatography, solid-phase extraction, and high-pressure gel permeation chromatography. High-performance LC has been used on a semipreparative scale in vitamin D and vitamin K assays to obtain purified fractions of sample extracts. This technique is discussed in Sec. V.B.3.

1. *Precipitation of Sterols*

The bulk of the sterols can be removed from the unsaponifiable fraction of the sample by precipitation from a freezing methanolic solution followed by filtration.

2. *Open-Column Chromatography*

The more recent applications of open-column chromatography in fat-soluble vitamin assays utilize liquid–solid (adsorption) chromatography using gravity-flow glass columns dry-packed with magnesia, alumina, or silica gel. Such columns enable separations directly comparable with those obtained by thin-layer chromatography to be carried out rapidly on a preparative scale.

a. **Magnesia**

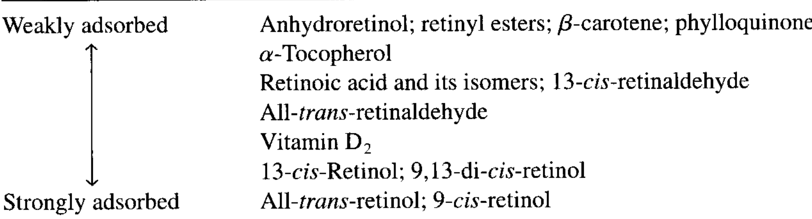
A glass column packed with 3–4 g of a mixture of magnesia and diatomaceous earth (1 + 1, w/w) was employed to remove interfering pigments from the unsaponifiable fraction of vegetables or fruits prior to the determination of carotenes and monohydroxycarotenoids by HPLC (93). The unsaponifiable residue was dissolved in hexane and applied in a total volume of 5 ml to the column. The carotenes and monohydroxycarotenoids were eluted with hexane/acetone (90 + 10 or 85 + 15) leaving residual chlorophylls and dihydroxy- and polyoxycarotenoids on the column. A magnesia column will also retain lycopene, which is a potential interference in tomato extracts (94).

b. **Alumina**

Alumina must be supplied in the neutral condition, i.e., pretreated with acid to reduce its basic behavior (95) and partially deactivated in order to provide the necessary chromatographic resolution. The practical working range for alumina is 2–10% water-deactivated, i.e., where the adsorbent is fully activated by driving out the water at 600°C and then deactivated by shaking with 2–10% of its weight of water. Alumina-column chromatography has found application as a cleanup step in vitamin D assays, with the chief aim of removing cholesterol, phytosterols, and carotenes; vitamins A and E will also be removed, if present.

For the determination of vitamin D in fortified milk (96), the unsaponifiable residue was dissolved in 5 ml of hexane, and 0.1 or 0.2 ml of a tracer solution (chlorophyll-a) and 1 g of dry 8% water-deactivated alumina were added. The solvent was evaporated off, and the dried alumina containing the sample was poured on top of a prepared column packed with 15 g of alumina. Elu-

Table 5 Relative Retentions of Fat-Soluble Vitamins on Silica Gel



Weakly adsorbed	Anhydroretinol; retinyl esters; β-carotene; phylloquinone
↑	α-Tocopherol
	Retinoic acid and its isomers; 13-*cis*-retinaldehyde
	All-*trans*-retinaldehyde
	Vitamin D_2
↓	13-*cis*-Retinol; 9,13-di-*cis*-retinol
Strongly adsorbed	All-*trans*-retinol; 9-*cis*-retinol

Source: Reprinted from Ref. 97, p. 345, by courtesy of Marcel Dekker, Inc.

tion of the column was effected with chloroform using the visible chlorophyll-a band to locate the purified vitamin D fraction.

c. Silica Gel

The weak adsorption of phylloquinone on silica gel (Table 5) provides the basis for silica purification of lipid extracts of milk and infant formulas in vitamin K assays. Haroon et al. (98) washed the hydrocarbons from a silica gel column with petroleum ether, after which the phylloquinone fraction was eluted with petroleum ether containing 3% diethyl ether; lipids that were more polar than phylloquinone were retained on the column.

3. Solid-Phase Extraction

a. General Considerations

Solid-phase extraction, a refinement of open-column chromatography, uses disposable prepacked cartridges to facilitate rapid cleanup of sample extracts prior to analysis by gas chromatography and HPLC (99). The full range of silica-based polar and nonpolar stationary phases encountered in HPLC column packings is commercially available, but only silica and C_{18}-bonded silica have so far found widespread application in fat-soluble vitamin assays. The average silica particle size is typically 40 μm (BondElut and Bakerbond) or 60 μm (SepPak) and allows easy elution under low pressure. The small mean pore diameter, which is typically 60 Å (1 Å = 10^{-10} m), excludes proteins of molecular weight higher than 15,000–20,000, and the large surface area (typically 500–600 m^2/g) confers a high sample loading capacity. The successive conditioning, loading, washing, and elution of solid-phase extraction cartridges are carried out by a step change in solvent strength using the smallest possible volumes of solvent. The cartridges may be operated under positive pressure using a hand-held syringe or under negative pressure using a vacuum manifold. The latter technique is preferable because multiple samples can be processed simultaneously and the solvent flow rate can be precisely controlled with the aid of a vacuum gauge.

Purification of the sample extract can be effected in two ways, after first conditioning the sorbent with an appropriate solvent in order to solvate the functional groups of the stationary phase. In the *sample cleanup mode*, a stationary phase is selected that has a very high affinity for the analyte and little or no affinity for the matrix; therefore the sorbent retains the analyte, and unwanted material passes through. After loading the sample, the cartridge is washed with an appropriate solvent to remove further unwanted material, and the analyte is finally eluted with the minimum volume of a solvent that is just strong enough to displace it from the sorbent. This technique provides the opportunity for trace enrichment, in which a large volume of dilute sample is passed through the cartridge, and the enriched sample can be displaced with a small volume of solvent.

In the *matrix removal mode*, the sample extract is simply passed through the cartridge. Unwanted material will be retained, while the analyte will pass through the sorbent. This strategy is usually chosen when the analyte is present in high concentration.

b. Vitamin D

Solid-phase extraction in the sample cleanup mode is proving to be an invaluable technique for purifying the unsaponifiable fraction of food samples in HPLC methods for determining vitamin D. The unsaponifiable residue is dissolved in a nonpolar solvent; the resultant solution is loaded onto a cartridge containing silica, a highly polar sorbent. The hydroxyl group on the vitamin D molecule bestows sufficient polarity to cause retention onto the silica surface. Nonpolar material in the sample solution has a greater affinity for the solvent and hence is unretained. The silica bed is then washed with a solvent that is sufficiently polar to remove further interfering material without displacing the vitamin D. The vitamin D is then eluted with a slightly more polar solvent, thus achieving isolation of the vitamin from its less polar coextractants.

This approach was used by Indyk and Woollard (100) to remove the interfering vitamin E from fully vitaminized infant formulas. After loading the sample unsaponifiable extract onto a silica cartridge, the sorbent was washed with 60 ml (or 65–75 ml) of hexane/chloroform (21.5 + 78.5) to remove the carotenoids and vitamin E. The carotenoids appeared in the first 10 ml of eluate; α-tocopherol appeared in the first 30 ml of eluate; and γ-tocopherol and some of the tocotrienols appeared in the following 30 ml. The vitamin D was then eluted with 10 ml of methanol, along with the retinols, sterols, xanthophylls, δ-tocopherol, and other unidentifiable polar excipients. Bui (68) removed the bulk of the sterols from high-fat vitamin D–fortified milk products and diet foods by washing the silica bed with hexane/ethyl acetate (85 + 15). The vitamin D was then eluted with hexane/ethyl acetate (80 + 20). The recovery of vitamin D_3 following solid-phase extraction was 98%.

Solid-phase extraction effectively separates vitamin D from its more polar 25-hydroxy metabolite. In the analysis of human milk (64), the dried lipid fraction of milk was dissolved in 35% dichloromethane in hexane and then applied to a preconditioned silica cartridge. The sample was fractionated using the following elution sequence: 9 ml hexane (discard), 3 ml 7% ethyl acetate in hexane (discard), 15 ml 7% ethyl acetate in hexane (vitamins D_2 and D_3), 25 ml 15% ethyl acetate in hexane (discard), and 9.5 ml 3% 2-propanol in hexane (25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3).

4. *Gel Permeation Chromatography*

High-pressure gel permeation chromatography, employing up to four 300 × 7.8 mm (length × internal diameter) μStyragel 100-Å columns connected in series, has been used in vitamin A, D, and E assays to remove glycerides in the analysis of oils and margarine (101), breakfast cereals (102), and infant formulas (103,104). μStyragel 100 Å is a semirigid gel composed of 10-μm particles of polystyrene crosslinked with divinylbenzene; it has an average pore size of approximately 40 Å (105).

V. HPLC SYSTEMS

A. The Column

The majority of published HPLC techniques used in fat-soluble vitamin assays have utilized 5- or 10-μm particles of porous silica or derivatized silica packed into stainless steel tubes of typical length of 250 mm and standard internal diameter (ID) of 4.6 mm. Radially compressed

cartridge-type columns (Waters Chromatography Division) manufactured from heavy-wall polyethylene of dimensions 10 cm × 8 mm have also found application. The insertion of a short guard column between the injector and the analytical column protects the latter against the loss of efficiency caused by strongly retained sample components and from pump or valve wear particles. The column-packing material is held in the column by fine-porosity frits of stainless steel or some other material. Membrane filtration of all test extracts is important for the removal of particulate material or macromolecules that might otherwise enter the guard or analytical column. Rabel (106) discussed the care and maintenance of HPLC columns.

Narrow-bore columns of between 1.0 and 2.5 mm ID are available for use in specially designed liquid chromatographs having an extremely low extracolumn dispersion. For a concentration-sensitive detector such as the absorbance detector, the signal is proportional to the instantaneous concentration of the analytes in the flow cell. Peaks elute from narrow-bore columns in much smaller volumes compared to those from standard-bore columns. Consequently, because of the higher analyte concentrations in the flow cell, the use of narrow-bore columns enhances detector sensitivity. The minimum detectable mass is directly proportional to the square of the column radius (107); therefore, in theory, a 2.1-mm-ID column will provide a mass sensitivity about five times greater than that of a 4.6-mm-ID column of the same length.

The enhanced detectability obtained using a 2.0-mm-ID column with respect to a 4.0-mm-ID column of the same length (250 mm) is illustrated in Fig. 7, which compares the HPLC-UV chromatograms of a standard solution of fat-soluble vitamins. The narrow-bore column was used with a 2-mm^3 volume flow cell and the standard-bore column with an 8-mm^3 cell. The flow rate of the narrow-bore system was adjusted to give the same linear velocity as the standard-bore system. Limits of detection using the narrow-bore system were between 1.5 (α-tocopherol) and 90 (retinol) times lower than those obtained using the standard-bore system (Table 6). For a component eluting with a k' (solute capacity factor) of 1, the two columns had comparable efficiency as determined by calculation of the number of theoretical plates (N). However, in the case of a late-eluting peak, i.e., the retinyl palmitate peak, the narrow-bore column proved to be more efficient ($N = 8215$) than the standard-bore column ($N = 3850$) (108).

Food analysts have been somewhat reluctant to convert to narrow-bore HPLC. However, several vitamin methods utilize 3.2-mm-ID columns, which in a properly designed system give a twofold increase in sensitivity over 4.6-mm-ID columns.

B. Chromatographic Modes

The chromatographic mode selected for analytical separations depends upon the extraction and cleanup procedures employed and the vitamers required to be measured.

1. Normal-Phase Chromatography

In the normal-phase mode, a polar (hydrophilic) stationary phase is used in conjunction with a nonpolar mobile phase. Separation is based on the relative polarity of the solutes and their affinity for the stationary phase. Relatively nonpolar solutes prefer the mobile phase and elute first; more polar solutes prefer the stationary phase and elute later.

a. Adsorption Chromatography

This is liquid–solid chromatography in which the surface of microparticulate silica or other adsorbent constitutes the polar stationary phase. The silica particles are characterized by their shape (irregular or spherical), size and size distribution, and pore structure (mean pore diameter,

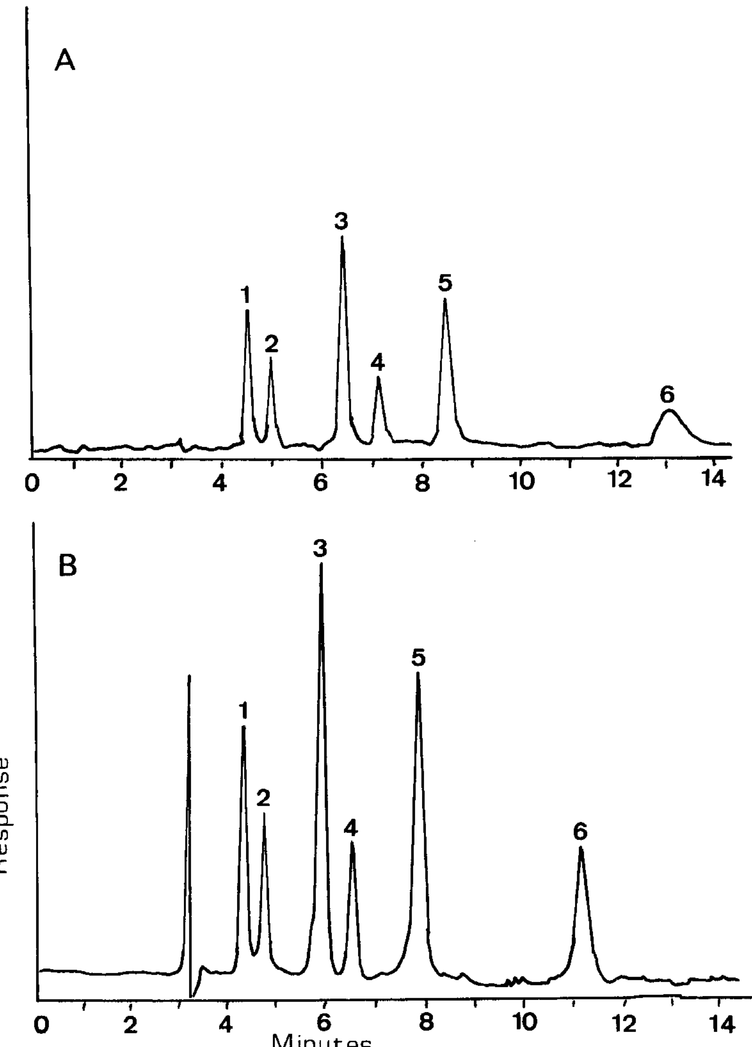


Fig. 7 Comparative HPLC separations of a standard solution of six vitamins using (A) 250 × 4.0-mm-ID standard-bore and (B) 250 × 2.0-mm-ID narrow-bore columns. Stationary phase (both columns), 5-μm Nucleosil-120-5 C_8 (octyl); mobile phase, methanol/water (92:8). Flow rate (A) 0.7 ml/min, (B) 0.2 ml/min. Injection volume, 1 μl. Wavelength-programmed absorbance detection. Peaks: (1) retinol; (2) retinyl acetate; (3) vitamin D_3; (4) α-tocopherol; (5) α-tocopheryl acetate; (6) retinyl palmitate. (From Ref. 108.)

specific surface area, and specific pore volume). The adsorption sites on the surface are silanol (≡Si—OH) groups, which are present both as isolated groups and hydrogen-bonded to one another. The nonpolar mobile phase is typically hexane containing a small percentage (usually <5% by volume) of a polar organic solvent (e.g., 2-propanol) to act as modifier. The modifier is preferentially adsorbed from the mobile phase by the hydrogen-bonded silanol groups and effectively deactivates these strong adsorption sites. The isolated silanol groups that remain are those responsible for the adsorptive properties of the deactivated silica. The dissolved solute molecules compete with mobile-phase molecules for interaction with the remaining, weaker adsorption

Table 6 Absolute Detection Limits (DLs) of Fat-Soluble Vitamins Using Standard-Bore (4.0-mm-ID) and Narrow-Bore (2.0-mm-ID) Columns and UV Detection

Vitamin	DL (ng)[a] 4.0 mm ID	DL (ng)[a] 2.0 mm ID
Retinol	0.090	0.001
Retinyl acetate	0.100	0.003
Retinyl palmitate	0.320	0.030
D_3	0.800	0.040
α-Tocopherol	0.900	0.600
α-Tocopheryl acetate	2.500	0.700

Source: Ref. 108.
[a] Detection limits based on a signal-to-noise ratio of 3:1.

sites, so solute retention can be increased by decreasing the polarity of the mobile phase. Solute retention is very sensitive to changes in temperature; therefore column thermostating is recommended, especially when peak height measurements are used in quantitative assays.

Adsorption chromatography is a powerful means of separating cis and trans isomers of unsaturated compounds, the separation mechanism being attributed to a steric fitting of solute molecules with the discrete adsorption sites. This is illustrated in the isocratic separation of six geometric isomers of vitamin A obtained by photolysis of all-*trans*-retinol (109).

Silica columns can tolerate relatively heavy loads of triglyceride and other nonpolar material. Such material is not strongly adsorbed and can easily be washed from the column with 25% diethyl ether in hexane after a series of analyses (83). Procedures for determining vitamins A and E have been devised in which the total lipid fraction of the food sample is extracted with a nonaqueous solvent, and any polar material that might be present is removed. An aliquot of the nonpolar lipid extract containing these vitamins is then injected into the liquid chromatograph without further purification. Direct injection of the lipid extract is possible because the lipoidal material is dissolved in a nonpolar solvent that is compatible with the predominantly nonpolar mobile phase. Procedures based on this technique are rapid and simple, because there is no need to saponify the sample.

An operational disadvantage of adsorption chromatography is the slow equilibration towards water, which is a very strong modifier in deactivating the silica surface. All organic solvents (unless specifically dried) contain an inherent amount of water in the parts-per-million range that is sufficient to affect solute retention. Gradient elution is best avoided, because it is difficult to ensure that equilibration between the silica adsorbent and the changing mobile phase is occurring sufficiently rapidly. In addition, the silica surface, even when deactivated, can tenaciously hold very polar compounds present as contaminants in the injected sample or as impurities in the mobile phase (110). This leads to an eventual loss of chromatographic efficiency as strongly adsorbed material accumulates with continued use. It then becomes necessary to regenerate the column by washing off the adsorbed material and then to re-equilibrate the column. This process may be carried out by pumping a sequence of solvents of increasing polarity through the column and then reversing the sequence (110).

b. Polar Bonded-Phase Chromatography

Bonded-phase column packings for use in normal-phase chromatography are available in which the stationary phase is a polar functional group chemically bonded onto the silica surface. One

[SILICA]–OH + CH_3O–Si(OCH_3)(OCH_3)–$CH_2CH_2CH_2$–O–CH_2–CH–CH_2 (epoxide, O)

Silanol

3-Glycidoxypropyltrimethoxysilane

1. Silylation of the silica surface
2. Hydrolysis
3. Thermal curing

[SILICA]–O–Si(O–)(O–)–$CH_2CH_2CH_2$–O–CH_2–CH–CH_2 (epoxide, O)

Epoxysilica

Fig. 8 Synthesis scheme to form an epoxysilica. (From Ref. 111. AOCS Press.)

such stationary phase that has been used as an alternative to silica for the separation of vitamin E vitamers is called a *diol* phase. The Supelcosil LC-Diol bonded phase is prepared by reacting 5-μm spherical silica particles containing surface ≡Si—OH groups with 3-glycidoxypropyltrimethoxysilane to furnish an epoxysilica (Fig. 8). The functional group of this phase should therefore be considered as an "epoxide" rather than as a "diol." The epoxy column reportedly gives more reproducible and constant separations than silica columns, owing to its stability to polar and nonpolar solvents, its incapability to form any strong hydrogen-bonded structures with proton-donor analytes, and its moderate polarity (111).

2. *Reversed-Phase Chromatography*

In this mode a nonpolar stationary phase is used in conjunction with a polar mobile phase. Solute elution is the reverse of that observed in the normal mode; i.e., polar solutes are eluted before nonpolar solutes.

The majority of reversed-phase HPLC applications utilize an octadecylsilane (ODS, C_{18}) stationary phase manufactured by reaction of an organosilane reagent with silanol groups on the silica substrate to form stable siloxane-bond linkages (Si—O—Si). The concentration of bonded ligands on the high surface area of the porous substrate is designated by the term *surface coverage* or *ligand density*, expressed in units of micromoles per square meter. In comparison, carbon loading is a measure of the percentage of carbon on a bonded stationary phase, and does not take into account the surface area of the substrate. *Phase coverage* is a general term usually taken to mean carbon loading. Owing to steric hindrance during the bonding process, not all sites on the

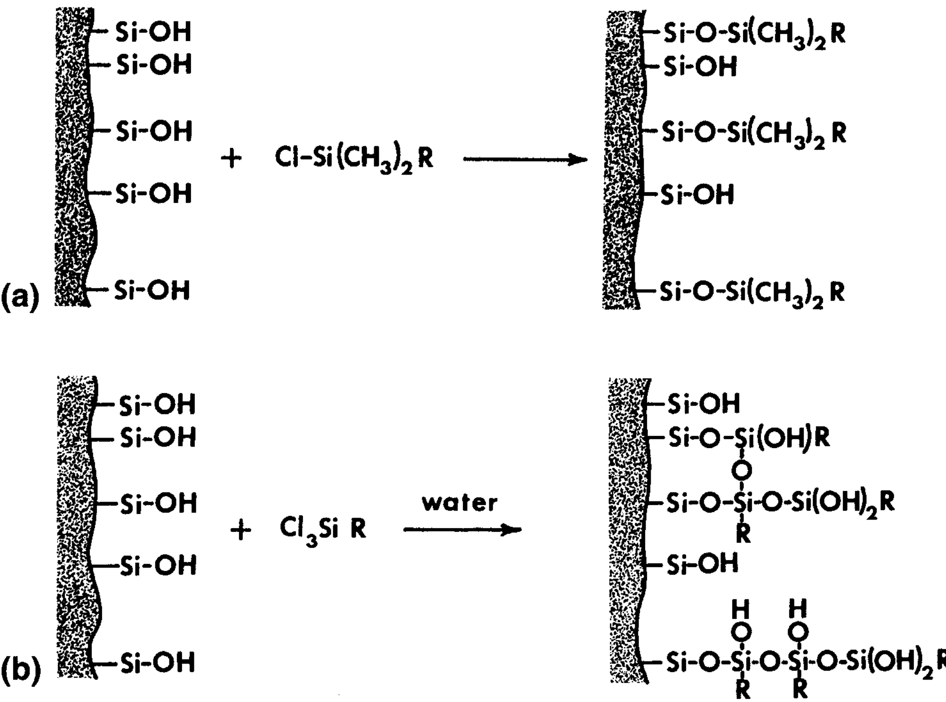


Fig. 9 Synthesis scheme for surface modification of silica. Formation of (A) a monomeric phase and (B) a polymeric phase. (From Ref. 112.)

silica surface react. Accessible residual silanols trapped by steric hindrance can be endcapped by carrying out a secondary silanization reaction using a small monofunctional silane, such as trimethylchlorosilane. Fully endcapped stationary phases are almost completely hydrophobic and exhibit true reverse-phase properties. Nonendcapped phases contain a percentage of accessible silanol groups that impart some secondary normal-phase properties.

Depending on the reaction conditions and the type of silane reagent used, both monomeric (monolayer) and polymeric stationary phases can be prepared (Fig. 9). Monomeric stationary phases result from the use of monofunctional organosilanes (e.g., dimethyl-*n*-octadecylchlorosilane); polymeric phases are manufactured by intentionally introducing a measured quantity of water into a synthesis involving polyfunctional silanes. Only siloxane bonds are involved in polymerization, and both linear addition and crosslinking reactions are possible (112a). (*Note*: The term *polymeric phase* should not be confused with polymer-based substrate phases, which have a polymer substrate instead of a silica substrate).

The physical structure of the stationary phase depends on the compatibility of the solvent with the bonded *n*-alkyl chains. Compatible nonpolar solvents tend to promote extension of the chains, allowing full penetration by the solvent. Conversely, fairly polar solvents tend to promote collapse of the chains upon each other, allowing negligible solvent penetration. The stationary phase therefore has the ability to adjust itself to maintain a relatively nonpolar character (113). Retention on monomeric bonded phases with octyl (C_8) or longer chains are dominated by a partitioning-like mechanism (114).

Martire and Boehm (113) concluded from a mathematical study that chemically bonded

phases exhibit molecular shape selectivity, which increases as the bonded chains become more fully extended. Solute retention in the collapsed-chain state resembles that of classical liquid–liquid partition chromatography. Wise and Sander (115) observed that polymeric phases exhibit a greater selectivity for molecular shape than monomeric phases and that shape selectivity increases with increasing phase coverage for polymeric phases. These observations suggest that polymeric phases may be more extended or rigid than monomeric phases.

Mobile phases used in reversed-phase chromatography are frequently composed of mixtures of methanol and water or acetonitrile and water. Increasing the proportion of water causes an increased retention of the more hydrophobic solutes relative to the more polar solutes. The surface tension of the mobile phase plays a major role in governing solute retention, so an increase in temperature, by reducing viscosity, increases column efficiency and shortens retention times.

Several methods for determining fat-soluble vitamins employ nonaqueous reversed-phase (NARP) chromatography as a means of increasing their solubility. A typical NARP mobile phase consists of a polar basis (usually acetonitrile), a solvent of lower polarity (e.g., dichloromethane) to act as a solubilizer and to control retention by adjusting the solvent strength, and, occasionally, a small amount of a third solvent with hydrogen bonding capacity (e.g., methanol) to optimize selectivity. To compensate for the increased affinity of the lipophylic compounds for the mobile phase, a highly retentive stationary phase, such as Zorbax ODS (20% carbon loading), is required.

The removal of triglycerides from the sample before injection is essential when using reversed-phase chromatography. Triglycerides are insoluble in water and only sparingly soluble in methanol and acetonitrile. If injected, they may not be completely eluted from the column, and the retained material would impair chromatographic efficiency, peak shape, and reproducibility. In the absence of nonpolar material in the final sample extract, reversed-phase chromatography exhibits improved reproducibility of solute retention times compared with normal-phase systems. This is largely because retention in reversed-phase chromatography is little affected by small variations in the mobile-phase composition, and, unlike adsorption chromatography, no significant effect is seen from slight changes in water content. Re-equilibration of reversed-phase columns is easier and quicker compared with silica columns, owing to the weaker interactive forces between the solute and the nonpolar stationary phase. Elution with several column volumes of methanol is usually sufficient to restore the column to its original condition.

3. Two-Dimensional HPLC

For determining the trace amounts of naturally occurring vitamins D and K in foods, it is often impossible to achieve an adequate separation for quantitation using a single column. This is because prior chromatographic cleanup techniques fail to remove lipoidal substances that are of similar polarity to the vitamin in question, and these substances interfere in the analytical HPLC. It has therefore been found necessary in such cases to perform the HPLC in two systems. A true two-dimensional combination involves two distinct chromatographic modes (e.g., normal phase and reversed phase) and can be expected to provide better selectivity than the use of two columns operated in the same mode (116). The first system (semipreparative HPLC) is designed to isolate and collect a fraction of the sample extract that contains the vitamin analyte and the internal standard. Ideally, the analyte and the internal standard should be unresolved from one another so that they can be collected by reference to a single peak in the chromatogram. If the analyte/internal standard peak is masked by coeluting peaks, the obvious method of collecting the fraction is to refer to the retention time of an analyte standard. However, this method may not be reliable if the chromatographic mode is normal phase, because retention times may vary from run to run. To overcome this problem, the chromatogram can be compared with one obtained by spiking a small

amount of the sample extract with the pure analyte. This enables the fraction to be collected by observing the analyte peak in relation to UV-absorbing contaminants in the sample extract.

The fraction of column effluent containing the analyte and internal standard can be either collected manually for subsequent reinjection onto the second (analytical) column (offline operation) or diverted directly onto the second column via a high-pressure switching valve (online operation). For manual collection, a drop-counter-fraction collecting system rather than a volume collection system has been recommended (117). The fraction is collected in a small tapered tube, and the solvent is carefully evaporated off under a stream of nitrogen. The residue is then dissolved in a small volume of a suitable solvent for the analytical separation. Because the sample is reconstituted in offline operation, the potential problem of mobile phase incompatibility between the two HPLC systems is avoided, and hence any semipreparative/analytical combination can be used.

Online operation has the potential for being completely automated, because the switching valve can be actuated by time-programmable events from a microprocessor-based chromatograph. Limitations are that the two mobile phases must be miscible with each other, and the mobile phase from the first column must be of weaker elution strength than that used for the second column. The latter criterion facilitates the concentration of solute onto the head of the second column, this being essential to maintain the effect of the first separation. If a stronger solvent is injected onto the second column, band spreading will occur, because the injected solvent will preferentially move the solutes down the column until the concentration of this solvent is diluted sufficiently that solutes begin to be retained (118).

C. Detection Systems

Three types of inline HPLC detector have been used to measure fat-soluble vitamin concentrations in food sample extracts: absorbance, fluorescence, and electrochemical detectors. Each of these detectors provides a continuous electrical output that is a function of the concentration of solute in the column effluent passing through the flow cell.

1. Absorbance Detection

Radiation absorption monitoring of the column effluent at an appropriate wavelength provides the most versatile means of detection for the fat-soluble vitamins. Vitamins A, D, E, and K exhibit characteristic absorption spectra in the UV region, whereas the carotenoid pigments absorb light in the visible region.

Absorbance measurement in HPLC is generally most frequently performed using a continuously variable-wavelength spectrophotometer, which permits any wavelength to be selected throughout the UV–visible range of the spectrum (i.e., 190–900 nm). This type of detector allows operation at the wavelength of maximum absorption (λ_{max}) of the analyte or at a wavelength that provides optimum selectivity. Fixed-wavelength photometers are suitable provided that the analyte displays sufficient absorbance to permit its measurement at the operational wavelength (most commonly 254 nm). The photodiode array detector can monitor absorbance at several wavelengths simultaneously and can record the complete absorption spectrum of a chromatographic peak in less than 1 sec. Peak purity, i.e., the presence or absence of a coeluting compound, can be assessed by recording spectra at the upstroke, apex, and downstroke of the peak. The three spectra, after normalization, should be superimposable if the peak is attributable to a single compound.

The selectivity of absorbance measurement for a given vitamin is dependent upon the wave-

length employed in the measurement and the strength of absorbance of the vitamin relative to the absorbance of accompanying substances. Obviously, it is desirable to isolate the vitamin analyte from interfering substances on the HPLC column. If this cannot be achieved, it is sometimes possible to select a detection wavelength that reveals the vitamin peak in the chromatogram but not the accompanying substances.

The relationship between the molar absorbance coefficient (ϵ) and the specific absorbance coefficient ($A^{1\%}_{1\,cm}$) is

$$\epsilon = A^{1\%}_{1\,cm} \times \frac{\text{Molecular weight}}{10}$$

The analytical techniques employed in the determination of fat-soluble vitamins in foods always involve extraction of the sample or unsaponifiable fraction of the sample into an organic solvent; therefore, accompanying substances in the final extract will be lipoidal or lipophylic in nature. Most lipids found in foods do not have chromophores that absorb strongly in the UV region above 220 nm. Exceptions are free or esterified conjugated fatty acids that occur in at least trace amounts in fats or oils that have undergone autoxidation or bleaching. Conjugated fatty acids exhibit very strong UV absorption, with λ_{max} of 230–235 nm for dienes and 260–280 nm for trienes (119). Glycerides and sterols exhibit weak, but measurable, UV absorbance within the spectral range of vitamins D and K; hence they constitute potential sources of interference in the determination of these vitamins. For example, the λ_{max} for triolein (265 nm in hexane; $A^{1\%}_{1\,cm}$ = 0.74) and for cholesterol (266 nm in chloroform; $A^{1\%}_{1\,cm}$ = 0.68) coincide with the λ_{max} (265 nm) for vitamin D (119). The absorption spectra of vitamins A and E lie beyond those for glycerides and sterols. Other potential sources of spectral interference include vitamers of low biological potency, vitamin oxidation and decomposition products, and added antioxidants.

a. Vitamin A

Retinol and its esters exhibit similar UV absorption spectra within a broad wavelength range and have practically equal molar absorptivities when dissolved in a given solvent. The ϵ value of crystalline all-*trans*-retinol in 2-propanol at the λ_{max} of 325 nm is 52,300 (120), which corresponds to an $A^{1\%}_{1\,cm}$ of approximately 1830. The on-column minimum detectable quantity of vitamin A using UV absorption is approximately 2 ng (121).

b. Carotenoids

Most carotenoids absorb radiation in the visible region of the spectrum, the chromophore being the conjugated double-bond system of the molecule. The absorption spectra of most carotenoids exhibit three maxima, whose positions are influenced by the solvent in which the spectrum is obtained. Spectra of carotenes (hydrocarbons) are usually determined in petroleum ether or hexane and those of xanthophylls in ethanol. The λ_{max} is mainly a function of the length of the conjugated double-bond system. A cyclic carotenoid in which the conjugation extends into the ring (β-ring) will normally have its λ_{max} at shorter wavelengths than an acyclic pigment with the same number of conjugated double bonds. Rings having nonconjugated double bonds (e.g., ϵ-ring) do not contribute to the chromophore. A hydroxy substitution, among others, has little effect on λ_{max}; therefore β-carotene, β-cryptoxanthin, and zeaxanthin have virtually identical spectra, with λ_{max} at 428, 450, and 476–478 nm in ethanol. The overall shape or fine structure of the spectra in most cases reflects the extent of planarity of the chromophore (122).

Formation of a cis isomer from an all-*trans* carotenoid results in a shift of the spectral bands

to shorter wavelengths (hypochromic shift) together with a loss of fine structure and a hypochromic effect on absorbance. These changes are accompanied by the appearance of a chromatographic "cis peak" in the UV region of the spectrum, generally between 320 and 380 nm.

Absorption maxima and $A^{1\%}_{1\,cm}$ values for a large number of carotenoids have been compiled by De Ritter and Purcell (123). The $A^{1\%}_{1\,cm}$ value for β-carotene dissolved in hexane or petroleum ether is 2592 at the λ_{max} of 453 nm.

c. Vitamin D

Vitamins D_2 and D_3 exhibit identical UV absorption spectra, with λ_{max} at 265 nm and an ϵ value of 18,000 in ethanol or hexane. The ϵ value is less than that predicted for a conjugated *cis*-triene structure because the degree of conjugation is reduced by the C-19 methylene group being above the plane of the other two double bonds. Reported on-column detection limits range from 1 to 10 ng (68,124,125).

d. Vitamin E

It is fortuitous that indigenous concentrations of vitamin E in the principal food sources are on the order of mg rather than μg/100 g, for the tocopherols and tocotrienols exhibit relatively low intensities of UV absorption. Individual vitamers are characterized by a slightly different absorption maximum within the wavelength range of 292–298 nm in ethanol. Published $A^{1\%}_{1\,cm}$ values for the tocopherols at their λ_{max} in ethanol are: α-T 70–73.7 at 292 nm, β-T 86–87 at 297 nm, γ-T 90–93 at 298 nm, and δ-T 91.2 at 298 nm (126). The different absorption characteristics among the vitamers necessitates the running of individual standards for accurate quantitation of each vitamer. The absorption intensity of α-tocopheryl acetate is lower still with an $A^{1\%}_{1\,cm}$ value of only 40–44 at the λ_{max} of 285.5 nm (44). Reported minimum detectable quantities of α-, β-, γ-, and δ-tocopherols at 295 nm are, respectively, 50 ng, 70 ng, 90 ng, and 130 ng (127).

e. Vitamin K

Phylloquinone and the menaquinones all possess the same chromophore and exhibit identical UV absorption spectra, which contain five maxima. The ϵ value at the λ_{max} of 248 nm is 18,900 (128). Hwang (129) reported an on-column detection limit of 0.3 ng for both cis and trans isomers of phylloquinone using photometric detection at 254 nm.

Photometric determination of phylloquinone has sufficient sensitivity for the analysis of green leafy vegetables and vitamin K–supplemented infant formulas, but it lacks the required sensitivity for the analysis of foods that contain smaller amounts of the vitamin.

2. *Fluorescence Detection*

Retinol and its esters and unesterified tocopherols and tocotrienols possess strong native fluorescence, but neither vitamin D nor vitamin K fluoresce. The carotenoids commonly associated with foods do not fluoresce to any significant extent, except notably phytofluene, which is found in considerable amounts in tomatoes (22) and in smaller amounts in carrots (130) and which fluoresces six times more intensely than retinyl acetate (131).

Fluorescence detection is more selective than absorbance detection because two wavelengths are required in the measurement, and the structural features necessary for a molecule to fluoresce are more limited. Most lipids, including glycerides and sterols, do not fluoresce. Maximum sensitivity is obtained by selecting the wavelengths corresponding to the intensity maxima

in the excitation and emission spectra. At other wavelengths, the sensitivity, although reduced, may still be adequate for measurement purposes. The fluorescence intensity of a compound is highly dependent upon the composition of the mobile phase. Coeluting compounds that absorb radiation at either the excitation or emission maximum of the fluorescent compound of interest can partially or even completely quench the fluorescence by absorbing the excitation or emission energy (132).

a. Vitamin A

The fluorescence excitation spectra of retinol and its esters correspond to their absorption spectra, with wavelength maxima in the 324–328-nm region; emission takes place between 470 and 490 nm (λ_{max} of 470 nm) (31).

For many applications, fluorescence detection offers no real advantages over absorbance detection, and the linear response range is more limited. Moreover, the fluorescence response of 13-*cis*-retinol is less than that of all-*trans*-retinol, the relative fluorescence depending upon the solvent (133,134).

b. Vitamin E

Unesterified α-tocopherol displays excitation and emission spectra with wavelength maxima at 295 nm and 330 nm, respectively. The fluorescence maxima of the other tocopherols are at longer wavelengths, in accordance with their absorbance spectra. The fluorescence activity of α-tocopheryl acetate is very weak, and the excitation and emission maxima of 285 and 310 nm are even closer together than those for α-tocopherol. Only those spectrofluorometers equipped with a high-energy (150-W) xenon lamp and narrow-band excitation and emission monochromators are capable of stimulating and measuring the weak fluorescence (86).

Thompson and Hatina (135) showed that the sensitivity of a fluorescence detector toward unesterified vitamin E compounds under normal-phase conditions was at least 10 times greater than that of a variable-wavelength absorbance detector. The relative fluorescence responses of the tocopherols at 290 nm (excitation) and 330 nm (emission), as measured by HPLC peak area, were α-T, 100; β-T, 129; γ-T, 110; and δ-T, 122. The fluorescence responses of the corresponding tocotrienols were very similar to those of the tocopherols, and therefore tocotrienol standards were not needed for calibration purposes. The fluorescence detector also allows the simultaneous monitoring of ubiquinone derivatives; for example ubiquinone-10 has been detected in tomato (136).

The fluorescent intensities of the E vitamers are highly dependent on the solvent. Polar solvents such as diethyl ether and alcohols provide greater intensities compared with hexane. The fluorescence is negligible when the compounds are dissolved in chlorinated hydrocarbons (137). The inclusion of an ether or an alcohol in the hexane mobile phase increases the sensitivity of vitamin E detection measurably in normal-phase HPLC.

The fluorescence detector response to α-tocopherol can be increased by up to 20-fold in some instruments by using short-wavelength excitation at 205 nm (138). A disadvantage of short-wavelength excitation is a marked loss of selectivity and an aggravation of quenching effects (139); hence, the longer wavelength (295 nm) is usually employed in food analysis applications.

c. Vitamin K

Phylloquinone can be detected fluorometrically after electrochemical (87,140) or chemical (141) postcolumn reduction to its hydroquinone (quinol) form. Indyk (142) reported that a commercial

fluorescence detector facilitates photochemical reduction and simultaneous detection during normal passage of the column effluent through the flow cell.

3. Electrochemical Detection

a. General Considerations

All of the fat-soluble vitamins, including provitamin carotenoids, exhibit some form of electrochemical activity. Both amperometry and coulometry have been applied to electrochemical detection. In amperometric detectors, only a small proportion (usually <20%) of the electroactive solute is reduced or oxidized at the surface of a glassy carbon or similar nonporous electrode; in coulometric detectors, the solute is completely reduced or oxidized within the pores of a graphite electrode. The operation of an electrochemical detector requires a semiaqueous or alcoholic mobile phase to support the electrolyte needed to conduct a current. This restricts its use to reverse-phase HPLC (but not NARP) unless the electrolyte is added postcolumn. Electrochemical detection is incompatible with NARP chromatography, because the mobile phase is insufficiently polar to dissolve the electrolyte. A stringent requirement for electrochemical detection is that the solvent delivery system be virtually pulse-free.

Amperometric detection in the oxidative mode produced on-column detection limits of 0.07, 4.3, and 0.19 ng for retinol, vitamin D_3, and α-tocopherol, respectively (143). A limitation of amperometric detection in vitamin E assays is that it cannot measure α-tocopheryl acetate, owing to the absence of the oxidizable hydroxyl group.

b. Vitamin K

The first combined HPLC–electrochemical measurements of vitamin K used the reductive mode, but this technique suffered from interference from the reduction of oxygen. A redox method was later developed that eliminated this interference, and provided a 10-fold increase in sensitivity over photometric detection and an improved selectivity. The coulometric detector employed in the redox mode is equipped with a dual-electrode cell in which phylloquinone is first reduced upstream at the generator electrode and the hydroquinone is reoxidized downstream at the detector electrode.

VI. APPLICATIONS OF HPLC

A. Vitamin A

Methods for determining vitamin A, and sometimes β-carotene as well, are summarized in Table 7.

1. Quantification

Several different quantification procedures for vitamin A have been described in the literature, some using retinol directly as a standard and some using retinyl acetate, which is converted to retinol by saponification. The latter approach is generally preferred, because crystalline all-*trans*-retinyl acetate is commercially available in high purity and is free from cis isomers. Commercial sources of retinol are oily preparations and are at best only about 70% pure. There are two ways of preparing a retinol standard from retinyl acetate.

1. A relatively large amount (typically 25 mg) of retinyl acetate is saponified and extracted, and the residue is dissolved in 2-propanol to give a stock solution of retinol,

Table 7 HPLC Methods Used for the Determination of Vitamin A and Carotene in Food

Food	Quantitative HPLC					Ref.
	Sample preparation	Column	Mobile phase	Compounds separated	Detection	
		Normal-phase chromatography				
Margarine	Dissolve sample in heptane containing 500 mg BHT and 200 mg α-tocopherol per L. Pass emulsion through glass column containing 10 g Na_2SO_4 and 20 g NaCl.	LiChrosorb Si-60 5 μm 250 × 4.6 mm	Heptane/diisopropyl ether, 95:5	Retinyl palmitate or retinyl acetate	UV 325 nm	144
Fortified fluid milk (whole, semiskimmed, skimmed)	Mix 2 ml milk and 5 ml absolute EtOH in a centrifuge tube, let stand 5 min. Vortex-mix with 5 ml hexane, let stand 2 min. Repeat mixing and standing procedure twice. Add 3 ml water, mix by inversion, centrifuge.	LiChrosorb Si-60 5 μm 250 × 3.2 mm	Hexane/diethyl ether, 98:2	All-*trans*-retinyl palmitate	UV 325 nm	83
Margarine	Dissolve sample in hexane, shake with 60% EtOH, centrifuge.	LiChrosorb Si-60 5 μm 250 × 3.2 mm	Hexane/diethyl ether, 98:2	Carotene, all-*trans*-retinyl palmitate	Vis 453 nm (carotene) UV 325 nm (retinyl palmitate)	83
Milk, infant formula	Saponify (ambient), extract unsaponifiables with hexane/diethyl either, 85:15.	Apex Silica 3 μm 150 × 4.5 mm	1–5% 2-PrOH in heptane	13-*cis*- and all-*trans*-retinol	UV 340 nm (filter)	145
Cheese	Saponify (ambient), extract unsaponifiables with hexane.	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/methyl ethyl ketone, 90:10	Carotene, all-*trans*-retinol	Vis 450 nm (carotene) UV 340 nm (retinol)	146
Milk	Dilute 400 mg milk with water/MeOH/EtOH (55:9:36) and saponify in a culture tube. Extract unsaponifiables with heptane/isopropyl ether (3:1), centrifuge. Repeat extraction.	Perkin-Elmer HS-5-Silica 125 × 4 mm	6% 2-PrOH in heptane	Retinol	Fluorescence: ex 344 nm em 472 nm	147

(continued)

Table 7 Continued

Food	Quantitative HPLC					Ref.
	Sample preparation	Column	Mobile phase	Compounds separated	Detection	
		Normal-phase chromatography				
Various foods	Saponify (ambient), dilute solution with water and absolute EtOH to yield a volumetric ratio of water to EtOH of 1:1. Pipet a 20-ml aliquot onto a Kieselguhr cartridge and elute with petroleum ether.	Spherisorb SW silica gel 3 μm 100 × 2 mm (narrow-bore)	0.3% 1-octanol in hexane	13-*cis*- and all-*trans*-retinol	UV 325 nm	148
		Reversed-phase chromatography				
All food types	Saponify (hot), extract unsaponifiables with diethyl ether/petroleum ether, 1:1.	μBondapak C_{18} 10 μm 300 × 3.9 mm	$MeOH/H_2O$, 90:10	Retinol	UV 325 nm	149
Unfortified fluid dairy products	Saponify (hot) 2-ml sample in a centrifuge tube, extract unsaponifiables once with diethyl ether/petroleum ether (1:1), centrifuge.	Nova-PAK C_{18} 150 × 3.9 mm	$MeOH/H_2O$, 95:5	Retinol	UV 325 nm	73
Fortified fluid milk (whole, skimmed), infant formula, margarine	Saponify (hot) 1-ml sample of milk or formula or 50 mg margarine in a centrifuge tube, extract unsaponifiables five times with hexane.	LiChrosorb RP-18 10 μm 250 × 3.2 mm	$MeOH/H_2O$, 90:10 (retinol) $MeOH/H_2O$, 99:1 (β-carotene)	Retinol and β-carotene (separate chromatograms)	UV 325 nm (retinol) Vis 453 nm (β-carotene)	150

Milk	Mix 3 ml milk with 6 ml 2-PrOH. Extract twice with hexane, centrifuge. Wash pooled extracts with 0.47 M Na_2SO_4, evaporate under nitrogen. Determine the lipid content of the extract by weight. Evaporate a portion of the hexane extract, dissolve residue in 1 ml 95% EtOH containing 12.5 mg/ml pyrogallol. Add 1 ml 60% KOH, saponify at 30°C, extract unsaponifiables with hexane, centrifuge.	ChromSep-ChromSpher PAH, 5 μm 100 × 3 mm (two columns connected in series)	MeCN/MeOH/ CH_2Cl_2, 80:14:6	β-Carotene	Vis 450 nm	151
Selected foods of animal origin and processed foods	Saponify (hot), extract unsaponifiables with hexane.	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeCN/MeOH/ ethyl acetate, 88:10:2	Retinol, α- and β-carotenes	UV 313 nm (retinol) Vis 436 nm (carotenes)	152
Breakfast cereals, margarine, butter	Saponify (hot), precipitate soaps with acetic acid in MeCN, dilute with water.	Vydac 201 TP C_{18} 10 μm 250 × 3.2 mm	MeCN/H_2O, 65:35	13-*cis*- and all-*trans*-retinol	UV 328 nm	153
Fortified cereal products	Supercritical fluid extraction	Altex C_8 (octyl) 5 μm 150 × 3.9 mm	MeCN/2-PrOH/aqueous 25 mM sodium perchlorate, 45:45:10	Retinyl palmitate	Amperometric (oxidative mode) glassy carbon electrode, +1.2 V vs saturated calomel electrode	90

BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; EDTA, ethylenediaminetetraacetic acid; MeOH, methanol; EtOH, ethanol; 2-PrOH, 2-propanol; BuOH, butanol; MeCN, acetonitrile; $CHCl_3$, chloroform; CH_2Cl_2, dichloromethane (methylene chloride); THF, tetrahydrofuran; DMF, dimethylformamide; DMSO, dimethylsulfoxide; MTBE, methyl *tert*-butyl ether.

which can be stored for 2–3 months in a refrigerator. This stock solution is diluted with 2-propanol to give a suitable working standard solution, whose concentration is determined spectrophotometrically ($A^{1\%}_{1\,cm}$ = 1830 at λ_{max} of 325 nm) immediately before use as an external standard in the HPLC procedure.

2. An accurately prepared standard solution of retinyl acetate (i.e., a solution of known concentration) is taken through the saponification and extraction procedure along with each batch of samples, and the resultant retinol solution is used as an external standard without spectrophotometric standardization. This technique, which is recommended by COST 91 (149), compensates for losses of vitamin A incurred during the saponification and subsequent manipulations (i.e., the calculated vitamin A value is recovery-corrected).

2. *Normal-Phase Separations*

Adsorption chromatography has been applied to the determination of added retinyl palmitate in margarine, fluid milk, and milk powder. The general procedure entails extracting the total lipid fraction of the homogeneous sample with a nonaqueous solvent, removing the polar material, and then injecting an aliquot of the resulting solution directly into the chromatograph (83,144,154). The vitamin A is maintained in its relatively stable ester form throughout the assay and is protected by the lipids to the point of chromatographic separation. A major practical advantage is that there is no need to evaporate extracts to dryness. If a foodstuff is supplemented with retinyl acetate, adsorption chromatography can be used to distinguish between supplemental vitamin A and indigenous vitamin A (mainly retinyl palmitate), as shown by Woollard and Woollard (154) in the analysis of fortified whole milk powder. Woollard and Indyk (155) extended the technique to identify the minor indigenous esters of vitamin A in milk and milk products.

Saponification of the sample simplifies the analysis by converting the vitamin A esters to retinol. The unsaponifiable material is extracted with hexane, or a predominantly hexane solvent mixture, which is compatible with the nonpolar mobile phase (146,153,156). In vitamin A–fortified foods there is no need to concentrate the unsaponifiable extract—an aliquot can be injected directly into the chromatograph (153).

3. *Reversed-Phase Separations*

The removal of triglycerides from the food sample by saponification provides the opportunity to utilize reversed-phase chromatography. The unsaponifiable matter is conventionally extracted into a solvent [e.g., diethyl ether/petroleum ether (50:50) or hexane] that is incompatible with a semiaqueous mobile phase. It then becomes necessary to evaporate the unsaponifiable extract to dryness and to dissolve the residue in a small volume of methanol (if methanol is the organic component of the mobile phase). For the analysis of breakfast cereals, margarine, and butter, Egberg et al. (153) avoided the time-consuming extraction of the unsaponifiable matter and the evaporation step by acidifying the unsaponifiable matter with acetic acid in acetonitrile to precipitate the soaps. An aliquot of the filtered extract could then be injected, after dilution with water, onto an ODS column eluted with a compatible mobile phase (65% acetonitrile in water).

Schneiderman et al. (90) extracted retinyl palmitate from commercial breakfast cereals using supercritical CO_2 and determined the vitamin by means of reversed-phase HPLC and electrochemical detection. Chromatograms of an unfortified wheat sample and a fortified bran-based cereal product are shown in Fig. 10.

Reversed-phase chromatography using semiaqueous mobile phases can separate all-*trans*-retinol from 13-*cis*-retinol, albeit rather poorly. Further separation of the minor cis isomers is not

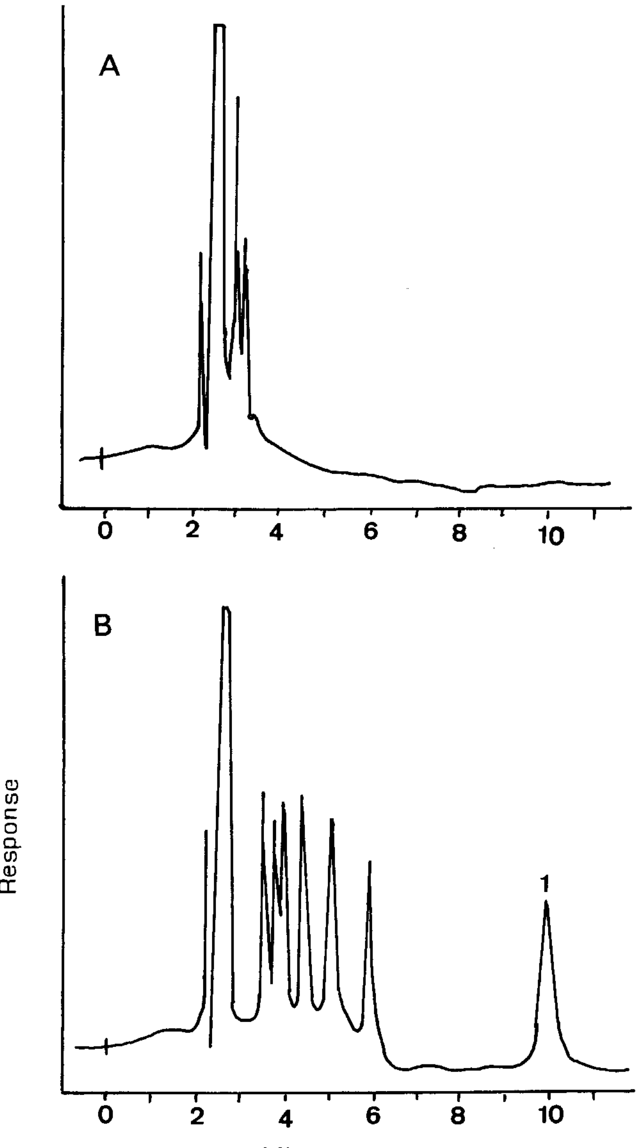


Fig. 10 HPLC chromatograms of supercritical fluid extracts of (A) an unfortified wheat sample and (B) a vitamin A–fortified bran-based ready-to-eat breakfast cereal. Column, 5-μm Altex C_8 (octyl) (150 × 4.6-mm ID); mobile phase, acetonitrile/2-propanol/aqueous 25 mM sodium perchlorate (45:45:10), 2.0 ml/min; amperometric detection (oxidative mode), glassy carbon electrode, +1.2 V, vs saturated calomel electrode. Peak: (1) retinyl palmitate. (Reprinted from Ref. 90, Copyright 1997, with the kind permission of Elsevier Science–NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

achieved, but this is of little practical concern in routine food analysis. In practice, it is convenient to adjust the elution conditions to obtain a single peak encompassing all of the geometric isomers, ignoring the reduced biopotency of the cis isomers. Alternatively, the peak areas of the all-*trans* and 13-*cis* isomers can be measured separately and then multiplied by their respective relative potencies of 100% and 75% and added.

B. Provitamin A Carotenoids

1. *Some Potential Problems*

It is important to correctly identify the provitamin A peak(s) of interest in the chromatogram. A tentative identification can be made by a combination of retention time and spectral characteristics, using a photodiode array detector. Identification is aided by comparisons with authentic carotenoid standards in more than one chromatographic mode. Because of the ease of cis-trans isomerization when solutions of carotenoids are exposed to heat, light, oxygen, etc., it is difficult to ascertain whether a cis isomer occurs in nature or whether it is formed during its isolation.

Chromatographic artifacts can be produced if the injection solvent is not compatible with the mobile phase. Khachik et al. (157) employed four mobile phases (eluents A, B, C, D; Table 8) and various injection solvents to study factors that produced artifacts in the reversed-phase separation of carotenoids. Using an injection volume of 20 μl, it was found that the injection solvents could be divided into two groups. The first group, comprising acetonitrile, acetone, methanol, hexane, and the four mobile-phase solvent mixtures, produced a single symmetrical peak for β-carotene and other carotenoids. The second group, comprising dichloromethane, chloroform, tetrahydrofuran, benzene, and toluene, produced artifacts such as peak splitting, peak distortion, and multiple peak formation. From solubility data (Table 8) and chromatographic profiles it appears that artifacts arose in cases where there was a dramatic difference between the relative solubilities of β-carotene in the injection solvent and in the mobile phase. Hence artifacts were produced when tetrahydrofuran and dichloromethane were used in injection solvents with eluents A and B. Conversely, the solubility of β-carotene in hexane and acetone is similar to those of eluents A and B and therefore no artifacts were observed. The injection of smaller (5- and 10-μl) volumes of sample eliminated the artifacts produced using injection solvents of the second group.

Owing to reported losses of carotenoids by rapid decomposition through oxidation in the presence of a stainless steel column frit (158), it has been recommended to purchase HPLC columns fitted with inert metal–free frits (159,160).

2. *Normal-Phase Separations*

Where silica columns have been utilized to determine vitamin A in margarine (83) and cheese (146), the sole carotenoid, β-carotene, which is eluted near the solvent front, can be determined at the same time. Apart from such applications, silica is an unsuitable stationary phase for carot-

Table 8 Chromatographic Conditions for the Separation of Carotenoids and Solubility Data

Eluent or injection solvent	% Composition	Solubility of β-carotene (mg/100 ml)
Eluent A	MeOH/MeCN/CH_2Cl_2, 25:55:20	22
Eluent B	MeOH/MeCN/CH_2Cl_2/hexane, 25:55:10:10	22
Eluent C	MeCN/THF, 78:22	No data
Eluent D	MeOH/MeCN/CH_2Cl_2/hexane (gradient)	No data
THF		930
CH_2Cl_2		325
Hexane		39
Acetone		21

Source: Reprinted with permission from Ref. 157. Copyright 1988, American Chemical Society.
Abbreviations: see Footnote to Table 7.

enoid analysis, because the carotenes are very weakly adsorbed and the xanthophylls are highly retained.

Microparticulate alumina specially prepared for HPLC is available commercially and facilitates the isocratic separation of all-*trans*-β-carotene from its lower potency cis isomers. Using an alumina column and a mobile phase of isooctane containing 0.5% stabilized tetrahydrofuran (161), the cis isomers of β-carotene are eluted before the all-*trans* isomer to form a single composite peak in the chromatogram. α-Carotene coelutes with the cis isomers of β-carotene and therefore cannot be accurately quantified. γ-Carotene, β-cryptoxanthin, and canthaxanthin are not eluted.

Calcium hydroxide is an excellent adsorbent for resolving carotenoid cis-trans isomers; compared with alumina, it is less retentive and less sensitive to temperature and moisture content of the mobile phase. However, calcium hydroxide columns are not commercially available, and separations using them can be difficult to reproduce, owing to extreme sensitivities to mobile-phase composition and temperature. Nine *cis*-β-carotenes were characterized from a chromatogram of 18 peaks after isocratic elution of a mixture obtained by thermal isomerization and photoisomerization of β-carotene (162). Chandler and Schwartz (10), using a calcium hydroxide column and a mobile phase of hexane containing 0.3% acetone, separated six carotene isomers from canned carrots. These were, in order of elution, two *cis*-α-carotenes, all-*trans*-α-carotene, 13-*cis*-β-carotene, all-*trans*-β-carotene, and 9-*cis*-β-carotene. A mobile phase of hexane modified with 2% *p*-methylanisole separated all-*trans*-β-carotene and its 9-, 13-, and 15-*cis* isomers in an iodine isomerized mixture (163).

Of the polar bonded-phase packings that have been investigated, the interactions between carotenes and nitrile stationary phases are very weak; thus the limitations described for silica apply (164). Amino-bonded phases eluted with iso-octane containing 0.5% stabilized tetrahydrofuran separate α- and β-carotene (unresolved) from γ-carotene; canthaxanthin and β-cryptoxanthin are not eluted (161). The cis isomers of β-carotene are separated from the all-*trans* isomer; thus amino columns offer an alternative option to alumina columns for determining all-*trans*-β-carotene without its cis isomers interfering.

3. Reversed-Phase Separations

Reversed-phase chromatography is generally preferred to normal-phase chromatography for the determination of provitamin carotenoids in foods, because the carotenes are retained and the separation of α- and β-carotene is easily achieved. Owing to the weak hydrophobic forces on which the separation depends, there is little risk of on-column degradation of carotenoids. The xanthophylls are eluted well before the carotenes, the latter requiring strong mobile phases containing little or no water to displace them.

The more recent published methods (Table 9) employ NARP chromatography, which overcomes the problem of poor solubility of carotenes in semiaqueous mobile phases. Recoveries of carotenoids are better with methanol-based mobile phases than with acetonitrile-based ones (178). Dichloromethane, tetrahydrofuran, and methyl *tert*-butyl ether appear to be the best modifiers for optimizing selectivities as well as reducing analysis times (179).

a. C_{18}-Bonded Phases

Nelis and De Leenheer (180) used isocratic NARP–HPLC with Zorbax ODS (a monomeric ODS stationary phase with a 20% carbon loading) and a mobile phase of acetonitrile/dichloromethane/methanol (70:20:10) to separate nine carotenoids spanning a wide polarity range. This classic separation was achieved by virtue of the fact that the Zorbax ODS material supplied at that time was nonendcapped. The carotenes were retained by hydrophobic interaction with the ODS

Table 9 HPLC Methods Used for the Determination of Provitamin A Carotenoids in Food

Food	Sample preparation	Quantitative HPLC				
		Column	Mobile phase	Compounds separated	Detection	Ref.
		Reversed-phase chromatography				
Vegetables, fruits	Extract sample with THF + Na_2SO_4 + $MgCO_3$.	Partisil ODS 5 μm 250 × 4.6 mm	MeCN/THF/H_2O, 85:12.5:2.5	α- and β-Carotenes, β-cryptoxanthin	Vis 470 nm	18
Vegetables, fruits	Extract sample with THF.	Vydac 218 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/MeCN/THF, 56:40:4	α-Carotene, all-*trans*-β-carotene (separated from *cis*-β-carotenes)	Vis 470 nm	165
Raw and cooked vegetables	Extract sample with THF + Na_2SO_4 + $MgCO_3$.	Spheri-5 ODS 5 μm 220 × 4.6 mm	MeCN/CH_2Cl_2/MeOH, 70:20:10	α-Carotene, all-*trans*-β-carotene (separated from *cis*-β-carotenes)	Vis 450 nm	166
Vegetables	Extract sample with THF + Na_2SO_4 + $MgCO_3$ + nonapreno-β-carotene (internal standard). Partition into petroleum ether and water.	Spheri-5 RP-18 5 μm 220 × 4.6 mm	MeCN/MeOH/CH_2Cl_2, 55:22:23	α- and β-Carotenes	Vis 470 nm	84
Raw and cooked vegetables, salads, fruits	Extract with MeOH, reextract with THF. Partition into petroleum ether and water.	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/THF, 95:5	Lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene	Vis 445 nm	167
Vegetables	Saponify (hot), extract unsaponifiables with diisopropyl ether.	Hypersil-ODS 3 μm 250 × 4.6 mm	MeCN/MeOH/$CHCl_3$/H_2O, 250 + 200 + 90 + 11	β-Carotene	Vis 445 nm	168
Vegetables, fruits	Saponify (hot), extract unsaponifiables with hexane. Magnesia column chromatography. Add Sudan I as internal standard to final extract.	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/$CHCl_3$, 94:6	Sudan I, zeinoxanthin, β-cryptoxanthin, α-carotene, all-*trans*-β-carotene (separated from *cis*-β-carotenes)	Vis 475 nm	93
Carrots, kale, tomato, papaya	Extract sample with cold acetone, partition into petroleum ether. Saponify (ambient; papaya samples only). Magnesia-column chromatography. Add Sudan I as internal standard to final extract.	LiChrosphere RP-18 5 μm 125 × 4 mm	MeOH/$CHCl_3$, 95:5	Sudan I, β-cryptoxanthin, α-carotene, β-carotene	Vis 470 nm	94
Olive oil	Saponify (ambient), extract unsaponifiables with diethyl ether.	Supelcosil LC18 5 μm 150 × 4.6 mm	MeCN/2-PrOH/1,2-dichloroethane, 92.5:5.0:2.5	α- and β-Carotenes	Vis 458 nm	169

Malaysian vegetables	Saponify (hot), extract unsaponifiables with hexane.	μBondapak C_{18} 10 μm 300 × 3.9 mm	MeCN/MeOH/ethyl acetate, 88:10:2	β-Cryptoxanthin, γ-, α-, β-carotene	Vis 436 nm	170
Vegetables	Supercritical fluid extraction.	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/MeCN/ CH_2Cl_2/hexane, 65:27:4:4	α- and β-Carotenes	Vis 450 nm	171
Vegetables, fruits	Extract sample with THF/MeOH (1:1) + $MgCO_3$ + internal standard (β-apo-8′-carotenal or, for green vegetables, echinenone). Filter. Partition into petroleum ether containing 0.1% BHT. Saponify (ambient), extract unsaponifiables with petroleum ether (saponification applied to fruit samples and peppers only).	Vydac 201 TP54 C_{18} 5 μm (metal frits replaced with Teflon frits) 250 × 4.6 mm	MeCN/MeOH/ CH_2Cl_2 (75:20:15) containing 0.1% BHT and 0.05% triethylamine (the MeOH contains 0.05 M ammonium acetate)	β-Cryptoxanthin, α-carotene, all-*trans*-β-carotene (separated from *cis*-β-carotenes)	Vis 450 nm	172
Leafy vegetables, sweet potatoes, mangos	Extract homogenized material with Na_2SO_4, $CaCO_3$, and THF containing 0.01% BHT, filter. Repeat extractions until extract is colorless.	Vydac 218 TP54 C_{18} 5 μm (metal frits replaced with PAT (Peek alloyed with Teflon) 250 × 4.6 mm	MeOH/THF, 98:2	All-*trans*-α-carotene, all-*trans*-β-carotene, *cis*-β-carotene	Vis 450 nm	173
Vegetables, fruits	Extract freeze dried material with THF/MeOH (1:1) + $MgCO_3$ + internal standard (β-apo-8′-carotenoate) at 0°C. Homogenize, centrifuge. Saponify if β-cryptoxanthin needs to be determined. Add 10% NaCl (w/v) to either saponified or unsaponified solution and extract with petroleum ether.	Vydac 201 TP C_{18} 5 μm (Hastalloy frits replaced with PAT (Peek alloyed with Teflon frits) 250 × 4.6 mm	MeOH/THF, 95:5	Internal standard, all-*trans*-α-carotene, all-*trans*-β-carotene (separated from their cis isomers)	Vis 450 nm	174
Chinese vegetables	Extract sample with hexane/acetone/EtOH/toluene (10:7:6:7) + $MgCO_3$ + internal standard (β-apo 8′-carotenal). Filter. Saponify (ambient), extract unsaponifiables with hexane.	Ultramex C-18 5 μm 250 × 4.6 mm	MeCN/MeOH/ethyl acetate, 75:15:10	β-apo 8′-carotenal (internal standard), β-cryptoxanthin, α- and β-carotenes	Vis 450 nm	175
Carrots	Extract sample with hexane/acetone/absolute EtOH/toluene (10:7:6:7) + 40% methanolic KOH. Leave for 16 h in the dark for saponification. Extract unsaponifiables with hexane.	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm	MeOH/CH_2Cl_2, 96:1	α- and β-Carotenes (separated from their 15-*cis* isomers and from 13-*cis*-β-carotene)	Vis 450 nm	176

(*continued*)

Table 9 Continued

Food	Sample preparation	Quantitative HPLC				
		Column	Mobile phase	Compounds separated	Detection	Ref.
Dark green vegetables	Blanch leaves in boiling water. Homogenize with water containing 0.5% ascorbic acid. Extract an aliquot of the resultant mixture with acetone/petroleum ether (3:2) containing 0.5% BHT by shaking for 10 min. Re-extract until colorless (3 extractions). Saponify (ambient) the combined solvent extracts.	Vydac 201 TP C_{18} 5 μm 250 × 4.6 mm	MeOH/CH_2Cl_2/H_2O, 80:15.2:4.8	All-*trans*-, 9-*cis*-, 13-*cis*-β-carotene, all-*trans*-α-carotene	Vis 450 mn	177
Fresh and processed fruits and vegetables	Homogenize pureed tissue with water, $CaCO_3$, and Celite. Extract with MeOH, filter. Extract filter cake with acetone/hexane, 1:1, until colorless. Wash hexane layer with water. *Separate extraction procedure for orange juice*: vortex samples with MeOH, centrifuge, filter. Extract pellets with acetone/hexane, 1:1, until colorless. Wash hexane layer with water. *Saponification*: applicable to extracts (prepared as just described) of broccoli, collards, orange juice, peaches, spinach, vegetable soup. All purified extracts passed through anhydrous Na_2SO_4. *Solid phase extraction*: applicable to extracts of carrot, orange juice, peach, tomato, vegetable soup. Load hexane extracts onto alumina *N* Sep-Pak cartridge. Elute α- and β-carotene with hexane/acetone, 96.5:3.5 and β-cryptoxanthin with acetone	Polymeric C_{30} 5 μm 250 × 4.6 mm	MeOH/MTBE, 89:11	All-*trans*-α-carotene, all-*trans*-β-carotene (separated from their cis isomers)	Vis 410 nm	12

Abbreviations: see Footnote to Table 7.

ligands, while retention of the xanthophylls was controlled by secondary interactions involving residual silanol groups. It should be noted that Zorbax ODS is now manufactured as a fully endcapped material, the nonendcapped form being no longer available. Craft (181) repeated the separation of Nelis and De Leenheer (but using the endcapped Zorbax ODS) and observed that zeaxanthin and lutein were no longer resolved and relative retention had drastically reduced.

Cis-trans isomers of carotenes cannot normally be separated on a monomeric ODS phase. However, the separation of all-*trans*-β-carotene from its principal 9-*cis* and 13-*cis* isomers has been achieved using silica-based polymeric ODS bonded-phase column packings, such as Vydac TP (93,161,165,182–184) and Spheri-5 ODS (166,185,186). Under the isocratic NARP conditions employed, 15-*cis*-β-carotene coelutes with the 13-*cis* isomer (186,187). Vydac TP and Spheri-5 ODS column packings have very different particle characteristics and surface coverages, as shown in Table 10. It appears that the polymeric surface configuration, which is common to Vydac TP and Spheri-5 ODS, is responsible for the separation of the cis-trans isomers of β-carotene, endcapping being unimportant.

b. C_{30}-Bonded Phases

Sander et al. (188) obtained a high degree of selectivity toward the cis-trans isomers of β-carotene using a 5-μm polymeric C_{30}-bonded phase prepared without endcapping. (The 250 × 4.6-mm-ID column used in this study is available commercially). The rationale for using alkyl chain lengths longer than C_{18} was based on previous observations (189) that molecular shape recognition for polycyclic aromatic hydrocarbons was enhanced with increasing chain length. Further studies (190,191) confirmed that the C_{30} phase was superior to C_{18} phases commonly employed for carotenoid analysis. Among reversed-phase materials, the C_{30} phase is uniquely capable of resolving geometric isomers of asymmetric carotenoids. For example, the predominant geometric isomers of α-carotene were separated in the following order of elution: 13-*cis*, 13′-cis, all-*trans*, 9-*cis*, 9′-*cis* (191). Chromatograms of carotenoids extracted from raw and thermally processed carrots are shown in Fig. 11. The 9′-*cis* isomer of α-carotene coelutes with all-*trans*-β-carotene under the chromatographic conditions employed. (*Note*: The thermal treatment used in the processing was more intensive than that used commercially.)

Retention characteristics and elution order of carotenoid cis-trans isomers with C_{30}-bonded phases are strikingly similar to those obtained with normal-phase systems using calcium hydroxide columns (190). Different carotenoids exhibit varying retention behavior in response to temperature changes for C_{30} and C_{34} polymeric stationary phases as compared with a C_{18} polymeric phase (179). These behaviors are believed to be related to conformational changes in the longer stationary phases with temperature. The "slot model" proposed for the retention of planar

Table 10 Properties of Polymeric ODS Bonded-Phase Column Packings

	Vydac 218 TP[a]	Vydac 201 TP[a]	Spheri-5-ODS[b]
Surface configuration	Polymeric	Polymeric	Polymeric
Endcapped	Yes	No	Yes
Percentage carbon loading (w/w)	9	9	14
Mean pore diameter (Å)	300	300	80
Specific surface area (m^2/g)	80	80	200

[a] Separations Group.
[b] Brownlee Laboratories.

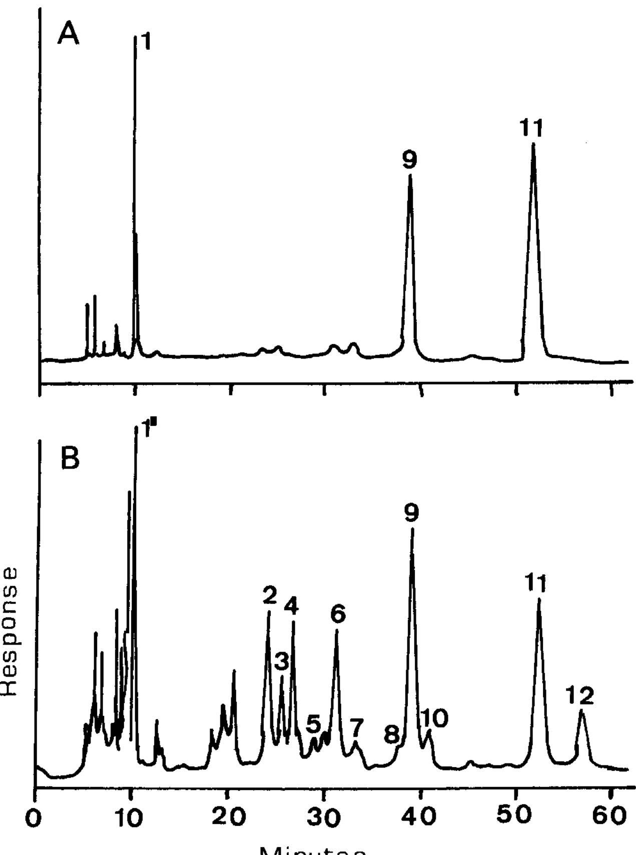


Fig. 11 HPLC of carotenoids solvent-extracted from (A) raw and (B) thermally processed carrots. Column, 5-μm polymeric C_{30} (250 × 4.6-mm ID); mobile phase, methyl *tert*-butyl ether/methanol (11:89), 1 ml/min; absorbance detection, 453 nm. Tentative peak identifications: (1) all-*trans*-lutein; (2) 13-*cis*-α-carotene; (3) a *cis*-α-carotene isomer; (4) 13′-*cis*-α-carotene; (5) 15-*cis*-β-carotene; (6) 13-*cis*-β-carotene; (7 and 8) *cis*-β-carotene isomers; (9) all-*trans*-α-carotene; (10) 9-*cis*-α-carotene; (11) all-*trans*-β-carotene; (12) 9-*cis*-β-carotene. (Reprinted with permission from Ref. 192. Copyright 1996, American Chemical Society.)

and nonplanar polycyclic aromatic hydrocarbons (193) could account for the excellent shape selectivity of the C_{30} polymeric phase toward carotenoid cis-trans isomers and the preferential retention of lycopene. Lycopene, being acyclic, is relatively planar compared to β-carotene and therefore fits more easily into slots within the rigid polymeric stationary phase. It is hypothesized that C_{18} polymeric phases possess insufficient thickness to permit complete penetration of carotenoid molecules; only part of the molecule interacts with the bonded phase, and poorer isomer separation results.

C. Vitamin D

Selected methods for determining vitamin D are presented in Table 11.

Table 11 HPLC Methods Used for the Determination of Vitamin D in Food

Food	Sample preparation	Semipreparative HPLC	Quantitative HPLC	Compounds separated	Ref.
Infant formula	Saponify (ambient), extract unsaponifiables with hexane. Convert vitamin D to isotachysterol with acidified butanol.	Supelcosil LC-18-DB 5 μm 250 × 4.6 mm MeCN/MeOH, 90:10 UV 301 nm	*Normal-phase* Spherisorb silica 5 μm 250 × 2 mm Hexane/ethyl acetate/MeOH, 97:2.5:0.05 UV 301 nm	Isotachysterols D_2 or D_3 (same retention time)	194
Fortified fluid milk (whole, low-fat, skimmed)	Saponify (ambient), extract unsaponifiables with petroleum ether. Coprecipitate sterols with digitonin. Extract with petroleum ether. Alumina-column chromatography.		*Reversed-phase* Vydac TP 201 C_{18} 10 μm 250 × 3.2 mm MeCN/MeOH, 90:10 UV 265 nm	Vitamins D_2 and D_3	91
Fortified fluid milk (skimmed), whole milk powder, milk powder with soybean, chocolate milk powder, diet food	Digest starchy samples with takadiastase before saponification. Saponify (hot), extract unsaponifiables with petroleum ether. Silica solid-phase extraction in the sample cleanup mode (high-fat samples only).		Hypersil ODS 5 μm 120 × 4 mm (two columns connected in series) 0.5% H_2O in MeOH UV 265 nm	Vitamin D_2 or D_3	68
Fortified whole milk powder	Add vitamin D_2 or D_3 to sample as internal standard. Saponify (ambient), extract unsaponifiables with petroleum ether/diethyl ether, 90:10. Precipitate sterols from a methanolic solution.		Radial-PAK cartridge containing either Resolve C_{18} or Nova-PAK C_{18} 5 μm (two cartridges connected in series) MeOH/THF/H_2O, 93:2:5 UV 254 and 280 nm (dual)	Vitamins D_2 and D_3	195
Infant formula	Add vitamin D_2 or D_3 to sample as internal standard. Saponify (ambient), extract unsaponifiables with petroleum ether/diethyl ether, 90:10. Silica solid-phase extraction in the sample cleanup mode.		Two Radial-PAK cartridges as in preceding entry MeOH/THF/H_2O, 92:2:6 UV 254 and 280 nm (dual)	Vitamins D_2 and D_3	100

Table 11 Continued

Food	Sample preparation	Semipreparative HPLC	Quantitative HPLC	Compounds separated	Ref.
Milk (unfortified)	Add vitamin D_2 to sample as internal standard. Saponify (ambient), extract unsaponifiables with hexane/diethyl ether, 90:10. Silica solid-phase extraction in the sample cleanup mode.	Radial-PAK cartridge containing Resolve silica 5 μm Hexane/2-PrOH, 99:1 UV 265 nm	Radial-PAK cartridge containing Resolve C_{18} 5 μm, column temperature 30°C MeOH/THF/H_2O, 93:2:5 UV 265	Vitamins D_2 and D_3	196
Milk	Saponify (ambient), extract unsaponifiables with petroleum ether/diethyl ether, 90:10. Silica solid-phase extraction in the sample cleanup mode.		Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm MeCN/MeOH, 90:10 UV 254 nm	Vitamins D_2 and D_3	197
Infant formula	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract unsaponifiables with hexane. Silica solid-phase extraction in the sample cleanup mode.		Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm, column temperature 27°C MeCN/MeOH, 91:9 UV 265 nm	Vitamins D_2 and D_3	198
Milk	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract unsaponifiables with hexane. Florisil solid-phase extraction in the sample cleanup mode.		Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm MeCN/MeOH, 97 + 3 UV 265 nm	Vitamins D_2 and D_3	199
Margarine, fats, and oils	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract unsaponifiables with diethyl ether.	LiChrosorb Si-60 7 μm 250 × 4.6 mm Hexane/2-PrOH/THF, 98:1:1 UV 264 nm	ChromSphere C_{18} 8 μm 100 × 3 mm MeCN/$CHCl_3$/MeOH, 91:6:3 UV 264 nm	Vitamin D_2 or D_3	200
Fortified fluid milk (whole)	Saponify (ambient), extract unsaponifiables with hexane.	Supelcosil LC-Si 5 μm 150 × 4.6 mm Cyclohexane/hexane, 50:50 containing 0.5% 2 Pr-OH UV 254 nm	Radial-PAK cartridge containing Resolve C_{18} 5 μm or Spherisorb ODS 10 μm MeCN/MeOH, 90:10 UV 254 nm	Vitamins D_2 or D_3	70

Margarine, infant formula	As in preceding entry, but with alumina-column chromatography before semipreparative HPLC.	As in preceding entry, but concentrations of 2-PrOH changed to 0.25%	As in preceding entry	Vitamins D_2 and D_3	70
Margarine, vegetable oils, fortified milk	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract unsaponifiables with hexane.	Polygosil 60 5 μm 300 × 8 mm, column temperature 30°C Isooctane/$CHCl_3$/THF/isobutanol, 94:3:2:1 UV 254 nm	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm, column temperature 30°C MeCN/MeOH/$CHCl_3$, 82:12:6 UV 265 nm	Vitamins D_2 and D_3	201
Infant formula	Add vitamin D_2 to sample as internal standard. Saponify (hot), extract unsaponifiables with petroleum ether/diethyl ether, 90:10.	Polygosil 60 5 μm 250 × 8 mm Isooctane/isobutanol, 99:1 UV 265 nm	Hypersil ODS 5 μm 250 × 4.6 mm (two columns connected in series) 100% MeOH UV 265 nm	Vitamins D_2 and D_3	202
Margarine	Add vitamin D_2 or D_3 to sample as internal standard. Saponify (ambient), extract unsaponifiables with hexane. Cleanup on an alumina–digitonin/Celite column.	Nucleosil 50-5 5 μm 250 × 4 mm Hexane containing 0.5% Pr-OH UV 265 nm	Spherisorb ODS-2 3 μm 250 × 4 mm MeCN/MeOH/$CHCl_3$, 91:3:6 UV 265 nm	Vitamins D_2 and D_3	203
Nutritionally complete liquid-formula diet	Saponify (hot), extract unsaponifiables with diethyl ether. Add vitamin D_2 as internal standard to the extracted ether solution.	Nucleosil 50-5 5 μm 250 × 4.6 mm Hexane containing 0.05% 2-PrOH UV 265 nm	Hitachi Gel 3056 reverse-phase column 5 μm 250 × 4.6 mm MeCN/MeOH/50% perchloric acid (970 + 30 + 1.2) containing 0.057 M sodium perchlorate Dual-cell electrochemical detector (redox mode): +0.65 V (oxidation) −0.20 V (reduction)	Vitamins D_2 and D_3	204

Table 11 Continued

Food	Sample preparation	Semipreparative HPLC	Quantitative HPLC	Compounds separated	Ref.
Raw meat and liver; milk and milk products	Add vitamin D_2 and $25(OH)D_2$ to homogenized sample as internal standards. Saponify (ambient), extract unsaponifiables with diethyl ether/petroleum ether, 1:1.	μPorasil silica 10 μm 300 × 3.9 mm Gradient elution with hexane/2-PrOH to obtain a vitamin D fraction and a 25(OH)D fraction UV 265 nm *Vitamin D fraction*: Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm MeOH/H_2O, 93:7 UV 265 nm *25(OH)D fraction*: Vydac 201 TP54 C_{18} 5 μm MeOH/H_2O, 83:17 UV 265 nm	*Vitamin D_3*: Zorbax ODS + Vydac 201 TP54 C_{18} (connected in series) MeOH/H_2O, 96:4 UV 265 nm *$25(OH)D_3$*: Spherisorb S5NH_2 5 μm 250 × 4.6 mm + μPorasil 10 μm (connected in series) Hexane/2-PrOH, 97:3 UV 265 nm	Vitamins D_2 and D_3 $25(OH)D_2$ and $25(OH)D_3$	205
Meat and fat from livestock fed normal and excessive quantities of vitamin D	Saponify (hot), extract unsaponifiables with hexane/CH_2Cl_2, 85:15. Alumina-column chromatography to obtain a vitamin D fraction and a 25(OH)D fraction.	*Vitamin D fraction*: Apex silica 3 μm 150 × 4.5 mm Cyclohexane/hexane (1:1) containing 0.25% 2-PrOH UV 254 nm *25(OH)D fraction*: Radial-PAK cartridge containing Resolve C_{18} 5 μm Dry 100% MeOH UV 254 nm	*Vitamin D_3*: Radial-PAK cartridge containing Resolve C_{18} 5 μm Dry 100% MeOH UV 254 nm *$25(OH)D_3$ by normal-phase HPLC*: Apex silica 3 μm Heptane/2-PrOH, 96:6 UV 254 nm	Vitamins D_2 and D_3 $25(OH)D_3$	206

Abbreviations: see Footnote to Table 7.

1. Analytical Considerations

a. Thermal Isomerization

The most effective means of removing the vast excess of triglycerides from samples of full-fat foods is saponification. Hot saponification promotes thermal isomerization of vitamin D with the formation of previtamin D. During subsequent chromatographic cleanup, either the previtamin D is separated and discarded, or it is retained in the final sample extract. In the latter event, the previtamin D peak in the chromatogram is far removed from the vitamin D peak when either normal-phase or reversed-phase chromatography is used (207). Unfortunately, the smaller previtamin D peak cannot usually be measured because of interference from coeluting contaminants. Thus, in practice the potential vitamin D content cannot be obtained by measuring the amounts of previtamin D and vitamin D and adding them together.

The reversibility of the isomerization reaction is very slow, and therefore the percentage of the previtamin will remain virtually unchanged during the subsequent stages of the analytical procedure. This equilibrium allows the potential vitamin D to be calculated from measurements of the vitamin D peak alone, and the same principle applies to the hydroxylated metabolites of vitamin D (208). The determination of potential vitamin D can be carried out by saponifying a standard vitamin D solution in parallel to the sample and using the resultant solution as an external standard in the quantification (117). Alternatively, if vitamins D_2 and D_3 can be separated during quantitative HPLC, one of these vitamers can be used as an internal standard to quantify the other supplemental vitamer. Because the isomerization rates of vitamins D_2 and D_3 are virtually equal, the previtamin D/vitamin D ratio will be the same for both vitamers at any given temperature. Therefore, the quantification will compensate for the formation of previtamin D and give a result for the potential vitamin D content of the sample.

Agarwal (194) circumvented the isomerization problem by converting previtamin D and vitamin D to a common derivative, isotachysterol, by treatment of the unsaponifiable residue with acidified butanol.

b. Cleanup Procedures

The removal of sterols, vitamin E vitamers, carotenoids, and other interfering material from the unsaponifiable fraction of food samples has been achieved using one or more of the following techniques: coprecipitation of sterols with digitonin (91), precipitation of sterols from a methanolic solution (195,209), adsorption chromatography on open columns of alumina (70,91,96), thin-layer chromatography on silica plates (209), and solid-phase extraction on silica (68,100) and reversed-phase (210) cartridges.

Semipreparative HPLC has been employed to obtain a vitamin D–rich fraction of the unsaponifiable matter for subsequent quantitative HPLC. Combinations of chromatographic modes used for offline semipreparative and quantitative analysis have included polar bonded-phase/adsorption (211,212), reversed-phase/adsorption (194,213), and adsorption/reversed-phase (70,125). An online two-dimensional HPLC technique using two polar bonded-phase columns has also been described (214).

2. Normal-Phase Separations

Normal-phase HPLC, using either silica or polar-bonded stationary phases, separates, isocratically, vitamin D_2 or D_3 from their respective previtamins and inactive isomers (207). Vitamin D ($D_2 + D_3$), 25-hydroxyvitamin D_2, and 25-hydroxyvitamin D_3 can be separated from one another and from other hydroxylated metabolites (215), but vitamins D_2 and D_3 cannot be resolved from one another. The inability to resolve vitamins D_2 and D_3 means that one vitamer cannot be used as an internal standard for the other.

3. *Reversed-Phase Separations*

Reversed-phase chromatography also separates, isocratically, vitamin D_2 or D_3 from their respective previtamins and inactive isomers (207), but, unlike normal-phase chromatography, it can separate vitamin D_2 from D_3 using nonendcapped stationary phases (198). The 25-hydroxylated metabolites of vitamins D_2 and D_3 can be separated from one another using a Vydac 201 TP column (37). The separation of vitamin D_2 from vitamin D_3, and 25-hydroxyvitamin D_2 from 25-hydroxyvitamin D_3, allows the D_2 form of the vitamin or its metabolite to be used as an internal standard for quantifying the corresponding D_3 form.

Normal-phase/reversed-phase chromatography is the ideal combination for semipreparative and quantitative separations in two-dimensional HPLC. Vitamins D_2 and D_3 coelute during the semipreparative stage, allowing a narrow retention window to be collected for analysis using internal standardization. By this means, Johnsson et al. (201) obtained a vitamin D_3 detection limit of 0.1 μg/kg for milk and milk products.

Indyk and Woollard (195) demonstrated that the removal of cholesterol from the unsaponifiable fraction of vitamin D–supplemented whole milk powder by methanolic precipitation and filtration was an adequate cleanup procedure, making semipreparative HPLC unnecessary. This simplified procedure was made possible by connecting two analytical columns in series. The tandem columns adequately separated vitamins D_2 and D_3 from one another and from vitamins A and E. The analysis of infant formulas (100) required cleanup by silica solid-phase extraction to remove the minor tocopherols and tocotrienols, which constituted potential sources of interference.

Mattila et al. (205) described a two-dimensional HPLC procedure for determining vitamin D_3 and 25-hydroxyvitamin D_3 in meat and milk products. Samples were saponified in the presence of vitamin D_2 and 25-hydroxyvitamin D_2 as internal standards, and the extracted unsaponifiable matter was subjected to normal-phase semipreparative HPLC to obtain a fraction containing 25-hydroxyvitamin D_2 + 25-hydroxyvitamin D_3 and a fraction containing vitamin D_2 + vitamin D_3. The collected fractions were evaporated and purified by reversed-phase HPLC. Fractions were again collected, after which vitamin D_3 was quantified by tandem-column reversed-phase HPLC and 25-hydroxyvitamin D_3 by tandem-column normal-phase HPLC. Analytical chromatograms of a purified extract of chicken are shown in Fig. 12.

The method developed by Bristol-Myers Squibb (198) for the determination of vitamin D in infant formulas and enteral products has been studied collaboratively (216) and adopted, first action, by AOAC International.

D. Vitamin E

Methods for estimating vitamin E values in foods are summarized in Table 12.

1. *Quantification*

External standard calibration is generally used. The quality of an α-tocopherol standard can easily be checked by making a solution in hexane and measuring the UV absorbance at minimum (A_{min}) and maximum (A_{max}) wavelengths of 255 nm and 292 nm, respectively. If the quotient A_{min}/A_{max} exceeds 0.18, the standard contains less than 90% α-tocopherol (232).

2. *Normal-Phase Separations*

Normal-phase HPLC is capable of separating isocratically all of the eight unesterified tocopherols (T) and tocotrienols (T-3) that occur in nature, and it has been utilized in determining the distribution of these vitamers in a wide variety of fats, oils, and foodstuffs. The elution sequence

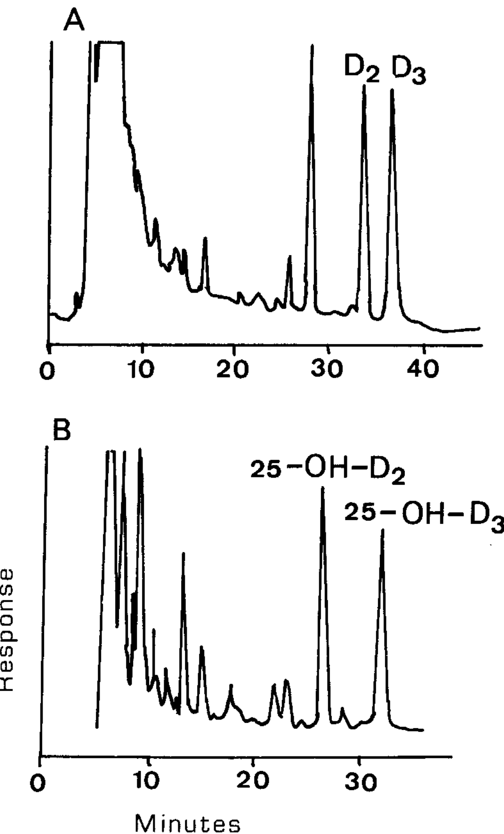


Fig. 12 Analytical HPLC of fractions from a saponified chicken sample isolated by semipreparative HPLC. (A) Vitamins D_2 (internal standard) and D_3. Tandem columns, Zorbax ODS + 5 μm Vydac 201 TP54 C_{18} (250 × 4.6-mm ID); mobile phase, methanol/water (96:4), 1 ml/min; absorbance detection, 264 nm. (B) 25-Hydroxyvitamin D_2 (internal standard) and 25-hydroxyvitamin D_3. Tandem columns, 5 μm Spherisorb $S5NH_2$ (250 × 4.6 mm) + 10 μm μPorasil (300 × 3.9 mm); mobile phase, hexane/2-propanol (97:3), 1 ml/min; absorbance detection, 264 nm. (Reprinted in part with permission from Ref. 205. Copyright 1995, American Chemical Society.)

is α-T, α-T-3, β-T, γ-T, β-T-3, γ-T-3, δ-T, and δ-T-3. Supplemental α-tocopheryl acetate is eluted before α-T, and the acetate and palmitate esters of vitamin A elute separately before α-tocopheryl acetate.

The distribution of naturally occurring E vitamers in oils and fats may be determined by simply dissolving the sample in hexane (typically 0.5 g in 50 ml) and injecting an aliquot of the solution, without concentration, on a silica column (219). Complex food matrices require a more rigorous initial extraction before partitioning of the total lipid into hexane. Fluorescence detection is usually obligatory when the total lipid fraction is analyzed, because absorbance detection reveals peaks of lipid origin that interfere with the peaks of the E vitamers. If the sample is saponified, absorbance detection can be utilized, since hexane extracts of the unsaponifiable matter are usually free from interfering lipoidal material. For the analysis of saponified vitamin E–supplemented foods, fluorescence detection provides a much higher sensitivity, because the liberated α-tocopherol fluoresces more strongly than α-tocopheryl acetate.

Table 12 HPLC Methods Used for the Determination of Vitamin E in Food

Food	Sample preparation	Quantitative HPLC				Ref.
		Column	Mobile phase	Vitamers separated	Detection	
		Normal-phase chromatography				
Seed oils	Dissolve oil in hexane.	Polygosil 60 5 μm 250 × 4.6 mm	Hexane/diisopropyl ether, 90:10	α-, β-, γ-, δ-T α-T-3	Fluorescence: ex 296 nm em 320 nm	217
Wheat flour, wheat germ	Hexane extraction in Soxhlet apparatus.	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/diisopropyl ether, 97:3	α-, β-, γ-, δ-T α-, β-T-3	Fluorescence: ex 290 nm em 330 nm	218
Seed oils, margarine, butter	Dissolve sample in hexane.	LiChrosorb Si-60 5 μm 250 × 4 mm (column temperature 45°C for separation of γ-T and β-T-3)	Diisopropyl ether gradient of 8%–17% in hexane	α-, β-, γ-, δ-T α-, β-, γ-, δ-T-3	Fluorescence: ex 290 nm em 325 nm	219
Cereals, flour foods (unfortified)	Extract samples with boiling 2-PrOH, reextract with acetone, partition into hexane.	LiChrosorb Si-60 5 μm 250 × 3.2 mm	0.2% 2-PrOH or 5% diethyl ether in dry hexane/water-saturated hexane, 1 + 1	α-, β-, γ-, δ-T α-, β-, γ-T-3	Fluorescence: ex 290 nm em 330 nm	135
Infant formula	Saponify (hot), extract unsaponifiables with diethyl ether.	As in preceding entry	As in preceding entry	Total α-T, β-, γ-, δ-T α-, β-, γ-T-3	As in preceding entry	135
Infant formula	Saponify (hot), extract unsaponifiables with hexane.	LiChrosphere Si-60 5 μm 120 × 4.6 mm	1% 2-PrOH and 0.5% EtOH in hexane	Total α-T, β-, γ-, δ-T	Fluorescence: ex 292 nm em 320 nm	220
Various foodstuffs, dairy products, infant formula	Saponify (hot), extract unsaponifiables with petroleum ether/diisopropyl ether, 3 + 1.	Radial-PAK cartridge containing Resolve silica 5 μm	1% 2-PrOH in hexane	Total α-T, β-, γ-, δ-T	Fluorescence: ex 295 nm em 330 nm	221

Infant formula	Disperse sample in nonaqueous solvent mixture (DMSO/DMF/ $CHCl_3$, 2 + 2 + 1) containing 0.1% (w/v) ascorbic acid. Partition total lipid fraction into hexane, centrifuge.	Radial-PAK cartridge containing Resolve silica 5 μm	0.08% 2-PrOH in hexane	α-Tocopheryl acetate	UV 280 nm	222
Pork (muscle, adipose tissue)	Saponify (hot), extract unsaponifiables with hexane/toluene, 1:1.	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/ethyl acetate, 95:5	α-T	Fluorescence: ex 295 nm em 330 nm	78
Beef (muscle)	Saponify (hot), extract unsaponifiables with isooctane.	Resolve silica 5 μm 150 × 3.9 mm	Isooctane/THF, 96:4	α-T	Fluorescence: ex 296 nm em 325 nm	223
Meat and meat products	Saponify (ambient), extract unsaponifiables with hexane.	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane/diisopropyl ether, 93:7	α-, β-, γ-T α-, β-T-3	Fluorescence: ex 292 nm em 324 nm	224
Fish and fish products	As in preceding entry	As in precedent entry	As in preceding entry	α-, β-, γ-T	As in preceding entry	225
Infant formula	As in preceding entry	As in preceding entry	As in preceding entry	Total α-T, β-, γ-T α-, β-T-3	As in preceding entry	226
Cereal products	As in preceding entry	As in preceding entry	As in preceding entry	α-, β-, γ-, δ-T α-, β-, γ-T-3	As in preceding entry	227
Rice bran	Saponify (hot), extract unsaponifiables with hexane.	Supelcosil LC-Si 5 μm 250 × 4.6 mm	Isooctane/ethyl acetate/2,2-dimethoxypropane, 98.15:0.9:0.85:0.1	α-, β-, γ-, δ-T α-, β-, γ-, δ-T-3	Fluorescence: ex 290 nm em 330 nm	228

(*continued*)

Table 12 Continued

Food	Sample preparation	Quantitative HPLC				
		Column	Mobile phase	Vitamers separated	Detection	Ref.
		Normal-phase chromatography				
Breakfast cereals, infant formula	*Cereals*: add water and MeOH to ground sample, shake, and sonicate. Extract with MTBE/petroleum ether, 10 + 14. *Formulas*: reconstitute sample with water, add dipotassium oxalate solution (35% w/v) and EtOH, extract with MTBE/petroleum ether, 25 + 35.	LiChrosphere 100 Diol 5 μm 250 × 4 mm	Two-step gradient composed of hexane and an increasing concentration of MTBE. 0–4 min hexane, 4–5 min up to hexane/MTBE (97:3), 5–41 min isocratic, 41–42 min up to hexane/MTBE (95:5), 42–50 min isocratic	α-Tocopheryl acetate α-, β-, γ-, δ-T α-, β-, γ-, δ-T-3 plastochromanol	Fluorescence: ex 295 nm em 330 nm	86
Margarine, infant formula, broccoli	Saponify (hot), extract unsaponifiables with hexane/ethyl acetate (90 + 10) containing 20 mg BHT per L.	LiChrosorb Diol 5 μm 250 × 4.6 mm	Hexane/MTBE, 14 + 6	α-, β-, γ-, δ-T α-, β-, γ-, δ-T-3	Fluorescence: ex 295 nm em 330 nm	229

Diets	*Unesterified vitamin E*: add cold EtOH, extract with hexane. *Esterified vitamin E*: saponify the remaining ethanolic mixture at 70°C, extract unsaponifiables with hexane.	Supelcosil LC-Diol 5 μm 250 × 4.6 mm	Hexane/2 PrOH, 99:1	α-, β-, γ-, δ-T α-, β-, γ-, δ-T-3	Fluorescence: ex 296 nm em 330 nm	111
		Reversed-phase chromatography				
All food types	Saponify (ambient), extract unsaponifiables with hexane.	Zorbax ODS 5 μm 250 × 4.6 mm	MeCN/CH_2Cl_2 containing 0.001% triethylamine/MeOH, 700 + 300 + 50	α-, ($\beta + \gamma$)-, δ-T	Fluorescence: ex 290 nm em 330 nm	230
Vegetable oils	Dilute oil with THF and further dilute with MeOH.	Spherisorb ODS 5 μm 250 × 4.6 mm	0.05 M aqueous sodium perchlorate/MeOH, 10 + 90	α-, ($\beta + \gamma$)-, δ-T α-, ($\beta + \gamma$)-, δ-T-3	Amperometric +0.600 V	231

Abbreviations: see Footnote to Table 7.

For the determination of supplemental vitamin E in infant formulas, Woollard and Blott (222) employed a radially compressed Radial-PAK cartridge. This enabled lipid material to be rapidly cleared by stepping up the mobile-phase flow rate from 2 ml/min to 10 ml/min after elution of the α-tocopheryl acetate. Fluorescence detection, using a filter-type fluorometer, allowed the indigenous α-tocopherol to be conveniently estimated, while UV absorbance detection was used to quantify the α-tocopheryl acetate. Supplemental retinyl acetate could be assayed simultaneously with either added or indigenous vitamin E using the appropriate detection mode. With the aid of a dual-monochromator spectrofluorometer, α-tocopheryl acetate and α-tocopherol could be determined simultaneously with wavelengths of 280 nm (excitation) and 335 nm (emission), but the increased selectivity eliminated detection of the vitamin A esters (233).

Shin and Godber (228) used a silica column and a mobile phase composed mainly of isooctane and small amounts of ethyl acetate, acetic acid, and 2,2-dimethoxypropane. The acetic acid component reduced retention times of the late-eluting E vitamers, presumably by competing with water and polar material for binding to silanol groups on the silica surface. 2,2-Dimethoxypropane reacts with water to form acetone and methanol, and its inclusion stabilized retention times and reduced the need for column regeneration. Chromatograms of vitamin E vitamers in a standard solution and in a saponified rice bran sample are shown in Fig. 13.

Balz et al. (86) utilized a diol stationary phase and a two-step gradient composed of hexane and an increasing concentration of methyl *tert*-butyl ether to separate α-tocopheryl acetate, plastochromanol-8, and the eight unesterified E vitamers. The system was applied to the analysis of infant formulas and breakfast cereals.

3. *Reversed-Phase Separations*

In reversed-phase HPLC, the E vitamers are eluted in the order δ-T-3, γ-T-3, β-T-3 (unresolved), α-T-3, δ-T, γ-T, β-T (unresolved), and α-T. This elution profile contrasts with that obtained using normal-phase chromatography, in which α-T is eluted first and δ-T-3 is eluted last. The positional β and γ isomers of tocopherols and tocotrienols cannot be separated using reversed-phase columns of standard dimensions, even if gradient elution is used. α-Tocopheryl acetate elutes immediately in front of α-tocopherol, with baseline separation.

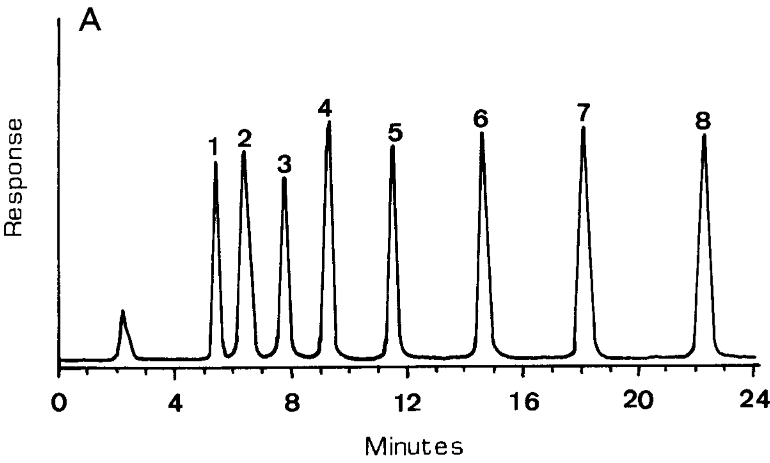
Reversed-phase HPLC with fluorescence detection is the preferred system for the routine determination of total α-tocopherol in vitamin E–supplemented foods after saponification. The use of NARP chromatography with a predominantly hexane mobile phase allows aliquots of hexane extracts of the unsaponifiable matter to be injected directly onto the column, thus avoiding the evaporation step necessary when a semiaqueous mobile phase is used (234).

E. Vitamin K

Selected methods for determining vitamin K in a variety of foods are summarized in Table 13. The photodiode array detector lacks the required sensitivity for identifying phylloquinone in foods other than green leafy vegetables. Careri et al. (248) reported on the use of particle beam mass spectrometry for the determination and unequivocal identification of phylloquinone in some vegetable samples. The proposed LC–MS method permitted phylloquinone assay at levels down to 0.1 μg/g with high specificity.

1. *Normal-Phase Separations*

Adsorption chromatography using a silica column facilitates the separation of the inactive cis isomer of phylloquinone from the active trans isomer. Hwang (129) applied adsorption HPLC to the



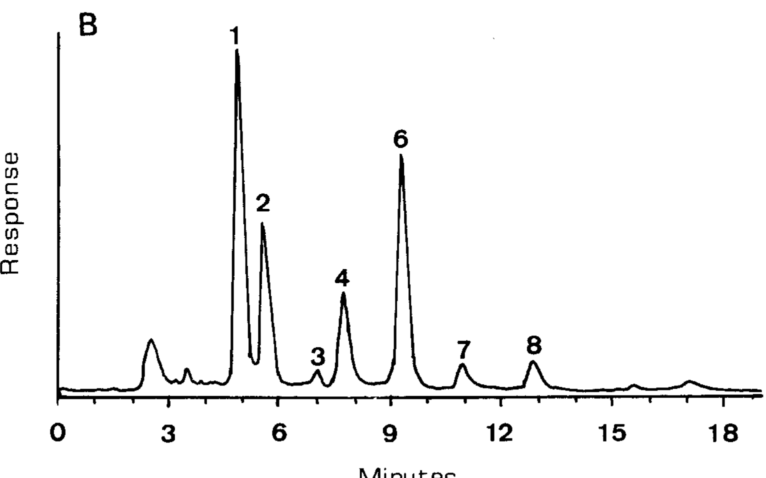


Fig. 13 HPLC of vitamin E. (A) Standards of vitamin E vitamers. Column, 5-μm Supelcosil LC-Si (250 × 4.6-mm ID); mobile phase, isooctane/ethyl acetate (97.5:2.5), 1.6 ml/min; fluorescence detection, excitation 290 nm, emission 330 nm. Peaks: (1) α-tocopherol; (2) α-tocotrienol; (3) β-tocopherol; (4) γ-tocopherol; (5) β-tocotrienol; (6) γ-tocotrienol; (7) δ-tocopherol; (8) δ-tocotrienol. (B) Saponified rice bran sample. Chromatographic conditions as in (A) except for mobile phase: isooctane/ethyl acetate/2,2-dimethoxypropane (98.15:0.9:0.85:0.1). (From Ref. 228. AOCS Press.)

determination of phylloquinone in infant formulas using photometric detection at 254 nm. Both the cis and trans isomers could be measured in standards and in liquid formulas, but matrix interferences prevented measurement of the cis isomer in powdered formulas.

2. *Reversed-Phase Separations*

Reversed-phase chromatography can separate phylloquinone from closely related structures, but it cannot separate *cis*- and *trans*-phylloquinone. The postcolumn reduction of vitamin K and

Table 13 HPLC Methods Used for the Determination of Vitamin K in Food

Food	Sample preparation	Semipreparative HPLC	Quantitative HPLC	Compounds separated	Ref.
Infant formula	Mix sample with concentrated NH_4OH/MeOH, extract with CH_2Cl_2/isooctane, 2 + 1. Silica-column chromatography.		*Normal-phase* Apex Silica 5 μm 250 × 4.6 mm Isooctane/CH_2Cl_2/2-PrOH, 70 + 30 + 0.02 UV 254 nm	*Cis-* and *trans-* phylloquinone	129
Infant formula	Digest sample with lipase, extract with pentane.		*Reversed-phase* μBondapak C_{18} 10 μm 250 × 4 mm MeOH/MeCN/THF/H_2O, 39:39:16:6 UV 254 nm	Phylloquinone	235
Soy bean oil	Digest sample with lipase, extract with pentane. Alumina-column chromatography.		Supelcosil LC-18 5 μm 150 × 4.6 mm MeOH/MeCN/H_2O, 81:10:2 UV 270 nm	Phylloquinone	236
Milk (human and bovine)	Deproteinize milk sample with EtOH at 4°C with added synthetic phylloquinone homolog (internal standard). Extract with hexane aided by sonication and vortexing.	μPorasil silica 10 μm 300 × 8 mm Heptane/ethyl acetate, 99:1 UV 254 nm	Resolve C_{18} 5 μm 150 × 3.9 mm 100% MeCN UV 270 nm	Phylloquinone	237
Infant formula, endogenous vitamin K in milk	Dissolve powders in warm water, add 0.8 M phosphate buffer (pH 8.0) and lipase, shake for 5 min. Incubate tubes at 37°C for 2 h in an ultrasonic bath, cool. Add EtOH/MeOH (95:5) + solid K_2CO_3 + cholesteryl phenylacetate (internal standard) and extract twice with hexane.	Radial-PAK cartridge containing Resolve silica 5 μm Hexane/2-PrOH, 99.1 + 0.1 UV 269 nm	Radial-PAK cartridge containing Resolve C_{18} 5 μm MeOH/2-PrOH/ethyl acetate/water, 450 + 350 + 145 + 135) Dual-wavelength UV 269 and 277 nm	Phylloquinone, cholesteryl phenylacetate (internal standard)	238
Infant formula	Supercritical fluid extraction.		μBondapak C_{18} 10 μm 150 × 3.9 mm MeCN/CH_2Cl_2/aqueous 0.025 M sodium perchlorate, 90:5:5 Amperometric detection (reductive mode), silver electrode, −1.1 V vs saturated calomel reference electrode	Phylloquinone	65

Milk (human)	Digest milk sample with lipase, extract with pentane.	Nucleosil C_{18} 5 μm 300 × 8 mm MeCN/MeOH, 1:1 Column temperature 35°C UV 248 nm	Partisil ODS-2 5 μm 250 × 4.6 mm MeOH/EtOH/60% perchloric acid (600:400:1.2) containing 0.05 M sodium perchlorate in the total solution Dual electrochemical detection (redox mode): −450 mV (generator) +350 mV (detector)	Phylloquinone, menaquinone-4	239
Milk (human)	Extract vitamin K with 3 vol of 2-PrOH/hexane (3:2), centrifuge. Evaporate three times from 10 vol of $CHCl_3$/MeOH, 2:1. Silica-column chromatography. Add menaquinone-7 as internal standard to purified fraction.	Radial-PAK cartridge containing C_{18} 5 μm Convex gradient of EtOH/H_2O (90:10) to EtOH/hexane (90:10) UV 254 nm	Radial-PAK cartridge containing C_{18} 10 μm EtOH/hexane/H_2O (90:6.5:3.5) containing 0.025 M tetrabutyl ammonium perchlorate Amperometric detection (redox mode): −600 mV (reductive electrode) +200 mV (oxidative electrode)	Phylloquinone, menaquinone-7 (internal standard)	240
Milk, milk powder	Digest milk sample with lipase over 90 min at 37°C, treat with alcoholic NaOH (15 sec contact time). Extract hydrolysate twice with hexane.		OD-224 RP18 5 μm 220 × 4.6 mm (Brownlee Labs) MeOH/H_2O (99 + 1) containing 2.5 mM acetate buffer Dual-amperometric detection (redox mode) glassy carbon dual electrode at −1.1 V and +0.7 V vs Ag/AgCl	Phylloquinone	241
All food types	*Vegetables, fruits, cereals, meat and fish*: extract sample with acetone, filter. Partition the phylloquinone into hexane. *Fats, oils, and dairy products*: digest sample with lipase, extract with hexane. Purify hexane extracts obtained from either extraction technique by silica solid-phase extraction in the sample cleanup mode. The internal standard is phylloquinone 2,3-epoxide (unlabeled for UV detection and tritium labeled for coulometric detection).	A. Partisil silica 5 μm 250 × 4.6 mm 50% water-saturated CH_2Cl_2/hexane, 15:85 UV 254 nm B. Spherisorb nitrile 5 μm 250 × 4.6 mm 50% water-saturated CH_2Cl_2/hexane, 3:97 UV 254 nm	A. Hypersil ODS 250 × 4.6 mm CH_2Cl_2/MeOH, 15:85 UV 270 nm B. Spherisorb C_8 (octyl) 5 μm 250 × 4.6 mm MeOH/50 mM acetate buffer pH 3.0, 97:3 containing 0.1 mM EDTA Dual-electrode coulometric detection (redox mode), porous graphite electrodes, −1.5 V (generator electrode) +0.05 V (detector electrode)	Phylloquinone	242, 88

(*continued*)

Table 13 Continued

Food	Sample preparation	Semipreparative HPLC	Quantitative HPLC	Compounds separated	Ref.
Oils, margarine, butter	Add menaquinone-4 to oil and margarine samples as internal standard. Dissolve sample in hexane, filter.	μPorasil 5 μm 300 × 3.9-mm ID 1% diethyl ether in hexane UV 248 nm	Vydac 201 TP54 C_{18} 5 μm 250 × 4.6 mm MeOH/0.05 M acetate buffer pH 3, 95:5 Dual-electrode coulometric detection (redox mode), porous graphite electrodes: −1.1 V (generator electrode) 0 V (detector electrode)	Phylloquinone, menaquinone-4 (internal standard for oil and margarine samples)	243
Vegetables, fruits, berries	Add 2-PrOH and internal standard (menaquinone-4) to homogenized sample, digest at 100°C for 5 or 10 min. Cool, partition into hexane.	As in preceding entry (when necessary)	As in preceding entry	Phylloquinone, menaquinone-4 (internal standard)	244
Various foods (vegetable juice, whole milk, raw spinach leaves, plain bagel, raw ground beef)	*Vegetable juice*: add dihydrophylloquinone (internal standard) to sample. Extract with 2-PrOH/hexane (3 + 2) and water aided by sonication and vortexing. Silica solid-phase extraction in the sample cleanup mode. *Milk*: as described for vegetable juice followed by liquid-phase reductive extraction for removal of lipids. *Spinach*: homogenize and grind sample with Na_2SO_4. Extraction and cleanup as for vegetable juice. *Bread*: add $K_{1(25)}$ (internal standard) to ground sample. Extraction and cleanup as for vegetable juice. *Beef*: as described for bread, followed by C_{18} solid-phase extraction in the sample cleanup mode.		Hypersil ODS 3 μm 150 × 4.6 mm MeOH/CH_2Cl_2 (90:10) containing 0.01 M $ZnCl_2$, 0.005 M acetic acid, and 0.005 M sodium acetate Fluorescence ex 244 nm em 418 nm Postcolumn chemical reactor column packed with zinc metal	Phylloquinone, dihydrophylloquinone (internal standard)	141

Egg, whole milk, yoghurt, cheese (emmentaler), oatmeal, carrot, potato, broccoli, cauliflower, edible oils	Blend samples with appropriate solvent* and added dihydrophylloquinone (internal standard). Centrifuge. Evaporate sample extracts to dryness (except edible oils) and dissolve residue in hexane. Add equal volume of $MeOH/H_2O$ (9:1), mix, and centrifuge. Remove upper hexane layer and evaporate to dryness. * *Cheese, oatmeal, broccoli, cauliflower*: $CH_2Cl_2/MeOH$, 2:1; *Egg, milk, yoghurt, carrot, potato*: 2-PrOH/hexane, 3:1; *Edible oils*: 100% hexane.	Hypersil ODS 5 μm 250 × 4.6 mm Mobile phase: 900 ml MeOH + 100 ml CH_2Cl_2 + 5 ml of a methanolic solution containing 1.37 g $ZnCl_2$, 0.41 g sodium acetate, and 0.30 g acetic acid Column temperature 40°C Postcolumn chemical reactor column packed with zinc powder Fluorescence: ex 243 nm em 430 nm	Phylloquinone, dihydrophylloquinone (internal standard)	245
Canola (rapeseed) oil	Digest oil sample + internal standard (menaquinone-4) with lipase in pH 7.7 buffer at 37°C. Extract with hexane. Silica solid-phase extraction in the sample cleanup mode.	PartiSphere C_{18} 5 μm 150 × 4.6 mm Mobile phase: 850 ml MeOH + 150 ml MeCN + 5 ml MeOH/MeCN (85:15) solution containing 2 M $ZnCl_2$, 1 M sodium acetate, and 1 M acetic acid (pH of final solution, 3.3) Postcolumn chemical reduction with zinc dust column Fluorescence: ex 254 nm em 400 nm (filter)	Phylloquinone, menaquinone-4 (internal standard)	246
Vegetables (raw and processed)	Homogenize with 2-PrOH, partition into hexane.	Hypersil-MOS 5 μm $MeOH/H_2O$ (92.5 + 7.5) containing 0.03 M sodium perchlorate Coulometric reduction: −500 mV Fluorescence: ex 320 nm em 430 nm	Phylloquinone	140

(continued)

Table 13 Continued

Food	Sample preparation	Semipreparative HPLC	Quantitative HPLC	Compounds separated	Ref.
Soybean oil	Shake oil sample. Add $K_{1(25)}$ (internal standard) to oil sample. Shake with 0.9% NaCl solution + EtOH and partition vitamin K into hexane. Centrifuge, evaporate to dryness. Silica solid-phase extraction in the sample cleanup mode.		Beckman XL C_8 (octyl) 3 μm 70 × 4.7-mm cartridge MeCN/EtOH (95:5) containing 0.005 M sodium perchlorate Electrochemical reduction followed by fluorescence detection	Phylloquinone, $K_{1(25)}$ (internal standard)	247
Vegetables (carrot, tomato, brussels sprouts, spinach)	Ultrasonically shake sample with MeOH, centrifuge. Mix with solid Na_2CO_3 and heat at 80°C for 1 h. Partition the alkaline solution with hexane by vortex-mixing, centrifuge. Extract three times with hexane.		LiChrosorb RP-8 (octyl) 10 μm 250 × 4.6 mm 100% MeOH UV 247 nm or particle beam mass spectrometry using negative-ion CI detection	Phylloquinone	248

Abbreviations: see Footnote to Table 7.

fluorescence detection permits the use of highly efficient NARP chromatography, which is incompatible with electrochemical detection.

In a method developed by Shearer (88) for determining phylloquinone, food sample extracts are purified by silica solid-phase extraction and semipreparative HPLC and then analyzed by reversed-phase HPLC. Either of two systems are used, one utilizing UV detection and the other utilizing electrochemical detection. The retention window for collecting the fraction containing the phylloquinone and internal standards excludes *cis*-phylloquinone. With UV detection, a nonaqueous mobile phase is used, and the phylloquinone is quantified by the method of peak height ratios using phylloquinone 2,3-epoxide as the internal standard. With electrochemical detection, a semiaqueous mobile phase is used in conjunction with a less retentive octyl-bonded (C_8) stationary phase. The addition of 0.1 mM EDTA to the semiaqueous mobile phase prevents the reduction of metal ions at the generator electrode. Phylloquinone 2,3-epoxide is electrochemically inactive; therefore quantification is accomplished by the technique of radioisotopic dilution using tritiated phylloquinone 2,3-epoxide as the internal standard. A chromatogram showing the analytical separation of the phylloquinone fraction from a sample of brown rice isolated by semipreparative HPLC is shown in Fig. 14.

In a method proposed by Booth et al. (141) for the determination of phylloquinone in various food types, extracted samples are subjected to silica solid-phase extraction followed, in the case of meat or milk samples, by further purification using reversed-phase solid-phase extraction or liquid-phase reduction extraction, respectively. The final test solution is analyzed by NARP–HPLC, and the fluorescent hydroquinone reduction products of phylloquinone and the internal standard are produced online using a postcolumn chemical reactor packed with zinc metal. 2′,3′-Dihydrophylloquinone, a synthetic analog of phylloquinone, is a suitable internal standard for the analysis of vegetable juice, whole milk, and spinach. Another synthetic analog, $K_{1(25)}$, is used for the analysis of bread and beef, because a contaminant in the test solution coelutes with dihydrophylloquinone.

F. Simultaneous Determination of Two or Three Vitamins

Examples of simultaneous determinations are shown in Table 14. Few such methods have been reported, partly because different physicochemical properties prohibit a common extraction procedure for certain vitamins. Other reasons are a lack of sensitivity due to relative differences in concentration and differences in the optimal absorption wavelengths for each vitamin.

Delgado Zamarreño et al. (143) proposed a rapid method for the simultaneous determination of vitamins A, D_3, and E in saponified milk and milk powders using reversed-phase HPLC and amperometric detection. The high sensitivity of amperometry allows the determination of vitamin D_3 in unenriched fluid milk; this determination is not possible using UV detection without a preconcentration step. A typical chromatogram showing the indigenous vitamins in fluid bovine milk is depicted in Fig. 15. A directly coupled sample treatment–HPLC system was later developed for the online automatic determination of the same vitamins in liquid and powdered milk (255) and butter and margarine (256). Alkaline hydrolysis of the samples was performed using two confluent channels through which the sample solution and hydrolysis reagent flowed for a given period of time. A third channel merged with the other two to neutralize the solution before it arrived at a C_{18} solid-phase extraction cartridge. The latter, inserted into a loop with a six-port injection valve, retained the fat-soluble vitamins. The vitamins were eluted with a stream of methanol, and a 100-μl loopful of eluate was automatically injected into the HPLC column. The problem of solubilizing butter and margarine was overcome by dissolving these samples in an aqueous micellar medium using the surfactant Triton X-100 before introduction into the online system.

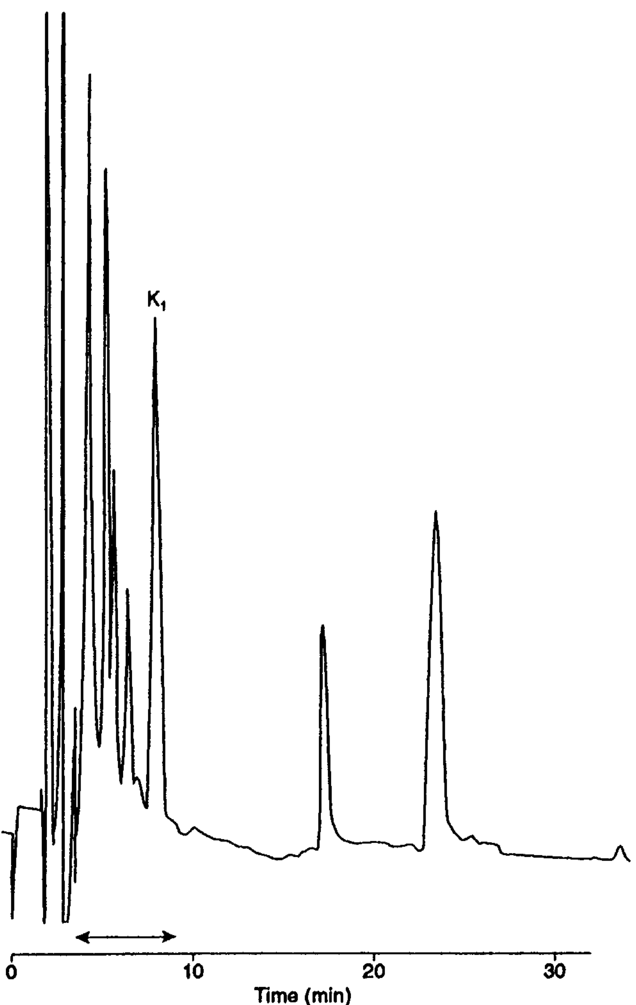


Fig. 14 Analytical HPLC of the phylloquinone fraction from an extracted sample of brown rice isolated by semipreparative HPLC. Column, Spherisorb C_8 (octyl); mobile phase, methanol/50 mM acetate buffer pH 3.0 (97:3) containing 0.1 mM EDTA, dual-electrode coulometric detection (redox mode), porous graphite electrodes, −1.5 V (generator electrode), +0.05 V (detector electrode). The arrows signify the fraction containing tritiated phylloquinone 2,3-epoxide (internal standard) and phylloquinone (analyte) that is collected for quantitation by radioisotopic dilution. (Courtesy of M. J. Shearer.)

VII. FUTURE DEVELOPMENTS

HPLC instrumentation and column technology have undergone major advances since the early 1970s, when HPLC made its debut in the field of vitamin analysis. Yet sample preparation in food analysis continues to rely largely on manual wet-chemical techniques, which are time consuming and labor intensive, require considerable analytical skill, and constitute the major source of error in the assay procedure. There is also the serious problem of environmental pollution and the exposure of laboratory personnel to toxic chemicals.

The ideal sample preparation technique for HPLC would be nontoxic and capable of direct coupling to the chromatograph, allowing complete automation of the analysis. A potential tech-

Table 14 HPLC Methods Used for the Simultaneous Determination of Two or More Fat-Soluble Vitamins in Foods

Food	Sample preparation	Quantitative HPLC				Ref.
		Column	Mobile phase	Vitamins separated	Detection	
		Normal-phase chromatography				
Breakfast cereals fortified with vitamins A and E	Extract sample with a solvent mixture of $CHCl_3$, EtOH, and H_2O at 50°C.	μPorasil 10 μm 300 × 4 mm	Hexane/$CHCl_3$ containing 1% EtOH, 85:15	Retinyl palmitate, α-tocopheryl acetate	UV 280 nm	249
Butter, whole milk powder, infant formula	Saponify (hot), extract unsaponifiables with diethyl ether.	LiChrosorb Si-60 5 μm 250 × 4 mm	Hexane containing 8% 1,4-dioxan	α-Tocopherol, all-*trans*-retinol	UV and fluorescence detectors connected in series UV (retinol) 325 nm Fluorescence (tocopherol): ex 293 nm em 326 nm	250
Italian cheeses	Saponify (ambient), extract unsaponifiables with diethyl ether.	LiChrosorb Si-60 5 μm 250 × 4 mm, column temperature 44°C	Hexane containing 0.8% 2-PrOH	Total carotenes, α-, β-, γ-, δ-tocopherols, 13-*cis*-, 9,13-di-*cis*-, 9-*cis*-, and all-*trans*-retinol	Programmable UV/Vis: 450 nm (carotenes) 295 nm (tocopherols) 328 nm (retinols)	251
Italian cheeses	Saponify (hot), extract unsaponifiables with hexane/ethyl acetate, 9 + 1.	Ultrasphere Si 5 μm 250 × 4.6 mm	(A) 1% 2-PrOH in hexane and (B) hexane in a multilinear gradient elution	Total carotenes, α-, β-, γ-, δ-tocopherols, 13-*cis*- and all-*trans*-retinol	Programmable UV/Vis and fluorescence detectors connected in series Vis (carotenes) 450 nm Fluorescence (tocopherols): ex 280 nm em 325 nm Fluorescence (retinols): ex 325 nm em 475 nm	252

Table 14 Continued

Food	Sample preparation	Quantitative HPLC				
		Column	Mobile phase	Vitamins separated	Detection	Ref.
		Normal-phase chromatography				
Dairy foods	*Butter, anhydrous milk fats*: dissolve in hexane. *Milk, milk powder, cream, infant formula*: reconstitute milk powder or infant formula. To a 5-ml sample, add 1 ml conc (25%) ammonia and 5 ml EtOH, mix. Add internal standard (BHA, retinyl acetate or α-tocopheryl acetate). Shake with diethyl ether containing 40 mg BHT per L, add hexane. After phase separation, remove aqueous layer and wash organic layer with 0.35 M $CaCl_2$. Evaporate under vacuum at 45°C, dissolve residue in hexane.	Econosphere silica 3 μm 150 × 4.6 mm	3% Di-isopropyl ether and 0.04% acetic acid in hexane. *For samples containing both retinyl acetate and α-tocopheryl acetate*: 1.2% diisopropyl ether and 0.016% acetic acid in hexane.	β-Carotene; 13-*cis*-, 9-*cis*-, and all-*trans*-retinyl palmitate; 13-*cis*-, 9-*cis*-, and all-*trans*-retinyl acetate; α-tocopheryl acetate, α-tocopherol	Programmable UV/Vis and fluorescence detectors connected in series Vis (β-carotene) 450 nm Fluorescence (vitamin A): ex 330 nm em 470 nm Fluorescence (vitamin E): ex 295 nm em 330 nm	253
		Reversed-phase chromatography				
Infant milk formula	Saponify (ambient), extract unsaponifiables with hexane.	Spherisorb ODS-2 5 μm 250 × 4.6 mm	MeOH/MeCN/H_2O, 95:1:4	Retinol, α-tocopherol	UV 323 nm (retinol), 292 nm (tocopherol)	254
Milk, milk powder	Saponify (hot), extract unsaponifiables with hexane.	Spheri-5 RP-18 5 μm 220 × 4.6 mm	MeOH/H_2O (99:1) containing aqueous 0.1 M lithium perchlorate	Retinol, vitamin D_3, α-tocopherol	Amperometric (oxidative mode) glassy carbon electrode, +1.05 V, vs silver–silver chloride reference electrode	143

Abbreviations: see Footnote to Table 7.

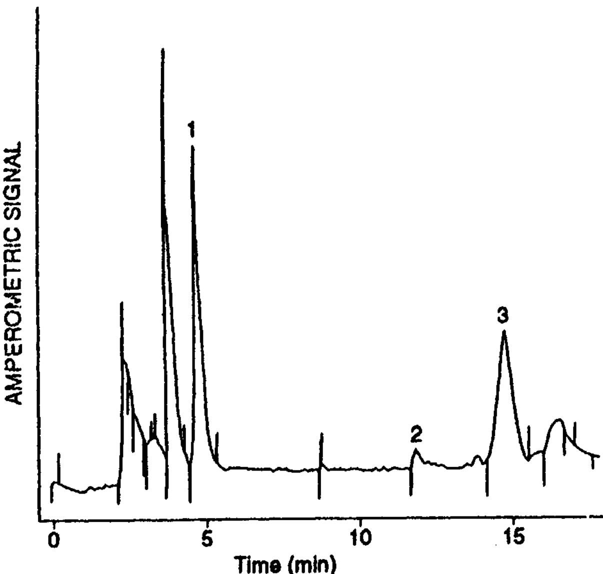


Fig. 15 HPLC of vitamins A, D_3, and E in the unsaponifiable fraction of milk. Column, 5 μm Spheri-5 RP-18 (220 × 4.6-mm ID); mobile phase, methanol/water (99:1) containing aqueous 0.1 M lithium perchlorate, 1 ml/min; amperometric detection (oxidative mode), glassy carbon electrode, +1.05 V, vs silver–silver chloride reference electrode. Peaks: (1) retinol; (2) vitamin D_3; (3) α-tocopherol. (Reprinted from Ref. 143 with the kind permission of Elsevier Science–NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

nique that could at least partly meet this dual requirement is supercritical fluid extraction, which has already proved successful in extracting vitamin A, carotenes, tocopherols, and phylloquinone from a variety of foodstuffs.

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10

Quantitative Determination of Water-Soluble Vitamins

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I. INTRODUCTION

According to the classical definition, a *vitamin* is an organic compound that the organism must obtain from exogenous sources and that is essential for the organism's normal metabolic and physiological functions (1). Its absence from the diet generally results in overt deficiency disease that can be reversed by administration of the vitamin. In recent years, additional nonvitamin health promotion functions, such as reduced risks of chronic diseases or birth defects, have been proposed for vitamin intakes in excess of those required to prevent overt nutritional deficiency. This has been accompanied by an explosion in the amount of nutrition information available to the consuming public, and has triggered consumers' demands for more and better nutrition information and food labeling. The role of vitamins in health promotion is now a major factor driving the need for reliable vitamin methodology.

A. Method Selection Criteria

The literature contains an abundance of high-performance liquid chromatography (HPLC) methods for determining the water-soluble vitamins, leaving little doubt that HPLC has become the most popular technique for their quantitation. The nonvolatile, hydrophilic nature of these compounds makes them particularly good candidates for reversed-phase HPLC analyses. A major advantage of HPLC is its ability to separate the vitamins from the interfering artifacts inherent in complex biological systems such as food. In addition, HPLC protocols are easily automated by using autosamplers or robotics. Compared to the biological, microbiological, and manual chemical methods of vitamin analysis, HPLC provides the advantages of increased speed of analysis, increased precision, reasonable accuracy, increased specificity, and the potential for the simultaneous quantitation of multiple vitamins, vitamers, and/or metabolites.

This chapter does not constitute a comprehensive review of all recently published HPLC methods for the analysis of water-soluble vitamins. It is a summary of selected methods and is intended to serve as a tool for the analyst in search of a method for quantitating one or more of the water-soluble vitamins in foods. The selected methods must:

AFHRC Contribution No. 2187

Provide a complete description of the HPLC separation, including complete chromatographic conditions and an example chromatogram
Include a detailed extraction protocol
Be accompanied by an indication of analytical reliability, in order to provide the reader with some means of evaluating and comparing different analytical procedures
Be applied to food samples
Be published between 1992 and 1997

B. Chapter Organization

The water-soluble vitamins are a highly diverse group of compounds with differing physicochemical properties. A single vitamin generally consists of several vitamers, or chemical species, each of which exhibits the same biological activity in vivo. Individual vitamers with the same biological functions often exhibit vastly different physicochemical properties. This necessitates unique extraction and separation procedures for each vitamin. As a result, each vitamin is considered individually in this chapter. A section on methods that determine multiple vitamins simultaneously is also included.

For each vitamin, the food sources, biochemistry, metabolism, and biological functions, including both vitamin and nonvitamin activities, will be discussed briefly. References to recent review articles are included for the benefit of readers requiring more detailed information.

The functions and requirements for many of the vitamins are being reexamined as a result of the health promotion benefits now being attributed to them (1–6). Furthermore, these nonvitamin functions are often controversial, since they are still in the process of being defined. This has triggered a revision of the nutrition recommendations and food labeling regulations. Since the nutrition recommendations and labeling regulations are specific to each jurisdiction, no attempt will be made to catalog them here.

The discussion of the physicochemical properties of each vitamin will highlight those properties having a significant impact on HPLC analysis. Recent methodological reviews will be listed for each vitamin.

1. *Extraction Techniques*

In general, it is very difficult reliably to extract and quantitate multiple vitamins from complex food systems, due to their diverse physical and chemical properties. Consequently, the extraction of the vitamins from the food matrix is usually the greatest challenge of vitamin analysis. This is especially true for the naturally occurring vitamins, which are often bound to other food constituents, such as carbohydrates or proteins. To prevent vitamin degradation or loss, the extraction conditions should complement the labile nature of the vitamins. Indiscriminate "mixing and matching" of extraction and quantitation methods is not recommended, since the extraction conditions can affect subsequent separation and quantitation steps.

2. *HPLC Methods*

The selected methods are presented in tables and have been grouped by analyte of interest, type of column, and detection method, which are often the principal criteria governing method selection. Because of the water-soluble nature of the vitamins, reversed-phase chromatography is the most common mode of HPLC. Ion exchange is used occasionally. When attempting to reproduce a published HPLC method, attention should be paid to both the type of column and the manu-

facturer. The same analytes chromatographed using identical conditions on the same type of column from different manufacturers often produce different separations (7,8).

Because the vitamins occur in food in trace quantities, detection sensitivity is often an issue. Ultraviolet absorbance is the most common detection method. Fluorescence and electrochemical detection are used in specific cases where physicochemical properties permit and where increased sensitivity and selectivity are desired. Refractive index is seldom used, due to its lack of specificity and sensitivity.

3. *Method Validation*

The most critical part of the method selection is determining the relative merits of seemingly diverse HPLC separations. Unfortunately, there is no standard against which all HPLC methods for vitamins are presently compared. However, there are indicators of assay reliability for which the analyst should look. These include: measures of precision, accuracy, and reproducibility; recoveries from spiked food samples; linearity of calibration; limits of detection; measures of peak purity; comparisons with existing recognized methods; and results of collaborative or interlaboratory trials (9,10).

The value of recovery data is strongly dependent on the point in the analysis at which the samples were spiked. An extract that is spiked just prior to injection into the HPLC would be expected to give very favorable recoveries, since the only variability being tested is that of the HPLC separation and detection. In contrast, a sample that is spiked at the beginning of the extraction should provide an indication of the loss or degradation occurring throughout the entire extraction, HPLC separation, and quantitation.

Great care should be exercised in applying a method to a food matrix for which it has not been validated. Complete extraction and the prevention of vitamin degradation/loss can be highly dependent on the type of food matrix. The AOAC International has developed a systematic approach for determining the types of food to which a method is applicable (11,12). In this approach, all foods are assigned to one of nine categories, based on their protein, fat, and carbohydrate content exclusive of water and ash. As a general rule of thumb, a method that has been validated for two foods within any given category can likely be applied successfully to other foods within the same category. In the absence of such information, a full validation of the method for the new food substrate is recommended.

A word of caution concerning the use of internal standards is also warranted. Detailed guidelines for the selection of internal standards have been published (13,14). The most crucial and most often overlooked of these criteria is the necessity for the internal standard and the analyte to possess similar physicochemical properties, including similar responses to the extraction and chromatographic conditions. Like recoveries from spiked samples, the internal standard should be added to the samples at the beginning of the extraction in order that it be subjected to the same extraction, separation, and quantitation conditions as the analyte of interest.

II. VITAMIN C

A. Occurrence

Fruits and vegetables are the most common food sources for vitamin C (15–18). In food, vitamin C exists as two vitamers: L-ascorbic acid (AA) and its oxidation product, dehydro-L-ascorbic acid (DHAA) (Fig. 1) (15,17–19). Total Vitamin C is the sum of the AA and the DHAA contents. A stereoisomer, isoascorbic acid (IAA) is often added to food as an antioxidant. Both IAA and its oxidation product, dehydroisoascorbic acid (DHIAA), can interfere in Total Vitamin C determinations.

L-Ascorbic Acid (AA) Dehydro-L Ascorbic Acid (DHAA)

Fig. 1 Vitamin C.

B. Biological Activity

Ascorbic acid and dehydroascorbic acid have equivalent biological activities, whereas isoascorbic acid has only 5% of the biological activity of AA (15,17,19). The absorption and metabolism of vitamin C were recently reviewed (17,20,21).

Present knowledge indicates several biochemical roles for ascorbic acid in vivo, including:

Participation as a cofactor in an number of enzymatic reactions, including the synthesis of collagen, carnitine, and norepinephrine; the metabolism of tryptophan, tyrosine, histamine, and cholesterol; the amidation of neuropeptides; and detoxification reactions in the liver

Antioxidant activity involving the transfer of two electrons between the ascorbate/dehydroascorbate redox couple or donation of one electron to inactivate highly reactive free radicals, e.g., protection of vitamin E by reduction of the tocopheryl radical

Competitive inhibition in substrate binding reactions, including inhibition of the formation of carcinogenic nitrosamines

Modulation of the gastrointestinal absorption of minerals including iron (3,15–18,22–24)

Vitamin C is well known for its role in the prevention of the deficiency disease scurvy (15,17–19). Its nonvitamin health promotion functions are still under investigation and remain controversial. They include the modulation of hypertension, ischemic heart disease, cancer, cataracts, immune responses, and the common cold (3,6,15,17,25–41).

C. Physicochemical Properties

From an analytical perspective, vitamin C's most important physicochemical properties are those that confer its notorious lack of stability. It is often used as an indicator of overall vitamin stability in foods because it is one of the most labile vitamins. In solution, AA and DHAA are readily oxidized, especially upon exposure to elevated temperatures, metal ions, dissolved oxygen, alkaline pH, light or naturally occurring degradative enzymes, such as ascorbic acid oxidase (15,17–19). The oxidation of AA to DHAA is reversible, but oxidation of DHAA is irreversible and produces biologically inactive 2,3-diketogulonic acid. Care must therefore be exercised throughout the extraction and analysis to prevent degradation and loss of vitamin C. Ascorbic acid is freely soluble in water and sparingly soluble in acetonitrile, acetic acid, and short-chain alcohols such

as methanol and ethanol (15,17). It is insoluble in ether, chloroform, benzene, petroleum ether, oils, and fats.

D. Methods of Analysis

1. *Extraction*

Vitamin C extraction procedures are governed by the need to prevent degradation and loss of the vitamers. The most popular extraction reagents are acids, which protect the C vitamers from oxidation and hydrolysis while precipitating the protein often associated with the endogenous C vitamers in foods (18,42,43). Most extractants contain metaphosphoric, trichloroacetic, or oxalic acid alone or in combination with other acids or short-chain alcohols such as methanol and ethanol. Additional protection against oxidation induced by metal cations such as iron and copper can be achieved using antioxidants and metal chelators such as EDTA and diethylenetriaminepentaacetic acid in the extraction solutions. Purging solutions with inert gases to reduce exposure to dissolved oxygen, conducting the extraction at low temperature, and reducing the exposure of sample solutions to white light will afford additional protection.

2. *Detection*

The detection of the individual C vitamers is complicated by their distinctly different properties. Although AA and DHAA are both ultraviolet (UV) absorbers, the absorbance maximum of DHAA is between 210 and 230 nm (15,18,42,43). For practical detection purposes, this makes DHAA particularly susceptible to interferences from a number of naturally occurring food constituents and limits the choice of reagents and solvents. In contrast, AA exhibits a pH-dependent absorbance maximum of 245–265 nm, which makes UV absorbance an ideal choice for detection. On the strength of its reducing capacity, AA can be detected electrochemically, but DHAA is electrochemically inactive. Neither AA nor DHAA fluoresce naturally. However, DHAA readily forms a fluorescent quinoxaline derivative upon reaction with *o*-phenylenediamine. As a result, chemical derivatization is often used to achieve the sensitivity needed to detect the naturally occurring vitamin C in food.

3. *HPLC Methodology Reviews*

Parviainen and Nyyssönen (18) reviewed selected methods for quantitating the C vitamers in foods, biological tissues, and pharmaceuticals, including paper chromatography, thin-layer chromatography (TLC), HPLC, and gas chromatography (GC) applications. Washko et al. (43) reviewed vitamin C analyses in foods and biological samples, and included a discussion of the principles, advantages, and pitfalls of various extraction protocols. In addition to HPLC, they covered colorimetric/spectrophotometric, fluorometric, GC, and enzymatic determinations. A summary of factors to consider when selecting an assay for vitamin C was also included. Russell (44) reviewed HPLC and flow injection methods for the C vitamers published between 1990 and 1994, including a brief discussion of standard or reference methods. Ball (45) reviewed HPLC methods published between the mid-1970s and the early 1990s. Other chromatographic and nonchromatographic chemical methods were also summarized.

A recent interlaboratory comparison of HPLC and fluorometric methods reported good agreement between laboratories for vitamin C in green beans (42).

4. *Recent Developments in HPLC Methodology*

Vitamin C continues to be the water-soluble vitamin upon which the bulk of the method development attention is focused. The HPLC methods published from 1992 to 1997 for total vitamin C

and the individual vitamers are summarized in Tables 1–5. Methods using C_{18} columns with UV absorbance detection continue to be very popular, although HPLC with fluorescence detection was preferred in a recent interlaboratory comparison (42). When using 4-hydroxyacetanilide (54) or α-methyl-L-DOPA (62) as an internal standard for ascorbic acid analyses, it should be noted the physicochemical properties of the internal standard and the analyte are significantly different. The use of a preliminary aqueous extraction (48) or maceration (56–58) prior to addition of a stabilizing acid may induce some degradation of the C vitamers, particularly in fresh produce that contains active ascorbic acid oxidase systems.

III. THIAMINE (VITAMIN B_1)

A. Occurrence

In decreasing order of concentration, yeast, meat (especially pork), cereal germs, whole cereal grains, fortified cereal and bakery products, nuts, and legumes are dietary sources of thiamine (19, 67). In general, the thiamine content of green vegetables, fruit, and seafood is low. Thiamine is absent from fats, oils, and refined sugar. In food, endogenous thiamine exists as four vitamers (67,68); the nonphosphorylated form, thiamine, and its phosphorylated esters, thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate (TTP) (Fig. 2). Thiamine predominates in plants, while the esters, especially TPP, are the principal forms in animal products. Much of the endogenous thiamine in food is bound to protein (67). Total thiamine is the sum of thiamine, TMP, TPP, and TTP.

B. Biological Activity

The diphosphate ester, TPP, is the physiologically active vitamer and functions as a coenzyme (67,68). The absorption, metabolism, and physiological functions of thiamine have recently been reviewed (20,67,68).

Biochemically, TPP acts as a coenzyme for several enzymes involved in carbohydrate and amino acid metabolism, including:

Decarboxylation of α-ketoacids such as pyruvate and α-ketoglutarate for the production of TCA or Krebs cycle intermediates;
Transketolase reactions leading via the pentose or hexose monophosphate shunt pathway of glucose oxidation to the eventual production of pentoses for RNA/DNA synthesis and NADPH for the biosynthesis of fatty acids
Involvement in nerve conductance or neural transmission, for which the exact mechanism is unknown (67,68)

Thiamine deficiency has been recognized for years as the cause of the deficiency disease beriberi (19,67,68). Its antipolyneuritic properties are also well known.

C. Physicochemical Properties

The thiamine vitamers are relatively stable in the dried state at low temperature in the dark (67–69). In solution, they are generally unstable at elevated temperatures or under alkaline conditions. Thiamine is stable to heat, including autoclaving, and oxidation below pH 5.0. It is most stable at pH 2–4. In solution, TPP is stable at pH 2–6 if it is stored at low temperature. The thiamine vitamers are also susceptible to degradation by endogenous thiaminase enzymes and other thiamine

Table 1 HPLC Methods for Quantitating Total Vitamin C in Foods (C_{18} Columns; Fluorescence Detection)

Analyte(s)	Type of food analyzed	Extraction conditions[a]	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
Total vitamin C	Canned vegetables; potato chips; fruit juice; infant formula	Metaphosphoric acid + acetic acid extraction; AA oxidized to DHAA using Norit; total vitamin C as DHAA derivatized with *o*-phenylenediamine	Analytical: μBondapak C_{18} (300 × 3.9 mm; Waters)	Isocratic: methanol + water (55+45, v/v) 1.0 ml/min.	Fluorescence; 350/430 nm (ex/em).	External standardization Linear range = 0.138–3.0 μg/ml Reproducibility: $CV \pm 1.67\%$ in infant formula, $\pm 1.08\%$ in canned corn ($n = 5$). LoD = 10 ng/100 μl injection. Good agreement with results from AOAC International microfluorometric method for all foods tested. Recoveries: 91–108%.	46
Total vitamin C	Cabbage; cauliflower; lettuce; onion; potato; tomato; sweet pepper	Trichloroacetic acid-octanol extraction; AA oxidized to DHAA using ascorbic acid oxidase; total vitamin C as DHAA derivatized with *o*-phenyldiamide	Analytical: Spherisorb (125 × 4 mm, 5 μm; Hewlett-Packard)	Isocratic: methanol + 80 mM potassium dihydrogen orthophosphate, pH 7.8 (20+80, v/v). 1.0 ml/min (47).	Fluorescence; 365/418 nm (ex/em).	External standardization. Recoveries: $99.4 \pm 0.1\%$ ($n = 2$) using fresh, raw potatoes.	48

[a] AA = L-ascorbic Acid; DHAA = dehydro-L-ascorbic Acid.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] n = number of determinations; LoD = limit of detection; CV = coefficient of variation.

Table 2 HPLC Methods for Quantitating C Vitamers in Foods (C_{18} Columns; UV Absorbance Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
AA, DHAA, and IAA simultaneously	Oranges; orange juice; beer; kiwi; tomato; camu-camu	Methanol + water (5 + 95 v/v) extraction; IAA added as internal standard when absent from sample; solid-phase extraction/cleanup with C_{18} Sep-Pak (Waters); derivatization of DHAA with 1,2-phenylenediamine hydrochloride	Precolumn: C_{18} (Waters). Analytical: μBondapak C_{18} (300 × 3.9 mm, 10 μm; Waters).	Isocratic: 5 mM cetrimide in methanol + 50 mM phosphate buffer, pH 4.59 (5 + 95 v/v). 1.8 ml/min.	UV absorbance; 261 nm for AA and IAA, 348 for DHAA (derivatized).	Internal standardization when IAA absent from sample, otherwise external standardization. Linear range = 0–20 mg/100 ml. Reproducibility: $CV \pm 0.80$–1.85% for DHAA standards ($n = 6$), ± 0.59–1.67% for AA standards ($n = 6$), ± 0.49–1.74% for IAA standards ($n = 6$).	49
AA & DHAA simultaneously	Citrus fruit juice; fresh fruit and vegetables (caramondin, mango, papaya, sweet pepper, tomato)	0.05 N phosphate buffer extraction; solid-phase extraction/cleanup using C_{18} Sep-Pak (Waters)	Analytical: Spheri-5 RP-18 (110 × 4.6 mm, 5 μm; Brownlee Labs) plus two Polypore H in series (110 × 4.6 mm + 220 × 4.6 mm; Brownlee Labs).	Isocratic: 2% potassium phosphate buffer, pH 2.3. 0.4 ml/min.	UV absorbance; 260 nm for AA. Differential UV absorbance spectrophotometry at 215 and 260 nm for DHAA.	External standardization. Reproducibility: $CV \pm 1.5\%$ for AA, ± 8.3% for DHAA ($n = 6$) using grapefruit. Recoveries: 91% for AA, 97% for DHAA in juice.	50
AA	Babaco; berries; currants; feijoa; passion fruit; tamarillos; medlar; persimmon	Metaphosphoric acid + acetic acid extraction	Precolumn: Newguard RP-18 (Brownlee Labs). Analytical: Spherisorb ODS (250 × 4.6 mm, 5 μm; Waters ?).	Isocratic: water acidified with sulphuric acid to pH 2.2. 0.4 ml/min.	UV absorbance; 254 nm.	External standardization. Linear calibration, $r = 0.9995$. Reproducibility: $CV \pm 1.7\%$ using babaco ($n = 10$). LoD = 0.8 mg/L. Recoveries: 94.8% (mean) in babaco.	51, 52
AA	Margarine; butter	Two procedures reported to be equally effective based on extraction into warm water or hexane	Analytical: Spherisorb-ODS (150 × 4.6 mm, 3 μm; Tracer Analytical).	Isocratic: water acidified with sulphuric acid to pH 1.95. 0.7 ml/min.	UV absorbance; 254 nm.	External standardization. Linear range = 5–100 μg/ml, r > 0.999. Recoveries: 96–101% ($n = 6$).	53

AA and IAA simultaneously	Fortified fruit juice	Metaphosphoric acid (2.5%) extraction; add 4′-hydroxyacetanilide as internal standard	Analytical: Spherisorb ODS (250 × 4 mm, 5 μm; Waters ?).	Isocratic: 5 mM cetyltrimethylammonium bromide in 50 mM potassium dihydrogen orthophosphate, pH 4. 1 ml/min.	UV absorbance; 254 nm.	Internal standardization. Linear range = 2–60 μg/ml juice, $r = 0.999$. Reproducibility: CV ± 3.5% for AA, ± 3.1% for IAA using fruit juice. Recoveries: 98.7% for AA, 98.9% for IAA in fruit juice ($n = 3$).	54
AA	Green beans	Metaphosphoric acid (4.5%) extraction	Precolumn: Newguard RP-18 (15 × 3.2 mm, 7 μm; Brownlee Labs). Analytical: Spherisorb ODS2 (250 × 4.6 mm, 5 μm; Waters ?).	Isocratic: water acidified with sulphuric acid to pH 2.2. 0.5 ml/min.	UV absorbance; 245 nm.	External standardization. Linear range = 0.5–4 mg/100 ml. Reproducibility: CV ± 1.2% using green beans ($n = 10$). LoD = 0.27 μg/ml (3 × standard deviation of the blank). Peak purity indices calculated from photodiode array spectrum indicated no coelution. Recoveries: 98.5%.	55
AA	Paprika	Maceration; metaphosphoric acid (4%) extraction	Analytical: Lichrosorb C_{18} (250 × 4.6 mm, 5 μm; Merck ?).	Isocratic: 0.75 mM tetrabutylammonium hydroxide in 10 mM phosphate buffer, pH 2.75 + methanol (97 + 3 v/v).	UV absorbance; 225 nm.	External standardization. Reproducibility: CV ± 2–9% using paprika ($n = 5$).	56, 57
	Tomatoes	Maceration; metaphosphoric acid (2%) extraction	Analytical: Lichrosorb C_{18} (250 × 4.6 mm, 10 μm; Merck ?).	Isocratic: 100 mM phosphate buffer, pH 2.75 + methanol + tetrabutylammonium hydroxide (97 + 3 + 0.05 v/v). 1 ml/min.	UV absorbance; 225 nm.	External standardization. Reproducibility: CV ± 2–3% using tomato ($n = 5$).	58

[a] AA = L-ascorbic acid; DHAA = dehydro-L-ascorbic acid; IAA = isoascorbic acid.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication; ? = column manufacturer not reported in original publication. Packing manufacturer suggested where possible.
[c] r = correlation coefficient; n = number of determinations; LoD = limit of detection; CV = coefficient of variation.

Table 3 HPLC Methods for Quantitating C Vitamers in Foods (C_{18} Columns; Electrochemical Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
AA and IAA simultaneously	Yeast	Metaphosphoric acid/cold perchloric acid extraction	Precolumn: ODS-10 (40 × 2.6 mm; Bio-Rad). Analytical: Spherisorb ODS-2 (250 × 4.6 mm, 5 μm; Rainin). 35°C.	Isocratic: 0.1 mM EDTA and 1.0 mM tetrabutylammonium phosphate in 0.08 M acetate buffer, pH 4.2 + methanol (95 + 5, v/v). No flow rate reported.	Electrochemistry; +0.72 V vs. Ag/AgCl reference electrode, glassy carbon working electrode.	External standardization. Linear range = 0–60 μg/g yeast (dry weight). Reproducibility: $CV \pm 2.3\%$ for IAA, ± 1.2% for AA. Recoveries: quantitative for both vitamers.	59
AA and IAA simultaneously; DHAA & DHIAA determined by difference	Processed meats; ground beef; applesauce; baby food (fruit, rice cereal, fruit juice, meat, vegetables); fruit juice; canned fruit; marmalade	AA + IAA: cold metaphosphoric acid extraction. Total AA + Total IAA: cold metaphosphoric acid extraction; DHAA & DHIAA reduced to AA and IAA with homocysteine.	Precolumn: Spheri-5-RP-18 (30 × 4.6 mm; Brownlee). Analytical: Three Supelcosil LC-18-DB (250 × 4.6 mm, 5 μm; Supelco ?) in series.	Isocratic: 5 mM tetrabutylammonium hydrogen sulphate and 0.15% metaphosphoric acid in 0.08 M acetate buffer, pH 5.4. 0.4 ml/min.	Amperometry; +0.6 V (oxidative) vs. Ag/AgCl reference electrode, glassy carbon working electrode.	External standardization. Nonlinear calibration curve, range = 0.5–2.5 ng. Reproducibility: $CV \pm 1.9\%$ for AA, ± 2.0% for IAA. LoD = 0.5 ng at SNR > 2. Recoveries: 94–107% for AA, 94–110% for IAA.	60, 61

AA and Total Vitamin C separately; DHAA determined by difference	Fruit juice (orange, apple, lemon, grape); Japanese tea	AA: Aqueous extraction; α-methyl-L-DOPA added as internal standard; AA stabilized by addition of 2% metaphosphoric acid. Total AA: aqueous extraction; α-methyl-L-DOPA added as internal standard; AA stabilized by addition of 2% metaphosphoric acid; DHAA reduced to AA using L-cysteine.	Analytical: Inertsil ODS-2 (150 × 4.6 mm, 5 μm; GL Sciences).	Isocratic: 1 mM ethylenediaminetetraacetic acid in 100 mM phosphate buffer, pH 3. 0.6 ml/min.	Electrochemistry; 0.3 V vs. Ag/AgCl reference electrode.	Internal standardization. Linear range = 0.1–10 ng AA. Reproducibility: CV ± 2.6–3.2% for AA, ± 2.1–3.2% for total AA using orange juice ($n = 3$). LoD ≈ 0.15 ng at SNR = 2. Recoveries: 92–100.5% for AA, 82% for Total AA using juices ($n = 3$).	62

[a] AA = L-ascorbic acid; DHAA = dehydro-L-ascorbic acid; IAA = isoascorbic acid; DHIAA = dehydroisoascorbic acid.

[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication; ? = column manufacturer not reported in original publication. Packing manufacturer suggested where possible.

[c] n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Table 4 HPLC Methods for Quantitating C Vitamers in Foods (Polymer Columns; Fluorescence Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification	Ref.
AA, DHAA, IAA, and DHIAA simultaneously; total vitamin C by addition (AA + DHAA)	Fresh and processed vegetables and fruit; fruit juice; ready-to-eat cereals; pizza; processed meat	Extraction with metaphosphoric acid/acetic acid containing EDTA; add IAA (or AA) as internal standard when absent from sample; cleanup as required with hexane to remove lipid material or *n*-butanol to remove starch	Precolumn: PLRP-S guard cartridge (Polymer Laboratories). Analytical: two PLRP-S in series (150 × 4.6 mm + 250 × 4.6 mm, 5 μm, 100-Å pore; Polymer Laboratories). 4°C.	Isocratic: 200 mM phosphate buffer, pH 2.14. 0.8 ml/min.	Online postcolumn reactions: AA and IAA oxidized to DHAA and DHIAA with $CuCl_2$; derivatization of DHAA and DHIAA with *o*-phenylenediamine. Fluorescence; 350/430 nm (ex/em).	Internal standardization with IAA or AA if absent from sample. External standardization if both AA and IAA are present in food. Linear range = 3–2,000 ng. Recoveries: 80–113% for total vitamin C (AA + DHAA) in a variety of food samples. Good agreement with results of AOAC International reference method.	63, 64

[a] AA = L-ascorbic acid; DHAA = dehydro-L-ascorbic acid; IAA = isoascorbic acid; DHIAA = dehydroisoascorbic acid.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.

Table 5 HPLC Methods for Quantitating Individual C Vitamers in Foods (Ion Exchange, Ion Exclusion, and Amino Columns; UV Absorbance Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
AA	Babaco	Aqueous extraction	Analytical: Lichrosorb-NH_2 (250 × 4.6 mm, 5 μm; Merck ?).	Isocratic: acetonitrile + 5 mM phosphate buffer, pH 3.5 (40 + 60 v/v). 1 ml/min.	UV absorbance; 268 nm.	External standardization. Linear range = 20–80 mg/L. Reproducibility: CV ± 5.14% using babaco. LoD = 4.4 mg/L. Recoveries: 65.9% (mean) in babaco.	51
AA and total vitamin C separately; DHAA determined by difference	Potatoes; strawberries	AA: Metaphosphoric acid extraction. Total AA: Metaphosphoric acid extraction; DHAA reduced to AA using 30 mM homocysteine.	Precolumn: Micro-Guard cation H^+. Analytical: Aminex HPX-87H (300 × 7.8 mm, 9 μm; Bio-Rad).	Isocratic: 4.5 mM sulphuric acid. 0.5 ml/min.	UV absorbance; 245 nm.	External standardization. Linear range = 1–20 ng/μl, r = 1.00. LoD = 1 ng/μl using a 10-μl injection. Reproducibility: CV ± 8.40%, for AA, ± 7.66% for Total AA in strawberries (n = 4). Recoveries: 96–99% for AA in potatoes and strawberries.	65
AA and total vitamin C separately; DHAA determined by difference	Orange juice	AA: Extraction with methanol + citric acid + EDTA. Total AA: extraction with methanol + citric acid + EDTA; DHAA reduced to AA with 0.8% homocysteine.	Zorbax NH_2 (250 × 4.6 mm, 5 μm; DuPont).	Isocratic: methanol + 0.25% phosphate buffer, pH 3.5 (40 + 60, v/v). 1.0 ml/min.	UV absorbance; 244 nm.	External standardization. Linear range = 1–100 mg/L. Recoveries: 99% for AA in orange juice.	66

[a] AA = L-ascorbic acid; DHAA = dehydro-L-ascorbic acid.
[b] Column specifications expressed as (length × id, particle size; manufacturer) when reported in original publication; ? = column manufacturer not reported in original publication. Packing manufacturer suggested where possible.
[c] r = correlation coefficient; n = number of determinations; LoD = limit of detection; CV = coefficient of variation.

Thiamine

Thiamine Monophosphate (TMP)

Thiamine Pyrophosphate (TPP)

Thiamine Triphosphate (TTP)

Fig. 2 Thiamine (vitamin B_1).

antagonists present in foods, such as raw fish, coffee, tea, and a variety of plants (67). Thiamine hydrochloride is readily soluble in water, partially soluble in alcohols and acetone, and insoluble in lipid solvents such as ether, benzene, hexane, and chloroform. The phosphate esters are soluble in water but insoluble in organic solvents.

D. Methods of Analysis

1. Extraction

The thiamine vitamers are normally determined as their thiochrome derivatives (42,67,68,70). Acid hydrolysis by boiling or autoclaving in 0.1–0.3 M hydrochloric acid or 0.05–0.1 M sulphuric acid for 10–60 min is usually employed to release the vitamers from the food matrix. For total thiamine determination, the phosphate esters are converted to thiamine using enzymatic hydrolysis with a phosphatase-containing enzyme such as claradiastase or takadiastase, alone or in combination with phosphatase. Typically the extract would be incubated for 1–18 hours at 37–50°C. Enzymatic hydrolysis has also been used to remove excess starch (β-amylase or amyloglucosidase) or protein (papain) in the food matrix. Ion-exchange column chromatography may be used for further cleanup of the extracts. The thiamine vitamers are oxidized with potassium ferricyanide under alkaline conditions to produce highly fluorescent thiochrome derivatives. Re-

moval of the derivatization reagents can be accomplished using solid-phase extraction or column chromatography with C_{18} or strong cation-exchange materials. Isobutanol can be used for selective extraction of thiochrome in the absence of derivatization reagents and the thiochrome phosphates (derivatized TTP, TPP, and TMP), which are insoluble in isobutanol.

2. *Detection*

Thiamine shows a pH-dependent UV absorbance range of 230–270 nm. However, its UV absorbance is prone to interference by other endogenous UV absorbers in foods, such as nucleic acids (67,68). In a recent interlaboratory comparison of thiamine methods (42), the results obtained from an HPLC method using UV absorbance detection were rejected due to the presence of peaks that interfered with thiamine. In the interests of increased sensitivity and selectivity, the thiamine vitamers are generally converted to their thiochrome derivatives by alkaline oxidation and determined fluorimetrically (42,70). The thiochrome derivatives of thiamine and its phosphate esters all fluoresce at nearly identical excitation (365–375 nm) and emission (425–435 nm) maxima at pH over 8. The thiochrome derivatives are all relatively stable in alkaline solution at pH greater than 9 and room temperature.

3. *HPLC Methodology Reviews*

Kawasaki (68) briefly reviewed HPLC methods for determining total thiamine alone and in combination with riboflavin. Russell (44) provided a more detailed summary of HPLC methods, published between 1990 and 1994, for thiamine alone and in conjunction with other vitamins. Ball (45) reviewed selected HPLC analyses for thiamine in various foods, as well as other chemical and microbiological assays.

Good agreement between laboratories was reported in two recent method intercomparison studies of HPLC, fluorometric, and microbiological assays for thiamine in milk powder, pork muscle, green beans, pork liver, mixed vegetables, and wholemeal flour (42,70). In general, the thiamine values obtained from microbiological assays tended to be slightly higher than those obtained by HPLC.

4. *Recent Developments in HPLC Methodology*

Recently developed HPLC methods determine thiamine either alone or concomitantly with other vitamins. Tables 6–10 review HPLC methods, published from 1992 to 1997, for the determination of total thiamine. All but one method (79) uses fluorescence detection of the thiochrome derivative. Those methods that determine thiamine simultaneously with other B vitamins are reviewed in Sec. XI of this chapter.

IV. RIBOFLAVIN (VITAMIN B_2)

A. Occurrence

Yeast, meat (especially organ meats), dairy products, milk, and eggs contain significant quantities of riboflavin (80–82). Foods derived from plants contain lower quantities of riboflavin; rapidly growing green, leafy vegetables are the best plant sources of this vitamin. Food contains three principal B_2 vitamers: riboflavin and its two coenzyme forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Fig. 3). The coenzymes are the predominant endogenous vitamers in foods and are usually bound to protein (81,82). Milk is the exception, containing free riboflavin as the predominant vitamer. Total riboflavin is the sum of riboflavin, FMN, and FAD.

Table 6 HPLC Methods for Total Thiamine in Foods (C_{18} Columns; Fluorescence Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total thiamine	Cheese; fortified, ready-to-eat breakfast cereal; pork; potato; wheat flour	Acid hydrolysis with 0.1 M hydrochloric acid; enzymatic hydrolysis of thiamine phosphates to thiamine with β-amylase and takadiastase; cheese protein precipitation with trichloroacetic acid; oxidation of thiamine to thiochrome with alkaline 0.25% potassium ferricyanide; C_{18} solid-phase extraction/cleanup.	Pre-column: guard column used—type not specified. Analytical: Novapak C_{18} (150 × 3.9 mm, 4 μm; Waters).	Isocratic: methanol + 50 mM phosphate buffer, pH 7.0 (30 + 70, v/v). 1.0 ml/min.	Fluorescence; 445/522 nm (ex/em).	External standardization. Linear range = 1.5–25 ng/injection (10 μl). LoD = 10 pg/30 fmol thiamine as thiochrome at SNR ≥ 2. Reproducibility: $CV \pm 3.4\%$ for thiochrome. Recoveries: 90–105% TPP as total thiamine from cheese, cereal, pork, and flour.	71
Total thiamine	Bread; flour; cereal; potato; meat; milk	Acid hydrolysis with 0.1 N hydrochloric acid at 125°C for 15 min (autoclaved); enzymatic hydrolysis of thiamine phosphates to thiamine with claradiastase at pH 4.0–4.5 and 50°C for 3 h; protein precipitation with trichloroacetic acid; oxidation of thiamine to thiochrome with alkaline 0.1% potassium ferricyanide; C_{18} solid-phase extraction/cleanup. Extraction carried out under dimmed lights.	Analytical: Bondapak C_{18} radial-pak cartridge (100 × 8 mm, Waters). 30°C.	Isocratic: 5 mM phosphate buffer, pH 7.0 + methanol (65 + 35, v/v). 0.8 ml/min.	Fluorescence; 360/425 nm (ex/em).	External standardization. Linear range 0–50 μg thiamine, $r = 1.000$. Reproducibility: $CV \pm 5.5\%$ for thiamine using rye flour ($n = 60$). Recoveries: 85–104% thiamine as total thiamine from bread, cereal, flour, potato, meat, and milk. Samples spiked before autoclaving.	72–75

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] r = correlation coefficient; n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Table 7 HPLC Methods for Quantitating Total Thiamine in Foods (Phenyl Columns; Fluorescence Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification	Ref.
Total thiamine	Milk	Enzymatic hydrolysis of protein with trypsin and thiamine phosphates to thiamine with claradiastase; oxidation of thiamine to thiochrome using ferricyanide (derivatization stopped with sodium sulphite); thiochrome extracted with 1-butanol	Analytical: Nucleosil Phenyl (150 mm, 5 μl; Macherey-Nagel).	Isocratic: methanol + acetonitrile + iso-butanol + water (80 + 10 + 10 + 5 v/v/v/v). 0.7 ml/min.	Fluorescence; 375/430 nm (ex/em).	External standardization. Recoveries: 95% thiamine as thiochrome from milk.	76

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.

Table 8 HPLC Methods for Quantitating Total Thiamine in Foods (TMS Columns; Fluorescence Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total thiamine	Rice	Extraction by refluxing at 60°C for 30 min in 0.1 M hydrochloric acid + methanol (60 + 40 v/v); homogenization; centrifugation	Analytical: Zorbax TMS (250 × 4.6 mm; DuPont). 55°C.	Isocratic: phosphate-perchlorate buffer, pH 2.5. 0.4 ml/min.	Online postcolumn derivatization with alkaline potassium hexacyanoferrate (III) to convert thiamine to thiochrome. Fluorescence; 375/435 nm (ex/em).	External standardization. Linear range = 0–35 ng thiamine; $r = 0.999$. Recoveries: 94–101% thiamine as thiochrome from brown rice. Reproducibility: $CV \pm$ 2–8% for total thiamine as thiochrome in 6 varieties of rice ($n = 6$). Good agreement between results by HPLC and AOAC International methods ($r = 0.958$).	77

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] r = correlation coefficient; n = number of determinations; CV = coefficient of variation.

Table 9 HPLC Methods for Total Thiamine in Foods (Anion Exchange and NH_2 Columns; Fluorescence Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total thiamine	Fortified, ready-to-eat breakfast cereal; cereal bars; dessert; yeast	Extraction with hydrochloric acid–perchloric acid; hydrolysis of thiamine phosphates to thiamine with takadiastase; oxidation of thiamine to thiochrome using alkaline 1% $HgCl_2$; thiochrome extracted with isobutanol.	Analytical: Lichrosorb NH_2 (250×4.6 mm, 5 μm; SFCC, France).	Isocratic: methanol + dichloromethane (10 + 90, v/v). 1.0 ml/min.	Fluorescence; 365/440 nm (ex/em).	External standardization. Linear range = 12–200 ng TPP/ml. Good agreement with results from microbiological method.	78

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] TPP = thiamine pyrophosphate.

Table 10 HPLC Methods for Total Thiamine in Foods (Anion Exchange and NH_2 Columns; UV Absorbance Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total thiamine	Baby food; cereals; cookies	Acid hydrolysis with 0.1 M hydrochloric acid at 100°C for 30 min; enzymatic hydrolysis of thiamine phosphates to thiamine with takadiastase at 47°C for 3 h; weak ion-exchange (methylcarboxylatein, acid form) solid-phase extraction/cleanup	Analytical: Lichrospher 100 RP-18 (125 × 4 mm, 5 μm; Merck ?).	Isocratic: methanol + 10 mM phosphate buffer, pH 2.8 containing 5 mM sodium hexanesulphonate + triethylamine (15 + 84.9 + 0.1, v/v/v). 1.0 ml/min.	UV absorbance; 254 nm.	External standardization. Linear range = LoD to 7 μg/ml thiamine, $r = 0.9995$. Reproducibility: $CV < 3\%$ using food samples ($n = 10$). Recoveries: 92.1–96.0% thiamine using baby food ($n = 3$). Samples spiked before hydrolysis.	79

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication; ? = column manufacturer not reported in original publication. Packing manufacturer suggested where possible.
[b] r = correlation coefficient; n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Riboflavin

Flavin Mononucleotide (FMN)

Flavin Adenine Dinucleotide (FAD)

Fig. 3 Riboflavin (vitamin B_2).

B. Biological Activity

The coenzymes, FMN and FAD, are the physiologically active vitamers. The biochemistry, absorption, metabolism, and physiological functions of riboflavin have been reviewed (80,81).

Biochemically, FMN and FAD act as cofactors, i.e., as covalently bound prosthetic groups, or as noncovalently bound coenzymes in a variety of biological oxidation–reduction reactions, such as:

Conversion of glucose to gluconic acid and hydrogen peroxide catalyzed by glucose oxidase
Oxidation of α-amino acids to keto acids catalysed by D- and L-amino acid oxidases
Oxidation of NADH via the cytochrome system catalyzed by cytochrome reductase
Energy production via the TCA or Krebs cycle catalyzed by succinate dehydrogenase
Fatty acid oxidation catalyzed by acyl-coenzyme A dehydrogenases
Synthesis of fatty acids from acetate (80,81)

FMN and FAD participate in one- and two-electron transfers and can react directly with molecular oxygen. As a result, flavoproteins are essential for the metabolism of lipids and carbohydrates; they are required for the conversion of pyridoxine and folic acid to their coenzyme forms; and they act as intermediates in the TCA or Krebs cycle.

Riboflavin deficiency is not associated with a specific disease per se (19,80,81). However, a lack of riboflavin in the diet produces changes in the eye, including photophobia, corneal opacity and ulceration, presenile cataracts, circumcorneal infections, and reduced tearing, as well as skin lesions, especially around the mouth, nose, and ears.

C. Physicochemical Properties

From an analytical perspective, the single most important physicochemical characteristic of riboflavin is its photosensitivity (80–82). Exposure of this vitamin to ultraviolet and visible light results in irreversible photoreduction to lumiflavin and lumichrome and loss of vitamin activity. In addition, the coenzymes are subject to hydrolysis by endogenous phosphatases that are present in a number of foods. Since these enzymes are generally inactivated by thermal processing, they are a concern only in the analysis of fresh products.

All of the B_2 vitamers are unstable at alkaline pH; the coenzymes are also degraded below pH 5.0 (80–82). As a result, analysis of the endogenous coenzymes needs to be carried out at pH 5.0–7.0, while total riboflavin (as riboflavin) is best determined at acidic pH. Riboflavin is soluble in water and short-chain alcohols, but insoluble in ether, chloroform, and acetone. The coenzymes are water soluble.

D. Methods of Analysis

1. Extraction

Due to their photosensitivity, the quantitation of the riboflavin vitamers must be carried out under subdued light using low-actinic glassware (82,83). For total riboflavin analysis, the coenzymes are first released from the protein to which they are bound and then hydrolyzed to riboflavin. This is usually accomplished with dilute mineral acids (0.1–0.3 M hydrochloric acid or 0.05–0.1 M sulphuric acid) at 100–121°C (boiling or autoclaving) for 15–60 minutes, with or without enzymatic hydrolysis of the coenzymes (19,42,70,80–82). Enzymatic hydrolysis involves the use of claradiastase or takadiastase alone or in combination with trypsin or papain at 37–50°C for 1–18 hours. Variability in the activity and specificity of takadiastase from different sources has been reported to affect total riboflavin analyses (42). Milder extraction conditions are required for the analysis of the endogenous coenzyme vitamers. Less aggressive conditions used for these purposes include (a) a shorter hydrolysis time with cold trichloroacetic acid in the absence of enzymatic hydrolysis, or (b) a two-step extraction using aqueous buffer and methanol.

Solid-phase extraction is routinely used to clean up extracts prior to quantitation (19,42,70, 80–82). Alternatively, endogenous fluorescent artifacts in food samples can be eliminated by oxidation with potassium permanganate/hydrogen peroxide/sodium metabisulphite. Benzyl alcohol has been used to extract riboflavin selectively without the coenzymes, permitting the determination of free riboflavin.

2. Detection

The riboflavin vitamers all have similar UV absorbance spectra. They all fluoresce naturally, although the quantum yield of FAD's fluorescence is 10 times less than that of riboflavin and FMN

(19,42,70,80–82). While both UV absorbance and fluorescence detection are feasible for these vitamers, fluorescence is generally the method of choice due to its greater sensitivity.

3. *HPLC Methodology Reviews*

Three recent reviews specifically cover HPLC methods for quantitating riboflavin in foods. In addition to HPLC methods, Nielsen (81) summarized paper chromatography, TLC, and open-column chromatography procedures for quantitating total riboflavin and the individual vitamers in foods, pharmaceuticals, and biological samples. Russell (44) included a brief discussion of the standard methods, along with HPLC and flow injection analyses published between 1990 and 1994 for total riboflavin and the individual vitamers in foods. Ball (45) reviewed HPLC methods for quantitation of riboflavin, as well as chemical and microbiological riboflavin assays for foods.

A recent interlaboratory comparison of HPLC and microbiological methods for total riboflavin revealed significant variability between the 13 participating laboratories (42). The extraction and hydrolysis of the riboflavin coenzymes were cited as the most likely sources of this variability. A later intercomparison (70) of riboflavin methods showed lower variability between laboratories, although coefficients of variability (*CV*) of 12–40% were still reported.

4. *Recent Developments in HPLC Methodology*

The HPLC methods published from 1992 to 1997 for total riboflavin and the individual vitamers are summarized in Tables 11–13. The simultaneous quantitation of riboflavin with one or more other vitamins is covered in Sec. XI of this chapter.

There have been reports of significant concentrations of isomeric artifacts in the commercial coenzyme standards (83–87). Because the coenzymes are the predominant vitamers in most foods, failure to account for these impurities during quantitation of the coenzymes could result in significant analytical errors. Several methods have been suggested to compensate for the lack of purity in the commercial coenzyme preparations (83–85,88,89).

V. NIACIN (VITAMIN B_3)

A. Occurrence

In general, niacin is widespread in foodstuffs (93,94). Cereals, seeds, meat, and fish are good food sources of niacin. The niacin content of coffee beans is primarily a product of the roasting process, which converts trigonelline (1-methylnicotinic acid) to nicotinic acid (95).

The niacin vitamers in foods include nicotinic acid and nicotinamide (Fig. 4), which occur in limited quantities in the free form, and their coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (93,96). The nicotinic acid analog of NAD as well as nicotinamide and nicotinic acid mononucleotides also occur in nature. In addition, niacin occurs as nicotinyl esters bound to polysaccharides, peptides, and glycopeptides, which are known as niacytin and niacynogens, respectively. In general, the niacin vitamers in cereal grains and other seeds are principally the nicotinic acid forms, whereas those in meat and fish are primarily the nicotinamide forms (94,95).

Humans can convert ingested tryptophan to niacin in vivo (19,93,96). Therefore, the niacin activity of food can be expressed in "niacin equivalents," which is the sum of the niacin content (nicotinic acid vitamers + nicotinamide vitamers) plus 1/60 of the tryptophan content (in mg). This section will be restricted to analyses for the niacin vitamers. Tryptophan methodology will not be covered.

Table 11 HPLC Methods for Quantitating Total Riboflavin in Foods (C_{18} Columns; UV Absorbance Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total vitamin B_2 as riboflavin	Ready-to-eat baby food (vegetables, milk, meat, liver, and/or fish)	Extraction by autoclaving with 0.1 M hydrochloric acid at 103.5 kPa for 20 min; enzymatic hydrolysis with claradiastase (B_2 coenzymes) and papain (protein) at pH 4.5 for 20 h at 37°C; protein precipitation with trichloroacetic acid at 100°C for 10 min; C_{18} solid-phase extraction/cleanup.	Precolumn: Nucleosil C_{18} (50 × 4.6 mm, 10 μm; Macherey-Nagel ?). Analytical: Nucleosil C_{18} (250 × 4.6 mm, 5 μm; Macherey-Nagel ?) at 41°C.	Isocratic: acetonitrile + 0.01 M potassium phosphate buffer, pH 7.0 (10.5 + 89.5 v/v). 1.0 ml/min.	UV absorbance; 268 nm.	External standardization. Linear range = 0.05–0.5 μg/ml, $r = 0.998$ LoD = 30 ng/ml at SNR = 3. Reproducibility: $CV \pm 10.5\%$ in foods tested ($n = 4$). Recoveries: 90 ± 7% from foods tested ($n = 4$). Good agreement with results from microbiological method ($r = 0.987$).	90
Total vitamin B_2 as riboflavin	Milk; nondairy imitation milk	Protein precipitation and extraction with 10% lead acetate at pH 3.2.	Precolumn: C_{18} Bondapak guard (5 μm; Waters ?). Analytical: Spherisorb ODS 2 (150 × 3.9 mm, 5 μm; Waters ?).	Isocratic: methanol + dilute acetic acid (30 + 70 v/v). 1.5 ml/min.	UV absorbance; 270 nm.	External standardization. Linear range = 0.0–50.0 μg, $r = 0.99732$). Reproducibility: $CV \pm$ 1.6–5.5% in foods tested ($n = 6$). Recoveries: 92.20% from milk ($n = 2$).	91

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication; ? = column manufacturer not reported in original publication. Packing manufacturer suggested where possible.
[b] r = correlation coefficient; n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Table 12 HPLC Methods for Total Riboflavin in Foods (C_{18} Columns; Fluorescence Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total riboflavin	Bread; flour; cereal; potato; meat; milk	Acid hydrolysis with 0.1 N hydrochloric acid at 125°C for 15 min (autoclaved); enzymatic hydrolysis of B_2 coenzymes with claradiastase at pH 4.0–4.5 and 50°C for 3 h; protein precipitation with trichloroacetic acid; addition of alkaline 0.1% potassium ferricyanide for concomitant extraction of thiamine; C_{18} solid-phase extraction. Extraction carried out under subdued lighting.	Analytical: Bondapak C_{18} Radial-pak cartridge (100 × 8 mm, Waters). 30°C.	Isocratic: 5 mM phosphate buffer, pH 7.0 + methanol (65 + 35, v/v). 0.8 ml/min.	Fluorescence; 440/520 nm (ex/em).	External standardization. Linear range = 0–40 μg riboflavin, r = 1.000. Reproducibility: $CV \pm 10\%$ for thiamine using rye flour (n = 60). Recoveries: 80–96% riboflavin as total riboflavin from bread, cereal, flour, potato, meat, and milk. Samples spiked before autoclaving.	72–74
Total riboflavin	Casein	Enzymatic hydrolysis with pepsin in 20 mM hydrochloric acid at 37°C for 24 h; enzymatic hydrolysis of B_2 coenzymes to riboflavin with takadiastase at pH 5.0–5.4 and 45°C for 18 h. Extraction carried out under subdued lighting.	Precolumn: ODS direct connect cartridge (30 × 3.6 mm, 5 μm; Alltech). Analytical: Spherisorb ODS (25 × 4.6 mm, 5 μm; Alltech).	Isocratic: methanol + 50 mM acetate buffer, pH 5 (35 + 65, v/v).	Fluorescence; 447/517 nm (ex/em).	External standardization. LoD = 0.06 mg/kg. Reproducibility: $CV \pm 3\%$ (n = 10). Recoveries: = 90–92% riboflavin as total riboflavin from casein (n = 10).	92

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] r = correlation coefficient; n = number of determinations; LoD = limit of detection; CV = coefficient of variation.

Table 13 HPLC Methods for Quantitating B_2 Vitamers in Foods (Polymer-Based Columns; Fluorescence Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
Riboflavin, FMN, and FAD simultaneously	Liver; beef; chicken breast; milk; eggs; ready-to-eat breakfast cereal; fast-food hamburger	Addition of 7-ethyl-8-methyl riboflavin as internal standard; extraction with methanol, methylene chloride and 100 mM phosphate buffer, pH 5.5; centrifugation. Extraction carried out under yellow light.	Precolumn: PLRP-S (5 × 3 mm; Polymer Labs). Analytical: Two PLRP-S 100-Å columns in series (150 × 4.6 mm + 250 × 4.6 mm, 5 μm; Polymer Labs). 40°C.	**A**: acetonitrile. **B**: 0.1 sodium azide in 10 mM citrate-phosphate buffer, pH 5.5. Gradient: • **A** + **B** (3 + 97, v/v) at $t = 0$ min; 1.2 ml/min. • linear gradient to **A** + **B** (6 + 94, v/v) at $t =$ 43 min; 1.2 ml/min. • linear gradient to **A** + **B** (14 + 86, v/v) at $t = 51$ min; 1.0 ml/min. • isocratic at **A** + **B** (14 + 86, v/v) until $t = 70$ min; 1.0 ml/min. • linear gradient to **A** + **B** (3 + 97, v/v) at $t =$ 80 min; 1.0 ml/min. • isocratic at **A** + **B** (3 + 97, v/v) until $t = 90$ min; convex flow rate gradient to 1.2 ml/min at $t =$ 90 min.	Fluorescence; 450/522 nm (ex/em).	Internal standardization using 7-ethyl-8-methyl riboflavin. LoD = 0.21 ng for riboflavin; 0.89 ng for FMN; 11.15 ng for FAD at SNR = 3. Reproducibility: CV ± 0.5–3.0% (n = 3). Recoveries: 92–115% from all foods tested. Good agreement with results from AOAC International fluorometric method, except for eggs, breakfast cereal, and fast-food hamburger.	83

[a] FMN = flavin mononucleotide; FAD = flavin adenine dinucleotide.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Nicotinic Acid

Nicotinamide

* Phosphate group in Nicotinamide Adenine Dinucleotide Phosphate (NADP)

Nicotinamide Adenine Dinucleotide (NAD)

Fig. 4 Niacin (vitamin B_3).

B. Biological Activity

The physiologically active forms of niacin are nicotinic acid, nicotinamide, and their coenzymes (93,96). Niacytin and the niacynogens appear to have limited bioavailability, although more work is needed in this area. The absorption and metabolism of niacin has been reviewed (20,93).

Biochemically, the niacin coenzymes function as cofactors for a number of dehydrogenases due to their oxidation–reduction capabilities (19,93,96). They are involved in the metabolism of carbohydrates, fatty acids, and amino acids. Nicotinamide can also participate in nonredox reactions, such as the ribosylation of ADP.

Niacin deficiency is known to produce pellagra, which presents dermatological, gastrointestinal, and neurological symptoms (19,93,96). Niacin has also been implicated in several nonvitamin functions, which are still under investigation. These include the reduction of serum cholesterol levels, vasodilation, and modulation of the effects of cancer and diabetes (93,96–99).

C. Physicochemical Properties

Niacin is one of the more stable water-soluble vitamins. Both nicotinic acid and nicotinamide are stable in air at ambient temperature (93,96). Aqueous solutions of nicotinic acid or nicotinamide can be autoclaved for short periods, e.g., 10 minutes at 120°C, without degradation. Nicotinic acid's stability in solution is independent of pH, but nicotinamide is stable only at neutral pH. Heating nicotinamide in 1 N acid or alkali at 100°C will induce its conversion to nicotinic acid.

Both nicotinic acid and nicotinamide are soluble in water, short-chain alcohols, and glycerol. Nicotinamide is sparingly soluble in diethyl ether; nicotinic acid is insoluble.

The niacin coenzymes are water soluble, but their stability is pH dependent (96). The reduced forms, NADH and NADPH, are stable in alkali but are rapidly decomposed in weak mineral acid solutions. In contrast, the oxidized forms, NAD and NADP, are unstable under alkaline conditions but are stable in acid.

D. Methods of Analysis

1. *Extraction*

Due to the relative stability of the niacin vitamers, either acid or alkaline hydrolysis can be used to convert nicotinamide to nicotinic acid for quantitation of both vitamers as nicotinic acid (9,44). Acid hydrolysis is used to quantitate biologically available niacin. Alkaline hydrolysis releases both the biologically available and the unavailable vitamers and provides an estimate of the total niacin content. Because alkaline hydrolysis is much faster than acid hydrolysis, the latter is usually supplemented with enzymatic hydrolysis. The most common enzymes are takadiastase, papain, and clarase. On occasion, organic solvents such as methanol have been used to extract free nicotinic acid.

Nicotinamide extraction requires more specialized conditions than nicotinic acid due to its relative instability. Aqueous extractants and dilute sulphuric or hydrochloric acid have been used to release the vitamers from the food matrix without degrading nicotinamide (9,44).

2. *Detection*

The niacin vitamers are UV absorbers and are detected accordingly (9,96).

3. *HPLC Methodology Reviews*

Several recent reviews cover the HPLC quantitation of niacin in foods. In addition to HPLC methods, Shibata and Shimono (96) summarized paper chromatography, thin-layer chromatography, open-column chromatography, and GC procedures for the determination of niacin in foods, pharmaceuticals, and biological samples. Russell (44) included a brief description of the reference methods along with selected HPLC methods published between 1990 and 1994 for the analysis of niacin in foods. Ball (45) summarized selected HPLC methods for niacin in foods as well as microbiological assays, other chromatographic methods, and nonchromatographic chemical analyses.

All but one of the seven laboratories that recently participated in a intercomparison of niacin assays chose to use microbiological assays (42). The results from the lone HPLC determination/UV absorbance determination were rejected due to lack of chromatographic resolution.

4. *Recent Developments in HPLC Methodology*

Although microbiological methods are widely used for quantitation of total niacin, they tend to be time consuming and labor intensive (100). Reproducibility problems have also been reported (96). The HPLC methods for foods generally determine total niacin rather than the individual vitamers. Table 14 summarizes a recent HPLC method for quantitating total niacin in foods. The simultaneous determination of niacin with one or more other B vitamins is covered later in this chapter in Sec. XI.

Table 14 HPLC Methods For Quantitating Total Niacin in Foods (Ion Exchange and NH_2 Columns; UV Absorbance Detection)

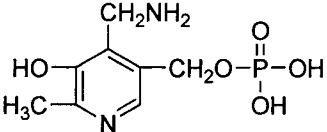
Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total niacin as nicotinic acid	Fortified foods (pasta, bread, ready-to-eat breakfast cereals, infant formula); unfortified foods (beef soup, tuna)	Sulfuric acid extraction at 121–123°C (autoclave) for 45 min; pH adjustment; Florisil solid-phase extraction/cleanup	Analytical: PRP-X100 (250 × 4.1 mm; Hamilton).	Isocratic: dilute acetic acid. 1.5 ml/min.	UV absorbance; 254 nm.	External standardization. Linear range = 0.24–0.80 μg nicotinic acid/ml. LoD = 0.11 μg nicotinic acid/ml. Reproducibility: $CV \pm 2.4\%$ for nicotinic acid in whey-based infant formula; ± 2.9% for nicotinic acid in macaroni ($n = 10$). Recoveries: 99.8 ± 7.7% from foods tested ($n = 15$). Results from microbiological method averaged 11% higher than those by HPLC.	101

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] n = number of determinations; LoD = limit of detection; CV = coefficient of variation.

Pyridoxine (PN)

Pyridoxine Phosphate (PNP)

Pyridoxal (PL)



Pyrodixal Phosphate (PLP)

Pyridoxamine (PM)

Pyridoxamine Phosphate (PMP)

Fig. 5 Vitamin B_6 (pyridoxine).

VI. PYRIDOXINE (VITAMIN B_6)

A. Occurrence

The six principal B_6 vitamers are widely distributed in foods (102,103). They include pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their 5′-phosphate esters, pyridoxine phosphate (PNP), pyridoxal phosphate (PLP), and pyridoxamine phosphate (PMP) (Fig. 5). The predominate B_6 vitamer in animal-based foods is PLP, whereas plant products generally contain PN and PM or their phosphorylated forms. Conjugated vitamers in the form of PN-glycosides have also been isolated from plant-based foods. Pyridoxal is readily converted to PM during cooking and food processing. Total vitamin B_6 is the sum of the six principal vitamers; inclusion of the conjugated forms depends on the extraction procedure.

B. Biological Activity

The conjugated forms of vitamin B_6 appear to be less available than the six principal vitamers (102,104–106); a relative value of 58% has recently been suggested for PN-glycoside in humans (107). In addition, food processing and storage can induce changes that reduce the bioavailability of vitamin B_6 (102). Thermal processing and low moisture conditions can induce reductive

binding between PL/PLP and the ε-amino groups of lysyl residues in protein and peptides. Storage of heat-treated milk may result in the formation of a disulphide derivative. The extent to which food processing induces the conversion of PN to 6-hydroxy-PN in the presence of ascorbic acid remains unclear. More detailed information on the absorption, metabolism, and bioavailability of vitamin B_6 is available from recent reviews (20,102).

Biochemically, PLP is the coenzyme form of vitamin B_6. As such it participates in many enzymatic reactions involved in amino acid biosynthesis and catabolism. Specific examples include:

1-Carbon metabolism leading to nucleic acid synthesis
Gluconeogenesis via transamination and glycogen phosphorylase reactions
Heme synthesis
Tryptophan metabolism and niacin formation
Synthesis of neurotransmitters and brain function via decarboxylase reactions (58,102)

Despite its considerable involvement in metabolic processes, no specific deficiency syndrome in humans has been attributed to vitamin B_6 (19,103). A considerable number of nonvitamin functions have been suggested, but they remain controversial (102,103,108–111). These include roles in coronary heart disease, immune response, premenstrual syndrome, sickle-cell anaemia, asthma, autism, gestational diabetes, carpal tunnel syndrome, and cancer.

C. Physicochemical Properties

Vitamin B_6 analysis is complicated by its occurrence as six vitamers of differing structures. The physicochemical properties of greatest significance for the analyst are those that define the instability of the B_6 vitamers (102,103). In general, vitamin B_6 is considered light-sensitive, although the extent of the photosensitivity is influence by pH. Analyses should therefore be carried out under subdued lighting using low-actinic glassware. The nonphosphorylated vitamers are heat stable under acidic conditions but are unstable when heated under alkaline conditions. For analytical purposes, the B_6 vitamers are water soluble but are insoluble in organic solvents. This excludes the use of organic solvents for sample extraction or extract cleanup/enrichment prior to analysis.

D. Methods of Analysis

1. *Extraction*

The total vitamin B_6 content of foods has traditionally been quantitated following extraction at elevated temperatures (autoclaving) in mineral acids, such as 0.0275–0.44 M sulphuric acid or 1 M hydrochloric acid (42,70,103). This results in hydrolysis of both the phosphorylated vitamers and the glycosylated forms. Since the glycosylated forms exhibit significantly lower bioavailability than the nonconjugated vitamers, the result is an overestimation of the available vitamin B_6 content. Milder conditions, such as the use of perchloric, trichloroacetic, or sulfosalicylic acid, are needed for nondegradative extraction of the six principal vitamers. However, the extraction efficiencies of these acids are variable; sulfosalicylic acid must be removed prior to analysis because it interferes with the fluorescence of the B_6 vitamers. Enzymatic hydrolysis with takadiastase or phosphatase has been used to convert the phosphorylated vitamers to PN, PM, and PL. The glycosylated vitamers can be determined either indirectly after hydrolysis with β-glycosidase or amyloglucosidase to release the bound vitamer or directly with HPLC separation and fluorescence detection.

2. *Detection*

Detection of the B_6 vitamers is complicated by the low levels at which they occur in foods (102,103). The sensitivity and specificity of the detection methods is therefore critical. All of the principal B_6 vitamers are UV absorbers (70). Although their spectra are similar in 0.1 M hydrochloric acid, this is not the case at higher pH. These vitamers fluoresce naturally in slightly acidic to neutral solution and under strongly alkaline conditions (42,70). However, the individual vitamers exhibit some qualitative dissimilarities in their fluorescence spectra and significant differences in the intensities of their quantum fluorescence response; PLP is significantly less fluorescent than the other five vitamers. In general, fluorescence is the preferred method of detection, due to its increased sensitivity and specificity relative to UV absorbance. Derivatization has been used to enhance and standardize the fluorescence properties of the B_6 vitamers. Detailed reviews of the spectral properties of the B_6 vitamers have been published (102,103).

3. *HPLC Methodology Reviews*

Several recent reviews include HPLC methods for vitamin B_6 in foods. Ubbink (103) reviewed HPLC, thin-layer chromatographic, and gas chromatographic methods for quantitating vitamin B_6 in foods, pharmaceuticals, and biological samples. Russell (44) summarized selected methods published between 1990 and 1994 for quantitating total vitamin B_6 and the B_6 vitamers in foods. Ball (45) summarized selected HPLC determinations for vitamin B_6 in foods, as well as other chromatographic methods, nonchromatographic chemical analyses, and microbiological assays.

Recent interlaboratory comparisons of HPLC and microbiological methods for vitamin B_6 revealed significant variability among laboratories (42,70). The extraction and hydrolysis of the B_6 vitamers, especially the pyridoxine-β-glucoside (PNG) in plant-based foods, were cited as problem areas. Other sources of analytical error included HPLC (mis)identification of the individual B_6 vitamers, and vitamer interconversion during extraction and analysis.

4. *Recent Developments in HPLC Methodology*

Total vitamin B_6 has traditionally been assayed microbiologically (112). The HPLC methods have recently gained popularity as a result of their ability to distinguish between individual vitamers, including the glycosylated forms. Recent publications include both HPLC methods for total vitamin B_6 in foods and those for the individual vitamers (Tables 15–17). Much of the recent work has been dedicated to the direct determination of the glycosylated forms in the presence of the other vitamers. The simultaneous determination of vitamin B_6 with one or more other vitamins is summarized later in Sec. XI.

VII. FOLACIN

A. Occurrence

Folacin occurs in a wide variety of foods, including green leafy vegetables, liver, yeast, beans, dairy products, and eggs (120,121).

Folacin consists of a large group of related compounds having the same basic structure as folic acid, or pteroylglutamic acid (PteGlu), but differing in the state of reduction and the number of glutamate residues (1–9+) (Fig. 6) (120–124). In theory, there are approximately 150 folacin vitamers, which can be divided into three basic groups: the oxidized monoglutamate forms, such as folic acid (PteGlu), and the two principal reduced forms, 5,6-dihydrofolate ($H_2PteGlu_n$) and 5,6,7,8-tetrahydrofolate ($H_4PteGlu_n$). In addition, the N-5 and N-10 positions of the tetra-

Table 15 HPLC Methods for Quantitating Total Vitamin B_6 in Foods (C_8/C_{18} Columns; Fluorescence Detection)

Analyte(s)	Type of food analyzed	Extraction conditions[a]	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
Total vitamin B_6 as pyridoxol	Unfortified foods (yeast, wheat germ, ready-to-eat breakfast cereal)	Extraction with 50 mM acetate buffer, pH 4.5; hydrolysis of phosphate esters with acid phosphatase at 37°C overnight; deamination of PM to PN using 1 M glyoxylic acid + ferrous sulfate catalyst at 37°C overnight; reduction of PN to PL with alkaline sodium borohydride	Precolumn: RP-18 guard column (10 μm; Merck). Analytical: Lichrospher 60 RP Select B (C_8) (250 × 5 mm, 5 μm; Merck).	Isocratic: acetonitrile + 50 mM phosphate buffer containing 5 mM sodium heptanesulfonate (4 + 96 v/v), pH 2.50. 1.0 ml/min.	Fluorescence; 290/395 nm (ex/em).	External standardization. Linear range = 0–405 μg PL/g. LoD = 0.02 μg PL/g. Reproducibility: $CV \pm$ 2.2–7.9% for foods tested ($n = 6$). Recoveries: 90–95% for PL, PM, and PN from foods tested. Good agreement with results from microbiological method. Collaboratively tested in 12 laboratories using 8 samples; reproducibility: $CV \pm$ 12–35%.	113, 114

[a] PN = pyridoxine; PM = pyridoxamine; PL = pyridoxal.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] n = number of determinations; LoD = limit of detection; CV = coefficient of variation.

Table 16 HPLC Methods for Quantitating B_6 Vitamers in Food (C_8/C_{18} Columns; Fluorescence Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
PN, PL, PM simultaneously; glycosylated forms determined indirectly as free PN after enzymatic hydrolysis	Legumes (lentils, chick peas, haricot bean flour)	Nonconjugated vitamers: trichloroacetic acid extraction; enzymatic dephosphorylation of PNP, PLP, and PNP with potato acid phosphatase at 37°C for 5 h. Glycosylated vitamers: trichloroacetic acid extraction; enzymatic hydrolysis to nonconjugated vitamers with β-glucosidase at 37°C for 5 h.	Precolumn: Porasil B C_{18} (20 × 3.9 mm; Waters). Analytical: Spherisorb ODS2 (300 × 3.9 mm, 10 μm; Sugelabor) at 17°C.	Isocratic: methanol + 33 mM phosphate buffer, pH 2.2 (2 + 98 v/v). 1.2 ml/min. Columns cleaned with methanol + water (30 + 70 v/v) for 5 min between injections, followed by 5 min re-equilibration with mobile phase.	Postcolumn reaction: 300 mM monobasic potassium orthophosphate (K_2HPO_4) to adjust the pH for enhanced fluorescence sensitivity of B_6 vitamers; 0.7 ml/min. Fluorescence; 328/390 nm (ex/em).	External standardization. Linear ranges = 0.04–0.6 ng PM/injection, 0.04–1.20 ng PL/injection, 0.06–1.70 ng PN/injection ($r > 0.990$). LoD = 0.2–0.4 ng/L. Recoveries: 93–107% for PN, PL, PM, PMP and PLP determined as the unphosphorylated vitamers using legume samples ($n = 3$). Samples spiked before extraction.	115

PN, PL, PM, PMP, and PLP simultaneously; PNG separately; total vitamin B_6 by addition	Wheat; flour	Aqueous extraction after addition of 4′-deoxypyridoxine as internal standard; protein precipitated with 5% metaphosphoric acid. Extraction carried out under yellow light.	Precolumn: Newguard RP18 (7 μm; Brownlee). Analytical: Ultremex C_{18} (150 × 4.6 mm, 3 μm; Phenomenex).	**A**: 8 mM 1-octanesulphonic acid in 33 mM phosphate buffer, pH 2.2. **B**: acetonitrile + 33 mM phosphate buffer, pH 2.2 (10 + 90 v/v). Gradient: • 100% **A** at $t = 0$ min. • linear gradient to 100% **B** at $t = 10$ min. • isocratic at 100% **B** until $t = 25$ min. • linear gradient to 100% **A** at $t = 29.5$ min. • re-equilibration with 100% **A** until $t = 35$ min. 1.2 ml/min.	Postcolumn reaction: 1.0 mg/ml sodium bisulphite in 1 M phosphate buffer, pH 7.5 to enhance fluorescence sensitivity of B_6 vitamers; 0.2 ml/min. Fluorescence; 311/360 nm (ex/em).	Internal standardization using 4′-deoxypyridoxine. Identity of PNG confirmed after hydrolysis with β-glucosidase. Recoveries: 34–101% for PN, PMP, PLP, PL, and PM in wheat ($n = 5$).	116, 117

(continued)

Table 16 Continued

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
Total PN, total PL, and total PM simultaneously	Fruit; fruit juice; vegetables; dairy products; snack foods; bakery products; meat (beef, pork, chicken)	Extraction with 5% trichloroacetic acid after addition of 4′-deoxypyridoxine as internal standard; enzymatic dephosphorylation of PNP, PLP, and PNP with takadiastase at 45°C for 3 h. Extraction carried out under subdued lighting. Status of PNG extraction unclear.	Analytical: Hypersil ODS (125 × 4.6 mm, 3 μm; Shandon Southern Products). Column packed in-house.	Isocratic: methanol + 1.25 mM 1-octanesulphonic acid (PIC-B8; Waters) in 100 mM phosphate buffer, pH 2.15 (3 + 97 v/v). 1.2 ml/min.	Postcolumn reaction: 1 M monobasic potassium orthophosphate (K_2HPO_4) to adjust the pH for enhanced fluorescence sensitivity of B_6 vitamers; 0.3 ml/min. Fluorescence; 333/375 nm (ex/em).	Internal standardization using 4′-deoxypyridoxine. Linear range = 0–0.4 ng PN, PL, PM, and 4′-deoxypyridoxine ($r > 0.98$). Reproducibility: CV ± 2.5–19.2% for PN, PL, and PM using rice (within batch) and corn flour (between batch) ($n = 4$). LoD = 0.02 μg/g for PN, PL, and PM at SNR = 3. Recoveries: 95.7–103.6% for PL, PN, PM, PLP, and PMP as the total nonphosphorylated vitamers using a variety of food samples ($n > 45$). HPLC results consistently higher than those from microbiological method.	118

[a] PN = pyridoxine; PM = pyridoxamine; PL = pyridoxal; PNP = pyridoxine phosphate; PMP = pyridoxamine phosphate; PLP = pyridoxal phosphate; PNG = 5′-*O*-(β-D-glucopyranosyl)pyridoxine.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] r = correlation coefficient; n = number of determinations; LoD = limit of detection; CV = coefficient of variation.

Table 17 HPLC Methods for Quantitating B_6 Vitamers in Food (Anion Exchange Columns; Fluorescence Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
PN, PM, PL, PMP, and PLP simultaneously; total vitamin B_6 by addition	Raw and fried chicken	Addition of 3-hydroxypyridine as internal standard; extraction with 5% sulfosalicylic acid and methylene chloride (lipid extraction); removal of sulfosalicylic acid on anion exchange resin (Dowex AG 2-X8; Bio-Rad).	Precolumn: Bio-Gel HPHT (50 × 4 mm; Bio-Rad). Analytical: two ion-exchange (diethylaminoethyl functionality on G500 PW) columns in series (750 × 7.5 mm; Bio-Rad).	Isocratic: 20 mM glycine buffer, pH 9.8 containing 120 mM sodium chloride. Column wash: 20 mM glycine containing 400 mM sodium chloride (used for 10 min after each chicken analysis). 0.8 ml/min.	Postcolumn reaction: 750 mM phosphate buffer, pH 4.90 containing bisulfite to enhance fluorescence sensitivity of PM, PMP, and PLP. Fluorescence time program: 330/400 nm (ex/em) for PM and PMP; 310/400 nm for PN, PL, and internal standard; 330/400 nm (ex/em) for PLP.	Internal standardization using 3-hydroxypyridine. Linear calibration, $r \geq 0.98-0.99$. LoD = 0.79 ng for PM, 0.78 ng for PMP, 2.68 ng for PLP, 1.26 ng for PM, and 1.24 ng for PL. Recoveries: 83.3–104.6% from raw and fried chicken breast and thigh ($n = 5$).	119

[a] PN = pyridoxine; PM = pyridoxamine; PL = pyridoxal; PMP = pyridoxamine phosphate; PLP = pyridoxal phosphate.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] r = correlation coefficient; n = number of determinations; LoD = limit of detection.

Folic Acid (PteGlu)

State of Reduction

7,8-dihydro, e.g. H_2PteGlu

5,6,7,8-tetrahydro, e.g. H_4PteGlu

One Carbon Substituent

5-methyl, e.g. 5-CH_3-H_4PteGlu

5-formyl, e.g. 5-HCO-H_4PteGlu

10-formyl, e.g. 10-HCO-H_4PteGlu

5,10-methylenyl, e.g. 5,10-CH_2-H_4PteGlu

5,10-methenyl, e.g. 5,10-CH=H_4PteGlu

Conjugation

n = number of glutamate residues

n = 1, e.g. 5-CH_3-H_4PteGlu

n = 5, e.g. 5-CH_3-H_4PteGlu$_5$

Fig. 6 Folacin.

hydrofolates can contain one-carbon substituents, such as 5-methyl-, 5-formyl, 10-formyl-, 5-formimino-, 5,10-methylene-, and 5,10-methenyl-tetrahydrofolate. Folic acid does not occur in nature, but it is the vitamer of choice for food fortification because of its relative stability. In nature the reduced forms, the dihydro- and tetrahydro-folates, usually occur as polyglutamates, most commonly with five or six glutamate moieties conjugated via a γ-peptide bond. In foods, the predominant endogenous folates are 5,6,7,8-tetrahydrofolate, 5,6-dihydrofolate, 5-methyl-tetrahydrofolate, and 10-formyltetrahydrofolate.

B. Biological Activity

Folacin bioavailability varies among the vitamers (120,125). Folic acid is more readily available than the naturally occurring food folates but may be less available from fortified foods than in aqueous solution or tablet form. Food folates have been reported to be 30–80% as available as folic acid. Folacin availability, absorption, and metabolism were recently reviewed (20,120,122).

Biochemically, folacin functions in vivo as coenzymes and carriers of one-carbon units for a number of enzyme reactions, including synthesis of amino acids, proteins, and nucleic acids (58,120,122). Folacin participates in both anabolic and catabolic reactions, and its metabolism is cyclic in nature. Greater detail on the biochemistry of folacin is available (120,122).

Folacin deficiency produces a megaloblastic anaemia not unlike that produced by vitamin B_{12} deficiency (120,122,126–130). This is a result of the interdependence of folacin and vitamin B_{12} in at least two biochemical pathways in vivo. Folacin deficiency also appears to affect neurological function.

Folacin has recently been implicated in a number of nonvitamin functions, including roles in various types of cancer, coronary heart disease, and the prevention of birth defects, such as neural tube abnormalities (109,121,131–144). Investigations into these functions are ongoing and have generated controversy concerning the exact nature of the nonvitamin functions, human nutritional requirements for folacin, and the wisdom of food fortification or supplementation of selected population groups with pharmacological doses of folic acid (131,132,145–150).

C. Physicochemical Properties

Folacin's most significant physicochemical properties are those relating to its lability. In general, the folacin vitamers are sensitive to heat, light, oxidation, and extremes of pH (120,122–124). Of these, $PteGlu_n$ and 5-formyl-$H_4PteGlu_n$ are the most stable. These two vitamers can withstand heating at 100°C in solutions at pH 4–12 for up to 10 hours; below pH 4 they become unstable. In the acid form, PteGlu is only slightly water soluble, but it is very soluble as a salt.

The reduced forms are more labile; the degree of degradation or interconversion depends on the environment and the vitamer (120,122–124). 5-Methyl-H_4PteGlu is most stable at pH 7.0 and can be converted to 5-methyl-H_2PteGlu in the presence of oxygen. In solutions containing the antioxidant dithiothreitol, 5-Methyl-H_4PteGlu is stable at pH 7.3 and 9.0, but not at pH 3.5 (151). Under acidic conditions, 10-formyl- and 5-formyl-H_4PteGlu isomerize to 5,10-methenyl-H_4PteGlu. Although 5,10-methenyl-H_4PteGlu is relatively stable to oxidation, at neutral pH it isomerizes to the relatively unstable 10-formyl-H_4PteGlu, which loses the formyl group under anaerobic conditions. 5-Formimino-H_4PteGlu is relatively resistant to oxidation, but it is readily hydrolyzed, with the production of ammonia. H_4PteGlu condenses reversibly with formaldehyde to form 5,10-methylene-H_4PteGlu; 5,10-methylene-H_4PteGlu is stable at pH 9.5 but exists in equilibrium with formaldehyde at pH of 7 or higher. A comprehensive review of the physicochemical properties of the folacin vitamers has been published by Hawkes and Villota (124).

D. Methods of Analysis

1. *Extraction*

The controversy surrounding the nutritional requirements for folacin and food fortification/supplementation is compounded by problems with the analytical methodology. It has been suggested that the folate levels reported in current tables of food composition may be inaccurate due to methodological problems, which, in turn, produces inaccurate estimates of intake (121,123,131). Folacin analyses are complicated by the low levels at which endogenous folacin vitamers occur in foods, the multiplicity of forms of the naturally occurring vitamers, their lack of stability, and the widespread presence of the conjugase enzymes that degrade the polyglutamyl chain. Naturally occurring folacin in food can occur bound to protein, from which it must be released before quantitation. Special care must therefore be taken to ensure quantitative extraction.

Folacin extraction from foods generally begins with homogenization in neutral or mildly acidic solution (122–124,152,153). One study (152) demonstrated that an extraction at pH 7.85 provided the most efficient recovery of endogenous folate from plant and animal tissues; a second extraction of the residual tissue significantly increased the completeness of the extraction procedure. The extractant should contain antioxidants, such as ascorbic acid, 2-mercaptoethanol, dithiothreitol, or a mixture thereof to protect the natural oxidation states of the folacin vitamers.

It should be noted that ascorbic acid is more stable at pH 4–5 than at pH 7, at which the folacin vitamers are more stable. Additional protection from oxidation can be achieved by degassing the extraction solution with an inert gas, such as helium. Homogenization is followed immediately by protein precipitation and release of bound folacin vitamers. This can be accomplished by mild acidification, heating, addition of organic compounds such as trichloroacetic acid, and/or enzymatic (e.g., papain) hydrolysis. The specific conditions used for homogenization and protein precipitation are dictated by the food matrix and the expected profile of folacin vitamers.

Endogenous food folacin occurs primarily in polyglutamate forms, which must usually be deconjugated to the corresponding monoglutamates prior to quantitation (122,123,152,153). This is accomplished with conjugase or pteroylpolyglutamyl hydrolase enzymes, the source of which is as critical as the conditions used for deconjugation. The folate conjugases differ in their pH optima, mode of action, and hydrolysis product. Chicken pancreas conjugase exhibits a neutral pH optimum and produces folate diglutamates. Hog kidney conjugase has an acidic pH optimum (pH 4.5–4.9) and yields monoglutamate products. Human and rat plasma conjugases exhibit slightly acidic pH optima (pH 6.0) and yield monoglutamates. Chicken pancreas conjugase tends to produce ambiguous HPLC results, since these methods are usually based on the separation and quantitation of monoglutamates. Due to its pH optimum, chicken pancreas conjugase produces low results when used with acidic foods such as citrus juices. Although hog kidney conjugase seems to be more suitable for food applications, there are reports of its inhibition by naturally occurring substances in food samples under conditions that did not inhibit chicken pancreas conjugase. It is therefore essential that deconjugation conditions, i.e., pH, conjugase source and concentration, and deconjugation time, be optimized for each type of food. A recent microbiological assay of the folacin content of four food composites demonstrated that a combination of chicken pancreas conjugase, protease, and α-amylase produced more accurate folacin values (154). Other recent methods have made use of conjugases from two sources (163,164).

Solid-phase extraction on anion- or cation-exchange resins can be used for sample cleanup prior to chromatographic separation and quantitation (122,152,153).

2. *Detection*

All biologically active folacin vitamers possess similar UV absorbance spectra (122). Fluorescence detection is a more selective and sensitive alternative for the tetrahydrofolates, which fluoresce naturally at acidic pH (e.g., pH 2.3). Other monoglutamates can be detected fluorimetrically after postcolumn oxidative cleavage to a pterin fragment. Although the folacin vitamers are electrochemically active, amperometric detection is limited by high background interference from other constituents in biological systems. Increasing use is being made of detection methods of higher specificity, such as fluorescence and electrochemistry, as a means of compensating for the low concentrations of endogenous folates in foods. The relative advantages and disadvantages of UV absorbance, fluorescence, and electrochemical detection have recently been demonstrated in conjunction with an HPLC separation of 11 folacin vitamers (155).

3. *HPLC Methodology Reviews*

Three recent reviews included HPLC methods for food folacin. Mullin and Duch (122) reviewed selected HPLC methods for determining the folacin vitamers in foods, biological tissues, and pharmaceuticals. They also included paper chromatography, TLC, and microbiological methods. Russell (44) reviewed selected HPLC methods published between 1990 and 1994 for folacin quantitation in foods, including a brief review of the reference methods. Ball (45) reviewed HPLC and microbiological methods for the quantitation of folacin in food samples.

4. Recent Developments in HPLC Methodology

At present, there are three basic types of folacin assay: microbiological, (radio-) protein binding, and chromatographic (121,123,124). In a 1993 interlaboratory comparison of microbiological, radioprotein-binding, enzyme protein–binding, and HPLC assays for food folacin, the microbiological methods gave the most consistent results; HPLC results for total folacin agreed satisfactorily with those obtained microbiologically (156). There were significant discrepancies in the relative proportions of the individual folacin vitamers reported from the HPLC analyses. A more recent interlaboratory comparison of HPLC procedures for individual folacin vitamers in milk powder and pork liver indicated that the 5-methyl-H_4PteGlu values were most successfully determined (157); considerable variability was reported for other folacin vitamers. One-third of the laboratories reported reasonable agreement between the HPLC and microbiological assays for total folacin in milk powder and pork liver. Low levels of endogenous folates, the presence of interfering artifacts in foods, lack of stability of folacin vitamers during extraction, and poor quality and stability of standards all contributed to the variability of the results. Calibration of HPLC methods is also complicated by the lack of suitable standards for the various folacin vitamers (123). Due to the number and physicochemical diversity of the folacin vitamers, a single comprehensive HPLC method for the folacin vitamers has yet to be developed.

Tables 18–20 summarize HPLC methods, published from 1992 to 1997, for folacin quantitation in foods. The simultaneous quantitation of folacin with one or more other vitamins is covered later in this chapter in Sec. XI.

VIII. VITAMIN B_{12} (CYANOCOBALAMIN)

A. Occurrence

Animal products are the principal food sources of vitamin B_{12} (19,167). Organ meats, such as liver, kidney, brain, and heart, are the best sources. Plant products contain very little vitamin B_{12}, which is an important consideration for strict vegetarians.

The B_{12} vitamers consist of a group of organometallic compounds that have a common corrinoid structure and vary in the substituent bound to the central cobalt atom (Fig. 7) (167,168). The principal naturally occurring B_{12} vitamers are hydroxocobalamin (HOCbl), methylcobalamin (MeCbl), and adenosylcobalamin (AdoCbl). Cyanocobalamin (CNCbl) is the form commonly used for clinical, pharmaceutical, and food fortification purposes, due to its greater relative stability.

B. Biological Activity

There are two biologically active coenzyme forms of vitamin B_{12}, MeCbl and AdoCbl (167,168). In humans, these coenzymes are presently known to participate in two biochemical reactions:

1. The formation of succinyl coenzyme A from methylmalonyl coenzyme A, part of the metabolism of odd-chain fatty acids, cholesterol, and the amino acids valine, isoleucine, threonine, and methionine
2. The methylation of homocysteine to form methionine, with the concomitant formation of tetrahydrofolate from 5-methyltetrahydrofolate (167,168)

More detailed information on the biochemistry, physiology, and metabolism of vitamin B_{12} is available from recent reviews (167–169).

Table 18 HPLC Methods for Quantitating Total Folacin in Foods (C_8/C_{18} Columns; UV Absorbance Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
Total Folacin as 5-methyl-H_4PteGlu, 10-formyl-H_2PteGlu, 10-formyl-PteGlu, 5-formyl-H_4PteGlu, and PteGlu	Unfortified white and wheat bread; rice; spaghetti	Extraction with boiling 50 mM Hepes/Ches buffer, pH 7.85 containing 2% (w/v) sodium ascorbate and 10 mM 2-mercaptoethanol (degassed); enzymatic deconjugation and hydrolysis with rat plasma conjugase, α-amylase, and protease for 4 h at 37°C; extract purification and concentration using affinity chromatography with immobilized folate binding protein. Extraction performed under yellow light.	Analytical: Ultremex C_{18} (250 × 4.6 mm, 5 μm; Phenomenex).	**A**: 33 mM phosphoric acid, pH 2.3. **B**: acetonitrile Gradient: • **A** + **B** (95 + 5, v/v) at $t = 0$ min. • isocratic at **A** + **B** (95 + 5, v/v) until $t = 8$ min. • linear gradient to **A** + **B** (82.5 + 17.5, v/v) at $t = 33$ min. • re-equilibrate at **A** + **B** (95 + 5, v/v) until $t = 45$ min. 1.0 ml/min.	UV absorbance; 280 nm (photodiode array).	External standardization. Linear range = 0.05–0.25 nmole/ml. LoD = 2–4 pmole/injection for 5-methyl-H_4PeGlu, 10-formyl-PteGlu, 5-formyl-H_2PteGlu, and PteGlu, and 7 pmole/injection for 10-formyl-H_2PteGlu at SNR ≥ 3. Reproducibility: $CV < 2\%$ ($n = 4$ injections). Recoveries: 84–107% for individual vitamers from bread, rice, and spaghetti. Good agreement with total folacin results from microbiological method.	158

Total folacin as: PteGlu, H_2PteGlu, H_4PteGlu, 10-formyl-H_4PteGlu, 5-methyl-H_4PteGlu, and 5-formyl-H_4PteGlu simultaneously	Egg yolk; lima beans; baker's yeast; bovine liver; soybean; wheat germ; cabbage; lettuce; orange juice; banana	Extraction with BIS-TRIS buffer, pH 7.8 containing 2% ascorbate and 10 mM mercaptoethanol at 120°C (autoclave) for 30 min; sample cleanup/folacin purification by affinity chromatography	Analytical: Econosphere C_{18} (100 × 4.6 mm, 5 μm; Alltech).	**A**: aqueous solution containing 5 mM tetrabutyl-ammonium phosphate, 0.5 mM dithioerytritol, and 25 mM sodium chloride. **B**: acetonitrile + water (65 + 35, v/v) containing 5 mM tetrabutylammonium phosphate, 0.5 mM dithioerytritol, and 25 mM sodium chloride. Gradient: • **A** + **B** (90 + 10, v/v) at t = 0 min. • isocratic at **A** + **B** (90 + 10, v/v) until t = 5 min. • linear gradient to **A** + **B** (64 + 36, v/v) at t = 15 min. • linear gradient to **A** + **B** (50 + 50, v/v) at t = 35 min. • linear gradient to **A** + **B** (40 + 60, v/v) at t = 52 min. 1 ml/min.	UV absorbance (diode array); 258 and 350 nm.	External standardization. Reproducibility: CV ± 5–19% for total folate in egg yolk, baker's yeast, lima beans, and bovine liver. Good agreement with results from microbiological assay (r = 0.966).	159–161

[a] PteGlu = folic acid; H_4PteGlu = tetrahydrofolic acid; H_2PteGlu = dihydrofolic acid.

[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.

[c] r = correlation coefficient; n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Table 19 HPLC Methods for Quantitating Added Folic Acid in Foods (Ion Exchange/C_8 Columns; UV Absorbance Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
(Fortified) folic acid	Infant formula; liquid diet	Aqueous extraction and hydrolysis with protease-papain at 40°C for 1 h	Precolumn: Bio Series SAX guard (DuPont). Analytical: Bio Series SAX (strong anion exchange) (80 × 6.2 mm; DuPont) and Zorbax RX (C_8) (250 × 4.6 mm; DuPont) at 35°C.	**A**: acetate–sulfate buffer, pH 5.30. **B**: acetonitrile + **A** (360 + 640 v/v). Linear gradient with column switching: • at $t = 0$ min: 100% **A** at 1.5 ml/min through SAX column. • at $t = 8$ min: add C_8 column to flow path, i.e., 100% **A** at 0.8 ml/min (through both columns). • at $t = 20$ min: remove SAX column from flow path, i.e., 100% **A** at 0.8 ml/min (through C_8 column). • linear gradient to **A** + **B** (86.1 + 13.9, v/v) at $t = 20.1$ min. 0.8 ml/min through C_8 column. • isocratic at **A** + **B** (86.1 + 13.9, v/v) until $t = 33$ min. 0.8 ml/min through C_8 column. • linear gradient to 100% **B** at $t = 40$ min. 0.8 ml/min through C_8 column. • linear gradient to 100% **A** at $t = 44$ min. 0.8 ml/min through C_8 column.	UV absorbance; 345 nm.	External standardization. LoD = 6 g/L folic acid. Reproducibility: $CV \pm 3.6\%$. Recoveries: 95.5–100.2% folic acid from milk- and soy-based infant formula.	162

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] LoD = limit of detection.

Table 20 HPLC Methods for Quantitating Folacin Vitamers in Foods (C_{18} Columns; Fluorescence and UV Absorbance and Fluorescence Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
H_4PteGlu, 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu, 10-formyl-PteGlu, and PteGlu simultaneously	Beef; pork; chicken; ham; sausage; liver; eggs; milk and dairy products; fish and fish products; raw and processed fruit and vegetables; wheat germ	Homogenization in inert (nitrogen) atmosphere with 1% (v/v) ascorbate and 0.1% (v/v) mercaptoethanol in 75 mM phosphate buffer, pH 6.0 (1-octanol added as needed to reduce foaming); extraction by rapid microwave heating in inert atmosphere; enzymatic deconjugation of polyglutamates using conjugases from hog kidney and chicken pancreas at 37°C for 2 h in inert atmosphere; strong anion-exchange solid-phase extraction/cleanup.	Precolumn: Nova-pak C_{18} Guard-Pak (4 × 6.5 mm, 4 μm; Waters). Analytical: Hypersil ODS (150 × 4.6 mm, 3 μm; Shandon). For peak purity and fruit/vegetable samples: Spherisorb ODS (250 × 4.6, 5 μm; Phase Separations) in series with Hypersil ODS (150 × 4.6 mm, 3 μm; Shandon). 30°C.	**A**: 30 mM phosphate buffer, pH 2.2 **B**: acetonitrile Analytical Gradient: • **A** + **B** (91 + 9, v/v) at t = 0 min. • isocratic at **A** + **B** (91 + 9, v/v) until t = 4 min. • linear gradient to **A** + **B** (76 + 24, v/v) at t = 12 min. • linear gradient to **A** + **B** (91 + 9, v/v) at t = 14 min. • isocratic at **A** + **B** (91 + 9, v/v) until t = 32 min. 0.8 ml/min. Gradient for peak purity and fruit/vegetable samples from **A** + **B** (91 + 9, v/v) to (75 + 25, v/v) in 14 min; analysis time = 30 min; total run time = 45 min. 0.5 ml/min.	Fluorescence; 290/356 nm (ex/em) for H_4PteGlu, 5-methyl-H_4PteGlu, and 5-formyl-H_4PteGlu. 360/460 nm (ex/em) for 10-formyl-PteGlu. UV absorbance; 290 nm for PteGlu.	External standardization. Linear range = 0–2.5 ng of folacin vitamers. LoD < 0.003–0.03, 0.002–0.02, 0.05–0.1, 0.1–1.4, 0.04 ng H_4PteGlu, 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu, 10-formyl-PteGlu, PteGlu at SNR = 2–3. Reproducibility: $CV \pm$ 4–14% for total folate and ± 17–19% for 5-methyl-H_4PteGlu in milk, pork liver, orange, and banana. Recoveries: 49–105% H_4PteGlu, 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu, 10-formyl-PteGlu, and PteGlu from wheat germ, beef steak, egg yolk, milk powder, and pork liver. Results slightly lower than those obtained by microbiological assay.	163–166

[a] PteGlu = folic acid; H_4PteGlu = tetrahydrofolic acid.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Cyanocobalamin -R = CN

Hydroxocobalamin -R = OH

Methylcobalamin -R = CH_3

Adenosylcobalamin -R =

Fig. 7 Vitamin B_{12} (cyanocobalamin).

A deficiency of vitamin B_{12} produces pernicious anemia similar to that from folacin deficiency. This is due to the interdependence of vitamin B_{12} and folacin in at least two biochemical pathways (126–128,130,167,168). Neuropsychiatric abnormalities have also been reported in conjunction with vitamin B_{12} deficiency. Vitamin B_{12} has also been implicated in certain nonvitamin functions, including coronary heart disease and neural tube defects (109,133,134,170).

C. Physicochemical Properties

Cyanocobalamin appears to be the most stable of the B_{12} vitamers (167,168). It can be autoclaved at 120°C in aqueous solution at pH 4–7. It is susceptible to degradation and loss of vitamin activity under alkaline conditions. Short exposure to UV or visible light causes conversion to HOCbl; prolonged exposure results in irreversible decomposition. CNCbl is soluble in water, short-chain alcohols, and phenol, but it is insoluble in acetone, chloroform, and ether.

AdoCbl and MeCbl are extremely susceptible to photodegradation (168). In the dark, they are relatively stable in neutral aqueous solution and can be heated for 20 minutes at 100°C. Although MeCbl is relatively stable in the presence of dilute acid or alkali, AdoCbl is unstable under acidic conditions. The chemistry of vitamin B_{12} is reviewed in greater detail by Lindemans (168).

D. Methods of Analysis

1. *Extraction*

Vitamin B_{12} must usually be extracted from protein-rich animal products under conditions that will destroy the protein but leave the vitamers intact (44), such as enzymatic hydrolysis with protease, extraction into ethanol at 80°C (171), or extraction into aqueous dimethylsulfoxide containing ammonium pyrrolidine dithiocarbamate and citric acid (172). For total vitamin B_{12} analyses, heating in a cyanide- or sulfate-containing buffer will extract the endogenous B_{12} vitamers and convert them to their more stable cyano- or sulfito-cobalamin form (173). Adsorption onto charcoal, anion-exchange chromatography, or solid-phase extraction on C_{18} cartridges has been used for further cleanup and concentration of the extracts (174). Extraction conditions should be optimized for each type of food sample, since the food matrix has a significant effect on the overall efficiency of the extraction process (173,175). Due to the photosensitivity of the B_{12} vitamers, extraction, and analysis should be carried out under subdued lighting using low-actinic glassware.

2. *Detection*

The B_{12} vitamers are all UV absorbers (168). However, their detection is complicated by differences in their absorbance spectra and the low levels at which they occur in foods.

3. *HPLC Methodology Reviews*

Common HPLC detectors lack the sensitivity and selectivity to determine the low concentrations of the endogenous B_{12} vitamers in foods. As a result, vitamin B_{12} method development for food samples has concentrated on ligand binding and microbiological assays; no reviews of HPLC methods have been published.

4. *Recent Developments in HPLC Methodology*

The first HPLC methods for quantitating vitamin B_{12} in foods are beginning to appear (Tables 21 and 22). The detection problems are being addressed by coupling HPLC with other analytical techniques. Further work is needed for routine analysis of these vitamers by HPLC.

Table 21 HPLC Methods for Quantitating B_{12} Vitamers in Foods (C_8 Columns; Detection by Radioassay)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
CNCbl, HOCbl, MeCbl, and AdoCbl simultaneously; total vitamin B_{12} by addition	Milk; dairy products	Extraction and protein precipitation at pH 4.6 (adjusted with 0.1 N hydrochloric acid) and 120°C (autoclave) for 10 min; protein removal by filtration; C_{18} solid-phase extraction/cleanup	Analytical: Lichrospher RP-8 (5 μm; BDH).	**A**: acetonitrile. **B**: 50 mM phosphoric acid: ammonium hydroxide buffer, pH 3.0 (176). Gradient: • **A** + **B** (95 + 5, v/v) at $t = 0$ min. • linear gradient to **A** + **B** (70 + 30, v/v) at $t = 16$ min. 1.0 ml/min.	Detection of vitamers in HPLC eluate by UV absorbance at 254 nm. Fractions of HPLC eluate collected and freeze-dried. Quantitation of B_{12} vitamers in freeze-dried fractions by radioassay kit (Bio-Rad).	External standardization. Recoveries: 93.7–96.4% individual B_{12} vitamers from dairy products ($n = 6$).	177

[a] CNCbl = cyanocobalamin; HOCbl = hydroxocobalamin; MeCbl = methylcobalamin; AdoCbl = adenosylcobalamin.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] n = number of determinations.

Table 22 HPLC Methods for Quantitating B_{12} Vitamers in Foods (C_{18} Columns; Detection by Electrothermal Atomic Absorption)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
HOCbl, CNCbl, MeCbl, and AdoCbl	Liver (beef, chicken); chicken	Extraction into 3% (w/v) trichloro-acetic acid	Precolumn: Spherisorb ODS-2 (Supelco). Analytical: Spherisorb ODS-2 (150 × 4.6 mm, 5 μm; Supelco).	**A**: 50 mM phosphate buffer, pH 4.2. **B**: methanol. Gradient: • **A** + **B** (74 + 26, v/v) at $t = 0$ min. • linear gradient to **A** + **B** (50 + 50, v/v) at $t = 8$ min. 1.5 ml/min.	Electrothermal atomization. Atomic absorption; 240.7 nm (cobalt hollow cathode lamp).	External standardization. Linear ranges = 0.3–6 μg HOCbl, CNCbl; 0.5–8 Fg AdoCbl, MeCbl ($r \geq$ 0.9875). LoD = 0.31–0.55 μg/ml for individual B_{12} vitamers (based on 3σ). Repeatability: CV ± 3.5–5.7% for individual B_{12} vitamers. Recoveries: 99.4 ± 5.8% (mean) for all B_{12} vitamers from liver and chicken ($n = 45$). B_{12} vitamers not found above detection limit in any food sample.	178

[a] CNCbl = cyanocobalamin; HOCbl = hydroxocobalamin; MeCbl = methylcobalamin; AdoCbl = adenosylcobalamin.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] r = correlation coefficient; n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

```
        O
        ||
        C
      /   \
   HN       NH
    |        |
   HC ------ CH
    |        |
   H2C      CH ---- (CH2)4 ---- COOH
      \    /
        S
```

Fig. 8 Biotin.

IX. BIOTIN

A. Occurrence

The best food sources of biotin (Fig. 8) include yeast, liver, soy products, rice, egg yolks, nuts, fish, and chocolate (179,180). Although many foods contain biotin, the levels are normally very low. Endogenous biotin in foods is usually protein bound; in general there is more free biotin in plant-based foods than in animal-based products.

B. Biological Activity

The relative bioavailability of free and bound biotin is not entirely clear (179,180); for example, the biotin present in many cereals and oilseeds is largely unavailable to animals. In addition, the bioavailability of biotin can be significantly impaired by avidin, a biotin-binding protein found in egg white. The physiology and metabolism of biotin has been recently reviewed (179,180).

Biochemically, biotin serves as a prosthetic group and a carboxyl carrier for a number of enzymes (179,180). In humans, biotin is required by four carboxylase enzymes:

Pyruvate carboxylase, which participates in gluconeogenesis and lipogenesis
Acetyl-CoA carboxylase, which participates in fatty acid biosynthesis
Propionyl-CoA carboxylase, which participates in isoleucine catabolism
3-Methylcrotonyl-CoA carboxylase, which participates in leucine catabolism

More detailed information on the biochemistry of biotin is available from recent reviews (179,180).

Although biotin deficiency is relatively rare, it is characterized by muscle aches, skin rashes, mild depression, slight anemia, and increased serum cholesterol (19,179,180).

C. Physicochemical Properties

Unlike the other water-soluble vitamins, biotin is relatively stable (179,180). It can tolerate extended periods of heating in strong acid, e.g., autoclaving in 4 M sulfuric acid at 120°C for 4 hours. However, it is readily oxidized to biotin sulfoxides in dilute aqueous solutions, which necessitates careful degassing of chromatographic solutions. Microorganisms can also significantly reduce the biotin concentration of nonsterile dilute solutions. Free biotin is soluble in dilute alkali and hot water, sparingly soluble in dilute acid, cold water, and alcohol, and insoluble in organic solvents.

D. Methods of Analysis

1. *Extraction*

Endogenous biotin in foods is predominately protein bound and is relatively stable (180). Consequently it can be extracted under fairly harsh conditions, e.g., autoclaving in 4 M sulfuric acid for 2 hours at 120°C. Enzymatic hydrolysis with papain will also release biotin from proteins (181). Potential sample-cleanup procedures include adsorption on charcoal and/or ion-exchange chromatography (182,183).

2. *Detection*

The biggest challenge in the HPLC analysis of biotin is its detection. Biotin does not exhibit UV absorbance or fluorescence, nor is it electrochemically active (180). Refractometry, a notoriously nonspecific technique, is the only means for direct detection of biotin. Fortunately, biotin can be converted to UV absorbing or fluorescent derivatives.

3. *HPLC Methodology Reviews*

Gaudry and Ploux (180) summarized recent HPLC, TLC, GC, column chromatography, paper chromatography, and LC-mass spectrometry techniques for biotin in biological and pharmaceutical samples. Russell (44) reviewed a recent HPLC determination for biotin in royal jelly.

4. *Recent Developments in HPLC Methodology*

The literature contains very little on methods for biotin determination in foods. Microbiological assays of biotin predominate in food work (19,180). Table 23 summarizes a recent HPLC method for the analysis of biotin in infant formula.

X. PANTOTHENIC ACID

A. Occurrence

Pantothenic acid is present in almost all foods, but usually at very low concentrations (19,185,186). Yeast, avocado, meat (especially organ meats), fish, several kinds of cheese, bran, whole-grain cereal products, and legumes are good food sources of pantothenic acid. In general, milk, fruit, and vegetables are relatively low in pantothenic acid.

Endogenous pantothenic acid occurs in food primarily in the bound form as a component of coenzyme A (CoA or CoASH), acyl-coenzyme A, and acyl carrier protein (ACP) (185,186). These are the principal vitamers in foods; free pantothenic acid (Fig. 9) is much less common. Only the D(+) or (R) enantiomer of pantothenic acid occurs naturally.

B. Biological Activity

The coenzymes, CoA, acyl-coenzyme A, and ACP, are the biologically active forms of pantothenic acid (185,186). Recent reviews of the absorption and metabolism of pantothenate are available (185,186).

Biochemically, the pantothenate coenzymes are integral in the metabolism of carbohydrates, lipids, and nitrogen-containing compounds (185,186). They participate in the TCA or Krebs cycle, as well as fatty acid, phospholipid, sterol (cholesterol), and heme synthesis. The coenzymes mediate the exchange of 2-carbon (acetyl) and other acyl groups. They can serve as

Table 23 HPLC Methods for Quantitating Biotin in Foods (C_{18} Columns; Detection by Fluorophore-Linked Protein Binding Assay)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Biotin and biotin analogs	Infant formula	Protein precipitation using concentrated hydrochloric acid; neutralization with 6 M NaOH; lipid extraction with *n*-hexane	Precolumn: Microsorb C_{18} (15 × 4.6 mm, 5 μm; Rainin). Analytical: Microsorb C_{18} (250 × 4.6 mm, 5 μm; Rainin).	Isocratic: 100 mM phosphate buffer, pH 7.0 + methanol (80 + 20, v/v). 0.4 ml/min.	Postcolumn reaction system: UV absorbance at 220 nm followed by streptavidin-fluorescein isothiocyanate (2.0 mg/L) knitted open tubular reaction system (10.0 m × 0.5-mm ID) at a flow rate of 0.1 ml/min. Fluorescence; 495/518 nm (ex/em) operated in photon-counting mode.	External standardization. Linear range = 0.08–1.00 μM biotin. LoD = 0.02 μM or 97 pg biotin at SNR = 3. Repeatability: *CV* ± 3.5% for biotin in infant formula.	184

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] LoD = limit of detection; SNR = signal-to-noise ratio; *CV* = coefficient of variation.

Pantothenic Acid

$HOH_2C-C(CH_3)_2-CH(OH)-C(=O)-NH-CH_2\text{-}CH_2-COOH$

Coenzyme A

Fig. 9 Pantothenic acid.

either hydrogen donors or acceptors. More detailed information on the biochemical functions of pantothenate is available (185,186).

Pantothenic acid deficiency appears only in cases of severe malnutrition, and it is usually characterized by a burning sensation in the feet and lower legs (19,186).

C. Physicochemical Properties

Pantothenic acid is relatively labile (185,186). In the dry form, it is hygroscopic and unstable; in solution its stability is strongly pH dependent, being greatest at pH 4–5. It is subject to hydrolytic cleavage to pantoic acid and β-alanine in more acidic or alkaline solutions. Pantothenic acid is very soluble in water, alcohols, and dioxane, less soluble in diethyl ether and acetone, and insoluble in benzene and chloroform.

Pantothenate salts are more stable and less hygroscopic than the free acid (185). In solution, pantothenate salts are most stable at pH 6–7. The pantothenate salts are very soluble in water. Coenzyme A is water soluble and stable at pH 2–6; it can be oxidized to coenzyme A disulphide.

D. Methods of Analysis

1. *Extraction*

Enzyme hydrolysis, with papain, diastase, clarase, takadiastase, intestinal phosphatase, or combinations thereof is most commonly used to release pantothenate from food proteins (186). A cold perchloric acid extraction was used to release pantothenic acid from tissue samples (187). Food spoilage prior to analysis may lead to inflated pantothenic acid levels (19).

2. *Detection*

Pantothenate detection presents several challenges. Direct detection is feasible only by means of refractometry, which is highly nonspecific, or by UV absorbance below 220 nm, which limits the

use of organic solvents in the HPLC mobile phase and is subject to interference from artifacts in complex food systems (185). Detection is further complicated by the low concentrations at which pantothenate occurs in many foods. Chemical derivatization of pantothenic acid enables detection by fluorescence or UV absorbance at wavelengths greater than 220 nm.

3. *HPLC Methodology Reviews*

There are few published HPLC methods for pantothenic acid in food samples. Velíšek et al. (185) reviewed nonchromatographic methods, column chromatography, paper chromatography, TLC, HPLC, and GC for pantothenic acid in biological and pharmaceutical samples. Russell (44) summarized a GC method for pantothenate in foods.

4. *Recent Developments in HPLC Methodology*

Microbiological assays are the most popular means of quantitating food pantothenate (188). Table 24 summarizes a recent HPLC method for determining pantothenic acid in infant formula.

XI. SIMULTANEOUS DETERMINATION OF MULTIPLE VITAMINS

A. Methods of Analysis

1. *Extraction*

In foods, the simultaneous HPLC analysis of several vitamins is feasible only under special conditions, for example, determination of fortified, free vitamins rather than endogenous ones, which are often bound to other food components, or determination of vitamins in relatively simple foodstuffs, such as fruit juice. The HPLC separation per se of multiple vitamins is not difficult; it is their simultaneous, nondegradative extraction from foods that is problematic. Developing a single set of extraction conditions that satisfies the diverse physicochemical properties and stability requirements of several vitamins is a challenge.

2. *HPLC Methodology Reviews*

Russell (44) and Ball (45) summarized multivitamin methods for foods that determined various combinations of thiamine, riboflavin, niacin, vitamin B_6, folacin, and biotin.

3. *Recent Developments in HPLC Methodology*

HPLC is the method of choice for multivitamin determinations. Tables 25–27 summarize simultaneous HPLC determinations of multiple B vitamins in foods, published from 1992 to 1997.

XII. FUTURE DIRECTIONS

A. Reference Materials

The lack of standardization during method verification is a long-standing problem in vitamin analyses. This makes it difficult, if not impossible, to assess the validity of individual methods and to compare different assays without extensive bench work. Although standard or certified reference materials are available for the minerals in foods, development of similar materials for the vitamins is being hampered by the lability of the vitamins as well as by inconsistent results from the various analyses for the same vitamin or, in certain cases, the interlaboratory variability for the same method.

Table 24 HPLC Methods for Quantitating Pantothenic Acid in Foods (C_{18} Columns; UV Absorbance Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Calcium pantothenate	Fortified infant formula	Extraction with 10% (v/v) acetic acid + 1 M sodium acetate (50 + 50, v/v); centrifugation to remove protein and fat	Analytical: Supersphere C_{18} (250 × 4.6 mm, 5 μm; Merck).	Isocratic: acetonitrile + 250 mM sodium phosphate, pH 2.5 (3 + 97, v/v). 1 ml/min.	UV absorbance; 197 nm (photodiode array).	External standardization. Linear range = 50–800 ng pantothenic acid (r = 0.9999). Reproducibility: CV ± 2.35–4.97% for pantothenic acid in infant formula (n = 4). Recoveries: 89–98% from infant formula (n = 5). Results slightly higher than those from microbiological method (r = 0.9278).	189

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] r = correlation coefficient; n = number of determinations; CV = coefficient of variation.

Table 25 HPLC Methods for Simultaneously Quantitating Thiamine and Riboflavin in Foods

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Thiamine and riboflavin simultaneously	Broccoli; cereal; flour	Extraction by autoclaving at 121°C for 30 min in 0.1 N hydrochloric acid; adjustment to pH 4.5 with 2 N sodium acetate; filtration; oxidation of thiamine to thiochrome with 1.0% potassium ferricyanide in 15% sodium hydroxide; C_{18} solid-phase extraction/cleanup	Analytical: μBondapak C_{18} (300 × 3.9 mm; Waters).	Isocratic: methanol + 5 mM ammonium acetate buffer, pH 5.0 (28 + 72, v/v). 1.5 ml/min.	Fluorescence; time-programmed wavelength switching using • 370/435 nm (ex/em) at $t = 0$ min. • 370/435 nm (ex/em) until $t = 10$ min (for thiochrome). • 370/520 nm (ex/em) for rest of analysis (for riboflavin).	External standardization. Linear calibration up to 100 ng on-column for each vitamin. LoD = 0.05 ng on-column for both thiamine and riboflavin at SNR = 12. Good agreement with results obtained from AOAC International method and for AACC check samples.	190

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] LoD = limit of detection; SNR = signal-to-noise ratio.

Table 26 HPLC Methods for Simultaneously Quantitating Thiamine, Riboflavin, and Pyridoxine in Food

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Thiamine, riboflavin, and added pyridoxine simultaneously	Milk- and soy-based infant formula	Extraction with perchloric acid; filtration; addition of *m*-hydroxybenzoic acid as internal standard	Analytical: Nova-pak C_{18} (150 × 3.9 mm; Waters).	Isocratic: acetonitrile + phosphate–ammonium hydroxide buffer, pH 3.60 containing 0.90 g/L 1-hexanesulfonic acid (9.0 + 91.0 v/v). 1.0 ml/min.	Thiamine and riboflavin: UV absorbance; 254 nm. Pyridoxine: fluorescence; 295/395 nm (ex/em).	Internal standardization using *m*-hydroxybenzoic acid. Linear range: 0.2–1.4 μg/ml for thiamine and riboflavin; 0.2–1.2 μg/ml for pyridoxine. LoD = 0.15 μg/ml for thiamine; 0.09 μg/ml for riboflavin; 0.01 μg/ml for pyridoxine. Reproducibility: CV ± 0.9% for thiamine; ± 1.4% for riboflavin; ± 1.8% for pyridoxine in infant formula (n = 10). Recoveries: 102% for thiamine and riboflavin from infant formula; 101% for pyridoxine from infant formula. Good agreement with results from AOAC International manual method for thiamine and with AOAC International automated and microbiological methods for riboflavin. Results were lower than AOAC International microbiological method for total vitamin B_6.	191

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

Table 27 HPLC Methods for Quantitating Riboflavin, Niacin, Pyridoxine, and Folacin in Food

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Riboflavin, niacin, vitamin B_6, and folic acid simultaneously	Eggs	Extraction with 0.1 N sulfuric acid; enzymatic hydrolysis with takadiastase and papain	Analytical: μBondapak C_{18} (300 × 3.9 mm; Waters).	Isocratic: methanol + aqueous 2.4 mM PIC-B (hexane sulfonic acid), pH 4.2 (22 + 78 v/v). 0.3 ml/min.	UV absorbance, 254 nm.	External standardization. Reproducibility: *CV* ± 7.9% and 8.1% for riboflavin; ± 10.9% and 14.5% for pyridoxine; ± 29.4% and 27.6% for folic acid; ± 25.5% and 24.1% for niacin in egg yolk and albumen, respectively. Recoveries: 102.1% and 102.5% for riboflavin; 96.6% and 98.4% for pyridoxine; 103.3% and 94.5% for folic acid; 92.7% and 92.4% for niacin from egg yolk and albumen, respectively.	192

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] *CV* = coefficient of variation.

Reference or control materials for use in method development or quality control should be made from a matrix that will mimic real samples as closely as possible, i.e., from foodstuffs rather than from standard solutions or pharmaceuticals (11). In order to reproduce the form and compartmentalization of vitamins in food samples, the analyte of interest should be endogenous in the selected reference material. A good reference material should be homogeneous in composition and should cover the range of concentrations of the analytes in the food samples. It should also be accompanied by instructions concerning proper storage, handling, and use. The concentration of the analytes in the reference material should be certified using definitive method(s). In addition, the analytes should be stable in the reference material to ensure that the certified concentrations remain valid over time. More detailed information on the development and use of reference materials is available from a recent review (11).

A number of reference materials for vitamins in foods are under development. Extensive analysis and stability testing have been conducted to assess the potential of vitamin-enriched milk powder, wholemeal flour, and margarine as well as lyophilized brussels sprouts, mixed vegetables, and pork liver for use as reference materials (193–196). The certification study for vitamin C in the brussels sprouts reference material has been completed. However, methodology problems continue to have a significant negative effect on the development of reference materials.

A standard reference material for infant formula has also been certified for vitamin C, riboflavin, niacin, and pyridoxine (197). Another recent study (198) indicated that "off-the-shelf" wheat germ is suitable for use as a secondary reference material for thiamine and niacin; riboflavin was also evaluated but found to be somewhat unstable over time. Powdered orange drink was deemed suitable as a secondary reference material for vitamin C.

B. Columns

Current trends in HPLC favor the development of faster methods that exhibit high resolution and use less mobile phase.

Microcolumn HPLC offers the advantages of more rapid analyses at high resolution, smaller sample volume, reduced solvent consumption/operating costs, and compatibility with mass spectrometric detection. However, conversion of conventional equipment to microcolumn use has not been successful, and considerable capital expenditure for dedicated hardware is usually necessary. This has had a negative impact on its introduction into food analyses. In the meantime, the use of narrow-bore columns or shorter conventional-diameter columns packed with small-diameter (3 μm) particles is becoming increasingly popular. These columns combine the advantages of rapid analysis, compatibility with mass spectrometry, and reduced solvent consumption with adaptability to existing, conventional HPLC hardware.

Development of new HPLC stationary phases is ongoing. A multimodal phenylpropanolamine-coated silica column was recently prepared and applied to the quantitation of ascorbic acid in orange juice and the separation of ascorbic acid from its epimer, isoascorbic acid (Table 28) (199). Other examples include new bonded phases and columns made of continuous porous rods that permit fast chromatography with low backpressure and high resolution (200–202).

C. Detectors

The availability of commercial bench-top mass spectrometry detectors for HPLC is facilitating the development of HPLC-MS methods for many analytes. This is more common in pharmaceutical than food applications. As is generally the case, mass spectrometry is first being applied to standard solutions and relatively simple samples before being applied to more complex food matrices. A standard mixture of ten vitamers, AA, DHAA, PN, PL, PM, thiamine, nicotinic acid, nicotinamide, pantothenic acid and biotin, were recently determined by HPLC-particle beam

Table 28 HPLC Methods for Quantitating Ascorbic Acid (Multimodal Phenylpropanolamine-Coated Silica Column; UV Absorbance Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	HPLC columns[b]	HPLC mobile phase and flow rate	Detection conditions	Method verification[c]	Ref.
AA and IAA simultaneously	Orange juice	Filtration to remove suspended material; dilution with 1% acetic acid	Analytical: Phenylpropanolamine-coated silica (150 × 4.6 mm, 5 μm; slurry-packed in-house).	Isocratic: methanol + 1% acetic acid (70 + 30 v/v). 1.0 ml/min.	UV absorbance; 244 nm.	External standardization. Linear range = 7–28 μg/ml ($r = 0.998$). Reproducibility: $CV \pm 3.77\%$ ($n = 5$).	199

[a] AA = L-ascorbic acid; IAA = isoascorbic acid.
[b] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[c] r = correlation coefficient; n = number of determinations; CV = coefficient of variation.

Table 29 HPLC Methods for Quantitating Total Thiamine (C_{18} Column; Mass Spectrometry Detection)

Analyte(s)	Type of food analyzed	Extraction conditions	HPLC columns[a]	HPLC mobile phase and flow rate	Detection conditions	Method verification[b]	Ref.
Total thiamine	Dried yeast	Hydrochloric acid extraction; enzymatic hydrolysis of thiamine phosphates to thiamine using diastase; thiamine extraction with isobutanol; sample purification with 1-octanesulfonate-isobutanol; *p*-chloroaniline added as internal standard	Analytical: Capcell Pak C_{18} (150 × 4.6 mm, 5 μm; Shiseido).	Quantitation: isocratic—0.15% (w/v) sodium 1-octanesulfonate in acetonitrile + 50 mM acetate buffer, pH 3.5 (15 + 85, v/v). Peak verification: isocratic—acetonitrile + 50 mM ammonium acetate buffer, pH 9.0 (10 + 90, v/v). 1.0 ml/min.	Quantitation: UV absorbance at 254 nm. Peak verification: UV absorbance at 254 nm followed by atmospheric pressure chemical ionization mass spectrometry.	Quantitation: • Internal standardization using *p*-chloroaniline. • Linear range = 25–300 μg/100 ml ($r = 0.998$). • Reproducibility: $CV \pm$ 7.3% total thiamine as thiochrome ($n = 5$). • Recoveries: 109.2% ($n = 5$). Good agreement with results from manual thiochrome method. Peak verification: • External standardization. • Linear range = 2–50 ng thiamine. • LoD = 2 ng thiamine at SNR = 3.	204

[a] Column specifications expressed as (length × ID, particle size; manufacturer) when reported in original publication.
[b] r = correlation coefficient; n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

mass spectrometry (203). High-performance LC–atmospheric pressure chemical ionization mass spectrometry has been used to the verify the identity of thiamine in dried yeast (204). Quantitation was carried out by HPLC with UV absorbance detection (Table 29). The physicochemical properties of the internal standard used in this assay, *p*-chloroaniline, are appreciably different from those of thiamine. In addition, the internal standard was added immediately before injection into the HPLC.

D. Capillary Electrophoresis and Capillary Electrochromatography

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are fast becoming viable alternatives to conventional, narrow-bore, and microcolumn HPLC. Capillary electrochromatography, which is CE using a capillary column packed with HPLC stationary phase, is still in its infancy. These techniques provide the same advantages as microcolumn HPLC, i.e., low solvent consumption, rapid analysis, high resolution, small sample volume, and compatibility with mass spectrometric detection. One of the biggest disadvantages has been the lack of detection sensitivity, although specialized designs in new detectors and flow cells are alleviating this problem. With respect to vitamin analysis, the CE literature contains a number applications for pharmaceuticals and the simpler food systems, such as fruit juices. Capillary electrophoresis is just beginning to be applied to more complex food samples (Tables 30–31).

METHODOLOGY REVIEWS

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Table 30 Capillary Electrophoresis Methods for Quantitating Vitamin C (UV Absorbance Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	Type of analysis amd column[b]	Analytical conditions	Detection conditions	Method verification[c]	Ref.
AA and IAA simultaneously	Lemon juice; orange juice	Juice expressed and filtered	Capillary zone electrophoresis. Coated capillary (20 cm × 25 μm; Bio-Rad).	Separation buffer = 0.1 M phosphate, pH 5.0. Separation voltage = 8 kV. Injection: on cathodic side by electromigration at 8 kV for 8 s.	UV absorbance; 265 nm.	External standardization. Linear range = 0–1 mg/ml for peak height vs. concentration, $r = 0.99$ for both AA and IAA ($n = 10$). LoD = 0.25 μg/ml for AA and IAA at SNR > 2. Reproducibility: $CV \pm 5\%$ for AA in fruit juice ($n = 5$). Recoveries: 97–101% AA from lemon and orange juice ($n = 5$).	205
AA and IAA simultaneously	Orange juice; apple juice; grapefruit juice; vegetable juice; cranberry cocktail; white wine	IAA added as internal standard; metaphosphoric acid (100 g/L) extraction	Capillary zone electrophoresis. Polyimide-coated capillary (37 cm × 75 μm; Polymicro Technologies). Length to detector = 30 cm.	Separation buffer = 100 mM tricine, pH 8.8. Separation voltage = 11 kV (297 V/cm). Pressure injection.	UV absorbance; 254 nm.	Internal standardization with IAA. Linear range = 1.6–480.0 μg/ml, $r = 0.9984$.	206

(continued)

Table 30 Continued

Analyte(s)[a]	Type of food analyzed	Extraction conditions	Type of analysis amd column[b]	Analytical conditions	Detection conditions	Method verification[c]	Ref.
AA and total vitamin C as AA separately	Orange juice	AA: extraction with 12.5% trichloroacetic acid. Total Vitamin C: extraction with 12.5% trichloroacetic acid; reduce DHAA with 0.8% DL-homocysteine (minimum 40:1 excess of homocysteine:DHAA). Phthalic acid added as internal standard; point of addition not specified.	Capillary zone electrophoresis. Acrylamide-coated fused-silica capillary (coating done in-house) (40 cm × 100 μm; Polymicro Technologies).	Separation buffer = 20 mM phosphate buffer, pH 7.0. Separation voltage = 6 kV (60 μA). Hydrostatic pressure injection on cathodic side for 6 s. Column flushed with separation buffer between runs.	UV absorbance; 254 nm.	Internal standardization with phthalic acid. Linear range = 3–80 μg/ml. Reproducibility: $CV \pm$ 2.9–3.0% in orange juice ($n = 6$). Good agreement with HPLC results.	207

Total vitamin C as AA	Fruit; vegetables	Metaphosphoric acid extraction; addition of IAA as internal standard and 0.2% dithiothreitol to reduce DHAA to AA; C_{18} solid-phase extraction/cleanup	Micellar electrokinetic capillary chromatography. Fruit: fused silica capillary (65 cm × 75 μm; Polymicro Technologies). Length to detector = 40 cm. Vegetables: fused silica capillary (75 cm × 75 μm; Polymicro Technologies). Length to detector = 50 cm. 28°C.	Separation buffer = 20 mM sodium borate + 20 mM potassium dihydrogen phosphate (50 + 50, v/v) containing 50 mM sodium deoxycholate, pH 8.6. Separation voltage = +25 kV. Vacuum injection (10 kPa/s). Columns flushed with separation buffer for 2 min between runs.	UV absorbance; 254 nm.	Internal standardization with IAA. Linear range = 0–60 μg/ml. Repeatability: CV ± 0.5–2.2% (n = 7). Good agreement with results from HPLC method.	208
AA and IAA simultaneously	Parsley; mushroom	Cryogenic freezing in liquid nitrogen; extraction with 3% metaphosphoric acid containing 1 mM EDTA; C_{18} solid-phase extraction/cleanup	Capillary zone electrophoresis. Fused silica capillary (57 cm × 75 μm; Beckman ?). Length to detector = 50 cm. 25°C.	Separation buffer = 200 mM borate, pH 9.0. Column rinsed hydrostatically with 200 mM sodium hydroxide (1 × 2 min), water (2 × 1 min), separation buffer (1 × 3 min). Separation voltage = +25 kV (439 V/cm; 103 μA). Hydrostatic injection (0.5 psi N_2) for 3–10 s.	UV absorbance; (photodiode array) 190–350 nm, 260 nm used for preparation of calibration curves for AA and IAA.	External standardization. Linear range = 15–2120 pg AA injected (r = 0.999). LoD = 15 pg AA injected at SNR 3. Good agreement with results from HPLC method.	209

[a] AA = L-ascorbic acid; IAA = isoascorbic acid.
[b] Column specifications expressed as (length x id; manufacturer) when reported in original publication.
[c] n = number of determinations; CV = coefficient of variation.

Table 31 Capillary Electrophoresis Methods for Quantitating Total Niacin (UV Absorbance Detection)

Analyte(s)[a]	Type of food analyzed	Extraction conditions	Type of analysis and column[b]	Analytical conditions	Detection conditions	Method verification[c]	Ref.
Total niacin	Yeast spreads	Extraction in aqueous calcium hydroxide at 121°C (autoclave) for 2 h to liberate bound vitamers and hydrolyze nicotinamide to nicotinic acid; solid-phase extraction/analyte concentration using C_{18} + strong cation-exchange cartridges in series; addition of saccharin as internal standard.	Capillary zone electrophoresis. Uncoated fused-silica capillary (75 cm × 75 μm; Polymicro Technologies). Length to detector = 50 cm. 30°C.	Separation buffer = 20 mM sodium tetraborate + 20 mM disodium hydrogen phosphate (50 + 50, v/v), pH 9.2. Column rinsed with separation buffer for 2 min between runs. Separation voltage = 20 kV. Vacuum injection (25 kPa/s).	UV absorbance; 254 nm.	Internal standardization using saccharin. Linear range = 0.5–15 μg/ml for nicotinic acid. Reproducibility: $CV \pm 3.3\%$ for yeast spread ($n = 7$). Recoveries: 96% for nicotinic acid in yeast spread ($n = 7$). Purity of nicotinic acid peak confirmed by photodiode array spectral analysis. Good agreement with results from AOAC International colorimetric method.	210

[a] Column specifications expressed as (length x id; manufacturer) when reported in original publication.

[b] r = correlation coefficient; n = number of determinations; LoD = limit of detection; SNR = signal-to-noise ratio; CV = coefficient of variation.

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HPLC Analysis of Organic Acids

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I. INTRODUCTION

Because they play a pivotal role in maintaining the quality and nutritional value of a variety of foods, organic acids are among the most frequently assayed substances in this type of substrate.

The organic acids present in foods originate from biochemical processes, from their addition as acidulants, stabilizers, or preservatives, or from the activity of some microorganisms (particularly yeasts and bacteria). They contribute to the sensory properties of foods. In fact, the role of organic acids in the taste and aroma of alcoholic beverages is well documented (1); the sour, sharp, and irritating flavors of cider are ascribed to lactic acid, total acidity, and acetic acid, respectively. Malic and acetic acid bear a negative though significant correlation to sweet taste and scented flavors; also, they taste equiacidic with solutions containing sucrose (e.g., 0.7% D-tartaric acid with a 0.8% concentration of this acid plus 2% sucrose) (2). Acetic acid and the global hedonic score for fermented beverages also bear a significant negative mutual correlation.

Organic acids may exhibit other sensory properties. For example, citric acid possesses sweet-and-sour sensory notes, and succinic acid has a salty–bitter taste. On the other hand, the typical taste and flavor of Emmental cheese can be ascribed to the propionic acid and a few other compounds, such as proline. In fact, taste and flavor result from the combination of different food constituents in definite proportions. Raw meat smells much like lactic acid, which arises from postmortem anaerobic glucolysis and determines the pH of meat, its final properties, and microbial stability. This same organic acid has been related to the inhibition of certain pathogenic bacteria in yogurt (3). Table 1 lists the reported threshold concentrations for various organic acids in different media (4–6).

Sour-tasting compounds are all acidic. The acidity of mineral acids is determined by their hydrogen ion concentration; that of organic acids, however, cannot be defined in such simple terms. Thus, a solution of acetic acid tastes more sour than one of a mineral acid at the same pH (5); however, the mineral acid tastes more sour than the organic acid in equimolar solutions. The threshold concentrations of organic acids are pH dependent, yet the relationship is usually quite complicated. The threshold concentrations of formic, malic, and succinic acid increase with increasing pH, whereas those of acetic, butyric, and lactic acid exhibit the opposite trend (7).

Organic acids are added to some foods in order to prevent sedimentation or darkening; thus, citric, malic, phosphoric, and ascorbic acid are used to reduce or delay enzymatic browning. Melanin formation following production of a quinone is a pH-dependent process (the rate of the former process increases with increasing pH) (8). Polyhydroxyacids such as citric and malic acid

Table 1 Typical Threshold Concentrations of Organic Acids in Various Media

Acid	Concentration (meq/L)
Acetic	1.800
Tartaric	1.200
Citric	2.300
Malic	0.500
Lactic[a]	0.400
Pyruvic[a]	2.800
Phenylacetic[a]	0.007
Propionic[b]	0.270
Valeric[b]	0.005
Isobutyric[b]	0.090
Butyric[b]	0.040
Acetic[b]	0.430
Isovaleric[b]	0.007

[a] Flavor threshold in beer.
[b] Flavor threshold in whiskey.

inhibit the polyphenol oxidase system by chelating the copper prosthetic group. On the other hand, nonenzymatic browning is inhibited by more acidic conditions, where the carbonylamino reaction is hindered. However, incorporation of sucrose into an acid medium fosters nonenzymatic browning.

Malolactic fermentation decreases the acidity of some alcoholic beverages, including wine and cider. Malic acid is converted to lactic acid, which possesses a weaker taste. This improves the flavor of these beverages and increases their biological stability. The biological advantage of malolactic conversion lies in the fact that it provides the energy required to raise the protonmotive force (Δp) and boost ATPase activity via an increase in the intracellular pH. Also, malolactic fermentation is typically associated with buttery, lactic, nutty, yeasty, oaky, and sweaty flavors as well as with fruitier notes in wines (9).

Several organic acids, including malic, pyruvic, and oxalacetic acid, take part in sugar metabolism in fruits. The vacuole malate pool and the malate produced by mitochondrial enzymes are used to synthesize sugars via gluconeogenesis (10). On the other hand, some organic acids in apples, i.e., propionic and butyric acid, are converted into ethylene, which acts as a trigger for ripening (11).

Some organic acids undergo major changes during ripening. The malic and citric acid contents, and those of major organic acids in most fruits, decrease concomitantly with a decrease in the starch content and an increase in sugar concentrations. The sugar-to-acid and sugar-to–dry matter ratios are frequently used as indicators of grape and orange ripeness. In addition, organic acid analyses can be used to reveal potential food adulteration. Genuine single-strength cranberry juice should contain an average $1.32 \pm 0.15\%$ quinic acid, $0.92 \pm 0.079\%$ malic acid, and $1.08 \pm 0.11\%$ citric acid (12). The presence of D-malic acid is a clear indication of adulteration, for this isomer does not occur naturally. The two isomers of malic acid can now be resolved chromatographically by nonenzymatic methods (13).

According to the food laws and regulations of the Food and Drug Administration (FDA), organic acids can be used as acidulants (e.g., citric, fumaric, malic, and sorbic acid), antimicrobial additives (e.g., propionic acid), and sequestrants (e.g., tartaric acid) (14). Most fatty acids of

1–14 carbon atoms possess bacteriostatic or fungistatic properties. According to European Union directives, organic acids used as food additives are legally classified as acidulants and/or acidity correctors (15). They are added to foods at widely variable concentrations (from 200 to 30,000 ppm). Lactic, citric, phosphoric, adipic, carbonic, fumaric, malic, and succinic acid, as well as glucono-δ-lactone, are typically used as acidulant additives.

The high relevance of organic acids to food technology has fostered the development of a host of methods for their determination; most such methods are volumetric, electrochemical, enzymatic, or chromatographic (paper, thin-layer, gas, or high-performance liquid chromatography) (16). However, many of them are inappropriate for the fast, sensitive, reliable determination of the organic acids usually encountered in foods and beverages. Volumetric and spectrophotometric methods are far from specific, which entails implementing time-consuming preliminary separations (precipitation, extraction, or ion exchange). On the other hand, like most biological compounds, organic acids can be assayed enzymatically, provided specific enzymes for their catalytic degradation are available. The specificity of these methods enables application to such complex media as foods and beverages with little sample pretreatment. However, enzyme assays call for separate kits for each individual acid, which detracts from throughput and raises costs per test in multideterminations. While still to a limited extent and at an early stage of development in this field, electrochemical methods are increasingly being used in connection with biosensors, particularly those involving immobilized oxidases and dehydrogenases, which are widely used for sensing some organic acids (especially lactic and malic acid) in foods. As far as chromatographic techniques are concerned, paper and thin-layer chromatography are scarcely sensitive and provide information of a qualitative rather than quantitative nature. Gas chromatography is occasionally used for determining organic acids. However, because most of them are not volatile enough, they require derivatization (conversion of the carboxyl group into a methyl or trimethylsilyl ester function), which complicates sample pretreatment. Therefore, gas chromatographic methods are not as straightforward, expeditious, or reliable as required for this type of analysis.

High-performance liquid chromatography (HPLC) has simplified the analysis for various food constituents, including organic acids. In fact, it allows the fast, sensitive, and nearly specific determination of organic acids in foods and beverages, and involves uncomplicated sample treatment. A number of ion-exchange, ion-exclusion, ion-pair, and reverse-phase chromatographic methods have to date been developed for the separation and determination of organic acids in a variety of samples of scientific and technological interest. The method of choice in each case is dictated by the types of acid to be determined and their proportions as well as by the nature of the matrix that contains them.

II. NATURE OF ORGANIC ACIDS

Organic acids are characterized by their carboxyl group (—COOH), which dissociates into a proton and the conjugate base and endows them with their acid properties. The acid properties of other compounds present in foods arise from other functions; thus, the acidity of ascorbic acid is due to its two enol groups. Like other families of organic compounds, organic acids can be classified according to the type of carbon chain (aliphatic, alicyclic, aromatic, heterocyclic) they bear, their extent of unsaturation (saturated vs. unsaturated) and substitution (substituted vs. unsubstituted), and the number of functional groups they contain (monocarboxylic, dicarboxylic, etc.). The lowest monocarboxylic aliphatic acids (C_1–C_4) are pungent, highly volatile liquids, whereas those with five or more carbon atoms are oily, slightly water-soluble liquids. Dicarboxylic acids are colorless crystalline solids with melting points in the region of 100°C. Alicyclic acids, which include at least one nonbenzene cyclic hydrocarbon skeleton, are less water soluble

Table 2 Dissociation Constants of Organic Acids in Aqueous Solutions at 25°C

Monocarboxylic		Di- and tricarboxylic			
Acid	pK	Acid	pK_1	pK_2	pK_3
Unsubstituted		Oxalic	1.20	3.67	
Formic	3.75	Succinic	4.22	5.70	
Acetic	4.53	Fumaric	3.09	4.60	
Propionic	4.87	Maleic	1.91	6.33	
Butyric	4.83	Malic	3.46	5.21	
Isobutyric	4.84	Mucic	3.08	3.63	
Substituted		Saccharic	3.01	3.94	
Glycolic	3.70	Citric	2.79	4.30	5.65
Lactic	3.83	Isocitric	3.28	4.71	6.39
Pyruvic	2.39	Oxalacetic	2.22	3.89	13.00
Glyoxylic	2.98				
Alicyclic					
Quinic	3.58				
Shikimic	4.76				

than the previous ones. As such, all these acids form more or less soluble metal salts and esters; the latter are sufficiently volatile for gas chromatography and have spectral absorbing or fluorescent properties that make them amenable to HPLC analysis. All organic acids occur naturally in a variety of plant and animal substrates. Table 2 lists some of the organic acids most frequently assayed in foods, as well as their dissociation constants (pK), which are of paramount significance to the chromatographic procedures typically used for their isolation.

III. SAMPLE PRETREATMENT

There is no procedure currently available for determining organic acids directly in their matrices, whichever their physical state, without altering or destroying the matrix. In fact, all available methods for the determination of organic acids call for dissolved analytes. Consequently, solid samples require a prior extraction. In addition, the typically high qualitative and quantitative complexity of the substrates to be analyzed (beverages, fruits, vegetables, sweeteners, dried foods, meat products, dairy products, processed foods, etc., which contain a number of organic acids in variable proportions) demands complex sample pretreatment in order to remove potential interferents and also occasionally to increase the solute concentration and hence precision, accuracy, and sensitivity. How complex the pretreatment need be will be determined by the nature of the sample to be processed and the chromatographic separation and detection method to be subsequently applied.

As a rule, beverages entail less complex pretreatment than do foods; such pretreatment usually involves the mere dilution in an appropriate solution or in the mobile liquid chromatographic phase, to an extent proportional to the solid content, viscosity, and acid concentration of the substrate as well as to the sensitivity of the sensing system used. The dilution is usually followed by centrifugation or passage through a filter of 0.45- or 0.22-μm pore size. Carbonated drinks should be degassed for 5 min in an ultrasonic bath. Such a straightforward procedure allows the organic acids present in apple juice (17), wine (18,19), fermented vegetables (20), and vinegar (21) to be determined by reverse-phase chromatography with UV detection, and those in soft drinks to be

measured by ion-exclusion chromatography (22). The adverse effects of some substances (e.g., phenol compounds) on the column lifetime can be lessened by replacing the guard column after every 100–200 injections. Alternatively, some workers purify samples prior to their chromatographic injection, thereby also improving resolution. Solid–liquid chromatographic procedures involving nonpolar phases or ion-exchange resins are the most frequently used for this purpose. The former use Sep-Pak C_{18}, Bond-Elut C_{18}, or similar types of cartridges (18,23), through which a sample volume of 5–10 ml is passed in order to retain neutral and highly lipophilic compounds in the solid phase. The latter procedures involve retaining organic acids on a strong ion-exchange resin from which they are subsequently eluted with 1 M HCl (24,25) or 0.5 M H_2SO_4 (26). If more thorough sample cleanup is required (27) or if the carbohydrate, polyphenol, and acid fractions are to be isolated, it is more practical to combine two cartridges (one of each type) and to pass the sample through both. The eluate will contain the carbohydrates, which are not retained by either cartridge; the polyphenols will be retained on the nonpolar support and can be reclaimed by elution with methanol; and the organic acids will be retained on the ion exchanger and can be reclaimed as just described.

On account of their high water solubility, organic acids in semisolid and solid samples can be most readily determined merely by cutting up an adequate portion (5–100 g), grinding the resulting pieces for 3–5 min at a high rate in a blender containing enough water (occasionally hot water, at 60°C), whether acidified or not, and 70–80% ethanol or acetonitrile, depending on the particular type of sample (e.g., the recoveries of citric, pyruvic, uric, butyric, and hippuric acids from yogurt (28) were more satisfactory with 0.01 N H_2SO_4 than with water and acetonitrile), and on whether other constituents (e.g., carbohydrates, phenol compounds) are to be extracted simultaneously. The acids should be completely extracted, so the slurry typically requires stirring for about 2 h. After centrifugation and filtration through Whatman paper (no. 3 or 4), the filter cake is washed once or twice with fresh portions of extractant, and the extracts are concentrated in a rotary vacuum evaporator; the sample is thus made ready for analysis after filtration or solid–liquid extraction as previously described for beverages. This general treatment procedure, with slight modifications as required, has been used for the determination of organic acids in apple (29), mango fruit (30), cotton leaves (31), cheese (32), sweet potatoes (33), and meat, dairy products, and pickles (34), among others.

IV. DERIVATIZATION TECHNIQUES

As a rule, online and offline pre- and postcolumn derivatization processes used in liquid chromatography are intended to improve the identification and detection of the different solutes to be determined. These processes can be mandatory in the determination of carboxylic acids on account of their structural similarities and the typically low molar extinction coefficients of their chromophors, on which their sensing usually relies, owing to the lack of more sensitive properties (e.g., fluorescence, electrochemical activity). This in turn may result in poor resolution in separations involving large numbers of acids in the same sample and inadequate UV detection limits. All these constraints have fostered the development of a number of methods for the derivatization of organic acids involving the obtainment of highly absorbing compounds or fluorescent or electrochemically active derivatives—the last two result in even lower detection limits, thanks to the higher intrinsic sensitivity of the two techniques used.

Offline precolumn derivatization is the most common alternative in this respect; it involves separating the esters obtained from the organic acids by reversed-phase chromatography, which amply surpasses solvophobic chromatography (i.e., the use of undissociated acids as such) and allows gradient elution techniques to be applied, thanks to the wider lipophilicity range covered by the derivatized compounds.

Of the different derivatization methods available for the absorptiometric determination of organic acids, those using organic compounds containing phenacyl (35–37), naphthacyl (38), and *p*-nitrobenzyl groups (39–41) are worth special note, as are those using 4-methyl-7-methoxycoumarin (42), 4-methyl-6,7-dimethoxycoumarin (43), and 9-methylanthracene (44) with fluorimetric detection. Some derivatives containing one of these functions in addition to one or two electroactive groups (e.g., —NO_2) have also been used with electrochemical detection (45,46).

As a rule, the derivatization of organic acids with phenacyl bromide, *o*-(4-nitrobenzyl)-*N,N'*-diisopropylurea and some other reagents is carried out in a solvent such as acetonitrile, dioxane, or acetone, in the presence of a catalyst or phase-transfer reagent (most often 18-crown-6 ether or one of its derivatives, though tetrabutylammonium hydroxide is preferred for dicarboxylic acids) if an adequate yield is desired (42). The reaction must be carried out at a high temperature (80–100°C) and allowed to develop for 60–75 min. It is advisable to remove any excess reagent prior to injecting the sample in order to improve the appearance of the resulting chromatogram by passing it through a Sep-Pak silica or a similar cartridge, or, as recommended by Badoud and Pratz (41), by adding a small amount of a strong ion-exchange resin to the sample in a 50:1 resin-to-reagent ratio and allowing it to stand for 15 min prior to injecting a portion of the supernatant into the chromatograph. Caccamo et al. (47) recommend keeping the reaction medium at pH 7 with 0.08 M phosphate buffer in order to ensure a high esterification yield (particularly in citric acid) when phenacyl bromide is used. This reagent was also selected by Marcé et al. (48) for the determination of carboxylic acids in wine following an optimization study (simplex and simultaneous modeling) involving various reagents and experimental conditions.

The esters produced by these derivatizing reagents usually enable the sensitive determination of their parent acids in foods, with detection limits occasionally comparable to those provided by intrinsically more sensitive techniques, such as electrochemistry. The UV (254 nm) and electrochemical detection (1.1 V) limits of the *p*-nitrobenzyl derivatives of lactic, formic, and acetic acid are the same, and are equal to 1.8, 0.9, and 1.1 ng, respectively (45).

V. SEPARATION TECHNIQUES

A scan of recent literature on the topic clearly reveals that organic acids in fruits, juices, fermented beverages, vegetables, dairy products, roots, and foodstuffs in general are being more and more frequently determined by use of HPLC preferentially over other methods of analysis. The choice of a given HPLC technique (ion-exchange, ion-exclusion, ion-pair, or reverse-phase chromatography of undissociated or derivatized substrates) for the separation of organic acids occurring in foods is dictated essentially by their nature and that of their matrix. The most usual method for the determination of organic acids in foods and beverages is ion-exclusion chromatography, followed by reverse-phase chromatography. Notwithstanding their extensive application in other fields (and also, formerly, in food analysis), ion-exchange and ion-pair chromatographic methods are gradually being superseded, because the previous two alternatives provide better, faster, and more reliable results. The better-shape peaks are obtained employing ion-exclusion columns, and faster analysis times are achieved using reverse-phase columns. However, for some applications, other organic compounds present in the samples may interfere if UV detection is used. For this reason, some authors recommended that ion-exchange columns be combined with a conductivity detector for the simultaneous determination of organic acids and inorganic anions (49).

The detectors most frequently used in HPLC are of the refractive index (RI), conductivity, and UV-Vis types. While RI detectors are probably the most flexible, they are somewhat sensitive to changes in pressure, temperature, and the composition of the mobile phase, which demand

strict control and the use of isocratic elution. However, they can be of use for determining other components of interest, including carbohydrates and alcohols, simultaneously in a single chromatographic analysis. Conductivity detectors were originally employed in ion chromatography for the determination or inorganic ions and, later, organic acids. Their inherent pitfalls have deterred potentials users from applying them to food analyses, because solute conductivity measurements require the prior elimination of the eluent background conductivity using a conventional suppressing column or a more modern alternative such as a cation-exchange membrane loaded with tetrabutylammonium ion, a hollow-fiber suppressor with a neutral or alkaline enhancer, or a redox suppressor (50–53). Ultraviolet-visible detectors are no doubt the most frequently used at present for determining organic acids in foods. For underivatized organic acids, detection at 206–220 nm usually poses no serious problem in the determination of major organic acids. By using a photodiode array detector, by optimizing the detection and quantitation conditions, and by checking chromatographic peak purity, determining organic acids is fairly easy, for a single sample injection provides all the spectral information required for a given wavelength range during elution, which is collected as a data matrix for subsequent processing.

High-performance LC is no doubt the most suitable choice for determining organic acids on account of its expeditiousness (a chromatogram typically takes about 15 min to record), sensitivity (detection limits, calculated as three times the standard deviation of background noise, range from 100 ng for species with low molar extinction coefficients, such as succinic acid, and 1 ng for shikimic acid, and they can be lowered by a factor of up to 4 using columns of 2-mm ID), selectivity (recoveries are usually close to 100%), and reliability (coefficients of variation are typically less than 5%). In addition, the analytical costs of HPLC are fairly low; in fact, the cost of determining six organic acids in wine or cider amounts to only about $3 (reagent and solvent consumption, filters, and column wear included).

A. Ion-Exchange and Ion-Exclusion Chromatography

The ready ionization of organic acids has long been exploited for their isolation by ion-exchange chromatography, which involves the use of an ion-exchange resin as stationary phase. Palmer and List (54) set the pace for their isolation from foods. This separation technique is rarely used nowadays, however, because it is clearly outperformed by ion-exclusion chromatography, which usually relies on the use of sulphonated styrene-divinylbenzene cation resin. The term *ion-exclusion chromatography* was coined by Wheaton and Bauman (55) to name the exclusion phenomenon undergone by coions on exchangers as a result of electrostatic repulsion forces, which allowed electrolytes to be readily separated from nonelectrolytes. This operational principle, based on the finding of Harlow and Morman (56) that the elution time of an acid from a Dowex 50W × 12 column in its hydrogen form depends on its dissociation constant(s) (pK), molecular weight, and water solubility [Tanaka et al. (57), using a strong cation-exchange column, found the capacity factors of various acids to depend linearly on their first dissociation constant; the retention volume of the weak acids increased with their first pK], was exploited for the separation of organic acids in a variety of matrices, including foods. The retention mechanism involved can be explained in the form of Donnan partition equilibria between the protons in the solute and the fixed hydrogen ions in the resin. The relative retention of a highly crosslinked ion-exchange resin is also conditioned by hydrophobic interactions and steric effects (58). Ion-exclusion chromatography is generally quite efficient; however, it has one major shortcoming: some neutral compounds, such as sugars and polyphenols, two usual components of foods that possess similar retention times to those of organic acids, are also retained by cation-exchange resins. As a result, samples must be pretreated prior to their chromatographic separation. The column most frequently used for this purpose is the Aminex HPX-87H 300 mm × 7.8-mm ID model from Biorad Laboratories. The

mobile phase is usually 0.0008–0.185 N sulfuric acid. The column temperature is maintained at 50–75°C, and a UV (206–220 nm) or refractive-index (RI) detector is normally employed. This technique fails to resolve shikimic and succinic acid (18), galacturonic and tartaric acid (24), pyruvic, citramalic and malic acid (24), and quinic and malonic acid (59). Also, the peaks for succinic, glycolic, and lactic acid, and those of propionic and glutamic acid, are extensively overlapped (59). In order to improve the chromatographic resolution and avoid the need for wine sample pretreatment, Frayne (19) approached the separation by using two serially arranged Aminex HPX-87H columns and recording the signals from a RI and a UV spectrophotometer simultaneously. For the RI recording of a dry wine, the malic acid peak is overlapped with those of glucose and fructose, although it is still resolvable, as are another two compounds of enormous relevance to the viticultural industry, namely, glycerol and ethanol. Because glucose has no absorbing properties, this peak can be removed from the UV recording to improve the resolution of malic acid. This separation method, like the previous ones, fails to resolve shikimic and succinic acid. In addition, the acetic acid peak is markedly impurified by phenol compounds that are eluted with the same retention time. Table 3 summarizes the most interesting applications of this technique to the determination of organic acids in foods.

B. Reversed-Phase Chromatography

Most stationary phases used in bonded-phase chromatography in its reversed-phase mode are based on octadecylsilane functionality (C_{18} columns). The mobile phases typically used in this context are water, aqueous buffers of a given pH and ionic strength, and mixtures of water and a miscible organic modifier, such as methanol or acetonitrile.

Horvath et al. (60,61) showed nonpolar stationary phases such as octadecyl silica to allow the separation of relatively polar substances, including organic acids, by using an aqueous phase at an appropriate pH to prevent the ionization of the species to be resolved. Under these conditions, retention is governed by hydrophobic interactions between the hydrocarbon rest of the solutes and the hydrocarbon chains of the stationary phase. The foundation of this hydrophobic chromatography lies in the solvophobic theory (62), according to which capacity factors, and hence the retention of the solutes to be resolved, depend essentially on the pH of the mobile phase, which influences their ionization. In addition, the capacity factor of ionic substances increases with the salt concentration or ionic strength of the mobile phase—the effect on acids was studied by Jandera et al. (63). On the other hand, the capacity factor decreases with the concentration of organic modifier (methanol or acetonitrile) and increases with the size of the aliphatic chain or the aromatic skeleton of the acid. Both are expanded on esterification, which results in highly hydrophobic compounds that can readily be resolved in this way—so much so that they require the use of an organic modifier in the mobile phase for elution—and allows gradient elution to be implemented and chromatographic resolution to be substantially improved as a result. The column type most frequently used for this purpose is a C_{18} 5-μm, 250 × 4-mm ID one, through which a 10^{-3}–10^{-1} M KH_2PO_4/H_3PO_4 mobile phase at pH 2.2–4.2 is passed. Underivatized acids require less than 5% organic modifier, whereas derivatized acids call for 35–85% of acetonitrile or methanol and isocratic or gradient elution.

In a critical comparative study of the separation of organic acids in wine by ion-exclusion chromatography and reverse-phase chromatography, Goiffon et al. (24) found the latter alternative to be the better for the resolution of galacturonic, tartaric, malic, shikimic, lactic, acetic, citric, succinic, and citramalic acids, though optimal results could be achieved only by using three serially arranged C_{18} columns. Some authors claim that all major and some minor acids can be resolved with a single column (25). The aforesaid authors compared their results with those obtained by the conventional enzymatic methods and found them to be quite consistent. In a similar study on the resolution of quinic, malic, shikimic, lactic, citric, and succinic acids in apple ex-

Table 3 Ion-Exchange and Ion-Exclusion Chromatographic Methods for Determination of Organic Acids

Acids	Sample	Column	Mobile phase	Detection	Ref.
Acetic, lactic, succinic, gluconic, pyroglutamic	Vinegars	TSK gel IC-Anion-PW (50 × 4.6 mm) 40°C	0.45 mM OABS[a], pH 3.5	UV, 302 nm	72
Oxalic, citric, isocitric, gluconic, lactic, malic, fumaric, quinic, acetic, succinic, formic, glycolic	Beer, wine, and fruit juices (concentrates)	AMINEX PHX 87H (300 × 7.8 mm) 65°C	0.005 M H_2SO_4, pH 3.02	UV, 210 nm	73
Seventeen acids involved in Krebs cycle	Dairy products	ION 300 (300 × 7.8 mm) 25–55°C	0.0065–0.008 N H_2SO_4	RI	74
Acetic, lactic, succinic, malic, tartaric, citric	Wine and grape juice	ION 300 (300 × 7.8 mm) 74°C	0.013 M H_2SO_4	RI	75
Acetic, lactic, succinic, malic, tartaric, citric	Wine	ION 300 (300 × 7.8 mm) 30–80°C	0.01–0.05 N H_2SO_4	RI	56
Citric, lactic, acetic	Cocoa	AMINEX HPX 87H (300 × 7.8 mm)	0.013 N H_2SO_4	UV, 215 nm, + RI	77
Acetic, lactic, succinic, malic, formic, citric	Vinegars	HPICE/AS 1	0.01 N HCl	Conducti-metric	78
Tartaric, malic, citric, pyruvic, succinic, lactic, acetic, shikimic, galacturonic, citramalic	Wine	AMINEX HPX 87H (300 × 7.8 mm) 45°C	0.0075 M H_2SO_4	UV, 210 nm	24
Citric, succinic, acetic, some fatty acids	Wine	Dionex HPICE-AS 1 (250 × 9 mm)	1–2 mM H_2SO_4	Conducti-metric	79
Citric, malic, lactic, acetic	Cucumber juice	AMINEX HPX 87H (300 × 7.8 mm) 60°C	0.013 N H_2SO_4	UV, 210 nm, + RI	80
Succinic	Orange juice	AMINEX HPX 87H (300 × 7.8 mm)	0.036 M H_2SO_4	UV, 210 nm	81
Citric, malic, lactic, tartaric, acetic	Sparkling wine fermenting	AMINEX HPX 87H (300 × 7.8 mm) 65°C	H_2SO_4, pH 2.15–2.25	UV, 210 nm	23
Pyruvic, succinic, lactic, formic, acetic, pyroglutamic	Cheese	AMINEX HPX 87H (300 × 7.8 mm)	0.1 N H_2SO_4	UV, 220 nm	32

[a] OABS: *o*-aminobenzenesulphonic acid.

tracts and ciders (29) carried out by the author's group by using ion-exclusion and reverse-phase chromatography they found the last to yield the best results.

In a collaborative study, 12 laboratories worked in conjunction to develop a standard method for the analysis of organic acids in fruit juices. Coppola and Starr (64) recommended pretreating the samples by passing them through disposable silica cartridges, followed by microfiltration, passage through two serially arranged C_{18} 250 mm × 4.6-mm ID columns and elution

with 0.2 M phosphate buffer of pH 2.4 flowing at a rate of 0.8 ml/min, with final UV detection at 214 nm. Under these conditions, resolution and reproducibility were excellent for quinic, malic, ascorbic, and citric acid.

Among the derivatization methods applied to the determination of organic acids in foods worth special mention are those of Badoud and Pratz (41), who use *o*-(4-nitrobenzyl)-*N,N'*-diisopropylurea (PNB) as esterification reagent for the HPLC determination of quinic, glycolic, pyroglutamic, lactic, formic, acetic, malic, phosphoric, and citric acids in coffee; quinic and malic acids in apple juice; malic and citric acids in orange juice; and lactic, acetic, tartaric, malic, succinic, and citric acids in white and red wine. Furan-2-carboxylic, malonic, itaconic, fumaric, and mesaconic acids are also detected in coffee by the same procedure. These separations can also be accomplished by using short, small-bore columns (100 mm × 2.1-mm ID), which result in considerably shorter analysis times (12 min), and linear water–acetonitrile gradients (0–70% acetonitrile at 0.4 ml/min for 10 min). Mentasti et al. (37) used phenacyl bromide as esterifying reagent to resolve the esters of glyoxylic, lactic, acetic, tartaric, malic, citramalic, succinic, glutaric, and citric acids in wines and those of malic and citric acids in orange juice by using Hibar RT 250-4 RP-18 7-μm columns and a water–methanol gradient (35–85% MeOH at 2 ml/mm for 20 min). The only significant differences between their results and those provided by traditional methods were encountered in the determination of tartaric acid by the Blouin–Rebelein method as a result of the interaction of vanadate ion with the vicinal diol moiety occurring in various wine components. Marcé et al. (65) optimized the separation of phenacyl derivatives of organic acids in wine using the surface-response modeling method. Table 4 shows selected applications of this technique to the determination of organic acids in foods.

VI. CONCLUDING REMARKS

As shown by the literature published on the topic in recent years, chromatographic techniques in general and HPLC in particular—the latter requires no derivatization and involves minimal sample treatment—are no doubt the most frequently used choices for determining organic acids in foods. The high sensitivity and selectivity of HPLC allows the effective determination of major organic acids. On the other hand, minor and, especially, trace organic acids pose a twofold problem. Currently available detectors are not sufficiently sensitive to them, so a derivatization reaction or preconcentration is usually needed. Mass spectrometers are still very expensive—unaffordable by most laboratories—and difficult to interface to liquid chromatographs. Also, the increased number of potentially detectable species in some foods makes available chromatographic columns inadequate. The problem can be addressed by using commercially available packed capillary columns (40–500-μm ID). Adapting ordinary equipment for this purpose is fairly easy and can provide increased efficiency and mass sensitivity and allow ready coupling to other separation and detection techniques (66–70). In addition, mobile phases are circulated at very low flow rates (up to 10 μl/min), which results in dramatically decreased operational costs. The sample volume used in each analysis ranges from 60–100 nl to ordinary volumes (e.g., 5 μl), provided the eluting power of the solvent containing the sample is much lower than that of the mobile phase. The technique is currently at a development stage. Our group has used it to quantify quinic, malic, shikimic, and citric acid in apple juice with the aid of a C_{18} 30-cm × 320-μm-ID column, 10^{-2} M K_2HPO_4/H_3PO_4 at pH 2.7 as the mobile phase ($q = 2$ μl/min), and UV detection at 206 nm. The detection limits thus achieved for an injected volume of 60 nl ranged from 2.9 ng for citric acid to 0.04 ng for shikimic acid, the coefficient of variation being less than 5% in all instances (71). To the authors' minds, these miniaturized systems will be routinely used in food analyses in the near future.

Table 4 Reverse Phase Chromatographic Methods for Determination of Organic Acids

Acids	Sample	Column	Mobile phase	Detection	Ref.
Citric, malic	Calamondin orange juice (Philippines citric)	Spheri-5RP-18 (110 × 4.6 mm) + 2 columns Polypore H (110 × 4.6 and 220 × 4.6 mm)	2% KH_2PO_4, pH 2.3	UV, 215 and 260 nm	95
Tartaric, citric, malic, sorbic, benzoic	Juices, nectars, and soft drinks	Spheri-5RP-18 (220 × 4.6 mm) 5 μm	Water (HPLC) pH 2.2, H_2SO_4	UV, 214 and 230 nm	96
Quinic, malic, citric	Kivi and babaco	Spherisorb ODS2 (250 × 4.6 mm) 5 μm	Water (HPLC) pH 2.2, H_2SO_4	UV, 214 nm	97
Citric, malic, oxalic	Fresh fruits and commercial orange juice	Spheri-5RP-18 (110 × 4.6 mm) + 2 columns Polypore H (110 × 4.6 and 220 × 4.6 mm)	2% KH_2PO_4, pH 2.3	UV, 215 nm	98
Pyruvic, lactic, acetic, citric, formic, orotic, uric	Dairy produce	Beckman C_8 (250 × 4.6 mm) 5 μm	0.5%$(NH_4)_2HPO_4$, pH 2.24:CH_3CN (99.55:0.45)	UV, 214 and 285 nm	99
Formic, acetic, pyruvic, uric, orotic, citric, lactic	Cheese	Beckman C_8 (250 × 4.6 mm) 5 μm	0.5%$(NH_4)_2HPO_4$, pH 2.24:CH_3CN (99.98:0.2)	UV, 214 nm	100
Quinic, malic, citric	Tropical fruits	Spherisorb ODS 2 (250 × 4.6 mm) 5 μm	Water (HPLC) pH 2.2, H_2SO_4	UV, 214 nm	101
Oxalic, glycolic, malic, shikimic, lactic, citric, fumaric	Cane juice	Sup Rs C_{18} 5 μm 32°C	2% $NH_4H_2PO_4$, pH 2.18	UV, 214 nm	102
Malic, shikimic, lactic, citric, succinic, tartaric, acetic, fumaric, citramalic, glucuronic	Grape juice	Beckman ODS Ultrasphere C_{18} (250 × 4.6 mm) 5 μ	0.02% HCOOH (v/v), pH 3.07	UV, 190 nm	103
Malic, shikimic, lactic, citric, succinic, tartaric, acetic, fumaric	Wines and fruit juices	Spherisorb ODS 2 (250 × 4.6 mm) 5 μm 35°C	Na_2SO_4 (0.1M)/ H_2SO_4, pH 2.45–2.50	UV, 210 nm	104
Oxalic	Carambola (*Averrhoa Carambola* L)	Zorbax NH_2 (250 × 4.6 mm)	NaH_2PO_4 (0.15M), pH 2.4	UV, 206 nm	105
Malic, citric, succinic	Orange and grape juices	a) Zorbax NH_2 (250 × 4.6 mm) b) Hamilton PRP-1 (150 × 4.1 mm) 10 μm	a) NaH_2PO_4 (0.075M), pH 4.4 b) $HClO_4$ (0.03N), pH 1.7	UV, 206 nm	106
Citric	Orange and grape juices	Waters RC-100 (100 × 8 mm) 5 μm	2% $NH_4H_2PO_4$, pH 2.7	UV, 206 nm + RI	107

Table 4 Continued

Acids	Sample	Column	Mobile phase	Detection	Ref.
Oxalic	Rhubarb	LiChrosorb RP-8 C_8 10 μm	0.5% KH_2PO_4/ 0.005 M TBA[a]/ H_3PO_4, pH 2	UV, 220 nm	108
Malic, citric, oxalic, tartaric, succinic, fumaric	Tropical fruits	Waters RCM-100 C_{18} (10 cm)	2% $NH_4H_2PO_4$/ H_3PO_4, pH 2.4	UV, 254 nm + RI	109
Tartaric, malic, citric	Orange, lemon, apple, and grape juices	Bondapak C_{18} (300 × 4 mm)	H_3PO_4(aq), pH 2.2	RI	110
Quinic, malic, citric	Blueberry juice	Bondapak C_{18} (300 × 4 mm)	2% KH_2PO_4/ H_3PO_4, pH 2.4	RI	111
Quinic, malic, citric	Apple juice	Supecosil LC-18 (250 × 4.6 mm) 5 μm	KH_2PO_4 (0.2M)/ H_3PO_4, pH 2.4	UV, 214 nm	64
Tartaric, malic, shikimic, lactic, acetic, succinic, citric, citramalic	Wine	3 colum. RP C_{18} (250 × 4.6 mm) 5 μm	0.15% H_3PO_4/ 25% CH_3CN	UV, 210 nm	24
Acetic, succinic, malic, tartaric	Vinegars	LiChrosorb RP-18 10 μm	Aqueous octylammonium salicylate	Conducti-metric	112
Citric, malic, tartaric, oxalic, ketoglutaric	Mango	Waters Radial-Pak C_{18} 10 μm	0.2M KH_2PO_4/ H_3PO_4, pH 2.4	UV, 214 nm	30
Malic, citric, quinic, succinic, lactic, shikimic	Apple juice and cider	Spherisorb ODS-2 C_{18} (250 × 4 mm) 5 μm	0.01 M KH_2PO_4/ H_3PO_4, pH 2.25	UV, 206 nm	29
Lactic, acetic, tartaric, malic, citric, succinic	Wines	Merck Lubar RT 240–4 RP-18 5 μm	H_2O/CH_3CN (65:35)[b]	UV, 254 nm	37
Lactic, acetic, tartaric, malic, citric, succinic	Wines	C_{18} (250 × 4.6 mm) 5 μm	H_2O/CH_3CN (linear gradient elution)[b]	UV, 254 nm	47
Lactic, acetic, quinic, glycolic, citric, others	Wines and fruit juices	(a) Nucleosil-5RP-18 (250 × 4 mm) (b) Hypersil ODS (100 × 2.1 mm)	H_2O/CH_3CN (linear gradient elution)[c]	UV, 265 nm	41
Lactic, succinic, malonic, malic, tartaric, maleic	Wine	Nova-pak C_{18} (150 × 3.9 mm) 4 μm coated with CPCl[d]	0.4 mM KHPh, pH 4.87	Indirect UV, 254 nm + conducti-metric	113
Glucuronic, γ-lactone, shikimic, dihydro-ascorbic, ascorbic, malic, tartaric, iso-citric, succinic, citric, oxalic, fumaric, gallic	Fruits, fruit juices, and vegetables	Spherisorb ODS2 (250 × 4.6 mm) 10 μm	0.01 M KH_2PO_4/ 0.8 M TBA[a]/ H_3PO_4:MeOH (97:3), pH 2.4	UV, 190–340 nm	114
Tartaric, malic, lactic	Wine	Lichrospher RP18 (100 × 4.2 mm) μm 40°C	0.2% H_3PO_4	UV, 220 nm	115

Table 4 Continued

Acids	Sample	Column	Mobile phase	Detection	Ref.
Oxalic, quinic, tartaric, malic, isocitric, ascorbic, citric, fumaric, succinic, propionic, *cis*-aconitic	Orange juice	YMC-Pack ODS-AQ (250 × 4.6 mm) 5 μm	20 mM KH_2PO_4, pH 2.8	UV, 214 nm	116
Galacturonic, gluconic, tartaric, pyruvic, quinic, malic, isocitric, succinic, fumaric, propionic, dimethylglyceric, 2-oxopentanoic, glutaric	Honey	Spherisorb ODS (250 × 4.6 mm) 5 μm	H_2SO_4, pH 2.45	UV, 210	26
Ascorbic, quinic, formic, shikimic, lactic, malic, tartaric, citric, isocitric, fumaric	Fruit juices	Spherisorb ODS2 (250 × 4 mm) 5 μm 40°C	0.01 M KH_2PO_4/ 5.5 mM TBAP[e], pH 7:MeOH (gradient elution)	UV, 210 –270 nm	117
Quinic, malic, shikimic, ascorbic, citric, fumaric	Strawberry	Spherisorb ODS2 (250 × 4.6 mm) 3 μm	0.01 M KH_2PO_4/ H_3PO_4, pH 2.5	UV, 206 nm	118

[a] TBA: tetrabutylammonium hydrogensulfate.
[b] Prior derivatization with phenacylbromide.
[c] Prior derivatization with *o*-(4-nitrobenzyl)-*N,N'*-diisopropylurea (PNBDI).
[d] CPCl: *n*-cetylpyridinium chloride.
[e] TBAP: tetrabutylammonium dihydrogenphosphate.

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Determination of Mycotoxins in Grains and Related Products

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I. INTRODUCTION

Toxigenic fungi can affect the most common and relevant crops during the growth, harvest, or storage steps and produce toxic substances known as mycotoxins. Over recent decades it has been recognized more and more that mycotoxin contamination of food and feed has a widespread significance on human and animal health, and the number of mycotoxins studied and considered is constantly increasing. Among the reasons for which mycotoxins contaminate approximately 25% of the world's food crops (1) are prominently inappropriate agricultural and storage practices and intercontinental shipping. As far as grains are usually concerned, technological procedures as well as domestic cooking procedures hardly ever reduce the level of the toxins, so it is likely that mycotoxins enter via the alimentary channel, especially in countries with environmental and climatic conditions favorable to mycotoxin contamination and poor hygienic and economic levels. Hundreds of papers have so far been published on the presence of mycotoxins in grains worldwide. Nevertheless this data, in terms of frequency and level of contamination, is greatly influenced by the reliability and detection limits of the adopted method of analysis and even more by the sampling procedures. Side-by-side evidence of the extent of mycotoxin contamination in the food channel is shown in studies on exposure biomarkers in human biological fluids (serum and milk), limited for now mainly to the frequency and level of ochratoxin A, one of the most toxic and widespread mycotoxins. A rather high diffusion of this toxin has been found, with the level of contamination being closely related to the origin of the samples (Canada, Italy, Sweden) and especially the detection limit of the method employed. Studies on biomarkers represent a valuable tool for the evaluation of exposure to mycotoxins and of the biological effects of mycotoxins. The issue has been reviewed by Wild et al. (2).

II. TOXICITY OF MYCOTOXINS

The different biosynthetic pathways through which mycotoxins are produced result in a broad diversity of chemical structures and, consequently, in a variety of toxic effects in humans and animals. An excellent review of the toxic effect of mycotoxins (3) is based on a "system approach," where the toxic effects are discussed on the basis of the body systems affected by the mycotoxins. Hepatotoxic, gastrointestinal, hematopoietic, and nephrotoxic effects are treated, as well as

immunotoxicity, teratogenicity, mutagenicity, and cancerogenicity. As far as the last effect is concerned, experimental and epidemiological evidence indicates that the most studied mycotoxins (aflatoxins, ochratoxin A, and fumonisins) are associated with different forms of human cancer, and this has largely influenced the risk management of those toxins. The mechanism of action of aflatoxin B_1, ochratoxin A, and fumonisin has been reviewed by Fink-Gremmels (4).

III. ISSUES RELATED TO RISK MANAGEMENT

In view of the global significance of the mycotoxins, a substantial number of programs dealing with the risk management of these toxic substances have been developed, both at the national and the international levels. This issue, which includes toxicological evaluations, has been reviewed by Pohland (5) and includes, among other initiatives, ones dealing with monitoring and education as well as the harmonization of regulatory levels and methods of analysis. All these topics are strictly interrelated, since mycotoxin contamination is a multifaceted problem, and technical and scientific issues have political, economic, and social ramifications. Nevertheless, the consumer expects that, following risk assessment, regulatory agencies will accomplish the appropriate risk management, in order to ensure a safe food supply. The issue of risk management for mycotoxins has been reviewed by Kuiper-Goodman (6).

A. Regulation of Mycotoxins

The generally recognized harm to health attributable to mycotoxins has prompted many countries to impose maximum levels that limit mycotoxins in food and feed commodities. The acknowledgment of limits is of extreme importance to the decisions of the analyst, especially when the testing for mycotoxins is carried out for control purposes or when evaluating the compliance of a food product with recommended or imposed limits.

As far as limits are concerned, many different issues have to be evaluated: Scientific demands deal both with risk assessment and with errors associated with the evaluation of the toxins in bulk, and interact with economic, political and trade considerations. Setting limits is necessary in order to protect the consumer and to support the producer both in trade and in maintaining a high standard of production. Unfortunately, the definition of limits on a strictly scientific basis is not yet fully pursuable, since many uncertainties still arise, mainly from incidence and consumption data, hazard analysis, and sampling procedures. Valuable consideration on defining the maximum limits has been done by Rosner (7), and information on worldwide regulation for mycotoxins is given by the U.N. Food and Agricultural Organization (FAO) (8). In 1998 (9) the European Union (EU) established maximum allowable limits for aflatoxin B (AFB_1) and total aflatoxins (AFLs) in peanuts (2 $\mu g/kg$ for AFB_1 and 4 $\mu g/kg$ for AFLs in peanuts intended for human consumption; 8 $\mu g/kg$ for AFB_1 and 15 $\mu g/kg$ for AFLs in peanuts intended for further processing) and in cereals intended for human consumption or as an ingredient (2 $\mu g/kg$ for AFB_1 and 4 $\mu g/kg$ for AFLs). Limits were also established for aflatoxin M_1 (AFM_1) in milk and milk products (0.05 $\mu g/kg$), and limits for others mycotoxins are currently under discussion. The advisory level for deoxynivalenol in wheat was set in the United States at 1 $\mu g/g$ in wheat products for human consumption (10).

B. Control of Mycotoxins in Food

Integrated strategies for the risk management of mycotoxins include, among others, the testing of food products, whatever the ultimate goal of the test is. The control of mycotoxins in grains is

performed with the chief aim of complying with guidelines, legal limits, or trade agreements or to check for the critical control points in a food chain, according to the principles of hazard analysis critical control points (HACCP) or to control finished products for the purpose of monitoring or punishment. The analytical methods should be chosen on the basis of such criteria as reliability and specificity. Furthermore, the ultimate scope of the analysis is often related strictly to the limits, so sensitivity is a driving factor in the selection of the analytical method. Whatever the aim of the test of mycotoxins in grains, testing is accomplished through the following analytical sequence: (1) sampling, which implies the collection of incremental samples and, depending on the desired sample size, reduction to a smaller test sample; (2) sample preparation, usually achieved by comminuting the test sample and removing a subsample; (3) analysis of the test portion.

Tests for mycotoxin contamination can be accomplished both on the finished product and on the raw form. The latter case prevents the manufacture of an unfit product, but it often implies trouble in the evaluation of the contamination in a batch. In this case, for a defined sample size, sample preparation, and analytical method, principles are available to evaluate the accuracy of the aflatoxin determination, depending on the availability of an accurate estimate of the variability associated with each step of the analytical sequence (11,12). A valuable effort to estimate the uncertainty of the analytical sequence as a whole was carried out by the FAO (13).

C. Sampling

Mycotoxin distribution in grains is largely nonhomogeneous, since often only a reduced amount of grains in a batch are contaminated at very high levels.

The total error associated with the analytical sequence combines the errors related to the three steps, i.e., sampling, subsampling, and analysis. Almost all reports on sampling are related to aflatoxins, for which sampling represents the largest source of error (the lower the level of contamination, the greater the error of sampling). The acceptability of 10 theoretical distributions was evaluated by Whitaker et al. (14), to simulate observed sample aflatoxin test results; they showed that obtaining a representative sample was an arduous and costly step, while improper sampling procedures were likely to result in the analysis of an unrepresentative sample of the batch. Nevertheless, quality control of sampling does not always receive the correct attention, and in many instances the analyst is unaware of the sampling procedures that have been adopted prior to delivery of the sample to the laboratory. The purpose of a sampling plan is to minimize the sampling error in the most pragmatic way possible. In other words, the requirement of a statistically based sampling plan has to match practical procedures. The decision of a sampling plan is largely dependent on the analytical procedure and the acceptance limit. Therefore the harmonization in sampling procedures among countries first requires the harmonization of legal limits or guidelines.

The uneven distribution of mycotoxins in grain has so far been statistically defined only for aflatoxins in a few matrices (corn, cottonseed, peanuts) (15,16), and sampling plans have been developed worldwide almost entirely for these toxins.

An effort to harmonize sampling procedures was made by the FAO (13) through specific recommendations for procedures in peanuts and corn, on the basis of two sample sizes, analysis by thin-layer chromatography (TLC), and five guideline levels ranging from 5 to 30 μg/kg AFLs.

In the United States, sampling plans were developed as a cooperative effort between the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and the Peanuts Administrative Committee (PAC). For peanuts they are based on the collection of a bulk sample (70 kg), obtained either from a stream or by probing every fourth bag, thoroughly

mixing it and subdividing it into three test samples of 21.8 kg each. After being comminuted, a 1100-g analytical sample is drawn for analysis using a subsampling mill. For other grains, different sampling plans are used depending on the nature of the food and the type of container. Suggestions for the preparation of lot samples are given in the AOAC Official Methods of Analysis (17).

In Europe many different sampling plans have been developed so far and been adopted by several countries, reflecting differences in national guideline standards and in the accepted compromise between procedures based on a sound statistical base and/or pragmatism.

During the year 2000, discrepancies at the EU level will be removed, since Directive 98/53/EC (18), laying down the sampling methods and the methods of analysis for the official control of levels for certain contaminants in foodstuffs, will be enforced. It contains rather pragmatic sampling procedures for aflatoxins both for bulk and for retail samples of dried fruits (figs and nuts) and cereals. As regards dried fruits and cereals, sampling criteria have been formulated correlating the number and weight of the final samples to be collected to lot sizes. For big lots, the number of incremental samples has been set to a minimum of 100, with weight ranging from 100 to 300 g. For smaller lots (15 ton), the number of incremental samples (100 g each) range from a minimum of 10 to 100.

Additional information on the general topic of sampling can be obtained in recently published reviews (19,20).

IV. MYCOTOXIN ANALYSIS

A. Laboratory Precautions

Despite data reporting occupational exposure that can occur through the handling and processing of mycotoxins and manipulation in the laboratory, little focus has been placed on those issues, and precautions to be undertaken in the laboratory are rarely mentioned in papers dealing with the analysis of mycotoxins. As far as the latter issue is concerned, safety measures include precautions in handling mycotoxins, and the decontamination and destruction of laboratory wastes. In addition, it is necessary to institute precautions aimed at avoiding the loss of accuracy.

1. Precautions in Handling and Decontaminating Mycotoxins

The weighing of standards in dry form must be avoided, since mycotoxins are highly electrostatic and can disperse in the work area. The handling of standards, either in powder or in concentrated and reference standard working solutions, must be performed with extreme care under a hood and while protecting the face of the operator with an appropriate mask and the operator's hands with gloves (vinyl gloves are strongly discouraged; latex items are recommended). The glassware must be cleaned with a powerful oxidant (bleach or sulfochromic mixture). Procedures for decontamination of the glassware and work areas include overnight treatment with acetone and sodium hypochlorite.

At the end of the workday it is recommended that work areas be decontaminated overnight with sodium hypochlorite and thoroughly washed and that the surfaces be checked for neutrality before starting a new analysis.

During the analysis, a flask containing a 5% aqueous solution of sodium hypochlorite should be kept near the bench, in case of emergency. The issue of safety precautions has been studied rather extensively for aflatoxins (21), ochratoxin A, citrinin, sterigmatocystin, and patulin (22).

2. *Precautions Aimed at Avoiding Loss of Accuracy*

Precautions must be undertaken to avoid degradation of the aflatoxins during the analyses. Filters for ultraviolet (UV) lights should be applied to the windows. Pretreatment of the glassware with sulphuric acid 2 N must be performed in order to neutralize the alkaline residue derived from the glassware, since alkali is responsible for aflatoxin destruction. The latter precaution is particularly necessary when using a new set; it is less crucial for glassware already used. General indications are also given in the AOAC *Official Methods of Analysis* (17).

In analyses using TLC, ad hoc precautions aimed at avoiding any loss of accuracy should be adopted (17).

The precautions to be undertaken when analyzing mycotoxins should be written down in ad hoc standard operating procedures.

B. Quality Criteria for Mycotoxin Analysis

The reliability of measurements plays a pivotal role in food and agricultural areas, particularly in the case of undesirable toxic compounds such as mycotoxins. Quality-control principles for mycotoxin analysis are common to other trace analyses, so good laboratory practices, such as EN 4500, represent the heart of quality assurance requirements. *Harmonized Guidelines for Internal Quality Control in Analytical Chemistry Laboratories*, published by IUPAC (23), also presents valuable guidelines for the determination of mycotoxins.

As far as aflatoxins are concerned, the variability associated with analytical methods in mycotoxin analysis was specifically pointed out by Horwitz et al. (24), demonstrating that notwithstanding the huge improvements in instrumentation and technology, among-laboratories precision has not improved significantly over the past 20 years, whereas within-laboratory precision has been greatly enhanced. Whitaker et al. (25), in evaluating a total of 1019 analytical precision estimates for total aflatoxins, stated that liquid chromatography (LC) had the lowest analytical variability, whereas ELISA had the highest. For a given method, among-laboratories variability was approximately twice the within-laboratory variability.

The working group CEN TC 275/WG 5 searched for performance criteria to be used in mycotoxin analysis and came up with a document reporting the criteria for the selection of methods (26). The criteria deal with limits of detection, minimum performance characteristics, extraction solvents, and applicability. Criteria for analytical methods in mycotoxin analysis are also included in Directive 98/53/EC (18).

Among the elements of quality control in mycotoxin analysis, proficiency tests, control materials (reference materials and certified reference materials), traceability in spiking, and recovery checks have been demonstrated to be particularly relevant.

C. Reference Materials

Analyzing control materials alongside the test samples greatly improves proficiency in mycotoxin analysis. Certified reference materials (CRMs) represent ideal control materials, due to their statement of uncertainty and traceability, and they should be routinely used as much as possible. Unfortunately, as outstanding as the improvements made in the last decade have been, even though the list of CRMs in the area of mycotoxins is rather long, it is still insufficient. A list of the available reference materials in the mycotoxins area is reported in Table 1; the issue has been reviewed by Boenke (27).

Table 1 Mycotoxin Certified Reference Materials (CRMs)—Grains ($\mu g/kg$)

Mycotoxin	CRM 262 Defatted peanut meal	CRM 263 Defatted peanut meal	CRM 264 Defatted peanut meal	CRM 401 Peanut butter	CRM 385 Peanut butter	CRM 375 Compound feed	CRM 376 Compound feed
Aflatoxin B_1	<3.0	43.3 ± 2.8	206 ± 13	<0.2	7.0 ± 0.8	<1	9.3 ± 0.5
Aflatoxin B_2				<0.1	1.1 ± 0.2		
Aflatoxin G_1				<0.1	1.7 ± 0.3		
Aflatoxin G_2				<0.1	0.3 ± 0.2		

Mycotoxin	CRM 471 Wheat	CRM 472 Wheat	CRM 377 Corn meal	CRM 378 Corn meal	CRM 396 Wheat meal	CRM 379 Wheat meal
Ochratoxin A	<0.6	8.2 ± 1.0				
Deoxynivalenol			<50	430 ± 40	<50	670 ± 20

IRMM, European Commission

D. Proficiency Testing

Proficiency testing represents a key issue in achieving external quality control. A sound quality assurance program should envisage the participation in national and/or international proficiency tests, aimed both at the improvement in quality of data and the updating of analytical methods applied on a routine basis by the laboratory. Furthermore, proficiency testing is gaining increasing relevance in the accreditation process. As far as mycotoxin analysis is concerned, the bodies presently involved in proficiency testing are FAPAS (Food Analysis Proficiency Assessment Scheme, UK MAFF) and the U.S. Department of Agriculture, Agricultural Marketing Service (limited to aflatoxins in peanuts).

E. Analytical Methods

Despite the fact that biological and predominantly immunoenzymatic methods have received increasing attention during the last decade for the determination of aflatoxins, chemical and immunochemical assays have to be preferred for their characteristics, including a lower limit of detection and high specificity.

Even though the attention of scientific literature to TLC methods has decreased steadily over the past few years, TLC is still a routinely used analytical technique, especially in developing countries. Improvements in the technique includes the use of a microcomputer interfaced with a fluorodensitometer to simplify data handling (28).

Gas chromatography (GC) is largely used for mycotoxins, such as zearalenone and especially trichothecenes, for which the use of mass spectrometry is crucial in the identification step.

High-performance LC is by far the most studied technique, and it is used whenever possible for aflatoxins, ochratoxin A, and zearalenone, especially in view of the improved rapidity attributable to the use of immunoaffinity in the cleanup step.

An excellent review of methods in mycotoxin analysis was published by the FAO (29), and yearly updates on analytical methods are provided by the General Referee Report, Committee on Natural Toxins, published in the *Journal of AOAC International.*

As far as the mycotoxin analysis based on HPLC techniques is concerned, most methods include the following steps.

Extraction: In order to extract the toxin from the matrix, solvents or mixtures of solvents (methylene chloride, bicarbonate solution, methanol–water, chloroform–water) are used. Two main types of apparatus are commonly used: the mechanical shaker (Ultra-Turrax homogenizer, multi-Wrist, magnetic stirrer) or High-Speed Waring Blenders. Other, rarely used extraction procedures are Soxhlet-type extractors and, more recently, supercritical fluid extraction. The time of extraction ranges from a few minutes (3–5) to 1 hour, depending on the procedure employed.

Cleanup: An additional separation of the mycotoxin from lipids and other components of the matrix is accomplished through the cleanup step. Most procedures include solid-phase extraction on stationary phases such as silica, C_{18}, florisil, and phenyl. Prepacked columns are largely used, with the variations between lots being recently ameliorated. Alternatively, the use of cleanup by immunoaffinity, based on the formation of mycotoxin–protein conjugate, is on the increase, since this is very rapid, selective, and usefully employed in various food matrices. One disadvantage is that the cost is still rather high, and cross-contamination phenomena (false-positive) can occur (30).

Determination: The last step in mycotoxin analysis includes an additional separation achieved by HPLC column, followed by detection and quantitative determination, performed on the basis of the physicochemical characteristics of the mycotoxin.

More details and references are given in the separate sections related to the individual mycotoxins.

V. AFLATOXINS

Aflatoxins are produced by species of *Aspergillus*, mainly *A. flavus* and *A. parasiticus*, ubiquitous in hot and humid climatic areas and occurring in a wide variety of commodities, including cereals, oilseeds, nuts (such as peanuts, walnuts, Brazil nuts), dried fruits (especially figs), spices, desiccated coconuts, and crude oils. Drought stress and insect injury represent the main causes of fungal contamination and aflatoxin production in the field; in storage, humidity higher than 85% and temperatures above 25% are favorable to the growth of fungi (31).

Aflatoxins are chemically related to difuranocoumarin, and out of 17 isolated aflatoxins, four naturally occurring aflatoxins—B_1, B_2, G_1, and G_2—are considered relevant, in view of their toxicity and diffusion. The G series contains a D lattone ring, instead of a cyclopentenone ring occurring in the B series, this difference being responsible for the higher toxicity of the B series (32,33). The most toxic and widespread metabolite produced by livestock fed with aflatoxin-contaminated feeds is aflatoxin M_1, excreted in milk, but other aflatoxins, such as aflatoxicol, aflatoxin Q_1, and aflatoxin P_1, have also been considered. Molecular structures of aflatoxins are presented in Fig. 1. Aflatoxins generally exhibit a strong fluorescence (Ex 223 nm, $\epsilon = 25{,}000$; 225 nm, $\epsilon = 13{,}400$; 362 nm, $\epsilon = 21{,}800$), widely used for analysis and detection in sorting. Chemical and physical data of aflatoxins is provided extensively by IARC (34).

Aflatoxin B_1 is regarded as hepatocarcinogen and genotoxic (2,9). Exhaustive reviews on toxicity, metabolism, and epidemiological studies are provided by IARC and more recently by several authors (2,4).

Procedures aimed at reducing or detoxifying aflatoxins and/or their effects have been reviewed by Phillips et al. (35), and include technological procedures for food and feeds and chemical degradation, as well as biocontrol and microbial inactivation, dietary modification and chemoprotection, and reduction in toxin bioavailability via selective chemisorption with clay.

Fig. 1 Molecular structure of aflatoxins B_1, B_2, G_1, and G_2.

A. Analysis

1. Preparation of Standards

Diluted solutions of aflatoxins are not very stable, since they are degraded under UV light. Therefore it is necessary to renew them regularly. It is worth preparing a concentrated stock solution [10 μg/ml in benzene:acetonitrile (90:10 or 97:3)] that is sufficiently stable and then preparing working solutions whenever necessary.

Generally, manufacturers supply aflatoxin as a preweighed dry film in a bottle. This standard can be dissolved in the volume of benzene–acetonitrile required to give a concentration of 8–10 μg/ml; the actual concentration is measured by UV spectroscopy. At the wavelength of maximum absorption (A_{max}) the aflatoxin concentration can be calculated using the following formula:

$$\mu\text{g aflatoxin/ml} = \frac{\text{optical density} \cdot \text{molecular weight} \cdot 1000 \cdot \text{CF}}{\epsilon}$$

in which ϵ is the molar coefficient of absorption and CF is the correction factor for the instrument.

In Table 2, the values of λ_{max} and ϵ for five aflatoxins in different solvents are given. The solutions are placed in water-tight containers, ambered or aluminium-foil covered, to protect from UV rays, and stored at 4°C. Stored, well-preserved stock solutions are stable for 1 year. Working solutions of 1 μg/ml can be prepared weekly in benzene:acetonitrile (90:10 or 97:3).

The CF can be determined according to AOAC *Official Methods of Analysis* (17) as follows: Determine the absorbance of the three solutions of $K_2Cr_2O_7$ in H_2SO_4 0.018 N (0.25, 0.125,

Table 2 Wavelengths of Maximum Absorption (λ_{max}) and Molar Coefficient of Absorption (ϵ) of Aflatoxins

Aflatoxin	Weight	Methanol (λ_{max}/ϵ)	Acetonitrile (λ_{max}/ϵ)	Benzene–acetonitrile[a] (λ_{max}/ϵ)	Chloroform (λ_{max}/ϵ)
B_1	312	360/21,800	355/20,600	348/19,800	360/20,600
B_2	314	362/23,800	358/22,500	348/20,900	360/24,300
G_1	328	362/18,000	360/17,800	355/17,100	360/19,500
G_2	330	362/20,900	358/20,700	355/18,200	360/20,900
M_1	328	357/19,000	350/19,850	345/17,450	357/19,950

[a] (97–3).

and 0.0625 mM), at maximum absorption near 350 nm, against 0.018 N H_2SO_4 as solvent blank. Calculate the molar coefficient of absorption (ϵ) at each concentration: $\epsilon = (A \cdot 1000)$/concentration in mM. If the three values vary by more than the guaranteed accuracy of the (Optical Density) O.D. scale, it is necessary to verify either the analyst or the instrument. Average the three ϵ values to obtain the final ϵ. Determine the correction factor for the particular instrument and the cells by substituting into the equation CF = $3160/\epsilon$, where 3160 is the value for ϵ of $K_2Cr_2O_7$ solutions. If the CF is less than 0.95 or greater than 1.05, verify either the analyst or the instrument to determine and eliminate the cause. The same set of cells in the calibration and determination of purity should be used.

More information on the preparation of standards is given by the AOAC *Official Methods of Analysis* (17).

2. *Extraction*

The extraction methods for aflatoxins are based on the solubility of these toxins in organic solvents, mainly chloroform, methanol, acetone, benzene, and acetonitrile. For more complex matrices, the addition of diatomaceous earth or citric acid is required. From matrices of vegetable origin, water is usually added in the extraction step, since it facilitates solvent penetration into substrates, improving the percentage of extraction of the toxin.

a. Extraction with Chloroform

A chloroform–water mixture is the extraction solvent for the contaminants branch (CB) method, proposed by Eppley (36). The CB method has been adopted by the Association of Official Analytical Chemists as the official method for determination of aflatoxins in groundnuts and their byproducts (17).

Extraction with a mixture of chloroform–water is also used in the EEC method (37) in feedstuffs, based on the Paulsch et al. method (38), and has been successfully adopted for various food matrices, such as rice, spices, grains, and dried fruit samples: 50 g of finely ground sample are mixed with 25 g of diatomaceous earth, moistened with 25 ml of water, and thoroughly shaken to obtain a homogeneous blend. Two hundred and fifty ml of chloroform are added and the whole shaken vigorously for 30 min on a vibrating shaker. Fifty ml of chloroform extract, recovered by filtering through a folded filter paper, is used for subsequent purification and assay.

The diatomaceous earth retains various substances, such as pigments, by adsorption, which simplifies subsequent purification and at the same time facilitates filtration by preventing the filter from clogging.

b. Extraction with Methanol

A methanol/hexane mixture is used in the best food (BF) method to extract the toxin from the substrate: 100 g of sample is added to 500 ml of a methanol–water (55:45) mixture and 200 ml of hexane. After 1 min of vigorous shaking, the resulting solution is centrifuged for 5 min at 2000 rpm. Twenty-five ml of the methanol phase is used for the TLC assay. The method has been adopted by the AOAC *Official Methods of Analysis* in peanut and peanut products (17).

Methanol–water is the extraction medium of the method recently tested for validation by the European Commission (39), for the determination of aflatoxins at the European regulatory limits for dried figs, pistachios, peanut butter, and paprika: 50 g of the test portion are extracted with methanol–water (80:20) for dried figs and paprika, and methanol–water (80:20) plus 100 ml of hexane for peanut butter and pistachio. After filtration, the filtrate is added to phosphate buffer saline (PBS) for the purification step.

c. Extraction with Acetone

A mixture of acetone/water is used in the Roch et al. method (40) for aflatoxins in groundnut cake: 1 kg of the sample is ground to a fine powder and mixed using a Hobert Vertical Cutter Mill (VCM 25). Sixty g of cake is extracted with 600 ml of acetone:water (85:15). The mixture is carefully shaken and filtered through a folded paper; 5 ml of the acetone extract is used for the subsequent purification assay.

d. Extraction with Acetonitrile

Acetonitrile–water is used in the Patey et al. method (41) for the determination of aflatoxins in peanut butter: 10 g of peanut butter and 30 ml acetonitrile/water mixture are shaken. Forty-five ml of water is added, and the mixture is shaken for an additional 30 min. After centrifugation at 4000 rpm at 30°C, the supernatant is filtered, added to PBS, and gently mixed for 15 s.

3. *Cleanup*

The cleanup step is generally based on two different approaches: (a) the use of solid-phase extraction (SPE) cartridges, (b) the use of immunoaffinity (IA) columns.

SPE cartridges: Commonly silica, Florisil, or C_{18} cartridges or a combination of these are used. The major advantages of this methodology are the elimination of the column chromatography cleanup step, the possibility of regenerating the cartridge for further analyses, and the low cost. The disadvantages are associated with the potentially low reproducibility of different batches of columns and/or low repeatability within a single batch.

IA columns: The use of antibody-based immunoaffinity columns has recently led to a considerable increase in the reliability of results, due to the high selectivity of this technique. Other advantages include the reduced time of analysis, and the possibility of analyzing more than one sample simultaneously. Basically the extract is purified into an immunoaffinity column containing antibodies specific to aflatoxins. The column is previously conditioned by applying 10 ml of PBS at a speed of 2–3 ml/min (gravity). The column is washed with water to remove impurities from the immunoaffinity column. Aflatoxins are separated from the antibody by passing methanol through the column. The resulting methanol solution can be injected into HPLC. An immunoaffinity column in the cleanup step is used in the method tested at the European Commission (39). More precision between samples results can be obtained using a

workstation in which sample preparation, derivatization, and HPLC injection is automated (42).

B. HPLC Analysis

To date, two main principles of liquid chromatography have been used: adsorption chromatography and liquid partition chromatography. In consideration of the wide development of reversed-phase columns and the consequent increasing applicability to various food matrices, adsorption chromatography has been almost entirely abandoned. Basically, in reversed-phase–partition chromatography, the mobile phase is more polar than the stationary phase, and combinations of solvents such as methanol, acetonitrile, water, and, rather infrequently, acetone are currently used as mobile phases. As for the stationary phase, the use of silica as support with chemically bonded chemical groups such as octadecyl or octyl is quite common. The residual free sylanolic groups are saturated with C-2 or other groups in order to increase the percentage of carbon loading. The main aim is to achieve a reduction of tailing peaks with a more symmetrical shape. Such columns are labeled as ODS1, ODS2, ODS3, or fully endcapped.

As regards the aflatoxin detection, UV spectrophotometry has been definitively abandoned, because of the lack of selectivity and the very poor analytical response, resulting in a very high detection limit. Since aflatoxins show fluorescence under UV light, spectrofluorimetric detection has been adopted in the last years.

The operating conditions range from an excitation wavelength of 360–365 nm and an emission wavelength 425–435 nm. The intensity of aflatoxin fluorescence depends strongly on the injected solvent, with a higher response if the sample is injected in the mobile phase (usually a ternary mixture of methanol/acetonitrile/water) and a lower one if injected in the methanol or acetonitrile only.

Furthermore, since aflatoxins B_1, G_1, and M_1 do not give a high response, it is necessary to derivatize them via pre- or postcolumn derivatization, in order to enhance their fluorescence.

Trifluoroacetic acid (TFA) precolumn derivatization: This reaction results in an aflatoxin hemiacetal formation (aflatoxins B_{2a}, G_{2a}, and M_{2a}) by the addition of a water molecule to the double bond of the furanic ring (43).

Postcolumn derivatization with iodine: The procedure has been developed by Tuinstra and Haasnoot (44); a freshly prepared aqueous saturated solution of iodine (2 g in 400 ml of bidistilled water) is pumped by an auxiliary HPLC pump simultaneously with the mobile phase (commonly water/methanol/acetonitrile). The iodine solution must be kept away from UV light. The reaction is carried out in Teflon tubing (0.5 mm × 3000 mm) thermostated at 60°C. A silicone oil bath is preferred to a water bath for a more stable temperature. The flow rates of the mobile phase and the derivatizing agent are generally 0.8 ml/min. and 0.7 ml/min, respectively. A scheme of the derivatization system is shown in Fig. 2.

Postcolumn derivatization with pyridinium bromide perbromide (PBPB): By this method (45) the brominating agent enhances the fluorescent signal of aflatoxins B_1 and G_1, increasing the sensitivity of the analytical method. The solution of PBPB (0.05 mg/ml in HPLC-grade water) is pumped at a flow rate of 0.3 ml/min, and the flow rate of the mobile phase is generally set at 1.0 ml/min. Typical chromatograms of standards and naturally contaminated pistachio samples are shown in Figs. 3 and 4, respectively.

Postcolumn derivatization with electrochemical-generated bromide (Kobra cell): Recently the fluorescence signal enhancement of aflatoxins was obtained by an online deriva-

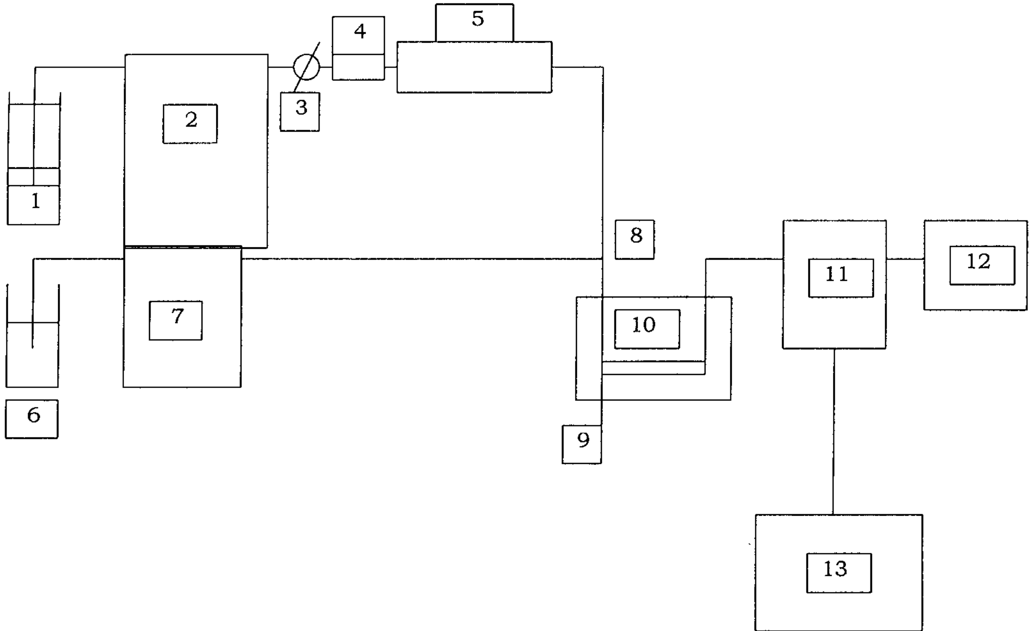

Fig. 2 Postcolumn derivatization scheme for aflatoxin analysis: 1, mobile phase; 2, HPLC pump; 3, injection valve; 4, precolumn; 5. analytical column; 6, derivatizing agent solution; 7, auxiliary HPLC pump; 8, T-valve; 9, oil or water bath; 10, reaction coil; 11, fluorescence detector; 12, waste; 13, chromatographic data handling system.

tization, with electrochemical-generated bromine. The reagent is produced in a postcolumn electrochemical cell from the bromide present in the mobile phase. The reagent concentration is controlled by the generating current. For electrochemical production of bromine, a Kobra cell is used. Reaction coils, providing reaction times of 4, 8, and 24 s at a flow rate of 0.5 ml/min, are used. The current is delivered by a variable DC supply with 100 kΩ of resistance in series with the Kobra cell. (46,47).

Liquid chromatography-MS: Mc Fadden and Schueler (48) performed the analysis of aflatoxins B_1, B_2, G_1, and G_2 by ion reconstruction at m/z 313, 315, 329, and 331 for the four aflatoxins, respectively, by using a moving-belt interface. Tiebach et al. (49) used a direct liquid induction (DLI) interface with a reversed-phase microbore LC system, and obtained positive-ion chemical ionization (PCI) and negative-ion chemical ionization (NCI) spectra for aflatoxin M_1 and reconstructed ion chromatograms for aflatoxins B_1, B_2, and M_1. Tateo et al. (50) used an MS/DI system for confirmatory analysis of aflatoxins. A liquid chromatography/mass spectrometry interfacing method was reported by Cappiello et al. (51). The chromatographic separation was performed with a reversed-phase packed capillary column coupled with a modified particle beam interface capable of handling microliter-per-minute flow rates.

VI. OCHRATOXIN A

A variety of fungal organisms included in the genera *Aspergillus* (mainly *A. ochraceus*) and *Penicillium* (mainly *P. verrucosum*) (52) are able to produce ochratoxins, with ochratoxin A (OA)

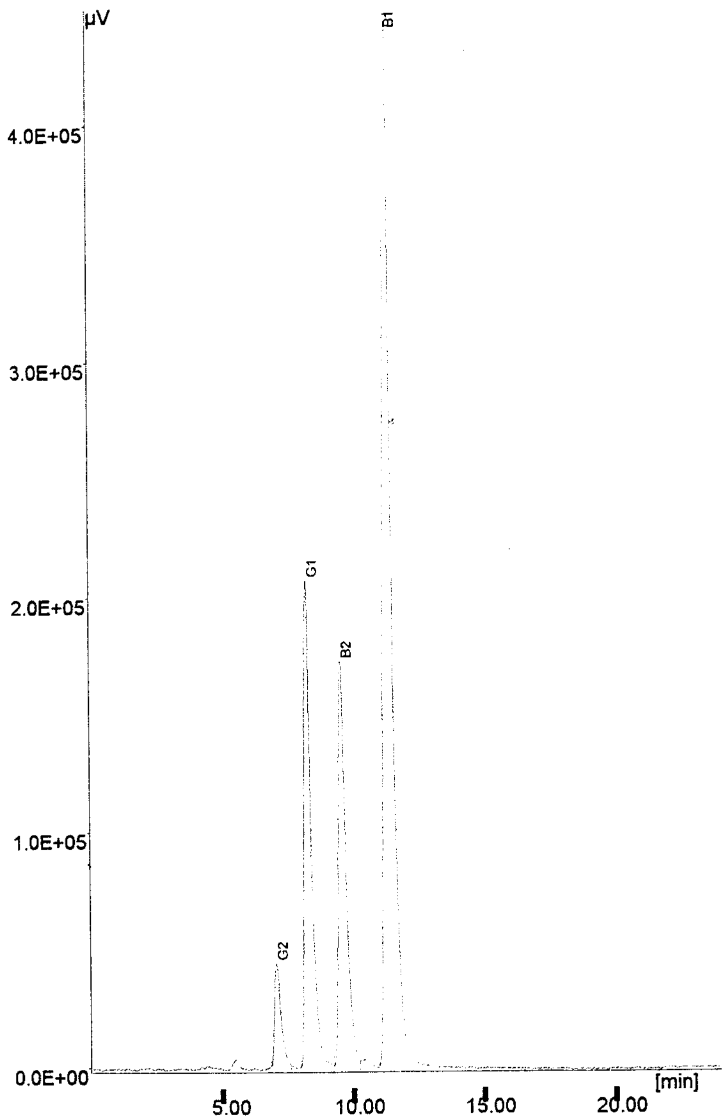

Fig. 3 Typical chromatogram of aflatoxins B_1 (0.2 ng), B_2 (0.04 ng), G_1 (0.2 ng), and G_2 (0.04 ng) standard. Mobile phase: water:methanol:acetonitrile 54:29:17. Pump A regulated at 1 ml/min; pump B (post-column derivatization with bromine) regulated at 0.4 ml/min.

being the most relevant toxin as far as toxicity and diffusion are concerned. At 24°C, optimum (Water Activity) Aw values for OA production are in the range 0.95–0.99, depending on the producing organism, while at optimum Aw the temperature range is 12–37°C and 4–31°C for *A. ochraceus* and *P. cyclopium* and *viridicatum*, respectively (53). *Penicillia* genera have been found to be responsible for OA-contaminated crops in colder climate areas (Scandinavia and Canada); *A. ochraceus* was isolated in warmer climatic zones, such as Yugoslavia and Australia, and in coffee bean–producing countries.

Ochratoxin A–contaminated crops include cereals, such as barley and maize, wheat, and oat, groundnuts, and beans (soy, coffee, and cocoa) as well as pulses. Grains contaminated by OA

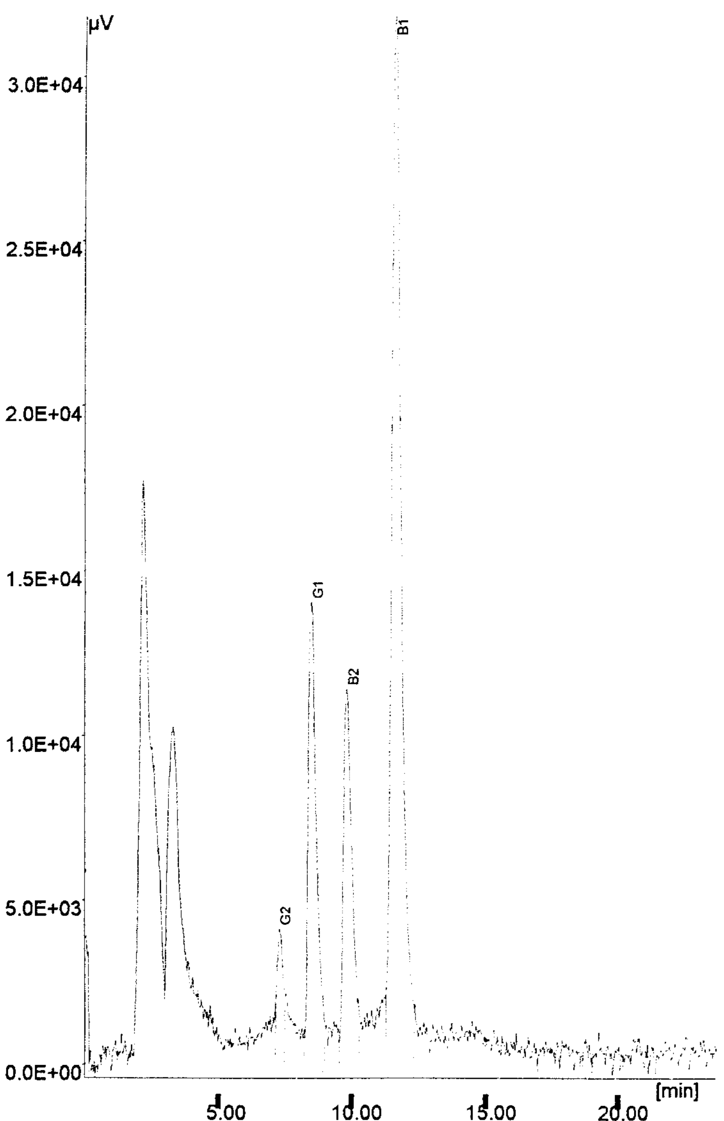

Fig. 4 Typical chromatogram of aflatoxins B_1, B_2, G_1, and G_2 naturally contaminated pistachio nuts sample. Aflatoxin B_1 (AFB1) 0.75 μg/kg, aflatoxin B_2 (AFB2) 0.12 μg/kg, aflatoxin G_1 (AFG1) 0.68 μg/kg, and aflatoxin G_2 (AFG2) 0.15 μg/kg. Mobile phase: water:methanol:acetonitrile 54:29:17. Pump A regulated at 1 ml/min; Pump B (postcolumn derivatization with bromine) regulated at 0.4 ml/min.

are likely to contain additional fungal metabolites, such as citrinin and penicillic acid (34). In livestock, OA residues are found only in pork and chicken products (mainly kidney and liver and possibly other edible parts), due to the OA presence in feed, since OA is degraded by rumen in dairy cattle. Ochratoxins are a group of closely related derivatives of isocoumarin linked to L-phenylalanine and classified as pentaketides, as reviewed by Steyn (54). On acid hydrolysis, OA degrades in phenylalanine and in ochratoxin α, which is nontoxic. An ethanolic solution of OA can be stored at 4°C for 1 year, if protected from light (55,56). The molecular structure of OA is given

Fig. 5 Molecular structure of ochratoxin A.

in Fig. 5. Ochratoxins A, B, and C and their esters have been identified, but ochratoxin B (OB) and ochratoxin C (OC) have not been found in naturally contaminated products.

Chemical, physical, and toxicological data of ochratoxins was reviewed by IARC (34). The kidney is the target organ, and OA in association with citrinin has been implicated in endemic nephropathy in the Balkans (57).

Although OA still does not appear to be a problem in the United States, interest around the world remains high (58).

A. Analysis

1. Preparation of Standard Solutions

A stock solution containing approximately 40 μg/ml of ochratoxin A in benzene:acetic acid (99:1) is prepared. The concentration as described for aflatoxins is determined by measuring the optical density at 333 nm (peak absorption wavelength for this toxin) and applying the following formula:

$$\mu\text{g of ochratoxin A/ml} = \frac{\text{OD} \cdot \text{molecular weight} \cdot 1000 \cdot \text{CF}}{\epsilon}$$

where the molecular weight = 403, ϵ = 5550, and CF = correction factor. This solution is diluted with benzene to obtain a working solution of 1 μg/ml. More information on the preparation of standards is given in the AOAC *Official Methods of Analysis* (17).

2. Extraction

The extraction of ochratoxin A from the ground product is consistent with the acid nature of ochratoxin A (pK_a = 7.1) due to the copresence of phenolic and carboxylic groups. It is thus possible to extract ochratoxin A with organic solvents in acid medium, especially when immunoaffinity columns are used for further purification. Extraction methods are similar to those described for aflatoxin, i.e., shaking (mechanical or magnetic) for 30–60 min or blending from 1 to 3 min with Ultra Turrax apparatus or Waring Blender. The most common extraction solvents are chloroform and 0.1 M H_3PO_4, toluene/2N hydrochloric acid/$MgCl_2$ or aqueous solutions in alkaline medium, e.g., bicarbonate.

3. Cleanup

Basically the cleanup step consists of one of the two approaches already described in the aflatoxin section: (a) the use of solid-phase extraction (SPE) columns, or (b) the use of immunoaffinity (IA) columns.

Purification by the immunoaffinity column can be carried out manually or by using a commercially available automated sample-preparation system. After the conditioning of the im-

munoaffinity column with PBS (20 ml), the sample extract (50 ml) is pushed through the column at a flow rate of approximately 5 ml/min. The column is washed with distilled water, and ochratoxin A is eluted from the column with methanol for HPLC over 2 min, with the eluate collected in a 4-ml amber vial. The sample is diluted with water before HPLC analysis. In some cases (e.g., beer and wine), the sample is loaded directly into the column without any extraction step. A unique IA column with a specificity both for aflatoxins and for ochratoxin A has also recently become available (59).

The main disadvantages of an immunoaffinity cleanup for OA could in some cases be the lack of specificity for ochratoxin A (since a cross-reaction with ochratoxin C can occur (60)), the potential contamination with release of ochratoxin A from the support of the IA (61), and the high cost of each column, although some authors considered the possibility of regenerating the column for further analyses (30).

B. HPLC Analysis

The general principles reported for aflatoxins are applicable also to ochratoxin A. More specifically, methods currently existing for the determination of ochratoxin A by HPLC are based principally on fluorescence detection with an excitation wavelength of 330–333 nm and an emission wavelength of 445–470 nm. The mobile phases most commonly used are a binary mixture of water: acid (acetic or phosphoric): acetonitrile in ratios such as 99:2:99, 100:1:100, 47:2:51, 220:2:99, and 43:2:57. Retention times vary from 6 to 14 min, depending on the type of LC column. Typical chromatograms of OA standard and OA naturally contaminated corn samples are shown in Figs. 6 and 7, respectively.

Three methods are usually adopted for confirmation of ochratoxin A: (a) methylation (methods A and B), (b) ammonia derivative formation, and (c) LC-MS confirmation.

Methyl ester formation:

Method A: 50 μl (ca 250 ng) of the stock solution or of the purified extract is transferred into a reaction tube. One ml of boron trifluoride is added to the methanol, the cap is closed, and the tube is kept on a block heater (80°C) for 10 min. Two ml of water and 2 ml of *n*-hexane are added. After thorough mixing, the layers are left to separate. Using disposable glass Pasteur pipettes, the upper layer is transferred to a clean, small vial. The extraction is repeated another two times with 2-ml portions of *n*-hexane. The hexane extracts are taken to dryness with a nitrogen stream, and the residue is dissolved in 1–2 ml of injection solvent. Ochratoxin A is confirmed by the presence of an OA–methyl ester peak at delayed retention time and the disappearance of the OA peak (62).

Method B: This method was originally published by Uchiyama et al. (63): two hundred microliters of the purified extract was evaporated to dryness and 2.5 ml methanol and 0.1 ml concentrated HCl were added. The solution was left standing overnight at room temperature. The methanol was evaporated and the residue was taken up in 200 μl of acetonitrile.

Ammonia derivative formation: The derivatization is carried out using a 10% ammonia solution mixed with the column effluent (64). The main advantages are an increase in sensitivity (1.7 times the signal of the underivatized ochratoxin A), and the availability of a confirmation test for ochratoxin A, as a consequence of this change of sensitivity. A second HPLC pump, similar to that described in Fig. 2 for aflatoxins, and a reaction coil of 10 cm are necessary. Conditions are as follows: 0.5 ml/min as flow rate, room temperature, excitation wavelength of 370 nm, and emission wavelength

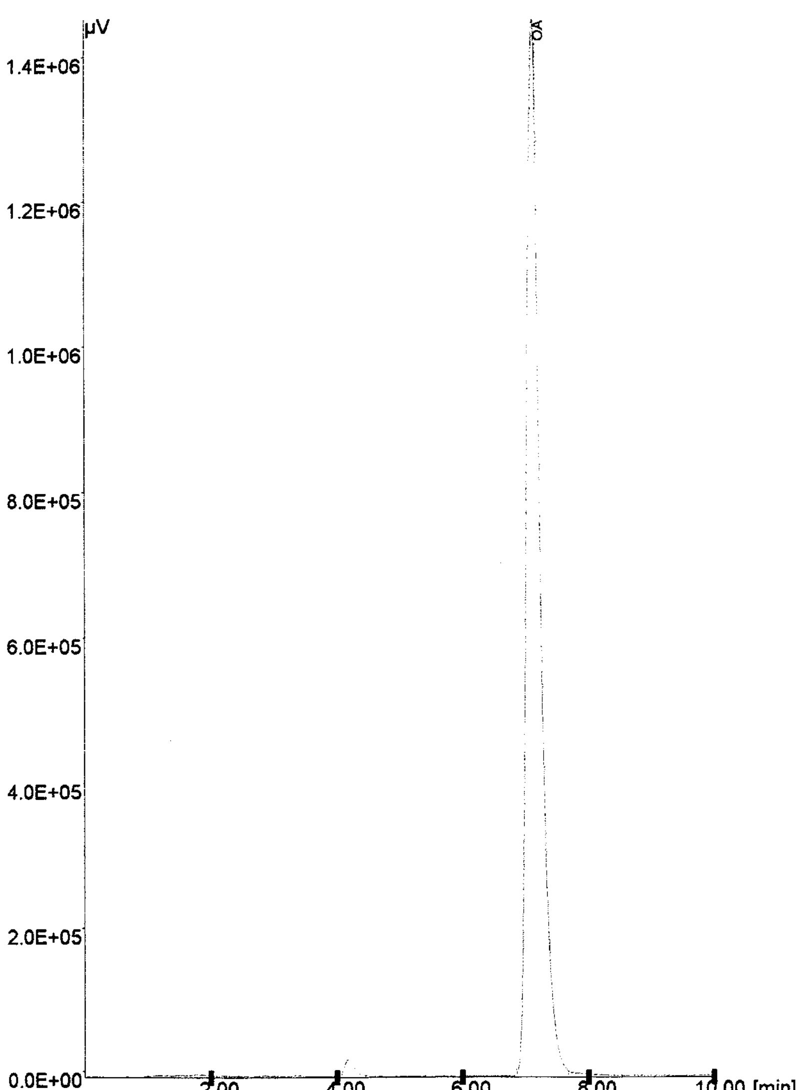

Fig. 6 Typical chromatogram of ochratoxin A standard (0.05 ng). Mobile phase: water (2% acetic acid) : acetonitrile 51 : 49; HPLC pump regulated to 1 ml/min.

of 460 nm. Further confirmation can be obtained by making a chromatographic run of the derivatized ochratoxin A, at the wavelengths normally used for underivatized ochratoxin A, i.e., 333 nm for excitation and 470 nm for emission. The disappearance of the derivatized ochratoxin A peak will act as a confirmation of its presence (65).

Confirmation by LC-MS: This method is not used much, since it involves expensive apparatus, and rather good experience in the technique is needed. Abramson (66) illustrated the advantages resulting from the combination of liquid chromatography and mass spectrometry for ochratoxin A in barley. Among the types of interfaces most used for ochratoxin A analysis were thermospray, direct liquid induction (DLI), and

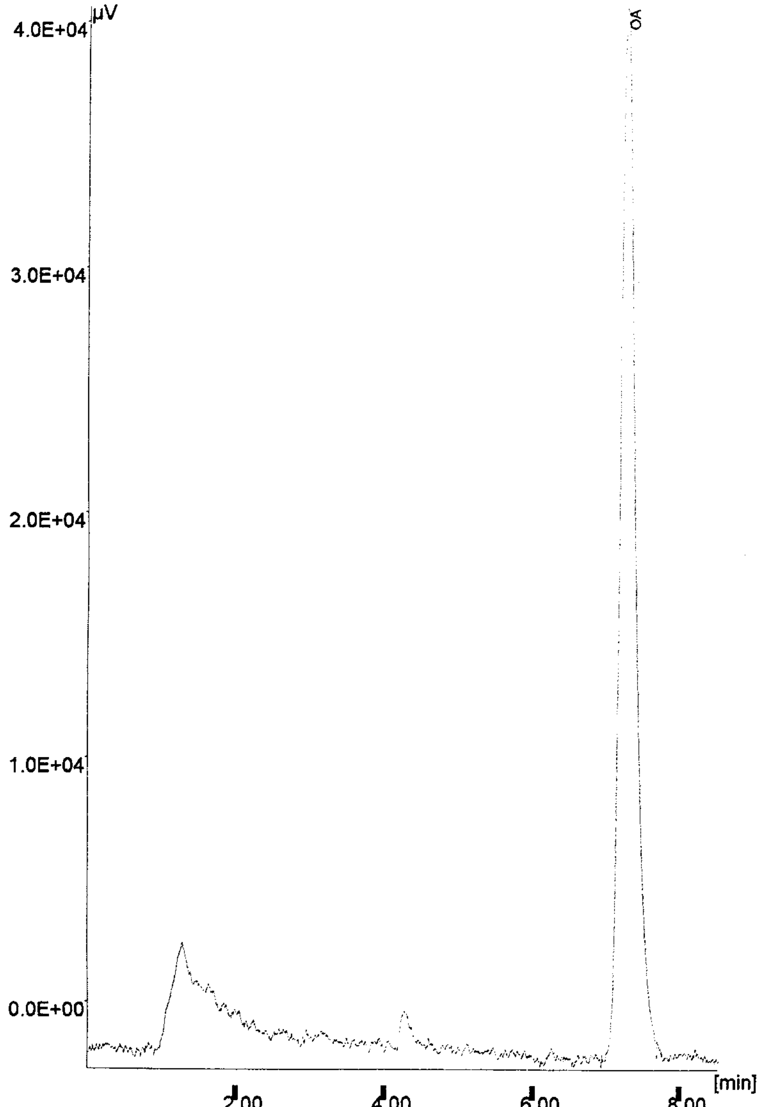

Fig. 7 Typical chromatogram of OA naturally contaminated corn sample at 0.22 μg/kg. Mobile phase: water (2% acetic acid) : acetonitrile 51 : 49; HPLC pump regulated to 1 ml/min.

the moving belt. Sensitivity is one of the weakest point of this technique, but it can be enhanced by operating MS in selected ion monitoring (SIM) mode and in some cases with negative-ion chemical ionization (NCI). Ominski et al. (67) used LC-MS for the confirmation of ochratoxin A in serum samples of swine. The quadruple mass spectrometer was equipped for negative-ion chemical ionization, and ochratoxin A standards were chromatographed and scanned from masses 150–450 to determine retention time and ion mass. For maximum sensitivity, sample extracts were analyzed by monitoring the ion of mass 403.1.

VII. FUMONISINS

Fumonisins, first isolated from corn cultures of *Fusarium moniliforme*, are also produced by several other *Fusarium* species (*F. anthophilum, F. proliferatum, F. dlamini, F. napiforme*, and *F. nygamai*) (68). From the seven structurally related fumonisin analogs, only fuminisins B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) are naturally occurring in corn (69,70). The first two invariably occur together and have been extensively studied. Molecular structures of fumonisins are shown in Fig. 8.

High levels of fumonisin B_1 have been found in corn worldwide (34,71), and high levels have also been found in milk (72). Corn naturally contaminated with fumonisins and aflatoxins has also been reported (73). A correlation between equine leukoencephalomalacia and consumption of mold corn has been known for a long time; more recently, fumonisin B_1 has been shown to be responsible for the production of lesions characteristic of the illness (74). Most recent interest has focused on fumonisins as a risk factor in oesophageal cancer, and Sydenham et al. (75) have shown that levels of fumonisins were higher in both moldy and healthy corn from high risk regions, compared to moldy and healthy corn from low-risk regions.

Liquid chromatography with fluorescence detection represents the most useful and studied analytical technique for the determination of fumonisins (76,77,59). Basic steps involve cleanup on C18, ion-exchange, or a combination of both, and fluorescence detection. At levels of less than 10 μg/kg it is necessary to derivatize the free amino group in order to enhance the fluorescence. Shephard's method in corn (78), following an AOAC-IUPAC collaborative study, was accepted by AOAC as an official method of analysis (17). The method includes cleanup of the aqueous extract with strong anion-exchange (SAX) media and *ortho*-phthaldialdehyde (OPA) as derivatizing agent. In its final version the method is suitable for the determination of FB_1, FB_2, and FB_3. In the last few years emphasis has also been given to capillary electrophoresis (79), to evaporative light-scattering detection (80,81), and to mass spectrometry (82-87), even though these tech-

CH_3 CH_3

$CH_3-CH_2-CH_2-CH_2-CH-CH-CH-CH_2-CH-CH_2-CH-CH_2-CH_2-CH_2-CH_2$

OR^1 OR^1 R^2

$CH_3-CH - CH-CH_2-CH$

NHR^3 OH OH

B_1: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$; $R^2 = OH$; $R^3 = H$

B_2: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$; $R^2 = R^3 = H$

Fig. 8 Molecular structure of fumonisins B_1 and B_2.

niques are generally useful for confirmatory but not for analytical work performed on a routine basis. Due to the possible citotoxicity of hydrolysis products derived from fumonisins following decontamination procedures, a method was developed by Shephard et al. (88) that also determines these fumonisin derivatives.

VIII. TRICHOTHECENES

Trichothecenes are a numerous group of sesquiterpenoids produced by various species of *Fusarium*, *Myrothecium*, *Trichoderma*, *Cephalosporium*, and other fungi.

Contamination occurs primarily in wheat, barley, rye, and maize. Type A trichothecenes include mainly T-2 toxin, HT-2, and diacetoxyscirpenol (DAS); mycotoxins of the group B include mainly 4-deoxynivalenol (DON), commonly known as vomitoxin, and nivalenol (NIV). Toxic effects include nausea, vomiting, visual disorder, vertigo, throat irritation, and feed refusal in farm animals. The most toxic is T-2, followed by DAS and NIV, with DON being the least toxic in acute toxicity studies but the most widespread in grains worldwide and therefore the most studied. Issues related to chemical and physical data, occurrence, toxicity, absorption, distribution, and metabolism of trichothecenes are reviewed in WHO (89) and IARC (34). Physicochemical data for some selected *Fusarium* toxins is given by Sydenham et al. (90). The molecular structures of the main trichothecenes are shown in Fig. 9.

Gas chromatography and capillary column GC represent by far the most developed and most used analytical techniques for the determination of multiple trichothecenes, especially in view of the possibility of employing MS for confirmation of the peaks. The available methods, such as the Scott and Ware methods (90,91), differ in sample extraction, sample cleanup, and the derivatization step. Heptafluorobutyryl (HFB), trimethylsilyl (TMS), and trifluoroacetyl derivatives are frequently used, coupled with electron capture detection. A GC method (92) has recently been collaboratively tested in barley and malt and accepted by the American Society of Brewing

T-2

Diacetoxyscirpenol

Deoxynivalenol

Fig. 9 Molecular structure of the main trichothecenes.

Chemists in its *Methods of Analysis*, 8th ed. Methods have also been developed for DON by TLC (93) and for several trichothecenes both by immunoassay (94,95) and by supercritical fluid chromatography (96). In comparison with LC, supercritical fluid chromatography is characterized by sharper peaks and faster analysis times, similar to those obtained by capillary GC.

Despite the fact that both GC and supercritical fluid chromatography give sharper peaks and faster analysis times, LC methods have also been published during the last few years for the determination of trichothecenes. An HPLC method was developed by Trucksess et al. (97) for DON in wheat finished products; the procedure involves extraction with acetonitrile–water (84:16), filtration, purification on charcoal, Celite, or other adsorbent columns, cleanup through silica-based chromatography determined in LC (C18, using a gradient of methanol and water), and UV detection at 220 nm. The limit of detection of the method was 0.5 μg/g. Improved sensitivity (in the μg/kg range of concentration) and selectivity was obtained (98) by the use of HPLC with on-line, postcolumn photolysis and oxidative amperometric detection (HPLC-hv-EC) (99).

IX. ZEARALENONE

Zearalenone is a nonsteroidal, estrogenic toxin produced mainly by *Fusarium graminearum*, *Fusarium culmorum*, and *Fusarium sacchari*. Zearalenone is frequently found associated with trichothecenes, and is reported as the second most frequently found *Fusarium* metabolite in maize (after DON). This toxin, a resorcylic acid lactone, is insoluble in water and heat stable. Maximum tolerated levels of zearalenone are specifically established in Austria (60 ng/g in wheat and rye) as guideline levels, in Brazil (200 ng/g in maize), in France (200 ng/g in cereals and vegetable oils), in Romania (30 ng/g in all foods), and in Russia (1000 ng/g in cereals, leguminous protein isolators, vegetable oils, and nuts). Zearalenone exerts an effect primarily on the reproductive system of animals, and is most evident in swine. Economic effects of the presence of zearalenone in feed are related to the production of premature estrous in young gilts. Detailed information on zearalenone structure, molecular data, chemical and physical properties, occurrence, and studies related to toxicity in animals and humans are available from IARC (34). The molecular structure of zearalenone is shown in Fig. 10.

Reliable analysis methods based on TLC, HPLC, GC, ELISA, and EIA are available for the determination of zearalenone, α-zearalenol, and β-zearalenol. As far as the application of HPLC in the analysis of zearalenone is concerned, the Bennett et al. method (100) has also been adopted as an official method of analysis by the Association of Official Analytical Chemists International (17). The method involves extraction with chloroform and purification with liquid/liquid partition into 2% sodium hydroxide. After additional purification steps, determination is performed by C18 with a fluorescence detection (236 nm excitation, 418 nm emission). The method also allows for the determination of α-zearalenone, and both analytes are determined in corn at levels

Fig. 10 Molecular structure of zearalenone.

as low as 50 ng/g. Other methods based on HPLC and fluorescence detection are reported by Tanaka et al. (101) and by Seidel et al. (102). A postcolumn derivatization with aluminium chloride, resulting in a more selective response for zearalenone and α-zearalenol in reverse-phase HPLC, has also been reported (103).This latter method allows for the simultaneous detection of ochratoxin A. An improved HPLC method has recently been published for the simultaneous determination of zearalenone, nivalenol (NIV), and deoxynivalenol (DON) (99). A TLC screening method has been adopted as the official method of analysis by the Association of Official Analytical Chemists International (17). The method is reliable at levels over 300 ng/g. The enzyme-linked immunosorbent assay (EIA) method has also been accepted as the AOAC official method of analysis; the TLC method is reliable at levels over 300 ng/g. Methods based on GC/MS have also been reported (104).

Typical chromatograms of zearalenone standard and naturally contaminated corn samples are shown in Figs. 11 and 12, respectively.

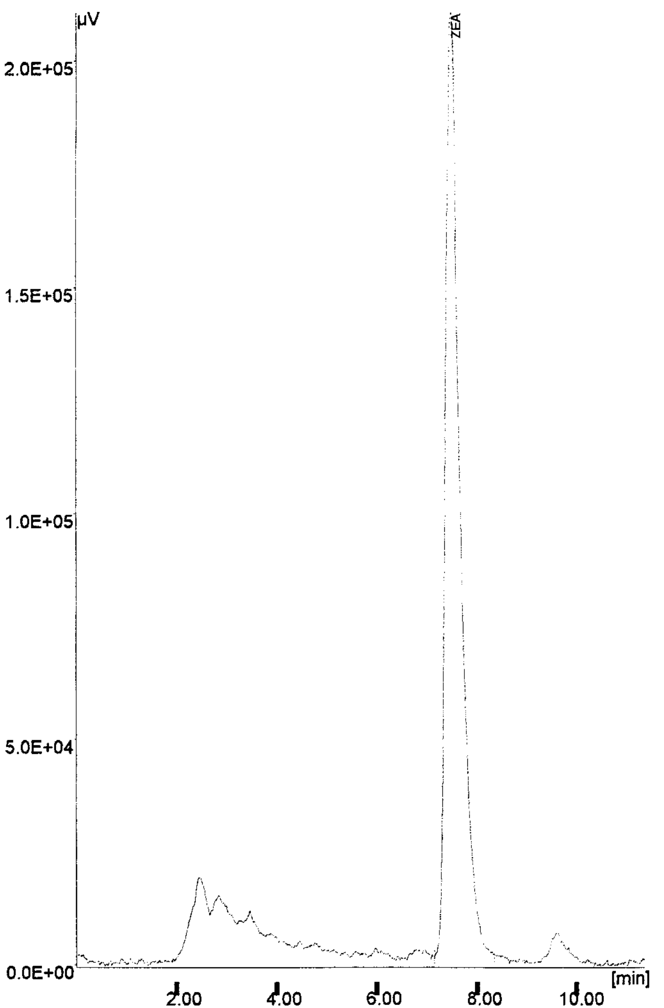

Fig. 11 Typical chromatogram of zearalenone standard (0.025 ng). Mobile phase: methanol: acetonitrile: water 1:1.6:2. HPLC pump regulated at 0.8 ml/min. ZEA = zearalenone.

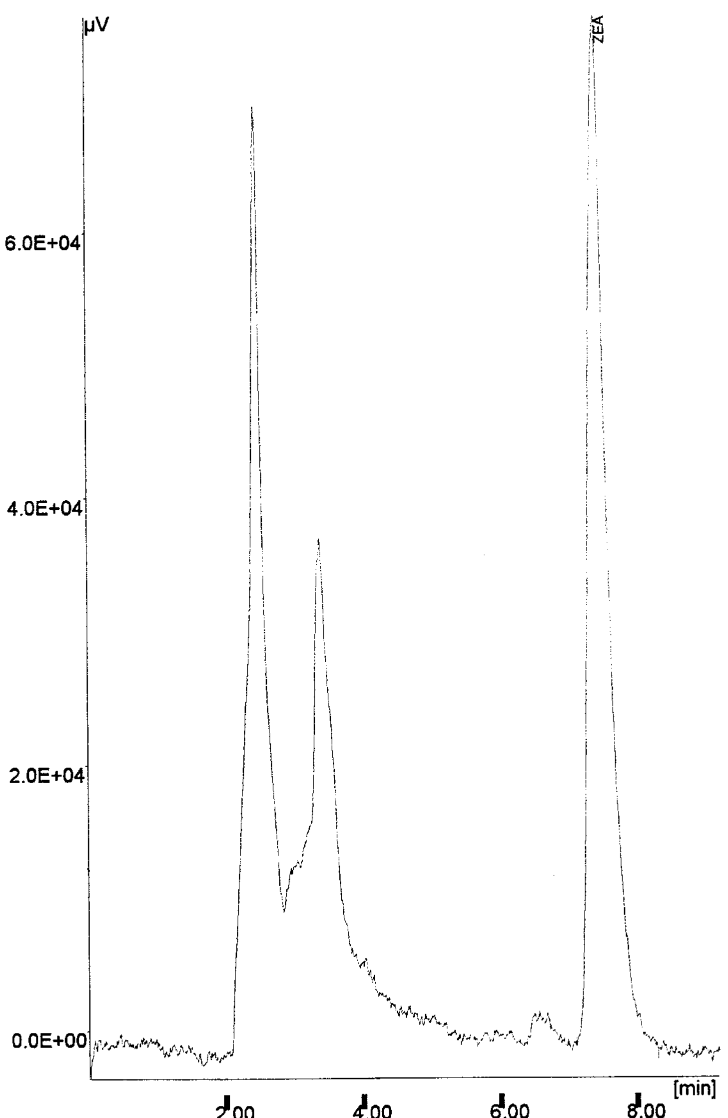

Fig. 12 Typical chromatogram of zearalenone naturally contaminated corn sample at 0.2 μg/kg. Mobile phase: methanol:acetonitrile:water 1:1.6:2. HPLC pump regulated at 0.8 ml/min. ZEA = zearalenone.

X. FOOD APPLICATIONS

In the previous sections, principles of the main methodologies for the determination of mycotoxins by HPLC in grains are given, along with information on the application on various matrices and references. In Table 3 a summary of methods for mycotoxins by HPLC is given.

Table 3 Main Food Applications

Mycotoxin	Analytical method	Food commodity	Ref.
Aflatoxin B_1, B_2, G_1, G_2	LC/iodine derivatization	Feeds	37, 38, 44
	CB method	Peanuts	17
	Automated/LC	Corn, peanuts	105
	Robotic system	Nuts	106
	SPE/LC	Peanut meal	40
	LC/TFA derivatization	Corn, tree nuts, peanuts	107
	LC/MS thermospray	Peanuts	108
	LC/TFA derivatization	Peanuts	109
	Immunoaffinity	Corn, peanuts	110
	LC/bromine	Feeds	47
	Immunoaffinity/LC	Peanut butter	41
Ochratoxin A	SPE/LC	Corn, barley	111
	Immunoaffinity/LC	Cereals	112
	SPE/LC	Maize	102
	LC/MS	Barley	66
	Immunoaffinity/LC	Green, roasted, and soluble coffee	113
	SPE/LC	Cereals	114
	LC/ammonia derivatization	Cereals	115, 116
	LC/immunoaffinity	Wheat	117
Fumonisins	SPE/LC-OPA	Corn	78
	LC/MS	Rice	87
	LC/OPA	Corn	77
	IA/LC/OPA		59
	C18 SPE/LC OPA	Corn, feed	118
Deoxynivalenol	Charcoal, Celite, SPE/UV	Wheat, bran	97
	Online, postcolumn photolysis; oxidative amperometric detection		98
	Cation-exchange/ alumina-carbon/LC/UV	Maize	99
Zearalenone	SPE/LC-FD		102
	Sephadex/LC-FD	Barley	101
	No cleanup/LC-FD	Maize	119
	LC-FD/postcolumn derivatization	Cereals	103
	BF method	Maize	120

LC: Liquid chromatography; CB: Contamination Bureau; SPE: solid-phase extraction; TFA: trifluoroacetic acid; MS: mass spectrometry; OPA: *o*-phthaldialdehyde; IA: immunoaffinity column; FD: fluorescence detector; BF: best food; UV: ultraviolet.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. Egle Cava and Dr. Francesca Romana Calfapietra for helpful discussion and technical assistance that contributed to this work.

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13
Intense Sweeteners and Synthetic Colorants

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I. INTENSE SWEETENERS

A. Introduction

Sweetness is one of the most important taste sensations for humans. Sucrose has been widely used for its sweetness as well as for functional properties such as texture, mouthfeel, bulking agent, and preservative. However, the specialized dietary requirements of diabetics and health concerns about obesity and dental caries have prompted a considerable research effort into the development of alternative sweeteners (1–6).

Sweeteners are widely used in the food, beverage, confectionery, and pharmaceutical industries throughout the world. Because of high consumer demand and acceptance of low-calorie products, the market for artificially sweetened foods has increased significantly and will continue to grow (2,4,7).

Sweeteners can be classified into two categories, bulk and intense. The bulk sweeteners are used in the food industry both as sweeteners and as bulking agents. They also offer preservative and bodying effect. They are metabolized by the body and provide calories. They include glucose, fructose, maltose, products hydrolyzed from starch, and sugar alcohols. These sweeteners vary in sweetness over a narrow range from 0.3 to 1.2 times the sweetness of sucrose. The bulk sweeteners are permitted in a number of specified foodstuffs at *quantum satis*—as much as needed (2,6,8–10).

The intense sweeteners have a sweet taste but are effectively noncaloric. These sweeteners are natural or synthetic compounds. They are intensely sweet, ranging from 30 to 3000 times the sweetness of sucrose. Therefore, the concentrations used for normal sweetness are very low (2,6,8,9). As indicated in Figs. 1 and 2 and Table 1, they have different chemical structures and properties. This category includes the following compounds: saccharin, cyclamate, acesulfame-K, aspartame, alitame, dulcin, sucralose, neohesperidin dihydrochalcone, glycyrrhizin, stevioside, and thaumatin. There are maximum levels allowed for intense sweeteners in each type of food, depending on legislation in any particular country. Schaller (10) provides a comprehensive review of the regulation for sweeteners in several countries and economic communities.

Since food laws in most countries regulate the use of intense sweeteners, analytical control for the presence and levels of sweeteners in food is essential. According to the FAO/WHO Joint Expert Committee on Food Additives (JECFA) it is also important to know the level of additives in food products in order to estimate the actual consumption by the population. This information will show the average intake in relation to the acceptable daily intake (ADI) over a period of time. Based on this knowledge, regulatory authorities can propose regulations to ensure intakes below

Saccharin

Sodium cyclamate

Acesulfame-K

Aspartame

Alitame

Dulcin

Sucralose

Neohesperidin dihydrochalcone

Fig. 1 Chemical structures of the intense sweeteners: saccharin, sodium cyclamate, acesulfame-K, aspartame, alitame, dulcin, sucralose, and neohesperidin dihydrochalcone.

Glycyrrhizin

Stevia sweeteners

Compound	R_1	R_2	R_3	Relative sweetness
Stevioside	gluc	gluc	H	150–300
Steviolbioside	H	gluc	H	100–125
Rebaudioside A	gluc	gluc	gluc	250–450
Rebaudioside B	H	gluc	gluc	300–350
Rebaudioside C	gluc	rham	gluc	50–120
Rebaudioside D	gluc-(1,2)-gluc	gluc	gluc	250–450
Rebaudioside E	gluc-(1,2)-gluc	gluc	H	150–300
Dulcoside A	gluc	rham	H	50–120

gluc = β-D-glucopyranosyl; rham = α-L-rhamnopyranosyl.

Fig. 2 Chemical structures of glycyrrhizin and of the sweet glycosides of *Stevia rebaudiana* Bert and relative sweetness of stevia glycosides compared to sucrose (4%).

Table 1 Some Properties of Intense Sweeteners

Sweetener (INS)[a]	Sweetness (sucrose = 1)	Sweetness characteristics	Synergism[b]	Solubility in water	Stability		ADI (mg/kg body wt)
					In solution	Heat	
Saccharin (954)	300 (200–700)	Slow onset, persistent, bitter metallic	cyc, asp, aces, sucral, alit, stev, suc	High Na-salt 667 g/L, 22°C	Stable pH 3–10	Good	0–5 (1993)
Cyclamate (952)	30 (30–140)	Slow onset, persistent; sweet-sour aftertaste	sac, asp, ace, sucr, ali, stev	Good Na-salt 200 g/L, 20°C	Stable pH 2–10	Good	0–11 as cyclamic acid (1982)
Acesulfame-K (950)	180 (125–250)	Rapid onset, prolonged sweetness	asp, cyc, sorb, alit	Good 270 g/L, 20°C	Stable pH ≥ 3	Good (up to 225°C)	0–15 (1990)
Aspartame (951)	200 (100–200)	Slow onset, clean sweet, sugarlike, prolonged sweetness	sac, cyc, aces, stev	Slightly soluble 10 g/L, 25°C, pH 4	Stable pH 3–5, hydrolysis pH 2–2.5	Unstable, sweetness may disappear	0–40 (1963)
Alitame	2000	Clean, no unpleasant aftertaste	sac, cyc, aces	Good 130 g/L at pH 5.6	Stable pH 6–8	Very good	0–1 (1996)
Sucralose (955)	400–800	Slow onset, clean sweet, sugarlike, prolonged sweetness	cyc, aces, nhdc	Good 280 g/L, 20°C	Stable pH > 3, loss of sweetness pH < 3	Very good	0–15 (1990)
Neohesperidin dihydrochalcone (959)	250–2000	Slow onset, lingering licorice, menthol aftertaste	sac, asp, ace, cyc, sucral, sugar alcohols	Low 0.5 g/L, 20°C 650 g/L, 80°C	Stable pH 2–6	Good	Not evaluated by JECFA 0–5 (1987, SCF, EC)
Glycyrrhizin (958)	50–100	Slow onset, long aftertaste, licorice flavor	stev, thau, asp	Good	Stable pH > 4.5	Good	Not evaluated by JECFA
Stevioside	100–300	Slow onset, prolonged sweetness, menthol at high levels, bitter	asp, cyc, ace, glyc	Low 0.8 g/L	Stable pH 3–10	Good	Not evaluated by JECFA
Thaumatin (957)	2000–3000	Slow onset, persistent, licorice-like	sac, aces, asp, cyc, stev, glyc	Good 600 g/L	Stable pH 2.7–6.0	Stable at neutral to low pH	Not specified (1985)

Source: Refs. 6, 8, 9, 11, and 12.

[a] INS = International Numbering System.

[b] sac = saccharin; cyc = cyclamate; ace = acesulfame-K; asp = aspartame; alit = alitame; sucral = sucralose; stev = stevioside; nhdc = neohesperidin dihydrochalcone; glyc = glycyrrhizin; thau = thaumatin; suc = sucrose.

the ADI (11,13). Furthermore, some sweeteners undergo decomposition during processing and storage of food products, forming a variety of degradation products, which may have sensory and toxicological significance. Therefore, rapid and accurate analytical methods are needed to check food labeling and to monitor the quality of the finished products (14,15).

The purpose of this section is to provide a review of HPLC methods available for the determination of sweeteners in foods. First, general information of the various modes of HPLC and on sample preparation procedures available for the determination of intense sweeteners is described. Then information is given on each individual sweetener.

B. HPLC Methods

The majority of recently published methods for the determination of sweeteners are based on high-performance liquid chromatography (HPLC), which offers great advantages over the more traditional methods of analysis. It prevents overestimation of sweetener content resulting from a nonspecific methodology. High-performance LC may not require tedious pretreatment of samples. It allows for the separation and quantification of several sweeteners simultaneously. It also allows detection and quantification at the very low levels normally used for sweetening purposes (16–20).

The first HPLC methods described for the determination of intense sweeteners were based on isocratic or gradient reverse-phase chromatography with ultraviolet (UV) detection (17). Ion-pair reverse-phase chromatography has also been used for the separation of sweeteners. Ion-pair reagent added to the mobile phase enhances retention of ionic compounds, improving separation of sweeteners from interfering compounds. Other advantages of ion-pair over reverse-phase chromatography are improved peak symmetry, greater selectivity and resolution, and reduced analysis time (21–23).

Several types of ion-pair reagents have been used; among them are petanesulfonate, triethylammonium, tetrapropylammonium, tetrabutylammonium, and cetyltrimethylammonium (hexadecyltrimethylammonium or cetrimide). The last two compounds are the most widely used. However, according to Herrmann et al. (24), tetrabutylammonium is the ion pair of choice, because it attains adsorption equilibrium more rapidly than cetrimide.

The latest methods described in the literature for the determination of sweeteners employ ion-exchange columns. This type of chromatography is becoming popular because it offers attractive alternatives to the reverse-phase methods. In contrast to organic solvent–mediated separations, chromatography is performed using aqueous buffers. The buffers are prepared from inexpensive salts instead of expensive organic solvents. In addition, the effluent is usually innocuous, since it is comprised of buffer salts that can be disposed at laboratory sinks. Hydroorganic solvents used in reverse-phase chromatography require costly special handling and disposal (17).

Even though there are several methods available in the literature for the determination of sweeteners, methods capable of determining a wide range of sweeteners simultaneously are required, since there is an increased variety of sweeteners available that are used in combination or as a blend (25). Therefore, there is a continuous search for improved, simpler, faster, more sensitive assays capable of detecting several sweeteners in a single run for routine analysis in the quality control of various food products (18,26,27).

C. Sample Preparation

A problem that may be encountered during analysis of sweeteners by HPLC is the diversity of products and the varied and complex matrices from which they are to be assayed (17).

In general, sample preparation will depend on the type of food matrix. Samples can be injected directly in the HPLC or after minimal pretreatment. However, extraction, cleanup, and/or purification might be necessary, depending on the complexity of the sample, in order to eliminate interference. Furthermore, according to Puttemans et al. (28), by extracting and purifying samples the analytical column will be protected and will have a longer life. Extraction methodology is the key to acceptable recovery (20).

Sweetener concentrates, tabletop sweeteners, and beverages can simply be diluted with HPLC-grade water or mobile phase and filtered through a 0.45 μm-pore membrane filter (16,29). Carbonated beverages are first degassed using an ultrasonic bath. Fruit juices, cocktails, and punches may require centrifugation (14) or filtration (16,30–34) to separate solids or insoluble matter. Yogurt, fruit syrups, hard or soft candies, powdered beverages, and solid foods are extracted with water at 40°C, homogenized, and filtered (17,24,35). Sweeteners in dairy products are extracted from samples by precipitating matrix components with 0.4% metaphosphoric acid (36) or with methanol (37). For matrices such as desserts and sweets, food thickeners and polysaccharides are precipitated with ethanol prior to extraction (16,38). In chocolate, defatting followed by extraction with hot water and clarification is necessary (35). In order to quantitatively extract sweeteners from chewing gum, it is necessary to disperse the gum in biphasic systems such as water:toluene, 25:40, v/v (39), or acetic acid:water:chloroform, 1:50:25, v/v (17). Extraction from different food samples can also be performed through dialysis with 1% phosphoric acid (40,41) or with 0.02 to 0.1 N hydrochloric acid (42,43).

Sample extracts can be clarified with Carrez I (potassium hexacyanoferrate II trihydrate) and Carrez II (zinc sulfate heptahydrate) reagents (14,24,33,35,44,45). Purification, isolation or concentration of extracts can be achieved through solid-phase extraction (20,46,47). The most commonly used cartridges are ODS-4, Sep-Pak C18, and Bond Elut C18 (33,40,47–49). Ion-pair reagents such as tri-*n*-octylamine, cetrimide, tetrabutylammonium, and *n*-propylammonium can be added to extracts prior to adsorption on cartridges to facilitate purification (28,40,48–50). Interference can also be reduced by using ion-exchange columns or cartridges (40,46,51), such as Bond Elut SAX (40). Before injecting into HPLC, all extracts must be filtered through 0.45 μm-pore membrane (33).

D. Detection Systems

For most sweeteners, detection has been performed at 254 nm (27,52). However, the trend now is to use lower wavelengths, 200–210 nm, in order to improve detection (16,34,40,47). When cyclamate is being analyzed, other means of detection, such as conductivity or indirect photometry, must be used because of its poor UV absorbing characteristics. Postcolumn derivatization of sweeteners and fluorometric detection has been used recently with increased selectivity and sensitivity (41,53,54).

Analytes are identified on the basis of retention time compared to standards and with the addition of the suspected compound to the sample (55). The diode array detector has been used recently as an additional aid in the identification of sweeteners and the determination of peak purity (56). Quantification is performed by the internal or external standard method on the basis of peak height or area.

E. HPLC Methods for the Determination of Specific Sweeteners

1. *Saccharin*

Saccharin is a general name used for saccharin, sodium saccharin, and calcium saccharin. Chemically, saccharin is 2,3-dihydro-3-oxobenzisosulfonazole (1,2-benzisothiazol-3-(2*H*)-one-1,1-dioxide, or *o*-sulfobenzimide) with the molecular formula $C_7H_5NO_3S$ and a molecular weight

of 183.18 (Fig. 1). It is a crystalline, colorless substance first synthesized in 1879 by Fahlberg (1,4,5,8,57,58). A gram of saccharin dissolves in 290 ml of water at 25°C or in 25 ml of boiling water. Saccharin is highly stable under food processing and storage conditions. It can withstand heating, baking, and acid media (Table 1). At very low pH, however, saccharin is hydrolyzed into 2-sulfobenzoic and 2-sulfamoylbenzoic acids (1,12,57,58).

Saccharin is about 300 times sweeter than sucrose. However, it has a bitter and metallic aftertaste that limits its use singly (Table 1). This aftertaste can be overcome by blending with other sweeteners. A mixture of saccharin with cyclamate (1:10) produces desirable sweetness. Saccharin has a slow onset of sweetness that gradually builds to a maximum intensity and then persists. Relative sweetness is affected by concentration, acidity, temperature, and type of food and flavor (3–5,8,9,58).

Saccharin is noncaloric and noncariogenic (2,3). The safety of saccharin for public health has been the center of several controversies. In 1970, saccharin at high dietary levels was observed to increase the incidence of urinary bladder cancer in experimental rats (1,59). However, extensive human epidemiological investigations showed that use of saccharin does not significantly increase the risk of bladder cancer (5,7,11,59). Saccharin is approved for use in several countries. Its use is not permitted in Canada, and a health warning on the label of saccharin-containing foods is required in the United States (7,8,10).

a. Sample Preparation

Sample preparation for saccharin analysis by HPLC can be performed as indicated in Section I.C. Methods for extraction have also been described for: desserts and sweets containing food thickeners (38); soy sauce, orange juice, and yogurt (60); chewing gum (17,39), and different food samples (42). Aminobenzoic acid, theophyllin, sodium fumarate, and adenine sulfate have been used as internal standards (17,31,39,44).

Clarification of extracts can be accomplished with Carrez reagents (44). Purification has been performed by solid phase or partition. Wu et al. (47) absorbed saccharin onto ODS-4 cartridges and then eluted with methanol:phosphate buffer. Puttemans et al. (28,50) extracted saccharin from soft drinks and yogurt by ion-pair extraction with tri-*n*-octylamine and back-extraction to an aqueous phase with perchlorate. Tereda and Sakabe (48) used cetrimide and Sep-Pak C18 in the cleanup of saccharin in coffee drink. The column was preconditioned with methanol, water, and 5 mM cetrimide. The sample, diluted in phosphate buffer pH 3.0 containing cetrimide was poured into the cartridge, washed with water, and eluted with acetonitrile:water (1:1, v/v). Moriyasu et al. (40) added *n*-propylammonium bromide to the extract, passed it through a Bond Elut C18 column, and eluted the sweetener with a mixture of methanol–water (4:6, v/v). The eluate was passed through a Bond Elut SAX column and washed with 0.5% phosphoric acid and water, and the sweetener was eluted with 0.3 N hydrochloric acid.

b. Separation Techniques

Reverse-phase chromatography has been used extensively for the determination of saccharin. Smyly et al. (30) and Eng et al. (39) used μBondapak C18 and 5% acetic acid for the determination of saccharin. Based on this work, an Association of Official Analytical Chemists (AOAC) collaborative study was conducted, and the method using a mobile phase buffered to pH 3 with sodium acetate and modified with 3% isopropanol was adopted. Webb and Beckman (61) used this method successfully for the separation of saccharin from aspartame, caffeine, sodium benzoate, and artificial colors and flavors. Veerabhadrarao et al. (27) added methanol to the mobile phase (methanol:acetic acid:water, 4:1:1, v/v) for improved separation of saccharin from caffeine, benzoic and *p*-hydroxybenzoic acids, vanillin, aspartame, acesulfame-K, and dulcin. Saccharin was also determined using LiChrosorb C18 and 4:6 v/v methanol:phosphate buffer,

pH 4.5 with ionic strength of 0.1 (50). Hannisdal (62) determined saccharin in samples containing acesulfame-K and benzoic and sorbic acids on C18 Spherisorb ODS-1 using 8% methanol in phosphate buffer (pH 6.7). Williams (26) separated saccharin from aspartame, caffeine, organic acids, and artificial colors using Spherisorb C8 and acetonitrile: methanol: phosphate buffer, pH 1.8 (17.5:12.5:70, v/v). According to Wu et al. (63), saccharin was separated from acesulfame-K on Bondclone 10 C18 using 20 mM potassium dihydrogen phosphate: acetonitrile (98:2, v/v). Separation of saccharin from organic acids, acesulfame-K, and aspartame was achieved on Superspher RP-select B using 20 mM phosphate buffer: acetonitrile, 9:1, v/v (44).

Hann and Gilkison (56) used a gradient of 10–60% methanol in 50 mM phosphate buffer pH 3.6 and Spherisorb 5 ODS for the separation of saccharin from aspartame, benzoic acid, and dyes in soft drinks. Ostermeyer (51) determined saccharin in samples containing acesulfame-K and aspartame on Nucleosil 100-5 C18 using a pH (4.8–6.7) and a mobile-phase gradient (7–20% acetonitrile in phosphate buffer). Lawrence and Charbonneau (16) separated saccharin from aspartame, alitame, acesulfame-K, sucralose, and dulcin using a gradient from 3% acetonitrile in 20 mM KH_2PO_4 (pH 5) to 20% acetonitrile in 20 mM KH_2PO_4 (pH 3.5) on Supelcosil LC18.

Ion-pair chromatography has been widely used for the determination of saccharin. Different types of ion-pair reagents were used, among them pentanesulfonate (52), triethylammonium phosphate (32), tetraethylammonium hydroxide (47), tetrapropylammonium hydroxide (40), and cetrimide (48,60,64,65). However, tetrabutylammonium (TBA) has been the most widely used. Herrmann et al. (24) used Hypersil MOS 3 and 12% methanol on 5 mM TBA *p*-toluenesulfonate, pH 3.5, and for the separation of saccharin from cyclamate, aspartame, and dulcin. Puttemans et al. (28) used a gradient of 30–60% methanol in phosphate buffer, pH 4.5, containing 5 mM TBA phosphate on LiChrosorb C18 for the determination of saccharin. Hausch (66) separated saccharin from acesulfame-K, aspartame, and neohesperidin dihydrochalcone using LiChrospher 60 RP-select and a gradient of 15–95% methanol in 10 mM TBA hydrogen sulfate. Suárez et al. (34) separated saccharin from aspartame, acesulfame-K, caffeine, and organic acids using Hypersil RP18 and a gradient of 17–40% methanol in Pic A Low UV (5 mM TBA phosphate).

Although reverse-phase columns are the most widely used for the determination of saccharin, there is a report on the use of an NH_2 column with a methanol: phosphoric acid mobile phase (42). Another type of column—Fractogel TSK HW-40—has been used for the determination of saccharin, aspartame, caffeine, and sodium benzoate. Separation is based on hydrophobic chromatography, using a mobile phase of 67 mM KH_2PO_4, pH 4.3 (67). A number of authors have described the use of ion-exchange columns. Nelson (68) used a strong anion-exchange resin consisting of a quaternary ammonium–substituted methacrylate polymer and a borate buffer, pH 9.2, mobile phase containing 0.1 M sodium nitrate as an ionic strength modifier for the determination of saccharin. Argoudelis (31) used Partisil-10 SCX with a mobile phase of 0.1 M ammonium dihydrogen phosphate at pH 4.5. Biemer (17) used Dionex AS4A-SC and a mobile phase consisting of 300 mg/L sodium carbonate solution for the determination of saccharin.

c. Detection Systems

Quantification of saccharin by HPLC was first performed by UV detection at 254 nm (32,39,50,52). However, recent methods have made use of lower wavelengths, among them 233–235 (48,60,65), 227–228 (62,63), 214–217 (31,56,66), and 200–210 nm (16,34,47). Saccharin has also been detected by a conductivity detector (17).

2. *Cyclamate*

Cyclamate is a name for a group of compounds that include cyclamic (*N*-cyclohexylsulfamic) acid, sodium cyclamate, and calcium cyclamate (Fig. 1). Audrieth and Sevda discovered this compound accidentally in 1937. It is a white crystalline powder with the molecular formula

$C_6H_{13}NO_3S$ and a molecular weight of 179.24. Cyclamates are 30–60 times sweeter than sucrose (Table 1). Its sweetness has a slow onset and persists for a period of time. A sweet-sour lingering aftertaste may be detected at high concentrations. It has the advantage of being sweeter when mixed with saccharin and of overcoming the bitterness of saccharin. The blend has a sweetness profile similar to that of sucrose (3,8,9,12,57).

Cyclamate is valued for its chemical inertness, stability, and solubility. The compound is stable at high temperatures. It is readily soluble in water, giving a clear, stable aqueous solution throughout the entire pH range used in food processing. Cyclamate is compatible with several food ingredients. It can intensify fruit flavors and give body to soft drinks. However, the use of the calcium salt of cyclamate in the presence of fruit pectin or in fruit concentrates of high acidity can cause gelling or precipitation problems (1,8,12,57,58).

Cyclamate is noncaloric and noncariogenic. In 1970 it was banned in the United States on the grounds of carcinogenicity. Rats fed a cyclamate: saccharin (10:1) combination at doses up to 5% of the diet for two years were found to develop bladder tumors (69). However, extensive human epidemiological investigation showed that users of cyclamate and saccharin do not have significantly increased risk of bladder cancer. Concern has also been raised from observations that some individuals and experimental animals can metabolize cyclamate to cyclohexylamine, which has been shown to produce testicular atrophy, reduced weight gain, and hyperactivity in experimental animals (70). After toxicological evaluation, JECFA established and ADI of 11 mg/kg body weight (11,70). While its use is restricted in the United States, it is permitted in several places, including European countries, Australia, Norway, South Africa, and Mercosur (7,8,10).

a. Sample Preparation

Sample preparation for the determination of cyclamate by HPLC can be performed as described in Sec. I.C. (17,24,35,43). Potassium bromide can be used as an internal standard (17). In order to reduce interference, extracts can be clarified with Carrez reagents (24,35) or by solid-phase extraction. Nakazato et al. (43) added tetrabutylammonium bromide to the extract, adjusted pH to 4.0–5.0, passed it through a Mega Bond Elut C18 cartridge, and eluted cyclamate with a mixture of acetonitrile: water (3:7, v/v). Lehr and Schmid (71) used an amino-anion–exchange column for the purification of cyclamate. After activation of the column with *n*-heptane, methanol, water, and 30% acetic acid, the sample was passed through the column and washed with water. Cyclamate was eluted with 2 M NaOH solution.

b. Separation Techniques

Reverse-phase chromatography was used by Lawrence and Charbonneau (16) for the separation of cyclamate from saccharin, aspartame, acesulfame-K, alitame, sucralose, and dulcin. The system consisted of a Supelcosil LC-18 column and gradient elution of 0–100% mobile phase B [20 mM KH_2PO_4 (pH 3.5): acetonitrile, 8:2 v/v] in mobile phase A [20 mM KH_2PO_4 (pH 5.0): acetonitrile, 97:3 v/v].

Herrmann et al. (24) used ion-pair chromatography for the determination of cyclamate. The efficiency of LiChrosorb RP-18 and Hypersil MOS 3 with a mobile phase of 5 mM tetrabutylammonium *p*-toluenesulfonate, pH 3.5, mixed with 12% methanol for the separation of cyclamate from other sweeteners was investigated. With the first column, cyclamate separated from saccharin, but the second was the recommended column for the analysis of cyclamate, saccharin, aspartame, and dulcin in a single run.

Determination of cyclamate using Dionex AS4A anion column and a mobile phase of 140 mg Na_2CO_3/L was described by Biemer (17). Wu et al. (47) also used Dionex AS4A-SC for the determination of cyclamate; elution, however, was performed with a 5 mM $Na_2B_4O_7$ solution.

c. Detection Systems

Since cyclamate has poor UV absorbing characteristics, HPLC methods for the analysis of this sweetener require specific detection systems, such as indirect photometry or conductivity. Herrmann et al. (24) used indirect photometry for the detection of cyclamate at 267 nm against a UV-absorbing mobile-phase component, *p*-toluenesulphonate. Biemer (17) and Wu et al. (47) used a conductivity detector for the determination of cyclamate. According to Biemer (17) the use of this detector offers distinct advantages, since compounds coeluting with cyclamate may not exhibit an electrochemical response and, hence, not appear in the chromatogram.

Another approach would be to derivatize cyclamate prior to analysis. Cyclamate can be determined by HPLC and UV detection at 314 nm after conversion to *N,N*-dichlorocyclohexylamine. Derivatization can be carried out directly in the sample or after extraction and cleanup. *N,N*-Dichlorocyclohexylamine is separated on a reverse-phase column (Nucleosil C18 or Finepak SIL C18 T-5) with a mobile phase of methanol: water, 8:2 v/v (43,46). Cyclamate can also be determined at 585 nm after postcolumn derivatization with methyl violet 2B as described by Lawrence and Charbonneau (16).

Cyclamate can also be determined after its oxidation to cyclohexylamine. Cyclohexylamine can be quantified by HPLC with trinitrobenzenesulfonic acid precolumn derivatization and UV detection (72). Cyclohexylamine can also be converted into a fluorescent derivative, separated on a C18 reversed-phase column with acetonitrile: Na_2HPO_4 buffer (64:36, v/v), and quantified at 350 and 440–650 nm of excitation and emission, respectively (73).

3. *Acesulfame-K*

Acesulfame-K was first prepared by Clauss and Jensen in 1967 and is available through Hoechst Ltd. under the brand name of Sunett or Sunette®. As indicated in Fig. 1, it is the potassium salt of 6-methyl-1,2,3-oxathiazine-4(3*H*)-one-2,2-dioxide or 3,4-dihydro-6-methyl-1,2,3-oxathiazine-4-one-2,2-dioxide, $C_4H_4KNO_4S$, with a molecular weight of 163.15. It is a white, crystalline, nonhygroscopic powder that decomposes at temperatures over 225°C. Its solubility in water is about 270 g/L at 20°C and 1 kg/L at 100°C (3,4,12,62). Acesulfame-K is 125–250 times sweeter than a 3% sucrose solution (Table 1). At higher concentration, the relative sweetness decreases. The sweetness onset of acesulfame-K is rapid, without unpleasant delay. It decreases slowly, without unacceptable lingering taste, persisting longer than sucrose. The sweetness quality of acesulfame-K can be improved by combining it with bulk or intense sweeteners (1,8,9,57,58). Under normal food processing and storage conditions, acesulfame-K is extremely stable. It withstands pasteurization and sterilization. However, below pH 3.0 and during prolonged storage at elevated temperatures, it can be hydrolyzed into acetoacetamide (12,33).

Acesulfame-K is not metabolized in the human body. It is not fermented by oral bacteria and produces no glycemic response. There is no evidence of any toxicological effect of acesulfame-K. An ADI of 0–15 mg/kg body weight has been allocated (2,7,57). Its use is approved in many countries in products, including soft drinks, juices, desserts, jams, marmalades, dairy products, baked goods, canned foods, candies, oral hygiene, and pharmaceuticals (7,10).

a. Sample Preparation

Sample preparation for HPLC analysis of acesulfame-K can be accomplished as described in Sec. I.C. A number of extraction procedures for acesulfame-K have been reported (16,17,33,34,40,44,49). Sodium fumarate and theophyllin can be used as internal standard (17,44).

Complex matrices may require a cleanup or purification step. Clarification can be performed with Carrez solutions (33,44,45,66). Highly colored solutions are decolorized by means of C18 disposable cartridges (33). Wu et al. (47) adsorbed acesulfame-K in ODS-4 cartridges and

eluted with methanol : phosphate buffer. Moriyasu et al. (49) purified beverage samples by adding McIlvaine buffer, pH 4.5, and 0.1 M tetrabutylammonium bromide and passing the mixture through Bond Elut C18. Interference may also be reduced on amino ion-exchange columns (40,46,51).

b. Separation Techniques

Reverse-phase and ion-exchange columns have been used for the separation of acesulfame-K. Veerabhadrarao et al. (27) and Hannisdal (62) separated acesulfame-K from other sweeteners and additives on reverse-phase C18 columns using methanol : acetic acid and methanol : phosphate buffer mobile phase, respectively. However, most of the reverse-phase methods for the separation of acesulfame-K use acetonitrile : phosphate buffer as the mobile phase (14,16,33,44,51,63). According to Prodolliet and Bruelhart (33), the use of acetonitrile in the mobile phase provides a better resolution for sweeteners than methanol.

Ion-pair reagents have been used for the separation of acesulfame-K on reverse-phase columns. Several types of reagents have been used: tetraethylammonium hydroxyde (47), tetrapropylammonium hydroxide (40,49), cetrimide (65), and pentanesulphonate (52). However, tetrabutylammonium (TBA) has been the most widely used. Grosspietsch and Hachenberg (74) incorporated 10 mM TBA hydrogen sulfate to a methanol : water (1 : 9, v/v) mobile phase for the determination of acesulfame-K using Lichrosorb RP-18. Hausch (66) used a gradient of 15–95% methanol in 10 mM TBA hydrogen sulfate on LiChrospher 60 for the separation of acesulfame-K from neohesperidin dihydrochalcone, aspartame, and saccharin. Suárez et al. (34) separated acesulfame-K from saccharin, aspartame, caffeine, and organic acids using ODS Hypersil RP18 with a gradient of 17–40% methanol in Pic A Low UV (5 mM TBA phosphate).

High-performance ion chromatography was used by Biemer (17) to separate acesulfame-K from saccharin and cyclamate. A Dionex AS4A anion separator column and a mobile phase of 300 mg Na_2CO_3/L were used for the separation of the sweeteners. The regenerate solution consisted of 25 mM sulfuric acid solution. Wu et al. (63) determined acesulfame-K on Dionex AS4A-SC and 0.5 mM Na_2CO_3 mobile phase.

c. Detection Systems

According to Prodolliet and Bruelhart (33) optimum sensitivity of acesulfame-K is obtained at 230 nm. However, quantification during HPLC analysis has also been performed at 254 (27,52), 233 (65), 227–228 (62,63,74), 217 (66), and 200–210 nm (16,34,47). Acesulfame-K has also been determined by a conductivity detector (17).

4. *Aspartame*

Aspartame is an intense sweetener first discovered in 1965 by J. Schlatter; it is available under the brand names of Nutrasweet®, Equal®, and Canderel®. Chemically, aspartame is N-L-α-aspartyl-L-phenylalanine methyl ester (Fig. 1), with a molecular formula of $C_{14}H_{18}O_5N_2$ (MW = 294.30). It is a white, odorless, crystalline powder. It is slightly soluble in water and sparingly soluble in alcohol. The solubility increases as the pH is lowered (2,6,57). It has 100–200 times the sweetness of sucrose and exhibits a sweet, clean taste and a sweetness profile similar to that of sucrose, without bitter or metallic aftertaste (Table 1). However, it displays a slow onset of sweetness coupled with lingering sweet taste. It extends and intensifies tastes and enhances fruit flavors. Aspartame exhibits synergism, a superior taste profile, and improved stability when used with other sweeteners (1,4,14,55,75).

In the dry state, aspartame is quite stable. However, decomposition occurs under certain conditions of moisture, pH, temperature, and length of storage of the food or beverage. Aspartame is stable in the pH range of 3–5; however, optimum stability is reported at pH 4.3 (9,57,76).

It has poor heat tolerance, but an encapsulated version with improved heat stability is now available (7). As indicated on Fig. 3, hydrolysis of either the methyl ester or the peptide bond forming aspartylphenylalanine, phenylalanine, aspartic acid, and methanol can occur (12,14,15,18,58). Also, methanol may be eliminated by cyclization to 5-benzyl-3,6-dioxo-2-piperazineacetic acid (diketopiperazine), which can be hydrolyzed to aspartylphenylalanine and then to phenylalanine and aspartic acid. Another degradative pathway involves the formation of phenylalanine methyl ester, which undergoes further hydrolysis to give phenylalanine (18,55). Racemization of L-α-aspartame and its degradation products has also been observed (77). According to Prodolliet and Bruelhart (14) and Tsang et al. (55), the major degradation products are diketopiperazine and aspartylphenylalanine. None of the decomposition products have a sweet taste or aftertaste. Therefore, when these compounds are formed in the food products, a loss of sweetness is perceived (77). Several investigators have monitored aspartame in different food products in the market and observed that levels varied from 52–107% of the value declared on the label (14,16,36,47,51,78).

Studies have shown that aspartame is rapidly digested and metabolized as the corresponding dipeptide, providing 4 kcal/g. However, because of its intense sweetness, the amount of energy derived from it is virtually negligible. It is noncariogenic and produces limited glycemic response (2,7,14). Products containing aspartame have to be labeled to warn people with phenylketonuria, i.e., those unable to metabolize phenylalanine. Methanol released from aspartame does not constitute an excessive metabolic load. Numerous studies have shown that aspartame and diketopiperazine are not associated with serious adverse health effects. An ADI of 0–40 mg/kg body weight was allocated by JECFA to aspartame. An ADI value of 7.5 mg/kg body weight was given for diketopiperazine. The use of aspartame appears to be unanimously authorized in different foods and beverages (1,7,9,10,57).

High-performance LC is by far the most frequently used technique to monitor aspartame, intermediates formed during its synthesis, and decomposition products formed during manufacture and storage of food products. It is also used to check for the amounts declared on food labels (14,23).

a. Sample Preparation

Samples should be handled according to the complexity of the food matrix. This is described in Sec. I.C (14,16,29,36,37). Aspartame has also been extracted from different food samples through dialysis with 1% phosphoric acid (40,41). Adenine sulfate and theophyllin can be used as internal standard (31,44).

Clarification with Carrez solutions can be used to eliminate interfering compounds (14,44,45). Purification, isolation, or concentration of the extract can also be performed by solid-phase extraction with C8 (53) or C18 (46,47,75). Hayakawa et al. (75) used Sep-Pak C18 cartridges for the separation of aspartame from its degradation products. The sample was applied to the Sep-Pak, and degradation products and aspartame were eluted with 10% and 30% methanol in acetate buffer, respectively.

Ion-exchange cartridges have also been used. Moriyasu et al. (40) used Bond Elut SCX in the purification of aspartame using a mixture of methanol : 15% sodium chloride (1 : 1, v/v). Bond Elut SCX connected to Bond Elut C18 was used in the purification of aspartame and diketopiperazine, which were eluted with 20% acetonitrile (40,80). Ibe et al. (81) adsorbed aspartame on Amberlite CG-120 and eluted with a 2 N HCl : methanol (1 : 1, v/v) mixture.

b. Separation of Aspartame from Other Sweeteners

During HPLC analysis of aspartame, the most commonly used stationary phase is reverse-phase C18. Two main types of mobile phase have been used: an alcohol (methanol or isopropanol)

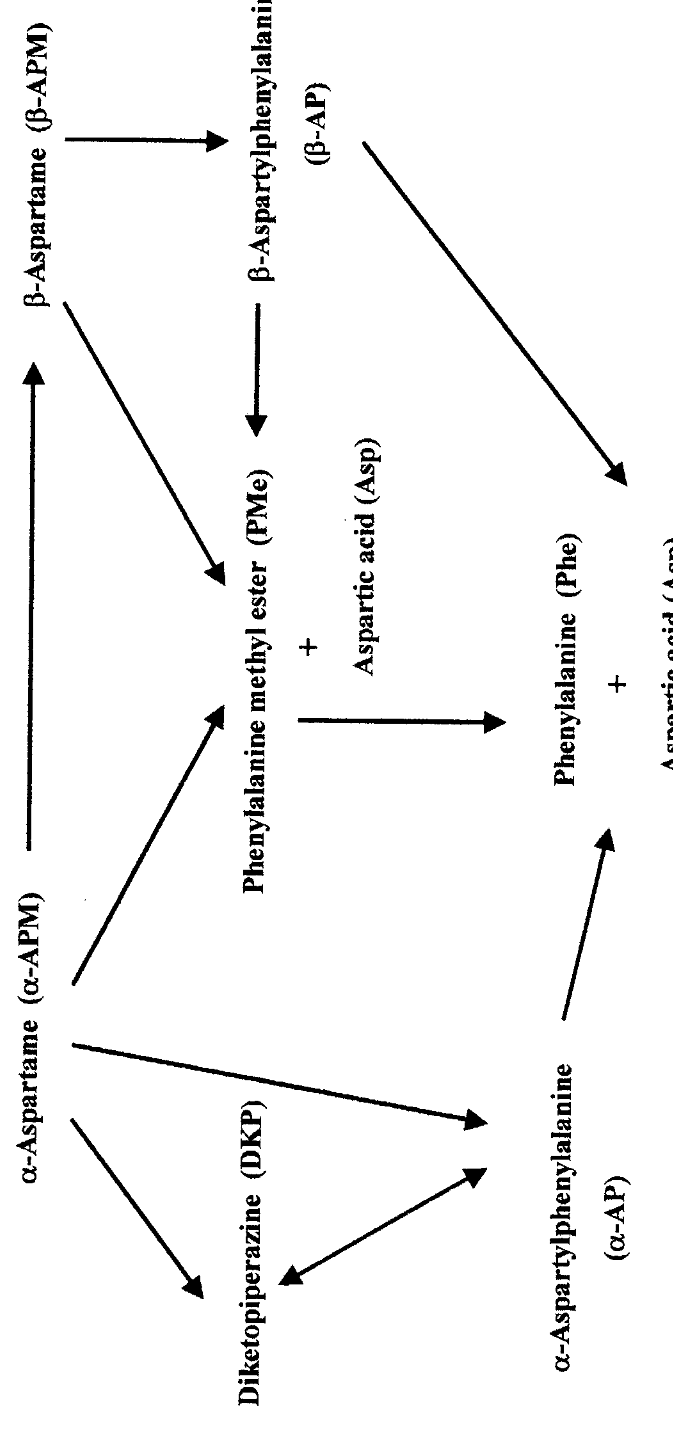

Fig. 3 Decomposition products of aspartame. (From Refs. 14, 55, 75, 77, and 79.)

associated with acetate or phosphate buffer (27,56,61) and acetonitrile associated with phosphate buffer (14,16,26,44,51,82). In both cases, the pH is adjusted to 3.0–4.5. According to Deborde et al. (36), the best results are obtained with the second mobile phase. Variation in pH and/or the acetonitrile content of the mobile phase greatly influences the retention times of aspartame and of interfering compounds present in the extract (14,44). Therefore, the proper mobile phase depends mainly on the analytes to be analyzed (14). Hagenauer-Hener et al. (44) separated aspartame from acesulfame-K, saccharin with 20 mM phosphate buffer: acetonitrile (9:1, v/v). Prodolliet and Bruelhart (14,33) separated aspartame and its degradation products from acesulfame-K, saccharin, alitame, dulcin, theobromin, caffeine, vanillin, hydroxymethylfurfural, and sorbic acid using 12.5 mM KH_2PO_4 (pH 3.5): acetonitrile (9:1, v/v). Ostermeyer (51) used pH (4.8 – 6.7) and acetonitrile (7–20%) gradient in a phosphate buffer mobile phase for the separation of aspartame from acesulfame-K, saccharin, benzoic, and sorbic acids. Lawrence and Charbonneau (16) separated aspartame from saccharin, cyclamate, acesulfame-K, alitame, sucralose, and dulcin with the gradient elution of 0–100%, B (20 mM KH_2PO_4, pH 3.5: acetonitrile, 8:2, v/v) in A (20 mM KH_2PO_4, pH 5.0: acetonitrile, 97:3, v/v).

Ion-pair chromatography has also been used for the separation of aspartame from other sweeteners. The ion-pair reagents commonly used are triethylammonium phosphate (32), tetraethylammonium hydroxyde (47), tetrapropylammonium hydroxide (40), pentanesulfonate (52), tetrabutylammonium phosphate (34), tetrabutylammonium hydrogen sulfate (66), and tetrabutylammonium *p*-toluenesulfonate (24).

Although reverse-phase columns are the most widely used, there is a report on the use of Fractogel TSK HW-40 for the separation of aspartame from saccharin, caffeine, and sodium benzoate. Separation is based on hydrophobic chromatography, using a mobile phase of 67 mM KH_2PO_4, pH 4.3 (67). A strong cation-exchange column (Partisil-10 SCX) eluted with 0.1 M ammonium dihydrogen phosphate at pH 4.5 was used by Argoudelis (31) for the separation of aspartame from saccharin.

c. Separation of Aspartame from Synthesis Intermediates, Stereoisomers, and Degradation Products

The most commonly used stationary phase for the separation of aspartame from synthesis intermediates, stereoisomers, and degradation products is the reverse-phase C18 column. As can be seen on Table 2, the main type of the mobile phase used is a phosphate buffer at pH ranging from 2.5 to 5.0 associated with acetonitrile (14,55,80,83). Reverse-phase HPLC with gradient elution of acetonitrile in phosphate buffer has also been used (16,78).

Ion-pair chromatography has also been used for the separation of aspartame from synthesis intermediates and degradation products. Lawrence and Iyengar (22) and Stamp and Labuza (23) used sodium heptanesulfonate to improve separation of aspartame from its decomposition products. Verzella et al. (21) used sodium hexanesulfonate and gradient elution to separate aspartame from two decomposition products and seven related synthesis by-products (Table 2). This method has been used routinely for the control of aspartame synthesis and as a check of purity of both finished bulk and mother liquor.

Chiral chromatography can also be used in order to obtain resolution of stereoisomers from aspartame, its precursors, and its degradation products. Lin et al. (84), using a Chiracel OD column and a mobile phase of 2-propanol: *n*-hexane (1:1, v/v), achieved complete separation of aspartame precursors, DD-, DL-, LL-, and LD-[(Z)-Asp(β-Bzl)-Phe-OCH_3]. Motellier and Wainer (85) separated four stereoisomers of aspartame, two of diketopiperazine, and three of aspartylphenylalanine using a stationary phase composed of a chiral crown either coated on a polymeric support—CrownPack CR(+)—a mobile phase of aqueous perchloric acid, pH 2.8, and modified

Table 2 HPLC Methods for the Simultaneous Determination of Aspartame and Its Synthesis Intermediates, Stereoisomers, and Degradation Products

Analyte[a]	Column	Mobile phase	Detection	Ref.
APM, DKP, AP, PMe, Phe, PA	Separon SI C18 250 × 6 mm	0.5 M NaH_2PO_4, pH 2.1 : methanol (85 : 15, v/v), flow rate 2 ml/min	UV—200 nm	79
APM, DKP	Nucleosil 5 C18 250 × 4.6 mm	10 mM KH_2PO_4 : acetonitrile, pH 4.0 (85 : 15, v/v), flow rate 0.7 ml/min	UV—200 nm	80
APM, Asp, Phe, PMe, DKP, AP	μBondapak C18 10 μm, 300 × 3.9 mm	12.5 mM KH_2PO_4, pH 3.5 : acetonitrile (90 : 10, v/v), flow rate 0.8 ml/min	UV—214 nm	55
α-APM, β-APM, α-AP, β-AP, DKP, PMe, PA, Phe, Asp	μBondapak C18 10 μm, 300 × 3.9 mm	12.5 mM KH_2PO_4, pH 3.5 : acetonitrile (85 : 15 or 98 : 2, v/v), flow rate 0.8 ml/min	UV—214 nm	14
α-APM, β-APM, DKP, AP, N-formyl-α-APM, N-formyl-β-APM	Hypersil MOS 5 μm, 120 × 4 mm	10 mM KH_2PO_4, pH 4.5 : acetonitrile (77 : 23, v/v), flow rate 2 ml/min	UV—215 nm	83
α-APM, β-APM	Supelcosil LC-18 5 μm, 150 × 4.6 mm	3–20% acetonitrile in 20 mM KH_2PO_4, pH 5.0, flow rate, 1 ml/min	UV—200 or 210 nm	16
APM, DKP	μBondapak C18 10 μm, 300 × 3.9 mm	5–20% acetonitrile in KH_2PO_4, pH 3.5	UV—214 nm	78
α-APM, β-APM, α-AP, β-AP, DKP, Phe, PMe, L-α-[(N-formyl)aspartyl]-L-Phe, L-α-[(N-formyl)aspartyl]-L-PMe, L-β-[(N-formyl)aspartyl]-L-PMe	Hibar RP-8 10 μm, 250 × 4 mm	10–30% acetonitrile in 2.5 mM K_2HPO_4, pH 2.5, with 10 mM Na hexanesulfonate, flow rate 2 ml/min	UV—220 nm	21
α-APM, β-APM	Spherisorb 5 ODS	acetonitrile : 20 mM KH_2PO_4 (2 : 8, v/v) pH 4.0, with 5 mM Na heptanesulfonate	UV—210 nm	22
α-APM, β-APM, α-AP, β-AP, DKP, Phe, PMe	NovaPak C18 4 μm, 150 × 3.9 mm	acetonitrile : 5 mM NaH_2PO_4 (2 : 8, v/v) pH 3.0, with 5 mM Na heptanesulfonate	UV—214 nm	23
LL-APM, LD-APM, DD-APM, DL-APM, L-Asp-D-Phe, DD + LL-DKP, DL + LD-DKP, D-Asp-D-Phe, D-Asp-L-Phe, L-Asp-L-Phe	Crownpack CR(+) 150 × 4 mm	perchloric acid, pH 2.8 + 1.5% 2-propanol 10–40°C temperature gradient	UV—210 nm	85
DD-, DL-, LL-, LD-[(Z)-Asp(β-Bzl)-Phe-OCH_3]	Chiralcel OD 250 × 4.6 mm	2-propanol : *n*-hexane (1 : 1, v/v) flow rate 0.5 ml/min	UV—254 nm	84
LL-α-APM, LL-β-APM, L-α-AP, L-β-AP, DKP	Chirobiotic T 5 μm, 250 × 4.6 mm	ethanol : H_2O (55 : 45, v/v) pH 3.85 flow rate 0.6 ml/min	UV—215 nm	15
APM, AP, Asp, Phe(1-cyano-2-substituted benz[f] isoindole derivative)	TSK ODS-120T 150 × 4.6 mm	30–80% acetonitrile in 50 mM acetate buffer, pH 6.0	Fluorescence λ_{ex} 420, λ_{em} 490 nm	75

[a] APM = aspartame, DKP = diketopiperazine, AP = aspartylphenylalanine, PMe = phenylalanine methyl ester, Phe = phenylalanine, PA = phenylalanine-aspartic acid, Asp = aspartic acid.

with 1.5% 2-propanol and a temperature gradient from 10 to 40°C. Aboul-Enein and Bakr (15) separated L-α-aspartame from L-β-aspartame, L-α-aspartyl-L-phenylalanine, L-β-aspartyl-L-phenylalanine, and diketopiperazine using a Chirobiotic T column and a mobile phase of ethanol: water (55:45, v/v), pH 3.85. These methods can be used to determine rapidly the stereochemical purity of aspartame in bulk material and the extent of its degradation in food products.

Aspartame and its degradation products aspartylphenylalanine, aspartic acid, and phenylalanine can also be separated after reaction with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide (sodium or potassium cyanide) in borate buffer (50 mM, pH 8). This reaction affords highly fluorescent and stable 1-cyano-2-substituted-benz[*f*]isoindole (CBI) derivatives that can be detected at 420 nm excitation and 490 nm emission. The CBI derivatives are separated on a TSK ODS-120T column using a gradient of 30–80% B (acetonitrile: water, 9:1 v/v) in A (50 mM acetate buffer, pH 6.0) (75).

d. Detection Systems

Aspartame has been quantified by UV detection at 254 nm and at 200–217 nm. However, because aspartame has a relatively low extinction at 254 nm, quantification at lower wavelengths provides increased response. Detection can also be performed with increased specificity by fluorescence after postcolumn derivatization with *o*-phthaldehyde (76).

In order to improve detection sensitivity, aspartame can also be separated by HPLC as a fluorescent derivative (41). Fluorescamine derivatives are separated on LiChrosorb RP-8 using acetonitrile in 50 mM acetate, buffer, pH 6, 22:78, v/v (53), or on Spherisorb S5 ODS-2 RP using 0.2 M phosphate buffer (pH 9): acetonitrile: methanol, 2:1:1, v/v (54). Detection is performed at 397 nm excitation and 482 nm emission (54).

5. Alitame

Alitame [L-α-aspartyl-*N*-(2,2,4,4-tetramethyl-3-thioethanyl)-D-alaninamide] is an amino acid–based sweetener developed by Pfizer from L-aspartic acid, D-alanine, and an amine 2,2,4,4-tetraethylthioethanyl amine (Fig. 1). Its formula is $C_{14}H_{25}O_4N_3S$ with a molecular weight of 331.06. It is produced under the brand name Aclame®. It is a crystalline, odorless, nonhygroscopic powder that is soluble in water (130 g/L at pH 5.6) and alcohol and significantly more stable than aspartame (Table 1). Alitame is 2000 times as sweet as sucrose and has a clean, sweet taste, with no unpleasant aftertaste. It blends with other sweeteners, such as acesulfame-K, saccharin, and cyclamate, to maximize the quality of sweetness (3,7–9).

Alitame offers some benefits, such as stability at high temperatures and a broader pH range. At a pH of 6–8 and at room temperature, it is stable for over a year. It is stable at high temperatures, withstanding pasteurization. However, prolonged storage of acidic solutions at high temperatures or the combination with certain ingredients (hydrogen peroxide and sodium bisulfite) may produce off-flavors. In the presence of high levels of reducing sugars, alitame can undergo Maillard reactions (3,4,8,9,57,58).

Alitame is noncariogenic (3). From an oral load of alitame, 7–22% is unabsorbed and excreted in the feces. The remainder is hydrolyzed to aspartic acid and alanine amide. The aspartic acid is metabolized normally, and the alanine amide is excreted in the urine as a sulfoxide isomer, sulfone, or conjugated with glucuronic acid. The incomplete absorption and metabolism results in a core value of 1.4 kcal/g (57). It was concluded by JECFA that alitame was not carcinogenic or mutagenic, and in 1996 an ADI of 0–1 mg/kg body weight was allocated. It is approved for use in Australia, New Zealand, Mexico, and China. Potential uses include baked goods, hot and cold beverages, beverage mixes, tabletop sweeteners, chewing gum, candies, frozen desserts, and pharmaceuticals (7,10).

a. Sample Preparation

Sample preparation for HPLC analysis of alitame will depend on the type of food (16,33), and complex matrices may be clarified with Carrez solutions or by means of C18 disposable cartridge (33), as described in Sec. I.C.

b. Separation Techniques

A reverse-phase column and a mobile phase of 12.5 mM KH_2PO_4 (pH 3.5): acetonitrile (9:1, v/v) was used by Prodolliet and Bruelhart (33) for the separation of alitame from saccharin, acesulfame-K, dulcin, aspartame and its degradation products, other food additives, and other constituents.

Lawrence and Charbonneau (16) used a gradient of 0–100% B (20 mM KH_2PO_4, pH 3.5: acetonitrile, 8:2 v/v) in A (20 mM KH_2PO_4, pH 5.0: acetonitrile, 97:3 v/v) and a Supelcosil LC-18 column for the separation of alitame from saccharin, aspartame, acesulfame-K, cyclamate, sucralose, and dulcin.

c. Detection Systems

Alitame can be detected by UV at 200 or 210 nm (16). However, according to Prodolliet and Bruelhart (33), optimum sensitivity was obtained at 205 nm.

6. Dulcin

Dulcin, 4-ethoxyphenylurea, *p*-phenetolcarbamide, and *p*-phenethylurea (Fig. 1) is a synthetic sweetener. Its chemical formula is $C_9H_{12}N_2O_2$, and it has a molecular weight of 180.20. Berlinblau first discovered it in 1884. Dulcin is 70–350 times sweeter than sucrose. However, it should not be used as a food additive, since it has been reported to cause cancer in laboratory animals (1,8,58).

a. Sample Preparation

Extraction of samples for dulcin analysis by HPLC is described in Sec. I.C (16,27,33). Purification can be performed with Carrez solutions or by means of C18 disposable cartridges (24,33).

b. Separation Techniques

Reverse-phase chromatography has been used for the determination of dulcin. Veerabhadrarao et al. (27) described a method for the separation of dulcin, acesulfame-K, and saccharin on μBondapak C18 using methanol: acetic acid: water (7:1:12, v/v). Prodolliet and Bruelhart (33) used the same column, but a mobile phase of 12.5 mM KH_2PO_4 (pH 3.5): acetonitrile (9:1, v/v) for the separation of dulcin from saccharin, acesulfame-K, alitame, aspartame and its degradation products, other food additives, and natural constituents. Lawrence and Charbonneau (16) used a gradient of 0–100% mobile phase B (20 mM KH_2PO_4, pH 3.5: acetonitrile, 8:2, v/v) in A (20 mM KH_2PO_4, pH 5.0: acetonitrile, 97:3, v/v) on Supelcosil LC-18 for the separation of dulcin from saccharin, aspartame, acesulfame-K, cyclamate, sucralose, and alitame.

Ion-pair chromatography has also been used for the determination of dulcin. Wu et al. (47) added the ion pair tetraethylammonium hydroxide to the mobile-phase methanol: 85% phosphoric acid, pH 6 (2:8), for the separation of dulcin from saccharin, cyclamate, aspartame, and acesulfame-K on μBondapak C18. Herrmann et al. (24) separated dulcin from saccharin, cyclamate, and aspartame on Hypersil MOS 3 using a mobile phase consisting of 5 mM tetrabutylam-

monium *p*-toluenesulfonate, pH 3.5, and 12% methanol. Chen and Fu (65) separated dulcin from saccharin and acesulfame-K using 2.5 mM hexadecyltrimethylammonium bromide in acetonitrile: 50 mM α-hydroxyisobutyric acid, pH 4.5 (2.2:3.4, v/v), and a C18 column.

c. Detection Systems

Detection and quantitation of dulcin can be accomplished by UV absorption at 254 nm (27) or at 210–220 nm (16,33,47). According to Prodolliet and Bruelhart (33), optimum sensitivity of dulcin was obtained at 205 nm.

7. *Sucralose*

Sucralose, 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl 4-chloro-4-deoxy-α-D-galacto-pyranoside or 4,1′,6′-trichloro-4,1′,6′-trideoxy-*galacto*-sucrose (Fig. 1), is a chlorinated derivative of sucrose discovered in 1976 and marketed under the brand name Splenda®. Its chemical formula is $C_{12}H_{19}O_8Cl_3$ (MW 397.35). It is a white, odorless, crystalline powder that is soluble in water (280 g/L at 20°C), methanol, and ethanol. Sucralose is 400–800 times sweeter than sucrose (Table 1). It has a clean, sugarlike taste and a time-intensity profile much like that of sucrose, although more persistent. It has no bitter or any other objectionable aftertaste. It is a flavor enhancer. It shows sweetness synergism with cyclamate, acesulfame-K, and neohesperidin dihydrochalcone (8,25,57,86).

Sucralose is extremely stable in the dry form (4 years at 20°C) and at a broad pH range. It withstands high temperatures, which makes it well suited for use in pasteurized, sterilized, cooked, and baked foods. However, under extreme conditions of pH, temperature, and time, sucralose can be hydrolyzed, producing 4-chloro-deoxy-D-galactose and 1,6-dideoxy-1,6-dichloro-D-fructose or degraded with elimination of hydrogen chloride in basic medium (2,9,58,86,87).

Sucralose is noncariogenic, produces no glycemic response, and is virtually noncaloric. Safety-testing and toxicological data has given promising results. It was reviewed favorably in 1990 by JECFA, which recommended an ADI level of 0–15 mg/kg body weight. Sucralose is approved for use in a wide range of food products in Canada, the United States, Australia, Mexico, Russia, Romania, China, the European Union, and Mercosur (Argentina, Brazil, Paraguay, and Uruguay). It has been used as a tabletop sweetener and in beverages, desserts, confectionery, bakery products, canned fruits and vegetables, condiments, dressings, and breakfast cereals (3,7,10,86,88).

a. Sample Preparation

Extraction of samples is described in Sec. I.C (16). Extracts might need purification or concentration, which can be performed by solid-phase extraction with Sep-Pak or Alumina Sep-Pak C18 cartridges (87).

b. Separation Techniques

Sucralose was determined by reverse-phase chromatography using a C18 Rad Pak column and a mobile phase of water: methanol, 7:3, v/v (87). Lawrence and Charbonneau (16) separated sucralose from saccharin, cyclamate, aspartame, acesulfame-K, alitame, and dulcin on Supelcosil LC-18 with gradient elution of 0–100% B (20 mM KH_2PO_4, pH 3.5: acetonitrile, 8:2 v/v) in A (20 mM KH_2PO_4, pH 5.0: acetonitrile, 97:3 v/v).

c. Detection Systems

Refractive-index detection can be used for the analysis of sucralose (16,87). Ultraviolet detection at 190 nm is also satisfactory. However, further sample cleanup and concentration is needed to

lower background interference (87). Lawrence and Charbonneau (16) suggested that detection be performed at 200 nm.

8. Neohesperidin Dihydrochalcone

Neohesperidin dihydrochalcone (NDHC) is a semisynthetic intense sweetener. It was first prepared by Horowitz and Gentili in 1963, by alkaline hydrogenation of the biflavanoid neohesperidin present in Seville (bitter) oranges (*Citrus aurantium*) to dihydrochalcone. It is 1-[4-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(3-hydrox y-4-methanoxyphenyl)-1-propanone with the molecular formula $C_{28}H_{36}O_{15}$ and a molecular weight of 612.60 (Fig. 1). It shows a slow buildup of sweetness, rising to 250 times that of a 5% sucrose solution, but more persistent (Table 1). It has a pleasant taste and a lingering menthol or licorice aftertaste. Neohesperidin dihydrochalcone has flavor-enhancing, flavor-modifying, bitterness and saltiness-suppression properties, an ability to improve sweetness quality and profile, and remarkable synergistic effects (89). The sweetness intensity of NHDC depends on many factors, such as concentration, pH, and the product to which it is added. It shows synergism with several intense sweeteners and sugar alcohol (9,20,57,90,91).

Neohesperidin dihydrochalcone is a colorless, crystalline solid that is sparingly soluble in water (0.40–0.50 g/L at 20°C) but highly soluble at 80°C (650 g/L) and in ethanol–water mixture. High NHDC stability is observed at pH 2–6. The sweetener is stable under most food processing and storage conditions. It withstands pasteurization, UHT processes, and the normal shelf life of soft drinks. It is stable during fermentation and pasteurization of yogurt. However, it undergoes hydrolysis at high acidity and elevated temperatures, yielding hesperetin dihydrochalcone, hesperetin dihydrochalcone-4′-β-D-glucoside, rhamnose, and glucose (12,20,90–93).

Neohesperidin dihydrochalcone is noncariogenic and has a caloric value of about 2 kcal/g (12). It is not absorbed in the small intestine, but can undergo glycosidic cleavage, releasing the aglycon. Several studies have confirmed its safety. In 1987, the Scientific Committee for Food of the Commission of the European Communities allocated an ADI of 0–5 mg/kg body weight (20,57,91,93). It is currently approved for use in the European Communities, Sweden, Switzerland, Morocco, Tunisia, and Mercosur. It has been used in juice, soft drinks, dairy products, desserts, confectionery, spreads, jams, chewing gum, chocolate-based products, and ice cream (10).

a. Sample Preparation

The solubility of NHDC in hot water, alcohol, aqueous alkali, acetonitrile, dimethyl sulfoxide, and alcohol/water mixture facilitates its selective extraction from food samples (20,91,94). It is extracted from jams, fruit juices, and dairy products with methanol (66,93) or acetone (95) and filtered or centrifuged. Chewing gum samples are dissolved in chloroform and extracted with water. The extract is centrifuged, and the clear supernatant is injected into the HPLC (95). If necessary, sample cleanup and concentration may be achieved by selective adsorption or desorption (20) on Sep-Pak C18 (96). Tomás-Barberán et al. (93) used Amberlite XAD-2 resin for purification of jam extract. Sugars, pectin, and other polar compounds were eluted with water, and NHDC was eluted with methanol. After concentration, the extract was further purified on a Sephadex LH-20 column prior to HPLC analysis.

b. Separation Techniques

Reverse-phase chromatography has been widely used for the determination of NHDC. Two main types of mobile phase have been used, methanol/water and acetonitrile/water. Schwarzenbach (95) separated NHDC from impurities such as phloroacetophenone-4′-β-neohesperidoside,

naringin, and neohesperidin on a octadecyltrichlorosilane-treated LiChrosorb SI60 column and a mobile phase of methanol: water (4:6, v/v). Canales et al. (92) used a reverse-phase column and gradient elution of methanol in phosphate buffer, pH 3.2, for the determination of NHDC. Two different gradient systems were used. For samples with pH 1–4, the gradient was from 50 to 70%, whereas for samples with neutral pH values, the gradient was from 40 to 70%. Tomás-Berberán et al. (93) used LiChrospher 100 RP-C18 and a gradient of 20–40% methanol in 5% formic acid solution for the determination of NHDC.

Fisher (96) determined NHDC in grapefruit juice on μBondapak C18 using water: acetonitrile (75:25, v/v). Montijano et al. (89) determined NHDC in dairy products using LiChrospher C18 and acetonitrile: acidified water (2:8, v/v). Montijano et al. (90) separated NHDC from its hydrolysis products, hesperetin dihydrochalcone and hesperetin dihydrochalcone-4′-β-D-glucoside, on NovaPak RP C18 with acidified water (5 ml acetic acid/L): acetonitrile (8:2, v/v) at 30°C. Castellar et al. (94) separated NHDC from phloroacetophenone neohesperidoside, naringin, neohesperidin, naringin dihydrochalcone, hesperidin dihydrochalcone, hesperitin dihydrochalcone glucoside, and hesperitin dihydrochalcone on LiChrospher C18 using acetonitrile: water acidified with acetic or phosphoric acid (2:8, v/v) at 25°C. The method was observed to be suitable for the determination of the purity of commercial NHDC.

Neohesperidin dihydrochalcone has also been determined by ion-pair chromatography on LiChrospher 60 with a gradient of 15–95% methanol in 10 mM tetrabutylammonium hydrogen sulfate. No interference was observed from acesulfame-K, aspartame, and saccharin (66).

c. Detection Systems

NHDC has been detected and quantified by UV detection at 285 nm (93), 282 nm (90,94), and 280 nm (95). However, when determining NHDC along with other sweeteners in the same run, a lower wavelength—217 nm—should be used (66).

9. *Glycyrrhizin*

Glycyrrhizin is found in licorice root of a small shrub, *Glycyrrhiza glabra* L., grown in Europe and Central Asia. Glycyrrhizin, glycyrrhizinic acid, glycyrrhizic acid, or 20β-carboxy-11-oxo-30-norolean-12-en-3β-yl-2-*O*-β-D-glucopyranurosyl-α-D-glucopyranosiduronic acid ($C_{42}H_{62}O_{16}$, MW 822.92) is a glycoside of the triterpene, glycyrrhetinic acid, which is condensed with *O*-β-D-glucuronosyl-(1′→2)-β-D-glucuronic acid (Fig. 2). It is present in the root as the calcium and potassium salts (97). A crude ammonium glycyrrhizin (AG) is prepared from the extract by precipitation with a mineral acid. Further treatment yields a white, crystalline mono-ammonium glycyrrhizin (MAG). Both derivatives have the same sweetness, but they differ markedly from each other in solubility and sensitivity to pH. Ammonium glycyrrhizin is relatively stable and highly soluble in hot or cold water. It withstands temperatures above 105°C for a short period of time. At pH values below 4.5, AG precipitates. Mono-AG is used in applications where low pH and color rule out AG (3,8,19,58).

Glycyrrhizin is 50–100 times sweeter than sucrose and has a slow onset of taste and a long aftertaste (Table 1). It exhibits a dark, sweet, woody flavor, which limits its use as a pure sweetener. Glycyrrhizin provides licorice flavor, enhances food flavors, masks bitter flavors, and increases the perceived sweetness level of sucrose. It also has the potential for providing functional characteristics, including foaming, viscosity control, gel formation, and possibly antioxidant characteristics (3,19,58).

Studies have focused on the many pharmacological effects of glycyrrhizin, including its antiulcer, anti-inflammatory, antiviral, and antispasmodic properties. It is also reported to have corticoid activity (2,9,97). It should be used in moderate amounts as a sweetener because of its phar-

macological action (58). Glycyrrhizin is anticariogenic. Metabolism studies have shown that glycyrrhizin can be hydrolyzed by human intestinal microflora to 18β-glycyrrhetinic acid (18β-GA) and two molecules of glucuronic acid. The ammonium salt of glycyrrhizin is approved as flavoring and flavor enhancer in the United States. It is on the FDA GRAS (generally recognized as safe) flavors list (57). The use of glycyrrhizin is permitted in Japan, Taiwan, and Mercosur. It has been widely used for several years as flavoring agent in candy, tobacco, confectionery, beverages, and pharmaceutical, and cosmetic preparations (3,10,19,97).

a. Sample Preparation

Several procedures for the determination of glycyrrhizin in licorice products are described. Liquid samples and carbonated beverages are analyzed with minimal sample treatment (see Sec. I.C). Solid samples are ground and extracted in an ultrasonic bath with double distilled water or NH_4OH (64). High levels of gums or sugars can be eliminated from samples by alcoholic precipitation and centrifugation (98). Lunder and Nielsen (99) extracted glycyrrhizin from licorice root by reflux with distilled water, centrifugation, and filtration. Spinks and Fenwick (97) extracted samples for 40 hours with 80% methanol in a Soxhlet apparatus. Glycyrrhizin can also be extracted from food samples (soy sauce, miso) by dialysis against 3% NH_4OH solution (100,101). Interfering substances and coloring material can be adsorbed on acid alumina. After washing the column with aqueous acetone followed by water, glycyrrhetinic acid is removed with chloroform and pure glycyrrhizin with 0.1% ammonia solution (102). Sep-Pak C18 can also be used (64).

b. Separation Techniques

Reverse-phase HPLC has been widely used for the determination of glycyrrhizin (99–104). Hurst et al. (101) determined glycyrrhizin on EM Lab, RP-18, and methanol: water: acetic acid (60:34:6, v/v). Lunder and Nielsen (99) determined glycyrrhizin on μBondapak C18 with gradient elution of 20–85% isopropyl alcohol in 0.01 N KH_2PO_4. Spinks and Fenwick (97) determined glycyrrhizin using Spherisorb 5 ODS2 and a gradient of acetonitrile in water: acetic acid. This method was subjected to collaborative testing and included in the 14th edition of the AOAC *Official Methods of Analysis*. Glycyrrhizin and its aglycon (β-glycyrrhetinic acid) were separated on Nucleosil RP18 with a gradient of 45–95% acetonitrile in 4% aqueous acetic acid (98). Beasley et al. (103) separated glycyrrhizin and glycyrrhetinic acid with a gradient of 0–60% acetonitrile in 2% formic acid solution using a C18-Corasil column. Tsai and Chen (104) separated glycyrrhizin from stereoisomers of glycyrrhetinic acid (GA), 18α-GA, and 18β-GA on LiChrospher RP18 with a linear gradient of 10–100% methanol in perchlorate buffer (pH 7.5–7.7).

Glycyrrhizin can also be determined by ion-pair chromatography (64,105). Matsunaga et al. (64) separated glycyrrhizin from saccharin on a reverse-phase column with ethanol: 50 mM sodium dihydrogenphosphate (2:3, v/v) containing 20 mM cetyltrimethylammonium chloride, pH 3.

c. Detection Systems

Detection of glycyrrhizin has been done by UV absorbance at 250 nm (97), 254 nm (64,99,103,104), or 258 nm (102).

10. Stevia Sweeteners

Stevia sweeteners are extracted from the leaves of *Stevia rebaudiana* Bertoni (Compositae), an herb native to Paraguay but cultivated in Southeast Asia, Japan, Paraguay, Brazil, Israel, and the United States. The sweet constituents of stevia include eight diterpene glycosides; stevioside, steviolbioside, rebaudiosides A, B, C, D, E, and dulcoside A, which collectively are 100–300 times

sweeter than sucrose. They are similar in structure, in that a steviol aglycon is connected at C-4 and C-13 to mono-, di-, or trisaccharides consisting of glucose and/or rhamnose residues, as shown in Fig. 2 (100,106). Stevioside is the major constituent, while dulcoside A and rebaudiosides A and C are the other main components. Stevioside is 300 times sweeter than sucrose (Table 1). It shows a sweetness profile similar to that of sucrose, except that it has an unpleasant, persistent, menthol, bitter aftertaste. However, development of new cultivars, derivatization, and incorporation of cyclodextrin, L-histidine, potassium phosphate, glucono-delta-lactone, and maltose in formulations have eliminated undesirable aftertastes. Rebaudioside A is more stable and much sweeter and has a better taste profile than stevioside. The remaining diterpene glycosides are not as sweet as stevioside (3,57,107–110).

Stevioside is a white powder that is highly soluble in water, ethanol, and methanol. It is nonfermentable. When heated at 100°C for 1 hour, solutions of stevioside at pH 3–9 show little loss in sweetness and no change at 22°C for 5 months (2,8,57,58). Chang and Cook (106) observed some degradation of stevioside and rebaudioside in carbonated beverages acidified with phosphoric and citric acids during storage at 37°C. Exposure to 1 week of sunlight did not affect stevioside, but resulted in approximately 20% loss of rebaudioside A. The high stability of stevioside makes it a suitable sweetener for cooked and baked foods and for beverages.

Stevioside and rebaudioside A provide very few calories and are noncariogenic (2,3,9,57). Evidence from exhaustive animal studies suggested that stevioside is not toxic, mutagenic, or teratogenic in a number of animal species (111–113). However, there are contradictory reports on the in vivo metabolism to steviol that is mutagenic (58,114). Since 1970, stevia sweeteners have been used in a wide range of food and beverage applications in Japan (9,18). It is currently approved for use in Japan, Taiwan, and Mercosur (10,115). Xili et al. (113) suggested an acceptable daily intake of stevioside for humans of 7.95 mg/kg body weight.

a. Sample Preparation

Extraction of stevia sweeteners from dried leaves can be accomplished with acetonitrile in the presence of calcium carbonate solution (116) or with boiling water adjusted to pH 9.0 (107). Ahmed and Dobberstein (117) extracted stevioside and rebaudioside A and C from dried leaves of *S. rebaudiana* in a micro-Soxhlet apparatus. They observed that chloroform/methanol provided the best results, compared to chloroform or to chloroform/methanol/water. Extraction of stevioside, rebaudioside A and C, and dulcoside A can also be performed by subcritical fluid extraction using CO_2 and methanol as a modifier. Such an extraction technique has been gaining popularity as an analytical tool because it is rapid, simple, and less expensive in terms of solvent cost (110). Beverages, tabletop sweeteners, beverages containing pulp, and candies are prepared as indicated in Sec. I.C (110,115,118).

According to Noda et al. (119), purification of extracts might be necessary, depending on the type of food sample. Stevia sweeteners can be purified through partition into *n*-butanol. However, a number of coexisting polar substances may be extracted at the same time (106,118). Interference can be eliminated by solid-phase extraction using C8 (110), Sep-Pak C18 (115), or Sep-Pak PLUS PS-1 (119) cartridges. Kitada et al. (115) activated Sep-Pak C18 with acetone, methanol, and water, applied the sample, and washed it with water. Stevioside, rebaudioside A and C, and dulcoside A were then eluted with 10 ml of acetonitrile: water (8:2, v/v). The sweeteners can also be purified on a Silica gel 60 silanized column with 80% methanol after washing the column with phosphate–borate buffer (pH 7), water, and 20% methanol solution (118).

b. Separation Techniques

High-performance LC is routinely used for the analysis of stevia sweeteners in plant material or in food and beverages. The first successful HPLC separation of stevioside and rebaudioside A

was reported by Hashimoto et al. (116) on a hydrophilic column (Shodex OHpak M-414) eluted with acetonitrile: water (4:1, v/v). However, NH_2 phase-bonded columns have been the most widely used. According to Noda et al. (119), the use of a polymer-based NH_2 column provides better resolution than silica-based ones. Chang and Cook (106) separated stevioside and rebaudioside A using μBondapak NH_2 and acetonitrile: water (8:2, v/v). Kitada et al. (115) obtained good resolution in the separation of stevioside, rebaudioside A and C, and dulcoside A using LiChrosorb NH_2 at 50°C and acetonitrile: water (8:2, v/v). Liu et al. (110) separated the same glycosides using a similar mobile phase; however, the column used was spherical (LiChrospher NH_2) at room temperature. Makapugay et al. (108) obtained rapid baseline separation of the eight sweet stevia glycosides on Zorbax NH_2 with a gradient of acetonitrile in water, pH 5. To minimize interference from the sample matrix, Fujinuma et al. (118) added 5 mM tetrabutylammonium phosphate to acetonitrile: water (200:45, v/v) during separation of stevioside and rebaudioside A on Finepak SIL NH_2.

Other types of column have also been used for the determination of stevia sweeteners. Nishiyama et al. (107) determined stevioside in leaves of stevia using LiChrosorb RP18 and methanol: NaOH 5 mM (65:35, v/v). Alvarez and Kusumoto (120) used this method to determine stevioside, rebaudioside A, steviolbioside, and three stevioside hydrolysis products—steviol, isosteviol, and steviolbioside—in dried stevia leaves and in beverages. Ahmed and Dobberstein (117,121) developed a method for the rapid and accurate separation of all eight sweet stevia glycosides using two Protein I-125 columns connected in series and *n*-propanol as the mobile phase. Kasai et al. (122) separated stevioside from rebaudioside A, C, D, and E on a hydroxyapatite column (Pentax PEC 101) using linear gradient elution of 77–70% aqueous acetonitrile in 30 min.

c. Detection Systems

Detection of stevia glycosides after separation by HPLC has been performed by high-sensitivity refractive index (106,116) and by UV at 210 nm (110,115–118). The use of UV at 210 nm allowed detection of 0.4 μg for stevioside, steviolbioside, rebaudioside B, and dulcoside A and 0.8 μg for rebaudiosides A and C–E. This sensitivity is higher than that obtained with the RI detector–2 μg of stevioside and rebaudioside A (116).

11. Thaumatin

Thaumatin is a mixture of sweet proteins originally isolated from the fruit of the West African plant katemfe (*Thaumatococcus danielli* Benth.). There are at least five thaumatins. Tate and Lyle Ltd. manufactures a mixture of two as talin. They are basic proteins, having isoeletric points of 11.5–12.5. Thaumatin consists of a single chain of 207 normal amino acid residues with eight disulfide bonds and a molecular weight of about 22,000 (123). It is very soluble in water (60%, w/w) but not in organic solvents, and is stable at pH 2.7–6.0 and also under pasteurization conditions (Table 1). Thaumatin can associate with negatively charged compounds, such as synthetic colors, acidic gums like xanthan, pectin, carrageenan, alginate, and carboxymethylcellulose, resulting in a loss of sweetness. Association with synthetic colorants may cause color loss (1,2,9,124).

Thaumatin is 1600–3000 times sweeter than sucrose. However, it has unusual taste profile: slow in onset, followed by intensification to lingering sweetness, with a licorice-type aftertaste (Table 1). To achieve a taste closer to that of sucrose, thaumatin must be blended with other intense sweeteners or with sugars. By combining thaumatin with alanine and organic acids, there is a doubling in sweetness and a reduction in the aftertaste and in the delay in sweetness. Thaumatin has the ability to enhance certain flavors and aromas, such as those in peppermint, spearmint, coffee, and ginger (8,57,58,123,125).

Thaumatin can be completely metabolized to its constituent amino acids, but, because of its high sweetness, it has a low-calorie value per unit of sweetness, less than 0.002 kcal (58). It is noncariogenic. Thaumatin has undergone several safety tests; results indicate that it is not allergenic, mutagenic, or teratogenic. Furthermore, it has a long history of use without adverse effect. In 1985 a "not specified" ADI was allocated by JECFA, indicating that it does not represent any hazard to health. Therefore it is safe, especially at the levels consumed (9). Thaumatin has been approved for use in Japan, Great Britain, Australia, Canada, South Africa, the European Union, Switzerland, Taiwan, Morocco, and Tunisia. In the United States and Switzerland it is permitted as a flavor enhancer in chewing gum. In the United States it has been classified as GRAS. The major applications include chewing gum, savory flavor, dairy products, dental and pharmaceuticals, and animal and pet foods. However, because of its high cost, it is not used to a great extent. Biotechnological alternatives to eliminate the uncertainties and variability associated with agricultural production and to lower costs are being investigated (1–3,8–10,123).

a. Sample Preparation

The extraction, purification, or concentration of samples for thaumatin determination will depend on the type of food matrix, as described in Sec. I.C. Purification can also be accomplished by partition (126). Ramsohoye and Kozlov (124) purified thaumatin on a cation-exchange cartridge (CM Sephadex C-50). After conditioning the cartridge with phosphate buffer, the sample is loaded and washed with buffer. Thaumatins are then eluted with 0.25 M sodium chloride. Mackenzie et al. (127) separated thaumatins on cellulose CM52. After equilibration of the column with buffer, pH 7.2, the sample was applied, washed with the buffer, and eluted with a gradient of 0 to 0.2 M NaCl in the same buffer.

b. Separation Techniques

Cascone et al. (126) used a reverse-phase HPLC system for the determination of thaumatin. It consisted of a Vydac 218TP54 column and elution with 10% acetonitrile in 0.1% trifluoroacetic acid.

c. Detection Systems

Thaumatin can be detected and quantified by UV absorption at 280 nm (126,127).

12. Multisweetener Methods

Today there is a tendency to use blends of sweeteners. When two or more sweeteners are combined, blends with increased stability, longer shelf life, lowered production costs, improved taste and flavor, and decreased side and aftertastes result. Also, mixtures of sweeteners can exhibit additive and synergistic effects. Furthermore, since lower amounts of each sweetener will be used, the average daily intake of each sweetener will decrease, minimizing the health risk from any one sweetener (2,4,9,25).

Various methods have been developed for the simultaneous determination of several sweeteners in a single run. Most of the methods described in the literature and summarized in Table 3 have been developed for the separation of three sweeteners, especially for saccharin, acesulfame-K, and aspartame. Herrmann et al. (24), Veerabhadrarao et al. (27), and Hausch (66) developed methods for the simultaneous determination of four sweeteners. Prodolliet and Bruelhart (33) and Wu et al. (47) separated five sweeteners. The most comprehensive method is the one developed by Lawrence and Charbonneau (16), which allows the simultaneous analysis of seven sweeteners. With the increased number of sweeteners available and their use being approved for use in specified food products and beverages by different countries, methods capable of separating several sweeteners simultaneously are still needed.

Table 3 HPLC Methods for the Simultaneous Determination of Intense Sweeteners

Sweeteners	Column	Mobile phase	Detection	Ref.
Acesulfame-K, aspartame, saccharin	Superspher RP-select B 4 μm	20 mM phosphate buffer: acetonitrile (9:1, v/v)	UV	44
Acesulfame-K, aspartame, saccharin	Nucleosil 100-5 C18	7–20% acetonitrile in phosphate buffer, pH 4.8–6.7	Diode array	51
Acesulfame-K, aspartame, saccharin	Finepak C18S	Methanol: water (2:8, v/v) pH 4.0 containing 0.1 M tetra-*n*-propylammonium hydroxide	UV—210 nm	40
Acesulfame-K, aspartame, saccharin	ODS Hypersil RP-18 5 μm, 100 × 4.6 mm	17–40% Methanol in 5 mM tetrabutylammonium hydrogen sulfate	UV—210 nm	34
Acesulfame-K, cyclamate, saccharin	Dionex AS4A anion separator column	Na_2CO_3 (140 mg/L)	Conductivity	17
Acesulfame-K, dulcin, saccharin	C18 column	Acetonitrile: 50 mM α-hydroxyisobutyric acid, pH 4.5 (2.2:3.4, v/v) with 2.5 mM hexadecyltrimethyl-ammonium bromide	UV—233 nm	65
Acesulfame-K, aspartame, cyclamate, saccharin	LiChrosorb RP-18	0–20% Acetonitrile in H_3PO_4 pH 2.34 with pentane-sulfonate (2 g/L)	UV—254 nm	52
Acesulfame-K, aspartame, dulcin, saccharin	μBondapak C 18 10 μm, 300 × 3.9 mm	Methanol: acetic acid: water (20:5:75, v/v)	UV—254 nm	27
Acesulfame-K, aspartame, dulcin, saccharin	μBondapak C 18 10 μm, 300 × 3.9 mm	Methanol: 85% phosphoric acid, pH 6 with 34 mM tetra-ethylammonium hydroxide (2:8, v/v)	UV—210 nm	47
Acesulfame-K, aspartame, saccharin, neohesperidin-dihydrochalcone	Lichrospher 60 RP 5 μm, 125 × 4 mm	15–95% Methanol in 10 mM tetrabutylammonium hydrogen sulfate, flow 0.8 ml/min	UV—217 nm	66
Aspartame, cyclamate, dulcin, saccharin	Hypersil MOS RP8 3 μm, 100 × 4 mm	5 mM Tetrabutylammonium *p*-toluenesulfonate with 10 mM glycine, pH 3.5: methanol (88:12, v/v)	Indirect photometry 267 nm	24
Acesulfame-K, alitame, aspartame, dulcin, saccharin	μBondapak C 18 10 μm, 300 × 3.9 mm	12.5 mM KH_2PO_4 buffer pH 3.5: acetonitrile (9:1, v/v)	UV—220 nm	33
Acesulfame-K, alitame, aspartame and its degradation products, cyclamate, dulcin, saccharin, sucralose	Supelcosil LC-18 5 μm, 150 × 4.6 mm	20 mM KH_2PO_4: acetonitrile in a linear gradient from 97:3 (pH 5.0) to 80:20, v/v (pH 3.5) Flow rate 1.0 ml/min, postcolumn derivatization with methyl violet 2B	UV—200 or 210 nm sucralose: RI cyclamate: 585 nm	16

II. SYNTHETIC COLORANTS

A. Introduction

Color is the first sensory quality by which foods are chosen; color helps us judge its wholesomeness, relish its flavor, and appreciate its texture. Therefore, food quality and flavor are closely associated with color (128,129). Since ancient times, colorants have been added to food. Colorants, with very few exceptions, do not contribute nutritionally to foods. They are used: to help preserve the identity or character by which foods are recognized; to intensify the natural color of food; to correct for natural variations in color; to ensure uniformity of color from batch to batch due to natural variations in color intensity; to restore the original appearance of the food when natural colors have been destroyed by heat during processing and storage; to enhance colors naturally occurring in foods but in less intensity than the consumer would expect; to give an attractive appearance to certain, otherwise virtually colorless foods; and to help protect flavor and light-sensitive vitamins during self-storage via a sunscreen effect. But colorants have also been used for adulteration purposes—to disguise food of poor quality, to mask decay, to redye food, to mask effects of aging, or to simulate a higher biological value (129–132).

Colorants can be natural or synthetic. Natural colorants or pigments will be described in Chapter 20. Synthetic colorants are attractive to the food industry because they are superior to natural colorants in tinctorial strength, hue, and stability. Synthetic colorants provide a larger spectrum of colors. Their colors are strong; therefore, very small amounts are required. They are also lower in cost and are more readily available (129,130,132–134).

Synthetic organic colors can be classified according to their chemical structures into the following classes: azo, triarylmethane, quinoline, xanthene, and indigoid compounds (128,135, 136). The chemical structures of some synthetic food dyes are shown in Figs. 4 and 5. The azo dyes give rise to colors in the yellow, orange, red, and brown range; the triarylmethane compounds are characteristically bright green or blue. The chromophoric group of the xanthene compounds imparts a brilliant red shade to erythrosine. It also exhibits strong fluorescence. Resonance hybrids are important in the colors of both quinoline and indigoid compounds (135,136). The chemical names, classes and formulas, molecular weights, wavelength of maximum absorbance at neutral pH, and color identification according to the International Numbering System (INS), European Economic Community (E number), and Color Index Constitution Number of some synthetic colors are shown in Table 4.

The physical and chemical properties of some synthetic colors are summarized in Table 5. The examination of the structural formula (Fig. 4) shows that water solubility is conferred by the presence of at least one salt-forming moiety. The most common is the sulfonic group, $—SO_3H$, but the additional presence of a carboxylic acid group, $—CO_2H$, in the molecular may also increase water solubility (135). Sulfonation decreases fat solubility (128). The physical properties of each form confer advantages and disadvantages to the dye, and thus fill a specific application need (129,130). Azo and triarylmethane dyes are susceptible to discoloration or precipitation in the presence of reducing agents (monosaccharides, aldehydes, ketones, and ascorbic acid) or heavy metals, exposure to light, excessive heat, or exposure to acid or alkali (Table 5). Free metals (iron and copper) can combine chemically with many dyes, causing loss of color. Azo dyes are reduced to the colorless hydrazo form or sometimes to the primary amine. Triarylmethane dyes are reduced to the colorless leuco base (136).

The safety of food colorants has been a matter of concern for several years. Most synthetic colors have been extensively tested in conventional toxicity studies. However, divergent views have often been expressed on the significance of the same toxicity data (128,132,142). Most of the questions have been associated with the azo colors with respect to hypersensitive reactions as well as to the nature and toxicological significance of reactions between colors and food components during processing and storage. In such conditions, azo dyes undergo fading, giving rise to

Triarylmethane

Food green S

Brilliant blue FCF

Xanthene

Erythrosine

Quinoline

Quinoline yellow

Fig. 4 Chemical structures of triarylmethane (food green S and brilliant blue FCF), xanthene (erythrosine), and quinoline (quinoline yellow) dyes.

Indigoid

Indigotine

Azo

Amaranth

Allura red

Black PN

Tartrazine

Fig. 5 Chemical structures of indigoid (indigotine) and azo (amaranth, allura red, black PN, and tartrazine) dyes.

Table 4 Generic Names, Code Numbers, Chemical Classes, Names, and Formulas, Molecular Weight, and Maximum Absorbance Wavelength of Some Synthetic Food Colors

Colors	INS, EEC[a]	Color index	Chemical class[b]	Chemical name	Chemical formula	Molecular weight	λ max[c] (nm)
Carmoisine, azorubine	122	14720	A	Disodium-4-hydroxy-3-(4-sulfo-1-naphthylazo)-naphthalene-1-sulfonate	$C_{20}H_{12}N_2Na_2O_7S_2$	502.44	510
Fast red E or S, naphthol red		16045	A	Disodium-2-hydroxyl-1-(4′-sulfo-1-naphthylazo)-naphthalene-6-sulfonate	$C_{20}H_{12}N_2Na_2O_7S_2$	502.44	505
Ponceau 4R, new coccine, cochineal red A	124	16255	A	Trisodium-2-hydroxyl-1-(4-sulfo-1-naphthylazo)-naphthalene-6,8-disulfonate	$C_{20}H_{11}N_2Na_3O_{10}S_3$	604.48	489–510
Amaranth, bordeaux S, FD&C red 2	123	16185	A	Trisodium-2-hydroxyl-1-(4-sulfo-1-naphthylazo)-naphthalene-3,6-disulfonate	$C_{20}H_{11}N_2Na_3O_{10}S_3$	604.48	519–525
Erythrosine, FD&C red 3	127	45430	X	Disodium-2-(2,4,5,7-tetraiodo-3-oxido-6-oxoxanthen-9-yl)-benzoate	$C_{20}H_6I_4Na_2O_5$	879.87	520–525
Allura red AC, FD&C red 40	129	16035	A	Disodium-2-hydroxyl-1-(2-methoxy-5-methyl-4-sulfophenylazo)-naphthalene-6-sulfonate	$C_{18}H_{14}N_2Na_2O_8S_2$	496.42	505
Sunset yellow S or FCF, FD&C yellow 6	110	15985	A	Disodium-2-hydroxy-1-(4-sulfophenylazo)-naphthalene-6-sulfonate	$C_{16}H_{10}N_2Na_2O_7S_2$	452.37	480–482
Tartrazine, FD&C yellow 5	102	19140	A	Trisodium-5-hydroxy-1-(4-sulfophenyl)-4-(4-sulfophenylazo)-pyrazole-3-carboxylate	$C_{16}H_9N_4Na_3O_9S_2$	534.37	426–435
Quinoline yellow[d]	104	47005	Q	Disodium salts of the disulfonates of 2-(2-quinolyl)indan-1,3-dione	$C_{18}H_9NNa_2O_8S_2$	477.38	415
Indigotine, indigo carmine, FD&C blue 2	132	73015	I	Disodium-3,3′-dioxo-2,2′-bi-indolylidene-5,5′-disulfonate	$C_{16}H_8N_2Na_2O_8S_2$	466.36	610
Brilliant blue FCF FD&C blue 1	133	42090	TAM	Disodium-α-[4-(*N*-ethyl-3-sulfobenzylamino)phenyl]-α-[4-(*N*-ethyl-3-sulfobenzylimino)cyclohexa-2,5-dienylidene]toluene-2-sulfonate	$C_{37}H_{34}N_2Na_2O_9S_3$	792.84	630
Fast green FCF, FD&C green 3	143	42053	TAM	Disodium *N*-ethyl-*N*-[4[[4-[ethyl[3-sulfophenyl)methyl]-amino]phenyl](4-*OH*-2-sulfophenyl)methylene]-2,5-cyclo-hexadienylidene]3-sulfobenzenemethanaminium hydroxide	$C_{37}H_{34}N_2Na_2O_{10}S_3$	808.84	628
Chocolate brown HT	E 156	20285	A	Disodium-4,4′-(2,4-dihydroxy-5-hydroxymethyl-1,3-phenylene bisazo)di(naphthalene-1-sulfonate)	$C_{27}H_{18}N_4Na_2O_9S_2$	652.57	460
Brilliant black BN, black PN	E 151	28440	A	Tetrasodium-4-acetamido-5-hydroxy-6-[7-sulfo-4-(4-sulfo-phenylazo)-1-naphthylazo]naphthalene-1,7-disulfonate	$C_{28}H_{17}N_2Na_5O_{14}S_4$	867.69	

Source: Refs. 10–13.

[a] INS = International Numbering System or European Economic Community number.

[b] Chemical class: A = azo, I = indigoid, Q = quinoline, TAM = triarylmethane, X = xanthene.

[c] Maximum absorbance at neutral media.

[d] Principal components.

Table 5 Chemical and Physical Properties of Synthetic Food Colors

Color	Tinctorial strength	Solubility 25°C (g/100 ml)		Stability to:					Compatibility with food constituents
		Water	50% Ethanol	Light	Oxidation	pH 3/4	pH 8/9	Heat	
Carmoisine		8	Slightly soluble	Very good		Good	Fair	Good	
Ponceau 4R		30	Slightly soluble	Good		Good	Poor	Good	
Amaranth	Good	7.2	Slight	Very good		Good	Fair	Good	
Erythrosine	Very good	9	8	Poor	Fair	Poor	Poor	Very good	Poor
Allura red AC	Very good	22.5	1.3	Very good	Fair	Good	Good		Very good
Sunset yellow S	Good	19	10	Moderate	Fair	Good	Good	Very good	Moderate
Tartrazine	Good	20	12	Good	Fair	Good	Good	Very good	Moderate
Brilliant blue FCF	Excellent	20	20	Fair	Poor	Good	Poor		Good
Indigotine	Poor	1.6	0.5	Very poor	Poor	Poor	Poor		Very poor
Brilliant green BS		5	0.2	Fair		Good	Fair	Very good	
Fast green FCF	Excellent	20	20	Fair	Poor	Good	Good		Good
Chocolate brown HT		20	Insoluble	Very good		Good	Good	Good	
Brilliant black BN		5	Slightly soluble	Excellent		Fair	Good	Fair	

Source: Refs. 129, 136, and 141.

colorless and colored subsidiary products whose toxic properties may not be known (142,143). It is also known that some individuals show several allergic reactions, such as urticaria (hives), asthma, and rhinitis, after ingestion of tartrazine (144,145). Therefore, the use of tartrazine in a food product should be declared on the label (136).

Carcinogenic potential has been confirmed for ponceau 3R, butter yellow, methyl red, soudam R brown, soudam 7B red, orange SS, and crisoidine (142,143). Erythrosine has been regarded as a probable adventitious source of iodine, and toxicological studies confirmed its carcinogenic action on thyroid of male rats (142,143,146). Studies on allura red indicated that there is no significant and consistent adverse effect. However, there is concern about the presence of impurities, such as *p*-cresidine, which has been proved to be carcinogenic (147,148). The International Life Sciences Institute/Nutrition Foundation's Catalog of Food Colors (149) provides information on the regulatory status of colorants in use throughout the world and on safety assessment data, including international expert committee evaluations. From toxicological data and no-effect levels, acceptable daily intakes (ADIs) have been established for synthetic colors as shown in Table 6 (129,136,143).

Previously, several different colorants were used in foods. However, there has been a gradual erosion of synthetic colors from the permitted list of many countries (142). Today, the types of colorants permitted for use varies greatly among countries. In the United States, seven synthetic colors are permitted for general use in foods. The European Economic Community allows much more than that. Norway, however, prohibits the use of any synthetic dye in the manufacture of foods. Therefore, color additives can become trade barriers for foods. Since international trade is becoming increasingly important, there is need of a worldwide list of permitted color additives (128,136,150).

In order to prevent indiscriminate use, regulations have been developed by many countries limiting the types, uses, and amounts of food colors permitted in foods (142,151). Since different countries allow the use of specific food colors, it is possible that foodstuffs may be imported into a country that forbids the coloring agent present in the product. Therefore, methods capable of identifying and quantifying several colors simultaneously are desired in order to verify compliance to regulations. Information on the levels of these compounds in foods is also important to assess where the dietary intake levels stand compared to the ADIs (131,152,153).

Table 6 Acceptable Daily Intake of Some Synthetic Food Colors

Food color	Acceptable daily intake in mg/kg body weight (last evaluation)
Allura red	0–7 (1981)
Amaranth	0–0.5 (1984)
Erythrosine	0–0.1 (1990)
Ponceau 4R	0–4 (1983)
Tartrazine	0–7.5 (1964)
Sunset yellow	0–2.5 (1982)
Quinoline yellow	0–0.5 (1981)
Fast green	0–25 (1986)
Brilliant blue	0–12.5 (1969)
Indigotine	0–5 (1974)

Source: Refs. 128, 142, and 143.

Regulation also limits the levels of permitted impurities in color additives. Detailed standards of quality and purity have been incorporated into regulatory requirements, establishing maximum amounts of organic impurities such as subsidiary dyes and residues of starting materials, intermediates, or other contaminants. Each batch of color made must be tested for compliance with chemical specifications in order to be certified (130,142,151). Therefore, there is a need for rapid and reliable techniques to separate the dyes from impurities and to monitor the quality of commercial dyes (154).

Some dyes react with food constituents or with metal packaging to yield colorless, sometimes toxic degradation products. Therefore, methods for determining the nature and amount of dye present in a food are essential for continuous quality control and to monitor the behavior of dyes during the various stages of processing (131,155,156).

Most of the procedures for the determination of food colors comprise several stages. First, the dye has to be extracted from the food matrix. At this stage, the main problem is to extract all the dye unchanged. The extract has to be purified to remove interfering coextractives. And, since many colors are used in very low amounts, a concentration step is necessary before separation, identification, and quantification (157).

Several hundred dyes are known, and this diversity has always posed a problem of identity for the analysis, for many dyes have similar characteristics and some occur as a mixture (158). Among the different techniques available for the analysis of food colors, HPLC can replace many of the traditional techniques, providing rapid results that are much more specific for the determination of colorants (131,142). The technique has been shown to have great potential for synthetic food color analysis in terms of simultaneous separation, qualitative identification, and quantitation (159).

The purpose of this section is to provide a review of HPLC methods available for the determination of synthetic colors in foods, including sample preparation, separation techniques, and detection systems.

B. Sample Preparation

Sample preparation for color analysis will depend on the type of food sample. Liquid samples such as beverages can be filtered through a 0.45 μm pore membrane filter and injected directly in the HPLC. Carbonated beverages are degassed. Beverages containing suspended solids are filtered or centrifuged to eliminate suspended solids (143,156,160).

Water-soluble solid samples, such as dehydrated drinks, jellies, jams, and sweets, are dissolved in water and warmed to 40–50°C, if necessary. At this point, they can be treated as liquid beverages or concentrated prior to HPLC analysis (156,157).

Solid foods can be extracted by means of partition into organic solvents (133). The samples are macerated with aqueous acetone, methanol, or ethanol. The extract is made alkaline with sodium tetraborate, and the solids are separated by filtration or centrifugation after the addition of Celite 545 (135). Foods can also be ground with Celite and 0.1 N hydrochloric acid and washed with chloroform (156).

For some foods, incomplete extraction of color is obtained, probably due to the high binding affinity of dyes to the bulk of the food matrix, especially to proteins, lipids, and carbohydrates (156,161,162). This problem can be overcome by the use of selected solvents or enzymes to digest the food prior to extraction. Petroleum ether can be used to extract lipids (163). Acetone can be used to remove lipids and coagulate protein (164). Enzymes, such as amyloglucosidase (165,166), papain (167), lipase, pectinase, cellulase, and phospholipase, added to the sample and incubated under optimum pH and temperature conditions release synthetic colors bound to or associated with the food matrix. Furthermore, enzyme digestion can solubilize some foods, enabling analysis to be continued (156).

Protein-rich foods can also be specially treated. According to Saag (135), in order to extract colorants from fish, samples are boiled, filtered, washed, with an ammonia solution to displace proteins, and then washed through Sephadex LH-20 with water. The colored zones are collected for HPLC analysis. Dairy products (ice cream, cheese, yogurt) are first mixed with acetone or ethanol to precipitate the protein, which is ground up with sea sand and Celite, and the slurry is placed in a column from which dyes are eluted with a solution of ammoniacal methanol (135,162).

Special care must be taken when analyzing for indigotine, due to its unstability to heat, acids, and alkalis. The sample should be crushed to facilitate rapid dissolution and then dissolved in water on a water bath at 55–60°C under nitrogen atmosphere (156).

Synthetic dyes may be isolated, purified, or concentrated from foods or from extracts by wool-dyeing procedures; column chromatography with polyamide; ion-pair or solvent extraction; reverse-phase cartridges; or ion-exchange resins (157,159,168). These techniques are discussed next.

1. *Wool Dyeing*

The use of wool fibers is one of the earliest methods available for extraction and cleanup of food samples. It consists of adding a prepared white knitting wool to an acidic solution of the food, which is boiled until the color is removed. After washing the wool with cold water, the dyes are removed with a hot solution of dilute ammonia. Natural colors may also dye the wool, but the color is not usually removed by ammonia. Basic dyes can be extracted by alkalinization of the food with ammonia, boiling with wool, and then stripping with acetic acid. The wool-dyeing procedure has several disadvantages; e.g., a number of dyes are taken up slowly from the acidic solution, and the adsorbed dyes often undergo changes due to extreme pH and temperature conditions (157,164,169). Furthermore, it is not effective for samples with a large amount of protein, lipids, and carbohydrates because it interferes with the binding of the colors with wool (133,157,164,170). This technique has been widely used for qualitative purposes (157,171,172).

2. *Adsorption on Polyamide*

Unlike the wool-dyeing technique, polyamide adsorption retains all acid dyes without altering their chemical composition (164,173). According to Davídek and Davidková (174), separation is achieved because sulfonic acid groups form strong hydrogen bridges with the polyamide. The separation of the synthetic dyes is accomplished via the presence of ammonia in the solvent system. Separation of dyes may be selective as a result of the difference in the nature and number of functional groups forming the hydrogen bridges.

Food samples are dissolved in water and acidified with acetic acid (135,157). According to Gilhooley et al. (157), excess methanol has to be removed from solutions before passing through polyamide because it impairs the adsorption of the dyes by the polyamide. The solution is stirred with polyamide powder, and the slurry is transferred to a microcolumn or it is passed through the column of polyamide. The latter is recommended since dyes are adsorbed as a narrow band at the top of the column. The column is washed with hot water to remove sugars, acids, and flavoring materials and with acetone to remove basic dyes, water-soluble carotenoids, and some anthocyanins. The adsorbed acid dyes are eluted with methanol: sodium hydroxide (164,172,175), with methanol: ammonia (176), or with acetone: ammonia (157). Acetone: ammonia is preferred because it can be removed in a water bath and, on addition of acid, no salts are formed that interfere with the adsorption of the dyes by the polyamide (157). The eluate is evaporated to dryness and redissolved in the HPLC mobile phase (156).

When using polyamide for purification of bakery product extracts, the addition of small

amounts of surfactant (polyoxyethylene sorbitan mono-oleate) helps disperse coextractives and prevent them from clogging the column (157).

The use of polyamide is advantageous because it separates natural from synthetic coloring material, removes sugars, acids, and flavoring materials, and concentrates dilute solutions of colors (156,167). However, the use of polyamide is not applicable to chocolate brown FB, chocolate brown HT, or indigotine, because the two chocolate browns are not completely eluted from the column and indigotine decomposes during extraction (157).

3. *Adsorption on Cellulose*

Column chromatography using cellulose and elution with alcohol: water: salt solutions has also been employed for the purification of food colors (161,177).

4. *Ion-Pair Partition*

The use of ion-pair extraction has been proposed for the isolation of synthetic colors from foods. Puttemans et al. (178,179) investigated the influence of the type and concentration of the counter ion (tetrabutylammonium—TBA—phosphate or tri-*n*-octylamine—TnOA), the pH, and the ionic strength of the aqueous phase and the composition of the organic phase on the recovery of six dyes during isolation from foodstuffs. According to Puttemans et al. (180) it is important to extract the analytes back to an aqueous phase by means of a displacement reaction in order to elute the adsorbed TnOA, which can disturb the elution of the solutes by HPLC. The influence of the type of back extractant was also investigated, among them sodium salts of chloride, bromide, iodide, nitrate, and perchlorate.

Boley et al. (177) and Bonato et al. (181) extracted colors from confectionery foods and beverages with cetyltrimethylammonium bromide (cetrimide) using chloroform: *n*-butanol or methanol. Synthetic dyes are extracted from soft drinks and juices with 10 mM TnOA at pH 5.5 and back-extracted to an aqueous phase with 0.1 M sodium perchlorate (182). Masiala-Tsobo (183) extracted dyes from liquid samples with TBA and chloroform: *n*-propanol (5:2, v/v), pH 7.5. De Beer and Dierickx (184) extracted dyes from urine and feces with TBA and dichloromethane followed by back-extraction into sodium perchlorate. Oi et al. (185) extracted dyes from soft drinks with 0.5% octadecyl-trimethylammonium bromide at pH 5.6 followed by extraction of the ion pair with *n*-butanol. The applicability of ion-pair extraction has also been successfully demonstrated on the determination of synthetic dyes in pickles (178), rice milk (186), alcoholic beverages (179), gelatin-containing sweets, soft drinks, and lemonade syrup (182).

Tetrabutylammonium phosphate was used for the extraction of dyes from grape beverages. Sample containing TBA was passed through a Sep-Pak C18 cartridge. The synthetic colors were retained on the column while most natural colors were not. The cartridge is washed with water and the synthetic colors are eluted with methanol: water, 1:1 v/v (159).

Ion-pair extraction has been especially valuable in the preparation of samples for the analysis of indigotine, due to its sensitivity to heat, acids, and alkalis. The cartridge is conditioned with methanol: water (7.8:2.2, v/v) containing cetrimide; the sample is applied and washed with water; and the color is eluted with methanol. The short analysis time and the mild conditions employed ensure maximum recovery of indigotine (177).

5. *Anion-Exchange Resins*

Purification of food dyes can also be accomplished by means of anion-exchange resins. Theoretically, the anionic resin combines with sulfonic acid groups in the dye molecule to form a complex. Since food dyes may be adsorbed onto carbohydrates or chemically bound to proteins, the resin must have greater affinity for the color than to any of these adsorbents (156,161). Usually

a liquid anion-exchange resin (Amberlite LA-2) dissolved in an organic solvent (hexane or *n*-butanol) equilibrated at a specific pH value is used (135,156,169,170,187).

According to Patel et al. (140), Amberlite XAD-2 can also be used to separate water-soluble food colors. Food colors were retained only with neutral eluent (methanol). By changing the concentration of methanolic sulfuric acid, selective elution of colors is achieved.

6. *Solid-Phase Extraction*

A popular method for the rapid isolation, cleanup, and concentration of synthetic colors involves the use of solid-phase extraction. Among cartridges available, Sep-Pak C18 has been observed to exhibit a great capacity for dyes (144,188–191). The cartridge is prewashed with isopropanol (2 ml) and aqueous 1% acetic acid (5 ml) to improve retention of the sulfonic acid dyes on the C18 packing. The sample is acidified with 1% acetic acid, applied, and flushed through the cartridge. The eluent containing sugars and flavorings is discarded. A sequence of solvent mixtures containing water: propanol is used to retain or elute the various dyes selectively. According to McKone and Ivie (188), aqueous solutions containing 18% of isopropanol extracted FD&C blue 2, yellow 5, yellow 6, red 2, and red 40 and 50% isopropanol extracted red 3, blue 1, and green 3. Sep-Pak separation shortens the analysis time considerably as compared to standard methods (188,190).

Ashkenazi et al. (131) developed a self-contained dynamic solid-phase extraction system for the extraction and concentration of synthetic food colors. In this system, the flow of the mobile phase is achieved by forcing a densely packed chromatographic column through the eluent in a completely closed system, except for the column outlet. Under such conditions, an intrinsic pressure develops in the system that causes the mobile phase to flow through the moving chromatographic bed in a direction opposite to the movement of the packed column. The extraction device is of polypropylene, and the column is packed with LiChroprep RP18. Conditioning of the column is done with 0.01 M HCl followed by citrate buffer. The liquid sample is applied, the colors are retained, and the liquid discarded. The analyte retained in the column is washed and then eluted with a suitable eluent. Advantages of this technique include: smaller volumes of chemicals required for elution and total recoveries, and reduced losses through nonspecific adsorption on the column and through accidental spillage since it is a closed system. Furthermore, there is no need for special equipment, and extraction and concentration can be performed in shorter period of time.

C. Separation Techniques

HPLC has been considered a powerful technique for the analysis of synthetic food color, for the detection of impurities in single dyes and also for the separation of a mixture of dyes (153). Reversed-phase ion-pair chromatography has been found particularly useful for the separation and detection of a large number of food colors (159).

1. *Separation of Impurities in Synthetic Food Colors*

During the synthesis of food dyes, as with any chemical reaction, the product that is formed contains impurities, since the reactants themselves are usually impure and side reactions can also occur. The impurities can be of three types: Uncombined intermediates are compounds from which a color is directly synthesized and do not impart color to the product; subsidiary colors are colored compounds that are structurally related to the product; other impurities include substances that may arise from contamination of the intermediates or other reagents used during synthesis

(151,192–194). The nature and amounts of these impurities are regulated by specifications that are enforced by batch analysis during color additive certification (192,195).

Reverse-phase and ion-exchange HPLC have been used for the determination of intermediates and subsidiaries of synthetic dyes. However, reverse-phase ion-pair HPLC has been found particularly useful for the separation and detection of these compounds (195).

a. Erythrosine

Automated HPLC methods were developed for the determination of subsidiary dyes, intermediates, and side reaction products of erythrosine. Peeples and Heitz (154) described a reverse-phase HPLC method to monitor the purity of erythrosine and a series of xanthene dyes involving a μBondapak C18 column and mixtures of methanol and ammonium acetate buffer.

Calvey and Goldberg (196) separated erythrosine from fluorescein and seven lower-iodinated fluoresceins-subsidiary colors (2′-iodofluorescein, 4′-iodofluorescein, 2′,5′-diiodofluorescein, 2′,7′-diiodofluorescein, 4′,5′-diiodofluorescein, 2′,4′,7′-triiodofluorescein, and 2′,4′,5′-triiodofluorescein) on Zorbax C8 by increasing the organic nature (methanol) of a buffered mobile phase (0.1 M ammonium acetate). Goldberg and Calvey (197), using the same column but a gradient of 20–100% solvent B (acetonitrile: methanol, 2:8, v/v) in A (0.2 M ammonium chloride), separated erythrosine from two intermediates, phthalic acid and resorcinol, and two side reaction products, 2-(2′,4′-dihydroxybenzoyl)benzoic acid and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid.

Lancaster and Lawrence (151) determined intermediates, side reaction products, and subsidiaries of erythrosine on Spheri 10 RP18 using methanol: water (36:64 and 64:36, v/v) containing 0.02 M KH_2PH_4. However, better separation of the subsidiaries from erythrosine was obtained by incorporating the ion-pair TBA phosphate (5 mM) into the mobile phase.

b. Amaranth

Ion-pair HPLC (194,195) was used to separate amaranth from its subsidiary dye 1-(4-sulfo-1-naphthylazo)-2-naphthol-6-sulfonic acid disodium salt (fast red E) and from its intermediates 1-naphthylamine 4-sulfonic acid (naphthionic acid) and 2-naphthol-3,6-disulfonic acid disodium salt (R-salt). Ion-pair HPLC was also used for the determination of total free and bound nonsulfonated aromatic amines in amaranth after diazotization and coupling with R-salt (198).

Bailey et al. (199) and Singh (200) used ion-exchange HPLC for the separation of amaranth from the intermediates naphthionic acid and R-salt, as well as the side reaction products 2-naphthol-6-sulfonic acid sodium salt (Schaeffer's salt), 2-naphthol-6,8-disulfonic acid disodium salt (G-salt), and 2-naphthol-3,6,8-trisulfonic acid trisodium salt (NTSA).

c. Ponceau 4R

Yamada et al. (201) developed a method for the determination of starting materials (7-hydroxy-1,3,6-naphthalenesulfonic acid, G-salt, R-salt, Schaeffer's salt, and naphthionic salt) and subsidiary colors (Ponceau 6R, amaranth, and fast red) in Ponceau 4R. It consisted of an ODS column and gradient elution of acetonitrile in 0.02 M ammonium acetate.

d. Allura Red

Free and bound nonsulfonated aromatic amines were determined in allura red by ion-pair chromatography after reduction with dithionite, diazotization with sodium nitrite, and coupling with R-salt (202).

Singh (203) separated allura red from cresidine sulfonic acid, Schaeffer's salt, and 6,6′-oxybis-2-naphthalenesulfonic acid (DONS) by ion-exchange HPLC using a strong anion-exchange column and a gradient of 0–60% solvent B (0.01 M $Na_2B_4O_7$ + 0.5 M $NaClO_4$) in 0.01 M $Na_2B_4O_7$. Bailey and Cox (204) used a strong anion-exchange column and a gradient of 0–100% 0.25 M sodium perchlorate + 0.01 M sodium borate in 0.01 M sodium borate for the separation of allura red from 4,4′-diazoamino-bis-5-metoxy-2-methylbenzenesulfonic acid (DMMA), cresidine sulfonic acid, Schaeffer's salt, and DONS.

p-Cresidine, a contaminant of cresidine sulfonic acid, was determined in allura red by reverse-phase HPLC after extraction with chloroform, diazotization, and coupling with the disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid (197). The isomers *p*- and *m*-cresidine can also be separated by HPLC using methanol:0.01 M ammonium acetate, 4:6, v/v (205).

e. Sunset Yellow

Lower-sulfonated subsidiary colors of sunset yellow, among them 5-(phenylazo)-6-hydroxy-naphthalene-2-sulfonic acid (ANSC) and 4-[(2-hydroxynaphthalene-1-yl)azo]benzenesulfonic acid (BNSC), were determined by reverse-phase HPLC using Novapak C18 and gradient elution with a water–tetrahydrofuran solvent system buffered with ammonium acetate (192).

Richfield-Fratz et al. (206) determined aniline, benzidine, 4-aminophenyl, and 4-aminoazobenzene in sunset yellow. The determination involved chloroform extraction followed by diazotization and coupling with the disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid and analysis by reverse-phase HPLC. Using a similar system, Peiperl et al. (207) determined benzidine in sunset yellow. Dithionite was used to reduce any combined benzidine present.

Sunset yellow was separated from its subsidiary dye 1-*p*-sulfophenylazo-2-naphthol-3,6-disulfonic acid trisodium salt (195) and from its intermediates sulfanilic acid and Schaeffer's salt (194) by means of ion-pair HPLC. Ion-pair chromatography has also been used to determine free and bound nonsulfonated aromatic amines in sunset yellow after reduction with dithionite, diazotization with sodium nitrite, and coupling with R-salt (202).

A strong anion-exchange column and a gradient of 0–100% of B (10 mM aqueous $Na_2B_4O_7$ in 0.5 M $NaClO_4.H_2O$) in A (10 mM $Na_2B_4O_7$) was used by Marmion (208,209) for the separation of sunset yellow from 4,4′-(diazoamino)-dibenzenesulfonic acid (DAADBSA). This same system was used by Singh (210) for the separation of sunset yellow from the intermediates sulfanilic acid, 6-hydroxy-2-naphthalenesulfonic acid, and DONS. Useful information on the identification and confirmation of products related to sunset yellow are described by Bailey and Calvey (211).

f. Tartrazine

Tartrazine was separated by ion-pair HPLC from its subsidiary dye 3-carboxy-5-hydroxy-1-*p*-sulfopheny-4-phenylazo-pyrazole disodium salt (195) and from its intermediates sulfanilic acid and 1-(4-sulfophenyl)-3-carboxy-5-hydroxypyrazolone (pyrazolone-T) by means of TBA hydroxide (194,212). Ion-pair chromatography was also used for the determination of free and bound nonsulfonated aromatic amines in tartrazine after reduction with dithionite, diazotization with sodium nitrite, and coupling with R-salt (202).

Prival et al. (213) determined combined benzidine in tartrazine. After reduction of combined benzidine with dithionite, benzidine was extracted, diazotized, coupled with pyrazolone-T, and analyzed by HPLC.

Tartrazine was separated from the intermediates sulfanilic acid, pyrazolone-T, and phenylhydrazine-*p*-sulfonic acid (PHSA) and from the side reaction products DAADBSA and 1-(4-sulfophenyl)-3-ethyl carboxy-5-hydroxypyrazolone (EEPT) by ion-exchange HPLC (199,214).

Using a Zipax SAX column, these compounds were eluted in a reproducible pattern by increasing the ionic strength of a mobile phase buffered with 0.1 M $Na_2B_4O_7$ (214).

g. Indigotine

Indigotine was separated from isatin-5-sulfonic acid (decomposition product), 5,7′-disulfoindigo (isomeric subsidiary color) and 5-sulfoindigo (lower sulfonated subsidiary color) by reverse-phase HPLC on Altex Ultraspher ODS using gradient elution (215).

2. *Separation of Synthetic Food Colors Mixtures in Foods*

Reverse-phase and ion-pair chromatography have been widely used for the separation and quantification of synthetic colorants. Because of the large number of dyes that may be present in food, it is preferable to use at least two quite different HPLC systems to ensure the identification of such compounds (168).

a. Reverse-Phase Chromatography

Synthetic dyes, because of their sulfonic and in some cases carboxylic acid functions, have short retention times in a reverse-phase HPLC system (168). Another problem encountered during reverse-phase HPLC is the tailing observed for compounds with sulfonic groups (216). Nevertheless, adequate pH and solvent composition have permitted the separation of some dyes in a reverse-phase system, as indicated on Table 7.

Based on the fact that aromatic sulfonic and carboxylic acids were successfully separated by reversed-phase chromatography in the presence of organic electrolytes, Chaytor and Heal (158) developed a method for the separation of 15 synthetic colors using a mobile phase containing *o*-phosphoric acid (Table 7). The presence of the electrolyte provided lower variation in response and retention over a period of time. Furthermore, eluted peaks were sharper than those seen in ion-pair chromatography.

b. Ion-Pair Chromatography

Because of their ionic nature, most synthetic colors require ion-pair reagents. When a hydrophobic ion of opposite charge (counter ion) is added, hydrophobic ion pairs are formed, and hence the retention of the ionized substance on a nonpolar reverse phase is enhanced to achieve higher selectivity and peak symmetry (135,168,188).

Retention of solutes and selectivity can be controlled by adjusting the type and concentration of the ion-pair reagent added and by selection of the type and concentration of the organic solvent in the mobile phase (135,168). The ion pair reagents most commonly used are tetra-alkylammonium salts such as cetrimide (155,156,177) and tetra-*n*-butylammonium (TBA). The TBA reagent can be used as TBA phosphate (159,184), TBA chloride (221), TBA hydrogen sulfate (188,189), or TBA hydroxide (168,175,183).

The application of ion-pair HPLC in the analysis of food colors is summarized on Table 8. As indicated, TBA has been the most widely used ion pair. It can be observed that using gradient mobile phase elution, a larger number of synthetic dyes can be separated. However, the mobile phase programming should include a return to the initial condition as well as reequilibration of the column by maintaining the initial composition for a period of time. This procedure provides reproducible result (222).

Table 7 Reverse-Phase HPLC Methods for the Simultaneous Determination of Synthetic Colors

Colors	Column	Mobile phase	Detection	Ref.
Citrus red no. 2	Ultrasphere ODS C18 5 μm, 250 × 4.6 mm	Acetonitrile : water (9 : 1, v/v)	504 nm	217
Patent blue	Supelcosil 5 μm, 150 × 4.6 mm	Gradient of methanol in phosphate buffer, pH 8	590 nm	163
Sunset yellow, ponceau	Hypersil 5 μm	Methanol: pH 6.7 phosphate buffer (1 : 3, v/v)	500 nm	220
Tartrazine, sunset yellow, ponceau 4R, carmoisine, erioglaucine	Val-U-Pak-5 C18 250 × 4.6 mm	Methanol : water (8 : 2, v/v)	254, 390, 430, 430, 590 nm, respectively	131
Tartrazine, sunset yellow, amaranth, allura red, ponceau 4R, erythrosine, brilliant blue, indigotine	Spherisorb C 18 5 μm, 150 × 4.6 mm	Water : methanol (7 : 3, v/v) + 0.1 M ammonium acetate, flow rate 0.7 ml/min	blue—595, red—525, yellow—450 nm	133
Amaranth, ponceau 4R, ponceau 6R, fast red E	ODS 250 × 4.6 mm	0–30% Acetonitrile in 0.02 M ammonium acetate	510 nm	201
Amaranth, ponceau 4R, allura red, tartrazine, sunset yellow	ODS 250 × 4.6 mm	0–40% Acetonitrile in 0.02 M ammonium acetate	510 nm	193
Tartrazine, amaranth, indigotine, ponceau 4R, sunset yellow, allura red, brilliant blue, orange I, eosine, acid red, auramine, orange II, erythrosine, ploxine, rhodamine, rose bengal	Zorbax ODS C8 250 × 4.6 mm	0–100% B (acetonitrile : methanol, 2 : 8, v/v) in A (1% ammonium acetate, pH 8)	UV—254 nm	170
Tartrazine, amaranth, sunset yellow, ponceau 4R	LiChrosorb RP-18 10 μm, 250 × 4 mm	30–60% Methanol in phosphate buffer pH 4.5	UV—254 nm	182
Xanthene dyes (erythrosine, eosin S-13, phloxin B, Eosin S-10, dinitrofluorescein, fluorescein, diiodofluorescein	Ultrasphere Altex C18 5 μm, 150 × 4.6 mm	30–100% Acetonitrile in acetic acid solution pH 3, 25°C, flow rate 2.5 ml/min	475, 525 nm	218
Brilliant blue, indigotine, amaranth, erythrosine, ponceau SX, allura red, tartrazine, sunset yellow, fast green	HP RP-8 10 μm, 250 × 4.6 mm	10–90% Methanol in 10 mM KH_2PO_4, flow rate 2.0 ml/min	UV—290 nm	219
Tartrazine, quinoline yellow, sunset yellow, yellow 2G, brown HT, amaranth, ponceau 4R, red 2G, carmoisine, erythrosine, indigotine, black PN, green S, brilliant blue, patent blue	Ultrasphere ODS 3 μm, 75 × 4.6 mm	0–100% B in 20 min, 100% B for 10 min A: 0.1 M Na sulphate, pH 2.5 with *o*-phosphoric acid B: solvent A : water : methanol (1.5 : 7 : 22, v/v) Flow rate 1.5 ml/min	All dyes—254 nm; yellow—430 nm; red—520 nm; blue/green—640 nm	158

Table 8 Ion-Pair HPLC Methods for the Simultaneous Determination of Synthetic Food Colors

Colors	Column	Mobile phase[a]	Detection	Ref.
Sunset yellow, orange GGN, ponceau 4R	Nucleosil 10 C18, LiChrosorb RP-8	Acetone: water (8:2, v/v) with 0.2 g/L TBA chloride	505 nm	221
Indigotine, tartrazine, amaranth, sunset yellow FCF, allura red AC, fast green FCF, brilliant blue, erythrosine, ponceau SX, fast red E, skyark, benzyl violet 4B	LiChrosorb RP-18 10 μm, 250 × 4.6 mm	Methanol: water (45:55, 6:4 or 7:3, v/v) with 5 mM TBA phosphate; flow rate 1–1.5 ml/min	red orange yellow—450 nm, blue green—610 nm	159
Tartrazine, sunset yellow, amaranth, allura red, indigotine	μBondapak C18 10 μm, 300 × 3.9 mm	2-Propanol: 5 mM TBA hydrogen sulfate, pH 6.5 (1:6, v/v); flow rate 1.0 ml/min	UV—254 nm	188
Tartrazine, amaranth, sunset yellow, orange GGN, allura red, ponceau 4R	μBondapak C18 10 μm, 300 × 3.9 mm	Methanol: phosphate buffer, pH 6.0 + 1 mM TBA hydroxide (41:59, v/v)	UV—280 nm	175
Sunset yellow, carmoisine, amaranth, ponceau 4R, erythrosine	NovaPak C18 4 μm, 150 × 3.9 mm	Methanol: NaH_2PO_4/Na_2HPO_4, pH 7 with 0.05 M TBA	520 nm	224
Tartrazine, sunset yellow, quinoline yellow, orange GGN, amaranth, carmoisine, allura red, ponceau 4R, erythrosine, patent blue, indigotine, brilliant green, black PN	RSILC18 10 μm, 250 × 3 mm	0–100% B (methanol: 5 mM TBA, 6:4, v/v) in A (methanol: 5 mM TBA, 4:6, v/v); flow rate 1 ml/min	UV—254 and 280 nm	168
126 colorants	Microspher C18 3 μm, 100 × 4.6 mm	25–100% B (0.5 M TBA hydroxide, pH 7.0: methanol, 7.7:3.3, v/v) in A (water), 30°C, flow rate 2 ml/min	Diode array (200–600 nm)	222
Amaranth, indigotine, tartrazine, sunset yellow, allura red, ponceau 4R, erythrosine, brilliant green, brilliant blue	Altex C18 5 μm, 250 × 4.6 mm	45–100% B (TBA hydrogen phosphate solution: methanol, 2:8, v/v) in TBA hydrogen phosphate solution; flow rate 1 ml/min	Diode array, UV—280 nm	223
Tartrazine, amaranth, ponceau 4R, sunset yellow, scarlet red, erythrosine	Hypersil BDS 3 μm, 125 × 3 mm	15–90% Acetonitrile in 10 mM NaH_2PO_4 + 1 mM TBA dihydrogenphosphate, pH 4.2	350, 465, 600, and 750 nm	216
23 dyes	SAS Hypersil RP-8 5 μm, 120 × 4.6 mm	Methanol: water: cetrimide (77:23:0.25, v/v/w); flow rate 1.0 ml/min	red—520, yellow—430, orange/brown—580, green/blue—640, black—600 nm	156
14 red, 9 orange and yellow, 5 green and blue, and 5 brown and black	SAS-Hypersil 5 μm, 120 × 4.6 mm	Isopropanol: water: cetrimide: glacial acetic acid (41:59:0.25:0.25, v/v/w/v); flow rate 1.0 ml/min	red—520, yellow—430, orange/brown—480, green/blue—640, black—600 nm	155

[a] TBA = tetra-*n*-butylammonium; cetrimide = cetyltrimethylammonium bromide.

3. Separation of Synthetic Food Colors from Metabolites

Ion-pair HPLC was used for the separation of dyes and their metabolites from rat feces using Hibar RP18 and a mobile-phase gradient of 15–100% methanol in water containing 5 mM tetrabutylammonium phophate. In this system, sunset yellow FCF was separated from sulfanilic acid, *N*-acetylsulphanilic acid, 1-amino-2-naphthol-6-sulfonic acid (ANSA), and *N*-acetyl-ANSA. And orange GGN was separated from metanilic acid, *N*-acetylmetanilic acid, ANSA, and *N*-acetyl-ANSA (184).

D. Detection Systems

During analysis of colorants by HPLC, the column eluate can be monitored by different detection systems. The most widely used is the UV-visible detector at different wavelengths in the visible range, depending on the investigated color. In general, 430 nm is used for yellow dyes, 520 nm for red dyes, 640 nm for blue/green dyes, 600 nm for black dyes, and 480 nm for orange/brown dyes (155,156,158). According to Wegener et al. (222), when analyzing a large number of colorants, four detection wavelengths should be selected to achieve selectivity and near-to-optimum sensitivity: 400 nm for the yellow colorants, 475 nm for the orange-red, 525 nm for the purple-red, and 600 nm for the blue and green colorants. Ultraviolet detection at 254 or 280 nm can also be used (189), especially when several colors are under investigation (158).

Confirmation of the identity of synthetic colors can be obtained by subjecting the purified extracts to UV-VIS spectrophotometric examination. As an aid to identification, Wadds (225) and Young (190) have reproduced the UV-VIS absorption spectra in neutral, acidic, and alkaline media of 16 commonly used synthetic colors.

The replacement of a conventional variable-wavelength detector by a rapid-scanning diode array detector has been very beneficial, because it allows the entire UV-VIS absorption spectra to be recorded rapidly and stored digitally in a microprocessor, from where they can be recalled or handled in several ways. Major uses include: (a) peak identification by having UV-VIS spectra of each HPLC peak; (b) determination of peak purity by absorbance rationing at two wavelengths; and (c) multiple chromatographic recording at several wavelengths, which can be displayed after the run to maximize sensitivity or minimize interference (135,222,223). Diode array equipment based on deuterium and tungsten lamps ensures the highest light output at 190–950 nm, which results in the lowest detection limits over the entire wavelength range (89). However, the limitations on the photodiode array detector include the high cost and the possibility of sample photodegradation while in the cell (135,222,223).

Eletrochemical detection has been used for the detection of synthetic dyes. Fogg et al. (226) described a method for the qualitative and quantitative determination of several synthetic dyes using polarographic detection. The system was a stationary mercury drop electrode operated in the differential-pulse mode. Ashkenazi et al. (131) used fast-scan square-wave voltammetry for the polarographic detection of five synthetic dyes. The voltametric mode was observed to be much faster than the differential-pulse method. Another advantage is that the experimental measurement produces, in addition to the peak current, the redox potential of the dye, which can serve to identify the analyte further.

The coupling of HPLC to a mass spectrometer (MS) has provided for the ultimate in detection systems in terms of sensitivity and versatility. Betourski and Ballard (227) have used the techniques of thermospray TSP-LC-MS and tandem LC-MS-MS to obtain positive-ion mass spectra of two cationic dyes, Basic red 14 and Basic orange 14. According to Yinon et al. (228), TSP-LC-MS has been found to be a suitable technique for the analysis of dyes. It is sensitive and specific, and the ionization process is soft. One of the drawbacks, however, is that one obtains

mainly molecular adduct ions, and this information may not be sufficient for the structural elucidation of dyes of unknown structure.

Yinon et al. (228) used an HPLC interfaced with a triple-quadrupole mass spectrometer by means of a particle beam for the identification of several azo dyes. Characterization of the dyes was achieved by observing typical fragment ions formed by cleavage of the N–C and C–N bond on either side of the azo linkage and/or cleavage of the N═N double bond with the transfer of two hydrogen atoms to form an amine. Sensitivity was observed to be two to three orders of magnitude worse than with thermospray ionization.

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14
Preservatives and Antioxidants

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I. PRESERVATIVES

A. Theory

Preservatives are chemical compounds that prevent or inhibit the activity and growth of microorganisms after adding to foods. The main aim is to prolong the shelf life of foods by protecting them against deterioration caused by microorganisms. Various kinds of microorganisms bring about food spoilage, and these microorganisms are also able to vegetate under different conditions, which can be measured by factors such as pH, temperature, water activity, relative humidity, and presence or absence of special growth factors (1,2). Physicochemical properties of selected preservatives are listed in Table 1; properties of commonly permitted antimicrobial preservatives are given in Table 2 (3,4).

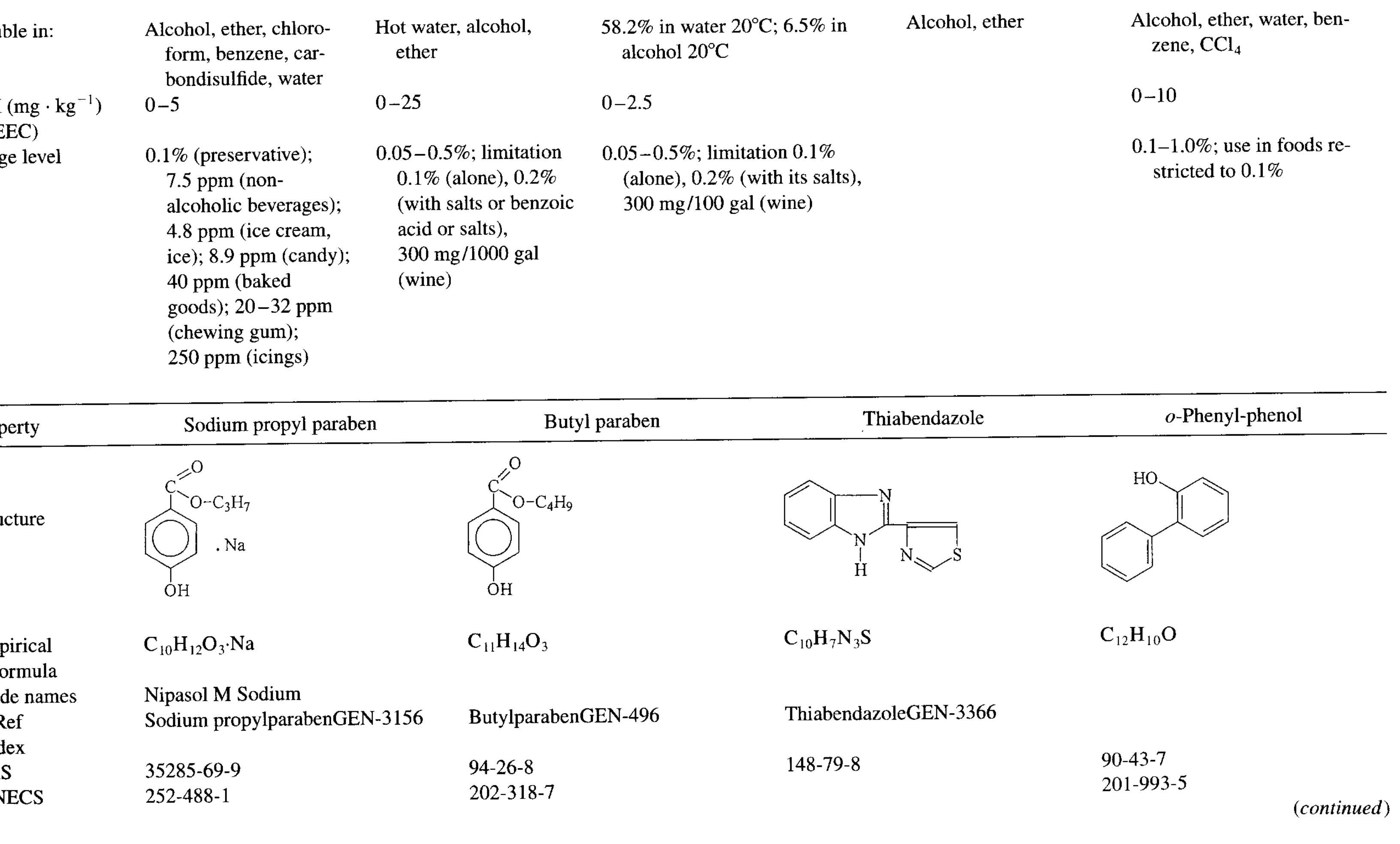
Sulfites and *sulfiting* agents are terms generally applied to a variety of sulfur-based compounds. Sulfites have been added to foods as preservative agents for centuries. The use of sulfating agents, or S(IV) oxoanion compounds, has been traced to antiquity, when SO_2 (from the fumes of burning sulfur) was used by the Egyptians and the Romans to cleanse and disinfect wine vessels (6). When added to a food matrix, some of the sulphating agent binds to the molecules of the food (aldehydes, ketones, sugars, anthocyanins and other pigments, tannins, etc.), and some of it does not. The portion of the sulphating agent that does not bind with the food is called *free sulfite*.

Free sulfite is a mixture of SO_2, bisulfite ion, and sulfite ion in dynamic chemical equilibrium. The percentage of each of the three chemical species in the matrix depends upon the pH (acidity) of the food (7). Two forms of bound sulfite have been observed: reversibly bound and irreversibly bound. Under certain conditions some of the bound sulfite molecules will dissociate (or break apart) and form free sulfite. The portion that dissociates is called reversibly bound sulfite. The portion that does not dissociate is referred to as irreversibly bound sulfite (8). Although sulfites had a GRAS (generally recognized as safe) status for a long time, the use of sulfites in foods has become an issue of concern recently to regulatory agencies, because sulfites have been implicated as initiators of asthmatic reactions. An asthmatic attack can be serious, and the ingestion of foods containing sulfite has been alleged to have caused several deaths in recent years (8). For this reason, the practice of adding sulfites to foods that are to be sold or served raw to the public was banned (9) by revocation of the GRAS status for use on fruits and vegetables

Table 1 Physicochemical Properties of Preservatives

Property	Benzoic acid	Sorbic acid	Potassium sorbate	Ethyl paraben	Methyl paraben
Structure	COOH (benzene ring)	$CH_3-(CH=CH)_2-COOH$	$CH_3-(CH=CH)_2-COOK$	C(=O)–O–C_2H_5 (benzene ring), OH; $C_9H_{10}O_3$	C(=O)–O–CH_3 (benzene ring), OH
Empirical formula	$C_7H_6O_2$	$C_6H_8O_2$	$C_6H_8O_2 \cdot K$	$C_9H_{10}O_3$	$C_8H_8O_3$
Trade names	Benzoic acid	Sorbic acid	Potassium sorbate	Nipagin A	Methylparaben
X-Ref	GEN-334	GEN-3181	GEN-2818	EthylparabenGEN-1267	GEN-2236
Codex					
CAS	65-85-0	110-44-1; 22500-92-1	590-00-1; 24634-61-5	120-47-8	99-76-3
EINECS	200-618-2	203-768-7	246-376-1	204-399-4	202-785-7
FEMA	2131				2710
ECC	E 210	E 200	E 202	E 214	E 218
Synonyms	Benzenecarboxylic acid; phenylformic acid; dracylic acid	2,4-Hexadienoic acid; hexadienic acid; (2-butenylidene) acetic acid; 2-propenylacrylic acid	2,4-Hexadienoic acid potassium salt; sorbic acid potassium salt; potassium 2,4-hexadienonate	Ethyl 4-hydroxybenzoate; 4-hydroxybenzoic acid ehyl ester; ethyl *p*-hydroxy benzoate; carboxyphenol	Methyl 4-hydroxybenzoate; 4-hydroxy benzoic acid, methyl ester; methyl *p*-hydroxybenzoate
State	Solid	Solid	Solid	Solid	Solid
Color	White	White	White	Ivory to white	Colorless, white
Molecular weight ($g \cdot mol^{-1}$)	122.13	112.14	150.23	166.18	152.14
Flash point	121.1°C; 260°F				
Boiling point (°C)	249.2	228		297–298	270–280
Melting point (°C)	121.25	134.5	270	114–118	125–128
LD_{50} in rate, oral ($mg \cdot kg^{-1}$)	2530	7360	4920		3000
Uses	Antimicrobial agent, flavoring agent, preservative	Preservative, acidulant flavoring antimycotic when migrating from food package	Mold retardant, preservative in baked goods, beverages, cakes, cheese, fish, fruit juice, margarine, pickled goods, salad dressings, fresh salad, wine; migrating to foods from paper/ paperboard	Antimicrobial; preservative (0.012–1 g/kg as *p*-hydroxybenzoic acid)	Antimicrobial agent, preservative, flavoring agent, for baked goods, beverages, food colors, milk, wine, antimycotic migrating from food package

Soluble in:	Alcohol, ether, chloroform, benzene, carbondisulfide, water	Hot water, alcohol, ether	58.2% in water 20°C; 6.5% in alcohol 20°C	Alcohol, ether	Alcohol, ether, water, benzene, CCl_4
ADI ($mg \cdot kg^{-1}$) (EEC)	0–5	0–25	0–2.5		0–10
Usage level	0.1% (preservative); 7.5 ppm (nonalcoholic beverages); 4.8 ppm (ice cream, ice); 8.9 ppm (candy); 40 ppm (baked goods); 20–32 ppm (chewing gum); 250 ppm (icings)	0.05–0.5%; limitation 0.1% (alone), 0.2% (with salts or benzoic acid or salts), 300 mg/1000 gal (wine)	0.05–0.5%; limitation 0.1% (alone), 0.2% (with its salts), 300 mg/100 gal (wine)		0.1–1.0%; use in foods restricted to 0.1%

Property	Sodium propyl paraben	Butyl paraben	Thiabendazole	*o*-Phenyl-phenol
Structure				
Empirical formula	$C_{10}H_{12}O_3{\cdot}Na$	$C_{11}H_{14}O_3$	$C_{10}H_7N_3S$	$C_{12}H_{10}O$
Trade names	Nipasol M Sodium			
X-Ref	Sodium propylparabenGEN-3156	ButylparabenGEN-496	ThiabendazoleGEN-3366	
Codex				
CAS	35285-69-9	94-26-8	148-79-8	90-43-7
EINECS	252-488-1	202-318-7		201-993-5

(continued)

Table 1 Continued

Property	Sodium propyl paraben	Butyl paraben	Thiabendazole	*o*-Phenyl-phenol
FEMA		2203		
ECC			E 233	E 231
Synonyms	4-Hydroxybenzoic acid, propyl ester, sodium salt; propyl-4-hydroxybenzoate, sodium salt; propyl-paraben, sodium salts	Butyl *p*-hydroxybenzoate; 4-hydroxybenzoic acid butyl ester; *n*-butyl *p*-hydroxybenzoate	Thiaben; 2-(thiazol-4-yl) benzimidazole; 2-(4-thiazolyl)benzimidazole	(1,1′-Biphenyl)-2-ol; 2-hydroxybiphenyl; 2-phenylphenol; *o*-Xenol
State	Solid	Solid	Solid	Solid
Color			Colorless	Nearly white
Molecular weight ($g \cdot mol^{-1}$)	202.19	194.22	201.25	170.22
Flash point				
Boiling point (°C)				280–284
Melting point (°C)		68–69	300 (subl.)	56–58
LD_{50} in rate, oral ($mg \cdot kg^{-1}$)			3600 (mice), 3100 (rats), >3800 (rabbits)	2480
Soluble in:		Very slightly in water, glycerin; freely soluble in acetone, alcohols, ether, chloroform, propylene glykol	Alcohol, esters, chlorinated hydrocarbons	Alcohol, sodium hydroxide solution, most organic solvents; insoluble in water
Usage level		0.001–0.2%; 0.05–0.1% in mixtures		

Table 2 Properties of Commonly Permitted Antimicrobial Preservatives

Preservative	Solubility at 25°C (g/L)	Solubility of salts at 25° (g/L)	Yield of active form (%)	pH range (pK_a)[a]
Sorbic acid C_5H_7COOH	1.6 (water)	Sodium 320 (water)	83.6	(4.8)
	0.7 (10% saline)	Potassium 1380 (water)	74.6	
	130 (ethanol)	Potassium 540 (10% saline)		
	5–10 (edible oil)	Calcium 12.0 (water)	74.2	
Benzoic acid C_6H_5COOH	21.0 (water)	Sodium 660 (water)	84.7	(4.2)
		Calcium 40 (water)	75.8	
Parabens ($(C_6H_4(OH)CO_2R)$)	2.5 (water)	Na readily	87.4	(8.5)
Where R = CH_3 (methyl)	520.0 (ethanol)	Water-soluble		
R = C_2H_5 (ethyl)	1.7 (water), 700.0 (ethanol)	Na readily water soluble	88.3	
R = C_3H_7 (propyl)	0.5 (water), 950.0 (ethanol)	Na readily water soluble	89.1	
R = C_7H_{15} (hepthyl)	0.015 (water)	Na readily water soluble	91.5	
Sulphur dioxide SO_2	110 (water)	K_2SO_3, 250	33.0	$H_2SO_3 \rightarrow HSO^- + H^+$ (1.76)
(sulphide)		$Na_2S_2O_5$, 540	67.4	$HSO^- \rightarrow SO_3^{2-} + H^+$ (7.21)
		$K_2S_2O_5$, 250	57.6	
		Na_2SO_3, 280	50.8	
		$KHSO_3$, 1000	53.5	
		$NaHSO_3$, 3000	61.5	
2(thiazol-4-yl)-benzimidazole $C_{10}H_7N_3S$	Insoluble in water, soluble in most organics	N/A	100	broad

[a] At pH above the pK_a, less than 50% of the active undissociated form is present.

intended to be served or sold raw to the consumer, except for potatoes and grapes. Also, the U.S. Food and Drug Administration (FDA) issued a rule on July 9, 1986, that required a sulfite declaration on the label of any food containing detectable (>10 ppm) amounts of sulfite (10) as well as regulations relevant to the use of SO_2 as a fumigant for table grapes (11).

Thiabendazole [2-(4-thiazolyl)benzimidazole] (TBZ), biphenyl (BP), and *o*-phenyl phenol (OPP) are postharvest fungicides used to protect citrus fruits, bananas, and vegetables against microbial spoilage. Most European nations (Austria, Belgium, France, Great Britain, Italy, The Netherlands, Switzerland) and North American countries (Canada, the United States) authorize the utilization of these stable products at a concentration of 6–10 mg/kg for citrus fruits and 3 mg/kg for bananas. Tribendazole has been in use since 1968. It is used primarily for postharvest treatment of fruits and vegetables to protect them from *Fusarium roseum*, *Collelotrichum musae*, *Verticullium theobromae*, *Thielaviopsis paradoxa*, *Deightoniella torulosa*, and *Nigrospora* spp. (12). Because of TBZ's frequent use and the quantities applied, and because of concerns about pesticides in the diets of infants and children, TBZ must be determined in all types

of foods (12). The substance used in the largest quantities for this purpose is also benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate (MBBC)]. Benzimidazole fungicides absorb strongly in the UV region of 280–310 nm, possess some native fluorescence, and are used mostly in combination. Either these preservatives are applied directly onto fruit surfaces or packaging materials are impregnated with them (1,5,13). Tribendazole is a postharvest fungicide used mostly to protect citrus fruits and bananas. In Japan, it has recently been registered as a food additive. The established tolerance for thiabendazole is 0.4 μg/g for the edible part of a banana, 3 μg/g for the whole banana, and 10 μg/g for citrus fruit (5). The most commonly used preservatives in the food processing industry are benzoic, sorbic, and propionic acids, along with sulfites and the methyl, ethyl, and propyl esters of 4-hydroxybenzoic acid (parabens). The most widely used preservatives in fruit juices and soft drinks, apart from sulfur dioxide, are sorbic and benzoic acids, either individually or in mixtures (1,2). The maximum permitted concentrations of benzoic and sorbic acids are controlled by legislation, so their determination is important to any analyst involved in the routine analysis of foods.

All chromatographic techniques have been applied to the determination of foods over the last few decades, but to date HPLC has emerged as one of the most powerful methods for the instrumental analysis of food additives. This technique allows fast quantitative determination of a wide range of constituents and, usually, the elimination of derivatization steps. Classical chemistry generally enables the determination and identification of one specific substance. In comparison, HPLC is well suited for the quantitative determination of complete groups of closely related compounds. Therefore, HPLC has largely replaced the older forms of chromatography as well as other analytical methods. For example, HPLC is superior to gas chromatography in separation and recovery for the detection of substances that are not volatilized. It is also preferred for molecules with high polarity, a number of ionic groups, or thermal instability. Thus HPLC has become more and more the cornerstone of foodstuff analysis (5).

Additives constitute large groups of different chemical substances with a tremendous diversity of properties and several modes of interaction between functional groups and solvent systems. Therefore, it is impossible to give general rules universally applicable to the chromatography of additives. High-performance LC is a powerful technique, but it has its limitations. No one method or set of operating conditions will apply to all situations; however, for each analytical problem a method may be found. These conditions should be as simple as possible, including room-temperature operations and UV absorbance (5).

One of the major advantages of HPLC in food additives analysis is the comparably simple possibility of automation. When there are many samples to analyze and quantify in routine analysis, an autoinjector usually is the additional component of choice. An autosampler may help to reduce costs, and the instrument may be left to run analyses overnight. Some autoinjectors can also be used to derivatize samples by adding the relevant reagent before injection into the chromatographic system (5).

This part of the chapter describes HPLC techniques for the separation and determination of preservatives, as follows: SO_2, postharvest preservatives (PPs), benzoic acid (BA), sorbic acid (SA), and the ethyl, methyl, propyl esters of 4-hydroxybenzoic acid (EsHBA) and 5-nitrofurylacrylic acid (5-NFA). The propionic, lactic, acetic acids are discussed in the chapter on organic acids.

B. Sample Preparation

1. Sulfites

A lot of analytical procedures have been followed to quantify the sulfiting agents, expressed as SO_2 in foods. With regard to the presence of many interference compounds in foods, indirect pro-

cedures have been used mainly for SO_2 determination, whereas the most used step for SO_2 separation is its separation from an acidified sample. While free SO_2 can be determined under acid conditions by aspirating a sample by air flow (15), heating to the boiling point brings about the release of both free and bound SO_2. Addition of base (e.g., NaOH) can also be used as the first step for the release of bound SO_2 (16–18).

Because sulfites are usually labile and can be lost during isolation and separation, their content may be stabilized by reaction with formaldehyde to form a stable derivate, hydroxymethylsulfonate (HMS) (19). Similarly, a highly alkaline medium is more suitable for the formation of the doubly charged sulfite anion, which is more stable than bisulfite, the dominant sulfite species found at the pH levels of normal foods (8).

2. *Postharvest Preservatives*

The samples (grapes) were extracted with petroleum ether and evaporated to dryness, and the residue was dissolved in acetonitrile and injected directly into the chromatographic system (20). Fruit nectars and purees, strawberry jams, and citrus juices were homogenized with 0.02 N HCl/methanol mixture for at least 5 min, centrifuged, and filtered under vacuum. The extract was diluted and filtered. An aliquot of this solution was brought to pH 7.5 with diluted NaOH and loaded onto an Extrelut 20 cartridge column. Elution was performed with dichloromethane and the eluate evaporated to dryness, and the residue was finally dissolved in mobile phase (21). Juice, fresh fruits, and potatoes were placed into a centrifuged tube, and extraction solvent (ethanol with 2 M ammonium chloride, adjusted to pH 9.5 with 14.5 M ammonium hydroxide) and methylene chloride were added. After centrifugation, the methylene chloride layer was evaporated to dryness (12).

3. *Organic Acids*

If necessary, organic acids (OAs) can be isolated from the matrix before determination by two isolation methods:

1. Steam distillation, following which volatile OAs are extracted from the aqueous distillate with organic solvents (22–25)
2. Direct extraction using diethyl ether and petroleum ether, chloroform, benzene, acidified methanol, or their mixtures as the most suitable organic solvents (26,27)

Usually, OAs present in beverages such as wine, beer, soft drinks, and fruit juices can be injected directly into the chromatographic system with no sample pretreatment other than dilution or filtration (5).

C. Separation Techniques

Modern HPLC techniques have resulted in a spectacular improvement in speed, resolution, and sensitivity. Separation times in HPLC are usually short, mostly in the interval of 10–20 min, with simpler separation problems being carried out in less than 10 min. This is due generally to the use of stationary phase that consists of very small and uniform porous particles with high ligand density. These conditions lead to better mass transfer and higher column efficiency. Consequently, high-performance separations can be obtained in short times (5).

Many column types are used for the separation and identification of foodstuffs. The nature of the separation dictates the parameters of the stationary phase as well as column and particle size. These last two characteristics are important, for they will determine the limits of sample loading and flow rate. Therefore, the specification of the selected column should be tailored es-

pecially for the intended analysis. Many factors influence the selection of a desirable column. For example, it is important to measure the capacity factor (k') when a new column is tested. The resolution may be increased by altering either the relative value (α) or absolute value of the capacity factor (the ratio of the amount of sample in the stationary phase to that in the mobile phase). The rule is that k' should be greater than 2 and lower than 10, which results in a clearcut boundary and acceptable peak broadening. The efficiency of the column is further affected by the solvent diffusion coefficient, column diameter, etc. Generally, the reverse-phase mode on a bonded-phase packing of microparticles is most common for the determination of food additives and preservatives (5).

D. Detection Systems

One limiting component of HPLC systems for the analysis of different food additives is the choice of the detector. In recent years, monitoring peak elution via the absorption of ultraviolet (UV) light has been the most common method, because the vast majority of compounds have some absorbance in the UV or the visible region. The popularity of this detection mode is primarily due to its sensitivity toward a large number of constituents in the range of 210–280 nm.

But there are a number of limitations to UV detection in the analysis of additives. Detection without derivatization relies on the presence of UV-absorbing functional groups. Although these groups are quite sensitive, UV absorption generally is not highly selective. Many compounds absorb in the UV range. So with many HPLC methods, the only information provided is retention time.

One method of overcoming this problem is to monitor the effluent at two wavelengths and to plot a wavelength ratio. If a peak is given by a single compound, a square wave will result; other picture forms signal the presence of another compound in the time monitored, i.e., approve insufficient seperation process. Another method is to stop the flow of the chromatogram and record the spectrum of the compound. More effective is the use of a diode array detector, which allows continuous spectral recording during the separation. Purity of peaks may be checked by matching spectra early and late in their elution. Spectra may also be matched with those of authentic compounds stored in the memory as an aid in identification (5).

Other techniques used for the assay of additives are based on fluorimetry, electrochemistry, or ratio activity, depending on the specific structure and concentration of the determined compound (5). Various detection systems are used for sulfite detection (1). An electrochemical detection is preferred to detection in the UV region (28,29) because of its better selectivity, although direct UV detection is also possible (30). Fluorimetric detection can be used after formation of the formaldehyde–bisulfite complex with 5-aminofluorescein, which gives a nonfluorescent product (1,31). Benzimidazole fungicides absorb strongly in the UV region of 280–310 nm, possess some native fluorescence, and are used mostly in combination; this makes HPLC the method of choice for their analysis (5). The simultaneous determination of benomyl (as total MBBC), methyl thiophanate, and TBZ residues was carried out by reverse-phase HPLC, using an ion-pairing agent in the mobile phase, with UV and florescence detectors coupled in tandem with the partition and/or column cleanup (12,21).

E. Applications in Foods

1. Sulfites

Roughly 10 years ago, an ion-pair HPLC was introduced for the determination of sulfites in foods (32). The method combined the chemical approach of the Monier–Williams technique for liberating the sulfite from the matrix with ion chromatography. The ion chromatographic procedure

offered several important advantages over the Monier–Williams method, including a lower detection limit (1 ppm). The analysis time was reduced from 2–3 h to 25 min, and the results were not affected by the presence of volatile acids or even organic sulfur compounds. After HPLC separation, various detection systems are used for sulfite detection. An electrochemical detection is preferred to detection in the UV region (19,28,29,33) because of its better selectivity, although direct UV detection (30) or even detection after postcolumn colorimetric reaction products (34) can also be used. A method based upon a combination of a modified Monier–Williams procedure and an HPLC separation and quantitation of sulfite has been developed (30). Its efficiency has been tested, in comparison with the Monier–Williams method, for SO_2 recovery in various foods (beer, jam, mustard, shrimps, wine, grapefruit juice, dried onions, potatoes). Desorption of SO_2 from different matrices was obtained by distillation in strongly acid solution. Gaseous SO_2, collected and oxidized to sulfate with hydrogen peroxide, was neutralized with sodium hydroxide. The neutralized solution was diluted and injected by an autosampling injector into an HPLC apparatus consisting of a strong anion-exchange column, eluted with a potassium hydrogen phthalate solution (0.15 g/L, pH 5.7), and detected at 280 nm. All results were obtained using a Supelcosil LC-SAX (250 × 4.6-mm, 5 μm) column, maintained at room temperature (30).

The reaction of sulfite with formaldehyde to form hydroxymethylsulfonate (HMS), which is very stable under the controlled conditions of this assay, was used as the first step in an analytical procedure to determine food-borne sulfite. The effect of mobile-phase pH on the stability of HMS during high-performance liquid chromatography was studied. It was found that on-column HMS dissociation to formaldehyde and bisulfite increased with the pH of the mobile phase; therefore the relatively low pH 4.7, at which the dissociation of HMS was approximately 2%, was selected for the analysis. In addition, the release of sulfite from its reversibly bound forms in wine and other foods was examined as a function of the pH of the extraction medium by following the appearance of HMS formed from the reaction of the freed sulfite with formaldehyde. The rate of dissociation of the reversibly bound sulfite was relatively slow at pH 3 but very rapid at pH 7.

This difference in kinetics was exploited to develop a procedure to determine free and reversibly bound sulfite in food. The mobile phase consisted of an aqueous solution of 0.05 M tetrabutylammonium hydroxide adjusted to the desired pH by the addition of glacial acetic acid (34). Fluorimetric detection is also possible, because a reaction of the formaldehyde–bisulfite complex with 5-aminofluorescein gives a nonfluorescent product. The sulfite is measured indirectly by its suppresion of the fluorescence of the reagent (31). This method is applicable to the determination of SO_2 at >10 ppm and is not applicable to dark-colored foods or ingredients where SO_2 is strongly bound, e.g., caramel color. This method does not detect naturally occurring sulfite. Sulfur dioxide is released by direct alkali extraction.

Diluted portions of liquid samples or diluted filtrates of solid samples are injected into a liquid or anion-exclusion chromatographic system equipped with an anion-exclusion column (sulfonated polystyrene/divinylbenzene) and an electrochemical (amperometric) detector. The electrochemical detector is set at +0.6 V on a platinum working electrode vs. Ag/AgCl reference electrode with 20 mM H_2SO_4 eluent. Dilute the liquid sample with pH 9 buffer so that the height of the sulfite peak from the sample is similar to that of a 0.60-ppm standard within 50%. For solid samples, homogenize the sample in 10–100-fold excess pH 9 buffer for 1 min with homogenizer, and filter (0.2–0.45 μm). Dilute the filtrate as necessary, comparing the signal intensity with that of the 0.60-ppm working standard solution. For acidic samples such as lemon juice, if the pH of the diluted samples is under 8, adjust to pH between 8 and 9 with dilute NaOH solution or perform extraction with 100 mM Na_2HPO_4, 10 mM D-mannitol solution. Inject the 0.60-ppm standard solution, and then inject the prepared, diluted test sample. Extraction, filtration, dilution, and injection should be done within 10 min, because sulfite concentration in extract tends to decrease gradually (35).

In one study, a modified Monier–Williams method has been utilized as a preparative procedure to obtain both the free and bound sulfite fractions. The two fractions were analyzed by HPLC with indirect photometric detection using a 250 × 4.6-mm LC-SAX column eluted with potassium hydrogen phtalate (0.15 g/L, pH 5.7) and detected at 280 nm. Levels of 5–10 ppm of SO_2 in foods, corresponding to 30–60 ng injected, were reliably detected by this method. The results confirmed that the chromatographic method, unlike the Monier–Williams method, is able to avoid the potential interference of volatile substances derived from matrices or utilized chemicals (36). The HPLC conditions are summarized in Table 3.

2. *Postharvest Preservatives*

Methods for determining MBBC residues in plant tissues involve isolation of the residue by extraction with ethyl acetate, hydrolysis of the extracted residue to 2-aminobenzimidazole, and final determination by HPLC using fluorimetric detection (37). TBZ is also determined by a similar procedure, without hydrolysis. The sensitivities of these methods are 0.02 ppm for benomyl and 0.001 ppm for TBZ. Recoveries of these compounds from various plant tissues were 90.5–102.9% for MBBC and 98.2% for TBZ, respectively (37). Tribendazole was repeatedly extracted from a sample with ethyl acetate, and the combined extract was evaporated under reduced pressure below 40°C to a small volume. Subsequently, 0.1 M HCl was added, and the evaporation was

Table 3 HPLC Systems Used for Determination of Sulfites

Type of sample	Sample preparation	Stationary phase	Mobile phase	Detector	Ref.
Fruit juices, dried bread, white wine	Monier–Williams distillation	Ion-exchange polystyrene with —NH^+_3 and —NR^+_3 groups	0.0024 M Na_2CO_3, 0.003 M $NaHCO_3$; flow rate 2 ml/min	Conductivity detector	32
Lemon juice, instant mashed potatoes, beer	Extraction with Polytron for 1 min, pH 2.0 for free and pH 8.9 for total sulfite	Anion exclusion	6 mM H_2SO_4	Electrochemical detection	33
White wines	Extraction procedure as in Ref. 33	Anion-exchange resin	0.04% H_3PO_4	Electrochemical detection	28
Foods and beverages	Liquid samples with pH 9 buffer; solid samples extraction in pH 9 buffer, filtered	Anion-exclusion column (sulfonated polystyrene/divinylbene) (4.6 × 100 mm)	20 mM H_2SO_4	Electrochemical detection, +0.6 V on platinum working electrode vs Ag/AgCl	35
Foods (beer, jam, juice, potatoes, dried onions)	Distilled, neutralized for titration; acidified with HCl, 3% hydrogen peroxide	Supelcosil LC-SAX (250 × 4.6 mm. 5 μm)	K_2HPO_4 (pH 5.7)	UV 280 nm	36

continued until the ethyl acetate was completely removed. The aqueous acid solution was washed with hexane, and NaOH was added to give a strongly basic solution. Finally, TBZ was extracted from this solution with ethyl acetate, and the combined extract was evaporated to dryness. The residue was dissolved in methanol and analyzed by HPLC. The effluent was monitored fluorimetrically. Because varying degrees of quenching were encouraged (38), the quenching factor of TBZ was determined by adding a known amount of TBZ to the untreated control extract and comparing the peak height obtained with that obtained from a standard solution under the same conditions. The results indicated that the factors ranged from 0.94 to 1.06. This meant that the quenching interferences were effectively removed by the procedure, and quenching factors were therefore omitted from the calculations. Recovery studies show that is possible to obtain more than 98% of TBZ by this procedure at a detection limit of 1 ppb.

Benomyl, BMC, methylthiophanate, and vinchlozolin were determined in grape (20). Sample was extracted with petroleum ether and evaporated to dryness, and the residue was dissolved in acetonitrile. The sample was injected directly into the chromatographic system, consisting of a reverse-phase column with acetonitrile–water mixture as mobile phase and UV detection at 221 nm. The detection limit of the method was between 0.01 and 0.05 mg/kg (5,20). Tribendazole was determined after extraction with methanol by HPLC using fluorometric detection (39). Fruits were homogenized, mixed with methanol, and filtered through a glass filter by suction. Filtrate was transferred into separatory funnel, and internal standard was added and diluted to volume with methanol. The excitation wavelength of the fluorimetric detector was set at 305 nm and emission at 350 nm (5,39). Tribendazole, benomyl, and carbendazim were determined in fruit (40). After extraction with ethyl acetate, the fungicides were partitioned into hydrochloric acid and then transferred into ethyl acetate at pH 6. The separation was carried out on a reverse-phase column using acetate buffer solution (pH 4.0) in acetonitrile as mobile phase. The eluent was monitored fluorimetrically when detection limits varied between 0.005 and 0.1 mg/kg (5,40).

Kitada et al. (5,41) used a rapid method for TBZ and BP in citrus fruits. The crops were extracted with ethyl acetate and separated on a reverse-phase C_8 column with UV and fluorometric detection, respectively. Methanol phosphate buffer mixture at a flow rate of 1.0 ml/min gave satisfactory results as the mobile phase. Recoveries of postharvest residue were greater than 93%. Carbendazim and thiophanate-methyl were determined in chicory, beans, lettuce, strawberries, and tomatoes (5,42). After extraction with methanol, the extract was cleaned up on a Sep-Pak C_{18} cartridge. The rapid method gives recoveries of between 90 and 100%.

Nakashima et al. (5,43) measured BP and OPP in citrus fruits by reverse-phase HPLC after steam distillation pretreatment. The chromatographic conditions included UV detection and a column temperature of 50°C.

Carbendazim and thiophanate-methyl were determined in cabbage and onions (5,44). Autors et al. (44) used the cation-exchange technique for the HPLC determination. The preservatives were detected at 275 nm, and tetramethylammonium nitrate in nitric acid was used as mobile phase; recoveries were 75% at a level of 0.08 mg/kg, when the determination limit of the method was 0.02 mg/kg.

High-performance LC was also used for determination of TBZ after its extraction from marmalades and curds with ethyl acetate (13). The use of a buffered mobile phase improved the response of the UV detector, and column performance remained constant throughout 2 months of daily use with a detection limit of 100 ppb. Three detectors (UV, fluorimetric, and electrochemical) were used for the determination of OPP, BP, and TBZ in plant materials (45). The compounds were extracted with dichloromethane and separated on an RP-18 column with a methanolic formic acid buffer as eluent. It was not possible to determine TBZ using an electrochemical detector, although the extraction recovery varied between 80 and 95%.

Cinquina et al. (46) used HPLC with a diode array detector for the simultaneous determination of benzoimidazoles (albendazole, oxibendazole, parbendazole, mebendazole, and tiabendazole) in sheep and goat milk. The milk sample was extracted with acetonitrile and after with dichloromethane; the organic phase was evaporated to dryness and the residue, dissolved in 0.2 N chloridric acid in ethanol, purified with solid-phase extraction, eluating with ethyl acetate. The eluate was evaporated and dissolved in methanol and 0.05 N phosphoric acid and detected by HPLC diode array detector at 295 nm. The method is simple and has an average recovery of 80% ± 5% for goat and sheep milk; furthermore it meets the requirements of analysis, especially in light of the EC regulation that establishes the benzoimidazole levels in milk.

The effects of domestic marmalade-making procedures on residues of imazalil, OPP, and TBZ in sweet oranges were investigated by comparing residue levels in raw fruit and in marmalades, prepared by heating the oranges with water and sugar for about 4 h in a preserving pan on a gas ring or for about 1 h in a microwave oven (47). Determination of residue in raw oranges and in marmalades was carried out by extraction with dichloromethane, followed by cleanup and reversed-phase HPLC with UV detection for imazalil and fluorescence detection for OPP and TBZ. Residue analysis showed that the extent of carry-through of imazalil, OPP, and TBZ residue into the marmalades depended on the method of marmalade-making and the nature of the fungicide.

Persistence of fungicides was higher in the marmalade prepared in the microwave oven than that prepared in the preserving pan. An increase (about 50%) in levels of imazalil in the fruit portions of marmalades compared with raw fruit residue levels indicated that imazalil was more readily extracted from oranges after processing. Comparison of OPP levels in raw fruit and fruit portions of marmalades showed that the longer exposure to heat needed for marmalade-making in a preserving pan reduced residue levels more severely (48%) than did cooking in the microwave oven (13%). Tribendazole levels in the fruit portion of marmalade prepared in the microwave oven stayed the same as in the raw fruit, but cooking in the pan decreased residue levels by about 22%.

Imazalil, OPP, and TBZ residues were determined using an HPLC system, consisting of a ternary HPLC pump attached to 250 × 4.6-mm-ID Hichrom RPB 5-μm column with a 10 × 3.2-mm-ID guard cartridge column. The packing material of both columns was base-deactivated silica, fully endcapped with a bonded phase of C_8/C_{18}. The mobile phase was methanol/water at a flow rate of 1 ml/min at ambient temperature. Imazalil in the eluate was detected with UV detector at 204 nm; a fluorescence detector was used to monitor OPP (excitation 285 nm, emission 350 nm) and TBZ (excitation 296 nm, emission 350 nm) (47).

High-performance thin-layer chromatography (HPTLC) was used to check for residues of benomyl (BYL), carbendazim (BCM), ethoxyquin (EMQ), and TBZ in apples and pears (48). The method used showed good precision, with a percentage coefficient of variation of less than 5%, and recoveries were higher 90%. High-performance TLC NH_2 plates derivatized with propylamine were used. Development was performend first with a mobile phase consisting of cyclohexane and chloroform (1.0:0.3) to elute ethoxyquin, which was read at 235 nm. The plate was eluted again with a mobile phase consisting of chloroform/cyclohexane/methanol (6.0:1.0:0.1) and carbendazim and TBZ read at 285 nm. For separation of compounds (BCM, EMQ, and TBZ), a Supelco LC_{18} column was used with a flow rate of 1.5 ml/min. For BCM and TBZ, the mobile phase was methanol/phosphate buffer (0.01 M, pH 6.6) (56:44) and at wavelength of 280 nm. For EMQ, a mobile phase consisting of acetonitrile/phosphate buffer (0.01 M, pH 6.6) (60:40) was used and the absorbance read at 235 nm. The limits of determination using HPTLC were always at least four times lower than Italian statutory limits. Selectivity with respect to other matrix components was excellent for all fruit varieties tested (48).

A novel method was developed for the determination of TBZ residues in whole green bananas and ripe banana pulp. Tribendazole was extracted from banana matrix with ethyl acetate, followed by cleanup of extract on a cation-exchange column with fluorescence detection. Recoveries of TBZ from whole green bananas fortified with TBZ at 0.05–10 ppm and from ripe banana pulp fortified with TBZ at 0.01–2 ppm averaged 93 and 95%, respectively (49).

A method has been developed for the analysis of the fungicide TBZ in fruits, potatoes, and their processed products. Extraction was performed with a basic solution (ethanol with 2 M ammonium chloride, pH 9.5, with 14.5 M ammonium hydroxide) and methylene chloride. Tribendazole was partitioned into methylene chloride with a polytron. No further cleanup was needed. Total analysis time, including extraction, was 25 min per sample. Recoveries ranged from 77 to 135%. Detection limits were 1.0 ppb for juice, fruits, and potatoes and 2 ppb for bulk concentrates (12).

An LC method using fully automated solid-phase extraction (SPE) sample cleanup and online analysis was developed for the determination of the benzimidazole fungicides, carbendazim, and TBZ in various crops (50). Automation of the total procedure was achieved by using a commercially available SPE cleanup apparatus. The cleaned-up extract was injected in a system with UV and fluorescence detection in tandem. Chromatographic separations were performed with methanol-phosphate buffer mobile phase (pH 7) and different polymeric stationary phases. Analytical columns were: PLRP-S (150 × 4.6 mm), PRP-1 (150 × 4.1 mm), ACT-1, C_{18}-derivatized (150 × 4.6 mm), Asahipak ODP-50, C_{18}-derivatized (150 × 4.6 mm). The polymer-based columns performed better than silica-based columns in separating the benzimidazole fungicides, and different columns were compared (50). A reverse-phase HPLC was used for the determination of benomyl and its metabolites carbendazim, TBZ, and methyl thiophanate in fruit products (nectars, purees, concentrates, and jams). The residues were extracted with an HCl–methanol mixture and partitioned into dichloromethane on an Extrelut 20 cartridge column. The fungicides were then separated on a reverse-phase HPLC column using an ion-pairing mobile phase and finally determined by UV and fluorescence detection. Positive samples were confirmed by normal-phase chromatography using a Diol column. The determination limit was 0.010 mg/kg for carbendazim and methyl thiophanate, and 0.001 mg/kg for TBZ (21).

A method for the determination of TBZ residues in or on whole green bananas and potatoes was applied to whole, unwashed citrus fruits. The method is applicable for determining TBZ residues in whole oranges, grapefruits, tangerines, and lemons (51). Tribendazole was extracted from citrus homogenate with ethyl acetate, and the extract was cleaned up on a cation-exchange, solid-phase extraction column. The purified extract was separated on a cation-exchange column, and fluorescence detection was used. Average recovery of TBZ from whole citrus fruits fortified with TBZ at 0.05–20 ppm was 96% (51).

Farré et al. (14) described the determination of 5-nitrofurylacrylic acid (5-NFA) in wines from different areas in Spain. Determination of (5-NFA) was achieved by optimalization of the mobile phase by reversed-phase HPLC. The mobile phases studied were 25% methanol or 23% acetonitrile, deionized water, alone or together with acetic acid–acetate buffer (pH 4.4) or glacial acetic acid. The 5-NFA was separated on a LiChrosorb RP-18 (150 × 3.2-mm-ID) column eluted with acetonitrile: water glacial acetic acid mixture (25:75:1.5) at the rate of 0.6 ml/min. The experiment was carried out at room temperature, and the detection wavelength was 360 nm.

A novel multiresidue method has been developed for quantitation of TBZ, the metabolite 5-hydroxythiabendazole (5-OH-TBZ) in raw cow's milk. The 5-HSO_4-TBZ was hydrolyzed quantitatively under acidic conditions to 5-OH-TBZ. The TBZ and 5-OH-TBZ were extracted from milk at pH 8.0 with ethyl acetate, followed by cleanup of the extract on a cation-exchange solid-phase extraction column. Analytes were separated with a cation-exchange stationary phase;

fluorescence detection (5-OH-TBZ detection: 318 nm excitation, 525 nm emission) was used. Recoveries from raw cow's milk samples fortified with TBZ and 5-OH-TBZ or TBZ and 5-HSO_4-TBZ at 0.05–2 ppm ranged from 87 to 103% for TBZ, 98 to 109% for 5-OH-TBZ, and 96 to 115% for 5-HSO_4-TBZ (quantitated as 5-OH-TBZ) (52).

An HPLC method was described for the residue analysis of TBZ in meat (53). The recovery varies from 62 to 75%. The TBZ was extracted from the tissue using 3 mol HCl, eluted from the Extrelut 20 column with dichloromethane and then injected onto a C_{18} column. The TBZ was detected with fluorescence and UV detectors. The sensitivity is such that TBZ can be determined at a level of 5 μg/kg meat (53). Table 4 summarizes the HPLC conditions.

3. *Organic Acids*

Formic acid (FA), benzoic acid (BA), sorbic acid (SA), and the methyl, ethyl, and propyl esters of 4-hydroxybenzoic acid (EsHBA) or their salts are the organic acids (OAs) most frequently used for the preservation of marmalades, jams, mustard, mayonnaise, margarine, wine, juices, sardines in oil, canned tuna, fish homogenate, diet foods, milk products, salad dressings, desserts, soft drinks, and other types of foods and beverages.

For the direct determination of OAs in beverages—for example, wine, beer, fruit juices, and soft drinks—the HPLC methods are very suitable. Sample treatment is usually limited to filtration (removal of solid particles) and sonication (removal of gases, mainly CO_2, from saturated drinks). The detection limit for individual OA compounds can be enhanced by monitoring at their absorption maximum. For BA this is 228 nm, for SA 259 nm, and for EsHBA 255 nm. But practical measurements are usually done, by compromise, at a wavelength of 235 nm (1,2).

When solid samples are analyzed, OAs are extracted from a matrix by the following procedures (1,2):

1. Direct extraction of an acidified sample by an organic solvent
2. Solid-phase extraction
3. Extraction as an ion pair
4. Steam distillation

Fruit juices (e.g., orange juice), wines, and other aqueous media generally do not need cleanup before HPLC analyses. In any case, filtration is recommended to eliminate any particulate matter. The detection limit for BA, which has the smallest absorption coefficient of the compounds analyzed, is far lower than the concentrations normally used in food chemistry (54).

Extremely fatty samples, such as margarine, have to be extracted by the following procedure: A sample is dissolved in diethyl ether and extracted twice in 0.1 N NaOH. The basic aqueous extracts are acidified with 5 N sulfuric acid and diluted to the appropriate volume with methanol (54).

The Extrelut cleanup method is suitable for most foodstuffs, such as cheese, yogurt, and other samples that tend to form emulsions during extraction. The prepacked or refilled Extrelut column in a plastic tube consists of a wide-pore kieselgel column. A sample is homogenized in 0.5 N sulfuric acid, diluted with water, and applied onto the Extrelut column for at least 15 min. The absorbed preservatives are eluted with a chloroform–isopropanol (9 : 1) mixture, and the eluate is collected and evaporated carefully nearly to dryness. The last few milliliters of solvent are removed with a gentle flow of nitrogen to prevent substantial losses of BA and SA, which have relatively high vapor pressures. The residue is transferred with methanol into a 10-ml volumetric flask and diluted to volume with methanol. To speed up the dissolution, the use of an ultrasonic bath is recommended. The filtered extract is analyzed on a μBondaPak C_{18} column, with a

Table 4 HPLC Systems Used for Determination of Postharvest Preservatives

Type of sample	Sample preparation	Stationary phase	Mobile phase	Detector	Ref.
Orange pulp	Extraction with ethyl acetate	Hitachi gel No. 3010–CH_2OH, 20–23 μm, column 500 × 2.1 mm	0.1% (v/v) acetic acid–methanol 1.2 ml/min	Fluorimetric, 37 ex. 305 nm, em, 355 nm	37
Curds (artificial marmalades), marmalades	Extraction with ethyl acetate	Polygosil 60/7.5/18	Aqueous 0.8% (m/v) NH_4NO_3 + 0.7% (v/v) NH_4OH–methanol (1:1), 1.0 ml/min	UV detection, 305 nm	13
Citrus fruits	Extraction with CH_2Cl_2	Nucleosil 7–C18 (Macherey–Nagel)	Formic buffer–methanol (40:60), 1.0 ml/min	UV 246 nm; fluorimetric, ex. 275 nm, em. 320 nm; electrochemical	45
Apples, pears	Homogenized, extraction with chloroform, 0.1 M HCl, neutralized Na_2CO_3, extraction chloroform	HPTLC NH_2 plates derivatized with propylamine, Supelco LC 18	Chloroform-cyclohexane–methanol (6.0:1:0.1), methanol–phosphate buffer (pH 6.6) (56:44)	UV 285 nm; UV 280 nm	48
Green bananas, banana pulp	Mixed, extraction with ethyl acetate, centrifuged, added Na_2SO_4, preconcentration PRS SPE	Parti-Sphere SCX (125 × 4.6 mm; 5 μm), 25°C	KH_2PO_4–acetonitrile (70:30), pH 3.8–4.0 with H_3PO_4	Fluorimetric, ex. 305 nm, em. 380 nm	49
Potatoes, fruits	Extraction with methylene chloride	Ultracarb 30 ODS (150 × 4.6 mm, 5 μm)	acetonitrile–methanol–water–monoetanolamine (260:70:500:0.1)	Fluorimetric, ex. 305 nm, em. 345 nm	12
Fruit, vegetables	Homogenized with acetone, extraction dichloromethane–petroleum eter, centrifuged	Shodex DE-613 (150 × 6.0 mm, 6 μm)	phosphate buffer–methanol (30:70), pH 7.0	Fluorimetric, ex. 235 nm, em. 280 nm	50
Commercial fruit products	Extraction with 0.02 M HCl/CH_3OH (80:20) centrifuged, umfuged, filtered, pH 7.5; Extrelud 20	Supelcosil LC-18-DP (250 × 4 mm, 5 μm), 40°C Lichrosorb DIOL (150 × 3 mm, 5 μm)	35% Methanol in ion-pairing solution (sodium decanesulfonate) isopropanol-n-hexane (18:85) mixture with 32% NH_4OH	UV 280 nm, 305 nm; Fluorimetric, ex. 280, em. 310 nm	21
Citrus, fruits	Homogenized with buffer pH 8, extraction–ethyl acetate preconcentration PRS SPE	PartiSphere SCX (125 × 4.6 mm, 5 μm), 40°C	KH_2PO_4–acetonitrile (75:25) pH 3.4 with H_3PO_4	Fluorimetric, ex. 305 nm, em. 380 nm	51
Milk	Hydrolyzed (HCl), pH 8, extraction–ethyl acetate, PRS SPE	PartiSphere SCX (125 × 4.6 mm, 5 μm), 25°C	KH_2PO_4–acetonitrile (75:25) pH 3.8 with H_3PO_4	Fluorimetric, ex. 305 nm, em. 380 nm	52
Meat	Hydrolyzed (HCl), pH 7.5, Extrelut-20, dichloromethane	μ-Bondapak C18 (300 × 3.9 mm, 10 μm)	0.03 mol/L sodium acetate–methanol (45:55, v/v), pH 7.6, with acetic acid	Fluorimetric, ex. 313 nm, em. 365 nm; UV 300 nm	53

variable-wavelength UV detector at 235 nm. For better resolution of EsHBA from BA and SA, it is necessary to use a gradient elution. While at the beginning the composition of the mobile phase consisted of 20% phosphate buffer in methanol, a linear gradient from 20% to 80% of phosphate buffer in methanol was reached within 1 min, which reduced analysis time and gave sharp peaks (54). The separation of the HBA esters themselves can be carried out by isocratic elution of 60% methanol in phosphate buffer. The determination of BA and SA at isocratic elution is also possible using a buffered mobile phase when the detector operates at 235 nm. The detection limit can be enhanced for a specific analysis by using the appropriate absorption maximum (54).

Benzoic acid, SA, and EsHBA can be also well separated using an isocratic elution. After separation on a Sep-Pak C_{18} cartridge, the sample was injected on a Rhodex RSpak DS-613 column containing a rigid polystyrene-divinylbenzene resin. An eluent, 0.05 M potassium dihydrogen phosphate–acetonitrile (60:40), was directed to the UV detector, which operated at 230 nm (2,55).

Perfetti et al. (5,56) developed an HPLC method for the analysis of EsHBA (methyl, propyl) in chopped ham, bologna, and chicken roll. Samples were extracted with acetonitrile, filtered, and analyzed on a reverse-phase C_{18} column. Acetonitrile as the extracting agent gave good recoveries of the parabens (88–99%) without the need for extracting higher amounts of fat. The researchers (5,57) used HPLC to measure the BA and SA content in yogurts. They extracted preservatives after precipitation of protein and performed chromatography on C18-bonded silica gel with metanol:phosphate buffer as mobile phase. The limit of detection was approximately 5 mg/kg for both compounds at 227 nm and 2 mg/kg for SA at 250 nm. Direct determination of BA and SA in beer using isocratic elution was elaborated. The preservatives were separated on a LiChrosorb RP-18 column eluted with 5.0 mM ammonium acetate buffer (pH 4.4)–acetonitrile (4:1, v/v), with UV detection at 233 nm (2,5,58). The sample was first acidified with 0.5 M sulfuric acid and applied to an Extrelut column. Elution of preservatives was done with a chloroform–methanol mixture that was then evaporated to dryness at 30°C. The residue was dissolved in methanol and filtered before analysis on a reverse-phase stationary phase eluted with a methanol–water mixture (65:35) as mobile phase, and detected at 254 nm (2,5,58).

Ali described the determination of BA, SA, and EsHBA (methyl, ethyl, propyl, butyl) in ground beef and pork sausage (2,5,59). The preservatives were extracted into a 70% ethanol solution and quantified by reverse-phase gradient elution HPLC. For the separation of OAs from a coffee drink, a steam distillation method and a Sep-Pak C_{18} cartridge method for the sample preparation were used. The steam distillation method had a few advantages; namely, it provided a cleaner sample solution that contained fewer interfering compounds, and it was applicable to samples of various types. The Sep-Pak C_{18} cartridge method generally provided better results and gave better recoveries of compounds added to coffee beverages at the 100-ppm level, ranging from 93.8 to 102.8%, with a coefficient of variation of 0.85–2.15% (60). For good separation of these compounds, an acetonitrile–water–0.2 M phosphate buffer at pH 3.6 (7:12:1) containing 2.0 mM cetyltrimethylammonium bromide as an ion-pair reagent and a Nucleosil 5 C_{18} column are required (60). For the determination of BA, SA, and EsHBA in fatty samples (mayonnaise, meat salad, meat salad with mayonnaise), samples were homogenized and acidified to pH 1–2 with 0.5 M H_2SO_4. The mixture was cleaned up by application on an Extrelut column, and the preservatives were eluted with a solvent of dichloromethane–diethyl ether (4:1). After removal of the solvent under reduced pressure, the fatty residue was dissolved in warm isopropyl ether or dichloromethane and diluted with heptane. The heptane solution was analyzed on a column of LiChrosorb Si 60 (150 mm × 4.6-mm ID) by consecutive isocratic elutions with various concentrations of mobile phase [heptane–diisopropyl ether–glacial acetic acid (88:12:0.1 or 90:10:0.5) at 230 nm]. The recoveries from food samples like meat, salad, mayonnaise, and canned

salmon were 83–97% for benzoic and sorbic acids and 86–113% for methyl, ethyl, and propyl 4-hydroxybenzoate (2,5,61).

Flak and Schaber (5,62) used reverse-phase HPLC for the quantitative and simultaneous determination of benzoic acid and sorbic acid, as well as 4-hydroxybenzoic acid, salicylic, 5-nitrofurylacrylic, and *p*-chlorobenzoic acid and the EsHBA (methyl, ethyl, propyl) in wines and beverages. The first five compounds can be determined by isocratic elution from a C_{18} column using 0.12 M acetate, pH 3.8 : acetonitrile (85 : 15), and all may be separated with gradient elution (increasing acetonitrile from 10 to 60%, with a simultaneous decrease of the pH of the acetate buffer from 3.9 initially to 3.3).

Ikai et al. (5,63) used an HPLC method for the simultaneous determination of SA, BA, dehydroacetic acid, EsHBA (ethyl, isopropyl, *n*-propyl, isobutyl, and *n*-butyl 4-hydroxybenzoate), and saccharin sodium using the ion-pair reversed-phase. The food additives from some foods (soy sauce, orange juice, and yogurt) were extracted twice with diethyl ether. The extracts were combined, washed twice with saturated sodium chloride solution, dried with anhydrous sodium sulfate, evaporated to 1–2 ml, and dried by blowing with air. Food additives were separated on a Nucleosil C_{18} column using methanol–acetonitrile and aqueous acetonic acid solution, (pH 4.5) containing 2.5 mM cetyltrimethylammonium chloride as the mobile phase, and detected at 233 nm. In beverages (orange squash, lemon-lime squash, carbonated cider, orange light) and jams (strawberry jam, cherry jam, raspberry jam), SA and BA were determined after filtration through a 0.45-μm filter, and carbonated beverages were decarbonated using an ultrasonic bath. Jams were homogenized and diluted in water. For the separation, the column was PhaseSep, C_{18}, Spherisorb ODS-1. The mobile phase consisted of methanol–phosphate buffer (8 : 92) adjusted to pH 6.7 (64).

Sorbic acid and BA in fruit juices (65) were determined after passing through a 0.45-μm filter to eliminate particulate matter. The preservatives were separated on a Waters μBondaPak CN column. The mobile phase was 2% acetic acid–methanol (95 : 5). The UV detector operated at 240 nm for BA and at 254 nm for SA. The minimum detection limits were 1 ng for BA and 0.5 ng for SA.

Simultaneous determination of SA and BA in citrus juices by HPLC was done using a μBondaPak CN column and 2% acetic acid–methanol (19 : 1) as mobile phase (66). Simultaneous determination of various artificial sweeteners and some preservatives (BA, SA) that are commonly used together in diet food is also possible.

Chromatograms and conditions are presented in Fig. 1 (64,67). Several diet foods, such as soft drinks, juices, milk products, desserts, and salad dressings, were investigated. For sample cleanup, a simple Carrez clarification proved to be sufficient. The preservatives were separated on a Superspher RP 4-μm column eluted with a 0.02 M phosphate buffer–acetonitrile (90 : 10), pH 4.2–4.4, and detected at 220 nm. Theophyllin was used as the internal standard. Reverse-phase HPLC was used for the determination of preservatives in an aqueous solution, using *n*-butyl-*p*-aminobenzoate as the internal standard (1). This method was applied to the analysis of preservatives in yogurt. The preservatives were previously identified by TLC on F_{254} polyamide plates after developing with benzene–ethyl acetate–acetic acid (85 : 10 : 5) and detected under a 254-nm UV lamp. The preservatives were then separated on a reverse-phase C_{18} analytical column using a methanol–water mixture as mobile phase.

Sorbic acid and BA were extracted from a yogurt sample and quantified via ion-pair formation (2,68). A sample of yogurt was shaken with a phosphate buffer solution at pH 5.5, and the combined supernatant solution obtained after centrifugation was shaken with tri-*n*-octylamine in chloroform and back-extracted into aqueous 0.1 M sodium perchlorate. The acid solution was analyzed on a C_{18} reverse-phase column with methanol–phosphate buffer (2 : 3) as mobile phase.

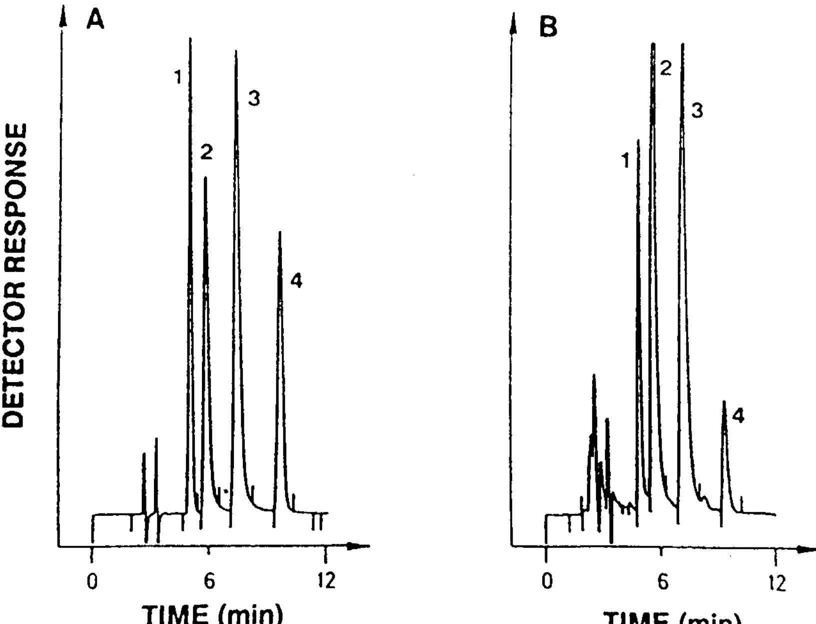


Fig. 1 Chromatograms of standard solutions (A) containing 1 mg/100 ml of each additive and a sample of raspberry jam spiked with acesulfame-K (B). 1—Acesulfame-K; 2—benzoic acid; 3—sorbic acid; 4—saccharin. Conditions: Column PhaseSep C18 (250 × 4.6-mm ID), mobile phase 8% methanol–phosphate buffer (8:92), flow rate 1 ml/min. Detection UV, 227 nm. (From Ref. 64.)

A fast, accurate HPLC determination of preservatives can be achieved by direct injection of an alkaline extract of the sample (69). The sample was acidified with 25% H_2SO_4 and then alkalinized with 2 N NaOH and extracted with diethyl ether (1).

Organic acids were separated on a LiChrosorb RP-18 (250-mm × 4.6-mm-ID) column, using the mobile phase 0.005 M ammonium acetate–acetic acid–acetonitrile, at a detection wavelength of 225 or 232 nm. This method can be used for vegetable oils, meat salads, jams, mayonnaise, and mustard (69). After derivatization (benzoylation), it is possible to separate EsHBA with either normal-phase or reverse-phase columns. The normal-phase method showed high efficiency and sensitivity at a detection limit of 0.2 ppm. The preservatives were separated on a LiChrosorb Si 60 column and eluted with isoacetate–diethyl ether–acetonitrile (500:35:0.3). On an RP-18 column, a methanol–water mobile phase was used. Eluents were monitored at 240 nm (70).

After benzoylation, it was possible to analyze together the food substances of varying chemical structures, such as alcohols, esters of 4-hydroxybenzoic acid, phenolic antioxidants, saccharides, and sugar alcohols. The method allowed the determination of these substances in different matrices by the same analytical procedure, using the same cleanup. The preservatives were separated on an RP-18 column. Acetonitrile–water (50:35) or acetonitrile–water–butylmethyl ether (110:35:40) were used as mobile phases. Detection was UV at 230 nm (71).

A simple isocratic technique was developed for the quantitative analysis of OAs in dairy products. An Aminex HPX-87 (300-mm × 7.8-mm ID) analytical column was eluted with 0.009 N H_2SO_4 mobile phase; UV detection at 220 and 275 nm was utilized. Lactic, citric, formic, acetic, propionic, and butyric acids were quantified for whole milk, skim milk powder,

sour cream, cottage cheese, yogurt, and cheddar cheese. Before analysis, samples were diluted in distilled water, and acetonitrile was added to a 50-ml glass centrifuge tube, shaken for 1 min, and centrifuged for 5 min. Over 90% recoveries of acids added to whole milk were observed for all acids except butyric acid (72).

An anion-exchange method for the separation and detection of BA and SA in citrus juice required only the filtration and dilution of samples with KH_2PO_4 before injection. The Partisil 10-SAX anion-exchange column was eluted with 0.225 M KH_2PO_4 at ambient temperature. Average recoveries for both BA and SA varied between 94 and 98% (73).

Preservatives in cheese may be separated after their isolation using a steam distillation (74) on an LiChrosorb RP-18 (25-cm × 4.0-mm ID) column with a mobile phase of methanol–0.02 M phosphate buffer (30:70) at pH 7.2 with 0.005 M tetrabutylammonia as the ion-pair reagent at UV 235 nm. Sorbic and dehydroacetic acids are determined by HPLC using a reverse-phase column (74).

Bui and Cooper (75) described a method applicable to many liquid and solid foods for the determination of BA and SA. A C_{18} column is used with 0.03 M phosphate buffer pH 6.5:methanol (95:5) as mobile phase, and 4-hydroxyacetanilide or 3,5-dinitrobenzoic acid was used as internal standard; detection is at 227 nm. Recoveries varied from 90 to 105% (75).

Veerabhadrarao et al. (76) used reverse-phase HPLC for the determination of some food additives (acesulfame, saccharine, BA, *p*-hydroxybenzoic acid). The samples (beverages, tomato sauce) were diluted and then separated on a μBondapak C_{18} column with methanol/acetic acid/water (20:5:75) or (35:5:60) as mobile phases. The determination was done at 254 nm. Recoveries varied from 98 to 106% for direct analysis and from 91.6 to 101.8% for extraction of samples (76).

The AOAC Official Method 994.11 (77) is applicable to the determination of 0.5–10-ppm BA in orange juice. Benzoic acid in solid-phase-extracted orange juice is separated by liquid chromatography on a C_{18} column, detected by ultraviolet absorbance at 230 nm, and quantitated by external standard. Operating conditions: flow rate 0.7 ml/min isocratic; column temperature, ambient; detector, 230 nm, 0.05 absorbance unit full scale; and injection volume, 10 μl. Benzoic acid elutes in 7.26 ± 0.05 min. The LC column was a 250 × 4.1-mm ID, 10-μm polystyrene divinylbenzene column PRP-1. The mobile phase was acetonitrile–phosphate buffer (0.05 M KH_2PO_4) (40:60). The orange juice samples were centrifuged, preconditioned on a C_{18} cartridge, and eluate adjusted with methanol. The calculation of recoveries was based on the difference between the amount determined in the control and the amount obtained after the C_{18} cartridge cleanup.

Mannio and Cosio (78) described a sensitive, specific, and rapid chromatographic procedure to determine BA and SA in different food products. Benzoic acid and SA were extracted from foods by a microdialysis probe connected online to an HPLC column that allows separation of BA and SA. Detection was done at 228 and 260 nm for BA and SA, respectively. The procedure was linear from 1 to 80 ppm, with a detection limit of 1 ppm for SA and 2 ppm for BA. The assay was successfully applied to soft drinks, fruit juices, and dairy products (cheese, yogurt, and cream).

A method is described for the determination of the preservatives SA and BA in foods (including yogurt, soft drinks, and fruit juices) based on HPLC on a hydrogen-sulfonated divinylbenzene-styrene copolymer column, isocratic elution with 0.01 N sulfuric acid/acetonitrile (75:25) mobile phase and UV detection at 220 nm (for BA) and 258 nm (for SA). Soft drinks and fruit juices merely require dilution and filtration before injection; yogurt samples require treatment with potassium ferricyanide (III) and $ZnSO_4$ before analysis. Recovery of SA from yogurt was 95–110%; the detection limit was 0.01 mg/kg. The recovery of BA from soft drinks and fruit

juices averaged 99.1%; the detection limit was 0.05 mg/L. No interference by other constituents of these samples was observed (79).

A method (80) is proposed for the determination of SA in wines using a hydrogen-sulfonated divinylbenzene-styrene copolymer (DVB-H) HPLC column and isocratic elution with 0.01 N H_2SO_4–CH_3CN mixture (75:25). Ultraviolet detection at 258 nm permitted analysis by direct injection of white and red wines without significant interference between the peaks of SA and other sample components. The samples analyzed included sweet commercial wines containing unknown amounts of SA and sweet wines and synthetic wines without sorbates to which known amounts of the preservative were added. An AMINEX HPX 87-H (BIO-RAD Labs) column (300 × 7.9 mm) was maintained at 65°C on a Jones Chromatography heating block. Isocratic elution was carried out at a flow rate of 0.6 ml/min using a mixture of H_2SO_4 in double-distilled water and CH_3CN. Each sample was diluted 50-fold with 0.01 N H_2SO_4 filtered through a membrane filter and injected into the column. Four mobile phases with different acid and acetonitrile concentrations in double-distilled water were tested: mobile phase A—0.03 N H_2SO_4: CH_3CN (80:20); mobile phase B—0.01 N H_2SO_4:CH_3CN (75:25); mobile phase C—0.03 N H_2SO_4:CH_3CN (75:25); and mobile phase D—0.01 N H_2SO_4:CH_3CN (80:20). Mobile phase B produced the best chromatographic results in terms of resolution and analysis time. The detection limit of the method was 0.01 mg/L, with an average recovery of 99.6% and an average percent RSD of 0.87.

Analyses of 30 wine samples by the proposed method and the official EEC spectrophotometric method show that there is a good correlation between the results of the two methods, although the HPLC analyses are clearly more precise and accurate. The use of a (DVB-H) HPLC column, which allows isocratic elution, together with the short analysis time (about 15 minutes) makes this method rapid and readily amenable to automation (80).

A paired-ion, reversed-phase high-performance liquid chromatographic method was developed for the simultaneous determination of sweeteners (dulcin, saccharin-Na, and acesulfame-K), preservatives (sodium dehydroacetate, SA, salicyclic acid, BA, succinic acid, methyl-para-hydroxybenzoic acid, ethyl-para-hydroxybenzoic acid, *n*-propyl-para-hydroxybenzoic acid, *n*-butyl-para-hydroxybenzoic acid, and isobutyl-para-hydroxybenzoic acid), and antioxidants (3-tertiary-butyl-4-hydroxyanisole and tertiary-butyl-hydroquinone). A mobile phase of acetonitrile–50 ml aqueous α-hydroxyisobutyric acid solution (pH 4.5) (2.2:3.4 or 2.4:3.6, v/v) containing 2.5 mM hexadecyltrimethylammonium bromide and a C_{18} column with a flow rate of 1.0 ml/min and detection at 233 nm were used. This method was found to be very reproducible; detection limits ranged from 0.15 to 3.00 μg. The retention factor (k) of each additive could be affected by the concentrations of hexadecyltrimethylammonium bromide and α-hydroxyisobutyric acid and the pH and ratio of mobile phase. The presence of additives in dried roast beef and sugared fruit was determined. The method is suitable for routine analysis of additives in food samples (81).

Commercial cheese samples were analyzed for the presence of organic acids (82). Sample preparation: cheese samples were added to buffer–acetonitrile mobile phase, homogenized, extracted for 1 h, and centrifuged. The supernatant was filtered and then used directly for HPLC analysis. A reverse-phase C_{18} column at room temperature, a mobile phase of 0.5% (w/v) buffer $(NH_4)_2HPO_4$ at 2.24 (with H_3PO_4) and 0.4% (v/v) acetonitrile, UV detector at 214 nm, and 0.3 ml/min flow rate were utilized. Formic, pyruvic, lactic, acetic, orotic, citric, uric, propionic, and butyric acids were quantitated for white, feta, kasar, canned tulum, and skinned tulum cheeses. Recoveries of more than 86.3% were observed for all acids. The organic acid content of kasar cheeses were generally found to be higher than that of the others (82).

A rapid method for the identification and quantitation of SA and BA in a variety of bever-

ages and foods by micellar electrokinetic capillary chromatography (MECC) was described (83). Dehydroacetic acid was used as the internal standard. The separations were achieved using a 68-cm fused-silica capillary column with a buffer comprising 0.05 M dodecylsulphate and 0.02 M disodium hydrogen phosphate, pH 9.2. The amounts of preservatives determined by MECC were in good agreement with those determined by the high-performance liquid chromatographic procedure currently used in the authors' laboratory. Here is how the sample was prepared for MECC: The juices and jams were mixed thoroughly. A suitable aliquot of the sample was added to 1.25 ml of the internal standard solution (dehydroacetic acid) and diluted with deionized water. The solution was basified to pH greater than 9 with 1 M NaOH. For cheese slices, the acid preservatives were extracted from the sample by steam distillation HPLC. The samples were prepared as described for MECC, except that the solutions were not basified. An internal standard was not used for the HPLC analysis.

The HPLC analyses were performed with a 10-um Spherisorb ODS2 C18 column (3.9 mm $\times$ 300 mm) equipped with a C_{18} μBondapak precolumn. The mobile phase comprised an aqueous solution of potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate, each at 2.5 g/L. The flow rate was 1 ml/min. The compounds were detected at 230 nm. A second system using an Exmere ODS2-8/5 C_{18} column (4.6 mm $\times$ 250 mm) with a C_{18} μBondapak precolumn and a mobile phase consisting of 5 g/L of dipotassium hydrogen orthophosphate adjusted to pH 8 with 1 M orthophosphoric acid was also used. This combination is not recommended, because the alkaline buffer substantially reduces the operating life of the column. The level of reporting for both HPLC and MECC was 0.1 mg/L. Benzoic acid was not detected by either MECC or HPLC. A sample spiked with BA at a level of 4 mg/L was also analyzed using this procedure; recoveries of 84% for MECC and 93% for HPLC were recorded. The MECC procedure has the same order of repeatability as the HPLC method and is also faster, more efficient, and less costly to operate. This procedure can also be used for the screening of SA, BA, and dehydroacetic acid in beverages using phthalate as the internal standard (83).

A reversed-phase liquid chromatographic method was developed for simultaneous determination of carboxylic acids, phenolic compounds, and SA in white wines (84). The diluted samples are injected into a Spherisorb ODS-2 column with a gradient of sulphuric acid (pH 2.5)/methanol as mobile phase. A diode array detector is used, set at 210 nm for carboxylic acids and altered to 278 nm, during the run, for phenolics and SA. The identification of compounds is based on retention time and UV spectra. Some cleanup methods (Sep-Pak C_{18} and an ion-exchange column) were tested and did not improve the results. The analysis was considered simple, with no sample preparation. Application of this method was illustrated by analyses of Brazilian Welchriesling wines (84).

A rapid and reliable HPLC method that allows the qualitative and quantitative determination of the most important acids (formic, ascorbic, lactic, citric) occurring naturally or by exterior influence in fruit juices is presented (85). This method is advantageous in quality control, because it brings about a time gain compared to single enzymatic analyses. To obtain the best separation with the RP18 column Spherisorb ODS2, various compositions of the liquid phase were tested (KH_2PO_4 0.01 mol/L, pH 7.0, TBAP 0.00055 mol/L; KH_2PO_4 0.01 mol/L, pH 7.0, TBAP 0.0055 mol/L; KH_2PO_4 0.01 mol/L, pH 2.5, without TBAP; KH_2PO_4 0.01 mol/L, pH 2.5, TBAP 0.0055 mol/L; KH_2PO_4 0.01 mol/L, pH 2.25, TBAP 0.0055 mol/L). The use of the ion-pair reagent tetrabutylammonium dihydrogenphosphate (TBAP) was decisive. Its concentration and the pH value influence the dissociation of the acids. This is essential for a good separation (85).

Reverse-phase HPLC with ion pairing and UV detection at 227 and 210 nm was used to determine sweeteners (acesulfame-K, saccharin, and aspartame), preservatives (BA and SA), and

caffeine simultaneously in 25 samples of soft drinks that had been decarbonated by ultrasound treatment for 15 min. Chromatograms were compared against those for standard solutions of all the additives. Results showed that citric acid could be identified but not quantitatively determined (86).

Ostermeyer (87) described the rapid and sensitive determination of acesulfame, saccharin, aspartame, SA, and BA in fishery products. Aspartame is determined directly in the aqueous extract of samples. For the other additives, cleanup of the extract is performed by anion-exchange chromatography. Ostermeyer used a Nucleosil 100-5 C_{18} (Macherey-Nagel) (250 × 4 mm, 5 μm) column and 0.02 mol/L KH_2PO_4 acetonitrile as mobile phase and 219 nm detection using a diode array detector. Recoveries of spiked samples at concentrations of 50–400 mg sweetener/kg and 50–4000 mg preservative/kg were in the range 84–102% for all analyzed substances (87). The HPLC conditions for the determination of OAs are summarized in Table 5.

II. SYNTHETIC PHENOLIC ANTIOXIDANTS

A. Theory

Antioxidants are compounds that prolong the shelf life of foods by protecting them against deterioration caused by oxidation, such as fat rancidity and color changes (88). Antioxidant technology plays an important role in the utilization of fats and oils as raw materials in food processing and in the marketing of foods containing fats. As such, the proper and effective use of antioxidants is dependent on a basic understanding of:

The chemistry of fats
The mechanism of oxidation
The function of an antioxidant in counteracting this type of deterioration

Antioxidants, to be effective, have to be used in conjunction with good materials, correct processes, and proper packaging and storage conditions, because they do not enhance mediocre-quality products. A good antioxidant system imparts no flavor, odor, or color to finished foods when used under the proper conditions (89).

In general, antioxidants are divided into natural and synthetic groups. Because natural antioxidants are discussed in other chapters ("The Fat-soluble Vitamins," "Analysis of Phenolic Compounds," and "Analysis of Organic Acids"), we deal here only with the determination of synthetic phenolic antioxidants (SPA).

1. History

The use of SPA in foods had its beginning in the late 1940s, when butylated hydroxyanizole (BHA) was found to have antioxidant effectiveness in fatty foods and toxicity studies proved it was safe for food use. Somewhat earlier, several alkyl (including *n*-propyl) esters of gallic acid had been investigated and approved for food use in a number of countries. It soon became apparent that combinations of BHA and propyl gallate (PG) could be use to take advantage of their different antioxidant properties as well as the synergism they exhibited when used together in food fats and oils. Also, at that time it became evident that something was needed to counteract the harmful effects of certain metals, such as iron and copper, in food. Not only are these metals strong catalysts of fat oxidation, but they also may react with antioxidants to cause discoloration. Certain acids were found to provide protective effects, and citric acid became widely used as metal deactivator or chelating agent, in combination with phenolic antioxidants (acid synergism). In 1954, butylated hydroxytoluene (BHT) was approved in the United States and came into use

Table 5 HPLC Systems Used for Determination of Organic Acids

Compounds determined	Sample preparation	Stationary phase	Mobile phase	Detector	Ref.
BA, SA, EsHBAs	Extraction of margarine, washing of diethyl ether, extraction with/NaOH and H_2SO_4	μBondaPak C18	Phosphate buffer–methanol (20:80) (v/v); phosphate buffer–methanol (40:60) (v/v)	UV 235 nm	54
BA, SA, EsHBAs	Filtration of orange juice	Rhodes RS pak DS-613 (150 × 6 mm) polystyrene-divinylbenzene resin RP-18 guard column (40 × 3.4 mm)	0.05 M KH_2PO_4 (pH 2.65)–acetonitrile (60:40) (v/v); 1.0 ml/min	UV 230 nm	2, 55
BA, SA, EsHBAs	Coffee drink, steam-distilled, cleanup—SPE technique	Nucleosil 5 C18 (150 × 4.3 mm): LiChromosorb RP-18 (150 × 4.3 mm); Develosil ODS 5	Acetonitrile–water–0.2 M phosphate buffer pH 3.6 (7:12:1) + 2 mM CTA	UV 235 nm	60
BA, SA, EsHBAs	Fatty samples cleaned on Extrelut column; elution with CH_2Cl_2–diethyl ether (4:1)	LiChrosorb Si 60 (150 × 4.6 mm); μBondaPak C18, 10 μm, (300 × 4.6 mm)	Heptane–diisopropyl ether glacial–acetic acid (88:12:0,1), (90:10:0,05) 1.8 ml/min; water–acetonitrile–H_3PO_4 (90:10:0.5), flow rate 0.8 ml/min	UV 230 nm	2, 61
BA, SA	Beverages, jams; filtration, degassing	PhaseSep C18; Spherisorb ODS1 (250 × 4.6 mm), 5 μm	Methanol–phosphate buffer (8:92) (v/v), pH 6.7 using H_3PO_4; 1.0 ml/min	UV 227 nm	64
BA, SA	Fruit juice; filtration	μBondaPak CN	2% Acetic acid–methanol (95:5) (v/v)	UV 254 nm	65
BA, SA	Citrus juices; filtration	μ-BondaPak CN	2% Acetic acid–methanol (19:1) (v/v) 0.02 M	UV 240 nm	66
BA, SA	Diet foods; clarifying with Carrez	SuperSpher RP-4 μm; LiChrosorb	Phosphate buffer–acetonitrille (90:10) (v/v); 1 ml/min	UV 220 nm	67
Fa, BA, SA, HBA, propionic acid	Vegetable oil, salad dresings, mayonnaise mustard; extraction with diethyl ether, alkalinization, and acidification	RP-18, 7 μm (250 × 4.6 mm)	Ammonia buffer (pH 4.4)–acetonitrile (80:20, v/v), 1–3 ml/min	UV 225 nm, UV 232 nm	69
EsHBAs	Fish products, mustard, juices; derivatization with benzoil chloride	LiChrosorb Si-60 5 μm; RP-18, 7 μm (300 × 3 mm)	Isoacetate–diethyl ether–acetonitrile (500:35:0.3, v/v/v), 1.2 ml/min, methanol–water (80:20, v/v) 0.6 ml/min	UV 240 nm	70
FA, acetic, propionic, lactic acids	Dairy products; centrifugation	Aminex HPX-87 (300 × 7.8 mm)	0.009 N H_2SO_4	UV 220 nm, UV 275 nm	72

(continued)

Table 5 Continued

Compounds determined	Sample preparation	Stationary phase	Mobile phase	Detector	Ref.
BA, SA	Orange juice, citrus juices; filtration	Partisil 10 SAX	0.225 M KH_2PO_4, 1.0 ml/min	UV 226 nm, UV 254 nm	73
SA, propionic acid	Cheese; steam distillation	Li Chrosorb RP-18 (250 × 4 mm)	Methanol–0.02 M phosphate buffer (30:70, v/v) + tetrabutylammonia, 1.0 ml/min	UV 235 nm	74
BA, SA	Various foods; centrifugation	RP-C-18	0.03 M phosphate buffer (pH 6.5)–methanol (95:5, v/v)	UV 227 nm	75
BA, HBA	Beverages, tomato sauce; extraction	μBondaPak C18	methanol–acetic acid–water (20:5:75, v/v/v), (35:5:60, v/v/v)	UV 254 nm	76
BA	Centrifuged, precondition C18 cartridge, adjusted methanol	10 μm polystyrene-divinylbenzene column PRP-1 (250 × 4.1 mm)	Acetonitrile–phosphate buffer (0.05 M KH_2PO_4) (40:60) (v/v)	UV 230 nm	77
SA	Sweet commercial wines; synthetic wines diluted with 0.01 N H_2SO_4, filtered 0.45 μm	AMINEX HPX 87-H (300 × 7.9 mm), 65°C	0.01 N H_2SO_4:CH_3CN (75:25)	UV 258 nm	80
BA, SA	Dried roast beef, sugared fruit, mixed with aqueous α-hydroxyisobutyric acid solution (pH 4.5) and HTA, sonicated/ A/residue washed with acetonitrile–H_2O (2:3, v/v), /B/, A and B combined	Sep-Pak C18, 5 μm	Acetonitrile–50 mM aqueous α-hydroxyisobutyric acid (pH 4.5) (2.2:3.4 or 2.4:3.6, v/v) containing 2.5 mM HTA	UV 233 nm	81
FA, LA, propionic acid	Commercial cheeses; extraction in mobile phase, centrifuged	Nucleosil C18 (120 × 5 mm)	0.5% $(NH_4)_2HPO_4$ buffer (pH 2.24 with H_3PO_4); 0.4% acetonitrile	UV 214 nm	82
BA, SA	Juices, jams, mixed, diluted with deionized water	Spherisorb ODS 2 C18 (3.9 × 300 mm); μBondapak C18 precolumn	KH_2PO_4–K_2HPO_4 2.5 g/L	UV 230 nm	83
	Cheese slices; steam distillation	Exmere ODS 2-8/5 C18 (4.6 × 250 mm)	K_2HPO_4 pH 8 with 1 M H_3PO_4		
FA, LA	Juices, standards of organic acids diluted, filtered	Spherisorb ODS 2 (250 × 4.0 mm, 5 μm) column, temperature 40°C	KH_2PO_4 (0.01 mol/L pH 2.5), TBAP (0.0055 mol/L)	UV 210 nm, UV 270 nm	85

with BHA and PG. The next major development in the area of SPA was marked in 1972, when *tert*-butyl hydroquinone (TBHQ) was commercialized as a food-grade chemical to fill the pressing need for an antioxidant for polyunsaturated oils, such as safflowerseed oil, being used in foods (90). Although a lot of antioxidants were approved for the use in foods, only five compounds find widespread usage throughout the world. These are BHA, BHT, PG, TBHQ, and tocopherols. The products, used either singly or in combination and often combined with acid synergists such as citric acid and ascorbic acid and their corresponding esters, fill the majority of the world's need for antioxidants in food products (91). Some physical properties of SPA are summarized in Table 6.

A special type of polymeric antioxidant is anoxomer, which consists of 1,4-benzenediol, 2-(1,1-dimethylethyl)-polymer with diethylbenzene, 4-(1,1-dimethyl-ethyl)phenol, 4-methoxyphenol, 4,4′-(1-methylethylidene)bis(phenol), and 4-methylphenol prepared by condensation polymerization of divinylbenzene (*m*- and *p*-) with *tert*-butylhydroquinone, *tert*-butylphenol, hydroxyanisole, *p*-cresol, and 4,4′-isopropylidenediphenol. Total monomers, dimers, and trimers below 500 are not more than 1%. Anoxomer is permitted in the United States as an antioxidant in food at a level of not more than 5 ppm of fat and oil content of the food.

2. *Mechanism of Effect*

Antioxidants for fats and oils function by interfering in the formation of the free radicals that initiate and propagate oxidation. Knowledge of the mechanism of antioxidant performance reinforces several important aspects of antioxidant usage.

- Antioxidants are not oxygen scavengers or absorbers. They function by reaction with free radicals.
- Antioxidants must be added to fats, oils, and food products as early as possible for maximum benefit. The addition of antioxidants to fats and oils with a substantial peroxide content will result in a loss of antioxidant performance.
- Antioxidants cannot rejuvenate fats or oils already oxidized.

The oxidation of unsaturated fats and oils is initiated with the formation of free radicals. This reaction occurs on the methylene groups adjacent to the carbon–carbon double bond:

$$[\!-\!C\!-\!CH_2\!-\!C\!=\!C\!-\!] \xrightarrow{-H\cdot} [\!-\!C\!-\!\dot{C}H\!-\!C\!=\!C\!-\!] \quad (1)$$

Fat → Fat Free Radical

The reaction proceeds when the fat-free radicals react with oxygen to form peroxide-free radicals:

$$[\!-\!C\!-\!\dot{C}H\!-\!C\!=\!C\!-\!] + O_2 \longrightarrow [\!-\!C\!-\!CH(O\!-\!O\cdot)\!-\!C\!=\!C\!-\!] \quad (2)$$

Fat Free Radical → Peroxide Free Radical

Table 6 Physical Properties of Synthetic Phenolic Antioxidants

Property	BHA	BHT	PG	OG	DG	TBHQ	NDGA	THBP	Ethoxyquin
State	Vaxy solid	Solid crystals	Solid	Solid	Solid	Solid	Solid	Solid	Liquid
Color	White	White	Cream	White	White	White	White	Yellow	Yellow
Melting point (°C)	48–55	68	146–148	101–104	96–97	126.5–128.5	184–185	149–153	0
Boiling point (°C)	264–270 (733 mm)	265	Decomposes over 148					Decomposes, emits acrid smoke and irritating fumes	125 (2 mm); decomposes, emits toxic fumes of NO_x
Molecular weight (g/mol)	180.27	220.39	212.22	282.34	338.49	166.24	302.36	196.22	217.34
Flash point (°C)						171			
Synergism with:	BHT, PG	BHA, not with gallates	BHA, BHT, not with NDGA			BHA, BHT	Not with PG		
LD_{50} in rats, mice (mg/kg)	2000	890	3800		1600	700		200	800
Soluble in:	Petroleum ether, 50% or higher alcohols, propylene glycol, fats, oils	Toluene, alcohols, methyl ethyl ketone, acetone, petroleum ether, benzene, fats, oils	Ethanol, oils, ether, fats, oils	Oil	Oil	Ethanol, ethyl acetate, acetone, ether; water only slightly	Ethanol, methanol, ether, acetone, glycerin, propylene glycol, diluted alkalies; slightly in hot water, chloroform	Alcohol, propylene glycol; water only slightly	Ethanol

Source: Refs. 3, 89, 90, 91, and 94.

The peroxide-free radicals capture a hydrogen ion from another fat molecule, forming a hydroperoxide and another fatty free radical:

$$[-C-C(H)(O-O\bullet)-C=C-] + [-C-CH_2-C=C-] \longrightarrow [-C-C(H)(O-OH)-C=C-] + [-C-\dot{C}H-C=C-] \quad (3)$$

Peroxide Free Radical; Fat; Hydroproxide; Fat Free Radical

This is the propagating step of free radical oxidation, making it a "chain"-type reaction. Antioxidants delay or inhibit the initiation step,

$$[-C-\dot{C}H-C=C-] + AH \longrightarrow [-C-CH_2-C=C-] + A\bullet \quad (4)$$

Fat Free Radical; Original Fat Molecule; Antioxidant Free Radical

as well as the propagation step:

$$AH + [-C-C(H)(O-O\bullet)-C=C-] \longrightarrow A\bullet + [-C-C(H)(O-OH)-C=C-] \quad (5)$$

Peroxide Free Radical; Antioxidant Free Radical; Hydroperoxide

Antioxidants have to form "stable" low-energy free radicals that will not further propagate the oxidation of fats and oils. From this point of view, the most convenient compounds are the phenolic compounds, which structure allows them to form low-energy radicals through stable resonance hybrids, as follows:

BHA + [—C—C—C=C—] (Fat Free Radical) ⟶

Stable Resonance Hybrid +

[—C—CH₂—C=C—] (Original Fat Molecule) (6)

3. Regulation of Synthetic Phenolic Antioxidants

In most countries of the world, food laws provide regulations for controlling the use of antioxidants in many food applications because of their possible toxicity. Such regulations identify specific approved antioxidants, establish permitted use levels, and include labeling requirements. However, differences among individual countries really exist, i.e., antioxidants permitted in one country may be prohibited in other countries. Moreover, the regulatory situation surrounding food additives is constantly changing. For illustration, Table 7 shows individual synthetic phenolic antioxidants approved for food use in various countries.

Internationally, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) periodically considers food additives, including antioxidants, on the basis of all available scientific data to establish acceptable daily intake levels (92) and specifications on the identity and purity of the additives. The conclusions of JECFA are published in numerous reports and technological summaries by the World Health Organization (WHO) as the WHO Technical Report Series and WHO Food Additives Series. Such information provided by JECFA is used by the Joint FAO/WHO Codex Alimentarius Commission in implementing the Joint FAO/WHO Standards Program, which was established to elaborate international standards for foods to protect the health of consumers, to ensure fair practices in food trade, and to facilitate international trade (90).

B. Determination of Synthetic Phenolic Antioxidants

Many research experiments have been conducted to determine the presence and quantity of SPA in foods. As literature reviews show, a particular emphasis has developed in the recovery procedures for antioxidants and in quantification procedures, including colorimetric methods, spectrophotometric methods in the UV region, paper and thin-layer chromatographic methods, gas

Table 7 Synthetic Phenolic Antioxidants Approved for Food Use in Various Countries/Communities

Country/ community	BHA	BHT	PG	OG	DG	TBHQ	NDGA	THBP	Ethoxy- quin	Ionox 100
Australia	+	+	+	+	+	+				
Brazil	+	+	+			+				
Canada	+	+	+							
Codex Alimentarius Commision	+	+	+			+				
European Union	+	+	+	+	+					
China	+	+	+	+	+					
Japan	+	+	+	+	+					
New Zealand	+	+	+	+	+	+				
Norway	+	+	+	+	+					
Philippines	+	+	+			+			+	
Slovak Republic	+	+	+	+	+					
Switzerland	+	+	+	+	+					
United States	+	+	+	+	+	+		+	+	+

chromatography methods for free antioxidants and their derivatives, methods based on high-performance liquid chromatography, electrochemical, and miscellaneous techniques. As stated, the earlier spectroscopic techniques have been replaced by chromatographic methods (93–96).

1. Determination of Synthetic Phenolic Antioxidants by HPLC

Determination of SPA by HPLC has became a dominant analytical procedure because of its advantages:

- Simple sample treatment
- Possibility of using a preseparation column in the HPLC system to remove impurities
- Possibility of changing the polarity of mobile phase during analysis to separate and elute SPA with different polarity
- Short time of analysis
- High reproducibility and sufficient detection limit

2. Sample Preparation

At the beginning of HPLC use, due to the complexity and variety of matrices that occurred in foods as well as their different properties and the evaluation level of techniques and equipment, it was practically impossible to use a single universal procedure for all SPA determinations. However, contemporary procedures and techniques make it possible to determine all SPA in one procedure.

Because SPA usually occur in foods at concentration levels up to 200 ppm, the appropriate extraction and isolation techniques for individual procedures depend on various factors:

- The character of the matrix (polar semipolar, nonpolar)
- The presence of interfering substances
- The number and physicochemical properties of SPA to be determined

Determination may be also affected by the choice of solvent to be used for extraction. Table 8 presents SPA in decreasing order of polarity.

Generally, effective extraction of SPA from samples might not be easy because of their relative low concentration in foods. Techniques for isolating SPA from the food matrix are based on direct organic solvent extraction (97–100), steam distillation (101–102), or solid-phase extraction (104). Problems of quantitative determination are usually associated with incomplete extraction of the SPA or with the coextraction of potentially interfering substances. For quantitative determination a particular emphasis has to be made during solvent evaporation in vacuum to prevent the loss of BHT (98) or the oxidation of TBHQ (105). The use of pure solvents for extraction purposes is also crucial, for trace impurities may cause losses of SPA (95). Also, the presence of peroxides in nonfreshly rectified solvents can cause loss of SPA. One widely used isolation technique is direct extraction with solvents (97,106), especially in the case of low-fat foods, with subsequent cleaning of the extracted matter. In this case, to eliminate interfering substances, a liquid–liquid partition (98), fractionation using gel chromatography (106), column cleanup using a silica gel column (107), thin silica gel layers (108,110), or a Florisil column (109) has been used.

The most suitable solvents for extracting SPA from fats are acetonitrile (113,125,126, 139,142) and water–alcohol mixtures. The fat is usually dissolved in hexane or petroleum ether, and SPA are extracted into acetonitrile (105,110–113,125,126,128). The disadvantages of acetonitrile extraction are that (a) BHT recovery is low and (b) moderately high levels of interfering compounds are coextracted. The advantage of aqueous methanolic extraction of SPA from nonpolar solvent is that the fat is mostly excluded (99,114). Hammond (99) described a methanolic extraction of a melted fat sample, heated to 40–50°C, followed by transfer of the mixture to a deep freeze for a few hours to aid the solidification of any excess fat from methanol. The methanol layer was then decanted and filtered prior to the addition of an internal standard and direct injection.

A procedure for extracting BHA and BHT from vegetable oils has been reported by Phipps (98). The oil was dissolved in *n*-heptane and extracted with dimethylsulphoxide (DMSO). Extracts of DMSO were mixed with aqueous sodium chloride solution, and the SPA were back-extracted into petroleum ether with subsequent concentration and analysis.

Mizutani et al. (144) derivatized BHT to 2,6-di-*tert*-butyl-4-methyl-4-methoxy-2,5-cyclohexadienone (BMMC) using up the quantitative Coppinger and Campbell reaction of BHT with bromine in methanol. Separation was carried out on a RadialPak μPorasil column using a hexane–2-propanol mixture as mobile phase. Detection of BMMC was monitored at 236 nm,

Table 8 Individual Synthetic Phenolic Antioxidants, in Decreasing Order of Polarity

Compound	Abbreviation
Propyl gallate	PG
2,4,5-Trihydroxybutyrophenone	THBP
t-Butylhydroquinone	TBHQ
Nordihydroguaiaretic acid	NDGA
2(or 3)(*t*-Butyl)-4-hydroxyanisole	BHA
2,6-di(*t*-Butyl)-4-hydroxymethylphenol	Ionox-100
Octyl gallate	OG
Dodecyl gallate	DG
3,5-(*t*-Butyl)-4-hydroxytoluene	BHT

Source: Ref. 95.

where BMMC gave a narrow symmetrical peak, the peak height was linearly dependent on the amount, and the minimum detectable amount was 0.5 ng. Galensa (71) converted food additives, including BHA, BHT, PG, octyl gallate (OG), and dodecyl gallate (DG), to their benzoylated derivatives using benzoyl chloride in pyridine. For separation of the derivatives, a concave gradient was used on a reverse-phase column. The method showed high selectivity and sensitivity, but it was a time consuming, because the elution of derivatives took 1 hour.

C. Separation Techniques

At present, in almost all cases, reverse phases made of chemically bonded octadecyl silica with a 5-μm particle size have been used for the separation of SPA. In special cases, i.e., for the effective separation of BHA isomers, γ-aminopropyl packing was used, modified with *N*-3,5-dinitrobenzoyl derivative of D-phenylglycine (116).

D. Detection Systems

The most common detectors used for the determination of SPA are the detectors that operate at the UV wavelength of 233 or 280 nm (104,111,113,115–119,126,144,71). Fluorometric detectors are used mainly for the determination of ethoxyquin (133–135), and it is also possible to use electrochemical detectors (123,137). A very progressive electrochemical detection system is based on measuring the "array potential" of SPA, with the previous oxidative removal of impurities (128).

E. Applications in Foods

A fast, sensitive, and convenient method for the determination of TBHQ in maize oils was described in which a sample was injected without any treatment (115). The sample was injected directly on a 25 × 0.4-cm-ID column packed with Lichrosorb SI 60. The mobile phase was a mixture of dioxane and *n*-hexane, and the eluent was directed to a fluorescence detector that operated at a 309-nm excitation wavelength and a 340-nm emission wavelength. A recovery of 98.5% was obtained, with a coefficient of 2.4% when the detection limit of 6 ng was reached; the average analysis time per sample was 5 min.

High-performance LC is also a suitable method for separating BHA isomers. Commercially available BHA is a mixture of two positional isomers, an approximately 85:15 ratio of 3-BHA and 2-BHA. The former is approximately 2.4 times more effective as a food antioxidant than is 2-BHA, and half as effective as an inhibitor of benzo(a)pyrene-induced for stomach neoplasia in mice. For the separation, Ansari (116) used isocratic elution with 7% of 2-propanol in hexane on a Pirkle Type I-A column packed with 5-μm γ-aminopropyl packing, modified with *N*-3,5-dinitrobenzoyl derivative of D-phenylglycine. Column eluent was monitored at 288 nm, with a detection limit between 1 and 2 ng. Under these conditions, isomers were separated without derivatization, where the phenolic group of 3-BHA was sterically hindered by an *o-tert*-butyl group and therefore could not interact with stationary phase that resulted in its rapid elution.

A rapid, sensitive, reversed-phase HPLC method for the separation of BHA isomers was described by Berridge et al. (117). Using a column packed with Hypersil ODS of 3-μm particle size and with a mobile phase consisting of an acetonitrile–water mixture, it was possible to detect less than 0.5 ng of the isomers injected. The procedure is reliable and robust and compared to another HPLC method, claimed to have a more stable, longer-lasting column requiring only occasional maintenance (117).

A procedure for determining BHT, BHA, and TBHQ in fats and oils onto either of two

normal-phase columns was elaborated by Indyk and Wolard (118). The procedure involves dilution of the sample in isocratic ternary mobile phase consisting of hexane–methylene chloride–acetonitrile, followed by HPLC on a silica or cyanopropyl column with UV detection at 280 nm. The method was successful in determining the three antioxidants in beef tallow and in palm, soy, and maize oils. The limits of detection were 3 ppm for BHT and BHA and 10 ppm for TBHQ. On the other hand, attempts to elute NDGA, THBP, and PG failed when peaks were broad, poorly defined, and hence unsuitable for quantification. Programmed solvent gradients also failed to achieve a satisfactory result.

Beker and Lovrec (119) developed the method for the determination of BHT in poultry premix. BHT was extracted from the sample with methanol, extract filtered, and injected onto HIBAR LiChrosorb RP-18 column. As a mobile phase, 5% water in methanol with a flow rate of 1 ml/min was used, and eluent was detected at 280 nm. The recovery varied from 98.7 to 101.8%, with a coefficient of variation from 1.58 to 3.05%.

Anderson and Niekerk (120) evaluated a simple HPLC procedure that requires neither extraction nor derivatization for the direct determination of six antioxidants: BHA, PG, OG, DG, TBHQ, and NDGA in edible oils and fats. Separations were achieved using a 25-cm × 0.45-cm column packed with 5 μm LiChrosorb DIOL using a mobile phase of hexane, 1,4-dioxane, and acetonitrile, with detection at 280 nm. The procedure may be applied to the analysis of groundnut, soybean, marula, sunflower, safflower, and rapeseed oils and beef fat. Interference from coeluting materials restricts application of the procedure to the determination of PG and NDGA in corn oil and only to NDGA in cottonseed oil. Under the HPLC conditions used, BHT is not resolved from the neutral lipids in the sample. The method is useful for the rapid determination of BHA, DG, TBHQ, OG, PG, and NDGA at relatively low levels in numerous types of oils and fat.

A qualitative and quantitative HPLC method for analysis of mixtures of 12 antioxidants was described Grosset et al. (121). For the identification of the components present, gradient elution with a convex profile from 35:65 water–methanol to pure methanol is used, on a Waters 5-μm C18 column, with UV detector. Propyl gallate was not separated by this system. For quantitative analysis, with UV and electrochemical detectors in series, the water–methanol mixture or pure methanol was used as the eluent, under isocratic conditions, with lithium perchlorate as supporting electrolyte. An applied potential ranging from +0.8 to +1.7 V allows detection of all the antioxidants tested. Both modes of detection were very sensitive, with limits of detection as low as 61 pg.

A combined system consisting of online nonaqueous size-exclusion chromatography (SEC) with reverse-phase chromatography was tested by Williams et al. (122) for the determination of BHT in extruded potato snacks. A sample was homogenized in an acetone–hexane mixture and then filtered and re-extracted with the same solvent. Combined filtrates were dried over sodium sulfate, and solvent was removed by rotary evaporation. Lipid residues were dissolved in toluene and injected into a poly(styrene-divinylbenzene) size-exclusion column using tetrahydrofuran as mobile phase. Separated fractions of crude lipid extract were analyzed on a reversed-phase Spherisorb ODS column using gradient elution. Butylated hydroxytoluene was detected at a wavelength of 254 nm. As found, coupled nonaqueous SEC and reversed-phase chromatography may be used for the determination in crude lipid extracts of analytes having polarity equal to or greater than that of phenol, with a detection limit of about 0.5 ppm.

Rustan et al. (123) used an isocratic HPLC method for the determination of alpha-, gamma-, and delta-tocopherol, BHT, BHA, PG, OG, DG, NDGA, TBHQ, ascorbyl palmitate, and beta-carotene in foods. An RP18 column was used in experiments, and seven mobile phases based on various combinations of acetonitrile, methanol, water, and tetrahydrofuran were tested. Trials with carrot juice, dried milk formula for infants, and aperitif cakes showed that all 12 antioxidants could be determined by a single isocratic HPLC analysis. The optimum mobile phase

was acetonitrile/tetrahydrofuran/water (65:20:2) for carrot juice and dried milk formula for babies, and acetonitrile/tetrahydrofuran/water (55:30:45) for aperitif cakes.

Andrikopoulos et al. (124) separated triglycerides, together with nine synthetic phenolic antioxidants most commonly used to prevent oxidation of edible oils and fats, as well as the natural antioxidants tocopherol and alpha-tocopherol acetate by HPLC using a reversed-phase C18 column and gradient elution with water/acetonitrile/methanol/isopropanol. Except for dilution of the oil with isopropanol/hexane, no further sample preparation was required. Ultraviolet detection was applied. The synthetic antioxidants PG, OG, DG, BHA, TBHQ, BHT, Ionox 100, THBP, and NDGA, as well as alpha- and delta-tocopherol and alpha-tocopherol acetate were separated.

Asap and Augustin (125) analyzed TBHQ content in frying oil. After solubilization in hexane, TBHQ was extracted into acetonitrile and analyzed on an ODS column using acetonitrile–*n*-butanol–water as mobile phase. Quantification of TBHQ was achieved at a wavelength of 292 nm.

Ten laboratories collaboratively studied an HPLC method for the determination of PG, OG, DG, THBP, TBHQ, NDGA, BHA, Ionox 100, and BHT in butter oil (126). A sample was mixed with hexane saturated with acetonitrile, and SPA were extracted with acetonitrile. After evaporation, the SPA were analyzed using a gradient liquid chromatographic system consisting of 5% acetic acid in water (A) and acetonitrile/methanol (1:1) (B). A linear gradient was run from 30% (B) in (A) to 100% (B) over 10 min, with a hold until the last antioxidant (DG) was eluted at a flow rate of 2 ml/min throughout a C_{18} bonded spherical silica column, and eluent was detected at 280 nm. Chromatographic separation of SPA standards is shown in Fig. 2. The effect of the stationary phase on the process of separation, on elution time, as well as on baseline is shown in

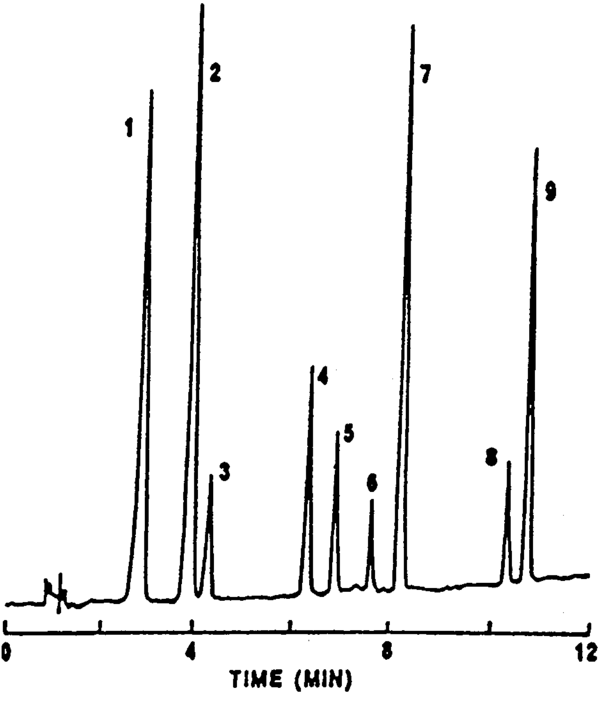


Fig. 2 HPLC chromatogram of SPA standards, ca. 0.1 μg each. 1—PG, 2—THBP, 3—TBHQ, 4—NDGA, 5—BHA, 6—Ionox 100, 7—OG, 8—BHT, 9—DG. (Reprinted from Ref. 126, Figure 983.15. Copyright 1993, by AOAC INTERNATIONAL.)

Fig. 3. Although the greater efficiency of the columns packed with the spherical material compared with the nonspherical material is evident, the results showed that separation using the latter material could also be acceptable. Therefore, the primary consideration in selecting the stationary phase should be that of acceptable separation, rather than the packaging material itself. Overall mean recoveries for PG, THBP, TBHQ, NDGA, BHA, OG, Ionox 100, BHT, and DG were 100.9, 97.8, 103.4, 95.4, 97.4, 93.6, 95.5, 79.0, and 96.2%, respectively. The overall reproducibility relative standard deviations were 8.55, 17.4, 25.6, 14.5, 6.60, 9.64, 10.8, 11.4, and 7.35%, respectively. As found in this study, TBHQ was rapidly oxidized, as had already been stated before (105). The oxidized product, with lower response, elutes between TBHQ and NDGA and increases as the parent TBHQ disappears. On the basis of the obtained results, the AOAC method 983.15 was modified (113). *tert*-Butyl hydroquinone and BHA were determined, together with preservatives and sweeteners, after their separation via SPE technique (104). Soy sauce was mixed with α-hydroxyisobutyric acid (HTA) solution, with pH 4.5, and hexadecyltrimethylammonium bromide as ion-pair reagent. After thorough mixing and cleaning on a C_{18} cartridge [additives were eluted with a mixture of water and HTA (first fraction) and acetonitrile–water (second fraction)], both were combined, diluted in a mixture of acetonitrile–water, filtered through 0.2-μm membrane filter, and analyzed. Dried roast beef and sugared fruit were ground and then mixed with α-hydroxyisobutyric acid solution, with pH 4.5, and hexadecyltrimethylammonium bromide. After sonication filtered and residue washed with acetonitrile–water. Combined filtrates were precleaned via SPE technique and analyzed. A mobile phase of acetonitrile–50 mM aqueous α-hydroxyisobutyric acid solution containing the ion-pairing reagent hexadecyltrimethyl ammonium bromide and a C_{18} column with a flow rate of 1.0 ml/min and detection at 233 nm were used to separate 15 food additives.

A specific reverse-phase method for the determination of seven antioxidants (GP, THBP, TBHQ, BHA, BHT, OG, DG) in margarines by means of internal standard was developed by Irache et al. (111). The method is based on extraction with acetonitrile, rinsing the extract with an acetonitrile–isopropanol mixture, and analysis by reverse-phase HPLC on a C18 Spherisorb ODS-2 column with an acetic acid–water–acetonitrile (5:70:25) mobile phase followed by a gradient to acetic acid–water–acetonitrile (5:5:90). A detector operated at 280 nm and trials with seven antioxidants showed a recovery of 73.7–100.2% and a detection limit of 0.5–1.1 μg/g. This procedure enables a good resolution between the peaks and avoids interferences due to the presence of some preservatives, i.e., sorbic and benzoic acids, and methyl paraben. This procedure was then applied to the determination of the stability of BHA, BHT, and DG in a commercial margarine during storage at -18, 6, 20, and 50°C.

Hall et al. (127) compared free solution capillary electrophoresis (FSCE) and micellar electrokinetic capillary chromatography (MEKC) techniques with HPLC analysis. Four major food-grade antioxidants, propyl gallate (PG), BHA, BHT, and TBHQ, were separated. Resolution of the 4 antioxidants was not successful with FSCE, but was with MEKC. Separation was completed with excellent resolution and efficiency within 6 min and picomole amounts of the antioxidants were detectable using UV absorption. In contrast, reversed-phase HPLC separation was not as efficient and required larger sample amounts and longer separation time.

An HPLC method using progressive electrochemical detection of SPA was described by McCabe and Acworth (128). Samples were mixed with hexane, and SPA were extracted with acetonitrile. An HPLC analysis of the extracts was performed, without an evaporation step, on a high-pressure Coul Array system in which analytes were detected on two coulometric array-cell modules, each containing four electrochemical sensors attached in series after the column. Analytes were separated on a Supelcosil LC-18, 5-μm column using gradient elution and detected at potentials of -50, 0, 70, 250, 375, 500, 675, and 825 mV. To remove oxidative impurities to be coeluted with BHT, a guard cell with applied potential of 900 mV was also placed in the system.

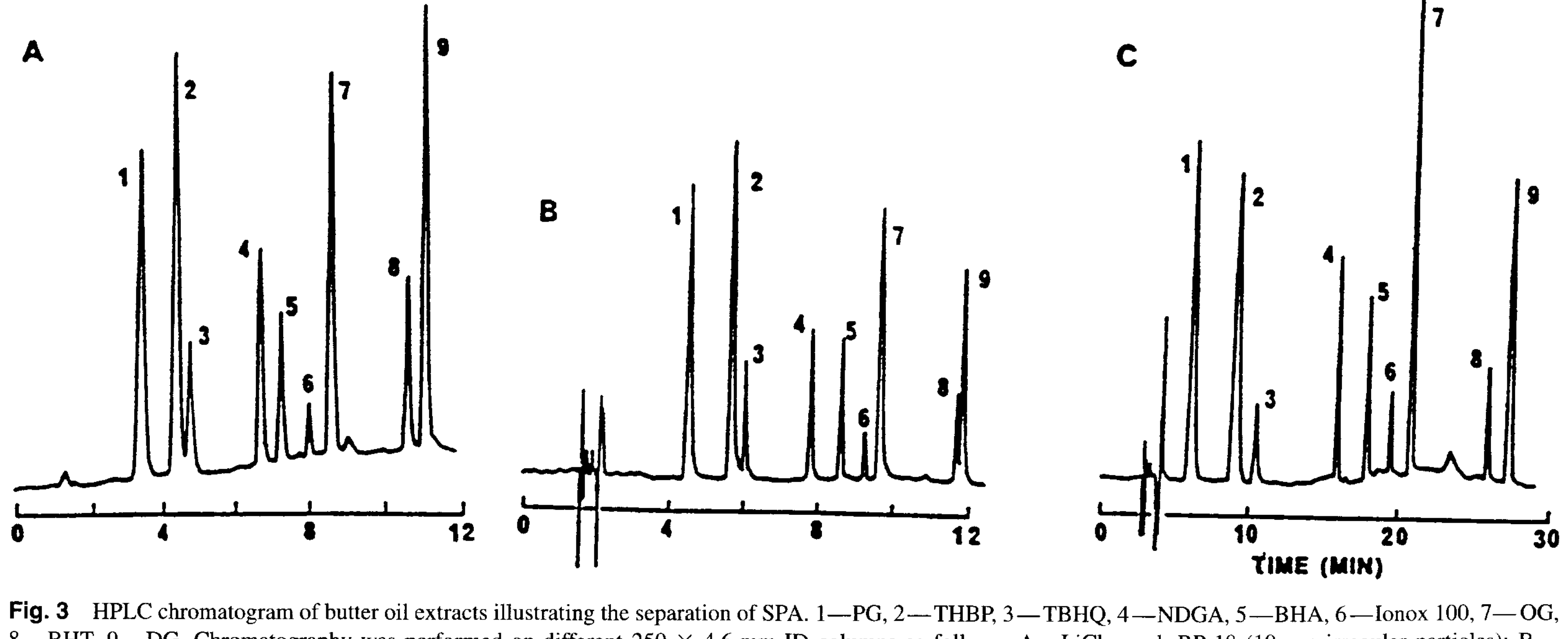


Fig. 3 HPLC chromatogram of butter oil extracts illustrating the separation of SPA. 1—PG, 2—THBP, 3—TBHQ, 4—NDGA, 5—BHA, 6—Ionox 100, 7—OG, 8—BHT, 9—DG. Chromatography was performed on different 250 × 4.6-mm-ID columns as follows: A—LiChrosorb RP-18 (10-μm irregular particles); B—Spherisorb ODS (5-μm spherical particles); C—TSK ODS-120T (5-μm spherical particles). (Reprinted from Ref. 126, Figure 1. Copyright 1993, by AOAC INTERNATIONAL.)

The method was applied to the simultaneous detection of PG, THBP, TBHQ, NDGA, BHA, Ionox 100, OG, BHT, and DG in butter, margarine, shortening, lard, and hand cream. The advantages of the present method over the conventional AOAC method include simpler sample preparation, lower detection limits (10–200 pg for HPLC–ECD and 2000–10,000 pg for HPLC–UV), wider linear responses of ECD (three–four orders of magnitude) to UV detection (two orders of magnitude), higher sample throughput, lower solvent consumption, and lower costs and time per analysis.

Ethoxyquin, a synthetic antioxidant, is not generally allowed for human consumption in foods, but it is being added to animal feed and to fruits as an antiscald agent (94,143). Ethoxyquin is also used in the spice industry to prevent carotenoid loss during postharvest handling. However, ethoxyquin-treated paprika is unacceptable for some markets and some consumers (129). Perfetti et al. (130) described a method for determination of ethoxyquin in paprika and chili powder. Ethoxyquin was extracted from the spice with hexane and partitioned into 0.3 N HCl. After adjusting the solution to pH 13–14, ethoxyquin was extracted into hexane, and the hexane layer was evaporated to dryness. An acetonitrile solution of the residue was then analyzed by reversed-phase HPLC, with detection at 254 nm. The mobile phase was water/acetonitrile with ammonium acetate buffer. Recoveries from samples fortified at 50, 100, and 200 ppm averaged 92%, with a coefficient of variation of 2.3%. The method was applied to a number of commercial samples of paprika and chili powder. Ethoxyquin was found in paprika samples at levels up to 63 ppm and in chili powder samples at levels up to 20 ppm.

Perfetti et al. (131) described a method for the determination of ethoxyquin in milk. Milk solids were precipitated by adding acetonitrile, and the water–acetonitrile supernatant was washed with hexane to remove fat. The addition of NaCl caused the water–acetonitrile solution to separate into an aqueous phase and an acetonitrile phase, thus separating ethoxyquin from most water-soluble impurities. A large volume of water was then added to the acetonitrile layer, and ethoxyquin was partitioned into hexane and removed at reduced pressure. The residue was dissolved in the mobile phase and analyzed on a 250-mm × 4.6-mm-ID. Ultrasphere ODS column using fluorescence detection with excitation of 230 nm, and emission of 418 nm, respectively. A mixture of water and acetonitrile with a diethylamine–acetic acid buffer was the mobile phase. Recoveries from milk samples fortified at 1, 5, and 10 ng/g averaged 78%, with a coefficient of variation of 5.0%. Low concentrations (less than 1 ng/g) of apparent ethoxyquin were detected in commercial milk samples analyzed by this method.

A procedure for the analysis of fruit for residues of postharvest preservatives and ethoxyquin was described by Gieger (132). Residues were extracted with dichloromethane via ultrasonic treatment. The extracts were analyzed on a C_{18} 5-μm column, with methanol/0.01 M ammonium acetate (61:39) as a mobile phase adjusted to pH 7.5–7.7. Recovery for ethoxyquin was 65–78%, with a detection limit of approximately 0.05 mg/kg.

A method for the determination of ethoxyquin in paprika that avoided the previous separation steps from other colored substances was proposed by Viñas (133). Analysis is carried out by reverse-phase HPLC using the gradient elution technique and UV detection at 270 nm. Using fluorimetric detection with excitation at 311 nm and emission at 444 nm, a detection limit of 0.2 μg/ml was reached. The method can be applied to the determination of ethoxyquin in commercial samples in the presence of paprika (*Capsicum annuum*) carotenoids.

A collaborative study for the determination of ethoxyquin in various meals and extruded pet foods was done under Schreier and Greene (134). Eleven laboratories took part in the study and determined ethoxyquin in acetonitrile extracts of ground samples using a column packed with C_{18} octadecylsilane and a mixture of acetonitrile and ammonium acetate solution as a mobile phase. The method was tested for the analysis of 16 samples that contained ethoxyquin from 0.25 to 289 ppm. Repeatability standard deviations ranged from 0.08 to 3.2 ppm, and repeatabil-

ity relative standard deviations ranged from 4.5 to 32%. Reproducibility standard deviations ranged from 0.12 to 13 ppm, and reproducibility relative standard deviations ranged from 4.5 to 55%. On the basis of the results it was recommended that the HPLC method for the determination of ethoxyquin in feeds be adopted per the first action method of the AOAC Official Methods.

Kato and Kanohta (135) dealt with the determination of degradation products of ethoxyquin using HPLC, GC-MS, and NMR spectrometer, respectively. For HPLC analysis, an ODS column was used, with a methanol–water mixture as mobile phase. Ethoxyquin and its oxidized products were detected at 254 and 380 nm, as well as fluorimetrically. As found, the main autooxidation product of ethoxyquin was its dimer, which converted into many other products upon exposure to daylight.

High-performance LC systems used for the determination of SPA are presented in Table 9.

Table 9 HPLC Systems Used for the Determination of Synthetic Phenolic Antioxidants

Antioxidant	Sample/treatment	Stationary phase	Mobile phase	Detection	Refs.
PG, THBP, TBHQ, NDGA, BHA, BHT, Ionox 100, OG, DG	Oils, fats, and butter oil; extraction with acetonitrile from hexane solution	C_{18}-bonded spherical silica	1. 5% Acetic acid in water 2. Acetonitrile/methanol (1:1) Linear gradient from 30% (2) in (1) to 100% (2), flow rate 2 ml/min	UV 280 nm	113, 126
TBHQ, BHA	Dried roast beef, soy sauce, sugared fruit; solid-phase extraction	5-μm C_{18} column 25 × 4.6-mm ID	Acetonitrile/aqueous α-hydroxyisobutyric acid solution (pH 4.5) (2.2:3.4 or 2.4:3.6) (v/v) containing ion-pairing reagent hexadecyltrimethyl–ammonium bromide	UV 233 nm	104
BHA, DG, BHT, THBP, PG, TBHQ, OG	Margarine; extraction with acetonitrile from hexane solution	C_{18} Spherisorb ODS 2 3 μm, 150 × 4 mm	Acetic acid/water/acetonitrile 5:70:25 (v/v/v), for 4 min isocratically, then linear gradient to acetic acid/water/acetonitrile 5:5:90 (v/v/v), flow rate 1 ml/min	UV 280 nm	111
TBHQ	Maize oil	25 × 0.4-cm-ID column packed with 5-μm Lichrosorb SI 60 (Merck)	Dioxane/*n*-hexane 24:76 (v/v), flow rate 3 ml/min	Fluorimetric detection: 309 nm excitation, 340 nm emission	115
Isomers of BHA	Mixture of 2-BHA and 3-BHA	5-μm λ-aminopropyl packing, modified with *N*-3,5-dinitrobenzoyl derevative of D-phenylglycine	2-propanol/hexane 7:93 (v/v), 1 ml/min	UV 288 nm	116
Isomers of BHA	Mixture of 2-BHA and 3-BHA	10 × 0.5-cm-ID column packed with 3-μm Hypersil ODS	Acetonitrile/water 2:3 (v/v), flow rate 2 ml/min	UV 228 nm	117
BHA, TBHQ, BHT	Oils, fats; solving in mobile phase	Waters 10-μm μPorasil column (30 cm × 3.9-mm ID), Rad Pak Cyano cartridge and Rad Pak silica cartridges (5 μm, 8-mm ID)	Hexane/methylene chloride/acetonitrile 85:9.5:5.5 (v/v/v), hexane/methylene chloride/acetonitrile 88.1:9.8:2.1 (v/v/v), flow rate 0.8 ml/min	UV 280 nm	118
BHT	Poultry premix; extraction into methanol, filtration, and dilution with mobile phase	HIBAR LiChrosorb RP-18, 25-cm × 4-mm-ID column	5% water in methanol, flow rate 1 ml/min	UV 280 nm	119

BHT	Extruded potato snacks; homogenization in aceton/hexane 1:1 (v/v)	Poly(styrene-divinylbenzene), PL gel, 100 Å, 5 μm, 300 × 7.7 mm, and Spherisorb ODS 5 μm, 250 × 4.9 mm	Gradient: 0–15 min; water, 15–17 min; 0–20% acetonitrile, 17–30 min; 20–80% acetonitrile, 30–35 min; 80–100% acetonitrile, 35–40 min; 100% acetonitrile	UV 254 nm	122
BHT, TBHQ, BHA, NDGA, PG	Carrot juices, powdered milk, appetizer cakes	LiChrocart RP 18	Acetonitrile/tetrahydrofuran/water	Electrochemical	123
PG, THBP, TBHQ, NDGA, BHA, BHT, Ionox 100, OG, DG	Oils; dDilution of the oil with isopropanol/hexane	Nucleosil C_{18}	Gradient elution with water/acetonitrile/methanol/isopropanol	UV diode array	124
TBHQ	Oils; solution in hexane and extraction with acetonitrile	Hypersil ODS	Acetonitrile/*n*-butanol/water	UV 292 nm	125
Ethoxyquin	Paprika; extraction into ethyl acetate	Spherisorb ODS-2 with particle size of 5 μm	Gradient: acetonitrile/water from 80:20 to 100% acetonitrile, acetonitrile/ethyl acetate (5:95); flow rate 2 ml/min	UV 270 nm Fluorimetric detection: 311 nm excitation, 444 nm emission	133
Ethoxyquin	Various meals and extruded pet foods; extraction of ground samples with acetonitrile	250 × 4.6-mm-ID column packed with C_{18} octadecylsilane, 5-μm spherical, 100-Å pore size	Acetonitrile/0.01 N ammonium acetate 70:30 (v/v), flow rate 1.3 ml/min	Fluorimetric detection: 360 nm excitation, 432 nm emission	134
Ethoxyquin	Standard of ethoxyquin	250 × 4.6-mm-ID column packed with ODS–silica	Methanol/water 90:10 (v/v), flow rate 1.0 ml/min	UV 254 and 380 nm Fluorimetric detection: 360 nm excitation, 440 nm emission	135
TBHQ, BHA, BHT, gallates	Fats and oils; extraction into methanol	BondaPak C_{18}	Gradient elution; water + 1% acetic acid/methanol + 1% acetic acid from 50:50 to 10:90 (v/v)	280 nm	136
BHA, BHT, TBHQ	Model system	Partisil PXS ODS 2	0.05 M $LiClO_4$ in methanol/water 30:70, 65:35, 85:15 (v/v)	UV, fluorimetric, and electrochemical in series	137

(continued)

Table 9 Continued

Antioxidant	Sample/treatment	Stationary phase	Mobile phase	Detection	Refs.
BHT, BHA, PG, OG, DG, ethyl gallate	Oils, fats; solution in methanol	ODS Sil XII	Methanol/water (10:0, 9:1, 8:2, 7:3)	UV 280 nm	138
BHT, BHA, TBHQ	Fats, oils; extraction from hexane solution with acetonitrile	LiChrosorb RP-18	Acetonitrile/water–phosphoric acid, gradient elution	UV 280 nm	139
BHT, BHA	Buter, meat products, bakery; extraction into methanol	ODS-Sil XI	Methanol/water (45:55, 65:25)	UV 280 nm	140
Ionox 330, Irganox 1076, BHT	Fats and oils; dissolution in chloroform	Nucleosil 10 C18	Methanol/water	UV 280 nm	141
BHT	BHT; derivatization to cyclohexadienone	10 × 0.8-cm ID RadialPak μPorasil	Hexane/2-propanol 99:1 (v/v), flow rate 1 ml/min	UV 236 nm	144
BHA, BHT, NDGA, PG, OG, DG	BHA, BHT, NDGA, PG, OG, DG; derivatization to benzoylated derivatives	Ultrasphere-ODS RP-18	1. Acetonitrile/water (50:35) (v/v) 2. Acetonitrile/water/*tert*-butylmethylether (110:35:40) (v/v/v), flow rate 1 ml/min, gradient 2% of 2, that to 100% of 2 during 40 min, then to 2% of 2 during 10 min and 10 min isocratically	UV 230 nm	71
PG, THBP, TBHQ, NDGA, BHA, Ionox 100, OG, BHT, DG	Butter, lard, margarine, shortening, hand creams; extraction into acetonitrile after mixing with hexane	Supelcosil LC-18 5 μm, 150 × 4.6-mm ID	A: Water that contained 25 mM sodium acetate and 25 mM citric acid–methanol 95:5 (v/v) B: Water that contained 25 mM sodium acetate and 25 mM citric acid–methanol–acetonitrile 20:40:40. Gradient: Initial 25% B with linear increase to 100% B over 12 min, hold for 8 min, return to 25% B and hold 10 min	Electrochemical, UV 280 nm	128

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HPLC Determination of Antimicrobial Residues in Edible Animal Products

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I. INTRODUCTION

Antimicrobial drugs are widely used for prevention and treatment in food-producing animals. They are applied as feed additives or therapeutically by injection. The antimicrobial applications could result in the presence of their residues in food chain samples, which very often could have a negative influence on human health. Therefore, the demands for the control of antimicrobial concentration levels in various food commodities are very realistic.

Antimicrobial agents are either completely natural (derived from living organisms—antibiotics) or synthetic. But both of these groups involve antimicrobial activities, and they have been used for many years in the treatment of diseases and as dietary supplements. Additionally, they may be used prophylactically, to prevent disease and to promote growth. The antibiotics fall into six classes: penicillins, tetracyclines, macrolides, aminoglycosides, polyethers, and amphenicols. Strictly speaking, sulphonamides, nitrofurans, and quinolones are not antibiotics, being synthetic (1).

To safeguard human health, the European Union (EU) has established safe maximum residue limits (MRLs) for residues of veterinary drugs in animal and food samples (2,3). The Council Directive 96/23/EU has established numbers of samples to be tested for each compound group (4).

The U.S. Food and Drug Administration (FDA) has also placed severe restrictions on the use of some classes of antibacterial drugs (5) because of concerns about drug-resistant bacteria (6).

A. Methods for Monitoring Residues of Antibacterial Drugs

Monitoring antibacterial agents in food samples involves analytical and bioanalytical methods. Immunological or microbial inhibition screening tests are commonly used for the determination of these drugs. Screening methods, except for a few immunoassays, are not able to identify individual antibacterial agents, and very often false-positive results are obtained. But some drugs could be detected under MRL values without the complicated, time-consuming sample preparation steps.

Analytical techniques have also been successfully developed to identify and quantify these compounds. The scientific literature is rich in descriptions of the various methods. Several chapters in a recently published multiauthor book (7–13) and articles (6,14,15) have provided excellent reviews of the methodologies available for the analysis of antimicrobial drugs from biomatrices.

For the characterization and quantitation of drug residues, compounds must be separated from one another and from the food matrix too. Both chromatographic and electrophoretic separations have been used to monitor antimicrobial agents. But capillary zone electrophoresis, an emerging technique, has not been successfully applied to the determination of antibiotic residues. Of chromatographic techniques, high-performance liquid chromatography (HPLC) is generally preferred for antibiotic residue analysis. There are also a few reports of the use of gas chromatography (GC) and thin-layer chromatography (TLC) as inexpensive alternatives. Supercritical-fluid chromatography (SFC), another emerging technique, does not work well with most antibiotics, but it has been applied to sulphonamide determination (16). Micellar electrokinetic capillary chromatography (MECC) has also been tested for some antibiotic analysis. Compared to HPLC, it offers the advantages of speed and better selectivity, but HPLC still performs better in quantitative analysis (17). Some papers reviewed recent applications of countercurrent chromatography (CCC) to the separation of antibiotics (18). This technique is a form of liquid partition chromatography that utilizes a separation column free of solid support matrix. This technique could also be applied to antibiotics that are strongly adsorbed to silanol groups on silica gel used in column chromatography. But this technique is beyond the scope of this chapter, which is devoted to HPLC, the analytical method widely applied as the comparative assay for antibiotic monitoring.

B. HPLC in Antibacterial Drug Analysis

The HPLC technique, with UV, DAD, fluorescence, or electrochemical detection or coupled to mass spectrometry (MS), has an important place in the field of residue analysis. It became widely applied as a determinative assay after positive results were confirmed by some of the screening methods. This technique gives a real chance to separate simultaneously all analyzed compounds, together with their metabolites and degradation products. In many cases this technique enables the determination of low concentration levels of one analyzed analyte in the presence of many other interfering and coeluting components. It is valuable especially in a combination with effective sample preparation techniques, mainly in on-line combinations. There are numerous advantages that led to the increased popularity of the HPLC method in this field: The fine choice of commercially available columns, the application of all chromatographic principles, including the special restricted-access material (RAM) sorbents, the possibility for use of two or more columns in a switching mode, combinations of different-sensitivity detectors, a great variety of applied mobile phases, gradient applications, and, of course, being able to manage the whole HPLC assay and results evaluation with a computer. Moreover, LC-MS is a powerful combination for the reliable determination and confirmation of veterinary drug residues in nearly all food samples. In the past 10 years on-line combination LC-MS has developed into an on-line approach for LC. Both electrospray ionization (ES) and atmospheric pressure chemical ionization (APCI) have replaced the use of thermospray in many applications of LC-MS in food analysis. They have actually made LC-MS in combination with tandem mass spectrometry the technique of choice in quantitative bioanalysis (15). Moreover, the preparation of samples before LC-MS or LC-MS-MS assays is not as complicated as before HPLC assays.

C. Sample Preparation Before HPLC Analysis of Antibacterial Drugs

Reviewed articles have presented many possibilities for effective preseparation procedures before HPLC monitoring drugs in biological materials and foodstuff samples. It is generally agreed that one of the most difficult steps required for the analysis of an antibiotic or any drug is the extraction and cleanup of the drug from the biomatrix. Screening methodologies (e.g., receptor and microbial screening tests) require often only simple extraction and cleanup procedures. Quantitative, comparative, and confirmative methodologies, such as chromatographic and spectrometric assays, particularly of residues in tissue matrices, require extensive treatment before they are ready for analysis (14).

The commonly utilized preparation procedures very often involve liquid–liquid extraction (LLE), since many of analyzed samples are not of liquid consistency for injection into an HPLC column. A drug component is partitioned between two immiscible phases to bring the drug or contaminants from one phase to another. With this technique, the most important points in the preseparation method development are the choice of solvent, the pH, the temperature, the sample-to-solvent-volume ratio, and the number and the time intervals of individual extraction steps. All these factors influence extraction recovery and, of course, the accuracy of the whole assay. Where analyzed compounds are highly polar, the choice of the solvent suitable for efficient extraction is very problematic, because the drugs of interest must be partitioned between two immiscible phases. Only a few solvents fulfill this condition, e.g., diethylether and ethyl acetate. Solvents that are medium polar, such as dichloromethane and chloroform, provide good results for medium-polar analytes, but highly polar compounds are extracted in these solvents yield extraction recovery values that are not very high. The choice of pH is also important, because analytes in nondissociated forms are extracted in a better way into the organic solvents. This means that basic antimicrobial drugs are extracted into an organic solvent using a higher pH and acidic ones using a lower pH. The choice of pH is not important for neutral analytes. In the opposite case, if ion-pair agents are used in the extraction procedure, the analyte molecules have to be dissociated to form the ion pairs. In many published papers, the LLE step is combined with precipitation of proteins present in biological matrices. In some cases the precipitation is performed simultaneously with LLE, e.g., adding the precipitation agent (MeCN, MeOH) directly to the extraction solvent (ethyl acetate, acetone). Of course, the protein precipitation is also realized by using mineral or organic acids. So acidified extraction solvents are also applied for the combined precipitation–extraction procedures. Recoveries of these assays are considerably higher.

Solid-phase extraction (SPE) is a good choice for the cleanup procedure for crude extracts of food samples. This technique, which applies all separation principles, became very popular in the past decade. In some cases more than single-functional group SPE cartridges are used. Their major weakness is that they cannot be readily used for multiresidue analysis, due to the widely varying chemistries of antibacterial drugs and their metabolites. So mixed-phase SPE cartridges have also been manufactured combining nonpolar and ion-exchange groups. New types of SPE cartridges were based on the use of porous graphitic carbon (PGC) (14). The advantages of SPE compared to LLE are that SPE is faster and more reproducible, cleaner extracts are obtainable, emulsion creation is avoided, and smaller sizes are needed. It has also been stated that SPE is cheaper. From an environmental point of view, a lower consumption of toxic solvents is used in most SPE procedures (14). Better precision and accuracy and higher and reproducible extraction recoveries are also typical for many SPE assays. The last but not least advantage is the possibility for combining the SPE procedure on-line with the HPLC equipment and realizing so-called direct sample analysis (DSA). New possibilities are in the application of RAM sorbents not only in packing analytical columns for the direct application of sample extracts (one-column system) but also for the column-switching mode (two-column system). In this case the precolumn packed

with the RAM sorbent is connected with the analytical column for the separation and determination of all analytes in food samples. The column-switching system could be automated using the autosampler incorporated into the HPLC system. The RAM sorbents characteristically have a hydrophobic interior and a hydrophilic barrier, which allows the passage of small molecules and restricts the access of large molecules. A hydrophobic interior retains small molecules. These sorbents combine both reversed-phase and size-exclusion principles. The RAM sorbents are divided into four groups (physical diffusion barrier, chemical diffusion barrier, monofunctional phases, and mixed functional phases) (19).

Matrix solid-phase dispersion (MSPD) is the next alternative preseparation procedure, convenient especially for liquid and semiliquid food samples. This technique is a multiresidual assay that may be used as an effective preparation procedure not only for HPLC but also for immunoassays, enzyme analysis, or microbial assays. The MSPD technique consists of matrix homogenization with a solid silica phase placed into a short column, as in SPE. Analytes and matrix interferences are retained on the mixed solid-phase material. Specific elution allows one to obtain analytes after the elimination of matrix compounds via washing steps (20). The technique combines homogenization, cellular disruption, extraction, fractionation, and purification in a single process. It was found that mixing biological samples with silica-bonded support causes a disruption of the sample structure by the mechanical blending (20). Reversed-phase materials (C18, C8) are suitable sorbents for MSPD. The washed sorbent is mixed with a food sample, and this mixture is packed into an empty SPE cartridge. After washing all interfering compounds, individual groups of analytes are eluted from a cartridge. The elution is realized with the experimentally determined solvent sequence. After evaporating solvents, residues are dissolved in mobile phase and injected into an HPLC system. The MSPD method has the following advantages: The sample is dispersed over a large surface area, the volumes of extraction solvents are low in comparison with LLE, the dispersion of the sample involves mechanical and hydrophobic forces, the technique is easy to perform without the special demands for the equipment, and it can be automated.

D. Applications

In this chapter, the latest applications published in the literature from 1994 to 1998 are reviewed. The following groups of antimicrobial agents are discussed: tetracyclines, penicillins, polyethers, aminoglycosides, macrolides, amphenicols, nitrofurans, sulphonamides, quinolones and other antimicrobials.

The chapter is divided into 10 parts devoted to individual groups of antimicrobial agents. The text is focused mainly on sample pretreatment, new achievements in method development, comparison with other techniques, and the factors limiting chromatographic analysis. The sample pretreatment and detection conditions (especially for MS detectors) are presented only in the main features, to make the text more concise.

The chromatographic conditions [analytes, samples, columns, mobile phases, detection, and limits of detection (LODs)] are summarized in the tables following each section. The names of compounds are abbreviated, and the abbreviations are explained in the first paragraphs of each section; other abbreviations are listed at the end of this chapter.

II. TETRACYCLINE ANTIBIOTICS

Tetracyclines (TCs) are chemically characterized by a partially conjugated octahydronaphtacene four-ring skeleton with a carboxyamide functional group. They are amphoteric compounds soluble in polar and moderately polar solvents, and they show the ability to form strong complexes

with divalent and trivalent cations. They are highly effective against a number of gram-positive and gram-negative bacteria; thus they are considered broad-spectrum antibiotics with a wide range of applications.

Oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) are the main representatives of this antibiotic class. In addition, demeclocycline (DMC), doxycycline (DXC), minocycline (MNC), and methacycline (MTC) can be found in food samples.

European Community Commission Regulation No. 2701/94 (21) states that the maximum residue level (MRL) for all substances belonging to the tetracycline group is the sum of the residues of all substances within the group. This legislative requirement necessitates the development and use of analytical methods capable of simultaneously detecting residues of more than one tetracycline. The MRLs for combined tetracycline residues are 600 μg/kg, 100 μg/kg, and 100 μg/kg in kidney, muscle, and milk, respectively (22).

The Center for Veterinary Medicine of the U.S. Food and Drug Administration has set safe levels for tetracycline residues in bovine milk of 30, 80, and 30 μg/l for OTC, TC, and CTC, respectively (23).

The HPLC methods for the determination of TCs in food samples can be divided into four groups according to the physicochemical principle used for sample cleanup or according to the type of detection:

Metal chelate affinity chromatography (MCAC)
McIlvaine/EDTA buffer homogenization/precipitation–SPE assays
LC-MS assays
Other assays

See Table 1 for the different analysis techniques of tetracyclines in food samples.

A. Metal Chelate Affinity Chromatography

A method based on a metal chelate affinity chromatography (MCAC) was described by Carson (24). Generally, it consists of extraction/precipitation with succinate buffer followed by cleanup on a Chelating Sepharose column preloaded with copper(II) sulphate when TCs are specifically adsorbed by chelatation with metal ions bound. Elution of TCs was achieved using EDTA-containing buffer.

This approach was used for the determination of OTC, TC, and CTC in milk samples. The centrifuged raw milk was mixed with succinate buffer (pH 4.0) to precipitate proteins. The clear supernatant was loaded directly on an MCAC column (1.5-ml Chelating Sepharose Fast-Flow) activated by passage of both water and 10 mM copper(II) sulphate solution. The blue-colored column was washed with both water and MeOH, and TCs were eluted with McIlvaine/EDTA/NaCl buffer. The extract was injected directly into the chromatographic system (25).

A previously reviewed method was applied for OTC, TC, CTC, DXC, and DMC analysis in tissue and egg samples; however, further optimization and improvement were necessary. The optimal recoveries from tissue were obtained using succinate buffer and MeOH as a deproteinization agent. The eluate from the MCAC column was acidified and further purified on an Empore disk equipped with a poly(styrene-divinylbenzene)-RP sulphonated membrane previously activated with MeOH and hydrochloric acid (pH 1.0). The elution of TCs was done with methanolic ammonia solution. The extract was evaporated under vacuum and reconstituted in oxalic acid solution. Even though the stability of TCs is poor under alkaline conditions, no influence on the recovery was observed (59–76% with RSD $< 6.5\%$ for kidney samples) (26).

The assay (26) was applied for the determination of degradation products of DXC (6-epiDXC and 4-epiDXC) in liver and muscle samples. A possible noninterfering by-product

Table 1 Analysis of Tetracyclines in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Refs.
OTC, TC, CTC	Liver, kidney, muscle	Ho, SPE	200 × 3 5-μm Chromspher C8	Gradient MeCN:20 mM oxalic acid	F post-der, ex 390, em 490	20–30 μg/kg muscle, 40–230 μg/kg kidney	22
OTC, TC, CTC, DMC, MTC, DXC, MNC	Milk	Pr, MCAC, UF	150 × 4.6 5-μm PLRP-S	Gradient MeCN:MeOH:10 mM oxalic acid	UV 355		23[a]
OTC, TC, CTC	Milk	Ho, Pr, MCAC	250 × 4.6 5-μm PLRP-S	MeCN:MeOH:10 mM pH 2.0 oxalic acid 15:10:65	F post-der, ex 410, em 515	1–4 g/L	24
OTC, TC, CTC, DXC, DMC	Kidney, liver, muscle, eggs	Ho, Pr, MCAC, SPE	250 × 4.6 5-μm PLRP-S	Gradient MeCN:10 mM pH 2.0 oxalic acid	F post-der, ex 406, em 515	0.21–3.0 μg/kg	25
DXC, 4eDXC, DMCTC	Liver, muscle	Ho, Pr, MCAC, SPE	250 × 4.6 5-μm PLRP-S	Gradient MeCN:10 mM pH 2.0 oxalic acid	F post-der ex 406, em 515	1–1.2 μg/kg	26
OTC, TC, CTC, DMC, DXC, MNC	Milk, shrimp tissue (OTC)	Ho, Pr, MCAC, SPE	150 × 3.9 5-μm PLRP-S	MeOH:5 mM oxalic acid 58:42	PB MS		27
OTC, TC, CTC, DMC	Liver, kidney (29) Egg, poultry, fish, venison (30)	Ho, SPE, on-line MCAC	Precolumn: 10 × 6 10-μm Anagel-TSK-Chelate-SPW, 150 × 4.6 5-μm PLRP-S	MeCN:MeOH:(10 mM KH_2PO_4, 10 mM citric acid, 10 mM EDTA) 25:10:65	UV 350	10–20 μg/kg (29), 3–6 μg/kg (30)	29, 30
OTC, TC, CTC, DXC, MNC, MTC, DMC	Milk	Ho, SPE	250 × 4.6 5-μm RP-18	Gradient MeCN:MeOH:10 mM oxalic acid with 1% THF, 1% TEA	UV 363	75 μg/kg	32
OTC, TC, CTC	Milk, meat, cheese	Ho, SPE, MSPD	250 × 4.6 5-μm LiChrosorb RP18	MeCN:MeOH:10 mM oxalic acid 17.5:17.5:65	UV 360	15–22 μg/kg (SPE), 75–200 μg/kg (MSPD)	33
OTC, TC, CTC	Muscle, eggs	Ho, SPE	250 × 4 5-μm Spherisorb ODS2	MeCN:MeOH:10 mM oxalic acid 20:35:45	UV 360	50 μg/kg	34, 35
OTC	Fish	Ho, Pr, SPE	250 × 4.6 5-μm YMC C18	MeCN:10 mM oxalic acid, 30 mM octanesulphonic acid 29.5:70.5	UV 355	3–6 μg/kg	36
OTC	Catfish muscle	Ho, Pr, SPE	120 × 4 5-μm LiChrosorb C8	MeCN:50 mM oxalic acid 14:86	UV 355	10 μg/kg	37

OTC, CTC	Tissue	Ho, SPE	250 × 4.6 5-μm ODP-50	MeCN : pH 12.0 Sörensen buffer 10 : 90	F, ex 374, em 508, ex 350, em 420	1–5 ng	38
OTC, TC, CTC	Kidney, muscle	Ho, SPE	250 × 4.6 5-μm RP8, de-activated silica	MeCN : MeOH : 10 mM oxalic acid 30 : 10 : 60	UV 350		39[a]
OCT, TC, CTC, DXC	Milk	Pr, SPE, TLC	250 × 4 5-μm Bakerbond C8	MeCN : MeOH : 10 mM oxalic acid 6 : 4 : 10	UV 350	10–20 μg/kg	42[b]
OTC, TC, CTC, DXC	Honey	SPE	250 × 4.6 5-μm Inertsil Ph (99.99% pure silica)	MeCN : MeOH : 5 mM TFA 2 : 2 : 11 with 1% thioglycerol	FFAB-MS	100 μg/kg	43
OTC, TC, CTC, DXC	Liver, muscle	Ho, SPE	100 × 4.6 2-μm TSK gel Super Octyl	MeCN : 0.05% TFA 1 : 4	ES-MS-MS	10 μg/kg (muscle), 20 μg/kg (kidney)	44
OTC, TC, CTC	Muscle, kidney	Ho, SPE	250 × 4.6 5-μm Inertsil ODS-2 (pure silica)	Gradient MeCN : 0.04% heptafluorobutyric acid, 20 mM oxalic acid, 0.01 mM EDTA	APCI-MS	50 ng/g	45
OTC	Muscle, tissue	Ho, UF	150 × 4.6 5-μm L-ODS	MeCN : 1 M pH 7.2 imidazole with 50 mM magnesium acetate, 10 mM EDTA 10 : 90	F, ex 380, em 520	4–8 μg/kg (milk), 20–50 μg/kg (tissue)	46
OTC, TC, CTC	Milk, muscle, liver, kidney	Ho/Pr	250 × 4.6 5-μm PLRP-S	MeCN : aqueous H_3PO_4 with decanesulphonate and dodecylsulphate (different ratio and concentrations)	UV 355	2–4 μg/kg	47
OTC, TC, CTC	Milk	Pr, LLE	150 × 4.6 5-μm PLRP-S	MeCN : 20 mM phosphoric acid with 10 mM sodium decanesulphonate 70 : 30	UV 380		48
TCs	Meat	Ho, Pr, MCAC, SPE	150 × 2.1 5-μm Symmetry C18	Gradient MeOH : 0.069% TFA	ES-MS-MS		51

[a] AOAC International–adopted method.
[b] Confirmation by TLC-FAB-MS.

(MTC) was detected, since DXC had been produced synthetically out of MTC, resulting in the possible presence of traces in the raw material (27).

Since TCs form highly fluorescent chelates with metal ions at the appropriate pH value, their complexation with 5% zirconium chloride solution added postcolumn to the eluate from HPLC was used for fluorescence detection. The highest yield of fluorescence was observed at pH 2.0; therefore a pH adjustment of the mobile phases was necessary (25–27). Moreover, using a mobile phase of pH 2.0, the formation of both the C4 epimers and anhydroTCs are minimized.

Preconcentration and desalting of the MCAC eluate were accomplished using a Supelclean ENVI-Chrom P SPE cartridge as an alternative to the Empore disk with poly(styrene-divinylbenzene)-RP sulphonated membrane. The TCs were eluted with MeOH, which was subsequently evaporated, and the residue was dissolved in water. The SPE cartridge with polymeric medium had recoveries superior to all those of the silica-based cartridges; furthermore it allowed the use of the elution solvent with no addition of nonvolatile salts (oxalic acid). A particle-beam MS with methane negative-ion chemical ionization was used for the detection of six TCs (OTC, TC, CTC, DMC, DXC, MNC), which produced greater fragmentation and richer spectra, providing higher specificity of residue identification than had been previously reported. The major drawback of the scan-monitoring mode—low sensitivity—was overcome by scaling up the method from 5 ml to 40 ml of milk used in the sample cleanup. This provided a nearly 40-fold enrichment of TCs in the final milk extract (28).

The assay described by Carson (24) was further improved by ultrafiltration of the MCAC eluate, on-column focusing in 10 mM oxalic acid, and subsequent gradient elution followed by UV detection. This assay was validated for OTC, TC, CTC, DMC, DXC, MTC, MNC residues in milk samples in a collaborative study that involved eight laboratories. Each laboratory was asked to prepare and analyze known control and fortified milk samples as well as 18 coded blind samples. Average interlaboratory recoveries ranged from 59% (MTC at 10 μg/L) to 78% (OTC at 60 μg/L). Average recoveries for each of seven residues were between 60 and 110%. Reproducibility for the known fortified samples varied from 11% to 39%, with six of seven residues at the 30-μg/L level having RSD values below 20%. The determination of MNC was the least reproducible; the authors suspected that the pH value of milk might be an important factor. No interference was detected in control milk samples. The MCAC-LC method for the determination of multiple tetracycline residues in milk has been adopted by AOAC International (23).

An on-line MCAC-HPLC method was developed for OTC, TC, CTC, DMC residues in both liver and kidney samples. The drugs were extracted with succinate buffer, and the extract was diluted with EDTA–pentanesulphonic acid buffer. Diluted extract was then purified on C8 or XAD-2 SPE cartridges previously activated with MeOH, water, and PSA-containing phosphate buffer. The TCs were eluted with MeOH, and the eluate was then injected onto an Anagel-TSK-Chelate-SPW column that had been preloaded with copper(II) sulphate. The TCs were eluted directly onto the analytical column in a column-switching system after washing with both water and MeOH (29).

An improvement was necessary when TCs residues were analyzed in egg, poultry, fish, and venison tissues. The SPE step was replaced by LLE with ethyl acetate. Initially, direct loading of an ethyl acetate onto an MCAC column gave high recoveries and clean chromatograms, but there was a tendency to block the precolumn. A solvent-exchange step (evaporation of ethyl acetate and dissolution in MeOH) was therefore introduced. The MCAC column performance was found robust under these conditions, and approximately 150 extracts could be processed before its deterioration (evident by a loss of response from tissue samples). An improvement in LOD and recoveries (42–104% with RSD $< 20\%$) was presented, and the chromatograms showed a generally flatter baseline than those obtained previously (29). This method was found inapplicable to milk and chicken-based baby food due to the presence of coeluting matrix interference (30).

Residues of TCs were quantified via the MCAC-HPLC method (26) in pork and chicken muscle tissue that had been previously screened with both a microbiological inhibition test using *B. subtilis* and an ELISA method. The correlation between the mean area of the inhibition zones and the DXC levels found in 28 samples by HPLC was 0.82; the correlation between the ELISA results and the DXC levels in the same samples was 0.73. The results indicated that an inhibition test was well suited to screen the mentioned samples for TCs residues. The authors found the more expensive ELISA screening test unnecessary, because only a minority of analyzed samples did not contain TCs. Confirmation with HPLC method was necessary because of the presence of some false-positive results. Moreover, the positive results from LC-fluorescence assay were confirmed using LC-MS-MS assay with electrospray ionization working in positive-ion mode (31).

B. McIlvaine/EDTA Buffer Homogenization/Precipitation–SPE Assays

The milk/tissue sample was homogenized/extracted with McIlvaine–EDTA buffer (pH 4.0). The pellet was resuspended and extracted again. The combined extracts were further filtrated on a paper disk to remove any solid particles that might result in the plugging of an SPE cartridge. The extract was loaded on a preconditioned SPE cartridge (with MeOH and water), and TCs were eluted with 10 mM oxalic acid solution in MeOH.

This assay was applied to the determination of OTC, TC, CTC, DXC, MNC, MTC, and DMC in milk samples using C8 cartridges in the SPE step (32) and OTC, TC, and CTC in milk and meat samples using a C18 cartridge (33). The recoveries ranged from 48% to 86% (RSD $<$ 15%), and LODs were sufficient for MRL demands. This procedure was compared to the MSPD assay in milk samples. Better recoveries were observed (89–93% with RSD $<$ 10%), though it is inapplicable for CTC residues at MRL concentrations due to the high LOD (33).

The homogenization with both McIlvaine–EDTA buffer (pH 4.0) and *n*-hexane: dichloromethane was presented for the determination of OTC in eggs and OTC, TC, and CTC in muscle. The proteins were further precipitated with the addition of TCA, and TCs were preconcentrated on a C18 SPE cartridge (34,35).

Further improvement to the method included incorporation of an additional deproteinization step (addition of TCA) and a silanization of the cartridge. This step alleviated the blockage of the SPE cartridge and significantly reduced chromatographic baseline noise. The extract was purified on a phenyl SPE cartridge, which showed greater retention of OTC. The elution of TCs was done with 300 mM oxalic acid in MeOH (36). The efficiency of SPE was enhanced by silanization of the cartridge with dimethyldichlorosilane in toluene prior to conditioning. This step probably prevents the irreversible adsorption of OTC to the free silanol groups of the cartridge (37). This study showed that the recovery of OTC in fillet tissue from six species of fish was strongly dependent on the proper preconditioning step (recoveries 59–90% with RSD $<$ 20%) (36).

An HPLC assay using fluorescence detection was presented for OTC and CTC residues in animal tissues. The sample cleanup was based on SPE on a C18 cartridge followed by an additional cleanup on an SCX cartridge. The TCs were eluted with MeOH: 1 M hydrochloric acid solution; the extract was alkalinized to pH 12.0 and allowed to stand for 1 hour when the fluorescence of CTC reached the maximal value. The influence of the pH value of the mobile phase on the fluorescence spectra of both OTC and CTC was studied. The reduction of fluorescence yields was explained by the decomposition to their iso-derivatives (38).

The assay based on a homogenization with McIlvaine/EDTA buffer followed by SPE on a C18 cartridge (as described earlier) was evaluated for the determination of OTC, TC, and CTC residues in pork/beef kidney and muscle samples in 13 laboratories, and the results were com-

pared with the alternative methodology—MCAC assay. The results suggested that the method could achieve reliable results for the analytes and matrices studied at concentrations from 100 to 600 μg/kg and higher in most laboratories. In general, this method provides better results at the higher concentrations than at concentrations near the detection limit, and there are fewer problems with interference in muscle tissue than in kidney. A comparison of the study mean results with those obtained using MCAC cleanup demonstrated that similar results might be obtained with both procedures and that they were similarly affected by interference. Moreover, participants were asked to verify their results below 500 μg/kg on a second LC column.

In this study, participants reported recoveries ranging from 17.8% to 126.6%. This is the main reason for the high RSD values (tenths of percents) obtained in this collaborative study. Due to the high variation of recovery values from SPE cartridge, users should pretest each lot of cartridges. Lots that do not yield reproducible results in the range 60–100% should be rejected. This method was adopted by AOAC International (39).

A radioimmunoassay test for TCs Charm II was compared with HPLC determination of OTC residues in milk samples. There was a significant difference between test methods with respect to the presence of OTC at the FDA safe concentrations. Using the HPLC test results as the standard with which Charm II test results were compared, 47 false presumptive-violative test results were obtained. These samples contained less than 30 μg/L of OTC, as evaluated by HPLC (40).

C. Liquid Chromatography–Mass Spectrometry Assays

Most of the LC-MS assays for the determination of TCs in food samples were based on a sample homogenization/precipitation with McIlvaine–EDTA buffer followed by SPE on a C18 cartridge. Only one paper presented a combination of MCAC assay with LC-MS analysis (28); thus, it was reviewed in the previous section.

The sample clean-up described in the previous section was used for the determination of OTC, TC, CTC, and DXC in milk. The TCs were analyzed in an LC-UV chromatographic system and confirmed by TLC-FAB-MS (fast-atom bombardment MS) assay. The eluate from the C18 SPE cartridge was evaporated to dryness, dissolved in MeOH, and further separated on an RP-8 TLC plate according to the procedure (41). The separation on a TLC plate yielded the reliable separation of all TCs, and the condensation technique of the spots improved the detection limits of FAB-MS by 50 times, with good reproducibility (42).

Most of previously reported LC assays were based on the use of mobile phases containing such nonvolatile compounds as oxalic or citric acids to control peak tailing. However, these could not be used in LC-MS analysis due to the clogging in the interface and due to the build-up of deposits in the ion source. Therefore, a mobile phase containing both TFA and thioglycerol were used in combination with a phenyl column with suppressed silanol activity (99.99% pure silica) for the separation of TCs residues in honey samples. Frit FAB-MS with the monitoring of individual protonated molecular ions (OTC m/z 461; TC m/z 445; CTC m/z 479; DXC m/z 445) was used, and TCs were confirmed by detecting the appropriate fragment ions. This type of MS ionization suffered from a lack of sensitivity and therefore could not be applied to the confirmation of tetracycline residues in tissue samples (LOD 100 μg/kg) (43).

An electrospray ionization LC-MS-MS assay was developed for the determination of OTC, TC, CTC, and DXC in bovine liver and muscle. The TCs gave three fragmentation ions in the electrospray mass spectra, except for DXC, and these ions are very useful for confirmation. In order to obtain optimal tandem MS conditions, the mass spectra were measured at different collision offsets, and the intensity of ions were measured. This method could confirm TCs below the 100-μg/kg level in the bovine kidney and muscle (44).

Extraction with glycine–hydrochloric acid buffer and protein precipitation using ammonia sulphate was used for the cleanup of muscle and kidney samples containing OTC, TC, and CTC residues. The extract was purified on an endcapped cyclohexyl SPE cartridge preconditioned with MeOH and water. The TCs were eluted with MeOH; the extract was evaporated and dissolved in mobile phase. Samples were analyzed using LC-atmospheric pressure chemical ionization MS with monitoring of at least three fragmentation ions. When incurred samples containing CTC were analyzed, CTC, 4-epi-CTC, and two additional compounds identified as keto-CTC and 4-epi-keto-CTC were observed (45).

D. Other Assays

A simple method for OTC residue analysis in swine tissue was based on homogenization with both mobile phase and hexane and ultrafiltration of the supernatant with Millipore Molcut II membrane. Since TCs formed fluorescing chelates when magnesium acetate was present in the mobile phase, the chromatographic separation was detected using fluorescence detector (46).

Precipitation/homogenization with hydrochloric acid and MeCN was presented for the milk, muscle, liver, and kidney samples. The extract was partially evaporated, and TCs were analyzed in separate ion-pair chromatographic systems, depending on the kind of sample and analyzed compound (47).

Deproteinization with hydrochloric acid and MeCN was reported for OTC, TC, and CTC residue determination in milk samples. The filtrate was extracted with hexane and dichloromethane, followed by partial evaporation of the aqueous layer. An addition of *t*-butyl alcohol to the aqueous layer during evaporation prevented foaming and bumping. Recoveries greater than 90% were achieved, and TCs were separated using the ion-pair chromatographic system (48).

The homogenization of liver, kidney, and muscle tissues with glycine–hydrochloric acid buffer was presented for the determination of OTC, TC, and CTC. The supernatant was purified on a cyclohexyl SPE cartridge previously activated with MeOH and water. The TCs were eluted with MeOH, which was evaporated at 65°C, and the residue was dissolved in mobile phase. Recoveries were achieved greater than 70% in muscle at the MRL concentrations and higher than 60% for kidney with RSD $< 11\%$. Gradient elution was employed to improve the separation of OTC and TC from interference found in kidney samples. The eluate from HPLC was mixed with aluminium chloride solution in a low-volume T-piece and delivered into the PTFE tubing 13.7 m $\times$ 0.3 mm immersed in an oil bath at 60°C followed by fluorescent detection (22).

A rapid and simple assay for the analysis of OTC, TC, CTC, and DXC in milk was established using an on-line sample cleanup. Coexisting substances in milk were removed by the precolumn head-cut-loading method, and TCs were rapidly loaded on an analytical column after changing the eluent (ion-pair mobile phase containing octanesulphonic acid). This method could be carried out after deproteinization with TFA only. The authors reported recoveries ranging from 80% to 104% (49).

The HPLC-receptorgram assay combined the advantages of HPLC separation with the multiresidue detection of the Charm II tests. The procedure was tested for identification and quantitation of the most common veterinary drugs at regulatory levels or lower. It was validated for 40 individual drugs from seven antibiotic families: 10 β-lactams, 13 sulphonamides, 8 tetracyclines, 4 macrolides, 3 amphenicols, and other miscellaneous antimicrobials. This procedure combined a simple aqueous extraction and SPE with HPLC fractionation of individual drugs. Final identification and quantitation was achieved with the Charm II test. A drug contaminant could be identified in less then 3 hours (50).

An interlaboratory study was organized for the determination of OTC and 4-epi-OTC in pig muscle tissue. Fourteen laboratories from EU member countries agreed to participate. They were

allowed to use the extraction method of their own choice, but they had to use HPLC as the analytical technique. The extraction cleanup procedures used could be divided into three main groups: MCAC-LC, McIlvaine/EDTA homogenization followed by C18 SPE, and tissue homogenization followed by cyclohexyl SPE and fluorescence detection, respectively. No outliers in results were found according to the Dixon test, and the RSD values ranged from 16.5% to 23.7%, depending on the concentration level. All RSD values were below the limits fixed for repeated analyses of a reference material for the appropriate concentration level (calculated with the Horwitz equation). The results of accuracy *z*-scores showed no significant differences between the analytical performance of the National Reference Laboratories participating in this test (51).

E. Stability

Under mildly acidic conditions, CTC can reversibly epimerize to form 4-epi-CTC. The existence of 4-epi-CTC has been recognized in European legislation—the MRL for CTC in edible animal tissue has been defined as the sum of the concentrations of CTC and 4-epi-CTC. Treatment of CTC and 4-epi-CTC with alkali resulted in the formation of iso-CTC and 4-epi-iso-CTC. The strong fluorescence of these species has been exploited in a number of published papers (52).

The heat stability of OTC in water and vegetable oil was investigated (54). Results showed that the drug was unstable in water at 100°C, with a half-life of about 2 min; it was more stable in oil at 180°C, but the half-life was about 8 min. The effect of a range of cooking processes, including microwaving, boiling, grilling, braising, and frying, on OTC residue stability was tested for incurred animal tissue samples. Substantial reductions were observed in OTC concentrations (35–94%); the reduction appeared to be related to the final temperature and the duration of cooking. Migration was observed from the tissue into the surrounding liquid or meat juices during the cooking processes.

These results corresponded with another work, where baking and smoking at 190°C were more effective in reducing OTC residues than frying (53). These results confirmed that common cooking procedures may not completely degrade OTC residues in tissues.

Diode array analysis of heat-treated OTC standard solution indicated that no individual closely related compounds, such as 4-epi-OTC or α- or β-apo-OTC, formed a significant proportion of the breakdown products (54).

III. β-LACTAM ANTIBIOTICS

β-Lactam antibiotics comprise several classes of compounds, among which cephalosporins and penicillins are the most important. Both classes contain bulky side chains attached to the 7-aminocephalosporanic acid or 6-aminopenicillanic acid nuclei, respectively. The penicillins are widely used for their microbial activity against both gram-positive and gram-negative organisms.

Amoxycillin (AMO), ampicillin (AMP), penicillin-G (PenG), penicillin-V (PenV), oxacillin (OXA), cloxacillin (CLO), and dicloxacillin (DICL) are the main representatives of penicillins; cephapyrin (CEP), ceftiofur (CEF), and cefadroxil (CFD) are the main representatives of cephalosporins. Other β-lactams could be found in the food samples, e.g., methicillin (MET), piperacillin (PIP), nafcillin (NAF), and ticarcillin (TIC), among others.

In order to protect consumers from risks correlated with drug residues in foods, the European Union has laid down MRLs for AMO, AMP, and PenG (4 μg/kg in milk and 50 μg/kg in tissues), for OXA, CLO, and DICL (30 μg/kg in milk and 300 μg/kg in tissues), and for NAF 30 (μg/kg in both milk and tissues) (2).

Due to the high complexity of the sample matrix, to the very low wavelength of the UV absorption maximums (penicillins), and to the low MRL values, six main approaches for sample cleanup and detection in the analysis of β-lactam residues were reported in the reviewed period of time:

LC-UV assays
LC-MS assays
LC-fractionation assay
Pre- and postcolumn derivatization assays
LC-ED assays
LC-microbial and immunoaffinity assays

In Table 2 the different methods are summarized.

A. Liquid Chromatography–Ultraviolet Detection Assays

Only a few works report a simple combination of conventional sample cleanup procedures with UV detection. Assay of CLO (OXA was internal standard) based on a protein precipitation using MeCN in acidic solution (pH 3.6) and LLE with chloroform was reported for milk samples. The organic layer was evaporated to dryness, and the residue was reconstituted in the mobile phase and injected into the chromatographic system with UV detection. Relatively clean chromatograms and high recoveries (75–84%) were obtained using this assay (55).

The protein precipitation with acetone followed by LLE with hexane was applied in the milk sample cleanup for the determination of PenG. The residue after LLE was further purified on a C2 SPE cartridge. The authors reported this method as simple and robust (recovery 82% with RSD 1%) (56).

The combination of protein precipitation with MeCN and SPE on a C18 cartridge was presented for the determination of CEF and CEP in milk samples. Although the addition of tetraethylammonium chloride (TEACl) in the protein precipitation step improved recoveries to nearly 100%, the high concentration of TEACl altered the ionic strength of the milk extracts, leading to competition between TEACl and the ion-pair reagent in the mobile phase and resulting in the shift of CEP retention time. Therefore, it was eliminated from the deproteinization step, and recoveries were remarkably lower (76–87%). Because of the low pH of the mobile phase (33 mM ortophosphoric acid), the analytical column should by flushed with at least 120 ml of water after a sample set (8–12 samples) (57).

A similar procedure based on a dilution of milk sample and SPE was presented for CEF residues. The authors reported that the contamination of CEF was avoided by sequentially washing the glassware with 1 M hydrochloric acid, water, and MeOH. The stability of the sample extract was studied, and a significant decrease in recovery was noted if samples were kept longer than 24 hours. In comparison to the previous procedure, a higher recovery was obtained (92% with CV 3.9%) (58). Both procedures were tested for possible interference of other drugs that may be found in raw milk. None of these compounds interfered with the analytes (57,58).

A simple and rapid ion-pair chromatographic method was developed for the determination of PenG, AMP, CLO, DICL, and NAF in milk samples. The milk proteins were precipitated by MeCN, and the supernatant was partially evaporated under reduced pressure. Analytes in the remaining aqueous solution were concentrated on a C18 SPE cartridge and eluted with MeOH. Although the authors achieved high recoveries ranging from 83% to 89%, high LOD values (30–50 μg/kg) made this assay not applicable for the monitoring of penicillin residues in milk under

Table 2 Analysis of β-lactams in Food Ssamples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Refs.
CLO	Milk	Pr, LLE	150 × 3.9 5-μm NovaPak C18	MeCN:20 mM pH 5.0 phosphate buffer 21:79	UV 225	LOQ 10 μg/L	55
PenG	Milk	Pr, LLE, SPE	250 × 4.6 5-μm Supelcosil LC-18DB	MeCN:10 mM pH 2.15 phosphate buffer with 20 mM HSA 34:66	UV 200	2 μg/L	56
CEF, CEP	Milk	Pr, SPE	150 × 4.6 5-μm Supelcosil LC-18	Gradient MeCN:33 mM phosphoric acid with 9 mM SDS	UV 290	1–2.5 μg/L	57
CEF	Milk	SPE	250 × 4.6 5-μm Supelcosil LC-18-DB	MeCN:100 mM pH 3.3 acetate buffer 20:80	UV 293	4 μg/kg	58
PenG, AMP, CLO, DICL, NAF	Milk	Pr, SPE	250 × 4.6 5-μm Kaseisorb LC ODS	MeCN:MeOH:50 mM pH 3.5 phosphate buffer with 5 mM sodium decanesulphonate 20:10:80	UV 210	30–50 μg/L	59
OXA, CLO, DICL	Bovine muscle	Pr, LLE	125 × 4 5-μm LiChrospher C18 endcapped	MeCN:MeOH:2% formic acid 10:50:40	PB-MS	40–50 μg/kg	60
PenG, PenV, OXA, CLO, DICL, NAF	Muscle, kidney, milk	LLE	150 × 4.6 5-μm Inertsil ODS2	MeCN:water:TEA 27:37:0.5	ES-MS	milk 2–10 μg/kg meat 25–100 μg/kg	61
PenG, AMP, AMO, CLO, CEP, CEF	Milk	UF, foc	150 × 0.32 Porous II R/H LC perfusion column	Gradient MeCN:water with 0.2% formic acid, 25 mM HFBA	ES-MS	10 μg/kg	63
CEF	Milk	UF, foc	75 × 3.9 4-μm NovaPak C18	Gradient MeCN:water with 1% acetic acid and 25 mM HFBA	ES-MS	10 μg/kg	64
PenG	Tissue, liver, kidney	Pr-LC-fract	150 × 4.6 5-μm Inertsil ODS-2	MeCN:phosphate buffer 32:68	UV 210		65
AMP, AMO, PenG, PenV, CLO, CEF, CEP	Milk	Pr, LC-fract	150 × 4.6 5-μm Supelcosil LC-18-DB	MeCN:phosphate buffer with dodecyl sulphate or sodium decane sulphonate (ratio depending on anal. compound)	UV 210–224 UV 290–295	>1 μg/kg	67
PenG	Milk	Pr, SPE, LC-fract	250 × 4.6 5-μm Kromasil 5C8	Gradient MeCN:10 mM ortophosphoric acid	UV post-der 325	LOQ 2 μg/kg	70

DFC, DFCC	Tissue	Pr, LC-fract	150 × 4.6 5-μm Supelcosil LC-18	MeCN: 15 mM phosphoric acid with 7.5 mM SDS 40:60 (DFCC), 44:56 (DFC)	UV 270	10 μg/kg	71
AMO	Salmon tissue	Pr, SPE, LLE, pre-der	250 × 4.6 5-μm Spherisorb S5 ODS2	MeCN: 50 mM pH 5.6 phosphate buffer 20:80	F, ex 258, em 440	0.5 μg/kg	73
AMP	Milk, tissue	Pr, pre-der	250 × 4.6 5-μm Prodigy ODS-3	MeCN: 10 mM pH 5.6 phosphate buffer 24:76 (25:75 [75])	F, ex 346, em 422	0.3–0.6 μg/kg	74, 75
AMO, AMP	Milk	Pr, SPE, pre-der	250 × 4.6 5-μm Prodigy ODS-3	MeCN: 20 mM pH 5.0 KH_2PO_4 32:68 (12 min), 50:50 (10 min)	F, ex 354, em 445	1–1.1 μg/L	76
PenG, CLO	Tissue	Pr, SPE, pre-der	150 × 3.9 4-μm NovaPak C18	MeCN: 100 mM pH 6.5 phosphate buffer with 15.7 mM sodium thiosulphate 22.5:77.5	UV 325	3–5 μg/kg	81
AMP, AMO	Milk	Pr, SPE, pre-der	150 × 4-μm NovaPak C18	MeCN: MeOH: 100 mM pH 6.5 phosphate buffer with 15.7 mM sodium thiosulphate and 20 mM TBA hydrogensulphate 18:12:70	UV 325	3 μg/L	82
PenG, PenV, AMO, AMP, CLO	Tissue, milk	Pr, SPE, pre-der	150 × 3.9 5-μm Symmetry C8	MeCN: 100 mM pH 6.5 phosphate buffer with 15.7 mM sodium thiosulphate 26:74	UV 325	2–5 μg/kg	83, 84
PenG, AMP, AMO, OXA, CLO, DICL	Milk	Pr, SPE, pre-der	150 × 3.9 4-μm NovaPak C18	Gradient MeCN: 100 mM pH 6.5 phosphate buffer with 15.7 mM sodium thiosulphate	UV 323	1.3–1.5 μg/L	85
OXA, CLO, DICL	Milk, tissue	Pr, SPE, pre-der	150 × 3.9 4-μm NovaPak C18	Gradient MeCN: 100 mM pH 6.5 phosphate buffer with 15.7 mM sodium thiosulphate	UV 345	5–7 μg/kg	86
OXA, CLO, DICL	Milk	Pr, SPE, pre-der	150 × 3.9 5-μm Symmetry C8	MeCN: MeOH: 100 mM pH 6.5 phosphate buffer with 15.7 mM sodium thiodulphate and 20 mM TBAHS 37:5:58	UV 340	2–5 μg/L	87

(*continued*)

Table 2 Continued

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Refs.
AMO, AMP	Tissue	Pr, SPE-SPE, pre-der	250 × 3.5 5-μm Kromasil KR100 C8	MeCN: 15 mM phosphate buffer with 15 mM sodium thiosulphate 20:80	UV 325	5 μg/kg	88
PenG, PenV, OXA, CLO, DICL	Tissue	Pr, LLE, online SPE, PD	250 × 4 5-μm LiChrospher 100RP-18 endcapped	MeCN: 200 mM pH 3.0 phosphate buffer with 2 mM Na_2EDTA	E + 650 mV glassy carbon electrode	1.2–4.6 ng	92
AMP, CEP	Milk	Pr, SPE	150 × 4.6 5-μm Luna C8	MeCN: water: 500 mM pH 3.75 acetate buffer 4:76:20	E gold electrode	5 μg/kg	93
PenG, PenV, AMO, AMP, OXA, CLO, DICL, MET, NAF	Milk	Pr, LLE, SPE	100 × 8 μBondaPak C18	Gradient MeCN: 20 mM acetate buffer	E gold electrode	0.2–0.3 mM	94
AMO, AMP, PenG, CLO, CEF, CEP	Milk	Pr, SPE	250 × 4.6 10-μm LiChrosorb RP-8	Gradient MeOH: 50 mM pH 6.0 phosphate buffer	Microbial test Charm II-Q	10 μg/kg	95

MRL values (59). However, a simple LC-UV analysis seems to be unsuitable for the determination of β-lactam residues (penicillin residues, especially), mainly when a multiresidue assay is required.

B. Liquid Chromatography–Mass Spectrometry Assays

A simple cleanup procedure based on acidic protein precipitation and LLE with ethyl acetate was used for the determination of OXA, CLO, and DICL in bovine muscle. The detection was accomplished with particle-beam MS with negative-ion chemical ionization, which was found more sensitive than electron-impact mode. Deviation from the linearity of response was observed, which could be caused by the phenomenon of coeluting "carrying" analyte particles through the particle beam (60).

The LC-electrospray MS determination of five β-lactam residues (PenG, PenV, OXA, CLO, DICL) was presented by Blanchflower et al. (61) in muscle, kidney, and milk samples. Both homogenized meat and acidified milk were extracted with dichloromethane and hexane. Low recoveries and reproducibility were prevented by the addition of an ion-pair agent (TBAHS) into the sample prior to the LLE (recoveries 89–117% with RSD $< 11\%$). One of the most critical steps of the sample cleanup was the final extraction of penicillins into dichloromethane and evaporation of the extract. The negative-ion measurement was found more sensitive toward the penicillins. This is in contrast to the results of Straub et al. (62,63), who found the positive-ion mode more sensitive. There was no explanation for this phenomenon except that it might be due to differences in the type of instrument used. It was found that for certain kidney and milk samples there could be a suppression of the signal during the first minutes because of the early-eluting matrix compounds. This problem was solved by eluting PenG and PenV using a mobile phase with a lower organic modifier content.

An ultrafiltration with a 10-kDa MWCO filter was used for the determination of six β-lactams (PenG, AMO, AMP, CLO, CEP, and CEF) in milk samples. The ultrafiltrate was injected onto the perfusive-particle column and preconcentrated by on-column focusing using the mobile phase consisting of 100% water with the addition of formic acid and an ion-pair agent (heptafluorobutyric acid, HFBA). This approach reduced the detection limits to 3–5 μg/kg. The linear calibration curve indicated that the perfusive-particle column was not overloaded and that the analytes were trapped on the column with high efficiency. The penicillins were eluted from the column by the gradient of MeCN, and they were detected by ultrasonic nebulization electrospray MS in positive-ion mode (MH^+ ions and several fragmentation ions). Even though all β-lactams could be detected using negative-ion operation mode due to the presence of carboxylic functional group, this was found to be about 3–4-fold less sensitive than positive-ion mode. The confirmation of early-eluting β-lactams (AMO, AMP) hindered interference from the milk ultrafiltrate eluted in the void volume (63).

A similar procedure based on ultrafiltration and LC-MS analysis was reported for CEF in milk samples. An addition of MeCN:water 1:1 to milk reduced protein–drug binding. On-column focusing with gradient elution was chosen to minimize sample handling. In order to achieve sufficient retention of CEF, an MS-compatible ion-pair agent, HFBA, was chosen for the separation. A postcolumn addition of propionic acid was reported to minimize the signal suppression from ion-pair formation with HFBA. The weaker ion-pair reagent, propionic acid, displaces the stronger one based upon volatility (the boiling point of propionic acid is much higher than the boiling point of HFBA). An increase was obtained in the signal intensity greater than a tenfold. This assay provided a simple cleanup with high recovery (95%) and sufficient LOD (10 μg/kg) (64).

C. Liquid Chromatography–Fractionation Assays

In this approach, residues from a sample extract were concentrated on a column in pure phosphate buffer and then eluted with a MeCN gradient. Fractions corresponding to each analyte of interest (1.5-ml fraction centered on the retention time) were collected and analyzed under different chromatographic conditions.

This analytical approach was described for the determination of PenG residues in tissue samples (65), AMO and AMP residues (66), and seven β-lactam residues (AMO, AMP, CEP, PenG, PenV, CEF, CLO) in milk samples (67). The milk proteins were precipitated by adding tetraethylammonium chloride and MeCN. An addition of an ion-pair agent improved recoveries during the extraction/deproteinization step. The clear supernatant was partially evaporated and fractionated with a gradient of MeCN in KH_2PO_4 from 0 to 40% over 30 minutes. The fractions were collected and partially evaporated to 1 ml and injected directly into the appropriate chromatographic system. In comparison to the previously published works (65,66,68), the gradient program was extended to accommodate the elution of compounds with neutral side chains. The recoveries for all analyzed compounds were 90–100%, except for AMP (80%) and AMO (60%) (67). Because the LC analysis of all fractions was tedious, screening of each fraction with the rapid commercial kits would greatly improve and speed up the identification and measurement of suspected β-lactam(s) (69).

The combination of both LC-fractionation and SPE techniques was reported for the determination of PenG in milk samples. The milk proteins were precipitated, and the supernatant was further purified on C18 SPE cartridge according to the method described by Boison et al. (80). The sample after SPE was focused on the top of the fractionation column by maintaining the gradient at 100% aqueous solution. The on-column focusing was further improved by bracketing of the injection (1 ml) on either side with 1 ml 10 mM ortophosphoric acid in the loop. No degradation effects were observed during the LC fractionation. The penicillin-G fraction was analyzed in a chromatographic system following the postcolumn derivatization with mercury chloride in the presence of both imidazole and nonionic surfactant BRIJ 35. A concentration of LOQ (2 μg/kg) lower than MRL for PenG in milk and recovery values of 70–73% were achieved (70).

A multiresidue procedure for β-lactams in milk (67) was adapted for the determination of free CEF metabolites—DFC dimer and DFC cysteine conjugate. The extraction and deproteinization procedure was modified for tissue samples. For LC analysis, an ion-pair chromatographic system gave satisfactory separation from the interference in the LC fractions. The recoveries of this procedure were 54–61% with CV $< 10\%$ (71).

A multiresidue procedure using LC fractionation for the identification and determination of 14 penicillins and cephalosporins and their metabolites in milk and tissue samples was presented (72). This procedure completed the previously published papers for the analysis of selected β-lactam residues in milk and tissue samples and gave an excellent tool for the determination of most antibiotic groups except for the tetracyclines.

The advantages of the proposed LC-fractionation system are: simplicity, high reproducibility, better selectivity in comparison to both SPE and LLE techniques, and low operating costs. However, high equipment cost is the main limiting factor of this approach. This method is intended for the examination of the small percentage of samples found to be positive for antibiotics by screening tests (72).

D. Pre- and Postcolumn Derivatization Assays

The combination of sample cleanup with precolumn derivatization enhances the selectivity of the determination and the sensitivity of the whole assay. Two different derivatization reactions were

used—reaction with aldehydes under acidic conditions forming highly fluorescent derivatives, and reaction with mercury chloride in the presence of triazole/imidazole under alkaline conditions, forming mercury mercaptide derivatives.

1. *Fluorescent Derivatization Assays*

The fish tissue sample containing AMO residues was homogenized with phosphate buffer (pH 4.5), followed by protein precipitation with TCA and SPE on a C18 cartridge. Trace amounts of nonpolar interfering substances present in the SPE eluate were removed by LLE using ether. The final extract was reacted with formaldehyde and TCA at 100°C for 30 min. A fluorescent derivative was extracted with ether three times, and the extracts were combined, evaporated, and reconstituted in the mobile phase. No interfering peaks from the control fish extract were observed. A proposed chemical structure of the fluorescent derivative was reported and confirmed by both MS and NMR experiments (73).

A simpler procedure consisting of protein precipitation using MeCN and TCA following formaldehyde derivatization was used for the determination of AMP in milk samples. The use of MeCN and TCA described in this method resulted in a clear supernatant and the highest recoveries (93% with CV of 6.1%). The derivatization reaction was done at 100°C for 30 min. The fluorescence of AMP derivatives was at least 20 times higher than that of AMO derivative; thus, no preconcentration step was needed, resulting in the simplicity of this assay. A coextracted interfering unknown compound was observed at or near the retention time of the AMP derivative. This did not affect the accuracy (less than 10%) of the determination (74). A similar procedure was reported for beef, pork, chicken, and catfish tissue samples (75).

A protein precipitation using sodium tungstate in the presence of sulphuric acid was combined with SPE on a C18 cartridge for milk samples. Sodium pentanesulphonate was added to the extract prior to the SPE in order to increase the retention of AMO and AMP. This significantly improved the recoveries of the whole assay ($>80\%$ with $CV < 5\%$). Highly fluorescent derivatives were formed when AMO or AMP was reacted with salicylaldehyde in the presence of TFA at 100°C for 45 min. Comparing the excitation and emission maximums, very similar results to those of the fluorescent formaldehyde derivatives were obtained. It was suggested that the salicylaldehyde derivatives of AMO and AMP may have similar fluorophores. The MeOH content in the eluate was partially evaporated under vacuum, since its presence seemed to affect adversely the derivatization reaction. After finishing the reaction, 2 ml of aqueous MeCN was added to dissolve the excess of unreacted salicylaldehyde (76).

The experiments showed that the derivatives were stable under refrigeration for at least 1 week (73), and they were stable at room temperature up to 48 hours (76). This analytical procedure is superior in terms of selectivity and sensitivity. The main disadvantage is that it is suitable for the analysis of amino-β-lactams only. No fluorescent derivatives were formed when other common β-lactam antibiotics were treated with salicylaldehyde or formaldehyde (76).

A comparison was made between this approach and the microbial inhibition method (*Bacillus stearothermophilus* disk assay according to [77]). No significant differences were found for AMO and AMP residues in milk within the reliable detection range of the microbial inhibition assay. The LC method was found more sensitive than the microbial inhibition method for residues lower than 10 μg/L (78,79).

2. *Ultraviolet Derivatization Assays*

This approach for sample cleanup was generally based on a protein precipitation using sodium tungstate under acidic conditions. The clear supernatant was further purified on a C18 or a trifunctional C18 SPE cartridge that had been previously conditioned with MeCN, water, and 2%

NaCl solution, improving the retention of penicillin residues. Penicillins were eluted from the SPE cartridge with MeCN or MeCN: phosphate buffer. The eluate was allowed to react with 1–10 mM mercury chloride in the presence of 2 M 1,2,4-triazole or imidazole under slightly alkaline conditions (pH 8.5–9). The derivatization was performed at 65°C for at least 30 min, yielding penicillinic acid mercury mercaptide with UV absorption maximum at 325–345 nm (80,81).

In the case of amino-β-lactams, an additional reaction step with acetic anhydride was necessary to protect the amino groups of AMO and AMP (82). This step was also incorporated into the assay for determination of five penicillins (AMO, AMP, PenG, PenV, CLO) (83). The previously described methods were modified by replacing the acylating agent, acetic anhydride, with benzoic anhydride; this permitted the elution of all derivatives under isocratic conditions within a very short period of time. This modification reduced analysis time per sample from over 1 hour to about 22 min without loss in detection sensitivity (84).

An LLE using dichloromethane was incorporated after the amino-penicillins had been reacted with benzoic anhydride. A cleaner chromatogram was observed with this improvement of the previous methods. The ruggedness of the different steps in the sample cleanup procedure was tested by a single-factor experiment and factorial design. The effect of the pH value of the sample extract on the recovery from a trifunctional C18 SPE cartridge was studied, and the strong influence on AMO was observed (recoveries 87–102%). This procedure described an LLE step for further sample cleanup. This was performed at pH 2.5 to obtain complete recovery of the penicillins, although PenG is not stable at this pH value. However, the breakdown was not significant within the first 5 min. The absorption effect of penicillins on the surface of glassware was eliminated by silanization with 10% dimethylchlorosilane in toluene (85).

When the composition of MeCN in the reaction medium was increased, the resulting mixture showed decreasing analyte sensitivity, usually accompanied by broad tailing and split peaks. Acetonitrile significantly suppressed the derivatization reaction between β-lactam and mercury chloride. Thus, it must be partially evaporated prior to derivatization (83), or the reaction time should be prolonged up to 180 min for the determination of OXA, CLO, and DICL (86).

An ion-pair separation system was used for the determination of AMO and AMP in milk samples (82), and the effect of TBAHS on the retention of OXA, CLO, and DICL was systematically studied. It was demonstrated that a concentration of 30 mM guaranteed an improved selectivity of the separation (87).

The combination of sodium tungstate protein precipitation and further purification on both strong-cation exchanger and porous graphitic carbon cartridges was published for the determination of AMO and AMP in tissue. There is an amino group in the analyte structure that is available for cation exchange at an appropriate pH value. Porous graphitic carbon offers a flat crystalline surface and a reverse-phase mechanism, which was particularly suited for the separation of planar highly conjugated organic compounds. This procedure not only removed more impurities but also exchanged an aqueous buffer for acetone, which could be more easily evaporated prior to derivatization (88).

A combination of MSPD and SPE was presented for the extraction of AMO, AMP, PenG, CLO, and DICL in porcine tissue samples. Tissue sample was mixed with prewashed endcapped C18 MSPD sorbent. The mixture was transferred to a 10-ml plastic syringe and covered by a paper disk. After compressing to 7.5 ml, the column was washed with hexane, and penicillins were eluted with MeOH. The extract was evaporated under nitrogen to near dryness and dissolved in 200 mM phosphate buffer. Direct derivatization was not possible due to the presence of proteins that inhibited the reaction because of bonding mercury chloride. Therefore, the MSPD eluate was further purified on a C18 SPE cartridge, followed by both acetylation of the amphoteric penicillins with acetic anhydride and reaction with mercury chloride in the presence of 1,2,4-triazole for 150 min. The mean recoveries of this procedure ranged from 25% to 120%, with a wide range of RSD values strongly depending on residue concentrations (89).

Sample cleanup based on the mercury chloride derivatization was compared to the antimicrobial inhibition tests. No significant differences were found between the methods when all data was included in the statistical computations (90).

An HPLC method was developed and validated for the determination and quantitation of both DFC cysteine and glutathione conjugates. This method was based on a cleavage of the disulphide and/or thioester bonds between the metabolites and their conjugate sulphur-containing moiety using dithioerythritol to yield DFC, which was then stabilized by derivatizing to DFC acetamide (91).

E. Liquid Chromatography–Electrochemical Detection Assays

Native penicillins, with the exception of AMO, cannot be detected electrochemically on glassy carbon electrode without prior photolysis. This was used for the PenG, PenV, OXA, CLO, DICL assay. Tissue sample was homogenized, and proteins were precipitated using MeCN under acidic conditions. The supernatant was extracted with both dichloromethane and light petroleum and re-extracted into the phosphate buffer. The extract was further purified using online SPE. The photochemical degradation was mediated using a transparent Teflon reaction coil woven to reduce band-broadening effects in a Beam Boost photoreactor equipped with a low-pressure mercury lamp. The penicillin photolysis products were detected on a glassy carbon electrode at +650 mV, with a 5–10-fold increase in sensitivity when compared with UV detection. It was important to add EDTA to the mobile phase, as it significantly reduced the baseline noise of the electrochemical detector (92).

Recently, it was shown that native penicillins could be oxidized on gold rotating-disk electrodes with a sensitivity comparable to that of direct UV measurement. Cyclic voltamperometry was used to study electrochemical responses of CEP and AMO, because the cyclic wave form used in cyclic voltamperometry mimics the cyclic scans performed during the detection in integrated pulsed amperometric detection. The potential was scanned between 240 and 1340 mV for four cycles to improve the signal-to-noise ratio in the shortest time. A potential of 1790 mV was chosen to achieve a fully formed gold oxide layer to allow complete cleaning of the electrode surface (93). The method was presented for milk samples that had been purified by MeCN precipitation and SPE (80) prior to the analysis. The NaCl solution was replaced by $NaNO_3$ due to the possible solubilization of the gold electrode in the presence of chloride ions (93).

A protein precipitation, LLE, and SPE followed by chromatographic separation with an indirect pulsed amperometric detection were used for the determination of nine penicillins in milk samples (94).

F. Liquid Chromatography–Microbial and Immunoaffinity Assays

This method combines the advantages of liquid chromatography with the selective and sensitive receptor assay Charm II-Q test. Milk sample previously precipitated (with McIlvaine buffer) and preconcentrated on a C8 cartridge was fractionated using an LC system. The fractions were collected according to the retention times and peak widths, and they were assayed directly with Charm II-Q test with small modifications. This method was suited for the AMO, AMP, PenG, CLO, CEF, and CEP residues in the milk samples and after small modifications for CFD, TIC, and NAF. A simple purification scheme gave recoveries from 50% for AMO to 80–90% for other β-lactams (95).

Multi-immunoaffinity chromatography columns were prepared by the coupling of monoclonal antibodies against AMP to activated sepharose. Both sensitivity and specificity were tested for the most commonly used penicillins (AMP, AMO, PenG, OXA, CLO, DICL). Recoveries ranging from 67% to 100% were obtained from phosphate buffer solutions, and it was assumed

that the capacity of the prepared immunoaffinity sorbents could be sufficient for the isolation and HPLC analysis of penicillins from milk and animal tissues at MRL levels (96).

G. Stability

The stability of PenG residues in both meat and liver tissues was tested in water, 5% ethanol, 5% bicarbonate, and sunflower oil. Penicillin-G residues were stable at 65°C in water. The half-time of decay in water at 100°C and 5% ethanol was approximately 60 min. The concentration reduced to about 40 min in acidic buffer and to 15 min in 5% sodium bicarbonate at 100°C. The half-times in sunflower oil at 140°C and 180°C were 45 and 20 min, respectively. This suggested that PenG was less stable in an aqueous than in a lipid environment and indicated that the degradation was caused by acid-and-base-catalyzed hydrolysis (97).

The effect of cooking on PenG residues in food was evaluated for different kinds of food preparation—boiling, frying, microwave cooking. The greatest losses were observed for processes that used the highest temperatures and the longest times. The PenG residues were found to be distributed between the solid cooked tissue and liquids used for cooking or that came from the meat as it was cooked. In some cases over a half of the total measured residue was present in the fluid (97).

IV. POLYETHER ANTIBIOTICS

Ionophores, or polyether (PET) antibiotics, produced by various species of *Streptomyces*, possess broad spectrum anticoccidial activities. They are chemically characterized by several cyclic esters, a single terminal carboxylic acid group, and several hydroxyl groups. Representative members of this class include salinomycin (SAL), monensin (MON), lasalocid (LAS), narasin (NAR), maduramicin (MAD), and semduramicin (SEM). The main chemical properties of interest in the extraction methodology are their low polarities and instability under acidic conditions. They are able to form stable complexes with alkaline cations. All of these compounds, with the exception of LAS, bind monovalent cations (e.g., Na^+ and K^+). Lasalocid has a tendency to form dimers and can form complexes with divalent cations such as Mg^{2+} and Ca^{2+}. The formation of metal complexes results in all of these compounds adopting a quasi-cyclic formation consequent to head-to-tail hydrogen bonding. No MRLs have yet been set by the EU for any of the carboxylic acid PETs (98).

The HPLC methods for the determination of PETs in food samples could be divided into two groups according to the physicochemical principle used for the sample cleanup or to the kind of detection:

LC-MS assays
Postcolumn derivatization and LC-UV assays

See Table 3.

A. Liquid Chromatography–Mass Spectrometry Assays

The LC-MS method was presented for the simultaneous determination of three PETs: MON, SAL, and NAR in muscle, liver, and eggs from domestic fowl. A relatively simple extraction and cleanup procedure was used. The best results were obtained by extracting PETs from tissues or

Table 3 Analysis of Polyethers in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
MON, SAL, NAR	Muscle, liver, egg	LLE	150 × 4.6 5-μm Inertsil ODS2	MeCN : MeOH : THF : water : TFA 67 : 10 : 10 : 13 : 0.1	ES-MS	0.5–1 μg/kg	99
NAR	Chicken fat	LLE, SPE	250 × 4.6 5-μm Jones C18	Gradient MeOH : water	ES-MS-LSC		100
SEM	Poultry liver	LLE, SPE	250 × 4.6 Zorbax silica	Ethyl acetate : isooctane : glacial acetic acid : TEA : MeOH 65 : 35 : 0.4 : 0.2 : 0.1	UV post-der 522	25 μg/kg	101
MON	Chicken tissues	LLE, SPE	250 × 4.6 5-μm Partisil ODS3	MeOH : water : acetic acid 94 : 6 : 0.1	UV post-der 520	5 μg/kg	102
MON	Bovine tissues, milk	LLE, SPE	250 × 4.6 5-μm Partisil ODS3	MeOH : water : acetic acid 94 : 6 : 0.1	UV post-der 520	LOQ milk 5 μg/kg, tissues 25 μg/kg	103
SAL	Chicken liver	LLE	150 × 2.1 5-μm Supelcosil LC-18	MeOH : 1% acetic acid 95 : 5 (UV), gradient MeOH : 1% acetic acid (ELISA)	UV post-der 520, ELISA	LOQ 100 μg/kg	104

eggs with MeOH without buffering. This was followed by a solvent-partitioning step, into toluene–hexane after the addition of NaOH to the extract. This resulted in better recoveries and cleaner chromatograms than did diluting the extracts in water. The extracts were evaporated, reconstituted in MeCN–water, and injected into a benchtop electrospray LC-MS system operated in the positive-ion mode for optimum sensitivity with PETs. No molecule ions are present in any of the spectra, and this reflects the high affinity of PETs to complex with cations, particularly sodium. The addition of TFA to the mobile phase maintained a low pH suitable for positive-ion EMS without causing significant ion-suppression effects, and the addition of MeOH increased the signal intensity by a factor of about 2. The THF was included in the mobile phase to improve the peak symmetry and to prevent tailing. The overall recoveries ranged from 77% to 113%, and the overall CV values were 4.4–15% (99).

An HPLC system with electrospray-MS and liquid scintillation counting (LSC) was used for assaying [^{14}C] NAR in chicken fat. The sample was combined with hexane and melted at 70°C, the hexane extract was reextracted with MeCN and evaporated to dryness, and the residue was resuspended in chloroform. Then it was passed through an unconditioned silica cartridge, and the retained radioactivity was eluted with MeOH. After the reconstitution and injection into the HPLC system, fractions for LSC were collected. Liver radioactivity was isolated by LLE and preparative silica LC (100).

B. Postcolumn Derivatization and Liquid Chromatography–Ultraviolet Detection Assays

Vanillin and 4-dimethylaminobenzaldehyde (DMABA) react with hydroxyl groups in the Komarowsky reaction. These postcolumn reagents, prepared in methanolic sulphuric acid, react with the carboxylic acid PETs at elevated temperatures. The PETs decompose in a poorly understood reaction, to form colored products detected at ca. 520 nm. Typically, the extraction step consists of either LLE or SPE (98).

The SEM was determined in poultry liver at μg/kg levels. The sample was extracted with ammonium hydroxide in MeOH–water, incubated at 55°C for 1 hour, centrifuged, and reconcentrated by evaporating. The SEM was separated from coextractives by reversed-phase (C8) and normal-phase (silica gel) extraction. After sample loading onto the C8 cartridge, this was washed with water and MeOH–water and eluted with ethyl acetate. The eluate was evaporated and reconstituted with methylenechloride–isooctane, loaded onto the silica gel column, and washed with methylenechloride–isooctane and ethyl acetate. The SEM was eluted with methylenechloride–MeOH, evaporated, and redissolved in ethyl acetate–isooctane. The derivatizing reaction was carried out at 95°C using EtOH–sulphuric acid–vanillin as a derivatizing agent. The mean recovery achieved was 95% (CV of 10% or better), and no interference was observed from the other commercial PETs (101).

Vanillin in MeOH and sulphuric acid was used for the derivatization of MON. Chicken tissue (muscle, liver, skin with adhering fat tissues) samples were homogenized with MeOH–water, NaCl was added to the supernatants, and MON was isolated and concentrated by liquid–liquid partition carbon tetrachloride and by SPE on the silica gel. Standard recoveries ranged from 82% to 96%. The method is specific for MON in the presence of closely related PETs—NAR and SAL. Lasalocid and other antibiotics, such as tylosin, nicarbazin, bacitracin, lincomycin, and bambermycin, do not react in the system and therefore do not interfere (102). A similar method was also used for the determination of MON in bovine tissues and milk. The homogenization of milk was performed by using MeOH. Recoveries achieved were 79–88% with RSD values of 4.6–9.1% (103).

A comparison of ELISA, HPLC, and HPLC-ELISA methods was published for the determination of SAL in chicken liver tissue. Samples were homogenized with MeOH and extracted with methylenechloride. Some samples were analyzed by HPLC using the isocratic solvent system and postcolumn derivatization (vanillin in MeOH containing sulphuric acid), with the eluent monitoring at 520 nm. The HPLC-ELISA system was used to characterize nonspecific effects analyzing column fractions by ELISA, since this detection is over 1000 times more sensitive than the spectrophotometric one. This alleviated the need to derivatize the drug prior to the detection (104).

A rapid preseparation technique was developed for the extraction of SAL from various chicken tissues using the irradiation of the sample in EtOH-2-PrOH for 9 s in a common household microwave oven. The extract was analyzed without further cleanup and detected via postcolumn reaction with DMABA at 86°C. Recoveries ranged between 87% and 100% (105).

C. Stability

The stability of SEM sodium in frozen (−20°C) poultry liver homogenates was assessed over a 40-day storage period. In this study, 2.5-g portions of control liver homogenate were fortified at the 60-μg/kg level, and samples were stored at −20°C in amber glass bottles. The samples after 5, 12, 19, 26, and 40 days gave respective mean levels of 56, 52, 54, 51, and 52 μg/kg, with an extrapolated zero time value of 55 μg/kg, indicating good stability of SEM in frozen poultry liver (101).

V. AMINOGLYCOSIDE ANTIBIOTICS

Aminoglycosides (AGLs) are broad-spectrum antibiotics with antibacterial and antifungal activities produced by *Streptomyces* spp. and *Micromospora* spp. They consist of an aminocyclitol ring (2-deoxystreptamine in most cases) connected to two or more amino sugars in a glycosidic linkage. All AGLs are potentially toxic compounds causing significant damage in vestibular and auditory functions in humans as well as in animals. The AGLs used in food-producing animals are apramycin (APR), bambermycin (BAM), dihydrostreptomycin (DIHS), gentamycin (GEN), hygromycin B (HYG), kanamycin (KAN), neomycin (NEO), spectinomycin (SPC), streptomycin (STR), and tobramycin (TOB).

The range between therapeutic effectiveness and toxicity is narrow; therefore, dosage must be monitored. The European Community has set MRLs for APR in swine at 1 mg/kg in muscle, fat, liver, and skin and at 5 mg/kg in kidney (106). The STR MRLs indicated in EU regulation 2377/90/EEC are: 200 μg/kg for milk (107), 300 μg/kg for muscle, 2.0 mg/kg for liver, and 5.0 mg/kg for kidney (108). The Swiss legal allowances for STR are 1.0 mg/kg for kidney and 500 μg/kg for liver (108). No MRL for STR in honey exists in the EU regulation. The STR Swiss allowance value is 100 μg/kg at this time, but it will be 10 μg/kg from January 2000 (109). The established U.S. tolerance for negligible residues of SPC in the edible tissues of turkey and chicken is 100 μg/kg, and 4.0 mg/kg for residues in kidney (110). The FDA has set tolerances of 100 μg/kg for SPC (110), 125 μg/L for DIHS, and 150 μg/L for NEO and a level of 30 μg/L for GEN in milk (5).

The HPLC methods for the determination of AGLs in food samples could be divided into three groups according to the physicochemical principle used for the sample cleanup or to the kind of detection:

LC-MS assays
Pre- and postcolumn derivatization assays
Other assays

In Table 4, the different methods of aminoglycoside analysis are summarized.

A. Liquid Chromatography–Mass Spectrometry Assays

Liquid chromatography–MS is often used as a confirmatory procedure. Mass spectrometry–MS detection was used in an analysis of SPC residues in bovine milk. Sample was extracted with TCA and centrifuged. The aqueous middle layer was removed, HFBA was added, and the mixture was applied onto a C18 SPE cartridge. The analyte was eluted with HFBA–MeOH, evaporated, and reconstituted in water. The extract was analyzed using ion-pair chromatography on a polymeric RP column and analyzed on a quadrupole ion-trap mass spectrometer equipped with an electrospray interface. The MS-MS data were acquired in the scan mode of product ions deriving from m/z 333, the protonated molecular ion. Several classes of veterinary drugs (AGLs, β-lactams, tetracyclines, and fluoroquinolones, among others) were also tested and did not interfere. This procedure was developed primarily as a qualitative confirmation of residue presence. However, since the linearity of response for standards was good, the authors also evaluated the ability of the ion trap, when used with this extraction, to determine the quantitative amount of residue present. Recoveries ranged from 69% to 93%, with RSD values of 6–18% (110).

A different procedure for the SCM confirmation was also published based on atmospheric pressure ionization with a collision-induced dissociation. The triple-quadrupole mass spectrometer operated in the Q1–Q3 product-ion mode. The preparation of tissue samples is described in the next section (111).

B. Pre- or Postcolumn Derivatization Assays

Aminoglycosides are basic, thermally labile, hydrophilic compounds that do not contain analytically useful UV-absorbing chromophores. Therefore pre- or postcolumn derivatization is necessary for their UV or fluorescence detection.

These highly polar compounds have a slight tendency to be bound to proteins and retained on nonpolar reversed-phase LC columns.

o-Phthalaldehyde (OPA) was used for the precolumn derivatization of APR. Any of four primary amines in APR could react to form fluorescence derivatives. The sample of swine kidney tissue was homogenized with ammonium hydroxide and MeOH, and the supernatant was evaporated. Phosphate buffer and ethyl acetate containing the ion-pairing agent (di-(2-ethylhexyl)phosphate) were added. The top of the organic layer was removed and combined with HCl, to break the ion pair, and heated at 60°C, and NaOH was added. The organic layer was aspirated, toluene was added, and the sample was centrifuged. Hydrochloric acid:NaOH (3:1) was added to the aqueous layer. The sample and OPA reacted together for 35 min prior to injection. Recoveries ranged from 76% to 86%, with CV of 4.8–14.3%. Mass spectrometry was also used for the detection of APR derivative, in addition to the fluorescence detection (106).

o-Phthaldehyde OPA was also used for the postcolumn derivatization of SPC in bovine liver. The sample was extracted with citric acid buffer (pH 4), TCA, and dichloromethane and treated by SPE. After washing the SPE cartridge (CBA) with phosphate buffer (pH 6.7), the analyte was washed with citric acid buffer (pH 2.6). The postcolumn procedure incorporated the oxidation of the secondary amines to the primary amines with sodium hypochlorite buffer pH 7–boric acid buffer pH 10.2–10.5 at 70°C followed by the derivatization with OPA at 26°C (111).

Table 4 Analysis of Aminoglycosides in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
SPC	Bovine milk	Pr, SPE	150 × 2.1 5-μm PLRP-S	MeOH : water : 20 mM HFBA 40 : 20 : 40	ES-MS-MS	LOC 100 μg/kg	110
SPC	Bovine tissues	Pr, LLE, SPE	150 × 2.1 Zorbax SB C18	MeOH : 1% acetic acid	APCI-MS-MS	LOC 100 μg/kg, kidney 50 μg/kg	111
SPC	Bovine liver	Pr, LLE, SPE	250 × 4.6 Zorbax SB C18	Gradient water : MeCN with 0.2% HFBA	F post-der, ex 340, em 455		111
APR	Swine kidney	Pr, LLE, pre-der	300 × 3.9 5-μm NovaPak C18	MeCN : water : acetic acid 40 : 60 : 2 with 5 mM OSA	F, ex 230, em 389, IS-MS		106
DIHS	Milk	Pr, LLE, SPE	150 × 4.6 5-μm Supelcosil LC-ABZ	MeOH : MeCN with 0.3% TEA : (pH 3.2 40 mM OSA with 20 mM EDSA, and 5 mM ninhydrin) 18 : 19 : 63	F post-der, ex 305, em 500	15 μg/L	112
DIHS	Kidney, meat	Pr, LLE, SPE	150 × 4.6 5-μm Supelcosil LC-ABZ + Plus	MeCN : pH 3.24 40 mM OSA with 400 mM NQS 32 : 68, flow gradient	F post-der, ex 375, em 420	20 μg/kg	113
STR, DIHS	Milk	Pr, SPE	125 × 3 4-μm Superspher 60 RP Select B	MeCN : NQS : pH 3.3 10 mM HSA with TEA	F post-der, ex 263, em 435	8–12 μg/kg	107
STR	Honey	Pr, SPE	100 × 4 3-μm Hypersil BDS	MeCN : 400 mM NQS in 20% MeCN with 10 mM HPSA 3 : 97	F post-der, ex 260, em 435	5 μg/kg	109
STR	Meat, milk, honey	Pr, SPE	100 × 4 3-μm Hypersil BDS	MeCN : 400 mM NQS in 20% MeCN with 10 mM HPSA 3 : 97	F post-der, ex 260, em 435	Honey 5 μg/kg, milk, meat 30 μg/kg, liver, kidney 100 μg/kg	109
STR, DIHS	Pork, bovine muscle, kidney	Pr, SPE, col-swi	250 × 4.6 5-μm LC8-DB	Water : MeCN 83 : 17 with 10 mM HSA and 0.4 mM NQS at pH 3.3	F post-der, ex 347, em 418	10–20 μg/kg	114

HFBA: heptafluorobutyric acid; EDSA: 1,2-ethanedisulphonic acid disodium; NQS: β-naphthoquinone-4-sulphonate.

Ninhydrin forms fluorophors of high intensity with guanidino compounds in alkaline media. Dihydrostreptomycin, which has two guanidino groups, yields similar fluorophors. Milk sample was treated with TCA, to precipitate proteins, and extracted with dichloromethane and NaOH, and the supernatant cleanup was performed using a C18 SPE column. The analyte was eluted with formic acid in MeOH. The postcolumn derivatization was performed at 80°C. The recovery from all procedures varied from 82.6% to 82.8% (only for two concentration levels), with RSD of 0.7–1.2%. This method can also be used for the determination of STR in milk (112).

A similar method was used for the determination of DIHS in kidney and meat from cow and swine. The derivatives were formed using 1,2-naphthoquinone-4-sulphonic acid (NQS) in NaOH. The temperature of the derivatization was lower (40°C) in comparison to the method described earlier. Recoveries achieved were 73.2–73.5% for kidney and 80.0–82.9% for muscle (for only two concentration levels), with RSD of 1.5–3.1% and 0.3–1.3%, respectively. After STR was added to the sample, the baseline resolution of the two drugs was not achieved (113).

The same derivatization was applied to the HPLC determination of STR and DHS in milk. The comparison of HPLC and ELISA methods was also performed for DIHS. After removal of fat by extracting a milk sample with oxalic acid and centrifuging, proteins were precipitated with TCA. The supernatant was treated by SPE on a C18 column. The cartridge was washed with water, and the analytes were eluted with ion-pair in MeCN. The eluate was reconcentrated by evaporating and dissolving in water. Postcolumn reaction took place at 65°C. Recoveries were dependent on the concentration level and the batch of SPE columns used, and independent of the fat content and homogenization. The sample cleanup was not sufficient for the analysis of cheese. The DIHS concentrations of incurred samples determined by ELISA were higher than those obtained by the LC method (107).

Streptomycin was also determined in honey, for it is an antibiotic used especially in beekeeping. Samples were mixed with $HClO_4$ (pH 2), centrifuged, and filtered. The cleanup of the filtrate was done by SPE, first on a cation-exchange cartridge (SCX), second on a C18 column. After the loading of the filtrate, the SCX column was washed with water and eluted with phosphate buffer (pH 8), the eluate was mixed with HPSA, pH adjusted to 2, and the mixture was loaded onto the C18 cartridge. This was washed with water and *tert*-butylmetylether-hexane (4:1). STR was eluted with HPSA in MeOH. The temperature of the derivatization was 40°C. Recoveries ranged from 86% to 94% (109).

The same authors also used the method just described to determine STR in meat and milk. For honey analysis, HPLC was compared with ELISA. The possibility of using the ELISA test was discussed for screening with and without SPE cleanup. A preliminary cleanup was absolutely necessary, because too many false positives (24%) were observed. After the SPE cleanup of the honey extract, ELISA results were quite in accordance with those observed by HPLC, and almost no false positives (4%) were recorded. However, because each positive ELISA result must still be confirmed by HPLC, the usefulness of this test is open to discussion. Considering the high financial cost and the time needed, this method really seems suitable only if a large number of samples must be analyzed. Comparison between the HPLC and ELISA results indicated a relatively good correlation between the techniques. However, the ELISA repeatability (CV of ca. 11%) was not as good as that of the HPLC (CV of ca. 6%) (108).

The column-switching system was applied to the determination of STR and DIHS in pork and bovine muscle and kidney. Perchloric acid was used to precipitate proteins and extract analytes from the tissue. The clear supernatant was further cleaned up by an offline SPE on a cation-exchange SPE column. After the washing of the cartridge with water, the analytes were eluted with phosphate buffer (pH 8.0) and diluted with HSA, perchloric acid, and water. The enrichment was achieved in the online mode. After loading the sample, the enrichment precolumn was flushed with HSA at pH 3.3 for 5 min. Using the ratio of MeCN to aqueous component of 83:17

in the mobile phase, the baseline resolution of the two drugs was not achieved. Changing it to 86:14 produced the baseline resolution but also resulted in long retention times. Because it was considered unlikely that both drugs would be encountered in the same sample, the first mobile phase was used. Mean recoveries were 61.1% for STR and 55.3% for DIHS, with CV 7.3% and 8.2%, respectively. The sample-enrichment precolumn began to deteriorate after about 150 injections (the injection volume was 2 ml) (114).

The direct extraction of gentamycin from milk was performed using a hydrophobic ion-exchange ($C_{18}COOH$ copolymer) SPE column. Gentamycin was eluted from the cartridge with buffer. In the presence of mercaptoacetic acid (MAA), OPA was used for the precolumn derivatization of the analyte, and the OPA–MAA derivative was detected by the UV detector at 330 nm. Tissue samples were homogenized in a buffer (pH 8.8) prior to SPE. Recoveries were greater than 90% (115).

C. Other Assays

A method capable of quantifying SPC in raw bovine milk was developed. In this procedure the sample was centrifuged at −4°C and the top fat layer removed. The defatted milk was deproteinated with TCA, and the supernatant was washed sequentially with dichloromethane, hexane, and ethyl acetate. An aliquot of the separated aqueous layer was prepared for the HPLC analysis by mixing with DSA and filtering. The analyte was quantified with an electrochemical detector. Recoveries achieved were 76–80% (116).

Monoclonal antibodies against STR were used for the preparation of an immunoaffinity chromatography column. Milk samples were defatted by centrifugation and diluted with phosphate-buffered saline. After loading onto the column, this was washed with saline, and STR and DIHS were eluted with the glycine–HCl buffer. The column bounded 80.4% and 88.7% of milk samples containing 100 ppb STR and DIHS, respectively (117).

VI. MACROLIDE ANTIBIOTICS

Macrolides (MACs) are chemically characterized by a macrocyclic lactone ring with isolated or conjugated double bonds, attached to amino sugars. Commonly used MACs consist of rings that are 12–16-membered structures. They are generally mixtures of more than one structural component. Representative members are erythromycin (ERY) and related substances, azithromycin (AZI), clarithromycin (CLA), dirithromycin (DIR), roxithromycin (ROX), flurithromycin (FLU), josamycin (JOS), rokitamycin (ROK), kitasamycin (KIT), mycinamycin (MYC), mirosamycin (MIR), oleandomycin (OLE), rosaramicin (ROS), spiramycin (SPI), tilmicosin (TLM), and tylosin (TYL). Macrolides are produced by various *Streptomyces* strains. They are active against gram-positive and some gram-negative bacteria.

Within the EU, MRLs for certain MACs has been set: for TYL, 50 μg/kg in milk (118) and 100 μg/kg in muscle, liver, and kidney for cattle, pigs and poultry (119); for TLM, 50 μg/kg in bovine muscle (120) and 1 mg/kg in porcine liver (1); for SPI, 50 μg/kg in bovine muscle (1) and 150 μg/kg in bovine milk (121); and for ERY, 400 μg/kg in bovine muscle (120). No MRL has been fixed for JOS (120).

Official U.S. and Canadian tolerance values are 50–300 μg/kg for SPI and 100–500 μg/kg for TYL in milk and different tissues from various species (122). Action levels for TLM have been established by Agriculture Canada for cattle tissues: 800 μg/kg in muscle and 2.4 mg/kg in kidney (123); and by FDA, 100 μg/L in milk (124).

The HPLC methods for the determination of MACs in food samples (Table 5) could be divided into three groups according to the physicochemical principle used for the sample cleanup or to the kind of detection:

LC-MS assays
LC-UV assays
Other assays

A. Liquid Chromatography–Mass Spectrometry Assays

Liquid chromatography–MS was used as a confirmatory technique in the determination of TYL in bovine muscle at the MRL level. The analyte was introduced into the ion source by a particle beam (PB) interface and identified by negative chemical ionization (NCI) with selective ion monitoring. The MS confirmation was carried out using this NCI mode because it provided the molecular ion and less fragmentation compared to electron impact (EI). Under the chosen LC-MS conditions, other MACs, such as SPI and ERY, do not interfere with TYL. The sample treatment was based on homogenization with the phosphate buffer (pH 8.5), extraction with chloroform, and a diol SPE cleanup of the chloroformic layer. Tylosin was eluted from the cartridge with the mobile phase (119). The same authors used a similar system to confirm the other four MACs (SPI, TLM, ERY, and JOS) in the same kind of matrix. The analytes were eluted from the SPE column with ammonium acetate–MeOH (50:50) and identified by negative and positive chemical ionization, with SIM of two ions in each mode. The use of TFA in the mobile phase instead of formic acid increased sensitivity. It was also essential for TLM, because it removed the memory effect of the chromatographic column (120).

B. Liquid Chromatography–Ultraviolet Detection Assays

The liquid–liquid partitioning method (LLE) is a cleanup method that has been used for many years for lipophilic compounds, such as MACs. These drugs are generally extracted from the biological matrix into organic solvents (chloroform, dichloromethane) at a pH at which the ionization of their basic function, amino sugar, is suppressed. Macrolides are also strongly retained on the C18 SPE cartridge. In the case of liver and kidney samples containing many contaminants, they cannot be completely eliminated (125).

A simple SPE was used in the assay for the determination of TYL. The milk sample was defatted by centrifugation, diluted with water, and loaded onto the C18 cartridge. The analyte was eluted with MeOH and filtered. Mean recoveries ranged from 81.7% to 87.6%, with CV of 7.32–17.1%. A small interfering peak occurred near the retention time of TYL (118).

Higher recoveries (80–106%) and higher CV values (3–9%) were achieved in the determination of TLM in bovine milk. Milk samples, defatted by centrifugation at −4°C, were treated by SPE based on removing matrix components with water, ammonium hydroxide–water, and water again, and eluting the drug with acetic acid–MeOH. To test method specificity, the LC system was applied to the analysis of some approved veterinary drugs to check for interference. The drugs analyzed include tetracyclines, sulphonamides, β-lactams, AGLs, MACs, corticosteroids, and diuretics. No peaks interfered with TLM. However, TYL had a peak that was very close to the TLM peak (124).

The combination of LLE and SPE was developed for the treatment of milk sample in the determination of SPI and neospiramycin (NEO). Milk was centrifuged at 10°C, and the aqueous layer was extracted with chloroform. The supernatant was loaded onto the diol cartridge. This was washed with chloroform, and the analytes were eluted with water–MeCN (121).

Table 5 Analysis of Macrolides in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
TYL	Bovine muscle	LLE, SPE	125 × 4 5-μm RP-18 endcapped	MeOH:MeCN:2% formic acid 45:20:35	PB-MS	10 μg/kg	119
SPI, TYL, TLM, ERY, JOS	Bovine muscle	LLE, SPE	125 × 4 5-μm LiChrospher RP 18 endcapped	Gradient 0.1% TFA:MeOH:MeCN	PB-MS		120
TYL	Milk	SPE	125 × 4 5-μm RP-18 LiChrospher	Gradient (100 mM ammonium formate:MeCN:MeOH pH 6.0):(water:MeCN:MeOH)	UV 287	12.5–15 μg/L	118
TLM	Bovine milk	SPE	250 × 4.6 5-μm Apex phenyl RP	Gradient (MeCN:water 50:50):pH 2.5 water:80 mM DBAP	UV 280	13 μg/L	124
SPI, NEOS	Milk	LLE, SPE	125 × 4 LiChrospher 100 RP-18	0.25% Sulphuric acid:MeCN 78:22	UV 231	LOQ 13–20 μg/L	121
MIR	Tissues	Pr, SPE	150 × 4.6 5-μm Puresil 5C18	MeCN:pH 2.5 50 mM phosphate buffer 30:70	UV 230	50 μg/kg	127
JOS, KIT, MIR, SPI, TYL	Meat	Pr, SPE	150 × 4.6 5-μm Puresil 5C18	Gradient pH 2.5 25 mM phosphate buffer:MeCN	UV 232 (TYL 287)	50 μg/kg	125
TYL, TLM	Bovine, porcine muscle, kidney	Pr, SPE	250 × 4.6 5-μm C18 Inertsil	pH 5 100 mM ammonium formate: MeCN:MeOH 60:30:10	UV 287	10–20 μg/kg	123
JOS	Porcine muscle, liver, kidney, fat	Pr, LLE, pre-der	125 × 4 5-μm LiChrospher 100 RP-18 endcapped	MeCN:MeOH:pH 6 10 mM phosphate buffer 45:5:50	F, ex 375, em 450	LOD 25 μg/kg	122
SPI, metabolites	Pig liver	Pr, LLE, col-swi	250 × 4.6 5-μm Kromasil C8	pH 2.3 50 mM phosphate buffer: MeCN 67:33 with 0.6 w.% $NaClO_4$	UV 232	LOQ 250 μg/kg	128

DBAP: dibutylammonium phosphate buffer.

A more difficult preseparation technique was used for the determination of TLM in ovine milk, combining several LLE procedures. Tilmicosin was extracted from milk into MeOH and acidified, and nonpolar coextractives were removed using hexane followed by carbon tetrachloride. pH was adjusted to 9.0, and TLM was partitioned into chloroform. Recoveries achieved were 84.3–104.8%, with RSD values of 6.6–12.9% (126).

The tissue sample containing MIR was homogenized and deproteinized with metaphosphoric acid–MeOH, and the extracts were cleaned up on SPE cartridges. Since MIR is a basic and lipophilic compound, a cartridge packed with an ion-exchange phase (benzenesulphonyl propyl) having cationic and lipophilic properties was selected. To elute MIR from the cartridge, MIR was first neutralized by washing the cartridge with K_2HPO_4 (pH 8.9) and then eluted with MeOH. Greater than 80% overall mean recoveries and ca. 5% RSD values were obtained. Under the chosen HPLC conditions, no interference was observed from the antibacterial substances (other MACs, penicillins, tetracyclines, sulphonamides, difurazon, and chloramphenicol), all having UV absorption in the vicinity of 230 nm. Only furazolidone eluted near MIR. However, furazolidone was not recovered at all by the proposed procedure. The HPLC was compared with bioassay, and excellent correlations were obtained (127). The same pretreatment procedure and a similar HPLC method were used in the assay of five MACs (JOS, KIT, MIR, SPI, and TYL) in meat. Greater than 70% overall mean recoveries and within 10% RSD were achieved with each sample (125).

Acetonitrile and phosphate buffer (pH 2.5)–MeCN were employed to homogenize and extract TYL and TLM from animal tissues (bovine and porcine muscle and kidney). Solid-phase extraction was utilized as the second preseparation step; MACs were eluted with ammonium acetate in MeOH. Mean recoveries were 79.9% (CV 8.1%) for TYL and 92.6% (CV 8.7%) for TLM, respectively (123).

C. Other Assays

In the presence of ammonia, cyclohexa-1,3-dione (CHD) reacts with the aldehyde group of JOS to give a heterocyclic structure having fluorescence properties. Reaction conditions (pH value, CHD, and an access of ammonium) were already optimized. The reaction rate was studied as a function of the temperature and the heating time. Maximum formation of the derivative was observed with incubation at 90°C for 2 h. The tissue sample preparation consisted of a homogenization step in MeCN–phosphate buffer (pH 6.0), centrifugation at 5°C, and LLE of the supernatant with iso-octane. The structure of the derivative was assessed using MS. Full selectivity was obtained in the HPLC system versus other MACs (TYL, SPI, and ERY), aldehydes (formaldehyde, acetaldehyde, and benzaldehyde), and endogenous compounds. Recovery was higher than 88% (RSD of 4.9%) (122).

D. Metabolites

A study of the metabolism of SPI in pig liver was conducted. The polar character of the cysteyl derivatives makes them difficult to extract in chlorinated solvents (dichloromethane, chloroform). As a consequence, extraction with pure MeOH was considered, because it extracted both SPI and its cysteyl metabolites. However, it also extracted other biomolecules interfering with the metabolites. Extractions with pure MeCN were unsuccessful, since liver tissues tended to agglomerate in this medium. Only 40% of the cysteyl conjugates were then extracted. The property of water to disperse the liver tissue was used to develop extraction conditions with MeCN–water (90:10). A good dispersion of the liver was then obtained, and pollution by polar interfering compounds coextracted from the liver was limited. Acetonitrile was evaporated and MeOH was added

to the aqueous residue. The mixture was sonicated and filtered. Two ml were injected onto the precolumn of the column-switching system, and it was flushed with phosphate buffer pH 2.3–MeCN containing $NaClO_4$ for 2 min. The HPLC system was operated at 60°C to avoid peak splitting due to the invertconvertion of thiazolidine isomers (128).

VII. AMPHENICOLS

Chloramphenicol (CAP), florfenicol (FLO), and thiamphenicol (TAP) are broad-spectrum antibiotics suitable for the treatment of a variety of infectious organisms (5). Chloramphenicol, which could produce aplastic anemia in a small percentage of humans, is not approved for use in food-producing animals in the United States (129). Florfenicol is allowed for the treatment of bovine respiratory diseases in the United States. The FDA has set a level of 10 μg/L in milk (130). The maximum residue level of CAP is set by the EU at 10 μg/kg (15). In Table 6, the different HPLC methods can be found.

A. Liquid Chromatography–Ultraviolet Assays

Chloramphenicol, FLO, and TAP have strong UV absorption, and they could be determined directly by HPLC (9). Unlike many of the more polar antibiotics, these three compounds could be extracted with an organic solvent. A single shake-out with ethyl acetate was sufficient for the quantitative extraction of CAP and FLO from milk (5). The extract was cleaned up using C18 and Florisil SPE cartridges. A review of methods for the determination of three amphenicol residues in food, including eight GC methods and six LC methods for CAP in milk, has also been reported (9).

The HPLC determination of CAP and TAP residues in gamebird (pheasant, mallard, and quail) was published (131). The drugs were extracted by LLE from the homogenized tissues (muscle, liver) by water, and the extracts were purified by subsequent partition with a ChemElut cartridge. The drug was eluted with ethyl acetate and the eluate after the evaporation was mixed with water and extracted with toluene. The aqueous phase was injected into an HPLC system. The recoveries were 67% for CAP and 72% for TAP. The purity of analyzed compounds was verified by DAD. The elimination kinetics of CAP and TAP from the quail tissues were also evaluated.

Another paper describes a modified HPLC assay for the determination of TAP residues in spiked milk at levels as low as 30 μg/kg (132). Milk spiked with TAP was extracted with ethyl acetate. Following the addition of hexane, the extract was cleaned up with a silica SPE cartridge. Just before use, the cartridge was pretreated by passing an ethyl acetate: hexane mixture. The TAP was eluted with methanol. The eluate was collected and evaporated. The residues were dissolved in mobile phase and injected into an HPLC column. The recovery was found to range from 68.0% to 90.0%, and precision data suggested that RSD ranged from 7.0% to 9.4%.

Determination of CAP in animal tissue using HPLC with a column-switching system and UV detection has also been published (133). The cleanup procedures described in this paper are rather complicated and vary for different tissues. The applicability of this method was tested by analyzing different tissues of pig (liver, kidney, muscle, fat, and skin) and trout (muscle, skin). Tissue samples were mixed with sand and dried. The sample was homogenized after adding MeCN. The procedure was repeated and the solvent evaporated. The residue was dissolved in MeCN and transferred to a C18 SPE cartridge, which was rinsed with the same solvent. The CAP was eluted with MeCN, the eluate was carefully evaporated, and the procedure was repeated with a silica cartridge, using ethyl acetate as a solvent instead of MeCN. The MeCN was evaporated, and hexane was added to the residue. An aliquot of the lower aqueous phase was injected directly

Table 6 Analysis of Amphenicols in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
CAP, TAP	Gamebird tissue	LLE	150 × 4.6 5-μm Hypersil RP-18	MeCN:50 mM pH 3 TEA–phosphate buffer 21:79 (CAP); 14:84 (FLO)	UV 278, 224	2 μg/kg	131
TAP	Milk	LLE, SPE	250 × 4.6 5-μm Hichrom	MeOH:water 30:70	UV 224	LOQ 30 μg/kg	132
CAP	Pig, turkey (liver, kidney, muscle, fat, skin), trout (muscle, skin)	Pr, SPE, col-swi	precolumn: 150 × 4.6 5-μm Hisep 150 × 4.6 5-μm Supelcosil LC-18 (A) 250 × 4.6 5-μm Supelcosil LC-18 (B)	MeCN:water:THF 80:18:2	UV 278	2 μg/kg	133
CAP	Fatty liver	LLE, SPE	150 × 3.9 4-μm NovaPak C-18	MeCN:5 mM pH 7.9 phosphate buffer 19:81	UV 278	1 μg/kg	134
CAP + other drugs	Tissue (veal, pork)	MSPD	250 × 4.6 5-μm Spherisorb C-18	Gradient MeCN-MeOH (70:30):10 mM pH 5.2, ammonium acetate buffer	UV 290	5.5–6.5 μg/kg	20

onto an HPLC equipped with a column-switching system. Three columns were connected in the HPLC system. A column packed with RAM sorbent (Hisep) was the first column in the system. Two C18 columns were applied as a trap and analytical columns, and they were combined with two switching valves. A RAM column was devoted to removing proteins and other compounds with high molecular mass from biological samples. The linearity and repeatability of the assay were evaluated. Extraction recoveries were calculated for all analyzed matrices. The application of HPLC with a column-switching system permits a significant simplification of the cleanup procedure. Furthermore, loss of CAP was minimized.

The HPLC with UV detection and scanning UV confirmation of CAP in fatty liver was discussed (134). After the addition of water, the sample was defatted with hexane, and CAP was extracted with ethyl acetate. The silica gel SPE assay was used to recover CAP from fatty liver extract. After washing the cartridge with hexane, CAP was eluted with phosphate buffer (pH 10) and then extracted from the eluate with ethyl acetate. After centrifuging, the acetate was evaporated and the residue dissolved in hexane : chloroform. After adding water and the centrifugation, the supernatant (aqueous phase) was injected into an HPLC system. The recovery was found to be 71% in the concentration range of 5–20 μg/kg. The SPE step was introduced to remove major interfering compounds. An additional step (LLE with hexane) was also necessary to eliminate other lipophilic interfering components. The fat content in fatty liver represents 50–60% of the total weight. The analyte identity was confirmed by the use of UV scanning detection and by the LC/MS method for concentrations of less than 10 μg/kg.

A multiresidue preparation technique—MSPD—has also been applied to the analysis of CAP residues in meat samples. Two fractions were collected by elution with methylene chloride and ethyl acetate. No additional purification was necessary. Diode assay detection and fluorescence detectors were recommended for the multiresidue analysis of sulfonamides, benzimidazoles, nicarbazin, furazolidone, and CAP. The percentage recoveries and linearity of the method were evaluated. The method was linear from 50 to 250 μg/kg of CAP. Not only do the authors recommend the MSPD multiresidue procedure for HPLC analysis, but it could be associated with several detection modes, such as immuno- or receptor assays. The MSPD technique represents a new approach in the field of biological-matrix extraction and provides a great possibility for the analysis of a wide range of compounds (20).

B. Pharmacokinetics

Studies have also been realized to determine the fate of C^{14}-labeled CAP in laying chickens. The elimination of CAP from tissue and eggs was evaluated. More than 95% of CAP was eliminated in 24 hours after dosing. Radiocarbon C^{14} was deposited first in yolks, compared to albumen or other tissues. Various metabolites were isolated and identified by TLC, LC, and LC/MS (135).

Tissue concentrations and pharmacokinetics of FLO in broiler chickens were studied (136). Florfenicol was administered to broiler chickens, and its concentrations in kidney, bile, muscle, intestine, heart, liver, and spleen were determined. Low concentrations were also found in brain, bone, and fat.

VIII. NITROFURANS

Nitrofurans (NFs) are synthetic chemotherapeutic agents effective in the prevention and treatment of gastrointestinal infections caused by *Escherichia Coli* and *Salmonella* spp. Administered orally, usually as feed additives, they are widely used in treating cows, cattle, pigs, and poultry.

Furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT), and nitrofurazone (NFZ) belong to this group of drugs.

Furaltadone, NFT, and NFZ are listed in Annex IV of EC Council regulation 2377/90; therefore no MRLs have been established for these drugs, and their use is prohibited in livestock production within the EU (2). Furazolidone was added to the list of prohibited compounds in 1995. This decision was taken because FZD residues, at whatever limit, in foodstuffs of animal origin constitute a hazard to the health of the consumer (137).

Nitrofuran assays are summarized in Table 7.

A. Nitrofuran Assays

Due to the high sensitivity of the UV-detection and "less problematic" UV-absorption maximum values (around 365 nm), simpler sample cleanup and preconcentration techniques were developed, as compared to the other antibiotic groups.

A simple assay based on a homogenization of muscle samples with MeCN was used for FZD in meat. The resultant supernatant was evaporated and dissolved in mobile phase, the lipidic compounds were removed with hexane, and an aqueous layer was injected into the ion-pair chromatographic system (138). Evaporation of the aqueous layer and dissolution in the mobile phase improved the previous assay by means of LOD. Ethanol was added during the evaporation to facilitate the removal of water residues—it also adjusted the surface tension of the extract and prevented strong bubbling, which could cause a loss of analytes (139). Single extraction gave sufficient recoveries for the NFs (NFZ, NFT, and FZD), varying in the range of 71–101% with RSD $< 20\%$.

The determination of all four NFs in bovine muscle tissue was used. The tissue sample was homogenized with MeCN, lipids were removed by washing with dichloromethane: ethyl acetate, and the concentrated aqueous layer was further purified by washing with hexane. Recoveries were obtained ranging from 60% to 110%, with RSD $< 18\%$ and a separation without interference (140).

An additional cleanup step of the final extract using SPE on C18 cartridges provided better removal of possible interference from meat in FZD, NFT, and NFZ assay. The extract after homogenization and *n*-hexane washing steps was loaded on a preconditioned cartridge, and NFs were eluted with ammonia in MeOH (141). The combination of alumina and C18 SPE cartridges was used for the determination of FZD in shrimp tissue (142). Recoveries ranging from 77% to 85% were obtained with sufficient LOD.

An LLE was used for the determination of FZD, FTD, and NFZ in avian eggs. Homogenized shelled eggs were mixed with MeCN, and the supernatant was extracted with dichloromethane. The extract was evaporated and dissolved and lipids were removed by washing with *n*-hexane prior to injection (143). The sample pretreatment of avian egg samples with FZD residues was simplified using an Extrelut-3 column, thereby avoiding tedious steps and significantly reducing the analysis time (less than 30 min, including sample cleanup) (144).

Although UV-VIS detection at 362–375 nm was shown to be suitable in the previously reviewed assays (138–141), deviations in UV-VIS spectrum of the FZD peak were observed in some cases. This indicated possible interference from matrix components, leading to false-negative results. Therefore, the chromatographic separation was detected using atmospheric pressure ionization MS in positive-ion mode with SIM of ions 199 (NFZ), 226 (FZD), and 325 (FTD) (143,144).

An LC-thermospray MS assay was used for the determination of FZD in porcine tissue. Frozen pulverized tissue was homogenized with MeOH: McIlvaine buffer, and the supernatant was extracted with dichloromethane. The organic layer, evaporated and reconstituted in

Table 7 Analysis of Nitrofurans in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
NFZ, FZD	Tissue	Pr	250 × 4.6 5-μm Supelcosil LC-ABZ	MeCN:25 mM pH 2.5 trisodium phosphate with 20 mM HSA 25:75	UV 365	LOQ 3 μg/kg	138
NFZ, NFT, FZD	Tissue	Pr, LLE	200 × 4.6 5-μm Hypersil C18	MeCN:1% acetic acid 25:75	UV 375	1 μg/kg	139
NFT, NFZ, FTD, FZD	Bovine tissue	Pr, LLE	100 × 4.6 5-μm Hypersil C18	MeCN:10 mM pH 4.5 acetate buffer 30:70	UV 365	1 μg/kg	140
NFT, NFZ, FZD	Meat	Pr, SPE	200 × 4 5-μm Hypersil C18	Gradient MeCN:20 mM pH 5.0 acetate buffer	UV 365		141
FZD	Shrimp	Pr, SPE	250 × 4.6 5-μm Ultrasphere C18	MeCN:0.01% phosphoric acid 30:70	UV 365	LOQ 2.5 μg/kg	142
NFZ, FTD, FZD	Avian eggs	Pr, LLE	150 × 4.6 5-μm Spherisorb ODS II	MeCN:20 mM pH 4.6 sodium acetate 21:79	UV 362, API-MS	1–3.2 μg/kg (MS) 2.5–5 μg/kg (UV)	143
FZD	Avian eggs	LLE	150 × 4.6 5-μm Wakosil 5WC18	MeCN: water 40:60	API-MS	100 g/L	144
FZD	Porcine tissue	Pr, LLE, SPE	125 × 4 5-μm LiChrospher RP18 endcapped	MeCN:100 mM ammonium acetate 1:3	TS-MS	1 μg/kg	145
NFZ, FTD, FZD	Porcine and poultry tissue, eggs	Pr, LLE, SPE	250 × 4.6 5 micro Spherisorb ODS2	MeCN: water 1:3	UV 362	LOW 0.5 μg/kg[a]	146
NFT, FTD, FZD	Milk	Pr, SPE	150 × 3.9 4-μm Novapak C18	MeCN:100 mM sodium perchlorate 28:78 with 0.5% glacial acid	E coulometric—600 mV	4 μg/kg	147
AOZ	Porcine tissue	Pr, LLE	125 × 4 5-μm LiChrospher RP18 (endcapped)	MeCN:100 mM ammonium acetate 35:65	TS-MS	10 μg/kg	148
AOZ, AMOZ	Tissue	Pr, LLE	UV: 250 × 4.6 5-μm Hypersil ODS MS: 30 × 3.9 10-μm Bondapak C18	UV: MeCN:10 mM pH 7.4 phosphate buffer 25:75 MS: MeOH:0.025% acetic acid 45:55	UV 275, APCI-MS	5–10 μg/kg (UV), 10 μg/kg (MS)	149
FZD + other drugs	Meat	MSPD	250 × 4.6 5-μm Spherisorb C18 ODS II	Gradient MeCN:10 mM pH 5.2 ammonium acetate buffer	UV 365	2.5–3.5 μg/kg	20

[a] Two times peak-to-peak noise value.

dichloromethane: hexane, was further purified on a preconditioned Bond-Elut NH2 cartridge in normal-phase mode. Furazolidone was eluted with chloroform: MeOH, and the eluate was evaporated to dryness and redissolved in mobile phase. Several other methods (including the application of an Extrelut column) were investigated. The assay with McIlvaine buffer and an NH_2 SPE cartridge provided the cleanest extracts (recoveries ranging from 66% to 70%), which were more amenable to LC-MS working in positive-ion mode with SIM of m/z 243. The variability of the assay was higher than that which would have normally been expected (RSD 7.4–18.4%). This might reflect the instability of FZD in tissue homogenates and the relative complexity of the extraction and cleanup procedures (145).

A similar procedure was developed for NFZ, FZD, and FTD residues in porcine muscle tissue and eggs. The tissue samples were extracted twice with dichloromethane: ethyl acetate, and the combined organic layers were evaporated to dryness. The residue was dissolved with dichloromethane and petroleum ether. Egg proteins were precipitated with MeCN, and the supernatant was extracted with dichloromethane and petroleum ether. The organic layers were applied directly on a silica SPE cartridge. The optimal recoveries (84–111%) were achieved after elution with dichloromethane: ethyl acetate (146).

Nitrofurantoin, FZD, and FTD were assayed in milk with an HPLC system following the electrochemical detection. Milk was deproteinized with TCA and the supernatant applied on a C18 SPE cartridge. Analytes were eluted with the mobile phase. The detection was accomplished using the coulometric detector COULOCHEM II, with two electrochemical cells at the working electrode potential of −600 mV. The coulometric detection seemed to be a less rugged detection technique for NF residues, even though high recoveries and excellent precision (RSD lower than 4% at 30 μg/kg) were presented. The authors observed a very high influence of the mobile phase composition and analysis conditions on background signal (147).

A multiresidue technique—matrix solid-phase dispersion (MSPD)—was used to purify the meat samples. The prewashed C18 bulk material was gently ground with the blended sample. The resultant C18/tissue matrix mixture was transferred to a 10-ml syringe barrel. The precolumn prepared in this way was washed with hexane, and FZD residues were eluted with dichloromethane, with 13 other veterinary drugs assayed. The recoveries varied from 44% to 87%, depending on the concentration level, and the LOD was established at 2.5 μg/kg (20).

B. Stability

Because NFs are light sensitive, all sample extraction and cleanup procedures must be performed under artificial yellow light. The stability of FZD residues in tissue was studied. The storage of a sample for 24 h at 4°C was sufficient to ensure the complete degradation of the FZD present in the tissue sample. The storage at −20°C resulted in degradation to 10%, at −70°C to 50% of original FZD residue concentrations after 1 week, respectively. Storage in liquid nitrogen provided full stabilization of this compound (145). According to a similar experiment, the level of FTD dropped to less than 25% of the original value after 4 days of storage in the dark at −18°C (146).

C. Metabolites

The data concerning the stability indicated that the analysis of FZD residues in tissue was of limited value. Consideration should be given to measuring 3-amino-2-oxazolidone (AOZ), FZD metabolite, and 5-morpholinomethyl-3-amino-2-oxazolidone (AMOZ), FTD metabolite, which were reported as possible mutagens and carcinogens. The administration of the drugs to animals can result in the occurrence of inextractable protein-bound residues releasing AOZ and AMOZ

under mildly acidic conditions for at least 1 month after the administration of FZD and FTD, respectively (148).

The frozen pulverized tissue was homogenized with water and MeOH. The homogenate was centrifuged, and both the bound FZD and FTD fraction (the pellet) and the extractable fraction (the supernatant) were treated differently. The supernatant was removed, the pellet was washed with MeOH, EtOH, and diethylether, and the fractions were added to the supernatant. The pooled supernatant was evaporated to dryness, and the residue was resuspended in water, hydrochloric acid, and 2-nitrobenzaldehyde (NBA) in DMSO. The remaining dry pellet was resuspended in water; hydrochloric acid and NBA in DMSO were added. The mixtures were incubated at 37°C for 16 h. Derivatives were extracted into the ethyl acetate at slightly alkaline conditions, and the organic layer was evaporated (148,149). The excess of derivatization agent was removed with hexane. The incorporation of a hexane cleanup step into the extraction method improved the method's ruggedness by decreasing the variability due to the NBA present in the final extracts and by significantly decreasing the NBA interference in the LC-UV assay of AOZ derivative (149). The derivatives were separated on C18 columns, and they were detected using thermospray ionization MS in positive-ion mode with SIM of m/z 253 (148) or 236 and 206 (149) for AOZ, SIM of m/z 335 and 291 for AMOZ (149).

The results of the stability studies showed that AOZ concentrations in the bound and extractable sample fractions of liver decreased by 22% and 27%, respectively, at +4°C after 48 h. However, liver samples that had been frozen at −20°C showed no significant difference in AOZ concentration after 6 months of storage (148). Therefore, AOZ seems to be a good marker residue for bound residues of FZD in tissue samples (149).

IX. SULPHONAMIDES

Sulphonamides (SAs) comprise a large group of synthetic N-derivatives of 4-amino-benzenesulphonamide. They are bacteriostatic agents, and they act by competing with *p*-aminobenzoic acid in the enzymatic synthesis of dihydrofolic acid. This leads to a decreased availability of the reduced folates that are essential for purine synthesis, methionine synthesis, etc. Sulphabrommetazine (SBM), sulphacetamide (SAC), sulphaclozine (SCL), sulphadiazine (sulphapyrimidine, SDZ), sulphadimethoxine (SDM), sulphadoxine (SDX), sulphafurazole (SFU), sulphaguanidine (SGU), sulphachlorpyridazine (SCP), sulphamerazine (SME), sulphamethazine (sulphadimidine, SMZ), sulphamethizole (SMT), sulphamethoxazole (SMX), sulphamethoxypyridazine (SMP), sulphamonomethoxine (SMM), sulphamoyldapsone (SD), sulphanilamide (SAA), sulphanitran (SN), sulphaperine (SPE), sulphaphenazole (SPH), sulphapyrazole (SPY), sulphapyridine (SPR), sulphaquinoxaline (SQX), sulphatiazole (STA), sulphatolumide (STO), sulphatroxazole (STR), and sulphisoxazole (SIA) belong to the group of SA compounds.

Trimethoprim (TMP) and ormethoprim (OMP) are synergists of SAs that operate by a mechanism of competitive inhibition of dihydrofolate reductase. Sulphonamides (and their synergists) are widely used in farm animal feedstuff and fish cultures; furthermore, they act as growth promoters at subtherapeutic concentrations.

European Union regulations, as well as the FDA, have set MRL values of 100 μg/kg in tissue and 10 μg/kg in milk samples for total SA content, respectively. There is a concern about the effects of SAs entering the human food chain, since there is a possibility that exposure to these drugs could reduce the effectiveness of human therapeutic drugs and that some SAs could be carcinogenic. The MRL values of 50 μg/kg in both tissue and milk samples were set for TMP and its congeners.

A wide variety of HPLC methods were used for the determination of SAs and their synergists, due to the high number of compounds belonging to this group of bacteriostatic agents (close to 30 compounds, two synergists). The assays reviewed in this part are divided into five groups, plus a separate section dealing with TMP and OMP analysis:

LC-UV assays
LC-MS assays
Pre- or postcolumn derivatization assays
SFE-LC assays
LC-microbial and immunoaffinity assays
TMP and OMP assays

See Table 8 for the different methods.

A. Liquid Chromatography–Ultraviolet Detection Assays

A simple assay was used for the determination of SGU, SDZ, STA, SPR, and SMX in honey, milk, and eggs. Honey sample was dissolved in water and homogenized and injected directly onto the column. Milk and egg proteins were precipitated with TCA, and the extract was diluted with TCA solution. It should be noted that the incomplete elution of some sample components (lipidic compounds) contributed to the steady deterioration of the chromatographic column. Therefore, washing the column with a mixture of ethyl acetate : MeCN was necessary at the end of each day. The extracts were separated using gradient elution and detected with UV detector. Small interfering peaks were observed equivalent to the LOD concentration levels (30–80 μg/kg), which were not totally resolved from either SDZ or SPR. The authors reported recoveries ranging from 90% to 110% (150).

Eight SAs (STA, SME, SCP, SMZ, SMP, SMX, SQX, and SDM) were analyzed in veal and pork meat using the MSPD technique. A homogenized meat sample was gently mixed with C18 sorbent, providing a tissue/sorbent phase. The resultant mixture was placed into a 10-ml syringe barrel, plugged by a paper disk, producing an SPE cartridge. The material was washed with hexane to remove lipidic compounds. Sulphonamides were eluted with dichloromethane, and the other simultaneously analyzed drugs were eluted with ethyl acetate. The SA fraction was evaporated and dissolved in the mobile phase. The chromatographic separation was carried out under gradient conditions followed by UV detection. Sufficiently low LOD values were found for all SAs (2–10.5 μg/kg) except SMP (23–42 μg/kg). The poor LOD value for SMP could be explained by coeluting compounds, which seemed to be natural components of both pork and veal meat. The recoveries of the MSPD assay varied from 39% to 88%, depending on concentration and SA concentration (20).

An MSPD reported by Long et al. for SDM residues (151) was modified in the elution step and applied to OMP, SDM, and SMX (internal standard). The drug residues were eluted from the column with dichloromethane and MeCN. Recoveries ranged from 86% to 95%, with CV < 18%. It should be noted that the LOD value was equal to the MRL value for TMP (50 μg/kg). This assay was applied to the assessment of the effect of cooking processes on OMP and SDM residue concentrations in catfish tissue (152).

The sample homogenization with an MeCN : THF mixture was used for the simultaneous determination of SMM, miloxacin, and oxolinic acid. The supernatant was filtered and injected directly into the ion-pair chromatographic system using a shielded hydrophobic phase. This method did not require time-consuming and complex extraction procedures; moreover, the use of a restricted-access-material column prevented both column clogging and peak broadening throughout the analysis. On the other hand, no preconcentration of the sample affected the LOD

Table 8 Analysis of Sulphonamides in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
SGU, SDZ, STA, SPR, SMX	Honey, milk, eggs	Pr/Ho	150 × 4.6 5-μm Spherisorb ODS-2	Gradient MeCN : water	UV 260	30–80 μg/kg	150
OMP, SDM, SMX	Catfish tissue	MSPD	250 × 4.6 5-μm Hypersil ODS	MeCN : 100 mM pH 4.5 phosphate buffer 19 : 81	UV 288	50 μg/kg	151
SMM + other drugs	Fish muscle	Ho	150 × 4.6 5-μm Hisep SHP	MeCN : (50 mM citric acid, 200 mM pH 2.5 phosphate buffer, 10 mM TBA bromide) 15 : 85	UV 265	100 μg/kg	153
21 SAs, TMP, OMP	Milk		50 × 4 3-μm ODS AQ	Gradient MeCN : water with 0.1% formic acid	ES-MS-MS	0.2–2 μg/kg	154
SDM, SMZ	Tissue	Ho, SPE	250 × 4.6 5-μm Spherisorb ODS-2	MeCN : 50 mM NaH_2PO_4 28 : 72 (UV), MeCN : 10 mM pH 4.6 ammonium acetate 28 : 72 (UV-MS)	UV 265, TS-MS	2 μg/kg (SMZ), 10 μg/kg (SDM)	156
13 SAs	Tissue, milk, eggs	Ho/Pr, LLE, pre-der	250 × 4.5 5-μm Nucleosil 100 RP-18	Gradient MeCN : 20 mM orthophosphoric acid	F, ex 405, em 495	5 μg/kg (10 μg/kg SQX)	157
SDA, STA, SMZ, SMM, SMX, SDM	Milk	Pr, pre-der	150 × 3.9 4-μm NovaPak C18	MeCN : 10 mM KH_2PO_4 30 : 70	F, ex 390, em 475	1 g/L	158
14 SAs	Salmon tissue	Ho, LLE	150 × 4.6 5-μm Symmetry C18	Gradient MeCN : MeOH : 2% acetic acid	F post-der, ex 400, em 495	1–5 μg/kg	159
SDZ	Salmon tissue	Ho, LLE, SPE	150 × 4.6 5-μm Inertsil ODS-2	MeCN : 2% acetic acid 10 : 90 (F), MeCN : 2% acetic acid 20 : 80 (MS)	F post-der, ex 400, em 495, APCI-MS	0.2 μg/kg (F), 2 μg/kg (MS)	160, 161
SAA, SGU, SDZ, SPR, SMX	Tissue, eggs, milk	Ho/Pr	150 × 4.6 5-μm Spherisorb ODS-2	Gradient MeCN : water	F post-der, ex 302, em 412	36–340 μg/kg (milk), 27–47 μg/kg (eggs), 37–60 μg/kg (tissue)	163

(continued)

Table 8 Continued

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
SMZ	Muscle, liver, kidney	MWE, Ho, SPE	250 × 4 5-μm LiChrospher 100 RP-18	MeOH : 100 mM pH 4.8 phosphate buffer 15 : 85	UV post-der 450	LOQ 2.5 μg/kg	164
SQX, SMX, SDM	Milk powder, egg yolk, liver	SFE, SPE	250 × 4.6 5-μm Deltabond ODS	MeCN : 8 mM ammonium acetate 15 : 85	UV 266		165
SQX, SMX, SDM	Eggs, liver	SFE, in-line SPE	250 × 4.6 5-μm Supelcosil LC-18	MeOH : 50(200) mM pH 7.2 phosphate buffer with 0.1% TBA hydroxide 32 : 68	UV 252–269	50 μg/kg	168, 169
SMZ, metabolites	Liver, kidney	SFE, LLE	250 × 4.6 5-μm Spherisorb C18	MeCN : 50 mM pH 5.0 acetate buffer with 10 mM TBA bromide 15 : 85	UV 266	mg/kg levels	170
SDM	Milk	LLE	300 × 4.6 5-μm Supelco LC-18-DB	MeOH : 100 mM KH_2PO_4 40 : 60	UV 269	2 μg/kg	171
TMP	Tissue, milk	Ho/Pr, SPE	150 × 3.9 4-μm NovaPak C18	MeCN : 5 mM pH 3.0 phosphate buffer with 5 mM PSA 12.5 : 87.5	UV 229, 280	5 μg/kg	176, 177
TMP	Tissue	Ho, LLE	250 × 4.6 5-μm Partisil ODS-3	MeCN : water : acetic acid 25 : 73.7 : 1.3 with 0.5 M ammonium acetate	ES MS	4 μg/kg	178
8 SAs	Veal and pork meat	MSPD	250 × 4.6 5-μm Spherisorb ODS-2	Gradient MeCN : MeOH : 10 mM pH 5.2 acetate buffer	UV 270 (365 SQX)	2–10.5 μg/kg (23–42 μg/kg SMP)	20

value for SMM (100 μg/kg), which is equivalent to the MRL value set for the total SA content in the food material (153).

B. Liquid Chromatography–Mass Spectrometry Assays

An HPLC coupled with electrospray MS and tandem MS was used for the determination of 21 SAs and their potentiators OMP and TMP in milk. The strategy used to detect, confirm, and quantify SAs was as follows: (a) prescreening by precursor ion-scan and multiple reaction-monitoring experiments using the group-specific ions at m/z 156, 108, and 92; (b) quantitation of identified compounds using SIM of their protonated molecules; and (c) further confirmation by selected reaction monitoring using ions characteristic of the *N*-heterocyclic moieties. Separation of all compounds was achieved within 6 min, with peak width less than 10 s, and the method LOD in the SIM procedure ranged from 0.2 μg/kg to 2 μg/kg (154).

Thermospray MS was used online, coupled with the UV detector, for the determination of SMZ and SDM in animal tissue. The tissue sample was homogenized with chloroform, and the supernatant was purified on the C18 SPE cartridge (recoveries $> 75\%$; RSD $< 10\%$). The changes made to the previous procedure (155) permitted the simultaneous determination of SDM and SMZ in animal tissue. The column effluent from the UV detector was introduced directly into the ion source of the quadrupole thermospray MS. The protonated molecular ions were monitored for each compound (m/z 279 for SMZ, 311 for SDM) and the internal standard (sulphaethoxypyridazine, m/z 295), respectively. The authors included an IS that was efficiently extracted from the matrix (recovery $> 80\%$); moreover, it had similar chromatographic characteristics to the SAs. This assay showed a good correlation between the UV and MS detection systems and allowed the detection of residues with concentrations of 2 μg/kg (SMZ) and 10 μg/kg (SDM) (156).

C. Pre- and Postcolumn Derivatization Assays

The derivatization reactions were chosen to improve the UV detection of SAs (reactions with DMBA, *p*-aminobenzoic acid) or to yield derivatives with high fluorescence intensity (reactions with fluorescamine, *o*-phthaldehyde).

1. *Fluorescamine Derivatization Assays*

This approach is based on the reaction of SAs with fluorescamine, yielding both highly fluorescent and relatively stable derivatives. The reaction conditions enable one to apply this technique in both pre- and postcolumn operation modes.

Sample cleanup following precolumn derivatization using fluorescamine was used for the simultaneous determination of 13 SAs (SAA, SDZ, SPR, STA, SME, SMZ, SMP, SMT, SDM, SQX, SDX, SMX, and STR) in tissue, milk, and egg samples. The tissue sample was homogenized with ethyl acetate, and the clear supernatant was evaporated to dryness. The residue was dissolved in acidic MeOH solution, and the lipidic part of the extract was partitioned with hexane. The milk or egg proteins were precipitated with TCA in ethyl acetate. The addition of TCA in this step improved the release of both STZ and SMZ residues from bonds with proteins. The mixture was shaken and centrifuged, and the organic layer was evaporated to dryness. Both the aqueous layer from the tissue extraction and the evaporated residue were from egg/milk sample cleanup treated with fluorescamine solution. The highest yield for derivatives was obtained for the 0.1% fluorescamine solution after 30 min of reacting. They were stable within 90 min; a significant decrease of the signal was observed after 90 min. Although the derivatives were separated using the

gradient chromatographic system, a long analysis time (approximately 1 h) was necessary to resolve some SA peaks (157).

A similar cleanup procedure was used for the determination of SDZ, STA, SMZ, SMM, SMX, and SDM in milk samples. Milk proteins were precipitated with TCA, and the supernatant was evaporated to dryness. The residue was dissolved in TCA solutions containing *p*-aminobenzoic acid as an internal standard. An aliquot was mixed with 0.1% fluorescamine solution, and the reaction was finished within 10 min. The derivatives were found to be stable for 180 min. Lower recoveries were obtained (63–84% with CV < 8%) in comparison with the previous assay, but better LOD results were reported (1 μg/L) (158).

A postcolumn derivatization with fluorescamine was another approach to the determination of 14 SAs (SAA, SDZ, SME, STA, SPR, SMZ, SMT, SMP, SCP, SMM, SDX, SMX, SDM, and SQX) in salmon tissue. The tissue sample was homogenized with acetic acid and MeCN. The supernatant was partitioned twice with dichloromethane, and combined extracts were partially evaporated. Sulphonamides were re-extracted with the phosphoric acid solution. The extract was separated under gradient elution conditions, followed by postcolumn derivatization with fluorescamine solution at 70°C. Although early experiments were performed on a 25-cm analytical column, a shorter column with smaller particles and gradient conditions reduced the analysis time for 25 SAs to less than 25 min. The 2% portion of acetic acid in the mobile phase maintained a pH of 3.0, which was optimal for the postcolumn derivatization reaction. No interfering compounds from fish tissue were present for 12 of 14 SAs; a small interference affected the quantitation of SAA and SMP to a greater degree. The average recoveries using this assay ranged from 78% to 88%, and they increased with the fortification level without exception. The recovery of SAA was significantly lower (58%) because of its greater polarity as compared to the other SAs (159).

The previous assay was applied to the analysis of SDZ in salmon tissue, with some modifications. The re-extraction with phosphoric acid solution was replaced by SPE on an SCX cartridge preconditioned with MeCN and phosphoric acid solution. The cartridge was washed with MeCN, and SDZ was eluted with an MeCN : phosphoric acid mixture. The eluate was injected directly into the chromatographic system, followed by postcolumn derivatization under similar conditions to the previous assay. The derivatization time was 1.2 min, and the fluorescence intensity was approximately a quarter of that for optimal conditions. However, the postcolumn derivatization was found to be considerably less labor intensive and was easily reproducible (recoveries 83–85%; CV < 7%). A significant improvement in the LOD value was obtained (0.2 μg/kg) (160). The SDZ residues from incurred salmon tissue were confirmed by MS detection; however, the sample cleanup should be improved due to the lack of sensitivity of MS. Therefore, SDZ residues were eluted from the SCX SPE cartridge with phosphoric acid, and the eluate was concentrated on a C18 SPE cartridge preconditioned with MeOH and water. The residues were eluted with MeCN, and the eluate was evaporated to dryness and reconstituted prior to the analysis. The column effluent was delivered into the atmospheric-pressure ion source, and SIM was chosen for positive ions at m/z 251, 158, 156, and 108, respectively (161).

2. *Other Derivatization Assays*

A new analytical method was based on the treatment of SAs with *p*-aminobenzoic acid, forming derivatives suitable for UV detection. The SAs were extracted from the kidney and liver samples, with recoveries ranging from 55% to 100%. The reaction yield was tested by microbial inhibition tests sensitive to low concentrations of SAs. After the incubation with *p*-aminobenzoic acid, none of eight SAs produced an inhibition zone on the assay medium, which showed 100% conversion to the appropriate derivatives (162).

A postcolumn derivatization using *o*-phthalaldehyde (OPA) in the presence of mercaptoethanol (ME), yielding fluorescence derivatives, was used for the determination of five SAs (SAA, SGU, SDZ, SPR, and SMX) in milk, tissue, and eggs samples. The sample extracts were injected directly into the chromatographic system after the proteins had been precipitated by treatment with TCA. A gradient elution was used, followed by postcolumn derivatization in the 2.5-m-long reaction coil maintained at 40°C. Concentrations of both OPA and ME were optimized to yield maximal intensity of fluorescence. The average recovery was 95%, and LOD values ranged from 27–47 μg/kg for eggs to 36–340 μg/kg for milk samples. Two small interfering peaks affected the determination of SGU and SAA, resulting in a higher LOD in milk samples (163).

Two different sample cleanup techniques followed by postcolumn derivatization with 4-dimethylaminobenzaldehyde (DMAB) were compared for SMZ assay in muscle, liver, and kidney. Methanol was added to thin slices of sample, and the container with the mixture was put into the center of a microwave oven and irradiated at maximum power for 25 s. The extraction mixture was centrifuged, and the supernatant was evaporated to dryness. The residue was dissolved in mobile phase and washed with hexane to remove lipidic fraction. A second sample cleanup was based on a homogenization of the sample with MeOH. The extract, evaporated and dissolved in mobile phase, was applied to a C18 SPE cartridge previously preconditioned with MeOH, water, and mobile phase. SMZ was eluted with MeOH; the eluate was evaporated to dryness and dissolved. In all cases, microwave extraction was superior to homogenization, with excellent recoveries obtained for muscle (95%), kidney, and liver (63–77%) samples. The SPE technique provided recovery values ranging from 60% to 74% with CV $< 25\%$. The postcolumn derivatization was performed on a 10-m-long Teflon-knitted reactor maintained at 40°C, followed by UV detection. A very long separation time for SMZ (43 min) was the main disadvantage of this assay (164).

D. Supercritical-Fluid Extraction–Liquid Chromatography Assays

Supercritical-fluid extraction (SFE) seems to be a powerful tool for the extraction of SAs from solid or semisolid matrices. The papers published in the reviewed period of time discussed the effects of SFE conditions, trapping conditions, and sample preparation on SA recoveries, accuracy, and precision.

The effects of both supercritical CO_2 and CHF_3 fluids and MeOH modifier content were investigated for SDM, SMZ, and SQX extraction from nonfat milk powder, egg yolk, and beef liver samples. Nearly quantitative recoveries for all SAs were obtained using CHF_3 with 10% of MeOH modifier content at 400 bar/40°C in an inert material (sand) within 60 min. Therefore, these parameters were used for SA extraction from food matrices. The SFE extract was trapped on a C18 SPE cartridge, followed by an MeOH trapping solution. The SAs were eluted with the mobile phase from the cartridge and mixed with MeOH trap solution. The replacement of solid trap material was required after each extraction because of the large amount of coextractives (mainly lipids) present in the extract. The nonfat milk powder seemed to be a relatively inert material, so extraction recoveries were similar to those of sand matrix (102–107%). Significantly lower values were observed for beef liver (69–74%) and egg yolk (24–57%), showing strong matrix/analyte interactions (165). The results of this work corresponded to that obtained for chicken liver samples (166).

A SFE-HPLC with an in-line alumina SFE procedure (167) was adopted for SQX, SDM, and SMZ in liver tissue (168) and egg samples (169). The egg or tissue samples were homogenized and mixed with the Hydromatrix sorbent. The SFE vessel was filled with a plug of polypropylene wool, alumina, and tissue/Hydromatrix mixture. The vessel was closed, and the

content was extracted at 680 bar/40°C using unmodified carbon dioxide. The SAs were in-line trapped on alumina sorbent. After finishing the extraction, the SAs were eluted from the alumina with the mobile phase and separated using the LC-UV chromatographic system. Recovery values for this SFE cleanup technique (72–98% with RSD < 12% for tissue and 84–99% with RSD < 7% for eggs, respectively) were comparable to those obtained by other SFE assays. In contrast, other investigators reported poor recoveries using unmodified supercritical CO_2 (166,170). The poor recoveries might be due to the lower pressure employed in those studies (<470 bar). In addition, off-line trapping might decrease recoveries and produce chromatograms difficult to quantify (168). The dehydration of the egg sample during SFE resulted in the adsorption of analytes, decreasing their extractabilities; therefore, the addition of a small amount of water prior to SFE increased the extractabilities by 12–20% (169).

The recoveries of SMZ and its five metabolites from swine liver and kidney were increased (72–97% with RSD 2–24%) when employing the ion-pair agent tetramethylammonium hydroxide (TMAH) in situ with SFE. The homogenized tissue sample was mixed with Hydromatrix and placed into the extraction vessel. A TMAH methanolic solution was added directly to the cell. The vessel content was extracted with supercritical MeOH–modified CO_2 at 400 bar/60°C, and the extract was poured through two vials containing MeOH. Lipidic endogenous material was partitioned with chloroform. An in situ addition of TMAH enhanced the extractability of the polar metabolites; the greatest improvement was observed for sulphate metabolite—from 0% to 72% (170).

E. Liquid Chromatography–Microbial and Immunoaffinity Assays

The milk sample containing SDM residues was extracted with dichloromethane : chloroform, and the organic layer was evaporated to just dryness. The residual fluid was dissolved with hexane, and SDM was reextracted into the phosphate buffer (171). The results of this procedure were compared to those from the FDA-recommended ELISA method, Charm inhibition, and Charm II assays. The HPLC method seemed to be more robust than the FDA methods because it allowed for easier calculation and interpretation of the results (172). Moreover, this method was found to be the most specific and had a sufficient LOD value (2 μg/kg) (171).

A relatively new approach to sample cleanup was based on immunoaffinity chromatography columns prepared from monoclonal antibodies against SMZ, SDZ, and streptomycin. The capacity of the resulting columns was approximately 250, 1050, and 1050 ng per column for SDZ, SMZ, and streptomycin, respectively. Spiked milk samples were used for performance testing under practical conditions. The column containing monoclonal antibody against SDZ bounded between 84.2 and 96.7% of SDZ, STA, and SME. About 90% of the applied SMZ was retained by the respective column. A multi-immunoaffinity chromatography column for SAs bound from 77% to 99% of a mixture SDZ, STA, SME, and SMZ (100 ng each). Although the tested values were higher than the MRL, the immunoaffinity chromatography seems to be a prospective and highly selective tool for sample cleanup (173).

The HPLC-receptorgram assay was used for 13 SAs and many other drug residues (50) (further details in Sec. II.D).

F. Trimethoprim and Ormethoprim Assays

Trimethoprim and OMP are potentiators of SAs, and they are administered in the mixed forms with the appropriate SA (Romet, e.g.) to the animals. The HPLC assays are reviewed in the fol-

lowing discussion; however, a few methods were also used for the simultaneous determination of TMP and OMP with SAs (151,152,154).

A simple assay for TMP in bovine tissue and plasma was used. The tissue sample was homogenized with alkaline phosphate buffer and partitioned with dichloromethane. The TMP was reextracted with sulphuric acid. The aqueous layer was mixed with phosphate buffer, and it was further cleaned by SPE according to the previously published assays (174). Sulphadoxine, administered together with TMP, was assayed using TLC densitometry. These assays were used for the study of adsorption and depletion of TMP and SDX from the healthy animals. It was observed that both TMP and SDX were readily adsorbed onto the tissues, but TMP was eliminated much faster than SDX (175).

A novel cleanup procedure was used for TMP residues in tissue and milk samples. Tissue/milk samples were homogenized/dissolved with phosphate buffer, and the supernatant was purified on a C18 SPE cartridge previously conditioned by MeOH, water, and phosphate buffer containing pentanesulphonic acid (PSA). The TMP was eluted with MeOH : phosphate buffer and injected directly into the ion-pair chromatographic system followed by dual-wavelength UV detection. The comparison of both signals ratio-enhanced the identification of the TMP peak besides the monitored UV spectra. Recoveries ranging from 73% to 98% were presented, and an LOD below the MRL set for TMP residues was achieved (176,177).

A simple assay was used for the separation and MS detection of TMP in tissue. The frozen pulverized tissue sample was homogenized with chloroform : acetone, and the extract was evaporated. The residue was dissolved with MeOH : acetic acid, and lipids were removed by washing with hexane. An aqueous layer was injected, and TMP was detected and identified using a thermospray LC-MS system. The MS detection was accomplished in the positive-ion mode with SIM of the ion m/z 291 (178).

G. Stability

The study involved spiking a bulk portion of homogenized pig liver tissue with STA, SCP, SMZ, SQX, and SDM. A significant SA residue degradation (10–25%; more than 50% for SQX) was observed during spiking and tissue homogenization, probably caused by the enzymatic activity. Storage stability at −20°C was conducted over a period of about 6 months. The decay half-lives ranged from 567 (SDM) to 271 (SQX) days. The results observed under accelerated decay conditions (4°C) showed a relative instability of SQX residues (half-life of 11 days) (179).

The stability of SMZ residues in bovine and porcine tissue was evaluated. The residue content decreased similarly in the different types of samples to about 50% during frozen storage at −20°C after 15 months. Storage at room temperature for 24 hours and at 20°C for 1 week did not affect the drug levels; however, they decreased significantly when the sample had been stored for 1 month (180).

Transferring and a decreasing profile of SQX in eggs was the aim of several studies (181,182). The drug content reached a plateau after 4 days of SQX feeding; the residue content began to decrease 1 day after the withdrawal of dietary SQX. However, the disappearance profile in albumen was different from that in egg yolk.

An MSPD sample cleanup was used for the assessment of the effect of cooking on SDM and OMP residues in catfish fillets. Fillets were cooked by one of four methods: smoked at 160–200°C, baked at 190°C, fried in canola oil at 190°C, or fried in vegetable oil at 190°C. The cooking processes reduced both OMP and SDM to a similar ratio, with concentrations in baked fillets being significantly lower (17–60% of original value) than in raw fillets. The same tendency was seen in smoked and fried fish fillets (7–84% of original value depending on the concentration

level). No statistical differences were observed among the cooking methods in the reduction of OMP and SDM in fish fillets. As a result, the cooking process, combined with a 3-day withdrawal period, provides consumers additional safety against exposure to OMP and SDM from catfish treated with Romet (152).

X. QUINOLONES

Quinolone (QUIN) and fluorquinolone (FQUIN) antibiotics are a group of highly potent synthetic antibacterial compounds. They actuate their antimicrobial effects by inhibiting DNA gyrase within the bacterial cell (183). The FQUINs are a relatively new class of drugs; they are quinole derivatives with a fluorine atom substituted normally at the 6-position. Substitutions in the chemical structure of a FQUIN influence the potency and biological spectrum of activity of the drug. Such FQUINs as ciprofloxin have been found to have activities approaching 1000 times higher that that of NALA. Foods that could contain QUIN residues include poultry muscle (chicken, turkey), egg, chicken liver, honey, cattle muscle, milk, and fish (184). These agents are widely applied in the treatment and prevention of veterinary diseases in food-producing animals and fish. The wide range of application and the extensive use of 4-QUINs in veterinary medicine represent a potential hazard, because residues of these drugs may persist in edible tissue or milk. Therefore the need to identify and determine 4-QUINs in various biological, veterinary, and food samples is obvious.

The following QUINs and FQUINs could be analyzed: pipemedic acid (PIP), enoxacin (ENO), norfloxacin (NOR), ciprofloxacin (CIPRO), lomefloxacin (LOME), desmethyl-danofloxacin (DES), danofloxacin (DAN), enrofloxacin (ENRO), sarafloxacin (SARA), cinoxacin (CINO), oxolinic acid (OXO), nalidixic acid (NALA), flumequin (FLU), piromidic acid (PIRA), marbofloxacin (MAR), difloxacin (DIFLX), decoquinate (DEC), and ofloxacin (OFLX).

The EU set MRL values for QUINs as follows: *Substances for which MRLs have been fixed*: ENRO in bovine muscle, porcine liver, poultry kidney 30 μg/kg, in milk 10 μg/kg; SARA in chicken liver 100 μg/kg, in chicken skin and fat 10 μg/kg; DIFLX in chicken liver 1900 μg/kg, in turkey kidney 600 μg/kg, in muscle 300 μg/kg, in skin and fat 400 μg/kg. *Substances for which provisional MRLs have been fixed*: DAN in bovine liver 900 μg/kg, in kidney 500 μg/kg, in muscle 300 μg/kg, in fat 200 μg/kg, in chicken liver 1200 μg/kg, in fat and skin 600 μg/kg; DEC in bovine and ovine muscle, liver, kidney 500 μg/kg; MAR in bovine and porcine muscle, liver, kidney 150 μg/kg, in fat 50 μg/kg, in milk 75 μg/kg; FLU in bovine muscle 50 μg/kg, in ovine liver 100 μg/kg, in porcine kidney 300 μg/kg, in chicken fat and skin 50 μg/kg, in Salmonidae muscle and skin 150 μg/kg.

Switzerland's limits for OXO are 10 μg/kg in fish, for ENRO 30 μg/kg in milk, eggs, and meat (185). The FQUINs ENRO and SARA have been approved for use in chickens and turkeys in the United States. The FDA has specifically prohibited the extralabel use of the FQUINs in food-producing animals (6).

The HPLC assays for QUINs in food samples use different sample-preparation procedures and various detection modes, and some of these complete methods are applied to pharmacokinetics studies or to stability tests:

LLE sample preparation
LLE-SPE sample preparation
LS-MS assays
On-line techniques

Pharmacokinetics
Stability

Analysis methods for quinolones are given in Table 9.

A. Sample Preparation Based on Liquid–Liquid Extraction

Liquid–liquid extraction has been used widely in many published papers as a simple and effective preparation procedure. This step has to be applied when nonliquid food samples are analyzed. It has often been applied in combination with the SPE technique.

An HPLC method in combination with a simple LLE has been described for the determination of SARA residues in channel catfish muscle tissue (183). Sarafloxacin was extracted from fillet tissue homogenized with MeCN:water (1:1). The extract was centrifuged, and the supernatant was partitioned with hexane. The aqueous fraction was filtered and evaporated to dryness. The residue was redissolved in 20% MeCN:MeOH (3:2) and 80% TFA (0.1%), centrifuged, and filtered to remove proteins. Samples were analyzed by HPLC with gradient elution and fluorescence detection. Mean recoveries ranged from 85.4% to 104.0%, and relative standard deviation (RSD) values were from 1.1% to 5.6% at sample concentrations of 10.0–863.8 μg/kg. The detection limit for SARA is 1.4 μg/kg. The results obtained were verified using an in-line radioactivity detector. The radioactivity in all samples was determined by liquid scintillation counting and quantified by disintegrations per minute. The total radioactivity extracted from individual spiked samples ranged from 79.4% to 89.9%, and the RSD ranged from 0.1% to 5.6%.

Flumequin, NALA, OXO, and PIRA were analyzed in catfish muscle. The identities of three of these compounds were confirmed by GC-MS. The extraction and cleanup procedures involve tissue homogenization with acetone, defatting of the acetone extract with hexane, and extracting the compounds into chloroform. The extract is further purified by first partitioning into a base and subsequently back-extracting into chloroform after acidifying the aqueous phase. After solvent evaporation, the residue was dissolved in the mobile phase and injected into an HPLC system with UV and fluorescence detections. Overall average recoveries were 83–94%, RSD of 5–7%. Fluorescence detection provided higher sensitivity than UV for three of four QUINs (FLU, NALA, and OXO). The presence in catfish muscle of incurred OXO, FLU, and NALA at the 10-μg/kg level was confirmed by analyzing the decarboxylated QUINs by GC-MS (186).

A previously published paper was applied to the determination of the same QUINs in salmon and shrimp using HPLC and GC-MS (187). The authors used the same preparation and separation conditions. Average recoveries and RSD values for salmon ranged from 75.9% to 90.8%, with RSD of 2.25–6.40%, and for shrimp from 81.3% to 91.2%, RSD of 7.34–10.7%. The identities of OXO, FLU, NALA, and PIRA were also confirmed by GC-MS.

An HPLC assay was described as a routine method for the determination of FLU and its hydroxylated metabolite 7-OH FLU in pig kidney tissues (188). The sample was extracted with ethyl acetate; after evaporation, the residue was dissolved in MeCN–oxalic acid (1:1). Analytical separation was performed using fluorimetric detection under gradient elution. The authors recommended an Ultrabase C-18 column, which allowed the work to be carried out at extreme pH values, ranging from 2 to 8. The assay was specific and reproducible within the range 50–2500 μg/kg; recovery was 94.8%.

A simple HPLC assay was described for the determination of OXO in tiger shrimp. The sample was simply extracted with ethyl acetate, with an extraction recovery of 97.4%, RSD of 10.3%. The detection limit of OXO was 3.5 μg/kg (189).

Oxolinic acid and oxytetracycline were analyzed in the shell of the blue mussel (*Mytilus edulis*). After roughly grinding the shell, drugs were extracted using a methanol–oxalic acid

Table 9 Analysis of Quinolones in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
OXO, SMM, MLX	Muscle, cultured fish	Pr, LLE	150 × 4.6 5-μm Hisep	MeCN:50 mM citric acid, 200 mM pH 2.5, Na_2HPO_4 in 10 mM TBAbromide 15:85	UV 265	0.05–0.1 μg/kg	153
SARA	Channel catfish	Pr, LLE	150 × 4.6 5-μm YMC ODS 120-A	Gradient MeCN:MeOH (3:2), 1% TFA	F, ex 280, em 389	1.4 μg/kg	183
Acidic QUIN, basic QUIN	Egg; honey; chicken liver; cattle, pig, chicken, turkey muscle	Pr, LLE, SPE	(1) 250 × 4.6 5-μm Zorbax C-8/basic, (2) 250 × 3.2 5-μm Kormasil C 8/acidic	(1) MeCN:10 mM pH 3 phosphate buffer 20:80, (2) MeCN:MeOH:10 mM oxalic acid 30:10:60	F, ex 278, em 445, UV 302	LOQ 5–10 μg/kg, 50 μg/kg (OFL ENO, LOM)	184
15 QUIN	Milk, salmon tissue	Pr, LLE	50 × 4 3-μm YMC/3-4-5 phenyl ODS-AQ	MeCN:water with 20 mM pH 2.75 formic acid 2:98	ES-MS/MS	1–2 μg/kg	185
FLU, LALA, OXO, PIRA	Catfish	Pr, LLE	150 × 4.6 5-μm PLRP-S	MeCN:20 mM phosphoric acid:THF 72:16:12	F, ex 325, em 365, UV 280	10 μg/kg	186
FLU, NALA, OXO, PIRA	Salmon, shrimp	Pr, LLE	150 × 4.6 5-μm PLRP-S	MeCN:20 mM phosphoric acid:THF 72:16:12		10 μg/kg	187
FLU, 7-OHFLU	Pig kidney	LLE	250 × 4.6 5-μm Ultrabase C-18	Gradient MeCN:2.7 mM pH 2.5 oxalic acid	F, ex 252, em 356	15–24 μg/kg	188
OXO, OTC	Shell	Pr, LLE	125 × 4.6 5-μm LiChrospher 100 RP-18	MeCN:20 mM pH 2.3 phosphoric acid 24:76	UV 262	8–12 μg/kg	190
FLU, 7-OHFLU	Sheep, muscle, liver, kidney, fat	LLE	150 × 4 5-μm LiChrospher RP Select B	MeCN:DMF:phosphoric acid 48:28:54	F, ex 320, em 365, UV 324	2–3 μg/kg	191
FLU, NALA, OXO, PIRA	Catfish	Pr, LLE	150 × 4.6 5-μm PLRP-S	MeCN:THF:water:H_3PO_4 80:13:70:0.02	F, ex 325, em 365, UV 280	5 μg/kg	192

OXO	Oyster	LLE, SPE	125 × 4.6 5-μm LiChrospher 100 RP-18	MeCN:20 mM pH 2.3 phosphoric acid 24:76	UV 262	10 μg/L	193
ENRO, CIPRO, SARA, DIFLX	Milk	LLE, SPE	150 × 4.6 5-μm Inertsil Phenyl	MeCN:2% acetic acid 15:85	F, ex 278, em 450	0.3–1.2 μg/kg	194
ENRO, CIPRO, MAR, DAN, SARA, DIFL	Pig muscle	LLE, SPE	125 × 4.6 5-μm LiChrospher 100 RP-18e	Gradient MeCN:1% formic acid in ammonium acetate pH 3	APCI-MS	2 μg/kg	196
Acidic QUIN, basic QUIN	Catfish muscle	LLE	150 × 4 5-μm DB, 250 × 4 10-μm	MeCN:4% THF in 25 mM acetic acid 22:78–38:62	APCI-MS	0.8–1.7 μg/kg	197
SARA	Catfish tissue	Pr, LLE, SPE	150 × 4.6 5-μm YMC-PACK	MeCN:citric acid in pH 2.4 buffer 30:70	ES-MS/MS, UV 280		198
FLU	Catfish muscle	Pr, LLE	150 × 2.1 5-μm PLRP-S	MeCN:2% formic acid 86:14	ES-MS		199
FLU, OXO	Chicken liver	LLE, on-line dialysis	150 × 4.6 5-μm PLRP-S	MeCN:THF:20 mM pH 5 sodium phosphate 20:15:65	F, ex 218, em 364	2.5–5 μg/kg	200
NOR	Fat, kidney, liver, lung, muscle	LLE	250 × 4.6 10-μm Vydac anion-exchange	MeCN:50 mM pH 7 phosphate buffer 20:80	UV 280	3 μg/kg	205
FLU, 7-OHFLU	Sheep tissue	Pr, LLE	250 × 4 5-μm LiChrospher, Select B	MeCN:DMF:water with 0.25% phosphoric acid 18:28:54	F, ex 324, em 365	1–4 μg/kg	207
OXO	Egg yolk, albumen	Pr, LLE	150 × 4 5-μm LiChrospher RP 18	MeCN:water:acetic acid 25:72:3	F, ex 334, em 382	LOQ 5 μg/kg	208

solution (190). Linearity and precision were checked over the concentration range 40–320 μg/kg. The limits of detection of OXO and oxytetracycline were 12 and 8 μg/kg, respectively. Extraction recoveries were 72.9 and 65.4%, respectively. This assay could also be applied to other animal calcified tissues (tooth, claw, hoof, etc.).

Residues of FLU and 7-OH FLU in edible sheep tissues (muscle, liver, kidney, and fat) were determined by HPLC with UV and fluorescence detection (191). Liquid–liquid extraction with ethyl acetate was described, with extraction recoveries of 90, 82, 89, and 82% for FLU in muscle, liver, kidney, and fat, respectively. Recoveries for 7-OH FLU were 91, 90, 86, and 84%. The method was validated for specificity, linearity, limits of detection and quantitation, and precision.

Simultaneous determination of FLU, NALA, OXO, and PIRA in catfish muscle was developed for HPLC with fluorescence detection (192). Sample workup involves homogenizing tissue with acetone, defatting with hexane, and extracting QUINs into chloroform. The sample was purified by partitioning into a base and back-extracted into chloroform after acidifying the aqueous phase. After evaporating and dissolving the residues in the mobile phase, the supernatant was injected into an HPLC system with UV and fluorescence detection (for PIRA). Extraction recoveries were FLU 79.7%, RSD 5.7%, OXO 80.8%, RSD 6.3%, PIRA 75%, RSD 5.9%, NALA 87.1%, and RSD 10.0%. The limits of quantifications were about 5 μg/kg for all analytes.

B. Sample Preparation Based on Liquid-Liquid Extraction–Solid-Phase Extraction

The determination of OXO in Japanese oyster was realized using reversed-phase HPLC. Samples were extracted with LLE and SPE; recoveries were 88.3% (193). Oyster samples were homogenized with a phosphate buffer adjusted to pH 7. After centrifugation, supernatants were concentrated using an SPE C-18 cartridge. Before use, the cartridge was activated with MeOH and phosphate buffer. After the sample had been passed, the cartridge was flushed with water and the analytes were eluted with MeOH–orthophosphoric acid (9:1). The eluate was evaporated, and the residues were dissolved in the mobile phase. The method developed was validated and the study of OXO stability was performed. The limits of detection and determination were 10 and 40 ng/ml, respectively.

A liquid chromatographic method with fluorescence detection was developed for the analysis of ENRO, CIPRO, SARA, and DIFLX in milk (194). Milk sample was extracted with acidified 95% EtOH; isolation and retention were achieved on a cation-exchange SPE cartridge. Acidification of acetone with a weak acid was selected over basic treatment because FQUINs, being zwitterions, are fully protonated at a low pH 3. The elution was realized with basic MeOH. Liquid chromatography was performed in isocratic mode. A target level of 10 μg/kg for each of the QUINs has been established. Average recoveries were in the range of 70–90%, with RSD lower than 15%. The limits of detection for CIPRO, ENRO, SARA, and DIFLX were 0.4, 0.3, 1.2, and 0.7 μg/kg, respectively. The limit of quantitation was 5 μg/kg for each of the four analytes.

The FQUINs are generally more basic than the other QUINs (except FLU). The multiresidual procedure for basic drugs has been evaluated for QUIN and FQUIN antibiotics (184). An SPE using cation-exchange cartridges was applied for basic QUINs. The approach for acidic QUINs (OXO, NALA, and FLU) was based on anion exchange. The samples containing basic QUINs are homogenized after adding MeCN with acetic acid. After homogenizing, sodium sulphate was added before the centrifugation. The supernatant was filtered into a conical flask through additional sodium sulphate, which was washed with MeCN. The samples containing acidic QUINs are extracted as before but without the addition of acetic acid. Following centrifugation, MeCN was evaporated to dryness and redissolved in disodium hydrogen phosphate (pH 11). An SPE of basic FQUINs was realized using the cation-exchange SPE. The cartridge

was conditioned with MeCN–glacial acetic, and acetic acid was also added to the extract before the application to the cartridge. The cartridge was washed sequentially with acetone, MeOH, and MeCN, and then analytes were eluted with MeOH–35% ammonia solution. The eluate was evaporated to dryness, and the rest was dissolved in the mobile phase. In the SPE procedure for acidic QUINs, an anion-exchange AGMP-1 resin was packed into an empty cartridge. The extract was applied to the resin, and the cartridge was washed with water; MeOH, MeCN, and analytes were eluted with MeOH–acetic acid. The eluate was evaporated, and the residue was dissolved in oxalic acid. The limits of determination were 5 μg/kg for DAN, SARA, and MAR, 50 μg/kg for OFLX, ENO, and LOM, and 10 μg/kg for OXO, NALA, and FLU.

Screening of ENRO, CIPRO, DAN, NOR, FLU, OXO, and NALA in pork muscle was based on HPTLC after the SPE procedure (195). Tissue samples were extracted with MeCN–NaOH, the supernatant dried, and the residues dissolved in dipotassium hydrogen phosphate buffer (pH 7.4); hexane was added. The aqueous phase was cleaned up using the SPE technique. After washing the cartridge, analytes were eluted with MeOH–ammonia solution (75:25). After the preparation step, extracts were spotted and eluted on silica gel plates. The plate is first inspected under UV illumination at 312 nm and then sprayed with terbium chloride. The method was validated at the levels of 15 μg/kg for ENRO, CPRO, DAN, and NOR and 5 μg/kg for FLU, OXO, and NALA.

C. Liquid Chromatography–Mass Spectrometry Assays

An LC-MS was described for the confirmation of six QUINs (ENRO, CIPRO, MAR, DAN, SARA, and DIFLX) in pig muscle (196). Analytes were extracted from muscle with phosphate buffer (pH 7.4). After centrifugation, the extract was purified on a C18 SPE cartridge. After washing a C18 cartridge, the QUINs were eluted twice with 0.1% TFA in MeCN. The collected eluate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in MeCN and the solution injected into an HPLC system. Extraction recoveries were 105% for CIPRO and 98% for ENRO (30 μg/kg). Sample extracts were analyzed by HPLC with gradient elution and detected by MS via atmospheric pressure chemical ionization (APCI). The method was specific at the concentration of 7.5 μg/kg in pig.

Multiresidue determination of QUIN antibiotics using liquid chromatography coupled to ACPI-MS and MS/MS was also described (197). In the source, collision-induced dissociation was used to optimize fragmentation to produce mass spectra consisting of the protonated molecule and two characteristic fragment ions of nearly equal intensity. Selected ion monitoring of three ions per QUIN yielded a sensitive detection in catfish muscle extracts (detection limits of 0.8–1.7 μg/kg). An MS/MS was used to increase the specificity and selectivity of analysis. The preseparation step was very simple; only the LLE procedure is required.

An LC/MS/MS assay has also been described for the confirmatory identification of SARA in catfish tissue (198). The ability to use HPLC in conjunction with an electrospray interface to produce a product-ion mass spectrum with ion intensities with variabilities of less than 7% for three product ions has been demonstrated. Sarafloxacin was extracted by homogenizing tissue with NaOH. Acetonitrile and phosphoric acid were added with mixing to precipitate proteins. After the centrifugation, the supernatant was acidified with phosphoric acid to remove salts that can suppress the MS signal. The homogenate was cleaned up through a C18 SPE cartridge, which was preconditioned with MeCN, MeCN–water, and ammonium acetate buffer adjusted with formic acid to pH 3.0. After the homogenate application, the cartridge was washed with water, MeCN–water (1:9), and ammonium acetate buffer. The SARA was eluted with MeCN–ammonium acetate buffer (1:1). A small aliquot of the eluate was diluted with buffer adjusted to pH 3 (for HPLC-UV). The rest was evaporated and submitted for LC/MS/MS.

Confirmation of FLU in catfish muscle by electrospray LC/MS was done (199). Residues of CIPRO, ENRO, SARA, and DIFLX were positively identified at 10–80 μg/kg. The extraction procedure was based on LLE with acidic ethanol. Extracts were cleaned up on a PRS SPE cartridge. Analytes were eluted with 30% ammonium hydroxide–MeOH (1:4). Chromatographic conditions were optimized to be compatible with the electrospray interface. To obtain maximum sensitivity, separate MS acquisition programs were developed for CIPRO/ENRO and SARA/DIFLX pairs. This method was used to confirm residues in tissues fortified in the 10–80-ppb range. All relative abundances were within 10% of the value calculated for standard compounds.

Simultaneous detection and confirmation of 15 QUIN antibiotics were accomplished by fast short-column liquid chromatography coupled with electrospray ionization tandem LC/MS/MS (185). To demonstrate the potential of the LC/MS/MS method, its application to trace analysis in several biological matrices, such as milk and salmon tissue, was investigated. Sample preparation of QUINs from milk was realized with MeCN and NaOH, the sample was mixed, and diethyl ether–hexane (3:2) was added. After centrifugation, the upper layer of organic solvent was discarded. The aqueous solutions were acidified with phosphoric acid, and MeOH was added, followed by centrifugation. After drying, the residues were dissolved in the mobile phase and the solution was injected. Recoveries were 65–86%. Fish samples were homogenized with acetone. The extract was defatted with hexane, and analytes were subsequently extracted into chloroform. After extract evaporation to dryness, the residue was dissolved in water. Recoveries of the procedure were 56–95%. This HPLC assay is very interesting with the application of short columns, which enable fast HPLC separation. Especially in LC/MS analysis, the short column has many advantages. The separation time is considerably shorter in comparison to conventional analytical columns. The highly efficient separation resulted in peaks of the order of 3–10 s for all compounds, thus requiring a fast scan of the mass spectrometer. All 15 QUINs were separated in 8 min. Electrospray MS and MS/MS were used for detection and identification. Electrospray data was acquired using triple-quadrupole MS. The ES mass spectra of the investigated QUINs under typical operating conditions were very simple, and they offer an advantage for quantification in the SIM mode. This method is sensitive and specific. The assay is suitable for a variety of different matrices (milk, fish), and it is also convenient for the rapid identification of metabolites. In this study, conventional, well-established sample preparation methods based on LLE were used. It will be more important in the future to develop efficient and effective sample preparation techniques that match the rapid turnaround time of the analytical procedures. Possible examples of such preparation techniques are: automated on-line SPE, the use of RAM materials, and immunoaffinity extractions with drug-specific monoclonal antibodies.

D. On-line Techniques

The isolation of FLU and OXO from chicken tissue was achieved using LLE, aqueous on-line dialysis, trace enrichment, and column switching using an ASTED system (200). The LLE assay was modified according to the results published in Ref. 186. In this paper, the multiresidue extraction method required 11 solvent extraction steps, 3 evaporation steps, and overall solvent consumption of more than 370 ml. In order to reduce the need for large amounts of solvents, the authors of this paper used smaller tissue samples. Recoveries were in the range of 94–96%, RSD 4–9%, for FLU and 98–99%, RSD 4–6%, for OXO. Clean chromatograms were obtained, allowing detection of 5 μg/kg FLU and 2.5 μg/kg OXO. Due to its lower organic solvent consumption, automation, and on-line capabilities, this method is very useful. Because FLU and OXO molecules are highly bound to proteins, it was necessary to include a deproteinization step prior to the extraction step.

The use of the HPLC technique with a programmable fluorescence detector was described for FLU analysis (201). This method was compared with on-line microdialysis used as the cleanup step for a chicken liver sample. After the on-line microdialysis sample cleanup, the resultant HPLC chromatograms were free of background interference, enabling the programmable detector to optimize the quantitation of the three analytes in a single run. The limit of quantitation was 1 μg/ml.

The simultaneous HPLC-UV determination of sulfamonomethoxine (SMM), miloxacin (MLX), and OXO in serum and muscle of cultured fish was developed (153). A sample of muscle was extracted with MeCN–THF (95:5); after centrifugation, the supernatant was injected into the HPLC system. A Hisep column, used in this study, is packed with restricted-access materials (RAMs) consisting of the polymeric hydrophilic/hydrophobic phase bound to silica gel. This column did not require time-consuming and complex extraction procedures. The RAM sorbent could also be applied in short precolumns, which are combined directly on-line with the HPLC equipment. This approach is much more convenient than that applied in the present paper. The guard column had to be changed very often in order to protect the analytical column in a sufficient way. The extraction recovery was 79.5%, RSD of 6.0%.

E. Stability

The stability of FLU (100 μg/kg) in sheep muscle, liver, and kidney samples was determined by analyzing spiked samples after 1 and 4 hours at room temperature and 1, 3, and 6 months at −20°C. No differences were observed in detector response in the HPLC analysis (191).

The solution of four QUINs (ENRO, CIPRO, SARA, and DIFLX) showed good stability in 1% acetic acid over a 6-month period (194). After several days of milk sample injection (about 24–36 samples), the chromatographic profile lengthened and resolution became poor. In this case a solution of MeCN–6% acetic acid (1:1) was flushed through the column for about 30 minutes.

It was found that ENRO and CIPRO are light sensitive, so the solutions must be protected from light, which was recommended in the screening study for some QUINs (195).

Stock standards of FLU, NALA, OXO, and PIRA are stable in MeCN at 4°C for 1 year. Working standard mixtures are stable for less than 2 months. Working standard mixtures in active method process are stable for at least 2 h in 100 mM NaOH and longer in all solvents used in the method (192).

F. Pharmacokinetics

Pharmacokinetic and residue analysis of ENRO and its metabolite CIPRO in chicken samples were performed using HPLC after extraction with dichloromethane and sodium phosphate buffer (pH 7.4) (202). After shaking and centrifuging, the organic phases were dried and the residues dissolved in the mobile phase. Extraction recovery was 87% for ENRO and its metabolite.

The distribution and elimination of SARA from tissue of juvenille channel catfish was evaluated as the loss of radioactivity of SARA C14 in liver, kidney, skin, and skinless fillet. An HPLC with fluorescence detection, in-line radiometric detection, and gradient elution was applied (203). The pharmacokinetics of ENRO in the milk of lactating cows was studied by HPLC, and it was found that a marked proportion of ENRO was metabolized to CIPRO (204).

The pharmacokinetics of NOR and its metabolites in tissue residues was also studied (205). Samples of fat, kidney, lung, and muscle were extracted in methylenechloride and sodium phosphate (pH 7.5). An HPLC with UV detector was used. Detection limits were 3 μg/kg and 5 μg/kg for NOR and its metabolites.

The pharmacokinetics of NALA in cultured fish (rainbow trout) was published (206).

The pharmacokinetics of FLU and its metabolite 7-OH FLU in sheep tissue was studied using the HPLC method with fluorescence detection (207). Tissue samples were extracted with ethyl acetate. After drying, phosphate buffer (pH 7.8) and hexane were added, and aqueous (lower) phase was injected into an HPLC system. Extraction recovery was 75% for FLU and 60% for 7-OH FLU. The limit of detection was 1 and 4 μg/kg for both compounds. The elimination of OXO in eggs (albumen, yolk) was described using an HPLC assay with fluorimetric detection. The limits of quantitation were 5 μg/kg in albumen and yolk. Of the overall oxolinic acid detected in eggs, 95% was concentrated in the albumen. Detectable residues persisted for 9 and 7 days, respectively, in albumen and yolk after the treatment was discontinued (208). Albumen sample was homogenized with water and hydrochloric acid and extracted with ethyl acetate. The supernatant was evaporated and the rest dissolved in mobile phase. Extraction recovery was 65.2%, RSD of 5.3, for a concentration of 10 μg/kg.

XI. OTHER ANTIMICROBIALS

The different methods are summarized in Table 10.

A. Pirlimycin

Pirlimycin (PIR) was recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of mastitis in lactating dairy cattle. Regulatory tolerances of 400 μg/L PIR in milk and 500 μg/kg in liver were established.

Determinative and confirmatory methods of analysis for PIR residue in bovine milk and liver have been developed, based on HPLC-TS-MS (209). Milk sample preparation consisted of precipitating the milk proteins with acidified MeCN followed by partitioning with a mixture of *n*-butylchloride and hexane, LLE of PIR from aqueous phase into methylene chloride, and SPE cleanup. The dry residue after methylene chloride extraction was dissolved in ammonium hydroxide, and this basic solution was transferred to the top of C18 SPE column. The PIR elution was accomplished with TEA in MeOH. For liver, the samples were extracted with trifluoroacetic acid (TFA) in MeCN. The aqueous component was released from the organic solvent with *n*-butyl chloride. The aqueous solution was reduced in volume by evaporation, basified with ammonium hydroxide, and then extracted with methylene chloride. The organic solvent was evaporated to dryness, and the residue was dissolved in ammonium acetate. The overall recovery of PIR in milk was 94.5%, RSD of 8.7%, for liver 97.6%, RSD of 5.1%. A chromatographically resolved stereoisomer of PIR with TS-MS response characteristics identical to PIR was used as an internal standard for the quantitative analysis of the ratio of peak areas of PIR and internal standard in the protonated molecular-ion chromatogram at m/z 411.2. The mass spectrometer was set for an 8 min SIM-MS acquisition. Six samples can be processed and analyzed in approximately 3 hours.

An interlaboratory study dealing also with the determination and confirmation of PIR in bovine milk and liver using LC-TS-MS has been described (210). Determinative procedures were validated by replicate analyses of negative control, fortified control, and residue-incurred milk. For the milk method, average corrected recoveries were evaluated. There was no interference in the control samples or in either matrix. Pirlimycin was confirmed by matching the retention time and relative abundance of four ions from sample extracts to corresponding values obtained for the PIR standard. The PIR was confirmed in all residue-incurred samples and all samples fortified at regulatory tolerances (400 μg/kg in milk and 500 μg/kg in liver) by two of the three laboratories and in most samples by the third laboratory. Analytical methods for PIR residues in milk or liver were developed and published by the authors of Ref. 209.

Table 10 Analysis of Other Antimicrobials in Food Samples

Compounds	Sample	Sample preparation	Column	Mobile phase	Detection	LOD (LOQ)	Ref.
PIR	Milk	Pr, LLE, SPE	250 × 4.6 5-μm CPS Hypersil 2(cyano)	MeCN:0.1 M ammonium acetate 30:70	TS-MS	LOQ 25–50 μg/L	209
PIR	Milk	Pr, LLE, SPE	250 × 4.6 5-μm CPS Hypersil 2(cyano)	MeCN:0.1 M ammonium acetate 30:70	TS-MS	LOQ 25–50 μg/L	210
PIR	Milk	Pr, LLE, der	100 × 5-μm Hypersil ODS	MeCN:MeOH:1% acetic acid 30:30:40	UV, MS	50 μg/kg	211
PIR	Liver	Pr, LLE, SPE	150 × 2.1 5-μm Zorbax C-18	MeCN:0.1 M ammonium acetate 30:70	APCI-MS	LOQ 25 μg/kg	212
FMG	Fish tissue	Ho, SPE	300 × 3.9 μBondapak C-18	MeCN:water:phosphoric acid 60:40:0.1	UV 350	LOQ 5 μg/kg	213
NBC	Milk	Pr	150 × 4.6 5-μm Supelcosil LC-18 DB	Gradient MeOH-5 mM phosporic acid (1:1):MeCN-5 mM phosphoric acid (80:20)	UV 340	7 μg/kg	214
LIN	Salmon tissue	Ho, Pr, LLE, SPE	250 × 4.6 5-μm Spherisorb S5 ODS2	MeCN:pH 4.5 phosphate buffer 22:88	E + 900 mV	10 μg/kg	215
COL	Milk	Pr, SPE, pre-der, col-swi	125 × 4 5-μm Nucleosil C-18 column-switching	MeCN:35 mM pH 2.5 TEA 17:83 (UV), gradient MeCN:water:TFA 60:39.9:0.1 (A-MS), MeCN:water:TFA 80:19.9:0.1 (B-MS)	F, ex 340, em 440, UV 210, ES-MS		216
IVER	Liver	Pr, Ho, online SPE	250 × 4 5-μm Lichrospher 100 RP-18	MeOH:water 98:2	F, ex 364, em 470	2.5 μg/kg	217

A procedure to determine PIR residues in bovine milk using HPLC with the derivatization step for UV detection has also been published (211). The PIR was extracted from milk after protein precipitation and a two-step LLE procedure. The extract was evaporated to dryness, dissolved in dilute base, and derivatized with 9-fluorenylmethyl chloroformate (FMOC). The derivatized extract was analyzed by reversed-phase HPLC. Overall recovery was 89%, with 4% for coefficient of variation. A linear regression analysis of HPLC/UV results was compared with the HPLC/MS assay (209,210). The procedure takes about 2.5 hours to complete six or eight samples. Pirlimycin is stable in milk frozen to −60°C or below for at least 3 months.

Two HPLC-MS methods were applied to examine the metabolite profile of PIR residues in bovine liver, kidney, and muscle (212). Investigations have demonstrated that the principal residues are the parent PIR itself and the sulphoxide metabolite that exists in two isomeric forms, a syn- and an anti-oxide configuration in liver. The sulphoxide metabolite is generally found in a higher proportion of the total residue by a factor 2 to 5 times that of the parent PIR. However, the microbiological activity of this metabolite is more than 100 times less. The tissues were assayed by HPLC-MS according to Ref. 209. The mass spectrometer was operated in the single-quadrupole mode, with Q3 as the analyzer set, on selected ion monitoring at m/z 411 and 413 for the parent PIR and m/z 427 and 429 for PIR sulphoxide.

B. Fumagillin

Fumagillin (FMG) is a metabolite of *Aspergillus fumigatus*, and it has a potent amoebicidal property. It has also been used against microsporodian in fish. An HPLC method for FMG determination in fish meat was described (213). Two different types of sample preparation were developed, cleanup and enrichment. The cleanup assay was recommended for the concentration range of FMG 0.1–5.0 μg/ml, and the enrichment for 0.01–0.1 μg/ml. In the cleanup procedure, MeCN was added to meat sample to precipitate proteins. After homogenization and centrifugation, the supernatant was passed through the SPE cartridge. In the enrichment procedure, after loading the supernatant on the SPE cartridge, FMG was eluted with MeCN: water (9:1). The recovery was 75%, CV 3%, in the concentration range 100–500 μg/kg. This work is the first one dealing with the SPE-HPLC assay for FMG determination in fish meat.

C. Novobiocin

Novobiocin (NBC) is used for the treatment of mastitis in dairy cattle. In 1982, the tolerance level was set at 100 μg/kg in milk from dairy animals. An HPLC assay was developed for NBC determination in bovine milk at a tolerance level (214). The milk sample was diluted with buffer, the proteins were precipitated with MeOH, and the solution was filtered. The filtrate was injected directly into an HPLC system with UV detection. The interlaboratory study was realized for the analysis of two concentrations of NBC in fortified control milk samples. Recoveries of NBC reported by the participating laboratories were 89–99% at 50 μg/kg, 93–101% at 100 μg/kg, and 89–100% at 2 mg/kg. The CVs ranged from 2.0% to 6.2%. All laboratories described the procedure as rapid and simple, allowing the preparation of 20 samples in less than 2 hours.

D. Lincomycin

Lincomycin (LIN) is an antibiotic used to treat human and animal diseases caused by gram-positive bacteria. As a growth promoter, it is also added in subtherapeutic amounts to feed or drinking water for animals. The FDA set tolerance levels for lincomycin residues at 150 μg/kg for milk and 100 μg/kg for edible tissues of chicken and swine. Lincomycin is not yet approved

for use in salmon, so no tolerance level for salmon has been established (215). It was documented that the microbial assay lacks specificity and selectivity because of the presence in samples of other microbial inhibitors, including other antibiotics, and it is also time consuming. Therefore alternative analytical methods have also been applied in LIN analysis. An HPLC assay was described for detecting and quantitating LIN residues in salmon muscle and skin tissue. Ion-pair chromatography with electrochemical detection at +900 mV was recommended. Lincomycin was extracted from tissue after homogenizing it with phosphate buffer (pH 4.5) and centrifuging the mixture. Water-soluble proteins were precipitated, by adding sodium tungstate and sulphuric acid, and removed by centrifugation. The buffer extract was then passed through a C18 SPE cartridge. The LIN was eluted with MeCN in water, and the eluate was extracted with ethyl acetate. After solvent evaporation, the residue was dissolved in mobile phase and analyzed by HPLC. Average recoveries were 85% for salmon skin (215).

E. Colistin

Colistin (COL) is a multicomponent antibiotic (polymyxins E) that is produced by strains of inverse *Bacillus polymyxa*. It consists of a mixture of several closely related decapeptides with a general structure composed of a cyclic heptapeptide moiety and a side chain acetylated at the N-terminus by a fatty acid. Up to 13 different components have been identified. The two main components of colistin are polymyxins E1 and E2; they include the same amino acids but a different fatty acid (216). A selective and sensitive HPLC method was developed for the determination of COL residues in milk and four bovine tissues (muscle, liver, kidney, and fat). The sample pretreatment consists of protein precipitation with trichloracetic acid (TCA), solid-phase purification on C18 SPE cartridges, and precolumn derivatization of colistin with *o*-phthalaldehyde and 2-mercaptoethanol in borate buffer (pH 10.5). The last step was performed automatically, and the resulting reaction mixture was injected into a switching HPLC system including a precolumn and the reversed-phase analytical column. Fluorescence detection was used. The structural study of E1 and E2 derivatives was carried out by HPLC coupled with an electrospray MS. Recoveries from the preseparation procedure were higher than 60%.

F. Ivermectin

Ivermectin (IVER) is a drug with a broad spectrum of activity against gastrointestinal nematodes and lung parasites in food-producing animals. In the EU, the maximum residue levels set for IVER in the liver of sheep, pigs, and cattle are 15, 15, and 100 μg/kg, respectively (217). The use of automated SPE equipment was described for IVER HPLC analysis in animal liver samples. This automated system, ASPEC, was used for the SPE purification step in order to provide more efficient and faster sample preparation. Some tests were performed to obtain recovery and repeatability data. The mean recovery for spiked samples was more than 90% in the concentration range of 7.5–30 μg/kg.

ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
ASTED	automated sequential trace enrichment for dialysates
CD	coulometric detection
CHD	cyclohexa-1,3-dione

col-swi	column switching
CV	coefficient of variation
DAD	diode array detector
DFC	desfuroylceftiofur
DMABA	4-dimethylaminobenzaldehyde
DMSO	dimethylsulphoxide
DSA	direct sample analysis
E	electrochemical detection
EDTA	ethylenediaminotetraacetic acid
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
EMS	electrospray mass spectrometry
ES	electrospray ionization
EtOH	ethanol
EU	European Union
F	fluorescent detection
FDA	U.S. Food and Drug Administration
FFAB	frit fast-atom bombardment
FMOC	9-fluorenylmethyl chloroformate
foc	on-column focusing
HFBA	heptafluorobutyric acid
Ho	homogenization
HPSA	heptanesulphonic acid
HSA	hexanesulphonic acid
HTACl	hexadecyltrimethylammonium chloride
IS	internal standard
LC-fract	LC-fractionation
LLE	liquid–liquid extraction
LOD	limit of detection
LOQ	limit of quantitation
LSC	liquid scintillation counting
MAA	mercaptoacetic acid
MCAC	metal chelate affinity chromatography
ME	mercaptoethanol
MeCN	acetonitrile
MeOH	methanol
MRL	maximal residual limit
MS	mass spectrometry
MSPD	matrix solid-phase dispersion
MWCO	molecular weight cutoff
MWE	microwave extraction
NBA	2-nitrobenzaldehyde
NCI	negative chemical ionization
NQS	1,2-naphthoquinone-4-sulphonic acid
OPA	*o*-phthalaldehyde
PB	particle beam
PD	photochemical degradation
post-der	postcolumn derivatization
Pr	protein precipitation

pre-der	precolumn derivatization
PrOH	propanol
PRS	propylsulphonic acid
PSA	pentanesulphonic acid
RAM	restricted access materials
RP	reverse-phase
RSD	relative standard deviation
SCX	strong cation exchanger
SDS	sodium dodecylsulphate
SFE	supercritical-fluid extraction
SIM	single ion monitoring
SPE	solid-phase extraction
TBA	tetrabutylammonium
TCA	trichloracetic acid
TEA	triethylamine
TEACl	tetraethylammonium chloride
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMAH	tetramethylammonium hydroxide
TS	thermospray
UF	ultrafiltration
UV	ultraviolet detection

ACKNOWLEDGEMENTS

The authors thank Dipl. Ing. Alica Čaniová for technical assistance.

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16
Pesticide Residues: Carbamate and Urea Pesticides

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I. THEORY

Carbamates and ureas constitute important groups of pesticides that are used as herbicides (phenylureas, sulfonylureas, phenylcarbamates, and alkylthiocarbamates), insecticides (benzoylureas, methylcarbamates, and oximemethylcarbamates), or fungicides (dithiocarbamates, alkylenebisdithiocarbamates, and benzimidazolecarbamates) in different crops to control various weeds, pests, and diseases.

Carbamates are substituted esters of carbamic acid (NH_2COOH) with aliphatic or aromatic substituents on the oxygen and nitrogen atoms. Carbamate insecticides have an aryl or oxime *N*-methylcarbamate structure, and their mode of action is based on the inhibition of the enzyme acethylcholine esterase (1). However, this inhibition is reversible, and recovery from sublethal doses occurs rapidly. Some carbamate fungicides have a dithio, bisdithio, or benzimidazole carbamate basic structure, and dithiocarbamate fungicides inhibit the enzyme aldehyde deshydrogenase (2). The herbicides have an *N*-alkylthiocarbamate or *N*-phenylcarbamate structure and interfere with photosynthetic activity or affect meristematic activity or lipid metabolism (3). Representative structures of carbamate pesticides are shown in Fig. 1.

A large number of substituted ureas has been synthesized since the discovery of the first urea derivative with herbicidal activity several decades ago. A main group of these herbicides are the *N*-phenyl-*N'N'*dialkylureas, such as chlorotoluron, diuron, and fluometuron. Other important groups are the *N*-phenyl-*N'*alkyl-*N'*metoxyureas (e.g., linuron, monolinuron, and metobromuron) and the substituted ureas with a heterocyclic group (e.g., methabenzthiazuron). These herbicides act by inhibiting photosynthesis at the photosystem II level (3). A new group of substituted ureas, named sulfonylureas, has been developed more recently and these compounds inhibit the acetolactate synthase, a key enzyme in the biosynthesis of branched-chain amino acids (3). A new insecticide activity acting on the molting process of insects was discovered in the study of biological activity of some benzoyl urea derivatives (4). Diflubenzuron and chlorfluazuron are examples of this group of insecticides. Chemical structures of representative urea pesticides are shown in Fig. 2.

Tables 1 and 2 show the chemical name, molecular formula, water solubility, and vapor pressure of representative carbamate and urea pesticides.

Insecticides

Aryl N-methylcarbamates

$OCONHCH_3$

Carbaryl

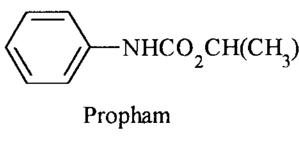
Oxime N-methylcarbamates

$CH_3NHCO_2N{=}C(SCH_3)CH_3$

Methomyl

Herbicides

N-Phenylcarbamates



Propham

N-Alkylthiocarbamates

$(CH_3CH_2)_2NCOSCH_2-C_6H_4-Cl$

Thiobencarb

Fungicides

Dithiocarbamates

$[(CH_3)_2NCS_2]_2\,Zn$

Ziram

Alkylenebisdithiocarbamates

$[\text{-}SC(S)NHCH_2CH_2NHCS_2Mn\text{-}]_x$

Maneb

Benzimidazole carbamates

H, N, N, $-NHCO_2CH_3$

Carbendazim

Fig. 1 Chemical structures of representative carbamate pesticides.

A. Regulations

The use of pesticides in agriculture has obvious advantages, such as an increase in the quantity and quality of food crops. Nevertheless, pesticides are toxic substances, and their residues can pose a risk to man and environment. Therefore, pesticide residues in food are regulated at the international and national levels according to the toxicity of the compound and the human intake of a particular crop. The acute oral toxicity for rats and the maximum residue levels (MRLs) of carbamate and urea pesticides are listed in Tables 1 and 2.

Insecticides

Benzoylureas

Diflubenzuron

Herbicides

Substituted ureas

Methabenzthiazuron

Phenylureas

Chlorotoluron

Sufonylureas

Triasulfuron

Fig. 2 Chemical structures of representative urea pesticides.

B. HPLC versus Other Analytical Techniques

High-performance liquid chromatography (HPLC) is the preferred technique for the determination of the nonvolatile, thermally labile pesticides, because this technique overcomes difficulties encountered with thermal stability in gas chromatography (GC) and with sensitivity in thin-layer chromatography (TLC). Since carbamate and urea pesticides have a tendency to decompose during GC, the main approach for their determination involves HPLC. In the case of methylcarbamates, a postcolumn alkaline hydrolysis to generate a primary amine, followed by derivatization and fluorescence detection, is the preferred procedure (8). Substituted ureas have been directly determined in foods via HPLC-UV with good sensitivity but with lack of selectivity, and the development of derivatization procedures provided a great improvement of selectivity (9,10).

Table 1 Properties of Carbamate Pesticides

Class	Compound	Molecular formula	Water solubility	Vapor pressure	Toxicity in rats (mg/kg)	MRL (mg/kg)	
						Fruits and vegetables	Cereals
Arylmethylcarbamates	Bendiocarb	$C_{11}H_{13}NO_4$	0.28 g/L (20°C)	4.6 mPa (25°C)	40–156	0.05	0.05
	Benfuracarb	$C_{20}H_{30}N_2O_5S$	8 mg/L (20°C)	26.6 mPa (25°C)	138	0.1–2.0	0.1–0.2
	Carbaryl	$C_{12}H_{11}NO_2$	120 mg/L (20°C)	0.4 mPa (24°C)	500–850	1.0–5.0	0.5–1.0
	Carbofuran	$C_{12}H_{15}NO_3$	320 mg/L (20°C)	31 mPa (20°C)	8	0.1–2.0	0.1
	Carbosulfan	$C_{20}H_{32}N_2O_3S$	0.3 mg/L (25°C)	41 mPa (25°C)	185–250		
	Cloethocarb	$C_{11}H_{14}ClNO_4$	1.3 g/L (20°C)	10 mPa (20°C)	35.4	0.05	0.05
	Diethofencarb	$C_{14}H_{21}NO_4$	26.6 mg/L (20°C)	8.4 mPa (20°C)	>5000	0.05–1.0	0.05
	Ethiofencarb	$C_{11}H_{15}NO_2S$	1.8 g/L (20°C)	0.45 mPa (20°C)	200	0.05–2.0	0.05
	Fenobucarb	$C_{12}H_{17}NO_2$	0.42 g/L (20°C)	1.6 mPa (20°C)	425–524		
	Fenoxycarb	$C_{17}H_{19}NO_4$	6 mg/L (25°C)	0.9 mPa (25°C)	>10,000	0.02–2.0	0.02
	Formetanate	$C_{11}H_{15}N_3O_2$	822 g/L (25°C)	1.6 mPa (25°C)	14.8–26.4	0.05–5.0	0.05
	Furathiocarb	$C_{18}H_{26}N_2O_5S$	11 mg/L (25°C)	3.9 mPa (25°C)	53	0.05	0.05
	Isoprocarb	$C_{11}H_{15}NO_2$	265 mg/L (25°C)	2.8 mPa (20°C)	450		
	Methiocarb	$C_{11}H_{15}NO_2S$	27 mg/L (20°C)	15 mPa (20°C)	20	0.05–1.0	0.05
	Metolcarb	$C_9H_{11}NO_2$	2.6 g/L (30°C)	145 mPa (20°C)	498–580		
	Pirimicarb	$C_{11}H_{18}N_4O_2$	3 g/L (20°C)	0.97 mPa (25°C)	147	0.05–0.5	0.05
	Propamocarb	$C_9H_{20}N_2O_2$	867 g/L (25°C)	0.8 mPa (25°C)	2000–8550	0.05–5	0.05
	Propoxur	$C_{11}H_{15}NO_3$	1.9 g/L (20°C)	1.3 mPa (20°C)	50	3.0	0.05
	Trimethacarb	$C_{11}H_{15}NO_2$	>58 mg/L (23°C)	6.8 mPa (25°C)	130		
	XMC	$C_{10}H_{13}NO_2$	0.47 g/L (20°C)		542		
	Xylylcarb	$C_{10}H_{13}NO_2$	0.58 g/L (20°C)	70 mPa (20°C)	325–375		
Oximemethylcarbamates	Alanycarb	$C_{17}H_{25}N_3O_4S_2$	20 mg/L (20°C)	<4.7 mPa (20°C)	440		
	Aldicarb	$C_7H_{14}N_2O_2S$	4.93 g/L (20°C)	13 mPa (20°C)	0.93	0.05–0.3	0.05
	Aldoxycarb	$C_7H_{14}N_2O_4S$	10 g/L (25°C)	12 mPa (25°C)	26.8		
	Butocarboxim	$C_7H_{14}N_2O_2S$	35 g/L (20°C)	10.6 mPa (20°C)	153–215	0.05–1.0	0.05
	Butoxycarboxim	$C_7H_{14}N_2O_4S$	209 g/L (20°C)	0.266 mPa (20°C)	458		
	Methomyl	$C_5H_{10}N_2O_2S$	57.9 g/L (25°C)	6.65 mPa (25°C)	17–24	0.02–2.0	0.02–0.05
	Oxamyl	$C_7H_{13}N_3O_3S$	280 g/L (25°C)	31 mPa (25°C)	5.4	0.05–3.0	0.05
	Thiodicarb	$C_{10}H_{18}N_4O_4S_3$	35 mg/L (25°C)	5.7 mPa (20°C)	66		
	Thiofanox	$C_9H_{18}N_2O_2S$	5.2 g/L (22°C)	22.6 mPa (25°C)	8.5	0.02–0.1	0.02–0.05

N-phenylcarbamates	Carbetamide	$C_{12}H_{16}N_2O_3$	3.5 g/L (20°C)	<1 mPa (25°C)	11000	0.05	0.05
	Chlorbufam	$C_{11}H_{10}ClNO_2$	540 mg/L (20°C)	2.1 mPa (20°C)	2380		
	Chlorpropham	$C_{10}H_{12}ClNO_2$	89 mg/L (25°C)	1.3 mPa (25°C)	5000–7500	0.05–0.1	0.05
	Desmedipham	$C_{16}H_{16}N_2O_4$	7 mg/L (20°C)	40 nPa (25°C)	>10250	0.05–0.2	0.05
	Phenmedipham	$C_{16}H_{16}N_2O_4$	4.7 mg/L (20°C)	1.3 nPa (25°C)	>8000	0.1	0.1
	Propham	$C_{10}H_{13}NO_2$	250 mg/L (20°C)	7 mPa (20°C)	5000	0.05	0.05
Alkylenebisdithiocarbamates	Mancopper				9600	0.05–5.0	0.05–0.2
	Mancozeb	$(C_4H_6MnN_2S_4)_x(Zn)_y$	6–20 mg/L	<1 mPa (20°C)	>5000	0.05–5.0	0.05–0.2
	Maneb	$(C_4H_6MnN_2S_4)_x$	Insoluble	<10 mPa (20°C)	>5000	0.05–5.0	0.05–0.2
	Metiram	$(C_{16}H_{33}N_{11}S_{16}Zn_3)_x$	2.7 mg/kg (20°C)	<10 mPa (20°C)	>10,000	0.05–5.0	0.05–0.2
	Nabam	$C_4H_6N_2Na_2S_4$	>200 g/L (20°C)	negligible	395	0.05–5.0	0.05–0.2
	Propineb	$(C_5H_8N_2S_4Zn)_x$	0.01 g/L (20°C)	<1 mPa (20°C)	>5000	0.05–5.0	0.05–0.2
	Zineb	$(C_4H_6N_2S_4Zn)_x$	10 mg/L (20°C)	<0.01 mPa (20°C)	5200	0.05–5.0	0.05–0.2
Dithiocarbamates	Ferbam	$C_9H_{18}FeN_3S_6$	130 mg/L (20°C)	46 mPa (20°C)	>4000	0.05–5.0	0.05–0.2
	Thiram	$C_6H_{12}N_2S_4$	18 mg/L (20°C)	2.3 mPa (25°C)	1800	3–3.8	0.3
	Ziram	$C_6H_{12}N_2S_4Zn$	0.03 mg/L (20°C)	<1 mPa	320	0.05–5.0	0.05–0.2
N-alkylthiocarbamates	Butylate	$C_{11}H_{23}NOS$	36 mg/L (20°C)	1.73 Pa (25°C)	>3500	0.05	0.05
	Cycloate	$C_{11}H_{21}NOS$	75 mg/L (20°C)	2.13 mPa (25°C)	2000–4000	0.05	0.05
	Dimepiperate	$C_{15}H_{21}NOS$	20 mg/L (25°C)	0.53 mPa (30°C)	959	0.01	0.01
	EPTC	$C_9H_{19}NOS$	375 mg/L (25°C)	0.01 mPa (25°C)	>2000	0.05	0.05
	Esprocarb	$C_{15}H_{23}NOS$	4.9 mg/L (20°C)	10.1 mPa (25°C)	3700		
	Molinate	$C_9H_{17}NOS$	88 mg/L (20°C)	746 mPa (25°C)	369–450	0.01	0.01–0.05
	Orbencarb	$C_{12}H_{16}ClNOS$	24 mg/L (20°C)	12.4 mPa (20°C)	1420		
	Pebulate	$C_{10}H_{21}NOS$	60 mg/L (20°C)	9 Pa (30°C)	1120	0.02–0.05	0.02
	Prosulfocarb	$C_{14}H_{21}NOS$	13.2 mg/L (20°C)	0.069 mPa (25°C)	1820–1950	0.02	0.02
	Pyributicarb	$C_{18}H_{22}N_2O_2S$	0.32 mg/L (20°C)	0.269 mPa (40°C)	>5000		
	Thiobencarb	$C_{12}H_{16}ClNOS$	30 mg/L (20°C)	2.2 Pa (23°C)	1300	0.10	0.10
	Tiocarbazil	$C_{16}H_{25}NOS$	2.5 mg/L (30°C)	93 mPa (50°C)	>10,000	0.01	0.01
	Triallate	$C_{10}H_{16}Cl_3NOS$	4 mg/L (25°C)	16 mPa (25°C)	1100	0.10	0.10
	Vernolate	$C_{10}H_{21}NOS$	90 mg/L (20°C)	1.39 Pa (25°C)	1500	0.05	0.05
Benzimidazolecarbamates	Benomyl	$C_{14}H_{18}N_4O_3$	4 mg/kg (25°C)	<4.9 mPa (25°C)	>10,000	0.1–5.0	0.1–0.5
	Carbendazim	$C_9H_9N_3O_2$	8 mg/L (24°C)	0.15 mPa (25°C)	15,000	0.1–5.0	0.1–0.5
	Debacarb	$C_{14}H_{19}N_3O_4$					

Source: Refs. 5–7.

Table 2 Properties of Urea Pesticides

Class	Compound	Molecular formula	Water solubility	Vapor pressure	Toxicity in rats (mg/kg)	MRL (mg/kg) Fruits and vegetables	MRL (mg/kg) Cereals
Substituted Ureas	Chlorbromuron	$C_9H_{10}BrClN_2O_2$	35 mg/L (20°C)	53 mPa (20°C)			
	Chlorotoluron	$C_{10}K_{13}ClN_2O$	74 mg/L (25°C)	17 mPa (25°C)	>10,000	0.05	0.05
	Diuron	$C_9H_{10}Cl_2N_2O$	42 mg/L (25°C)	1.1 mPa (25°C)	3400	0.02–1.0	0.05–1.0
	Fenuron	$C_9H_{12}N_2O$	3.85 g/L (25°C)	21 mPa (60°C)			
	Isoproturon	$C_{12}H_{18}N_2O$	65 mg/L (22°C)	3.3 mPa (20°C)	2417	0.05	0.05
	Linuron	$C_9H_{10}Cl_2N_2O_2$	81 mg/L (25°C)	51 mPa (20°C)	4000	0.02–1.0	0.05–1.0
	Methabenzthiazuron	$C_{10}H_{11}N_3OS$	59 mg/L (20°C)	5.9 mPa (20°C)	>2500	0.05	0.05
	Metobromuron	$C_9H_{11}BrN_2O_2$	330 mg/L (20°C)	0.4 mPa (20°C)	2623	0.02–0.1	0.02–0.1
	Monolinuron	$C_9H_{11}ClN_2O_2$	0.7 g/L (25°C)	1.3 mPa (20°C)	2215	0.02–0.2	0.02
Benzoylureas	Chlorfluazuron	$C_{20}H_9Cl_3F_5N_3O_3$	<0.01 mg/L (20°C)	<10 nPa (20°C)	>8500	0.05	0.05
	Diflubenzuron	$C_{14}H_9ClF_2N_2O_2$	0.08 mg/L (20°C, pH 5.5)	0.1 mPa (25°C)	>4640	0.05–1.0	0.05
	Flufenoxuron	$C_{21}H_{11}ClF_6N_2O_3$	4 mg/L (25°C)	6.5 pPa (20°C)	>3000	0.01–0.5	0.01
	Hexaflumuron	$C_{16}H_8Cl_2F_6N_2O_3$	27 mg/L (18°C)	59 mPa (25°C)	>5000	0.05–0.5	0.05
	Teflubenzuron	$C_{14}H_6Cl_2F_4N_2O_2$	19 mg/L (23°C)	0.8 nPa (20°C)	>5000	0.05–0.5	0.05
Sulfonylureas	Bensulfuron-methyl	$C_{16}H_{18}N_4O_7S$	120 mg/L	2.8 pPa	>5000	0.05	0.02–0.05
	Chlorsulfuron	$C_{12}H_{12}ClN_5O_4S$	27.9 g/L	3 nPa	>5000	0.05	0.05–0.1
	Nicosulfuron	$C_{15}H_{18}N_6O_6S$	12.2 g/kg	<75 mPa (110°C)	>5000	0.05	0.05–0.1
	Rimsulfuron	$C_{14}H_{17}N_5O_7S_2$	7.3 g/L	1.5 mPa	>5000	0.05	0.05
	Thifensulfuron-methyl	$C_{12}H_{13}N_5O_6S_2$	6.3 g/L	17 nPa	>5000	0.02	0.02–0.05
	Triasulfuron	$C_{14}H_{16}ClN_5O_5S$	0.8 g/L	<2 mPa	>5000	0.05	0.02–0.05
	Tribenuron-methyl	$C_{15}H_{17}N_5O_6S$	280 g/L (pH 6)	52 nPa	>5000	0.05	0.05
	Triflusulfuron-methyl	$C_{17}H_{19}F_3N_6O_6S$	110 mg/L	<10 mPa			

Source: Refs. 5–7.

II. SAMPLE PREPARATION

A. Extraction

The usual method for the determination of carbamate pesticides and their toxic carbamate metabolites in crop samples involves blending or homogenizing the analytical sample portion in the presence of an organic solvent. Various solvents of different polarities, e.g., low-polarity solvents such as benzene and chloroform or water-miscible organic solvents such as acetonitrile, acetone, and methanol, have been used to extract carbamate pesticides. The selection of solvent or solvent systems for the extraction will depend on the nature of the substrate (11–20). Other extraction procedures have been introduced lately. Supercritical-fluid extraction is an alternative technique to the classical extraction procedures based on organic solvents (21–23). Another interesting and promising technique is based on the extraction by matrix solid-phase dispersion (MSPD), which includes sample homogenization, cellular disruption, extraction, and purification in the same process (24–25). Table 3 lists the different solvents and extraction procedures used in the analysis of carbamate pesticides. For aqueous matrices, solid-phase extraction (SPE) has been used (26).

Several solvents of different polarities have been used to extract urea pesticides from crops, including acetonitrile, acetone, ethanol, and methanol (29–40). The selection of the extracting solvent will depend on the polarity of the substituted urea and on the nature of the sample. Solvents used in extraction should be pesticide-residue grade or be redistilled in all-glass apparatus. Various procedures have been used to extract urea pesticides from crop samples. Extraction can be done with a Soxhlet apparatus, but prolonged heating with a polar solvent may produce the breakdown of some compounds (35). Satisfactory recoveries from different type of samples have been achieved by using various extraction apparatus, such as a Waring Blendor (37–38), a Polytron homogenizer (35) or a Sorvall Omnimixer (29,32–34,36). After extraction, the solvents used in the procedure are evaporated by means of a Kuderna–Danish or a rotary evaporator. Recently, other alternative techniques have been employed in the extraction of substituted ureas from foods, e.g., supercritical-fluid extraction (41) and matrix solid-phase dispersion (25). Table 4 shows the different solvents and extraction procedures used in the analysis of urea pesticides.

Table 3 Extraction of Carbamate Pesticides from Foods

Compound	Solvent, Procedure[a]	Food	Refs.
Carbaryl	Acetonitrile, homogenizer	Corn	11
Aldicarb	Acetone-water, homogenizer	Potatoes	12
Carbaryl, carbofuran	Chloroform, homogenizer	Apples, potatoes, sugarbeets	13, 14
Methomyl	Acetone, homogenizer (Omnimixer)	Citrus fruits	18
Carbaryl	Benzene, homogenizer	Honeybees	15
N-methyl carbamates	Acetonitrile, homogenizer	Crops	17
Carbamates	CO_2 + modifiers, SFE	Meat, cereals	22, 23
Thiocarbamates	CO_2 + modifiers, SFE	Apples	21
Benzimidazoles	Acetone, homogenizer	Fruit and vegetables	20
Oxamyl, methomyl	MSPD	Apples, oranges	24
Dithiocarbamates	HCl–Stannous chloride	Vegetables	27
Mancozeb	EDTA	Leaves	28
Multiresidue	MSPD	Fruit and vegetables	25
N-methyl carbamates	Methanol, homogenizer (Omnimixer)	Crops	19

[a] SFE: supercritical fluid extraction; MSPD: matrix solid-phase dispersion; EDTA: ethylenediaminetetraacetic acid.

Table 4 Extraction of Urea Pesticides from Foods

Compound	Solvent, Procedure[a]	Food	Refs.
Chlorotoluron	MeOH:H_2O (80:20), homogenizer (Sorvall)	Cereals	29
Diuron	Acetone, homogenizer	Fruit and vegetables	30
Diuron, monuron	Hydrolysis, steam distillation	Fruit and vegetables	31
Linuron	Acetone, homogenizer (Sorvall)	Cereals and vegetables	32, 33
Multiresidue	MeOH, homogenizer (Sorvall and Polytron)	Wheat and vegetables	34, 35
	Acetone, homogenizer (Sorvall)	Cereals and vegetables	36
	EtOH, homogenizer (Waring Blendor)	Fruit and vegetables	37
	CH_3CN, homogenizer (Waring Blendor)	Fruit and vegetables	38, 59
	Hydrolysis, steam distillation	Vegetables	39, 40
Phenylureas	MeOH–sodium acetate, mixing	Milk	42
	MSPD	Fruit and vegetables	25
Sulfonylureas	CO_2 + modifiers, SFE	Wheat	41

[a] MSPD: matrix soild-phase dispersion; SFE: supercritical fluid extraction.

B. Cleanup

A preliminary cleanup procedure is often required for most food sample extracts before determination by HPLC. The goal of cleanup is to remove as much interfering coextracted material and as little analyte as possible. Due to the wide range of polarities of carbamate pesticides, it is difficult to develop one cleanup procedure equally effective for all of them. Therefore, the cleanup procedure will depend on the type of compound, the kind of sample to be analyzed, and the selectivity of the analytical equipment used in the determination. The use of selective detectors can reduce or even eliminate the need for cleanup procedures in some cases.

A variety of adsorbents, such as alumina, silica gel, Florisil, and various activated carbons, have been employed for carbamate residue cleanup (43). Liquid chromatography on alumina (44–46), silica gel (14), or Florisil (47) has also been used as a cleanup step.

Liquid–liquid extraction has also been employed as a cleanup step, with separations being made between an acid or alkaline aqueous phase and an organic solvent (48). This procedure takes advantages of differences in the physical and chemical characteristics between the carbamate and the substrate. Another commonly used procedure is based on the generally high solubility of carbamates in polar solvents and their low solubility in saturated hydrocarbons. Table 5 summarizes the use of the various cleanup procedures in the determination of carbamate pesticides.

Several cleanup methods have been developed for the determination of urea pesticides, involving different basic procedures, such as liquid–liquid partition (30–32,34,36,37), steam distillation (31), and liquid–solid chromatography (9,30,32,34,36,38,56–58). Different factors, e.g., water solubility, ionic and polarity properties, thermal stability, and the molecular weight of the compounds, determine the choice of the cleanup method. Moreover, micro-cleanup procedures and online enrichment techniques have been introduced for the automated determination of phenylureas (60). Table 6 summarizes the use of the different cleanup procedures in the determination of urea pesticides.

C. Derivatization

Ultraviolet (UV) absorbance has been the most commonly used detection method in HPLC determination of carbamate pesticides (18,61–64). Normally, the absorption maxima occurred at

Table 5 Cleanup Procedures for Sample Extracts Used in the Determination of Carbamate Pesticides

Compound	Cleanup procedure	Sample	Method	Ref.
Carbaryl, carbofuran	Column (Florisil, alumina, activated carbon)	Apples, potatoes, sugarbeets	GC	13
Carbaryl	Partition (acetonitrile–hexane)	Milk	GC	48
Carbofuran, phenolic metabolite	Hydrolysis, distillation, column (alumina)	Corn	GC	44
Aldicarb, metabolites	Hydrolysis, partition, column (silica gel)	Sugarbeets	GC	14
Carbaryl, carbofuran	SPE (Florisil)	Tobacco	HPLC	49
Carbofuran	Cartridge	Tomatoes	HPLC, GC	50
Carbamates	Column (Florisil)	Corn, potatoes, wheat	HPLC	51
Methomyl	Gel permeation chromatograpy (GPC)	Citrus fruits	HPLC	18
Triallate	Column (alumina)	Cereals	GC-MS	46
Aldicarb, ethiofencarb, methiocarb, and metabolites	Sep-Pack aminopropyl cartridges	Fruits and vegetables	HPLC	53
Chlorpropham, propham	Silica TLC	Potatoes	HPLC	54
Benzimidazoles	SPE-diol bonded silica cartridges	Fruits and vegetables	HPLC	20
Dithiocarbamates	Acidic digestion	Grain	Colorimetry	55
Carbaryl	Column (silica gel) C18 cartridge	Apples	HPLC	52

Table 6 Cleanup Procedures for Sample Extracts Used in the Determination of Urea Pesticides

Compound	Cleanup procedure	Sample	Method	Ref.
Chlorotoluron	Column (silica)	Cereals	GC	56
Diuron	Hydrolysis, distillation, partition	Vegetables	GC	31
	Column (Florisil)	Wheat	GC	57
	Partition, column (silica)	Fruits and vegetables	GC	30
	Partition, column (alumina)	Fruits	HPLC	59
Linuron	Column (Florisil)	Vegetables	GC, HPLC	58
	Partition, cartridge (silica)	Potatoes	HPLC	32
Metoxuron	Column (silica)	Potatoes	TLC	9
Multiresidue	Column (MgO : celulose : Florisil)	Fruit and vegetables	TLC	38
	Partition, column (Florisil)	Wheat and vegetables	HPLC	34
	Partition, column (Florisil)	Cereals and vegetables	HPLC	36
	Partition	Fruit and vegetables	GC	37
	Precolumn online	Water	HPLC	60
	Partition, cartridge (Florisil)	Milk	HPLC	42

202 nm or less (65); this is a region where plant coextractives also commonly show high absorption. In order to overcome these limitations, it is possible to obtain derivatives of carbamate pesticides for HPLC determination, followed by chromatographic separation (52,66–69). A more recent method employs a postcolumn derivatization reaction for UV detection. The derivatizing reaction is based on the alkaline hydrolysis of carbamates and subsequent coupling with diazo-

tized sulphanilic acid, the products of which are monitored photometrically in the region 400–506 nm (70–72).

Fluorescence detection is not as widely applicable as UV detection, since most carbamate pesticides do not posses native fluorescence. However, for those exhibiting fluorescence (73,74) or that can be made fluorescent by derivatization (75), fluorescence detection offers a degree of selectivity and sensitivity often an order of magnitude or more over that given by UV. The most important development has been a postcolumn derivatization, for the determination of methyl carbamates and their toxic metabolites by HPLC-fluorescence detection. The analytes, after elution through the HPLC system, were hydrolyzed by aqueous sodium hydroxide, the primary amines were then derivatized, forming the highly fluorescent isoindole derivative using *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (MCE), and their fluorescence was measured by the online detector (8,22,23,53,76–82).

Substituted ureas have commonly been analyzed by direct HPLC-UV, showing this method, in general, to have high sensitivity but low selectivity. In order to overcome this problem, pre- or postcolumn derivatization and fluorescence detection has been proposed. Precolumn derivatization has been carried out via the formation of derivatives with 5-dimethylaminonaphtalene-1-sulphonyl (Dns) chloride of anilines obtained from phenylureas by catalytic hydrolysis on silica TLC plates (9). A postcolumn procedure using derivatization with *o*-phtalaldehyde (OPA) was also proposed (10,34). In this procedure, phenyl ureas were photochemically degraded using a UV lamp, and the resulting amines were reacted with OPA to give fluorescent derivatives that were detected spectroflorometrically. Table 7 summarizes the derivatization procedures employed. Other type of derivatives of carbamates and urea pesticides have been obtained with the aim of reducing the polarity and increasing the thermal stability of these compounds, thereby allowing the analysis of derivatives by GC. The main classes of reaction used are: acylation (56,84–94), alkylation (37,95–103), and silylation (104).

III. SEPARATION TECHNIQUES

High-performance LC is the favored technique for the determination of carbamates, since many of them lack the thermal stability necessary for gas chromatographic analysis. Most HPLC methods for methyl and phenyl carbamate pesticides have employed reversed-phase chromatography with C18 or C8 columns and aqueous mobile phases (47,50,105,106). Two different solvent sys-

Table 7 Derivatization Methods for HPLC Determination of Carbamate and Urea Pesticides

Compound	Reaction	Reagent[a]	Detector	Refs.
Carbaryl	Precolumn alkaline hydrolysis	4-AAP	UV	52
Carbofuran	Precolumn alkaline hydrolysis	FDNB	UV	68
Methyl carbamates	Postcolumn alkaline hydrolysis	DSA	UV	70–72
Methyl carbamates, metabolites	Postcolumn alkaline hydrolysis	OPA, MCE	Fluorescence	8, 22, 23, 53, 76–82
Carbamates	Enzimatic thiolysis	Glutathione and cysteine	UV	83
Phenylureas	Precolumn catalitic hydrolysis	Dns chloride	Fluorescence	9
Phenylureas	Postcolumn photolysis	OPA	Fluorescence	10, 34

[a] (4-AAP): 4-aminoantipyrine; FDNB: 1-fluoro-2,4-dinitrobenzene; DSA: diazotized sulphanilic acid; OPA: 2-*o*-phthaldehyde; MCE: 2-mercaptoethanol; Dns: 5-dimethylaminonaphtalene-1-sulphonyl.

tems, methanol–water (107–111) and acetonitrile–water (47,59,64), have been used. In general, acetonitrile–water gave the best overall results and provided the lowest detection limits (112).

The carbamate pesticides can also be determined by normal-phase chromatography on different columns. Silica (113–116), amine (51), and cyanopropyl (112) columns have been used for the determination of carbamate residues. These columns were used with different solvent systems, with isopropanol–heptane being the mobile phase producing generally better results and methylene chloride–heptane the preferred solvent system when nonpolar carbamates were present in the sample. Another mobile phase used was iso-octane-dioxane (113). Although normal-phase mode was for the most part satisfactory, reversed-phase mode gave generally superior results.

Salts of ethylenebisdithiocarbamates (EBDCs) and dithiocarbamates were transformed into water-soluble salts, which were subsequently converted to the methyl esters and determined by HPLC with UV detection (28). Dithiocarbamates have also been determined by HPLC with electrochemical detection using a graphite polytetrafluoroethylene electrode (122). Ethylenethiourea (ETU), a degradation product of the EBDCs, can be successfully chromatographed by HPLC without derivatization; therefore this would be the chromatographic technique of choice. ETU has generally been chromatographed on a C8 or C18 reversed-phase silica-based HPLC columns with little or no organic modifier in the mobile phase and determined by an amperometric EC detector equipped with an Hg/Au working electrode (19).

HPLC analysis of thiocarbamates was carried out by means of reversed-phase HPLC, C18 column, with UV detection using a mixture of methanol–water or acetonitrile–water as the mobile phase (123,21).

Benzimidazole carbamates are usually analyzed by HPLC. Different stationary phases have been assayed, and alkyl-bonded-silica–based phases have generally been considered the most useful separation mode, although polymer-based columns performed better in separating benzimidazole fungicides (20). Buffered water and methanol (124,20) or acetonitrile (125) have been used as mobile phases.

Substituted ureas can be analyzed by normal- and reversed-phase HPLC. Alkyl-bonded stationary phases are the ones most commonly employed with aqueous methanol or acetonitrile mobile phases (9,34,35,60,127–129).

Tables 8 and 9 summarize the separation conditions used in HPLC analysis of carbamate and urea pesticides.

IV. DETECTION SYSTEMS

A. History

Polarographic, thin-layer chromatographic, and spectrometric techniques have been reported for the determination of carbamate pesticides (131–135). These compounds are thermally labile and thus tend to degrade at the elevated temperature of a GC column. Nevertheless, some carbamate insecticides can be determined, directly or indirectly, by GC (44,136–138). The obtaining of thermally stable derivatives has generally been limited to the aryl-*N*-methylcarbamates. The most difficult carbamate insecticides to chromatograph by GC are the oxime *N*-methylcarbamates (139–141).

Dithiocarbamates and alkylenebis-dithiocarbamates can also be analyzed by GC (142–145). The dithiocarbamates are acid hydrolyzed to carbon disulfide, which is then determined by colorimetry (55) or by headspace GC (27).

For the determination of ETU, paper chromatography, TLC, and GC have been used (146). TLC generally does not provide the desired precision for the quantitation of ETU, for this com-

Table 8 HPLC Determination of Carbamates in Food Samples

Compound	Column, mobile phase	Detector	D.L.[a]	Refs.
Carbaryl, carbofuran	Reversed-phase-RP-18, acetonitrile–water	UV diode array	0.02–0.004 mg/kg	47, 50, 105, 106
Carbaryl, carbofuran	Reversed-phase-RP-18, methanol–water	UV diode array	0.02 mg/kg	49, 107
Methomyl, carbaryl	Reversed-phase-ODS-C18, methanol–water	UV	10 ng/mL	108, 109
Phenmedipham, desmedipham	Reversed-phase-RP-18, methanol–water	UV	0.09 μg/L	110, 111
Methiocarb	Reversed-phase-C18, acetonitrile–water	UV	0.2 ppm	59, 64
Carbamates	Normal-phase-LichrosorbSi-60, iso-octane/dioxane, TMT–isopropanol	UV	0.1–1 ng	113, 114
Phenmedipham	Normal-phase-Nucleosil50, isopropanol–cyclohexane	UV	0.01–0.1 mg/kg	115
Thiophanate-methyl	Normal-phase-NucleosilNH_2, *n*-hexane–ethanol	UV	5 ng	51
Carbamates	Normal-phase-CN, Si, NH_2, isopropanol–heptane, methylene chloride–heptane	UV	0.5–60 ng	112
Carbendazim, phenmedipham	Reversed-phase-RP18, acetonitrile–water	UV	0.04–0.08 ng/mL	62
Carbamates	Reversed-phase-C8, acetonitrile–water	UV	0.02–0.92 μg/L	63
Carbaryl	Reversed-phase-phenyl column, methanol–water	UV	3 ng, 0.01 mg	52, 117
Carbofuran	Reversed-phase-C8, methanol–water	UV	2–4 ng, 0.05 ppm	68, 69
Carbamates	Reversed-phase-C18, acetonitrile–water	UV		22, 70–72, 118
Thiabendazol, chlorpropham	Normal-phase-ODS, acetonitrile–water–methanol	UV, fluorescence		74
Carbamates	Reversed-phase-ODS, methanol–water	Fuorescence	1 ppb	53, 79
Carbamates	Reversed-phase-RP8, RP18, acetonitrile–water	Fluorescence	0.1 ng	8, 76, 81, 82
Methyl-carbamates	Reversed-phase-RP18, methanol–water	Bioluminiscence	ppb	119
Carbaryl	Reversed-phase-ODS, acetonitrile–acetic acid–potasium chloride	Electrochemical	1 ng/ml	120
Carbamates	Reversed-phase-C18, acetonitrile–water–ammonium acetate	MS (thermospray)	0.025–1 ppm	121
Oxamyl, methomyl	Reversed-phase-ODS, acetonitrile–water	Fluorescence	20 ng/g	24
Thiram, disulfiram	Reversed-phase-RP18, acetonitrile–phosphate buffer	Electrochemical	0.5 ppm	122
Mancozeb	Normal-phase-NH_2, acetonitrile–methanol–borate buffer	UV	10 ppm	28
Thiocarbamates	Reversed-phase-C18, methanol–water–acetate buffer	UV	0.02–0.07 mg/mL	123
Thiocarbamates	Reversed-phase-C18, methanol–water–acetonitrile–KH_2PO_4 buffer	Chemiluminiscence, UV	48 pg	21
Benzimidazole	Reversed-phase-C18, acetonitrile–water–phosphate buffer	UV	0.01 μg/g	125
Benzimidazole	Reversed-phase-ODS, methanol–ammonium acetate	MS	0.02 ppb	126
Benzimidazole	Reversed-phase-C18, methanol–phosphate buffer–triethylamine	UV	50 ppb	124
Phenyl carbamates	Reversed-phase-C18, methanol–phosphate buffer	UV	0.5 μg/kg	54
Benzimidazole	Several columns, methanol–phosphate buffer	UV, fluorescence	0.05 mg/kg, 0.02 mg/kg	20

[a] D.L.: detection limit

Table 9 HPLC Determination of Urea Pesticides in Food Samples

Compound	Column, mobile phase[a]	Detector	D.L.[b]	Ref.
Chloroxuron	Spherisorb C18, $CH_3CN:H_2O$	UV	0.2 mg/g	127
Diuron	Porasil, C_8H_{18}:MeOH:PrOH	Photoconductivity	10 ppb	130
Linuron	Lichrosorb NH_2, isoPrOH:C_8H_{18}	UV	0.01 mg/g	32
	Lichrosorb Si60, isoPrOH:C_8H_{18}	UV		33
Metoxuron	Spherisorb C18, $CH_3CN:H_2O$ MeOH:H_2O	Fluorescence	0.02 mg/g	9
Multiresidue	Lichrosorb Si60, isoPrOH:C_8H_{18}	UV	0.1 mg/g	36
	Spherisorb C18, MeOH:H_2O	UV	0.02 mg/g	35
	Econosphere C18, MeOH:H_2O $CH_3CN:H_2O$	Fluorescence	0.01 mg/g	34
	Econosphere C18, Buffer pH 10.2	Fluorescence	ng	10
	Zorbax CN, MeOH:H_2O	Photoconductivity	ppb	128
	RP-8, RP-18, MeOH:H_2O	UV	ppb	60
	CP-Sher C18, MeOH:Phosphate buffer pH 7	Electrochemical	0.4 ppb	129

[a] C_8H_{18}: isooctane; isoPrOH: isopropylalcohol.
[b] D.L.: detection limit

pound can be degraded on the TLC plate. Gas chromatography is a less than satisfactory technique, because ETU generally chromatographs poorly on a GC column unless a derivative is formed.

Alkylthiocarbamate or thiocarbamate herbicides have been determined by various analytical methods, e.g., UV spectrometry, thin-layer chromatography, IR spectrometry, and gas chromatography.

Benzimidazole carbamates have generally been determined by HPLC (20,124–126), although other methods have been reported based on enzyme immunoassay (147) or in gas chromatography (124). The commercial availability of high-performance thin-layer chromatography (HPTLC) plates with layers of very different polarities has improved the sensitivity, selectivity, and analytical precision of TLC, which has led to a reconsideration of the use of this technique for qualitative and quantitative analysis (54).

Substituted ureas were determined, at the beginning, by colorimetric analysis. This determination is based on a basic or acidic hydrolysis leading to the corresponding aniline, followed by diazotation and coupling with *N*-(1-naphthyl)-ethylendiamine (40,58,148–152), 1-naphthol (153), or *N*-ethyl-1-naphthylamine (154) to obtain an azo-dye. Nevertheless, this method is time consuming and does not differentiate between the pesticide and metabolites, unless they are previously separated (154).

Thin-layer chromatography has also been employed in the analysis of these compounds (9,38,150,155–159). Several developers, such as silver nitrate (150,158), *p*-dimethyl-aminobenzaldehyde (38), or UV light (150,158) have been used. Derivatization techniques, based on the urea hydrolysis to the corresponding aniline and ulterior reaction with dansyl chloride, have also been carried out (9,159). Moreover, TLC is frequently employed in studies of metabolization with radiolabeled compounds (155–157).

Determination of urea pesticides has been performed by gas chromatography (37,56,160, 161), a technique that has highly sensitive and selective detectors. Nevertheless, some substituted ureas are thermolabile and decompose during the analysis by GC (102). Therefore, direct determination of unchanged compounds by GC is possible only for some urea pesticides under determined chromatographic conditions (161). In other cases, a degradation product is quantified instead of the parent compound (29,162–164), or these substituted ureas must be derivatized before GC determination (37,102).

B. Recent and Future Developments

Carbamate pesticides can be determined using different detectors in GC or HPLC analysis. A characteristic feature of a carbamate molecule is the nitrogen atom, which can form the bases for quantitation; and some carbamates also contain chlorine, sulfur, or other heteroatoms in the molecule. This allows the use of various detection techniques for their determination (139,140), such as electrical conductivity (165), alkali flame (141) photometry, and mass spectrometry (44,166).

Enzymatic techniques have also been employed in the analysis of these compounds. The toxicity of carbamate insecticides is due to the inhibition of the enzyme acetylcholine esterase, so the determination of these compounds can be achieved by enzyme inhibition (2,83,119), bioassay (118,167), or enzyme-linked immunosorbent assay (ELISA) (168–171). In the detection of carbamates by fluorimetric enzyme inhibition, the effluent from a reversed-phase chromatographic column was incubated with cholinesterase, which was introduced via a postcolumn reagent delivery pump. Then, the resulting partially inhibited cholinesterase was reacted with *N*-methyl indoyl acetate to produce a fluorophore and a reduction in the baseline fluorescence (172).

Electrochemical detection is also possible for the HPLC determination of carbamate compounds capable of being oxidized or reduced at moderate electrode potentials (120).

Recent developments in HPLC-MS for carbamate analysis in food samples have the advantage of not requiring a derivatization step while providing a higher degree of confirmation of molecular identity than methods based on fluorescence or ultraviolet detectors (121,173,174). These HPLC methods show detection and quantitation limits at low parts per billion (ppb) for food matrices. The limits of residue detection in foods for these methods are usually below tolerance levels, which generally range from 0.1 to 50 ppm; in addition, the mass spectral fragmentation can also be obtained (166). Table 8 shows the detection systems used in carbamate analysis by HPLC.

Direct determination of urea pesticides by high-performance liquid chromatography has been widely reported in the literature (10,32–36,127–130). Ultraviolet detection has often been used (32,33,35,36,60,127) with usually acceptable sensitivity, although this detector is nonspecific and the sensibility is, in general, low. To overcome this problem, several techniques have been assayed, such as precolumn enrichment (60), postcolumn derivatization (34,10), and the use of other detection techniques such as the electrochemical (129), photoconductivity (128,130), and fluorescence detectors (9,10,34). Table 9 summarizes representative papers using these techniques in HPLC analysis.

The successful combination of mass spectrometry with gas chromatography (GC-MS) and, subsequently, with liquid chromatography (HPLC-MS) allowed not only the determination of urea pesticides in food but also the identification of their residues at trace level. Mass spectrometry is a technique that can be used as a general detector, with cyclic scanning. The selectivity and sensibility of analysis can be enhanced using characteristic ions of the molecule, with selected ion monitoring (SIM). Urea pesticides have been determined by HPLC-MS directly (175–180), without the thermal instability problems of GC analysis.

The application of immunoassays to the determination of various urea pesticides have been reported (181,182), and this technique has a great potential for residue analysis by using rapid, simple, and cost-effective tests (183,184).

V. APPLICATIONS IN FOODS

High-performance LC is the technique of choice for residue determination in foods of nonvolatile, thermally labile carbamate and urea pesticides. National programs for monitoring pesti-

cide residues in foods are established in many countries, and carbamates and substituted ureas are normally included in these monitoring programs. In general, the obtained results evidence that a variable percentage of samples have detectable residues and that pesticide concentrations found in these samples are generally well below maximum residue levels fixed for the particular commodity analyzed (185–187).

VI. CONCLUSION

Carbamates and substituted ureas are a numerous group of pesticides widely used to control weeds, pests, and diseases in fruit trees, vegetables, and cereals. Carbamate residues in foods are commonly extracted with water-miscible solvents and determined by using a liquid chromatograph equipped with a sensitive detector, frequently a UV detector. In addition, to obtain adequate detection selectivity, the postcolumn fluorimetric labeling technique is used for methyl carbamates. Substituted ureas are normally extracted from foods with organic solvents, and they can be determined directly by HPLC-UV or after postcolumn derivatization by fluorescence determination of their derivatives.

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17

Pesticide Residues: Organochlorine and Organophosphates

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I. THEORY

Published information shows that the total amount of pesticides used in agriculture and industry is increasing (1,2). As a consequence of the large quantities used, pesticides are present in all areas of the natural environment. Monitoring pesticides in food poses substantial analytical problems as the demand increases for more sensitive methods, in response to recent legislation in many countries (3).

Water pollution may be caused by agricultural activities and has increased continually over the last few years. A wide variety of pesticides reach the soil in the agricultural areas where they are applied, but they can also be carried off by leaching as a consequence of water displacement in the soil environment or runoff (4–6). Some of this surface and groundwater is supplied as drinking water; because of this, it is necessary to screen them for pesticide contamination.

Contamination of vegetables may result from agricultural treatment as well as from improper use of pesticides, the residues of preceding treatments in the soil, and cross-biocontamination (particularly during harvesting). The sources of residues in products of animal origin include contaminated water or feed, pesticide-treated housing, and contaminated milk (during weaning) (7,8).

Many organochlorine pesticides (OCPs), which have shown undesirable effects in humans and the environment, have been banned in developed countries. Even so, OCPs deserve particular attention, for they are very stable and can accumulate in food chains (9). Products of animal origin as well as human mother's milk almost always contain residues of organochlorine compounds. The residue content of this milk is 10–30 times higher than that of cow's milk (10,11).

Organophosphorus pesticides (OPPs) have been used extensively as insecticides, fungicides, herbicides, and animal pesticides for more than four decades. They have been used instead of organochlorine pesticides because their relatively rapid decomposition makes them less persistent in the environment and because their accumulation in the biological food chain is limited. Basic to the proper assessment of the potential health hazards of OPPs is the knowledge of their fate in biological systems and in the environment (12,13).

The methods most frequently used to monitor for pesticides are high-performance liquid chromatography (HPLC) and gas chromatography (GC) (14,15). On the other hand, multiresidue analytical techniques are preferred to single-residue methods, because multiresidue methods provide the capability to determine different pesticide residues in a single analysis (12,16–18). As

newer analytical instruments allow the detection of ever lower residue levels, the opportunity for more accurate residue determination increases proportionately. Although capillary column gas chromatography remains the major determination technique, the number of publications describing HPLC methods for pesticides is steadily increasing (13).

High-performance LC is widely used, offline or online, in the determination of pesticides, either as a final measurement step or as a separation technique. The increase in the use of HPLC is mainly the result, on the one hand, of its suitability for determining thermally labile and polar pesticides that require derivatization prior to GC, and, on the other, of its compatibility with online precolumn extraction and cleanup and with MS systems (19).

The purpose of this chapter is to review the use of HPLC to determinate OPPs and OCPs. Primarily, the use of HPLC with UV and diode array detectors and developments in automation and HPLC-MS are reviewed. The use of HPLC to clean up and fractionate extracts from fatty samples prior to determination by GC is also reviewed.

A. Physicochemical Properties

Organochlorine pesticides involve organic molecules that contain several halogenated atoms, and OPPs involve esters of phosphoric, phosphonic, phosphorothioic, or related acids (20,21). Figure 1 shows the chemical structures and names of some OCPs and OPPs.

The water solubilities of the two types of compounds differ. The OCPs are practically insoluble in water. Most OPPs present low solubility, but a few of them are completely soluble in water and can formulate in aqueous medium (20–22).

Another difference has to do with their stability. The OCPs are chemically stable and do not degrade in environmental conditions. In contrast, OPPs suffer water hydrolysis, oxidation, and isomerization induced by light and temperature. These reactions can take place in the P atom or in the alkylic chain and in general induce a loss of pesticide action. Aqueous hydrolysis is favored by an alkaline pH (20). The physicochemical properties of organochlorine and organophosphorus pesticides are presented in Table 1.

It should be noted that a connection between hydrophobicity and fat-soluble partition coefficients (*n*-octanol/water) (P_{ow}) has been proposed. The ratio is reported as a logarithm (log P_{ow}) that can be considered a quantitative measure of the hydrophobicity of a compound. Hence, log P_{ow} could be used to predict useful operating HPLC conditions. It would be applicable to hydrophobic compounds, but it may not apply to hydrophilic compounds with a log P_{ow} of less than 2 (23).

Organochlorine pesticides are defined as fat soluble. Most have a log P_{ow} over 5 (aldrin, DDE, DDT, dieldrin, endrin, and heptachlor), and the HCH isomers have a log P_{ow} in the 3–4 range. The majority of the OPPs are also classified as fat soluble but present lower values of log P_{ow}. Chlorfenvinphos, fenitrothion, and diazinon, with a log P_{ow} in the 3–4 range, are examples of this kind of OPP. A few of these compounds cannot, however, be considered fat soluble (Mevinphos or Trichlorfon) (23).

B. HPLC versus Other Analytical Techniques

Organochlorine pesticides and OPPs have been determined mainly using GC, because of the stability and volatility that most of them show under chromatographic conditions and, particularly, the availability of element-selective detectors that display high selectivity for OCPs (electron-capture detector, ECD), and OPPs (flame photometric detector, FPD, and nitrogen phosphorus detector, NPD). Mass spectrometry–based detection is also more popular in GC than in HPLC (1,2,12,16).

Chemical structure	Name
Organochlorines	
	Aldrin 1,2,3,4,10,10-hexachloro-1α,4α,4αβ,5α,8αβ- -hexahydro-1,4:5,8-dimethanonaphthalene.
	Captan 3a,4,7,7^a-tetrahydro-2-[(trichloromethyl)thio]-1H- -isoindole-1,3-(2H)-dione.
	DDT 1,1'-(2,2,2-trichloroethylidene) bis [4-chlorobenzene].
	Dieldrin 3,4,5,6,9,9-hexachloro-1αa,2β,2αa,3β,6β,6αa -octahydro-2,7:3,6-dimethanonaphth[2,3-*b*]oxirene.
	Endosulfan 6,7,8,9,10,10 hexachloro-1,5.5a,6,9,9a-hexahydro-6,9- -methano-2,4,3-benzodioxathiepin-3-oxide.
	Endrin 3,4,5,6,9,9-hexachloro-1αa,2b,2ab,3α,6α,6ab,7b,7αa- octahydro-6,7-epoxy-1,4:5,8-dimethanonaphthalene
	HCB hexachlorobenzene.
	HCH 1α,2α,3β,4α,5α,6β-hexachlorocyclohexane
	Heptachlor 1,4,5,6,7,8,8-heptachloro-3^a,4,7,7^a-tetrahydro-4,7 methano-1*H*-indeno.

Fig. 1 Chemical structures and names of several OCPs and OPPs.

Chemical structure	Name
Organophosphorus	
	Azinphos methyl *O,O*-dimethyl-S-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl) methyl]phosphorodithioate
	Chlorfenvinphos 2-chloro-1-(2,4-dichlorophenyl)ethenyldiethyl phosphate
	Chlorpyriphos *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate
	Dichlorvos 2,2-dichloroethenyl dimethyl phosphate
	Dimethoate *O,O*-dimethyl S-[2-(methylamino)-2-oxoethyl] phosphorodithioate
	Fenamiphos Ethyl 3-methyl-4-(methylthio) phenyl (1-methylethyl) phosphoramidate
	Fenitrothion *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate
	Malathion Diethyl [(dimethoxyphosphinothioyl)thio] butanedioate
	Mevinphos Methyl-3-[(dimethoxyphosphinyl) oxy]-2-butenoate
	Parathion methyl *O,O*-dimethyl *O*-(4-nitrophenyl)phosphorothioate
	Parathion ethyl *O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate
	Trichlorfon Dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate

Fig. 1 Continued

Compared with the GC, LC has some limitations with respect both to its ability to separate individual members of OCPs and OPPs and to its selectivity and sensitivity. Mass spectrometry or diode array detection can be used to overcome these latter two disadvantages (13).

The main advantage of the HPLC over GC is that whereas GC can be used only for analytes capable of existing at some temperature in the gas phase, HPLC can be used for virtually

Table 1 Physicochemical Properties of Representative OCPs and OPPs

Pesticide	Molecular weight	M.P. (°C)	Solubility: At 25°C in water (mg/L)	Solubility: Benzene (g/L)	Solubility: Acetone (g/L)	Solubility: Hexane (g/L)	Solubility: Methanol (g/L)	log P_{ow}
Organochlorines								
Aldrin	364.93	104–105	<0.05	3500	1590	980	90	5.0–7.4
Captan	300.61	174–178	3.3	21	30	—	—	2.35–2.54
o,p'-DDE	318.00	88–90	0.1–0.25	Soluble	Soluble	Soluble	Soluble	5.63
p,p'-DDE	318.00	—	—	Soluble	Soluble	Soluble	Soluble	5.69–6.09
o,p'-DDD	320.00	—	—	Soluble	Soluble	Soluble	Soluble	—
p,p'-DDD	320.00	112	—	Soluble	Soluble	Soluble	—	—
o,p'-DDT	354.51	73–75	—	Soluble	Soluble	Soluble	—	5.75
p,p'-DDT	354.51	108.5–109	1.2 × 10–3	770	500	—	40	6.19–6.91
Dieldrin	380.93	176	0.19	400	220	—	10	4.32–5.40
Endrin	380.93	226–230	Insoluble	Moderate	Moderate	—	Slight	4.56–5.20
α-Endosulfan	406.96	109.2	0.32	—	—	24	—	—
β-Endosulfan	406.96	213.3	0.33	—	—	24	—	—
HCB	284.81	226	Insoluble	Soluble	—	—	—	5.47–6.18
α-HCH	290.8	—	—	—	—	—	—	3.78–3.81
β-HCH	290.8	—	—	—	—	—	—	3.78–3.84
γ-HCH	290.85	112.5–113.5	10	289	435	—	74	3.66–3.72
δ-HCH	290.8	—	—	—	—	—	—	4.14
Heptachlor	373.34	95–96	0.056	750	1060	—	—	5.27–6.06
Organophosphorous								
Azinphos-methyl	317.24	72.4–74	28–33	Soluble	Soluble	<1	Soluble	2.69
Chlorfenvinphos	359.6	−22.8–18.9	105	Soluble	Soluble	Soluble	Soluble	3.10–3.85
Chlorpyrifos	350.62	41.5–43.5	2	7900	6500	—	450	4.96–5.27
Dichlorvos	220.98	<−18	10,000	—	—	—	—	1.47
Dimethoate	339.30	51–52	23,300	Readily soluble	Readily soluble	Readily soluble	Readily soluble	0.50–0.79
Fenamiphos	303.4	49.3	700	Very soluble	Very soluble	Very soluble	Very soluble	3.23–3.28
Fenitrothion	277.24	0.3–3.4	21–30	Very soluble	Very soluble	Very soluble	Very soluble	3.38–3.47
Malathion	330.36	2.85–3.7	145	Very soluble	Very soluble	Very soluble	Very soluble	2.76–2.94
Mevinphos	224.15	21	Soluble	Soluble	Soluble	Soluble	Soluble	0.13
Parathion methyl	263.21	35–35	55–60	—	—	10–20	—	1.8–3.04
Parathion ethyl	291.27	6.1	12.4	Soluble	Soluble	50–100	Soluble	2.15–3.93
Trichlorfon	257.44	83–84	154,000	—	Soluble	0.1–1	Soluble	0.43

M.P.: melting point; log P_{ow}: partition octanol–water logarithm

any analyte, regardless of its volatility or stability. The basic purpose of pesticide residue analysis is therefore to provide reliable and cost-effective methods for the identification and determination of more than 400 trace analytes present in a variety of matrices. All pesticides can be separated and detected efficiently by HPLC, and the increasing interest in determining the largest possible number of pesticides in a single analysis favors the use of HPLC, and in particular of reversed-phase HPLC (RP-HPLC), instead of GC. This is especially true when one is dealing with the analysis of water samples. Another advantage of the HPLC over GC is the simplicity and robustness of the system (14,15).

Another technique is supercritical fluid chromatography (SFC), which is a chromatographic technique that in many ways is a hybrid of GC and HPLC. It is recognized as a valuable technique for the analysis of thermolabile compounds, which would not be amenable to analysis by GC or HPLC. Few applications have been reported for SFC in the field of OCP and OPP determination (16). The advantages reported for SFC are versatility in separation (by the addition of modifier or the choice of stationary phase) and detection (with LC or GC detectors). However, SFC is a little-used technique because it still presents a wide range of instrumental problems (14–16).

Although chromatographic techniques are sensitive and reliable, they have important drawbacks: they are labor intensive and expensive and require expert supervision. These methods have been used by regulatory agencies for random surveys and for risk-assessment programs. However, time and cost constraints make routine testing impractical for industrial applications such as raw material quality. If appropriate rapid-screening methods are made available, such as tests to detect the cumulative levels of OPPs or immunochemicals methods for OPPs and OCPs, it would give food producers and processors the ability to screen many samples (17,18).

Immunoassay (IA) techniques provide rapid, sensitive, and cost-effective analyses for a variety of pesticide residues. However, rapid progress in the analytical determination of pesticides by HPLC separation and selective detection clearly demonstrates that IA can not compete in terms of the information obtained about the sample composition. The main disadvantage of IA is that only one compound at time can be determined (24).

For sampling purposes, IA can be used to screen out negative samples for single-analyte determination, while the positives can be retained for further chromatographic analyses that will quantify and identify the incurred residues (16,24). Immunoassay techniques could serve as a tool for improving the screening of samples, but they must always be used together with chromatographic techniques.

C. Toxicity and Regulations

Pesticides and their metabolites are one of the most important classes of environmental pollutants, because, as their name indicates, they are chemicals designed to kill pests and, therefore, must possess inherent lethal activity. The high levels of pesticides applied to the environment, estimated at 3 million tons per year worldwide in the middle of 1980s, suggest the potential for significant exposures of human populations (25).

The entry of pesticide residues into the diet results primarily from the use of these chemicals in agricultural production, but many residues also result from the treatments of warehouses, food-processing and food-preparation facilities, and households. There are a variety of methods for estimating dietary exposures to pesticides, but all of them are hampered by the relative lack of accurate data on the pesticide residues in food. In terms of the number of people potentially exposed to pesticides, the dietary route is the most significant route of exposure. Even though the magnitude of this exposure is generally lower than that resulting from the occupational or house-

hold use of pesticides, the effects of pesticide residues in food and water probably cause the greatest public concern (26).

Many insecticides affect the nervous system of insects, since many have some activity against the mammalian nervous system. In humans the neurotoxic effects of insecticides are substantial. There are many energy changes that organisms exhibit in response to stress induced by toxics. Normally, chronic stress can induce compensatory physiological adjustments, such as changes in respiration and energy consumption, among others, that can be related to the growth of organisms based on the concept that energy in excess will be available for growth. It may impinge on commercial fishery and on the culture of several organisms. Among the main groups of insecticides are organochlorines and organophosphates (27).

The OPPs inhibit acetylcholinesterase (AChE) by phosphorylating the esteratic site of the enzyme. As a result of AChE inhibition, ACh accumulates and binds to muscarinic and nicotinic receptors throughout the nervous system. Transformation of OPPs in the organisms takes place by conversion of the phosphorothioate (P=S) group to oxon (P=O) analogs. These oxo compounds are of concern because they are the activated forms of the OPPs, with a considerably stronger inhibition of acetylcholinesterase activity (27).

Despite the fact that OCPs are used much less than formerly, their toxicological properties are still important, because they continue to be present in the environment and because the foodstuffs are imported into developed countries. Their residues continue to be present presumably as a result of either contamination from environmental sources or continued use in developing countries. Recent studies in several areas have found critical concentrations of restricted or forbidden OCPs in water (5). The OCPs are neurotoxics that stimulate sensory and motor nerve fiber and the motor cortex. These compounds alter the movement of sodium and potassium across neuronal membranes and affect membrane-related enzymatic reactions adversely. As a result of their lipid solubility, OCPs are stored in adipose tissue and accumulate in organisms that have relatively poor metabolic and excretory capabilities, leading to biomagnification along the food chain to top-level predators, such as fish-eating birds and many marine mammals (27). The toxicity values for several species are given in Table 2.

In developed countries, there are laws banning the marketing and use of some OCPs: aldrin, chlordane, dieldrin, DDT, endrin, HCH (mixture of isomers), heptachlor, HCB, and toxafene. However, they are still used in the developing countries.

To protect consumers' health, many countries have restricted the use of pesticides by establishing legal directives on maximum residue levels (MRLs) to control their levels in food (28). These MRLs sometimes cause conflicts, because residue levels acceptable in one country may be unacceptable in others. This problem has revealed the need to harmonize the different MRLs, which have been dealt with mainly by two international organizations, the European Union (EU) at the European Level, and the Codex Alimentarius Commision of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (28,29).

The EU has adopted several directives setting the MRLs for pesticides, including OCPs and OPPs, in fruit and vegetables (90/642/EEC), cereals (86/362/EEC), and foods of animal origin (86/363/EEC). One of the main concerns in pesticide residue analysis is to reach detection limits as low as 0.1 $\mu g/L$, which is the MLR established by the EU for drinking water (80/779/EEC) (28,29).

In the United States, food safety is an important responsibility not only of the Food and Drug Administration (FDA), but also of the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and other agencies at the federal, state, and local levels. The EPA registers or approves the use of pesticides and establishes a tolerance level if the use of a pesticide may lead to residues in food (30).

Table 2 Toxicological Values Reported for OPPs and OCPs

Pesticides	LD_{50} oral, rat (mg/kg)	LD_{50} dermal, rabbit (mg/kg)	Chronic	Other chronic effects	Bird, LD_{50} (mg/kg)	Fish, LC_{50}, 96 h mg/L	Bee, μg/bee	Use
Organochlorines								
Aldrin	38–67	15–25	In rat, diet LOAEL: 0.025-mg/kg/day.	In United States considered a potential carcinogen, because in mice has produced significative tumoral effects,	6.59–52	0.0026–0.053	Toxic	Insecticide
Captan	9,000	4,500 > 22,600	Rat, 2-year diet, NOAEL: 1000-mg/kg diet	NOAEL carcinogen, NOAEL teratogen	—	0.047–0.111	215	Fungicide
DDT	113	2,800	Rat, 160-day diet, NOAEL: 1 mg/kg	Accumulated in body fat and adipose tissues and excreted in milk	—	Toxic for fish and aquatic organisms	5.946	Insecticide
Dieldrin	38	—	Rat and dogs, diet, NOAEL: 0.1-mg/kg diet	Good skin absorbance; accumulated in adipose tissues and in lipid-rich organs	—	0.008–0.037	0.139	Insecticide
Endrin	7	—	Rat and mice, 2-year diet, NOAEL: 1-mg/kg diet	Rapidly absorbed by skin and accumulated in adipose tissues	Very toxic	Very toxic	2.018	Insecticide
α- and β-Endosulfan	22.7–160	359	Rat and dog, 2-year diet, 30-mg/kg/diet Dog, 1 year diet, NOAEL: 3-mg/kg diet	Without substantial accumulation in tissues, and not much elimination in milk; stimulation of the nervous central system	205–1000	0.002	4.4–7.8	Insecticide
HCB	10,000–40,000		—	Probable carcinogen for humans	—	0.02–0.5	—	Fungicide
γ-HCH	88–125	300	Rat, 2-year diet, NOAEL: 25-mg/kg diet Dog, 2-year diet; NOAEL: 50 mg/kg diet	Carcinogenesis in mice 2 year diet, at 400 ppm appears liver tumors	120–130	—	0.562	Insecticide
Heptachlor	100	—	Rat, diet, NOAEL < 5-mg/kg diet	Carcinogenesis promoter	17.5	7.4–13.1	0.525	Insecticide

Organophosphorus								
Azinphos-methyl	16	—	Dog, 1-year diet, NOAEL: 5-mg/kg diet Rat, 2-year diet, NOAEL: 5 ppm	Carcinogenesis In mice, NOAEL: 0.86 mg/kg/day Teratogenesis In mice, rat, and rabbit, NOAEL: 1 mg/kg/day	32.2–500	0.001–>1	0.1	Insecticide, acaricide
Chlorfenvinphos	10–39	—	Rat, 2-year diet, NOAEL: 1-mg/kg diet Dog, 2-year diet, NOAEL: 1-mg/kg diet	—	13.8–107	0.32	4.1	Insecticide
Chlorpyriphos	135–163	>2000	Rat, 2-year diet, NOAEL: 0.1-mg/kg/day Dog, 2-year diet, NOAEL: 0.03 mg/kg/day	Without effects, except cholinesterase action	17.7–102	0.033–125	0.114	Insecticide
Dichlorvos	56–80	—	Rat, 2-year diet, NOAEL: 10-mg/kg diet Dog, diet, NOAEL: 3.2 ppm (0.08-mg/kg/day)	Rat, 2-year diet, doses of 100 mg/kg diet without effects, except the acetil-cholinesterase inhibition. Classified in US as probable carcinogen.	14.8–26.8	0.1–0.23	0.495	Insecticide, acaricide
Dimethoate	290–425	—	Rat, 2-year diet, NOAEL: 1-mg/kg diet (0.05 mg/kg/day)	For EPA, it is considered carcinogenic, mutagenic, fetotoxic and with reproduction effects. However, potential doses are higher than those that the people can be exposed to.	15–108	0.2–60	0.188–0.9	Insecticide, acaricide
Fenamiphos	6–19.4	178–225	Rat, 2-year diet, doses of 3 mg/kg diet: without effects. Rat, 2-year diet, NOAEL: 1 mg/kg diet	—	1.6–12	0.0096–10.5	1.87	Nematicide, insecticide

(*continued*)

Table 2 Continued

Pesticides	LD_{50} oral, rats (mg/kg)	LD_{50} dermal, rabbit (mg/kg)	Chronic	Other chronic effects	Bird, LD_{50} (mg/kg)	Fish, LC_{50}, 96 h mg/L	Bee, μg/bee	Use
Fenitrothion	250–740	—	Rat, diet: 4-month, 80-mg/kg diet: without effect. 21-month, NOAEL: 5-mg/kg diet NOAEL: 10 ppm (EPA) Dog, diet, NOAEL: 5 ppm (EPA)	—	23.6–1190	1.7–3.8	0.288	Insecticide
Malathion	885–2800	4100 >2000 EPA	Rat, diet 21-month, doses of 100-mg/kg: weight gain normal	—	—	103–285	0.709	Insecticide, acaricide
Mevinphos	3.7–12	16–33.8	Rat, 2-year diet, doses of 4 mg/kg: without effect Dog, w-year diet; doses of 2 mg/kg: without effects	—	1.34–7.52	—	0.36	Insecticide
Parathion methyl	6–24	300–420	Rat, 2-year diet, NOAEL: 2-mg/kg diet	Rat: embriotoxicity and feto-toxicity at 1 mg/kg. Some test have demonstrated certain evidence of geno-toxicity.	6.6–7.6	2.7–6.9	0.291	Insecticide, acaricide
Parathion ethyl	2–32	—	Rat, 2-year diet, NOAEL: 2-mg/kg diet; doses of 10–25 mg/kg. At doses of 10 and 25 mg/kg, no effect was observed.	Higher doses show damage in retin and ciatic nerve. Classified in US as a potential carcinogen. Positive damage in DNA.	1.3–12.4	0.0178–2.7	0.07–7	Insecticide, acaricide
Trichlorfon	250–630	—	Rat, 2-year diet, NOAEL: 500-mg/kg diet. Mice, 2-year diet, NOAEL: 300-mg/kg/diet Dog, 4 year diet, NOAEL: 50-mg/kg diet	At 1000-mg/kg diet, inhibi-tion of 20% of the cholinesterase in rat.	110–720	0.2–10	59.83	Insecticide

LC_{50}, 96 h: lethal concentration fifty, after 96-hour exposure; LD_{50}: lethal dose fifty; LOAEL: lowest observable adverse effect level; NOAEL: no observable adverse effect level; EPA: Environmental Protection Agency; US: United States

The FDA is responsible for enforcing the EPA-established pesticide tolerances for foods shipped interstate, with the exception of meat and poultry, which are the responsibility of the USDA (30).

In Canada, the Health Protection Branch (HPB), Health and Welfare, fulfills the responsibilities by establishing MRLs for pesticides in foods and by establishing programs to ensure compliance with these MRLs (30).

In Japan there is no specific regulation for biocides, and new substances are regulated under the Industrial Chemical Control Law (ICCL). The scheme is based on an inventory for existing substances and notification for new substances. The fact that three different agencies are involved slows down the approval system considerably (28).

The toxicological risks from residues of synthetic pesticides in food are minimal because of careful food safety legislation and regulation.

II. SAMPLE PREPARATION

Sample preparation is usually necessary prior to the actual determination step, because of the complexity of the samples and the low determination levels that have to be achieved.

Water samples are stored at 4°C and filtered through 0.45-μm acetylcellulose filters prior to analysis. Cereal, fruit, and vegetable samples are chopped and homogenized carefully to obtain a portion for analysis that is representative of the whole. Lean and fatty animal tissues are analyzed for moisture and fat content prior to OPP and OCP determination.

A. Extraction

1. Water

One advantage of HPLC is that the analysis of unstable pesticides may be performed directly in aqueous medium without the extraction step or following extraction and concentration. Although the direct approach is quite useful for formulations or for kinetic studies to monitor the parent compounds in the presence of degradation products, its usefulness is limited in the case of environmental samples, where the concentration is usually in the parts-per-billion range (31).

High-performance LC determination is also compatible with the extraction of environmental water with an organic solvent such as methylene chloride or ethyl acetate (31,32) and solid-phase extraction (SPE) (33,34). Solid-phase extraction and liquid–liquid extraction (LLE) have been compared with respect to their ability to preconcentrate pesticides prior to HPLC analysis. The reproducibility of the method is better when C_{18} cartridges are used than with conventional LLE, but LLE sometimes gives better recoveries, for example, for dimethoate, chlorpyriphos ethyl, and carbofenothion (35).

Of the sorbent materials available for SPE of OCPs and OPPs from water, octadecyl-bonded silica (C_{18}) has become by far the most popular (33,36). The OPPs in surface water samples can be measured using SPE with C_{18}, and the recoveries are over 80% at sub-ppb levels (33,34,37). However, there is an increasing awareness, supported by experimental data, that C_{18} cartridges are inadequate to solve the problems of isolating polar contaminants from large water volumes. This failure has led investigators to evaluate alternative sorbent material, such as styrene divinylbenzene copolymer (PRP-1) and highly crosslinked styrene-divinylbenzene copolymers (PS-DVB). Another emerging material is graphitized carbon black (GCB).

Di Corcia and Marchetti (38) evaluate the performance of Carbopack cartridges in rapid and quantitative extraction for large water volumes containing pesticides with highly varied

chemical composition. For this purpose the Carbopack cartridge was compared with the 500-mg C_{18} bonded-silica cartridge. With the carbopack cartridge, the mean measurement accuracy of the 35 pesticides considered (six of them organophosphorus) was 95%. With the C_{18} cartridge, the mean measurement accuracy of analytes was 76%. The same method was modified to make it suitable for monitoring 89 pesticides (19 OPPs and 2 OCPs) in ground- and river water (39). For extracting very polar compounds from water, two new types of GCB, namely, Carbograph 4 and 5, are more effective than the older GCB, Carbograph 1 (40).

The use of a solid-phase extraction (SPE) cartridge is an alternative to storage of the original matrix. Handling, transport, and storage of the samples are also improved because of the small volume of such cartridges as compared with the more common, large volumes collected in environmental programs. In this way, the stability of four pesticides (desethylatrazine, fenamiphos, fenitrothion, and fonofos) was examined under different storage conditions after concentration in disposable SPE cartridges containing new polymer sorbent materials (Hysphere-1, IST EnvironLut, and LiChrolut). Complete recovery for all the compounds was observed in precolumns kept at −20°C for 1 month when preconcentrating 26 ml of groundwater sample spiked at 10 μg/L (41).

An automated system for offline SPE is ASPEC XL. The main advantage of using offline SPE is that the analytes can be stored in disposable preconcentration cartridges. Polymeric sorbent Lichrolut EN or ENV was used for groundwater preconcentration (42,43).

Another alternative to the use of cartridges is the Empore C_{18} extraction disk (44). These flat discs have large cross-sectional areas that provide advantages for online preconcentration and cleanup methods with respect to sorption, capacity, backpressure, and stability after repeated use.

Solid-phase extraction could be coupled online with HPLC. This is becoming increasingly important, because it makes it possible to handle all the situations in which large series of samples have to be analyzed routinely, and therefore rapid, (semi-) automatic, and unattended analysis is an aspect of major concern. Moreover, sensitive trace level determination requires the analysis of total samples or sample extracts rather than aliquots, under conditions in which analyte losses, due to evaporation or irreversible sorption to the vessels walls, and contamination, caused by the solvent or reagents used, laboratory air, and/or sample manipulation in general, must be rigorously minimized (45,46).

A method using online trace-enrichment technology on C_{18} for the quantitative analysis of OCPs in water samples by HPLC has been reported to be a good alternative to GC (47). The same system was adapted to the determination of OPPs. With 200 ml of tap water, the recoveries were ca. 90%. The detection limits ranged from 0.03 to 0.2 μg/L (48).

For online coupling of SPE with HPLC it is possible to use more automated equipment, such as the Prospekt (Spark Holland, Netherlands) or the OSP-2 (Merck, Darmstadt, Germany). Both systems have been validated for determining 11 OPPs at a level of 0.1 μg/L or lower in water, with good results (49–52).

The development of online sample procedures is critically important, for with them sensitivity increases, losses are prevented, and automation is made easy, as is demonstrated by the development of the system for automated measurement of organic micropollutants in surface water (SAMOS) LC and SAMOS GC (45,46,53,54). The SAMOS LC is based on SPE sample preparation, column LC, and diode array detection (53). Moreover, all types of MS detectors for HPLC can be coupled to the SAMOS.

Some attention has been devoted to the proper selection of the sorbent used for SPE. However, researchers soon realized that many early-warning and monitoring programs aim primarily at the isolation of the widest possible range of microcontaminants. Nonselective and preferably highly hydrophobic copolymers such as PLRP-S and PLRP-L, virtually became the best choice, when rapid screening of large series of sample is the main goal. One promising approach is the

so-called single-short-column technique, by which one high-pressure packed 20-mm × 4-mm-ID column is used for both trace enrichment and separation.

In summary, SPE methods, including extraction using both online and offline cartridges or more automated equipment such as the ASPEK, PROSPECT, or the OSP-2, are, without doubt, the preferred technique for the isolation and concentration of OPPs and OCPs from water samples prior to HPLC determination.

2. *Foods*

Vegetal materials, such as wheat (55), corn (56), fruit, and vegetables (33,57–59), and animal materials, such as beef tissues (60,61), or blubber samples from marine mammals (62) can be extracted by organic solvents using a Waring blender or Soxhlet extraction. The organic solvents that have been used for this purpose are acetonitrile, ethyl acetate–methanol, ethyl acetate, hexane, acetone, and benzene.

The determination of OCP residues in milk has always presented problems, because the most common approach has required the total extraction of fat, together with lipophilic compounds, including organochlorine pesticide residues. Only one procedure for the extraction and separation of OCPs directly into an HPLC system has been described (11). The direct procedure injects the samples into an internal-surface reversed-phase C_{18} column connected online with the analytical column.

B. Cleanup

1. *Cleanup Prior to HPLC Determination*

A cleanup procedure for sample extraction is often required, depending on the type of compound and the kind of sample to be analyzed and on the selectivity of the analytical equipment used in the determination. The use of a selective detector, such as a diode array, or a mass spectrometer can reduce the need for a cleanup procedure in some cases.

The use of commercially available SPE cartridges is an attractive technique, because the volume of elution solvent is reduced and the cartridges can be used in different cleanup methods. C_{18} retains OPPs because of its apolar characteristics, and it allows polar analytes to pass. This method has been used to clean up extracts from beef tissues (60,61,63). The opposite is true of polar stationary phases such as Florisil, which has been used to clean up extracts from marine mammal tissues (62).

2. *Cleanup and Fractionation Using HPLC*

Determination of pesticide residues in fatty samples by GC requires the elimination of interfering compounds, mainly lipids, from the extracts before sample injection into the chromatographic system. Even small amounts of lipids can cause damage to the column and contaminate the detector. The effectiveness of HPLC techniques for the separation of different molecules makes this technique adequate for the cleanup of this type of samples.

High-performance LC coupled to capillary GC is a technique in which fractions from one or more LC columns are transferred into the GC column for further separation. This coupled technique is used more to separate a particular compound and/or class of compounds from an unknown matrix. Another field in HPLC serves for the preseparation of closely related classes or subclasses of compounds (64).

In most cases, HPLC improves the laborious procedures usually applied to the analysis of OPPs and OCPs in fatty samples. Moreover, fully automated cleanup procedures would be very

helpful in minimizing the analysis time and avoiding human errors associated with sample handling. Therefore, HPLC is very suitable for the development of automated techniques.

The presently used HPLC cleanup methods preseparate the food extracts, either by polarity (usually using NP-HPLC, but also RP-HPLC) (65–68), by molecular size [gel permeation chromatography (GPC)] (65,69–76), or by a combination of both (77). Two types of preseparations could be termed "horizontal" and "vertical," as shown in Fig. 2.

Table 3 shows the cleanup and fractionation techniques.

a. Normal-Phase HPLC and Reversed-Phase HPLC

Sample cleanup with an NP-HPLC column has been shown to be an efficient, robust way to separate triglycerides from organochlorine compounds for analysis in a wide range of fatty samples, such as milk, pork fat, animal feed, and cod liver (67). Complete fat–OCP separation is obtained in a small fraction volume. The method showed average recoveries of 80–110% in the concentration range of 1–510 μg/kg, with relative standard deviations of less than 10%. The limits of detection ranged from 0.5 to 50 μg/kg. The process can be monitored online with a UV detector.

The major difficulty in analyzing OPPs in fatty samples has to do with the wide polarity range for both pesticides and lipids present in the matrix. Normal-phase HPLC is an adequate technique for cleaning up this type of sample using silica gel and modifiers with different polarity. In fact, an automated sample-cleanup system based on normal-phase HPLC using a silica gel column has been reported efficiently to clean up and fractionate chlorpyriphos, chlorpyriphos methyl, and their metabolites in molluscs. The system presents several advantages: The procedure is fully automated, from the injection of the extract to the collection of fractions, which are injected directly into the GC system, and a diode array detector (DAD) allows online monitoring of the elution of lipids (68).

However, NP-HPLC has been criticized because of the apparent similarity in polarity between many OPPs, and the lipids of both animal and plant origin precluded the development of

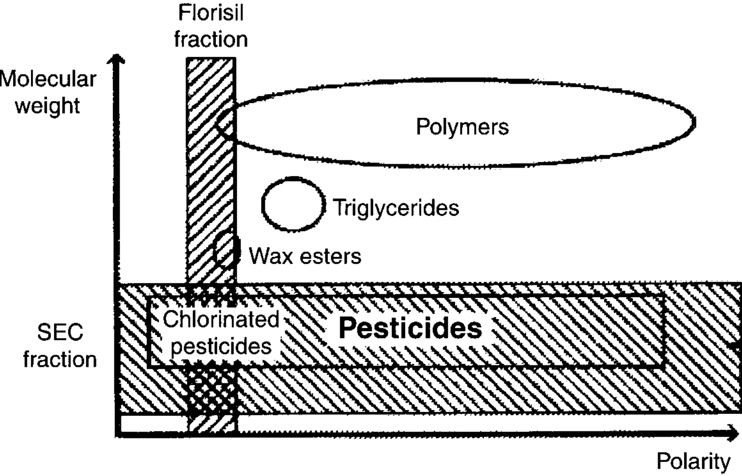

Fig. 2 Two methods of sample preparation: selectivity by polarity or by molecular size. Fractionation by polarity, e.g., using Florisil (vertical fraction), selects a limited range of the pesticides but does not remove high-molecular-weight materials of similar polarity. GPC (horizontal fraction) removes primarily material of high molecular weight, leaving all the pesticides (and other compounds of similar molecular weight) in the fraction. (From Ref. 19.)

Table 3 HPLC Cleanup of Food Samples

Food	Technique	Column	Mobile phase	Pesticide	Added (ppm)	Recovery (%)	Determin.	Ref.
Soybean oil, butterfat, cottonseed oil	RP-HPLC, offline	9.4-mm-ID × 25-cm Zorbax ODS, 6 μm	Cl_2CH_2 with back-flush to remove lipids. Acetonitrile to elute the analytes	Acephate	0.1–0.2	81.1–104.6	GC-ECD	78
				Diazinon	0.86	84.5–90.0		
				Malathion	0.52	91.8–108.9		
				Methamidophos	0.1–0.2	78.0–91.0		
				Monocrotophos	0.12–0.23	97.0–107.8		
				Omethoate	0.16–0.32	79.5–96.6		
				Parathion methyl	0.40	83.0–104.6		
				Aldrin	0.19–0.31	79.7–90.6		
				Dieldrin	0.21–0.32	93.1–105.6		
Baby foods	RP-HPLC, offline	4.6-mm-ID × 25-cm Lichrosorb RP-18, 5 μm	Gradient: methanol/water	44 OPPs and metabolites	—	—	GC-MS	66
Milk, pork fat, cod liver oil	NP-HPLC, offline	4.6-mm-ID × 6-cm Lichrosorb, 10 μm	*n*-Hexane to remove pesticide Cl_2CH_2 with back-flush to remove lipids	HCB	0.014–0.26	83–101	GC-ECD	67
				α,β,γ-HCH	0.024–0.51	90–107		
				β-Heptachlorepoxide	0.08–0.19	94–98		
				Dieldrin	0.051–0.20	92–107		
				p,p'-DDE	0.093–0.26	92–101		
				Endrin	0.31	80		
				p,p'-TDE	0.079–0.45	93–96		
				p,p'-DDT	0.19–0.39	94–98		
				o,p'-DDT	0.086–0.18	90–102		
				α,γ-Chlordane	0.044	89–95		
				Oxychlordane	0.068	84		
				Transnonachlor	0.056	88		
				o,p'-DDE	0.069	92		
				o,p'-TDE	0.175	93		
Mussels	NP-HPLC, online	3.9-mm-ID × 15-cm Silica Nova-pack, 4 μm	*n*-Hexane to elute lipids; hexane–ethyl acetate to remove pesticide	Chlorpyriphos	0.20–0.100	98–104	GC-NPD	68
				Chlorpyriphos methyl	0.20–0.100	96–101	GC-MS	
				Chlorpyriphos methyl oxon	0.40–0.200	91–95		
				3,5,6-Trichloro-2-pyridinol	0.40–0.200	88–90		

(continued)

Table 3 Continued

Food	Technique	Column	Mobile phase	Pesticide	Added (ppm)	Recovery (%)	Determin.	Ref.
Adipose tissue	NP-HPLC, online	1-mm-ID × 5-cm Hypersil Silica, 3 μm	*n*-Hexane	*p,p'*-DDE	0.22–22	91%	GC-ECD	79
Bovine liver Alfalfa hay Tomato	GPC	25-mm-ID × 18-cm 38-g SX-3, BioBeads, 200–400 mesh	30% ethyl acetate in hexane	Aldrin	0.1–0.125	88–100	GC-ECD GC-FPD	65
				α-BHC	0.07–0.09	86–106		
				γ-Chlordane	0.1–0.125	96–103		
				p,p'-DDD	0.1–0.125	96–100		
				p,p'-DDE	0.1–0.125	94–104		
				p,p'-DDT	0.1–0.125	98–101		
				Dicofol	0.5–0.6	93–101		
				Dieldrin	0.2–0.25	90–113		
				Endosulfan I	0.1–0.125	93–102		
				Endosulfan II	0.1–0.125	95–100		
				Endrin	0.1–0.125	81–102		
				HCB	0.5–0.6	88–112		
				Heptachlor	0.1–0.125	87–90		
				Heptachlorepoxide	0.1–0.125	87–93		
				Lindane	0.05–0.6	88–109		
				p,p'-Metoxichlor	0.2–0.25	94–101		
				Mirex	0.2–0.25	82–105		
				Acephate	0.125–0.5	84–103		
				Azinphos methyl	0.125–0.5	108–110		
				Carbophenothion	0.125–0.5	98–99		
				Chlorfenvinphos	0.125–0.5	99–103		
				Chlorpyriphos	0.125–0.5	94–97		
				Coumaphos	0.125–0.5	98–103		
				Crufomate	0.125–0.5	95–96		
				Crotoxyphos	0.125–0.5	98–106		
				DDVP	0.125–0.5	83–100		
				DEF	0.125–0.5	95–97		
				Demeton	0.125–0.5	82–94		

Diazinon	0.125–0.5	91–96
Dicrotophos	0.125–0.5	96–98
Dimethoate	0.125–0.5	81–100
Dioxathion	0.125–0.5	98–106
Disulfoton	0.125–0.5	87–92
EPN	0.125–0.5	97–104
Ethion	0.125–0.5	96–104
Ethoprop	0.125–0.5	82–93
Fenamiphos	0.125–0.5	78–103
Fensulphothion	0.125–0.5	97–100
Fenthion	0.125–0.5	85–103
Fonophos	0.125–0.5	89–96
Isofenphos	0.125–0.5	96–99
Malation	0.125–0.5	97–99
Merphos	0.125–0.5	99–105
Methamidophos	0.125–0.5	77–113
Methidathion	0.125–0.5	97–103
Methyl parathion	0.125–0.5	96–98
Mevinphos E	0.125–0.5	82–96
Mevinphos Z	0.125–0.5	89–98
Monocrotophos	0.125–0.5	93–100
Naled	0.125–0.5	84–109
Parathion	0.125–0.5	94–97
Phorate	0.125–0.5	84–93
Phosalone	0.125–0.5	99–105
Phosmet	0.125–0.5	104
Phosphamidon	0.125–0.5	88–107
Profenophos	0.125–0.5	96–100
Propetamphos	0.125–0.5	85–95
Ronnel	0.125–0.5	92–96
Terbuphos	0.125–0.5	86–94
Tetrachlorvinfos	0.125–0.5	98–99
Triazopos	0.125–0.5	98–107

(continued)

Table 3 Continued

Food	Technique	Column	Mobile phase	Pesticide	Added (ppm)	Recovery (%)	Determin.	Ref.
Animal tissues, liver, brain	GPC	15-mm-ID × 50-cm 10-g SX-3, Bio Beads, 200–400 mesh	Hexane–chloroform–acetone (75 + 20 + 5)	Fenitrothion	0.25–2.5	97.1–101.3	UV-DAD GC-ECD	69
				Azinphos ethyl	0.25–2.5	96.0–104.6		
				Chlorfenson	0.25–2.5	96.0–108.5		
				Chlorobencilate	0.25–2.5	97.3–113.9		
				Tetradifon	0.25–2.5	93.3–100.0		
				Dicofol	0.25–2.5	95.6–107.2		
Olive oil	GPC, online GC	7.5-mm-ID × 30-cm PLGel, 5 μm particle size and 50-Å pores	Ethyl acetate–cyclohexane 6%	Sulphotep	0.02–0.06	96	GC-NPD	70
				Diazinon		100		
				Fenamiphos		—		
				Disulfoton		100		
				Fenchlorfos		100		
				Paraoxon-ethyl		103		
				Fenitrothion		97		
				Chlorpyriphos		95		
				Fenthion		100		
				Parathion		97		
				Bromophos methyl		96		
				Isofenphos		93		
				Bromophos ethyl		83		
				Ethion		96		
				Carbophenothion		162		
				Azinphos methyl		92		
				Azinphos ethyl		95		
Fats	GPC and NP-HPLC	25-mm-ID × 34-cm 10-g SX-3, Bio Beads, 200–400 mesh	Ethyl acetate/cyclohexane (50:50)	HCB	0.02	77.3	GC-ECD	77
				α-HCH	0.02	80.9		
				β-HCH	0.02	82.4		
				γ-HCH	0.02	89.1		
		4-mm-ID × 25-cm silica Hypersil, 5 μm	10% Toluene and 2% acetone in hexane	δ-HCH	0.02	85.7		
				ϵ-HCH	0.025	83.5		
				o,p'-DDE	0.03	84.9		
				p,p'-DDE	0.03	87.1		

				p,p'-DDD	0.03	86.0		
				o,p'-DDT	0.03	87.8		
				p,p'-DDT	0.03	89.0		
				Dieldrin	0.03	86.1		
				Endrin	0.03	89.7		
				Cis-heptachlorepoxide	0.03	86.2		
				Oxichlordane	0.03	85.6		
				Transnonachlor	0.025	85.6		
Human milk	HPGPC	7.5-mm-ID × 30-cm PLRP-S	20% propan-2-ol in heptano	HCB	0.005	83.2–99.8	GC-MS	71, 72
				α-HCH		87.2–106.9		
				β-HCH		88.4–90.9		
				γ-HCH		90.9–106.5		
				δ-HCH		63.2–67.3		
				o,p'-DDE		86.7–91.5		
				p,p'-DDE		92.7–100.1		
				o,p'-DDT		91.7–94.6		
				p,p'-DDT		97.8–98.9		
				Dieldrin		87.8–94.1		
				Endrin		96.0–98.9		
Total Diet Study	GPC	25-mm-ID × 30-cm 10-g SX-3, Bio Beads, 200–400 mesh	Methylene chloride–hexane (1:1)	Malaoxon	0.004	81–101	GC-FPD	73
				Fentoate	0.004	86–100	GC-NPD	
				Diazinon	0.006	85–110	GC-ECD	
				Dimethoate	0.006	83–113		
				Fenitrotion	0.006	86–97		
				Metamidophos	0.008	80–104		
				Monocrotophos	0.008	85–114		
				Omethoate	0.008	81–107		
				Chlorpyriphos	0.010	84–110		
				Acefate	0.010	63–118		
				Malathion	0.010	89–112		
				Fonofos	0.010	83–100		
				Ethion	0.012	86–114		
				Methidathion	0.012	83–114		

(continued)

Table 3 Continued

Food	Technique	Column	Mobile phase	Pesticide	Added (ppm)	Recovery (%)	Determin.	Ref.
				Parathion	0.016	85–115		
				Ronnel	0.020	80–102		
				Phosalone	0.040	95–102		
Olive oil, mixed seed oil, artichoke in oil, mixed vegetables in oil, meat sauce, tomato sauce, salami	GPC	25-mm-ID × 30-cm 50-g SX-3, Bio Beads, 200–400 mesh	Methylene chloride-cyclohexane (15 + 85)	Dichlorvos	0.025–0.250	52.8–57.4	GC-FPD GC-MS	74
				Mevinphos		89.2–95.0		
				Thionazim		86.0–89.8		
				Ethoprophos		90.3–92.4		
				Sulfotepp		84.9–91.2		
				Phorate		78.0–82.8		
				Dimethoate		101.6–107.2		
				Fonophos		86.1–90.0		
				Diazinon		84.1–89.6		
				Disulfoton		84.9–91.0		
				Formothion		83.9–95.5		
				Methyl parathion		94.7–98.2		
				Chlorpyriphos methyl		91.1–94.2		
				Malaoxon		127.5–140.3		
				Paraoxon		119.1–122.1		
				Fenchlorfos		90.7–95.6		
				Fenitrothion		98.6–100.0		
				Pyrimiphos methyl		92.4–96.9		
				Malathion		95.5–98.9		
				Fenthion		92.7–96.7		
				Chlorpyriphos ethyl		93.1–95.6		
				Parathion		96.1–98.8		
				Bromophos		90.2–95.5		
				Chlorfenvinphos		92.6–98.3		
				Isofenphos		88.8–92.5		
				Mecarbam		92.9–97.0		
				Quinalphos		95.6–97.5		
				Methidathion		98.0–100.5		

				Bromophos ethyl		88.3–93.7		
				Tetrachlorvinphos		99.5–102.2		
				Ethion		91.6–95.4		
				Carbofenothion		87.3–90.8		
				EPN		96.1–98.2		
				Phosalone		90.3–96.8		
				Azinphos methyl		93.7–100.7		
				Pyrazophos		94.5–98.2		
				Azinphos ethyl		94.2–98.6		
				Dialifos		95.1–96.1		
				Coumaphos		92.1–98.9		
Olive oil, fat extracts of chicken and fish, lettuce	GPC, online	3-mm-ID × 25-cm PSS SDV, 5 μm	Cyclohexane–ethyl acetate (1:1)	Bromopropylate	0.01–0.05	—	GC-ECD	75
				Dieldrin				
				HCB				
				HCH				
				Heptachloroepoxide				
				DDE				
Fruits	GPC, online GC	3-mm-ID × 25-cm PSS SDV, 5 μm	Cl_2CH_2–MTBE	Phorate	10 ng/g	82.3–88.5	GC-FPD	80
				Fonofos		80.3–89.4		
				Diazinon		94.2–96.3		
				Parathion methyl		88.8–93.5		
				Fenchlorphos		92.4–94.8		
				Fenitrothion		90.7–98.6		
				Pirimiphos methyl		91.2–94.5		
				Malathion		88.7–97.1		
				Parathion		89.5–97.3		
				Bromophos methyl		90.5–97.7		
				Quinalphos		88.8–98.6		
				Methidation		93.7–97.4		
				Bromophos ethyl		92.3–98.8		
				Tetrachlorvinphos		86.8–93.3		
				Ditalmifos		94.4–97.5		
				Isoxathion		97.6–98.2		

(continued)

Table 3 Continued

Food	Technique	Column	Mobile phase	Pesticide	Added (ppm)	Recovery (%)	Determin.	Ref.
				Ethion		89.3–98.4		
				Carbophenthion		83.5–96.4		
				Pyridaphenthion		95.4–99.3		
				Azinphos methyl		94.7–99.5		
				Azinphos ethyl		88.9–96.6		
				Pirazophos		90.3–97.2		
Bovine liver, bovine rumen	GPC	25-mm-ID × 30-cm 60-g SX-3, Bio Beads, 200–400 mesh	Hexane-ethyl acetate (60 + 40)	Acephate	0.05–1.0	72–95	GC-FPD GC-NPD	76
				Azinphos-methyl		86–113		
				Carbophenothion		84–102		
				Chlorfenvinphos		88–108		
				Chlorpyriphos		97–105		
				Coumaphos		110–114		
				Crotoxyphos		102–110		
				Crufomate		100–105		
				DEF		100–103		
				Demeton		72–86		
				Diazinon		88–95		
				Dichlorvos		74–102		
				Dicrotophos		90–106		
				Dimethoate		88–101		
				Dioxathion		87–102		
				Disulfoton		90–109		
				EPN		84–107		
				Ethion		93–115		
				Ethoprop		84–95		
				Fenamiphos		87–111		
				Fensulfothion		100–107		
				Fenthion		81–103		

Fonophos	85–95
Isofenphos	92–95
Malathion	100–92
Merphos	107–115
Methamidophos	78–91
Methidathion	100–107
Methyl Parathion	91–100
Mevinphos	83–94
Monocrotophos	86–101
Naled	87–97
Parathion	97–103
Phorate	78–89
Phosalone	92–106
Phosmet	101–107
Phosphamidon	91–114
Profenophos	100–108
Propetamphos	89–96
Ronnel	93–103
Terbufos	84–90
Tetrachlorvinphos	101–109
Triazophos	100–111

MTBE: methyl *ter*-butyl ether.

practical separation schemes for these compounds using this approach (78). The potential of RP-HPLC was therefore tested. It was postulated that the lipids, consisting primarily of long-chain fatty acids and esters, would be highly retained in this system, while pesticides, which have a relatively low alkyl content, would be expected to elute rapidly and thus to be separated from the bulk of the sample.

The RP-HPLC system was tested on the fractionation of OPPs and OCPs, from edible fats and oils of both animal and vegetal origin. When acetonitrile was used, the most polar OPPs eluted rapidly but tailed on this column, whereas the relatively nonpolar OCPs were retained the longest. Additional cleanup on miniature Florisil columns is required for the "dirtier" samples. A UV detector was used to determine the elution patterns of standards (78). The same system was used for the fractionation of OPP residues in processed bay foods prior to the final determination by GC without further cleanup but using a MS detector (66).

b. Gel Permeation Chromatography

Gel permeation chromatography (GPC), also called size-exclusion chromatography, is the most widely used cleanup technique for pesticides in fatty foods. It is the method of choice for rapid cleanup of biological extracts, especially from high-fat samples, to determine pesticide residues, since separation occurs on the basis of molecular size (7).

Gel permeation chromatography is an excellent technique for the practically quantitative separation of compounds up to a molecular mass of 400 u (e.g., OCPs) from macromolecular compounds, such as lipids (600–1500 u). The GPC column, which consists of porous polymer beads, retains molecules that are small enough to enter the pores. Lipid molecules that are too large to enter these pores are not retained and are therefore eluted from the column first. Separation is generally performed using divinylbenzene-linked polystyrene gels, mostly Bio-Beads SX-3. It is suitable for OCPs and OPPs and nearly all other types of pesticides and does not involve any losses by adsorption.

There are two main reasons why it is important to monitor the contamination of human milk. Firstly, the ability and high lipophilicity of OCPs mean that relatively high levels can accumulate in human lipids. Secondly, infants receiving milk have poorly developed defenses and may be more susceptible to toxic effects. The analysis of human milk for organochlorine pesticide residues holds considerable problems. Most of the individual residues are present at low ng/ml levels and have to be separated from about a 107-fold excess of lipid, in addition to the other constituents of milk, prior to quantification. The GPC using a semipreparative Polymeric PLRP-S provides a low-volume lipid fraction, for a gravimetric determination of lipid content, and an organochlorine residue fraction that, after reduction in volume, is suitable for GC determination. The elution is done with 20% propanol in heptane and is monitored using a refractive index detector (71,72).

The GPC has been effectively applied for quantitative determination of OPPs in the Food and Drug Administration's Total Diet Study. Fats in spiked samples were removed with methylene chloride–hexane (1:1). This procedure was used to analyze fatty foods for OPPs. This procedure also worked well in removing a large portion of the coextractive materials from nonfatty foods (73).

Gel permeation chromatography has also been used to clean up extracts from olive oil, mixed seed oil, mixed vegetables in oil, artichokes in oil, tomato sauce, meat sauce, and salami. The recovery of 39 OPP compounds was examined (74). With the same GPC column, a multiresidue determination of 43 OPPs (76) and 17 OCPs (65) was described in plant and animal tissues.

Complex samples, therefore, very often require a two-step cleanup that combines different chromatographic techniques in series. In most cases, in the second step the extracts are separated

on various adsorbents according to their polarity. The combination of these two techniques results in a powerful two-dimensional cleanup by molecular size and polarity of the compounds. Although various adsorbents have been applied for this step, the GPC/silica gel column combination is the best known and almost universally utilizable cleanup technique. This system using S-X3 Bio-Beads produces very clean extracts and delivers high recovery rates for many OCPs from various fat-containing biological matrices (77).

Important advantages of this technique are that it can easily be automated and it has a large scope for pesticide residue analysis. However, an important drawback of GPC is that the triglycerides, which are usually present at the milligram level, elute before the pesticides, which are present at the microgram-to-nanogram level. Some tailing of the large triglyceride peak is inevitable and makes online combinations of GPC and capillary GC difficult to perform (75).

For online coupling to GC, GPC columns must be smaller than those currently used. A column packed with polystirene PSS SDV was used to measure chlorinated pesticides in olive oil, fat extracts of chicken and fish, and lettuce (75). GPC can be used for the automated online cleanup and determination of 22 OPPs in various fruits, such as apples, grapes, and kiwi fruit (80).

The preparation of oil samples for the determination of pesticides by GC requires the complete removal of fat. The online combination of GPC and the GC method allowed trace-level determination of 17 OPPs in olive oil samples. Sample preparation was negligible when a column packed with PLGel for GPC and UV detector was used (70).

III. HPLC DETERMINATION

High-performance LC methods for pesticide residue analysis were first developed for nonvolatile or thermally labile compounds, such as carbamate insecticides. Because HPLC offers a simpler and/or faster approach to analysis for a wide range of other compounds, it is becoming more and more widely accepted, and its applications are steadily increasing in number. Although HPLC has been used in the analysis of OCPs and OPPs, the literature on its application in food is scarce. The methods reported have been summarized in Table 4.

A. Columns and Mobile Phases

The most commonly encountered columns used in HPLC are still 4.6-mm ID and 10–25 cm in length. The HPLC support is generally fine-particle (3–5-μm diameter) silica with various chemical groups attached to it. The nature of the chemical group varies according to the type of analyte to be separated. A commonly used group is octadecylsilano (C_{18} or ODS), which is attached to the hydroxyl groups of the silica support. This makes a very hydrophobic column. Other stationary phases include C_8 and CN. Unmodified silica columns are also commonly used, and in this case the technique is designated NP-HPLC.

Pesticide analysis in water can be simplified considerably if a single short column (20-mm $\times$ 4.0-mm ID) is used. Use of the short column (high-pressure packed column) permits baseline separation of over 20 analytes. The column can be reused for some 20 real samples without any decrease in performance.

Usually, when an HPLC method is developed, an acceptable degree of separation for all the components of interest in our sample is required in a reasonable time. The mobile phases more frequently used are the classical mixtures of methanol–water and acetonitrile–water in different proportions. If a satisfactory separation cannot be achieved using a binary solvent mixture as mobile phase, a ternary composition may be used.

Table 4 HPLC Determination of OPPs and OCPs

Matrix	Pesticide	Other pesticides	Column	Mobile phase	Detector	Refs.
Water	Azinphos methyl, coumaphos, crufomate, diazinon, dicapthon, fechlorphos, fenitrothion, fensulfothion, fenthion, fonophos, gophacide, parathion methyl, phosmet, temephos, zytron	—	25-cm × 4.6-mm-ID Altech C_{18}, 10 μm 25-cm × 4.6-mm-ID Altech C_8, 10 μm	Isocratic 65% H_2O in acetonitrile	UV at 254 nm	31
Water	Mevinphos I, dimethoate, mevinphos II, dichlorvos, diazinon, paraoxon, azinphos-methyl, fenamiphos, parathion-methyl, malathion, fenitrothion, azinphos ethyl, fenthion, parathion-ethyl, coumaphos, phorate, phoxim, disulfoton, chlorpyriphos, omethoate, monocrotophos, vamidothion, bromophos ethyl, metoxichlor, DDT	Carbamate, thiocarbamate, phenylureas, triazines, acetanilide, uracil, propionalide, benzonitrile, benzamide, dicarboximide, dinitroaniline, pyrethroid, thiadiazinone, metoxybenzoic, phenol, phenoxy acid, coumarin	Primary column: 25-cm × 4.5-mm-ID 5-μm LC-18 DB Confirmatory column: 25-cm × 4.5-mm-ID 5-μm LC-CN	Gradient: initially: 80% H_2O containing phosphate buffer at pH 6.7 and 20% acetonitrile; finally 80% acetonitrile after 45 min	UV 220 nm	38–40, 81
Water	*p,p'*-DDT, aldrin, dieldrin, heptachlor, endrin	—	25-cm × 4.6-mm-ID 5-μm Spherisorb ODS	Isocratic methanol–water (75:25)	UV 220 nm	47
Water	Diazinon, azinphos-methyl, fenthion	—	22-cm × 4-mm-ID RP18	Gradient: methanol–water	UV 220 nm	82
Water	Azinphos methyl, phosmet, parathion methyl, azinphos ethyl, fenitrothion, parathion, diazinon	Carbamate	15-cm × 4.6-mm-ID 5-μm Nucleosil C_{18}	Gradient: acetonitrile–water	UV 254 nm	48
Water and fruits	Paraoxon, guthion, methyl parathion, fenitrothion, ethyl parathion	—	25-cm × 4.5-mm-ID 5-μm Spherisorb RP-18 22-cm × 4.6-mm-ID 5-μm Spherisorb RP-8	Isocratic methanol–water (70:30)	UV 260 nm	33

Fruit and vegetables	Azinphos methyl, captan, chlorpyriphos, chlorpyriphos methyl, *p,p'*-DDE, *p,p'*-DDT, dicofol, dieldrin, dimethoate, α-endosulfan, endosulfan sulfate, β-endosulfan, phosalone, tetradifon, tolclofos-methyl, ethion, folpet, lindano, parathion-ethyl	Carbamates, fungicides	25-cm × 4-mm-ID 5-μm Spherisorb RP-18	Gradient: acetonitrile–water	UV 220 nm	57
Wheat	Malathion	—	25-cm × 4.0-mm-ID 10-μm ODS	Acetonitrile–0.1% acetic acid (1:1)	UV 350 nm	55
Corn, beans	Malathion, malaoxon, isomalathion, malathion, monocarboxilic acids	—	7-cm × 2.1-mm-ID Co-pell ODS	Acetonitrile–water (70:30)	UV variable wavelength, 215–280 nm	56
Milk	DDT, endrin, aldrin, methoxychlor, heptachlor	30-cm × 4.6-mm-ID 3-μm Hypersil ODS		Gradient: H_20–acetonitrile	UV 254 nm	11
Water and beef muscle	Chlorpyriphos, fenphur, fenthion, fenthoxon, 3-methyl-4-(methylthio) phenol, parathion, paraoxon, *p*-nitrofenol, ronnel, ronnel oxon, 3,4,5-trichlorophenol stirophos	—	25-cm × 4.6-mm-ID 5-μm Bio-Sil C_{18}	Acetonitrile–water Gradient: acetonitrile–0.1 M ammonium acetate	UV variable wavelength, 190–350 nm DAD TSP/LC-MS	61, 63
Marine mammal blubber	*p,p'*-DDE	PCBs	25-cm × 4.0-mm-ID 5-μm LiChrosob Si 60	Isocratic hexane–chloroform (95:6)	UV 205 nm	62
Animal tissues, liver and brain	Fenitrothion, azinphos ethyl, chlorfenson, tetradifon, dicofol	Carbamate	12-cm × 4.0-mm-ID 5-μm Lichrosphere 100 RP-18	Gradient: water–acetonitrile methanol	UV variable wavelength, diode array (DAD)	69
Water	Dimethoate, dichlorvos, folpet, triazophos, chlorfenvinphos, chlorpyriphos-methyl, endosulfan sulfate, tetradifon, α-endosulfan, β-endosulfan, carbofenothion	Triazines, carbamates, ureic derivatives, imidic derivatives	15-cm × 3.0-mm-ID 5-μm Hypersil C_{18}	Gradient: water–acetonitrile–methanol	DAD 200–280 nm	34, 35
Water	Azinphos methyl, dichlorvos, fenitrothion, malathion, mevinphos cis, mevinphos trans, chlorfenvinphos, diazinon, azinphos ethyl, fenthion, parathion ethyl, parathion methyl	—	25-cm × 4.0-mm-ID 4-μm RP-8	Gradient: acetonitrile–methanol	DAD 220–280 nm	49

(continued)

Table 4 Continued

Matrix	Pesticide	Other pesticides	Column	Mobile phase	Detector	Refs.
Water	Chlorpyriphos-methyl, fenitrothion, fenchlorfos, parathion-ethyl	Triazine, phenylureas	25-cm × 4.6-mm-ID 5-μm ODS	Gradient: phosphate buffer–acetonitrile	DAD	83
Water	Paraoxon, methyl parathion, fenitrothion, ethyl parathion	—	22-cm × 4.6-mm-ID 5-μm ODS	70:30 methanol water with acetate buffer 4.8	Electrochemical detection with dual glassy-carbon electrode	84
Water	Azinphos methyl, captan, dicofol, dimethoate, malathion, tetradifon	Acetamide, triazine, carbamate, pyrethroid, urea derivatives, triazinone, acylalanine	15-cm × 4.6-mm-ID 5-μm Spherisorb ODS-2	Gradient: acetonitrile–water	DAD	37, 85
Water	Methyl parathion, fenitrothion, ethyl parathion, fenitrooxon, paraoxon ethyl	Phenolic transformation products	15-cm × 4.6-mm-ID 5-μm Hypersil C_{18}	Gradient: water–acetonitrile	DAD, ACPI-MS using FIA	42
Green bean	Chlorfenvinphos, chlorpyriphos-methyl, endosulfan sulfate, tetradifon, α-endosulfan, β-endosulfan, chlorpyriphos-ethyl, carbophenothion	*N*-Methyl carbamate, triazine, phenylurea	15-cm × 3-mm 5-μ C_{18}	Gradient: acetonitrile–methanol–water	DAD 200–350 nm	58
Beef tissue	Fanphur oxon, paraoxon, fentoxon, coumaphos oxon, famphur, ronnel oxon, stirophos, parathion, fenthion, coumaphos, chlorpyriphos methyl, ronnel, chlorpyriphos	Phenolic derivatives	25-cm × 4.6-mm-ID 5-μm Bio-Sil C_{18}	Gradient: acetonitrile–water	DAD 202–314 nm	60, 86
Water	Fenamiphos, fenitrothion, fonophos	Triazine	25-cm × 4.6-mm-ID 5-μm C_8	Gradient: acetonitrile–water	UV 254–270 nm, APCI-MS	41
Water	Fenitrothion, malathion, parathion ethyl, vamidothion	Triazines, thiocarbamate	20-cm × 4.6-mm-ID 5-μm Spherisorb ODS-2	Gradient: methanol–acetate buffer 4.5	APCI-MS, PB-MS	51
Water	Chlorpyriphos methyl, diazinon, disulfoton, fenamiphos, fenthion, isofenphos, malathion, methidathion, pyridafenthion, temephos	—	25-cm × 4.0-mm-ID 4-μm Superspher C_8	Gradient: acetonitrile–water with 0.05 mM ammonium formiate	TSP-MS	50, 87

Water	Fenthion, temephos	—	12.5-cm × 4.0-mm-ID 5-μm LiChrospher 100	Gradient: acetonitrile–water	DAD, APCI-MS	44
Water	Trichlorfon, dichlorvos, mevinphos, demethon-*S*-methyl, dimethoate, oxydemeton-methyl, fenamiphos, fenthion	—	15.0-cm × 2.1-mm 5-μm Zorbax cianopropyl	Gradient: methanol–water	ISP-MS	88
Water	Acephate, azinphos-ethyl, fenitrothion, fensulfoton, fenthion, methamidophos, naled, paraoxonmethyl, parathion methyl, trichlorfon, vamidothion, vamidothion sulfoxide	—	25-cm × 4.6-mm-ID 5-μm C_8 Waters	Gradient: methanol–water–0.1 M acetic acid	APCI-MS	43
Water	(E) Mevinphos, (Z) mevinphos, dichlorvos, azinphos methyl, parathion methyl, malathion, fenitrothion, azinphos ethyl, parathion, fenthion, chlorfenvinphos, diazinon	—	25-cm × 4.6-mm-ID 5-μm Waters C_8	Gradient: methanol–water acidified with 1% acetic acid	APCI-MS	52
Water	Dimethoate, metramitron	Triazines, phenylurea herbicides, acetanilides	15-cm × 2.0-cm-ID 4-μm Novapack C_{18}	Gradient: methanol–water–acetic acid	APCI-MS	32
Water	Fenchlorphos, chlorpyriphos, bromophos, tetrachlorvinphos, coumaphos	Triazines, anilides	25-cm × 4.6-mm-ID 5-μm Supelco LC-18-DB 15-cm × 4.6-mm-ID 3-μm Supelco LC-18-DB	Acetonitrile–water	DAD 210 nm, PB-MS	89, 90
Water	Metamitron, dimethoate, mevinphos, malathion, foxim	Urea derivatives, anilides, carbamates	25-cm × 4.6-mm-ID 5-μm Supelco LC-18-DB	Acetonitrile–phosphate buffer pH3	DAD 210 nm, PB-MS	53, 91, 92
Water	Dimethoate, fenamiphos, bromophos ethyl, fenthion, coumaphos, fenchlorfos, chlorpyriphos, monocrotophos, mevinphos, fosphamidon, paraoxon, azinphos methyl, foxim, carbophenothion, bromophos methyl	Carbamates, phenyl urea, anilides, nitrofenoles	15-cm × 4.6-mm-ID 3-μm Zorbax C_{18} 20-mm × 4.6-mm-ID 5-μm Supelcosil LC-18-DB	Acetonitrile–water, acetonitrile–phosphate buffer pH3	ACPI MS-MS, PB-MS	54, 93, 94

B. Detectors

1. UV-VIS Detector

The most commonly used detector for pesticide residue analysis by LC is UV-VIS. It includes fixed wavelength and variable wavelength. Most OCPs and OPPs absorb appreciably only at wavelengths below 250 nm, the same spectral region where many solvents, solvent impurities, and matrix-derived interferences absorb. Analysis of these compounds by HPLC is still possible with very clean environmental substrates such as water.

High-performance LC in the reversed-phase mode (RP-8 or RP-18 column) with UV detection (254 nm) and isocratic conditions was evaluated for the analysis of 15 organophosphorus pesticides. Typically the method could be used to analyze azinphosmethyl in water at 0.5 μg/L. This compares favorably with GC, since this compound is very difficult to analyze by that method. For other pesticides, such as fenitrothion, which is readily analyzed by GC, the HPLC method can be used for confirmation purposes (31).

A multiresidue HPLC method for monitoring polar pesticides in natural water has been proposed. After extraction with GCB, the analysis was carried out using a C_{18} column and UV detection (38–40,81).

The biotransformation of fenthion in animals and plants leads to five major metabolites. According to the FAO, the maximum residue limit in different commodities includes the sum of the active ingredient and the metabolites, expressed as fenthion. A satisfactory separation was achieved with an RP-18 column and UV determination (95).

Isocratic conditions have been established for HPLC with ultraviolet detection to determine trace levels of five pesticides frequently used in fruits, e.g., paraoxon, guthion, methyl-parathion, ethyl-parathion, and fenitrothion, in fruits and surface water (95). And HPLC was adapted for the determination of trace concentrations of seven OPP pesticides in drinking water. The analytes concentrated on the precolumn were eluted and separated on a C_{18} analytical column with an elution gradient program and determined by measuring their UV absorption (48).

A gradient HPLC procedure with UV detection, for determination of pesticide residues in fruit and vegetables such as apples, potatoes, and carrots, was used by Beil et al. (57). Figure 3 shows a clean chromatogram of many compounds that was obtained by HPLC and UV detection in apple.

The OPP malathion is widely used because of its low persistence in the environment and its high insecticide activity. Pure malathion has moderate toxicity, but crude malathion and its formulations contain impurities, which are far more toxic to mammals. These impurities not only are formed during commercial production but can also develop in the grains during storage. The most toxic of these products is the oxidation product malaoxon. An isocratic HPLC method indicated the degradation of malathion in stored wheat (55) and the presence of malathion and malaoxon in maize and bean samples (56). Isomalation and malation monocarboxylic acid metabolites were also detected.

Extraction in liquid/liquid segmented flow has been developed. A continuous-flow extraction system coupled online with an RP-HPLC with C-18 and a UV detector was used to study the extraction of fenthion and azinphos methyl in water (82).

A method for the quantitative analysis of the OCPs in water using the HPLC technique of online trace enrichment was developed. The pesticides were concentrated onto an ODS column, and the analysis was performed on an ODS column with UV detection (47).

High-performance LC was used to determine residue levels of *p,p*-DDE in marine mammal blubber. This compound can absorb UV well enough at shorter wavelengths. With normal-phase LC after derivatization to convert the *p,p*-DDE to *p,p*-DCPB (dichlorobenzophenone), a semiquantitative determination of this compound was done in the presence of PCBs (62).

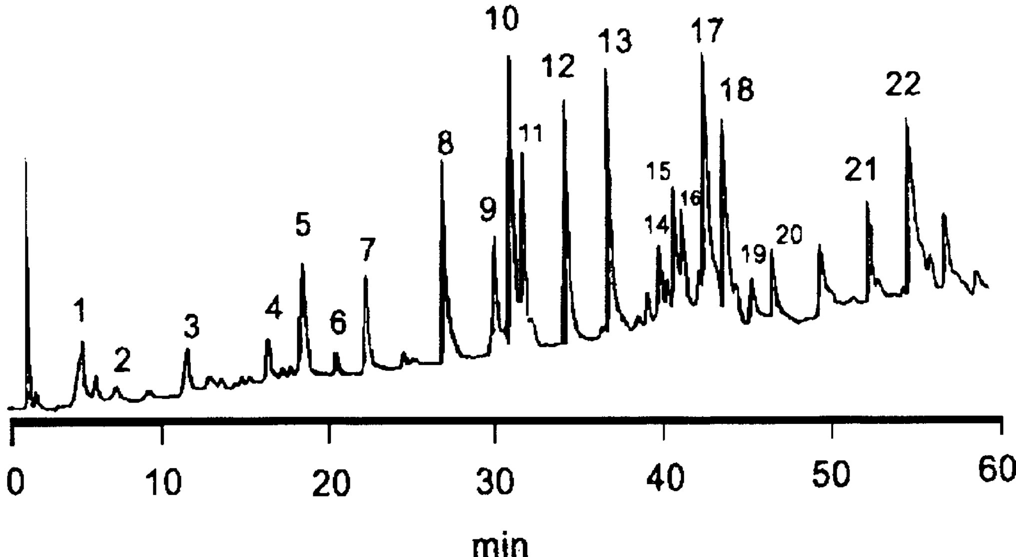

Fig. 3 HPLC chromatogram of spiked apple extract. Peaks: (1) folpet, (2) dimehoate and benomyl, (3) aldicarb, (4) bendiocarb and pirimicarb, (5) carbaryl, (6) tolylfluanid and thiram, (7) propham, (8) captan and azinphos methyl, (9) chlorpropham, (10) iprodione, (11) procymidone, (12) vinclozolyne, (13) parathyon ethyl, (14) endosulfan sulfate, (15) toclophos methyl and chlorpyriphos methyl, (16) dicofol, (17) β-endosulfan, (18) tetradifon, (19) α-endosulfan, (20) chlorpyriphos and dieldrin, (21) p,p'-DDT, (22) p,p'-DDT. 0.1 AUF: concentrations 10–20 ng/20 μl; injected volume 20 μl. (From Ref. 57.)

2. *Diode Array Detector*

The advent of multichannel detection methods, such as photodiode array detection (DAD), for monitoring chromatographic processes has opened up new prospects for the resolution of overlapping peaks.

Diode array detection allows collection of full-spectra data at rates of up to several scans per second. With this data it is possible to construct three-dimensional plots of absorbance, wavelength, and time. This methodology was used to determine mixtures of analytes for their agricultural interest, operating with a gradient program. The use of DAD allowed selection of the wavelengths employed for the determination of each pesticide in order to obtain an improvement in sensitivity and selectivity. This procedure was applied to determine OCPs and OPPs in several ground- and drinking water samples (35). The method was applied to the determination of folpet, procymidone, and triazophos in groundwater samples (34).

Liquid–solid extraction methods coupled to LC-DAD are gaining interest for the screening of pesticides in waters. Automated precolumn technology involving disposable solid-phase extraction precolumns has proved to be a useful tool. A C_{18} precolumn was used online by LC-DAD. In these conditions, 11 OPPs were analyzed in groundwater (49).

The widespread agricultural use of the OPPs and their potential mammalian toxicity has dictated the development of several methods for isolating and detecting the parent compounds. A method that allows the extraction and simultaneous detection of OPPs and their primary and secondary metabolites from beef tissue was developed using HPLC-DAD with a gradient mobile phase (96). Information on the thermal decomposition of OPPs in meat and other food products is still very limited. The temperature and pH stabilities of OPPs were investigated in lean beef

muscle. Compounds were extracted from fortified raw and cooked meat samples and analyzed by HPLC-DAD on a C_{18} column. The findings give some insight into the persistence of residues of parent OPPs in cooked meat product (86). Liquid chromatography–DAD was used to study the degradation of fenitrothion, ethyl-parathion, and methyl-parathion and the presence of various transformation products.

A multiresidue method for analyzing 28 common organophosphorus pesticides and three of their main metabolites (paraoxon-ethyl, paraoxon-methyl, and malaoxon) in a variety of crop samples has been developed (59). The use of LC-DAD techniques allowed identification of both organophosphorus pesticides and metabolites by means of standard and spectral comparisons, respectively. A typical chromatogram for fortified apple extract is shown in Figure 4.

3. *Mass Spectrometry Detector*

The use of liquid chromatography–mass spectrometry (LC-MS) is becoming more popular because of the increasing number of LC-MS interfaces commercially available: thermospray (TSP), particle beam (PB), and atmospheric pressure ionization (API). Coupled with mass spectroscopy, HPLC provides the analyst with a powerful tool for residue determination.

Interfacing these devices at present is most commonly done by the thermospray technique. The thermospray interface uses heated nebulization of the LC column effluent and extra pump capacity for effective desolvation. The TSP-MS configuration allows the molecular mass characterization of many members of the OP class of pesticides and metabolites. Numerous researchers have investigated the application of TSP-MS to OPP analysis and have attempted to evaluate and optimize several parameters, including interface temperature, mobile-phase buffer, and system stability for pesticide determination. Exploring the use of nonpolar mobile phase for TSP-MS of several pesticides, including OPPs, was the focus of a study by Barceló et al. (97). In another study, Barceló et al. (98) investigated the effect of chloride adition to the HPLC mobile phase for detection and enhancement using online LC-MS for OPPs. Modern instrumentation allows the option of monitoring the formation of negative ions (NIs) as well as positive ions (PIs) (50,99,100).

The ion formation characteristics of a group of OPPs and some of their primary or secondary metabolites were determined using TSP-MS. Full-scan mass spectra using a variety of ionization techniques, including filament-off, filament-on, and discharge-on, and two detection modes (PI and NI) were collected for reference standard. The majority of the OPPs and metabolites investigated produced ions in greater abundance in the NI discharge-on mode (61). Seventeen OPPs and metabolites were extracted from lean and fatty beef muscle samples. Five ions per compound were monitored in the selected ion mode (SIM), and their intensities were suitable for the confirmation of compound identity. Selected ion mode chromatograms obtained for reference standards and an extract of spiked lean muscle are given in Fig. 5.

The introduction of the PB interface originally known as the "monodisperse aerosol generating interface" for chromatography permits the coupling of a wide range of LC separations to conventional MS procedures. Desolvation occurs in steps by leading the column effluent through several differentially pumped chambers. An attractive feature of the PB interface is that it is the only interface that can generate electron impact (EI) and solvent-independent chemical ionization (CI) spectra.

Particle beam is especially useful in identifying unknown compounds, despite its limitations with respect to the quantification and its lack of sensitivity at trace levels (51). Particle beam–MS is generally carried out in the positive EI, because EI-MS provides very reproducible mass spectra, and a wide variety of mass spectra libraries, containing up to 130,000 entries, are available for fast identification. Chemical ionization with positive (PCI) or negative (NCI) ion de-

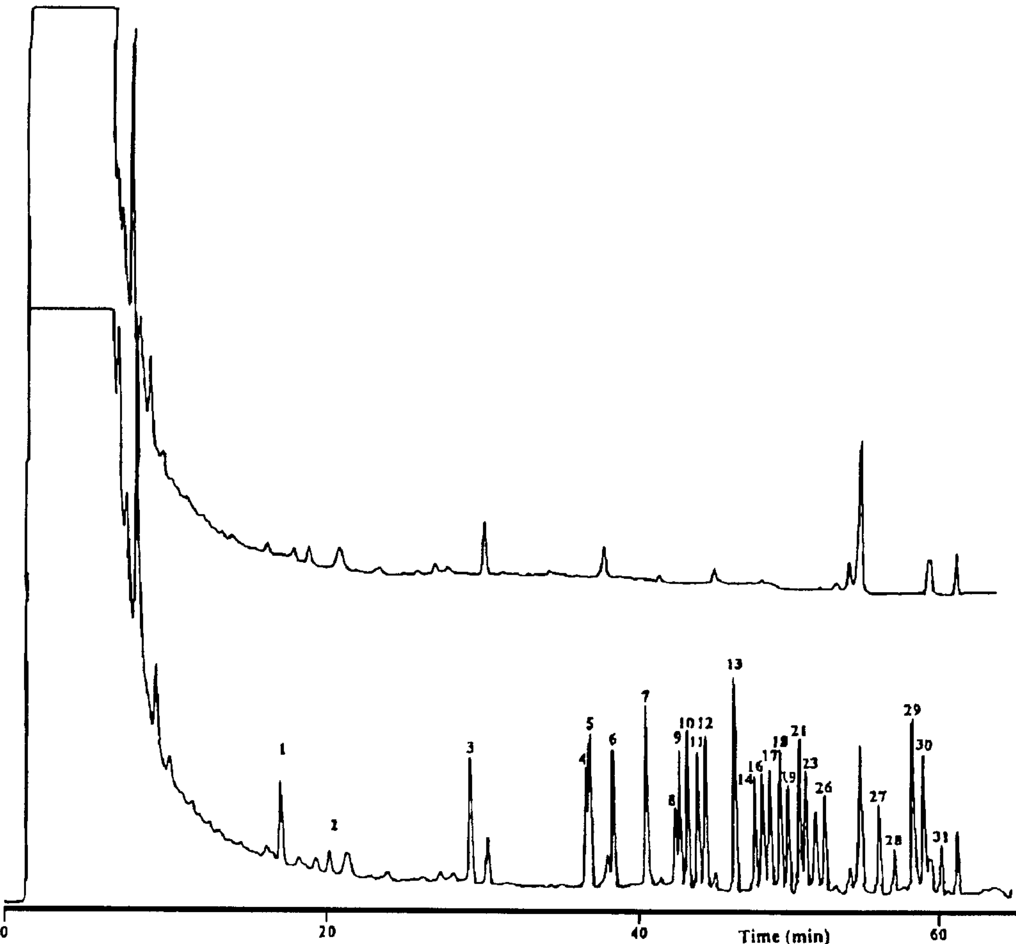

Fig. 4 Chromatograms of a blank apple sample (top) and the same apple sample spiked with 27 of the 31 organophosphorus at the following concentration levels (bottom). 1 = paraoxon methyl 500; 2 = malaoxon 6400; 3 = paraoxon ethyl 800; 4 = metidathion 500; 5 = azinphos-methyl 200; 6 = phosmet 200; 7 = parathion methyl 800; 8 = malathion 2000; 9 = triazophos 200; 10 = fenitrothion 800; 11 = azinphos ethyl 200; 12 = chlorfenvinphos 200; 13 = quinalphos 200; 14 = parathion-ethyl 400; 16 = ethrimphos 1000; 17 = diazinon 800; 18 = coumaphos 800; 19 = fonophos 200; 21 = phoxim 400; 23 = chlorpyriphos-methyl 500; 24 = disulfoton 2000; 26 = isofenphos 500; 27 = fenchlorphos 500; 28 = temephos 400; 29 = chlorpyriphos ethyl 200; 30 = pyrimiphos-ethyl 200; 31 = carbophenothion-ethyl 400. The chromatography was carried out with the "Altima" column using CH_3CN/H_2O as mobile phase in gradient elution CH_3CN from 28 to 39% in 20 min, and then from 39 to 88% in 40 min. (From Ref. 59.)

tection can provide additional information for the provisional identification of unknowns when no relevant information is contained in the libraries. Negative-ion chemical ionization is especially recognized for its improved selectivity and sensitivity in the detection of chlorinated pesticides. Only a few highly abundant ions are usually observed in the NCI mass spectra. This enhances analyte detectability if the selected SIM is applied. This system was used to analyze surface water samples; several pollutants—among them OPPs—were detected and identified (53,89–91).

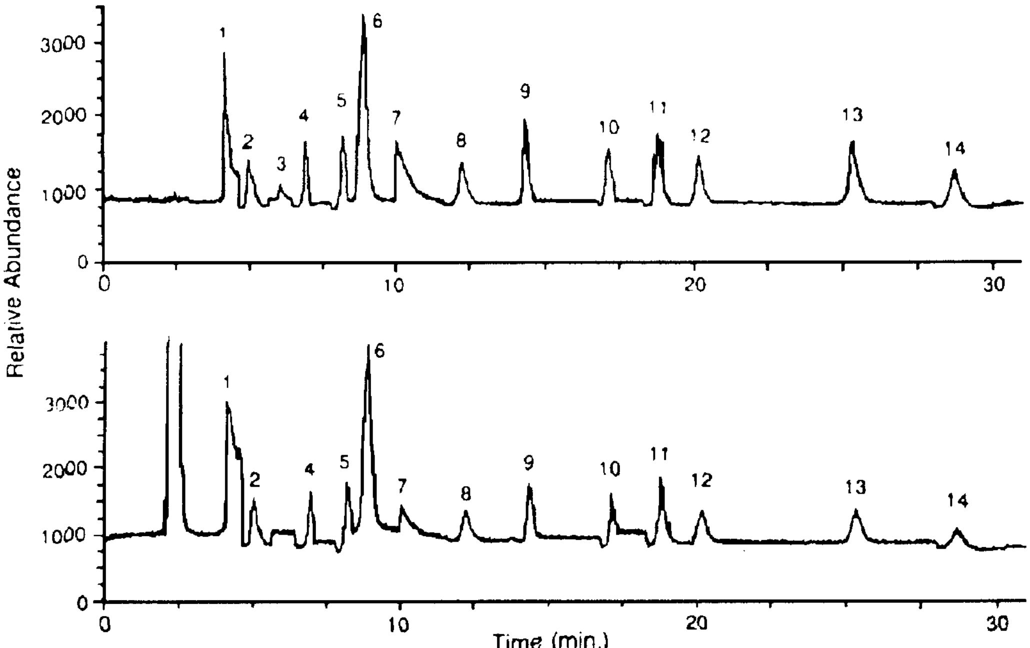

Fig. 5 SIM chromatograms obtained in NI discharge-on TSP/LC-MS of mixed reference standards (5 ppm) (top) and of a spiked (1 ppm) lean beef muscle sample (bottom). Peak identities: (1) *p*-nitrofenol, (2) 3-chloro-4-methyl-7-hydroxycoumarin, (3) 3-methy-4-(methylthio)phenol, (4) paraoxon, (5) coumaphos oxon, (6) famphur, (7) 2,4,5-trichlorofenol, (8) ronnel oxon, (9) stirofos, (10) parathion, (11) coumaphos, (12) chlorpyriphos-methyl, (13) ronnel, (14) chlorpyriphos. (From Ref. 75.)

In the field of LC-MS coupling there is currently a great deal of interest in API methods (32,42,44,51,54,88,93,94). In an API-MS system, the ion source region (located outside the mass spectrometer at ambient pressure) is separated from the high-vacuum mass analyzer region by a small-sampling orifice, which must be large enough to permit the introduction of a large proportion of ions in the atmospheric pressure region into the vacuum region while maintaining the low pressure required for mass analysis. Ionization takes place at atmospheric pressure by electrospray, ion-spray (ISP), or atmospheric pressure chemical ionization (APCI). Until now, only ISP and APCI applications for the analysis of pesticides have been described, probably because of the flow-rate limitations imposed on LC associated with electrospray.

A method for the determination of 11 OPPs in a water sample involved SPE followed by LC-ISP-MS and was developed with a limit of detection between 0.01 and 0.2 μg/L (88). The authors observed that ISP spectra display diagnostic ions of OPPs, which could be used for the identification of unknowns. Comparison of LC-ISP-MS with TSP-MS analysis of the same compounds showed that ISP is to be preferred: It has about 100-fold better sensitivity, and the thermal degradation of trichlorfon observed with TSP was not found with ISP. Liquid chromatography–ISP-MS is sometimes better when it comes to distinguishing isomers like *trans*- and *cis*-mevinphos, which is difficult to do under current LC-DAD conditions owing to the poor chromophore of *cis*-mevinphos.

Liquid chromatography–APCI-MS is applicable to many different types of pesticide structures, such as triazines, phenylurea herbicides, acetanilides, and OPPs. A study of 12 pesticides and pesticide degradation products demonstrated the sensitivity of the technique for OPP determination, with detection limits for water samples of about 0.001–0.005 μg/L (32).

The use of LC-APCI-MS makes it possible unequivocally to identify various degradation products from fenitrothion, ethyl-parathion and methyl-parathion, fenthion, and temephos (42,44). Moreover, LC-APCI-MS provides the best information on the transformation products.

Liquid chromatography–APCI-MS with NI and PI was used for the trace determination of several OPPs in groundwater. The limit of quantification varied between 5 and 37 ng/L in PI. Under the NI mode of operation, only the parathion group (both parathions and fenitrothion) had a better sensitivity than with the PI mode, with quantitation limit of 5–15 ng/L, whereas the rest of the pesticides had 2–4 times higher limits as compared to those in PI mode (52).

The same authors have also studied the effect of the probe temperature and the extraction voltage on the sensitivity and fragmentation of several OPPs. As a general rule, it was found that the probe temperature has a greater effect on compound sensitivity than on fragmentation. A compromise between sensitivity and structural information has to be defined; as a result, the probe temperature and extraction voltage have to be optimized, depending on the aim of each study (43).

The stability of four pesticides was examined under different storage conditions after preconcentration in disposable SPE cartridges. All the compounds studied were confirmed by coupling a cartridge online with the LC-APCI-MS system in the PI mode and under scan conditions. By analyzing the blank cartridges stored at 4°C with LC-APCI-MS, four interfering peaks were detected. These peaks were unknown compounds and appeared at the same retention time as the two first peaks obtained after the LC-DAD analysis. When analyzing cartridges preconcentrated with groundwater directly online by LC-APCI-MS, this interfering peak did not appear in the chromatogram obtained (41).

Single short, high-pressure packed columns coupled with MS or tandem MS detection permits the rapid trace-level determination and identification of environmental pollutants in water samples. Using an ACPI interface, a mixture of 17 pesticides, including OPPs, can be successfully used to identify pesticides at the level of 0.1 μg/L in tap water (93,94).

A study using APCI and PB-MS in both the NI and PI modes coupled to HPLC was used to determine four OPPs. The results demonstrated the higher sensitivity of HPLC-APCI-MS compared with HPLC-PB-MS and the potential of both techniques for confirming the presence of pesticide (51).

Liquid chromatography with ISP or ACPI followed by tandem MS-MS was used to analyze a 17-pesticide mixture. Both approaches gave similar product ion spectra from protonated molecules; and an MS-MS library was set up for more than 60 pesticides and their degradation products. The library was successfully used for searching product ion–ion spectra from SPE/LC-APCI-MS-MS at low levels (10 ng/L) in tap water (54).

IV. APPLICATIONS IN FOOD

A bibliographic search has shown that the majority of the HPLC techniques for determining OPPs and OCPs have been applied to the determination of residues in surface, ground- and drinking water. Table 5 lists pesticides determined, extraction and cleanup methods used, HPLC conditions, contaminated matrix and analyte detection limits taken from the literature for water, animal tissues, milk, fruit and vegetables, and cereals. The majority of the studies were done on spiked samples, and in the best of cases there were few real samples analyzed.

Driss et al. proposed an analytical procedure that, when applied to tap water, gives coextracted compounds with a large number of unresolved peaks at the start of the chromatogram, the intensity of which depends on the untreated water volume (48).

Parrilla and Vidal performed a study on drinking, ground- and seawater samples from *Almeria* (35). The chromatograms corresponding to drinking and seawater samples did not show

Table 5 HPLC Methods for OCP and OPP Residues in Food

Pesticides	Determination	Amount	Recovery (%)	LOD	Refs.
Water samples					
14 OPPs	Directly or after LLE with ethyl acetate *o*-dichloromethane, RP-HPLC C_{18} or C_8, UV 254 nm	1 ppm	73–99	0.1 ng/mL	31
3 OPPs	Continous-flow extration (heptane) online with RP-HPLC C_{18}, UV 220 nm	0.5–20 mg/L	70–90	0.04–0.09 mg/L	82
19 OPPs, 2 OCPs, other pesticides	SPE with Carbograph RP-HPLC C_{18}, UV 220–230 nm	20–300 μg	85–102	<0.1 μg/L	38–40, 81
4 OCPs	SPE with C_{18} online, RP-HPLC C_{18}, UV 220 nm	13–23 μg	60–77.3	0.1–1 μg/L	47
7 OPPs, 1 carbamate	SPE with C_{18} or PRP-1 online, RP-HPLC C_{18}, UV 254 nm	μg/L levels	54–100	0.003–0.2 μg/L	48
5 OPPs	SPE C_{18}, RP-HPLC C_{18}, UV 260 nm	13.7–46.7	79.3–100.3	0.26–0.57 ng/mL	33
4 OCPs, 8 OPPs, other	SPE or C_{18}, LLE dichloromethane, RP-HPLC C_{18}, DAD 206–245 nm	0.1–1 μg/L	24–112	<0.1 μg/L	34, 35
1 OPP, 3 degradation products	SPE C_{18}, RP-HPLC C_{18}, DAD, TSP/MS	0.5–40 μg/L	90	50 ng/L	87
3 OPPs	SPE Lichrolut EN, RP-HPLC C_{18}, DAD, APCI/MS	0.1–1.5 μg/L	—	0.05–0.1 μg/L	42
10 OPPs and transformation products	SPE C_{18} online, RP-HPLC C_8, DAD, TSP/MS	0.025–0.2 μg/L	—	0.01–0.1 μg/L	50
11 OPPs	SPE C_{18} online, RP-HPLC C_8, DAD 220–280 nm, APCI-MS	30–600 μg/L	—	<0.1 μg/L	49, 52
4 OPPs, other	SPE C_{18} online, RP-HPLC C_{18}, APCI/MS, PB/MS	—	—	0.08–200 μg/L	51
10 OPPs	SPE C_{18} online, RP-HPLC C_{18}, APCI/MS	9.09–62.9 ng/L	94–120	5–37 ng/L	52
2 OPPs	SPE C_{18}, RP-HPLC C_8, DAD, APCI/MS	—	—	—	44
10 OPPs	SPE (Amberchrom, Lichrolut EN, cyclohenyl, SDB, C_{18}, Isolute ENV) online, RP-HPLC C_8, DAD, ISP/MS	0.2 μg/L	8–133	0.01–0.2 μg/L	88
12 OPPs	SPE Lichrolut EN and Isolut EN online, RP-HPLC C_8, DAD, TSP/MS	0.2 μg/L	0–132	30–250 pg	43
2 OPPs, other	LLE with dichloromethane, SPE C_{18}, RP-HPLC C_{18}, APCI/MS	0.05 μg/L	77–102	0.001–0.005 μg/L	32
5 OPPs, other	SPE PLRP-S online, RP-HPLC C_{18}, DAD 210 nm	—	—	0.1–200 ng/L	89–92, 53
2 OPPs	PB/MS				
7 OPPs	SPE and separation is performed in the same column 2-cm × 4.6-mm-ID RP-HPLC C_{18}, DAD, PB/MS, APCI-MS/MS	—	—	50–1500 ng/L	93, 94
7 OPPs, other	SPE PLRP-S online, RP-HPLC C_{18}	—	—	0.1–3000 ng/L	54
	Compared with SPE, and separation is performed in the same column 2-cm × 4.6-mm-ID RP-HPLC C_{18}, PA-ESP-MS/MS, APCI-MS/MS				

Fruit and vegetables					
4 OCPs, 13 OPPs, carbamates	LLE acetone/water, partition with CH_2Cl_2, clean up with silice, RP-HPLC C_{18}, UV 220 nm	2–100 μg/20 μL	38–112	0.01 mg/kg	57
5 OPPs	LLE benzene, RP-HPLC C_{18}, UV 260 nm	1.15–19 μg/g	86.3–100.6	0.046–0.099 μg/g	33
4 OCPs, 8 OPPs	LLE acetone, partition with CH_2Cl_2, clean up with C_{18} and silice cartridges, RP-HPLC C_{18}, DAD 204–245 nm	0.4–2.0 mg/kg	74.7–110.1	0.01–0.1 μg/kg	58
28 OPPs and 3 metabolites	LLE acetonitrile, clean up with GCB, RP-HPLC C_{18}, UV 220–280 nm	100–100 ng/g	61–96	3–493 ng/g	59
Cereals					
1 OPP and its degradation products	LLE acetonitrile/water, partition with CH_2Cl_2, clean up with carbon, RP-HPLC C_{18}, UV 350 nm	10 ppm	—	—	55
1 OPP and its degradation products	Soxhlet with methanol, clean up with Florisil, RP-HPLC C_{18}, UV 220–220 nm	10–36 mg/kg	—	—	56
Milk					
5 OCPs	ISRP C_{18} online, RP-HPLC C_{18}, UV 254 nm	50 μg/μl	99.3	—	11
Animal tissue					
6 OPPs and their metabolites	LLE ethyl acetate–methanol, cleanup C_{18}, RP-HPLC C_{18}, DAD 190–350 nm, TSP-MS	50–1 ppm	45–95	—	63, 60, 61, 86
1 OCPs and PCBs	LLE hexane–acetonitrile, cleanup Florisil, RP-HPLC C_{18}, derivatization to *p,p*-DCBP, UV 205 nm	1 μg-1 mg/g	81.6	—	62
4 OCPs, 2 OPPs	LLE chloroform–acetone, cleanup: GPC and silica, RP-HPLC C_{18}, DAD	0.25–2.5 mg/kg	93.6–102.1	0.05–0.23 mg/kg	69

peaks. However, in groundwater, tetradifon, dimethoate, chlorpyriphos, and endosulfan were found in different samples at low levels.

The River Ebre (Spain) was investigated under APCI and PB in order to confirm unequivocally the absence of pesticides (50,51). The study demonstrated the absence of OPPs and OCPs, although other classes of pesticides were present.

A surface water sample from the Nitra River (Slovakia) was analyzed by online SPE-LC-API with MS and MS-MS detection. At least 10 unknown compounds were detected in the sample, but OPPs were not found (54,89). The authors indicated that SPE-LC-API-MS is ready to be used on a routine basis.

The SAMOS is based on SPE-HPLC-DAD. In one study, which applied SAMOS in analyzing samples from six European rivers, nearly all cases found one or more pesticide in concentrations over 1 μg/L; in many cases the pesticides were OCPs or OPPs (53).

Temephos was applied in a rice field of the Ebre Delta (Tarragona, Spain) via aircraft spraying at a rate of 250 ml/Ha. The calculated concentration range in the rice crop field water was between 41 and 125 μg/L. Only a maximum of 5.5% of the applied temephos was detected immediately following application. The analytical determination was performed using SPE followed by HPLC-TSP-MS in the PI mode (87).

Spliid and Køppen described a method using LLE and LC-APCI-MS for the analysis of water. The method proposed was then used to investigate the contamination of Danish groundwater with pesticides. More than 200 samples of groundwater collected from various areas of the country were analyzed. Metramitron was detected one or more times in concentrations ranging from the detection limit level to 19 μg/L (32).

Studies of the marine mammals stranded on the Oregon coast during 1991–95 indicate that organochlorine pollution locally was quite low. The values obtained range from 0.5 to 53.9 μg/g (wet weight) for *p,p'*-DDE. The animals thought to come from Californian waters exhibited higher levels of DDE. Even though Oregon's waters appeared to be relatively unpolluted with organochlorine contaminants, such pollution is still a problem in neighboring areas and poses a risk to these wide-ranging marine animals (9).

The FDA Pesticide Program of Residue Monitoring for Infants 6 to 11 months old in 1991, which tested mostly fresh foods, showed carbaryl consumed at over twice the level of any other.

A procedure for determining pesticide residues in fruit and vegetable (apples, potatoes, and carrots) using HPLC-UV detection has been reported. A monitoring study in which 40 samples of different origin were examined was carried out. α-Endosulfan was detected in one apple sample, and tetradifon was detected in a potato sample (57).

V. CONCLUSION

The combined HPLC fractionation–GC determination methods provide very low detection limits, clean chromatograms, and good identifications for OPP and OCP residues in fatty foods.

Ultraviolet-VIS is still the most common detector employed for OPP- and OCP-residue determination in clean matrices such as water. The methods utilizing straightforward extraction procedures and the improved optics of diode array detection demonstrate the applicability of HPLC for analysis of OPPs and their metabolites in food.

In food analysis, sensitivity is not the only requirement for analytical method development. Besides confirmation of the identity of pesticides, the identification of nontarget analytes is also important. One powerful tool is LC/MS, especially when it is combined with appropiate sample-treatment procedures; it allows one to obtain detection limits adequate for trace-level analysis. Liquid chromatography–MS has demonstrated that it is an effective way to obtain both qualitative and quantitative information.

In summary, the potential of the methods presented here for the analysis of real samples has not been thoroughly demonstrated. More studies on the presence of pesticide residues and their transformation products in real food samples will be needed.

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Liquid Chromatography of Hop Resin Components and Related Substances

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I. INTRODUCTION

The cultivated hop (*Humulus lupulus* L.) belongs to the botanical family Cannabinaceae. It is grown in a number of areas worldwide, principally in the United States, Europe, Australasia, and South America (1). Hops have been added to beer since at least the 16th century; today this is still essentially their only application. The agronomic properties of hops dictate that they grow most readily between latitudes of 35 and 55° of both hemispheres (2). This, together with economic considerations, has led some brewing nations to consider alternatives to hops for brewing, such as *Garcinia kola* (3).

This chapter will focus on the liquid chromatographic analysis of the commercially relevant nonvolatile material in hops and their processed counterparts, together with their subsequent derivatives in beers. As yet, there do not appear to be any reports of the chromatographic properties of the bittering components of *Garcinia kola*, so these will not be discussed further here.

II. HOP COMPOUNDS AND HOP-DERIVED COMPOUNDS

The major components of hop resin are the α- and β-acids (Fig. 1: I, II). They are termed *acids* because they have appreciable proton-donating tendencies: Humulone (Fig. 1: Ia) has a pKa of 5.1, and colupulone (Fig. 1: IIb) has a pKa of 6.1 (4). There are three major components of each, the variation being due to the presence of different carbon skeletons in the saturated acyl side chain. By inspection of their structures, it is clear that they possess a β,β'-triketone moiety, which betrays a propensity to chelate a wide range of metal cations. All of the hop compounds and hop-derived compounds considered here are UV-active and indeed demonstrate maximal molar absorption coefficients of the same order of magnitude as simple aromatic compounds (i.e., of the order of 10^4), so at the levels found in beers they can be readily detected using the UV detectors available today. The α- and β-acids demonstrate UV maxima at wavelengths of around 310–340 nm. These four features (acidity, the presence of homologues and structural isomers, metal-chelating properties, and UV activity) are common to many of the compounds considered here and dictate the chromatographic approaches for their isolation, separation, detection, and quantification.

α-acids (I)

β-acids (II)

trans-allo-iso-α-acids (V)

cis-anti-iso-α-acids (VI)

trans-iso-α-acids (III)

cis-iso-α-acids (IV)

hulupones (VII)

3-methyl-2-butene-1-thiol (VIII)

a) co-	R =	$-CH(CH_3)_2$
b) n-	R =	$-CH_2CH(CH_3)_2$
c) ad-	R =	$-CH(CH_3)CH_2CH_3$

tetrahydroiso-α-acids (IX)

ρ-iso-α-acids (X)

hexahydroiso-α-acids (XI)

Fig. 1 Hop resin components.

When added to the wort boiling stage of beer production, or when processed out with the brewing process, the α-acids undergo an isomerization reaction. An intramolecular rearrangement results in two series of five-membered ring compounds, the *trans*-iso-α-acids (Fig. 1: III) and the *cis*-iso-α-acids (Fig. 1: IV). It is these compounds that give beer much of its bitterness, that stabilize beer foams (5), and that are potent antibacterial agents (6). The concentrations of these iso-α-acids in beers typically range from 5 to 50 mg/L, but lower levels may be found in beers supplemented by their chemically modified counterparts, which are discussed in more detail later. There are a number of structural variants based on the iso-α-acids, in particular the *allo*-iso-α-acids (Fig. 1: V) and the *anti*-iso-α-acids (Fig. 1: VI). Though these may occur in beer, they are of relatively little importance and will not be considered further here. A minor series of bitter compounds can also be derived from the nonbitter β-acids. The hulupones (Fig. 1: VII) are generated in low yields by ill-defined degradation reactions that may be present in beer, typically at concentrations of 1–2 mg/L.

One difficulty with the presence of iso-α-acids in beer is their limited stability in visible light. Photochemical degradation of iso-α-acids in beer results in the highly flavor-active 3-methyl-2-butene-1-thiol (MBT; Fig. 1: VIII). A number of aspects related to this reaction have been reviewed by Templar et al. (7). Although canned and kegged products are immune from MBT formation, only brown glass bottles afford adequate protection from light. Clear or green glass bottles are poor barriers to the light required for MBT formation. One option that has become increasingly popular in recent years is the use of chemically modified variants of the iso-α-acids. Hydrogenation reduces both of the side-chain carbon–carbon double bonds. This results in the tetrahydroiso-α-acids (Fig. 1: IX), which are not susceptible to photochemical breakdown because the isohexenoyl double bond is necessary for MBT formation. Similarly, selective borohydride reduction of the isohexenoyl carbonyl to its corresponding carbinol gives the ρ-iso-α-acids (Fig. 1: X). These compounds also protect beer from the photochemical formation of MBT. Combining both hydrogenation and borohydride reduction yields the hexahydroiso-α-acids (Fig. 1: XI), which are, perhaps unsurprisingly, also light-stable. All of these chemically modified products can be added as a partial or total replacement for native iso-α-acids. The iso-α-acids and their chemically modified counterparts are UV-active at wavelengths around 254 nm. However, it is important to recognize the sensitivity of the UV spectra of these compounds to the presence of polyvalent metal cations (8) and pH (9).

It is worth pointing out that though hop-derived components might appear to be restricted solely to the brewing industry, much effort has been made to develop reliable chromatographic strategies for hop compounds and hop-derived compounds. This is because the hop and brewing industries rely on quantification data on which they can trade, and also so that brewers might effectively maintain beer quality. Furthermore, it is likely that the analysis of hop-derived compounds will be required outside its current sphere as these components penetrate new markets.

The scope of this review is limited to the hop-borne α- and β-acids and to products derived from them that have appreciable commercial relevance. Thus, the liquid chromatography of the iso-α-acids and their chemically modified counterparts as well as of the hulupones will be considered. By far the major focus here will be HPLC analyses, but other methodologies that have been applied (countercurrent distribution, gas chromatography, supercritical-fluid chromatography, thin-layer chromatography, and micellar electrokinetic chromatography) will also be briefly considered.

III. HISTORICAL ANALYTICAL APPROACHES

A comprehensive history of the methodology employed prior to 1960 for the analysis of hops has been compiled by Hudson (10). Particularly pertinent even then was an appreciation of the

difficulties inherent in handling hops and their extracts. Particular problems included the susceptibility of the α- and β-acids to oxidative degradation, the nontrivial problem of producing a representative sample of hops, and the hygroscopic nature of hop cones. Early applications of the chromatographic separation of hop extracts include those of Verzele and Govaert cited elsewhere (10), who used a silica gel column to prepare α-acid solutions for subsequent polarographic analysis. The development of high-performance liquid chromatography (HPLC) analyses for hop compounds and hop-derived compounds has been reviewed by Verhagen (11).

IV. COMPLEXITY OF HOP-COMPOUND AND HOP-DERIVED-COMPOUND MIXTURES

As mentioned earlier, the α- and β-acids occur as mixtures of homologues and structural isomers. There are three major components of each, traditionally labeled as the co-, *n*-, and ad- species.* The co-variants, bearing one methylene group less than the *n*- and ad-variants, are more polar and easily resolved from their less polar counterparts. However, it proves more challenging to resolve the *n*- and ad-variants, which have the same molecular formula and vary solely in the location of a side-chain branching point. Indeed, of the methods recommended for the reliable quantitation of the α- and β-acids, some are based on the coelution of the *n*- and ad-variants. This means that there is an implicit assumption that these compounds have the same response factors under the chromatographic conditions employed. In fact, generally, the co-, *n*-, and ad-variants are assumed to have the same response factors. This is not ideal, but such a compromise is necessary because purification of the individual compounds free from their homologues and congeners and free of degradation products is time-consuming, although the use of centrifugal partition chromatography (12,13) or fractional crystallization (14) has proved valuable in this respect. Indeed, when such studies have been attempted, the variation of molar absorption coefficients with solvent composition and pH makes the transfer of individual response factors from one laboratory to another a complex exercise. Even maintaining reproducible analyses within the same laboratory requires diligence.

Extending this issue to the iso-α-acids, the situation is complicated by the fact that each α-acid yields two stereoisomeric iso-α-acids, varying in the stereochemistry about one carbon. The stereoisomers come about because of the presence of two adjacent, optically active centers, induced by the ring contraction of the optically active α-acids. Resolution of the *cis*-iso-α-acids from their trans counterparts requires exacting chromatography, so in almost all cases chromatographic analyses are based on the coelution of the two co-variants and on the elution of the other four iso-α-acids as either one or two peaks. This puts an additional strain on the notion of equivalent response factors, but similar arguments about the difficulties inherent in the preparation of the pure single substances (16) and the dependence of response factors on the pH and composition of the mobile phase apply here. Thus, it is still by far the most common practice to assume equal response factors.

Consideration of the tetrahydroiso-α-acids depends on their precursors. They are manufactured commercially from, ultimately, either α- or β-acids. The chromatogram of those from the α-acid route demonstrate a chromatographic profile similar in detail to conventional iso-α-acids, with similar ratios of stereoisomers. Those derived from β-acids look quite different. Because the β-acids are racemic, there is a random distribution of orientations about the two adjacent chiral centers, which in practice results in the two peaks of about equal area for each of the

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* Although this nomenclature is rightly discouraged (15), it is used here strictly for ease of reference.

co-, n-, and ad-variants. However, whereas there will be six major tetrahydroiso-α-acids from an α-acid source, formally from β-acids there will be 12 components. Thus, the preparative work required to derive individual response factors for each of these compounds would be even greater. Again, most often a single response factor is assumed for both the α- and β-acid–derived tetrahydroiso-α-acids.

The complexity of the tetrahydroiso-α-acids from β-acids extends to the hexahydroiso-α-acids (also derived from β-acids), with the added complication that borohydride reduction of a carbonyl to a carbinol moiety results in yet another racemic, optically active center, theoretically giving rise to eight hexahydroiso-α-acids for each of the co-, n-, and ad-variants. This number of compounds has, as far as the author is aware, precluded the derivation of individual response factors for each of these compounds. Indeed, identification of all of the significant bands evident in a chromatogram of the hexahydroiso-α-acids has not yet been reported in the public domain.

The ρ-iso-α-acids are derived from the borohydride reduction of the iso-α-acids, so there are nominally 12 compounds—two from each of the iso-α-acids brought about by the formation of a racemic, optically active carbinol center from a carbonyl moiety. In practice, however, this does not occur as expected, with the *trans*-ρ-iso-α-acids being essentially absent from a ρ-iso-α-acid preparation. This is thought to be due to the steric hindrance experienced by the borohydride moiety when iso-α-acids are in the trans configuration. To justify the substantial losses of the trans species, a scheme by which the *trans*-isomers undergo retroisomerisation to α-acids and subsequent reisomerisation to the more borohydride-reactive cis orientation has been invoked (17). More recently, Ting and Goldstein have verified that, regardless of the initial cis/trans ratio of the iso-α-acids, *cis*-ρ-iso-α-acids are by far the major products (14).

In summary, then, the ideal situation by which each analyte is baseline resolved and quantified on the basis of unique response factors is as yet untenable. Even for the simplest case of the α- and β-acids this has proved difficult, and it becomes a more complex issue for the isomerized and chemically modified components. A number of research papers indicate that though there may be variation in response factors, these variations can, to a first approximation, be overlooked if there is agreement on methodology and the application of standard materials.

In fact, there are other considerations that complicate the compositional issue still further. The ad-variants bear a further optically active center as a result of the chain-branch position, which is likely to be racemic (it is adjacent to a carbonyl moiety). Because it is remote through space from other optical centers in α-acids and other optically active hop-derived components, it is unlikely to have a practical bearing on the properties and therefore the application of these compounds. More relevant though is the observation of minor components of the α-acids that have both shorter and longer side chains than the more abundant co-, n-, and ad-variants. Given that hydrophobicity is related to the potency of the brewing value of the hop-derived components, there is justification for the quantification of particularly the more hydrophobic species, as recently exemplified by Wilson et al. (18).

V. HPLC ANALYSES OF THE α- AND β-ACIDS

A. Recommended HPLC Analyses for the α- and β-Acids

There are currently a number of recommended methods of analysis for the determination of α- and β-acids in hops by HPLC (e.g., Fig. 2). The methods recommended by the Institute of Brewing, the American Society of Brewing Chemists, and the European Brewery Convention are summarized in Table 1. The method recommended by the latter two is in fact the same and is thus considered to be an international method. There are some key differences worthy of note here. Both clearly rely on the use of phosphoric acid to suppress the ionization of the acidic compo-

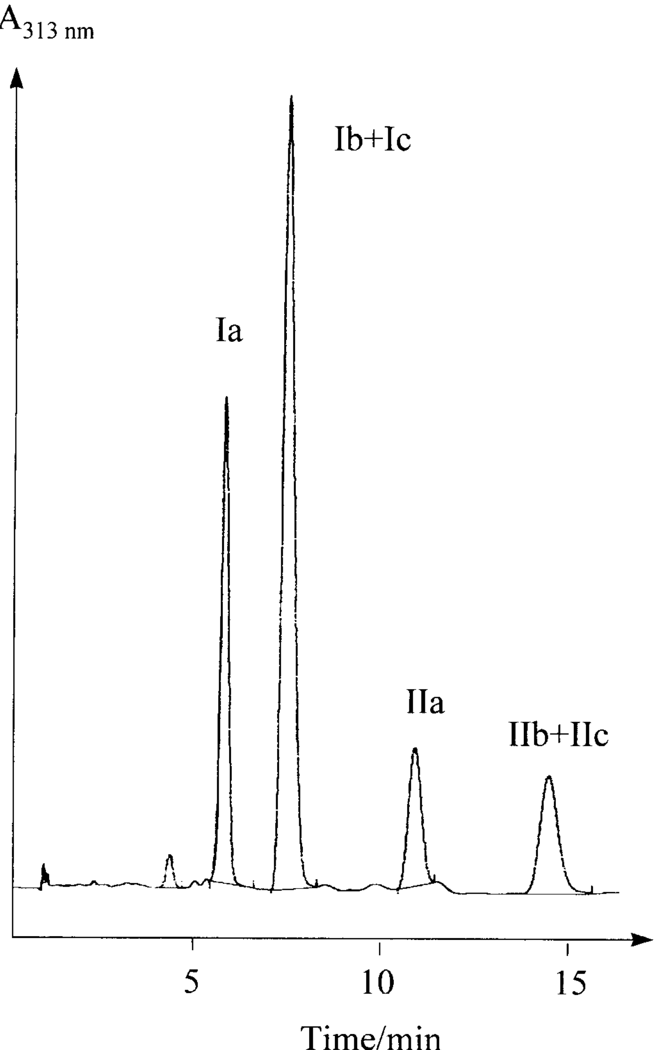

Fig. 2 Typical separation of α- and β-acids by reverse-phase HPLC. Note the coelution of the α-acids humulone and adhumulone (Ib and Ic) and the coelution of the β-acids lupulone and adlupulone (IIb and IIc).

nents, but the international method uses levels of phosphoric acid around 10 times that recommended by the Institute of Brewing. Such levels may result in a shorter column life. The international method also is less proscriptive concerning the column used, but it recommends the use of a Nucleosil C18 5-μm Macherey and Nagel, Düren, No. 720014 "Hop Analysis." This is because of the recognized effect on chromatographic behavior that trace levels of metal ions, such as iron(III), can have. This column is considered to be sufficiently low in levels of iron to enhance chromatographic reproducibility. In this respect, columns supplied by other manufacturers, such as the Merck Purasil cartridges, should also perform acceptably.

The European Brewery Convention also recommends a method for the simultaneous analysis of α-, β-, and iso-α-acids in a single chromatographic run. The method is more complex than the international method, because of differences in the UV maxima and in the polarities of the an-

Table 1 Recommended Methods for the Analysis of α- and β-acids

	Institute of Brewing	American Society of Brewing Chemists (ASBC)	European Brewery Convention
Method number (year)	6.5 (1997)	Hops-14 (1990)	7.7
Scope	α- and β-Acids in extracts and solutions	α- and β-Acids in hops, hop powders and conventional extracts	Same as ASBC
Column	Waters Nova-Pak C18, 5 μm, 100 × 8-mm ID	C18, 5 μm, 250 × 4.6-mm ID	Same as ASBC
Temperature (°C)	30 ± 1	Ambient	Same as ASBC
Detection (nm)	313	314	Same as ASBC
Standardization	*p*-nitroanilide of myristic acid	Calibration hop extract	Same as ASBC
Mobile phase	Methanol: water (85: 15 v/v) phosphoric acid (85%; 0.025% v/v)	Methanol: water: phosphoric acid (85%) 85:17:0.25 (v/v/v)	Same as ASBC
Flow-rate (ml min^{-1})	2.0	0.8	Same as ASBC
Sample size	20.0	10.0	Same as ASBC

alytes, and thus it requires gradient elution and detector wavelength switching during the run. The method is summarized in Table 2. The method also considers that there can be difficulties in obtaining satisfactory resolution of the components, recommending the addition of ethylenediaminetetraacetic acid (EDTA) to the mobile phase to bind any metal ions that may be present.

Calibration extracts are available from both the American Society of Brewing Chemists and the European Brewery Convention. In each case the extracts are calibrated for cohumulone, colupulone, (n+ad)humulone and (n+ad)lupulone on a % (w/w) basis. As with all hop extracts, it is essential to ensure that the calibration extracts are homogenized before use, because they do have a tendency to separate into layers during storage. This homogenization may be achieved by gentle warming of the extract (typically to 30–40°C), to decrease the extract viscosity, and then thorough stirring.

B. Alternative HPLC Analyses of the α- and β-Acids

There are many methods available in the scientific literature describing the separation and quantification of the hop acids. They may be classified according the mobile phase employed, for most if not all the reverse-phase methods are based on octyl (C8) and octadecyl (C18) columns (Table 3).

VI. HPLC ANALYSES OF THE ISO-α-ACIDS

A. Recommended HPLC Analyses for the Iso-α-Acids

Just as there are recommended methods of analysis for the α- and β-acids, so are there for the iso-α-acids (Table 2). Of these, two use tetraalkylammonium salt as an ion pair, whereas the other two rely on ionization suppression with phosphoric acid. Also evident is the variation in the de-

Table 2 Recommended Methods for the Analysis of Iso-α-Acids

	European Brewery Convention	American Society of Brewing Chemists		
Method number (year)	7.8	Hops-9C (1987)	Hops-9D (1988)	Hops-15 (1993)
Scope	α-, β-, and iso-α-acids in hop and isomerized hop extracts	Iso-α-acids in isomerized extracts	Iso-α-acids in isomerized extracts	Iso-α-acids in isomerized pellets
Column	250 × 4-mm-ID 5-μm ODS RP18 or "Grom Bitter Bier" column, 125 × 4.7-mm-ID 7-μm RP18	250 × 4.6-mm ID 10-μm reverse-phase column, e.g., Alltech RSL C18 No. 3427	Shim-Pack CLC-ODS/HP/N 228-00808-92, 250 × 4.6-mm-ID or equivalent	250 × 4.6-mm-ID 5-μm Nucleosil C18 column or equivalent
Temperature (°C)	35 ± 1	Ambient	Vary for optimal resolution	Ambient (20–25)
Detection (nm)	270 then 314	280	280	270
Standardization	Calibration extract (α-, β-acids) dicyclohexylamine : iso-α-acids complex	Internal: 4-methylbenzophenone; external: calibration extract	Internal: β-phenyl chalcone; external: calibration extract	Iso-α-acid standard (e.g., magnesium salt)
Mobile phase	Gradient elution: dependent on column; e.g., elute iso-α-acids with methanol : water : phosphoric acid (75 : 24 : 1 v/v/v), then increase proportion of methanol	Water : ion pair[a]:methanol : acetonitrile : THF (320 : 9.9 : 580 : 40 : 50 v/v/v/v/v)	Methanol : water + ion pair[b] (725 : 275 v/v) or methanol : water + ion pair[b] (780 : 220 v/v)	Methanol : water : phosphoric acid : 0.1 M EDTA (aq) (812 : 184 : 2.5 : 1 v/v/v/v)
Flow rate (ml min^{-1})	1.0	1.0	1.5	1.0
Sample size (μl)	10.0	10.0	10.0	10.0

[a] For every 320 ml water, add 9.9 ml 1 M tetrabutylammonium hydroxide and adjust to pH 6.8 with 85% (w/v) phosphoric acid.
[b] For each liter of mobile phase, add 17 g of 85% (w/v) phosphoric acid and 29.5 g of tetraethylammonium hydroxide.

Table 3 Representative Reverse-Phase HPLC Methods Cited in the Literature for α- and β-Acid Analysis

Mobile phase[a]	Column	n/ad separation	Temperature (°C)	Wavelength (nm)	Ref.
Methanol/ion pair/ water gradient	250 × 4.6-mm-ID Zorbax ODS or Shimpack ODS	No	Ambient	270	26
Methanol/ion pair/ water	Merck RP-8	No	35	314	27
Methanol/water/ phosphoric acid	100 × 8-mm-ID NovaPak C18 5-μm cartridge under radial compression	No	Ambient	313	28
Methanol/water/ phosphoric acid	250 × 4.5-mm-ID Alltech 10-μm #600RP	No	40	334	29
Methanol/acetate buffer	250 × 4.5-mm-ID Alltech 10-μm #600RP	Yes	40	334	29
Methanol/water/ phosphoric acid	250 × 4.6-mm-ID Alltech Rosil C18 D 5-μm	No	Ambient	270	30
Acetonitrile/water/ phosphoric acid gradient	250 × 4-mm-ID 5-μm Grom-Sil ODS-0 AB	Partial	Ambient	314	31
Methanol/water/ phosphoric acid	Zorbax SB C8	No	Ambient	MS detector	32

[a] All methods are isocratic unless stated otherwise.

tection wavelengths: 270 or 280 nm. The ion-pair systems are used with the longer detection wavelength. Verzele and de Keukeleire (33) consider that the chromatography of the iso-α-acids is much more sensitive to trace-metal ions than that of the α- and β-acids, and they recommend caution when evaluating columns and chromatographic systems for these analyses.

B. Alternative HPLC Analyses of the Iso-α-Acids

The requirements for the analysis of the iso-α-acids are diverse and, to a large extent, dictate the resolving power required of the chromatography. The requirement to separate cis/trans pairs of the iso-α-acids is often driven by research projects. Hughes (16) used a multicomponent mobile phase and a NovaPak C18 cartridge to obtain resolution of the five iso-α-acids in beer within 7 minutes (Fig. 3). The separation of *cis*-isohumulone (often the major iso-α-acid in beer) from *trans*-isoadhumulone (most minor of the six iso-α-acids) usually proves to be difficult. Other workers have successfully resolved the six compounds, and micro HPLC has proved particularly effective (34).

The preparation of the iso-α-acids as single components (for example, to derive individual response factors) by preparative or semipreparative HPLC is, unsurprisingly, difficult. Hughes (16) initially separated *cis*- and *trans*-iso-α-acids by forming salts of the latter with dicyclohexylamine (35) and the subsequent semipreparative purification of the *cis*- and the *trans*-isomers separately. To gain selectivity, ethanol was used as the organic modifier, although further gains in selectivity were not observed with propan-1-ol or propan-2-ol (36). Schwarzenbach (37) found that

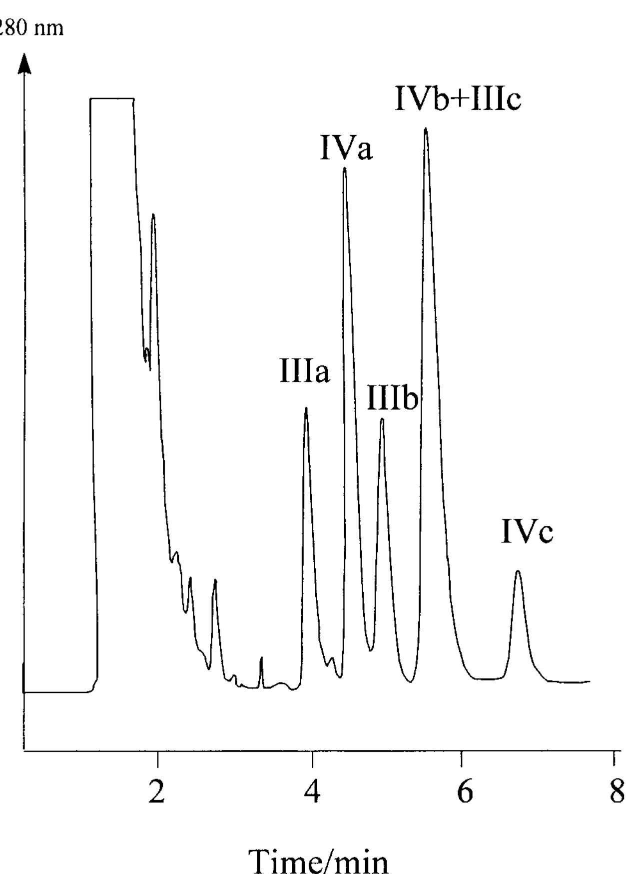

Fig. 3 Resolution of the six major iso-α-acids in beer into five bands by reverse-phase HPLC. Mobile phase: acetonitrile:water:methanol:0.2 M magnesium acetate (aq):formic acid:trifluoroacetic acid (840:490:480:24:3.6:1.2 v/v/v/v/v/v). Column: NovaPak C18 5 μm cartridge (100 × 8 mm i.d.) under radial compression. Mobile phase flow rate = 2 ml min^{-1}. (Chromatogram redrawn from original).

buffered normal-phase columns enabled the separation of *cis*- and *trans*-iso-α-acids, and Goldstein and Ting (14) consider that a combination of both normal- and reverse-phase chromatography is the most effective route to the preparation of single iso-α-acids.

VII. HPLC ANALYSIS OF THE CHEMICALLY MODIFIED ISO-α-ACIDS

The HPLC analysis of the chemically modified iso-α-acids is, in isolation, less problematic, for they tend to be more stable in solution than any of the native iso-α-acids, the α-acids, or the β-acids. However, quantification is not as straightforward because, at the time of writing, there are no independently calibrated standards available. Furthermore, the analytical requirements often include the analysis of mixtures of more than one class of hop-derived compounds, such as beer containing both iso-α-acids and tetrahydroiso-α-acids. There are no recommended methods of analysis, but a number of reverse-phase HPLC chromatograms have been published (14,38,39), although the actual chromatographic conditions required for these analyses are generally lacking.

Goldstein and Ting (14) reported that a chiral Cyclobond I column (Advanced Separation Technologies, Inc.) eluted with methanol/50 mM sodium citrate buffer (pH 4) could partially resolve the enantiomers that result from the conversion of α-acids to tetrahydroiso-α-acids. Clark et al. (40) considered in some detail the choice of column for the analysis of the tetrahydroiso-α-acids. They separate iso-α-acids and tetrahydroiso-α-acids in less than 30 minutes using a Zorbax XDB-C8 column, 250 × 4.6-mm ID, with a 5-μm packing and a gradient of methanol and acetonitrile/citrate buffer. Beer extracts of 50 μl were injected. Cope et al. (41) reported an automated method for the automated cleanup and isocratic separation of beer samples containing both iso-α-acids and their tetrahydro- counterparts. Cleanup was effected using 7-mm Empore Disc Cartridges (3M), and the eluted samples were subsequently chromatographed on a 150 × 4.6-mm-ID 5-μm Hypersil BDS C18 column at 40°C. The mobile phase consisted of methanol/phosphoric acid (7% v/v) with 0.5 mM aqueous EDTA (26:74) and an unusually large injection volume of 100 μl. This methodology was reported then to be in use at seven different Bass sites for routine quality assurance.

VIII. HPLC ANALYSIS OF THE HULUPONES

These components are relatively minor bittering agents in beers; nonetheless they are present at levels that can have a significant effect on the overall bitterness of beer (42). They are more polar than the iso-α-acids and therefore tend to elute earlier during a reverse-phase chromatographic run. This can make hulupone quantification in beer difficult unless some form of sample cleanup (e.g., solid-phase extraction) is employed. James et al. (43) described the synthesis and separation of cohulupone and hulupone using a NovaPak C18 column and isocratic elution with acetonitrile:water:phosphoric acid (55:45:1 v/v/v). Forster (44) reports that hulupones in beer are very susceptible to photochemical degradation, so it is prudent to protect samples for hulupone analysis from light.

IX. OTHER ANALYTICAL APPROACHES TO THE ANALYSIS OF HOP COMPOUNDS AND HOP-DERIVED COMPONENTS

Prior to the advent of HPLC, countercurrent distribution was the method of choice for the study of the individual hop acids (45). However, it was recognized that this was a time-consuming method and not suitable for routine application. There have been attempts to apply gas chromatographic separation to the analysis of a number of hop-derived compounds. Their low volatility and often thermal instability dictated that derivatization was required. Krueger et al. (46) generated the bis(trimethylsilyl) derivatives of the α-, β-, and iso-α-acids and achieved some degree of success. The author considers that, with the substantial improvements in gas chromatographic hardware that has occurred since this paper, the capillary GC approach ought to be revisited, especially for the tetra- and hexahydroiso-α-acids, which are likely to be more resistant to thermal deterioration.

Excellent chromatographic performance using micellar electrokinetic chromatography (MEKC) has been demonstrated for the α-, β-, and iso-α-acids. Thus the six major components of a mixture of α- and β-acids can be baseline resolved within 10 minutes (47). Similarly, the six major iso-α-acids can be baseline resolved within 20 minutes (48). De Keukeleire presented MEKC separations of the ρ- and tetrahydroiso-α-acids that compared well with conventional HPLC analyses (17).

Thin-layer chromatography (TLC) is a technique that has found extensive application for the analysis of hop compounds and hop-derived compounds (for example, Refs. 49–52), in common with other techniques, though this has fallen into disuse. Nevertheless, the discipline of planar chromatography has undergone radical development, and the author has found good agreement between HPLC and high-performance TLC (HPTLC) analyses of beer bitterness (53). Furthermore, wavelength scanning each band by UV gives scope for quantification that is independent of mobile phase.

Supercritical-fluid chromatography (SFC) has been demonstrated effective in the analysis of α-, β-, and iso-α-acids (54). However, few analytical laboratories have SFC installations, so its use as an analytical tool for the analysis of hop compounds is extremely limited. Interestingly, SFC and GC provide the opportunity to move away from the problems associated with differential response factors, because these methods rely on flame ionization detection.

X. THE FUTURE

The science of HPLC analysis of α-, β-, and iso-α-acids is mature, as indicated by the existence of collaboratively tested recommended methods. However, the recent increase in the popularity of the chemically modified compounds, with their wide-ranging effects on beer foam, bitterness, and antibacterial activities, means that it is difficult to rely on gross tests, such as spectrophotometry, for beer quality control. Although HPLC can be used effectively to achieve this, substantial coelution often occurs, particularly when more than one class of compounds is used (e.g., tetrahydro- and ρ-iso-α-acids).

Verzele and de Keukeleire have reviewed the difficulties associated with the standardization of hop acid analyses (33). In particular, at the time of writing, standardization for isomerized and chemically modified components is highly topical. As mentioned earlier, the *trans*-iso-α-acids specifically precipitate when a mixture of iso-α-acids is treated with dicyclohexylamine, and it has recently been shown that this behavior extends to the hexahydro-, ρ-, and (with difficulty) tetrahydroiso-α-acids (55). An international subcommittee has recently been convened to establish standards for the full range of chemically modified iso-α-acids to encourage international standardization for ensuring beer quality control and to provide an agreed basis on which these products can be traded.

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HPLC Analysis of Phenolic Compounds

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I. INTRODUCTION

Phenolic compounds are important components of many fruits, vegetables, and beverages, to which they contribute to flavor, color, and sensory properties such as bitterness and astringency. Recent interest in functional foods and the medicinal use of phenolic compounds have also stimulated interest in their chromatographic separation.

Due to the large number of and the structural variations in closely related food phenolic compounds, analytical procedures for the analysis of individual phenolic compounds have been relatively difficult and complicated. The analysis of phenolic compounds in food products can vary from simple colorimetric tests for detection to the use of sophisticated instrumentation for separation, quantitation, and characterization of individual components. In contrast to other liquid chromatographic methods (paper, thin-layer, and column chromatographies), HPLC approaches can provide a rapid response offering both high sensitivity and separation efficiencies through the use of tightly packed columns with small particles. There are no restrictions on sample volatility and derivitization requirements as there are in gas chromatography (GC). High-performance LC also provides a wide range of selectivity through the many solvent combinations and column packings available. Detection of phenolic compounds by paper chromatography (PC) and thin-layer chromatography (TLC) requires, in comparison with HPLC, considerably greater concentrations of extracts. For a simultaneous measurement of several phenolic compounds, either a gradient HPLC method or several isocratic methods using various combinations of mobile phases and/or analytical columns can be employed. Thus, in recognition of the complexity of food composition, HPLC with isocratic or, more commonly, gradient elution has invariably been the method of choice for separation of phenolics in food products.

Many excellent discussions of natural occurrence, structure, characterization, and analysis of phenolic compounds are available in the literature, and a series of books devoted to flavonoid chemistry has also been published. Detailed discussions on various chromatographic modes, including HPLC, GC, column chromatography (CC), capillary electrophoresis (CE), PC, and TLC, of simple phenolics and polyphenols are also presented in the recent book, *Handbook of Food Analysis, volume 1*, edited by Nollet (1). Due to their diversity and the chemical complexity of phenolic compounds, this chapter is limited to phenolic compounds that are considered to be important to foods and the food industry.

II. PHENOLIC COMPOUNDS IN FOODS

Phenolic compounds are a wide range of compounds that possess an aromatic ring bearing a hydroxyl substituent, including their functional derivatives, such as esters, methyl ethers, and glycosides (2). The phenolic compounds present in foods show considerable diversity in their structure and are divided into several different classes of compounds. In foods, flavonoids and related phenolic compounds exist in a multiplicity of complex conjugates with sugars and organic acids. Among the classes of simple monocyclic acids are phenolic acids. Phenolic acids embrace the benzoic acids (C_6–C_1) and cinnamic acids (C_6–C_3), as presented in Fig. 1. Phenolic acids occur naturally in a wide range of bound forms, such as esters, glycosides, and conjugated with organic acids. Phenolic acids are commonly found in fruits and vegetables, and the concentration varies greatly, depending on the tissue, cultivar, maturity, season, and various other factors (3). Another important class of phenolic compounds found in foods is flavonoids. Flavonoids are C_{15} compounds (C_6–C_3–C_6), present as the aglycone or in a glycoside form bound to various sugars, such as arabinose, glucose, galactose, rhamnose, and xylose. These polyphenols form a diverse range of compounds and can be classified into many classes (flavone, flavonol, flavanone, flavanol, anthocyanin, chalcone, isoflavanone, and isoflavone), as presented by Lee and Widmer (1), and each of the flavonoid classes can probably be further broken into subclasses based on their chromatographic behavior.

Most phenolic glycosides are water soluble, but the corresponding aglycones are usually less so. Phenolic substances are aromatic and therefore show intense absorption in the UV region of the spectrum (2). Most of the benzoic acid derivatives displayed their maxima at 246–262 nm, with a shoulder at 290–315 nm, except gallic and syringic acid, which have absorption maxima at 271 and 275 nm, respectively (4). The cinnamic acids absorb in two regions in the ultraviolet, one

Benzoic acid derivatives:

COOH

R_1 R_3

R_2

Gallic acid, R_1 = R_2 = R_3 = OH

p-Hydroxybenzoic acid, R_1 = R_3 = H, R_2 = OH

Protocatechuic acid, R_1 = R_2 = OH, R_3 = H

Vanillic acid, R_1 = OMe, R_2 = OH, R_3 = H

Syringic acid, R_1 = R_3 = OMe, R_2 = OH

Cinnamic acid derivatives:

H COOH

C=C

H

R_1 R_3

R_2

Caffeic acid, R_1 = R_2 = OH, R_3 = OH

p-Coumaric acid, R_1 = R_3 = H, R_2 = OH

Ferulic acid, R_1 = OMe, R_2 = OH, R_3 = H

Sinapic acid, R_1 = R_2 = OMe, R_3 = OH

Fig. 1 Structures of phenolic acids. (From Ref. 28.)

maximum occurring in the range 225–235 nm and the other between 290 and 330 nm (5). The four commonly occurring cinnamic acid derivatives—ferulic, sinapic, caffeic, and *p*-coumaric acids—have maxima at 300 nm (4). Chlorogenic and *p*-coumaric acid exhibited absorption maxima at 310 nm, with a shoulder between 270 and 290 nm, and flavonoids at 275–285 nm (6). Figures 2 and 3 present the spectra of common benzoic and cinnamic acids measured in MeOH solution.

Flavonoids typically have two major ultraviolet absorption bands, the first between 320 and 380 nm (band 1) and the second between 240 and 270 nm (band 2). Band 1 arises from the absorption of the B-ring portion of the flavonoid molecule; band 2 comes from absorption of the A-ring. Addition of substituents to an aromatic ring capable of electron donation, such as hydroxyl or methoxyl groups, usually induce strong (10–15 nm) bathochromic shifts for the absorption band associated with that ring (1). In the case of flavanones and isoflavones (5), absorption band 1, which is due to the conjugation between the B-ring and the carbonyl group of the pyrone ring, is lacking, or reduced to no more than a shoulder, as can be seen for the absorbance spectra of flavanone rutinosides hesperidin, narirutin, and didymin (Fig. 4), which are typical flavonoids in sweet oranges. In flavones, band 1 is most intense, occurring at 320–350 nm (1), and the absorption spectra of flavones may have a subsidiary peak in the form of a shoulder at 300–310 nm, which may be useful for their identification (5).

Phenolic compounds are of interest due to their potential contribution to the taste (astringency, bitterness, and sourness) and formation of off-flavor in foods, including tea, coffee, and various fruit juices, during storage. Their influence on the appearance of food products, such as haze formation and discoloration associated with browning in apple and grape products, is also significant. Furthermore, analysis of these phenolic compounds can permit taxonomic classification of the source of foods. The importance of each phenolic compound and its association with the quality of various foods is described further in Sec. IV, on food applications.

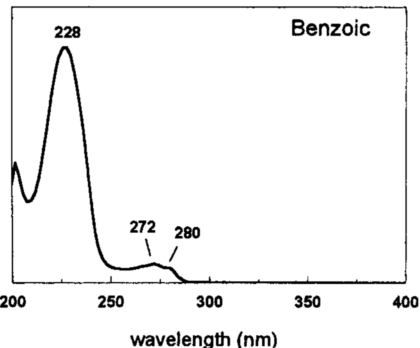
III. HPLC SYSTEMS FOR PHENOLIC COMPOUNDS

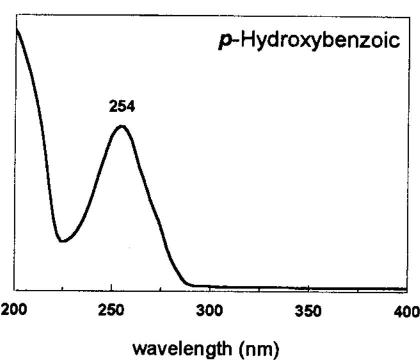
A. Chromatographic Conditions

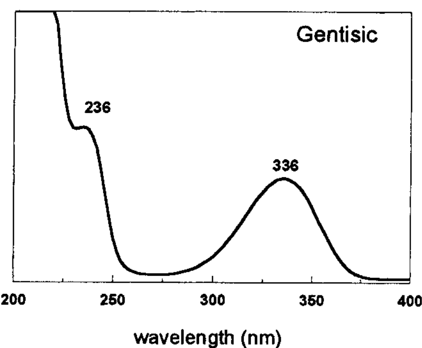
Reversed-phase chromatography is the most popular mode of analytical liquid chromatography for phenolic compounds. In most cases, the reported systems for the separation of phenolics and their glycosides in foods are carried out on reversed-phase chromatography on silica-based C_{18} bonded-phase columns. Occasionally, silica columns bonded with C_8 were applied in the analysis of phenolic acid standards and coumarins (7), and C_6 columns for the analysis of ferulic acid in wheat straw (8).

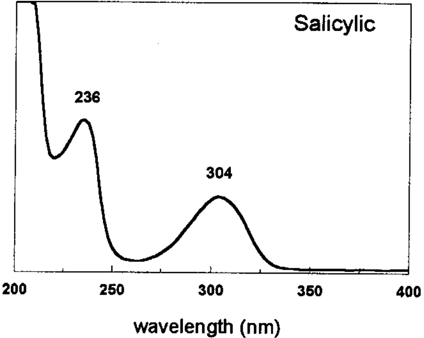
A reversed-phase HPLC method for food phenolics requires optimizing a wide variety of mobile-phase conditions (ionic strength, pH, ion pair, organic modifier, etc.) and column parameters. The majority of published HPLC techniques for phenolic compounds utilize a silica packing material with a particle size range from 3 to 10 μm in stainless steel columns having a typical length of 25 cm (or 15 cm) and a standard diameter of 4.6 mm, ID. Columns of smaller particle size (<3 μm) usually provide a larger number of plates per unit time than do columns with a larger particle size. However, 3-μm columns are somewhat more difficult to work with and are easily plugged.

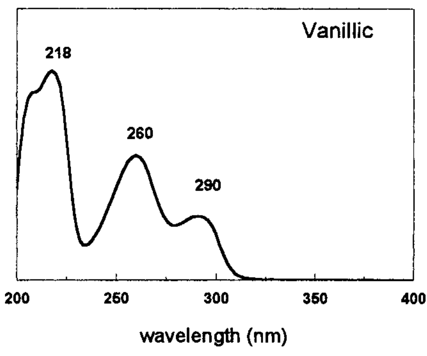
Most analytical columns for phenolics contain silica-based packing, and the choice of pore size and surface area of the column packing varies. The nature of the silica is one of the largest variables in reversed-phase stationary phases. As well as pore diameters and volume, pretreatment procedures make a large difference, even when the exact same bonding chemistry is used. A high-surface-area packing provides very good resolution for a wide range of phenolic











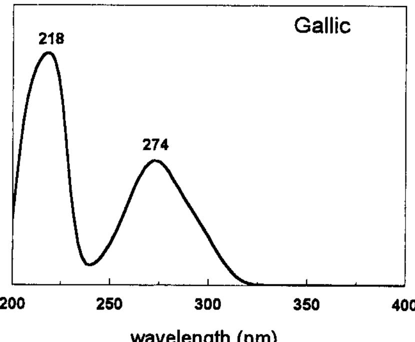
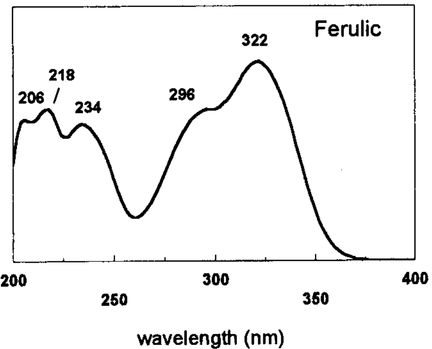
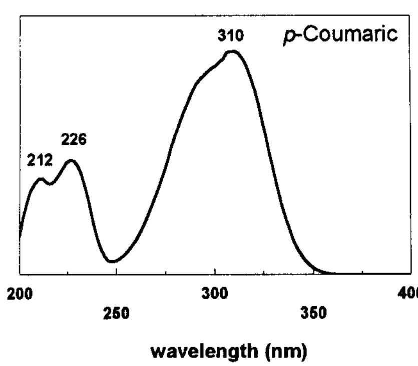
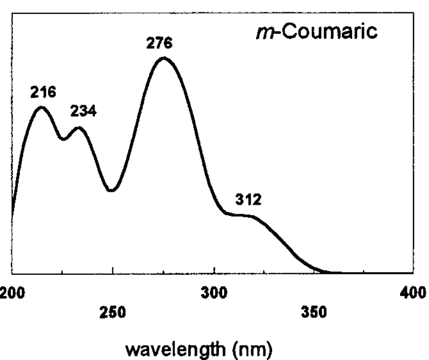
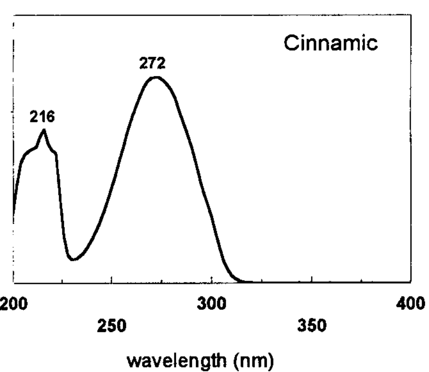


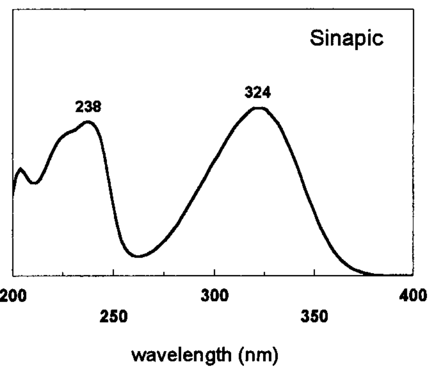
Fig. 2 Absorption spectra of benzoic acids.











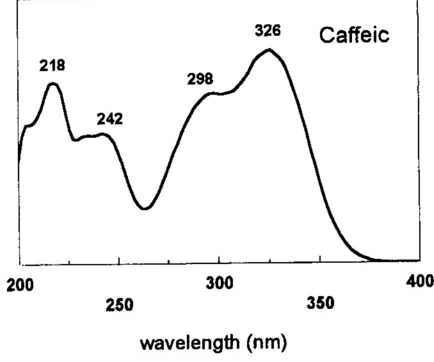


Fig. 3 Absorption spectra of cinnamic acids.

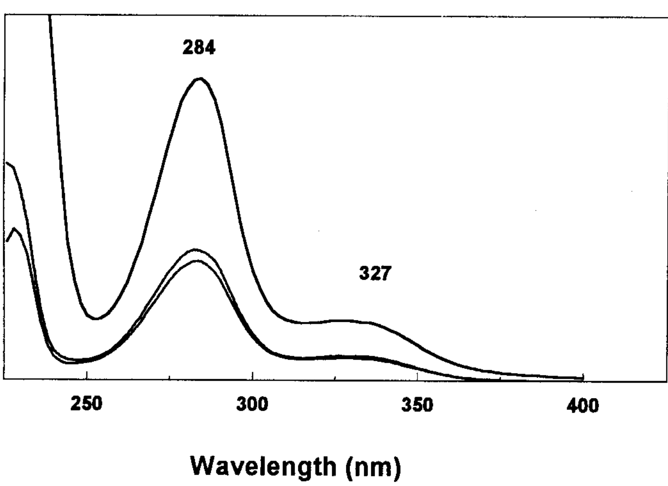


Fig. 4 Absorption spectra of flavanone glycosides.

compounds compared to a column with a lower surface area of packing (9). Also, chromatography with an HPLC column with a nonsilica polymer column based on polystyrene (PRP-1), which has no surface hydroxyl groups or surface metals for phenolics, provided lower resolving power and different selectivity than silica-based columns.

The use of short columns packed with very small particles can allow for very fast separations with considerable resolving power. A recent technical brochure from MAC-MOD Analytical, Inc. (1998), demonstrated high-speed and high-resolution chromatography with a short LC/MS cartridge column. Eight phenolic acids (gallic > protocatechuic > hydrocaffeic > gentisic > vanillic > syringic > sinapic > salicylic) were separated by gradient HPLC on a short column (Rapid resolution, Stablebond SB-C_{18}, 2.1-mm-ID × 30 mm) within 70 seconds of run time with 35 seconds of equilibration time. Thus, the analysis time was less than 2 min. This rapid-resolution LC/MS cartridge column is very short (15 and 30 mm) but packed with high efficiency; 3.5-μm particles are capable of providing both high-speed and high-resolution separations.

As higher efficiency and shorter columns translated into smaller peak volumes, HPLC systems required adaptation to avoid any extra column band broadening. Small-diameter connecting tubing as well as UV detection cells with reduced volume operating at the fastest response settings are some of the important considerations for maintaining the resolving power of the columns. Small-internal-diameter columns can reduce solvent consumption. However, the use of smaller columns would require further instrument optimization similar to a micro-column HPLC application. Also, short, narrow-bore columns can provide faster separations, but they do not have the separation power of a longer column. Thus, injection volume and solvent strength should be optimized to enhance the column efficiency when using low-volume columns.

The majority of reported HPLC methods for food phenolics, which include binary gradient elutions or occasionally isocratic elution, used solvents of aqueous acetic, formic, or phosphoric acids with methanol (MeOH) or acetonitrile (ACN) as an organic modifier (1). The range of solvent strengths used in gradient elutions and the time required for an analytical separation can be dependent on the number and type of phenolics in the food matrix. Often, multiple-step gradients are employed with complex mixtures or, even further, with a combination of linear, concave, and convex gradient programs. More sophisticated gradients, as presented in the food applications

section of this chapter (Sec. IV), are required for crude extracts of foods containing many types of phenolics, while isocratic methods can be employed for partially purified extracts or crude extracts containing only a few components of similar polarity.

The nature of the solvent, such as the solvent strength and the viscosity of the organic modifier, has an important influence on chromatographic separation. A water–acetonitrile gradient is often employed to reduce the elution time and to sharpen the peak shape for the late-eluting phenolics and to improve quantitation at low concentrations. However, methanol seemed preferable to acetonitrile because of its nontoxic nature and because a higher percentage of organic solvent could be used in the mobile phase to prevent deterioration of the reversed-phase column (10). Acetonitrile (ACN) gave better resolution in a shorter analysis time than methanol (MeOH), and tetrahydrofuran (THF) produced even better resolution for cinnamic acids in orange juice compared to systems employing methanol or acetonitrile (11). Generally, acetonitrile was found to give better and sharper peak shapes, resulting in a higher plate number than provided by methanol (12). Tetrahydrofuran has a higher solvent elution strength value (eluotropic strength) compared to acetonitrile, and acetonitrile has a higher value than methanol. Tetrahydrofuran was often chosen as the organic modifier instead of methanol or acetonitrile for phenolics (1). In some cases, the substitution of methanol with tetrahydrofuran improved resolution.

The pH range most often used for RP-HPLC for phenolics is low, between 2 and 4. The pH and ionic strength of the mobile phase are known to influence the retention of phenolics on the column, depending on whether there is protonation, dissociation, or a partial dissociation (12). Thus, pH plays a crucial role in determining retention and selectivity and in controlling the reproducibility and ruggedness of a method in RP-HPLC separations of phenolics. A change in pH that increases the ionization of a sample could reduce the retention in a reversed-phase separation. Thus, small amounts of acetic acid (2–5%), phosphoric, or trifluoroacetic acid (TFA) (0.1%) are included in the solvent system to suppress ionization of phenolic and/or carboxylic groups, improving the resolution and reproducibility of successive analyses. A relatively high concentration of acetic acid could result in a noisy baseline; using 0.1% TFA as the aqueous modifier could result in a more transparent mobile phase and could permit phenolic acids to elute as a very symmetrical band. Low pH has the advantage of creating an environment in which peak tailing is minimized.

Instead of using an organic modifier and an aqueous buffer in RP-HPLC, an ion-pairing agent such as 10 mg/L of SDS (sodium dodecyl sulfate) was also added to control the retention of the phenolic acids and flavonoids in fruit juices with little cleanup procedure (13). Ion-pair chromatography (IPC) is a useful HPLC technique for the separation of acids and bases, and can offer several advantages over RP-HPLC. However, a column that has been exposed to ion-pairing reagents can behave differently, altering the selectivity of the column. Ion-pairing agent is often used as a mobile-phase additive with samples that contain ionizable compounds, providing greater retention and higher selectivity than are afforded by the column and organic solvents alone.

When separating phenolic compounds, a buffered mobile phase such as phosphate buffer, ammonium acetate buffer, or citrate buffer is often used to maintain consistent retention and selectivity. A high salt concentration in the mobile phase can improve peak shape by suppressing solute and silica ionization as well as the secondary interactions between them. Also, a buffered mobile phase resists changes in pH to provide reproducible chromatography. Since a good pH control for buffers is limited to a pH range of 3.7–5.6 for acetate buffers, pH 2.0–8.0 for citrate buffer, and pH 1.8–3.5 and 5.8–8.0 for phosphate buffer, the pH of the aqueous mobile phase should be adjusted to optimize the analysis time and separation, depending on the buffer used. Buffer strength also should be high enough to enhance the separation; however, varying buffer concentration did not significantly affect the resolution or the retention times (10). Ionic strength

of these buffers can vary from 10 to 100 mM (1), but a buffer concentration range of 10–50 mM is generally recommended for most analyses.

Most HPLC applications used for phenolic analysis simply allow the room temperature to determine the operating temperature of the column, but elevated temperatures of between 30°C and 40°C are often applied for phenolics and derivatives in apples (14), carrots (15), apple juice (6,13), bilberry juice (16), and for cis-trans isomers of caffeic and *p*-coumaric acids in wines (17). Generally, a change in temperature has only a minor effect on band spacing in reversed-phase HPLC and has essentially no effect in normal-phase separations. Thermostatic control of the column temperature is generally recommended to provide reproducible retention.

Compound retention during RP-HPLC depends on the relative hydrophobicity of the sample compounds. As expected, the elution of phenolics for reversed-phase HPLC is in the order of decreasing polarity. Polarity is increased most by hydroxyls at the 4-position, followed by those at the 2- and 3-positions. Availability of the methoxy group and the acrylic substitution reduces polarity and increases retention times (4). Loss of polar hydroxy groups and/or addition of methoxy groups can decrease the polarity within each class of benzoic and cinnamic acid. Also, the presence of the ethylenic side chain in the cinnamic acids can reduce their polarity compared with similarly substituted benzoic acids (6). The elution order for benzoic acids is as follows (Table 1): gallic $>$ α-resorcylic $>$ protocatechuic $>$ γ-resorcylic $>$ gentisic $>$ *p*-hydroxyben-

Table 1 Chromatographic Retention and UV Data for Phenolic Acids

Phenolic acids	t_R (min)	Absorbance maxima (nm)	260/320 (ratio)	270/300 (ratio)
Benzoic acids				
4-hydroxybenzoic (*p*-hydroxybenzoic)	21.5	256	407	143
3-hydroxybenzoic (*m*-hydroxybenzoic)	29.4	237, 296	1.4	0.3
2-hydroxybenzoic (salicylic)	57.3	242, 301	0	0.2
2,6-dihydroxybenzoic (γ-resorcylic)	17.8	247, 306	0.9	0.1
2,6-dimethoxybenzoic	39.6	243, 280	14	2.2
2,5-dihydroxybenzoic (gentisic)	20.6	236, 327	0.1	0.1
β-resorcylic (2,4-dihydroxybenzoic)	26.1	256, 294	32	1.1
2,4-dimethoxybenzoic	61.7	256, 292	30	1.4
2,3-dihydroxybenzoic	29.2	245, 314	0.8	0.1
3,4-dihydroxybenzoic (protocatechuic)	10.1	258, 294	38	1.5
4-hydroxy-3-methoxybenzoic (vanillic)	30.9	260, 292	77	1.8
3,4-dimethoxybenzoic	56.3	260, 292	94	1.9
3,5-dihydroxybenzoic (α-resorcylic)	13.0	249, 306	2.2	0.5
3,5-dimetoxybenzoic	<70	249, 305	2.2	0.6
3,4,5-trihydroxybenzoic	6.5	271	27	2.8
4-hydroxy-3,5-dimethoxybenzoic (syringic)	36.4	275	31	2.2
2,4,6-trihydroxybenzoic	10.2	256, 293	41	2.1
Cinnamic acids				
3,4-dihydroxycinnamic (caffeic)	27.5	234, 290	0.3	0.4
4-hydroxycinnamic (*p*-coumaric)	47.7	232, 298	0.2	0.4
4-hydroxy-3,5-dimethoxycinnamic (sinapic)	58.7	238, 323	0.1	0.2
4-hydroxy-3-methoxycinnamic (ferulic)	55.0	236, 292	0.2	0.4
3-hydroxycinnamic (*m*-coumaric)	54.7	235, 279	2.5	2
2-hydroxycinnamic (*o*-coumaric)	65.4	234, 276	1.2	2.1

Source: Adapted from Ref. 27.

zoic > syringic > vanillic > *m*-hydroxybenzoic > β-resorcylic > benzoic > salicylic acid. Salicylic acid was retained longer than its para isomers, which may be due to intramolecular hydrogen bonding (18). Under the same condition, the elution order for cinnamic acids is caffeic > *p*-coumaric > sinapic > ferulic > *m*-coumaric > *o*-coumaric (19). Ferulic acid eluted after *p*-coumaric, which indicates that the methoxy ($—OCH_3$) substituent is nonpolar, for it increases in retention. Figure 5 presents the separation of some common phenolic compounds found in foods as analyzed by RP-HPLC. Phenolic compounds were separated using a binary gradient of 10 mM KH_2PO_4, pH 3.1 (eluent a) and 70% ACN (eluent b) on a Supelcosil LC-18 column (150 × 4.6-mm ID). The method described here has been shown to be applicable for the simultaneous determination of phenolic compounds and browning index compound in apple juice, as presented in the section on food applications of HPLC (Sec. IV).

Separation of geometric isomers of some phenolic compounds in standards as well as in food products is also possible by RP-HPLC. Reversed-phase HPLC based on a C_8 column has shown potential for the separation of geometric isomers of caffeic, ferulic, and isoferulic acids (20) using aqueous THF as a mobile phase, but this analysis is demonstrated only with standards corresponding to trans isomers in reversed-phase HPLC (21), which is a simple matrix compared to the complicated matrix of a food. Chromatographic methods enabling the separation of geometric isomers of caftaric, coutaric, and *p*-coumaric acid present in grapes and wine involved

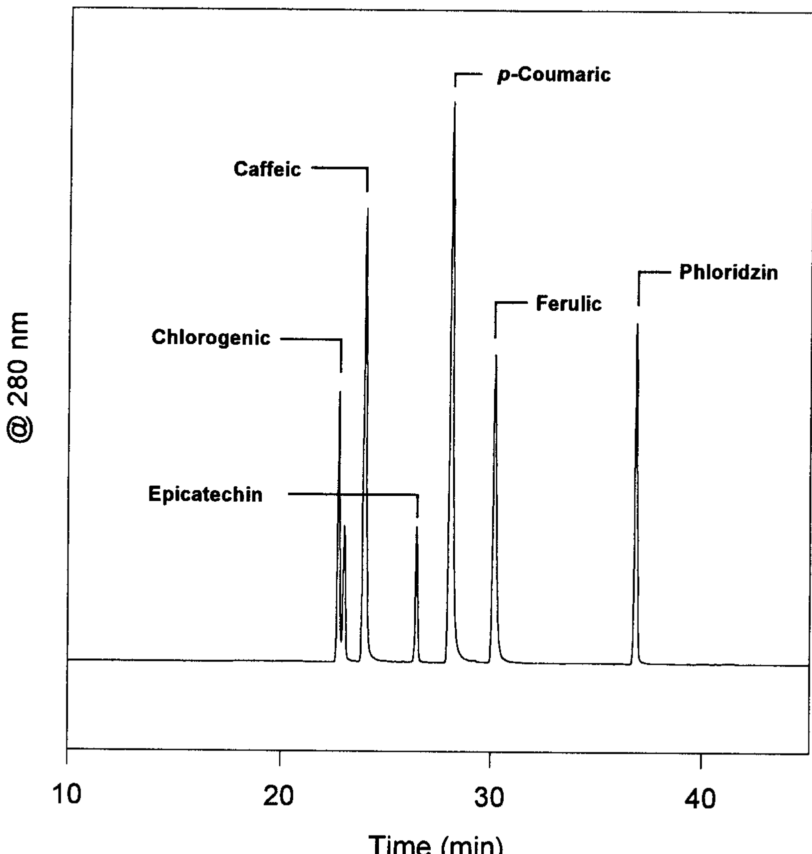


Fig. 5 HPLC separation of standard mixtures of phenolic acids.

reversed-phase separation based on C_{18} columns by binary gradient elution (17, 22), or simply by isocratic elution with 16% aqueous MeOH at pH 2.6 (23). The cis isomers of cinnamic are present in grapes and are known to elute before the corresponding trans isomers in RP-HPLC (21).

B. Mode of Detection and Identification

The variety of detection modes available for HPLC analysis that provide additional information about the eluent as it exits the column greatly facilitates unknown characterization. The majority of analytical methods for phenolic compounds includes HPLC with spectrophotometric-based detection techniques (UV absorption, fluorescence, photo diode array—PDA) as well as HPLC with electrochemical detection.

1. HPLC-UV

Phenolics absorb well in the UV region, and the most commonly used detector for HPLC is a variable-wavelength UV detector. This detector offers a high degree of sensitivity and is capable of collecting data from one or more wavelengths simultaneously. No single wavelength is ideal for monitoring all classes of phenolics, since they display absorbance maxima at different wavelengths (6). For maximum sensitivity, usually a wavelength near the maxima is desired; however, in practice, since absorption maxima can differ greatly between compounds, wavelengths are set for the best overall detection of all components.

The common phenolic acids have been detected at a single wavelength of 254 nm or 280 nm, by dual monitoring at 254 nm and 280 nm or at 280 nm and 320 nm, and by multiple-wavelength monitoring at 254 nm, 275 nm, and 300 nm. With detection at 320 nm, cinnamic acid derivatives can be detected without any interference from benzoic acid derivatives, which have a higher response at 254 nm. However, detection at 280 nm is at the best wavelength for the determination of both classes of phenolic compounds (10).

2. HPLC-Fluorescence

Fluorescence detectors are also used for phenolics (10,11) but have not been applied widely to the detection of phenolics. No advantage has been found for the detection of phenolic acids in beverages by fluorescence over UV detection (24). But in the analysis of orange juice, fluorescence detection offers some major advantages over UV detection in terms of enhanced selectivity and greater sensitivity. Dual fluorescence and absorbance detection have been used simultaneously to detect hydroxycinnamic acids and flavanone glycosides in orange juice (11), and for ferulic acid in ground wheat and flour (4). In orange juice, hesperidin and narirutin, major flavanone glycosides, do not fluoresce; therefore, they will not interfere with strongly fluorescing hydroxycinnamic acids.

Fluorescence detection was employed for quantitation of coumarins because of sensitivity and specificity. However, monitoring the fluorescence at one wavelength was not sufficient to observe all of the coumarin derivatives. A wide range of fluorescence maxima (314–346 nm for excitation and 420–517 nm for emission) for coumarins are also utilized possibly to identify the individual components and selectively to enhance the fluorescence of coumarins in citrus oils (25).

3. HPLC–Photo Diode Array

Spectrophotometers were improved somewhat by dual-wavelength detectors, but it was not until the development of the photodiode array (PDA) that spectrophotometric techniques were revolutionized. The photodiode array detector, which can acquire data in both the time and spectral domains, has led to considerable improvements in HPLC food analysis for the purpose of identifi-

cation and has demonstrated the usefulness of qualitative information in phenolic analysis based on the absorption spectrum (1).

Photodiode array detection has three major advantages for HPLC analysis (26): (a) multiple-wavelength detection, (b) peak identification, and (c) peak-purity determination. Since PDA can record the characteristic UV spectra of the different phenolics as they elute from the column, characterization and peak-purity information can be facilitated through comparison of the spectra at the front, the apex, and the tail of each peak. Furthermore, the rapid calculation of absorbance ratios between different wavelengths is possible, which can be used to classify the spectra by functional groups or by other criteria (Table 1).

A PDA can be employed to obtain spectral data and to study further some structural features, such as functional group conjugated with aromatic ring, degree of substitution, and position of substitution of unknown peaks of phenolics (27). The flavonoids consist of dozens of structurally similar compounds, typically differing only in the degree of ring substitution, the type of substitution (hydroxyls, methoxyls, etc.), and the type and degree of glycosylation. Thus, the addition of a second hydroxyl group to the basic structure has little effect on its spectrum by PDA. With their potent resolution, PDAs overcame many of the disadvantages of single- and dual-wavelength approaches; but even today, they still lack sensitivity. The PDA is by far the most commonly used detector for food phenolics, but, unlike electrochemical detection, it suffers from a lack of both discrimination and sensitivity. For a more in-depth look at the theories and benefits of HPLC-PDA, the reader can refer to the recent chromatographic science series book, *Diode Array Detection in HPLC* by Huber and George (26).

4. HPLC–Electrochemical Detection

HPLC-based electrochemical detection (HPLC-ECD) is very sensitive for those compounds that can be oxidized or reduced at low voltage potentials. Spectrophotometric-based HPLC techniques (UV absorption, fluorescence) measure a physical property of the molecule. Electrochemical detection, however, measures a compound by actually changing it chemically. The electrochemical detector (ECD) is becoming increasingly important for the determination of very small amounts of phenolics, for it provides enhanced sensitivity and selectivity. It has been applied in the detection of phenolic compounds in beer (28–30), wine (31), beverages (32), and olive oils (33). This procedure involves the separation of sample constituents by liquid chromatography prior to their oxidation at a glassy carbon electrode in a thin-layer electrochemical cell.

Electrochemical detection offers the advantage of superior sensitivity and selectivity over other HPLC detectors, which is very useful when analyzing real samples, reducing matrix effects, and consequently improving the quantitation and identification of analyte peaks and caffeic acids (28). Furthermore, ECD is almost insensitive to the changes in mobile-phase conditions associated with gradient elution. Thus, steady baselines can be achieved at high detector sensitivity settings. Besides sensitivity, EC detection in conjunction with PDA can be used in series to classify on the basis of conjugation pattern and hydroxyl substitution of wine and grape flavonoids (32). However, fouling of the working electrode by adsorbed phenols or their oxidation products has been encountered (33), which necessitates the removal of the electrode for cleaning purposes.

Many published articles on HPLC-ECD refer to the use of one of three voltammetric detectors (amperometric, coulometric, or polarographic). More detailed information on principles and techniques of various electrochemical detection modes can be obtained from the recent book, *Coulometric Electrode Array Detectors for HPLC* (34). There are also two electrode array detectors, the coulometric electrode array system and the CoulArray detector, currently available. Both detectors offer the qualitative data of PDA and the extreme sensitivity of ECD (34). The

coulometric array detector has added a new dimension to the HPLC analysis of food phenolics. The use of an array of up to 16 coulometric sensors in series allows increased resolution for HPLC analysis of phenolics based on differences in their voltametric properties. This technique may be utilized to detect adulteration and source classification in many fruit juice products (35). The basis for differences in the ease of oxidation within and among compound classes corresponds to the patterns of aromatic substituents. Differentiation within this group by HPLC-ECD could be based on variations in the substitution pattern of alkoxy, alkyl, hydroxyl, and glycosidic groups on the aromatic rings. Based on an informative brochure from ESA, Inc., the HPLC-ECD method permits the sensitive and selective measurements of catechins in tea and complex biological matrices such as human plasma and urine samples without the need for extensive sample preparation. The resolution of the array permitted online voltammetric validation of the analytes, and it is able to measure tea catechins in human plasma with very low limits of detection.

5. HPLC–Mass Spectrometry

Numerous papers have relied on only UV-visible spectra for their identification of phenolics, but for positive identification purposes, HPLC–mass spectrometry (MS) is another detection mode that can provide detection of all phenolic compounds in foods. This technique involves a hyphenated instrument that uses a mass spectrometer as a detector for HPLC or uses HPLC as cleanup step for mass spectrometry. After preparative HPLC, the MS technique has frequently been employed for structural identification of phenolics in many foods and essential oils because of its sensitivity and selectivity and its ability to provide structural information.

Recent advances in electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), thermospray, and particle beam LC-MS have advanced the analyst toward the universal HPLC detector, but price and complexity are still the primary stumbling blocks. Thus, HPLC-MS remains expensive and the technology has only recently been described. Early commercial LC-MS uses particle beam and thermospray sources, but ESI and APCI interfaces now dominate. Liquid chromatography MS can represent a fast and reliable method for structural analyses of nonvolatile compounds such as phenolic compounds (36,37), especially for low-molecular-weight plant phenolics (38), but the limited resolving power of LC hinders the widespread use of its application for phenolics as compared to GC-MS.

Since flavonol glycosides are thermolabile compounds, fragmentation patterns were used to identify the structure. Liquid chromatography–thermospray (TSP) ionization furnished parent species with few and diagnostic fragment ions, thus allowing structure elucidation as well as discrimination between different glycosylation sites. Since TSP provides information on molecular ions, sugar sequence, and glycosylation site, TSP can be of high value for the identification of phenolic compounds occurring in foods (37). Moreover, simultaneous detection by flow splitting or connecting serially with PDA can also provide confirmatory data based on UV spectra (38). A diode array detector supplements the single-quad MS in most benchtop systems to provide information on peak purity and compound identity. The LC-MS method is reliable and rapid although not particularly sensitive (detection limits were of the order of 1 μg for phenolic acids), and it has an important future for analysis of food phenolics. A recent application report, #262 from Finnigan, also demonstrated the utility of electrospray ionization of a tandem mass spectrometry (MS/MS) detector for HPLC in both detecting and characterizing polyphenolic compounds in tea and many fruits. The more expensive LC-MS-MS mode measures the mass of the primary ions as well as the pattern of masses of progeny ions for detailed information on the molecular structure of unknown compounds.

Other modes of detection, such as nuclear magnetic resonance (NMR) and Fourier transform infrared (FT-IR), could be advantageous detection techniques, especially to validate the HPLC of phenolics in various foods, but the cost and complexity of the instrumentation limit the

widespread utilization of this detection mode. Nuclear magnetic resonance (^1H and ^{13}C) spectroscopy is one of the most valuable methods for the identification and characterization of structural analysis. The structures are elucidated mainly on the basis of one- and two-dimensional NMR spectroscopy, and in addition to electron impact (EI) or fast-atom bombardment (FAB) mass spectra, the NMR spectrum was recorded to support further confirmation of the positions of the substituents in the phenolics.

C. Sample Preparation Techniques for HPLC

The separation between glycosides and aglycones, which can be successfully accomplished by RP-HPLC, is not always possible in a complex mixture containing many compounds from each group. Thus, an initial extraction step is often required to separate the different classes of phenolic compounds. Column flushing has been utilized in the analysis of glycosides to remove the remaining groups of compounds at the end of the chromatography (39) as an alternative to the extraction procedure.

Except where crude estimates of total phenolics are desired, the initial procedure for the analysis of phenolics of raw or processed food matrices will be extraction. The extraction procedure is highly dependent on the type of food to be analyzed, what phenolic compounds are of interest, the chemical properties of the phenolics (i.e., polarity, acidity, and hydrogen-bonding capacity of the hydroxyl groups to the aromatic ring), and the procedure utilized for analysis. For the analysis of phenolic compounds in foods, extraction and purification have been accomplished by a wide variety of sample preparation techniques. However, of primary importance is the increase of surface area of the food by crushing, milling, macerating, or grinding, which will allow for better contact of the extraction solvent with the sample.

Commonly used extraction solvents are ethyl acetate, diethyl ether, methanol, and aqueous methanol, but the majority of the free phenolic compounds can be extracted with alcohols (methanol or ethanol) or alcohol–water mixtures (1). Due to the differences in polarity between components (40), neither diethyl ether nor ethyl acetate are able to extract completely all the phenolic compounds in a liquid–liquid extraction. Thus, successive extraction with diethyl ether and then ethyl acetate has been used for phenolics in fruit juices (41). When using alcohol–water mixtures (40), repeated extraction or reflux for 1 h are necessary to extract free phenolic acids as well as their glycosides.

In some circumstances, hot water (42) is often useful in the extraction of polar flavonoids that occur in the form of glycosides or are bound to some other polar acyl group (i.e., organic acid). However, organic solvents (methanol, ethanol, isopropanol, and acetone) are usually necessary for complete extraction. Less polar solvents, such as the chlorinated hydrocarbons (CH_2Cl_2 and $CHCl_3$), benzene (Bz), diethyl ether (Et_2O), and ethyl acetate (EtOAc), are used for the extraction of less polar aglycones and highly methoxylated aglycones, which are usually located on the exterior surfaces of fruits and vegetables. Often, dipping the fruit or plant part in solvent for several minutes can efficiently extract apolar components.

For beers, wines, and similar alcoholic beverages, it is necessary first to remove the ethanol by degassing (27) or evaporation (17). The extraction efficiency of another organic solvent (ethyl acetate, ether, or chlorinated solvents) will be reduced by the presence of alcohol, changing the partitioning characteristics of the solvent, which can result in incomplete extraction and the additional extraction of potentially interfering compounds along with the phenolics of interest. When the alcohol is removed, care should be taken to minimize the effects of heat and air, which could cause degradation of flavanols, anthocyanins, and some phenolic acids.

The determination of the free phenolic acids in foods requires hydrolysis, because the phenolics are usually found in the conjugated form and rarely in the free state. When the separation

and determination of the phenolic esters in foods are required, the hydrolysis step is omitted. The determination of phenolics can employ both acid and alkaline hydrolysis, or enzymatic hydrolysis with β-glucosidase (43). Rupturing of glycosidic linkages can be performed by acid hydrolysis, heating in a water bath to 100°C with 2N HCl for 2 h or more. Alkali hydrolysis of benzoic and cinnamic acid esters can be accomplished utilizing 2N NaOH for 4–24 h at room temperature (4,44) or for 90 min at 60°C (45) under N_2. Attaining optimum hydrolysis can be accomplished by holding for 6 h at room temperature in 2N NaOH (44). To prevent oxidation, it is important to perform alkaline hydrolysis reactions in an inert atmosphere, nitrogen gas.

Sample cleanup of extracts has become a critical part of the methodology prior to analysis by HPLC, allowing for the removal of potential interferences. Cleanup procedures can vary according to the food matrix. These techniques include liquid–liquid partitioning with a nonmiscible solvent and gel chromatography based on the hydrogen-bonding properties of Sephadex LH-20, and open-column chromatography on polyamide (such as polyvinylpyrrolidone, polyvinylpolypyrrolidone, and nylon), or Amberlite XAD-2; prep-HPLC; and solid-phase extraction utilizing commercially available disposable cartridges. An insoluble form of polyvinylpyrrolidone (PVP) can adsorb larger quantities of polyphenols compared to the same weight of nylon, such as nylon 66 powder.

The preferred method of efficient sample cleanup of crude extracts has become the use of small, disposable solid-phase extraction (SPE) cartridges containing a variety of HPLC packing materials, which include a full range of silica-based polar and nonpolar stationary phases. Solid-phase extraction on C_{18} bonded phase is commonly used for the isolation of phenolics, replacing the use of column chromatography on polyvinylpolypyrrolidone (PVPP) or Sephadex in the purification step. Sample cleanup using the SPE disposable cartridge prior to the HPLC analysis of beer (30), wine (17,31), and other beverages (46–48) is proving to be an invaluable technique.

IV. APPLICATIONS IN FOOD ANALYSIS

Chromatographic analysis of food phenolics is a useful tool in such areas as characterization, chemotaxonomic studies, varietal and source classification, the study of processing and subsequent storage-related variability, and even for the study of food phenolics relating to health benefits. While the natural occurrence of some phenolic compounds is common in many different plants, some are specific to one class of plant or even a single species. Phenolic analyses are also applied to determine the authenticity of raw materials and processed products in terms of species, variety, and geographical origin. Since the application of reversed-phase HPLC in 1976 by Wulf and Nagel (18) with chemically bonded silica-gel, μBondapak C_{18} column with isocratic elution with aqueous acidified methanol (methanol–acetic acid–water, 30:5:65) for separating phenolic compounds, HPLC is the most commonly applied chromatographic method for the variety of phenolic compounds in foods. Since many of the references on chromatographic applications for food phenolics have been tabulated in a previous author's work (1), this chapter will focus on more recent advances in HPLC technique for phenolics found in food materials. Also, discussions about anthocyanins are not included with flavonoids because this subject is covered in Chapter 20.

A. Fruits and Fruit Products

Since phenolic compounds occur in many fruits and most of them contribute to color and taste, phenolic analysis of fruits has been an active research area, especially in apple, grape, and citrus fruits and their products, such as juice, cider, and wine.

For fruits and their products, HPLC techniques for phenolics have been used to study the effect of processing, concentration, and storage on the phenolic composition of juices as well as a potential precursor for an off-flavor compound in juices. Phenolic analysis has been further applied to the detection of economic adulteration and especially to verify the authenticity of fruit juices. This is especially important when cheaper fruits can be added to more expensive ones in a fraudulent manner. In most fruits, the nonanthocyanin flavonoids consist mainly of flavonols and flavanols, with trace amounts of flavones. Glycosides are the predominant forms present. These most often are separated by reversed-phase HPLC on C_{18} columns with gradients consisting of acidified H_2O and ACN, MeOH, or EtOH.

Fruits are commonly classified as pomaceous fruits, stone fruits, berries, tropical and subtropical fruits, hard-shelled dry fruits, and wild fruits, but grapes are presented separately with their major consumption form, wine. Citrus fruits are also presented separately from tropical/ subtropical fruits.

1. Pome Fruits

High-performance LC analysis of phenolics in apples and pear juices are often performed by gradient procedures (49–54), due to the presence of several phenolic classes. Most of the HPLC methods applied to apple phenolics are very similar to that of Oleszek et al. (55).

The phenolic composition of apple consists of cinnamic acids, flavonols, dihydrochalcones, and flavan-3-ols (50,56). In the apple fruit processing industry, hydroxycinnamic acid derivatives and flavan-3-ols are important due to their contribution to the astringency, haze, and browning in apple juice and cider. Chlorogenic acid represents the major hydroxycinnamic acid derivative. The flavan-3-ols (catechins) are present in the monomeric form as well as in oligomeric and polymeric forms (procyanidins) in apple and apple products (56).

Many different sample preparation procedures have been employed, ranging from simple filtration of juice products to solvent extraction, and extraction by SPE using C_{18}, Sephadex LH-20 (49,50,52), and Amberlite XAD-2 (51,54,57). The Amberlite XAD-2 cleaning step has been used for many phenolic extracts, especially for fruit purees, to remove the sugars and other polar compounds. However, due to the low recovery rate with Amberlite XAD-2 for certain phenol glycosides, a modified sample preparation technique is needed, especially for quantification of arbutin in pear juice and blends (54). Figure 6 describes the fractionation procedure for phenolics using a Sephadex LH-20 column (58).

For phenolics in fruit juices (apple and pear), phenolic acids and flavonol glycosides are prepared by simple filtration of the juice, while procyanidins are isolated by SPE on Sephadex LH-20 (49,50). A gradient of 2–40% MeOH in 70 mM phosphate buffer (pH 2.5) on a Supelcosil C_{18} column was used, which was also applied to grape juice phenolics (22). Phenolic acids in pear juice eluted in the first 50 min, followed by rutin, quercetin galactoside, quercetin glucoside, phlorizidin, quercetin xyloside, quercetin arabinoside, quercetin rhamnoside, and three glycosides of isorhamnetin. Using the same mobile phase, the procyanidins in the Sephadex LH-20 fraction were separated along with (+)-catachin and (−)-epicatachin. A similar HPLC condition was also applied to study the effects of phenolics on haze development in apple juice (52). Phenolic acid (20% MeOH) and procyanidin (100% MeOH) fractions were separated from Sephadex LH-20 (2-ml bed volume), and analyzed by gradient HPLC using methanol (eluent A) and 10 mM KH_2PO_4, pH 2.5 (eluent B) on RP-18 Spheri 5 column. Phenolic compounds appear to be one of the major determinants of haze potential in clarified apple juice. A quantitative polyphenol profile in apple juice by HPLC was also applied to monitor the changes in polyphenol composition throughout the membrane clarification process. Separation of apple juice phenolics was achieved by using a C_6 column at 34°C, with 2 mM phosphoric acid (eluent A) and methanol (eluent B).

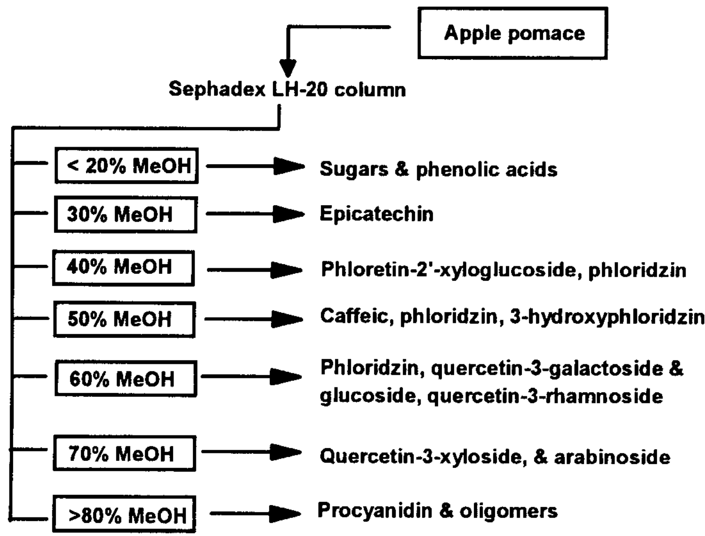


Fig. 6 Chromatographic fractionation of phenolics by Sephadex LH-20. (From Ref. 58.)

Table 2 Gradient HPLC Procedure for Phenolic Acids, Flavonol Glycosides, and Procyanidins in Fruit Juices

HPLC condition

Column: Supelcosil LC-18 (250 × 4.6-mm ID, 5 μm)

Mobile phase: Eluent (A): 70 mM KH_2PO_4, pH 2.5; eluent (B): methanol

Time (min)	0	5	45	57
A (%)	98	98	60	60
B (%)	2	2	40	40

Flow rate: 1.0 ml/min

Column temperature: Ambient

Detection: Diode array detection (UV 280 nm and 320 nm)

Sample preparation

Fractionate phenolic acids and flavonoids using Sephadex LH-20 column. For phenolic acids and flavonol glycosides, filter the fruit juice through 0.45-μm (type HA) filter. For procyanidins: apply juice onto the Sephadex LH-20 column, wash with 20% MeOH (30 ml), then elute with MeOH (15 ml), concentrate to dryness, redissolve in 2 ml H_2O, and filter through 0.45-μm (type HA) filter.

Source: Refs. 22, 49, and 50.

Table 2 describes the HPLC procedure for phenolics in fruit juices, which is based on previous works (22,49,50). A typical extraction procedure for obtaining phenolics from fruit juices is shown in Figure 7.

For phenolics in fruit purees and jams (54), an HPLC condition similar to that used for apple juice, but with acidified water (5% formic acid) and methanol, was utilized as a solvent system. In most cases, detection was achieved with diode array detection, at UV 280 nm and 320 nm. The different phenolic compounds were identified by their UV spectra and by chromatographic comparisons with authentic standards. Several classes of phenolic compounds (cinnamic acids, catechins, dihydrochalcones, and flavonol glycosides) could be detected along with arbutin in

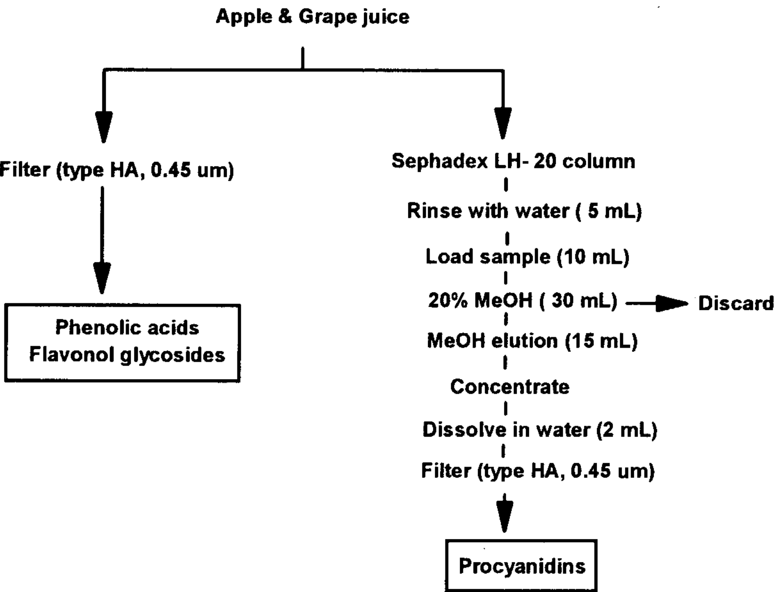


Fig. 7 Sample preparation procedure for phenolics in fruit juices. (From Refs. 22, 49, and 50.)

fruit puree. Arbutin, the characteristic hydroxyquinone glucoside, occurs in pear. Thus, the addition of pear to the other fruit puree and jams could easily be detected by the presence of arbutin (54).

For dihydrochalcone glycosides in fruit jams, HPLC with a binary gradient elution solvent system consisting of 5–80% MeOH and 95–20% of an aqueous solution containing 5% formic acid is also applied in apple juice and jam (51). A series of extractions with 50% MeOH and BuOH and preparative XAD-2 column chromatography procedures are utilized to isolate the phenolic compound fraction from apple jam. For dihydrochalcone glycosides (phloretin glucoside and phloretin xyloglucoside) in apple juice, nectars, and mashes, HPLC/PDA using a direct injection technique is applied (59). This direct injection method, including centrifugation (3000 rpm for 15 min) and filtration through a 0.22-μm cellulose-acetate filter, provided excellent recovery and precision, with a detection limit of 1.6 mg/L for dihydrochalcones. Dihydrochalcone glycoside is suggested as a quality-marker compound for apple juice identification, since there are no dihydrochalcone derivatives found in pear, strawberry, or raspberry samples (59). The presence of phloridzin (phloretin glucoside) has been regarded as characteristic of apple fruit and apple juice and has been employed in determining the authenticity of apple products (58,60).

For the simultaneous detection of fruit juice phenolics as well as browning reaction degradation products such as HMF (5-hydroxymethylfurfural) and furfural, an HPLC method with a combination of UV and EC detectors is useful (53). The HMF and furfural are not phenolic compounds but are known as quality indicators in various fruit juices. A gradient elution solvent system consisting of 2–40% acetonitrile and 98–60% of an aqueous solution containing 0.75% TFA was developed. A combination of two detectors (UV and EC) could increase the sensitivity significantly, especially for minor compounds such as caffeic, *p*-coumaric, ferulic, gallic, and protocatechuic acids and catechins in apple juice with simple filtration of juice. Figure 8 presents the HPLC chromatography of apple juice phenolics as well as HMF conducted in the author's lab. The chromatogram of apple juice stored at 50°C for 5 days was compared with the control apple

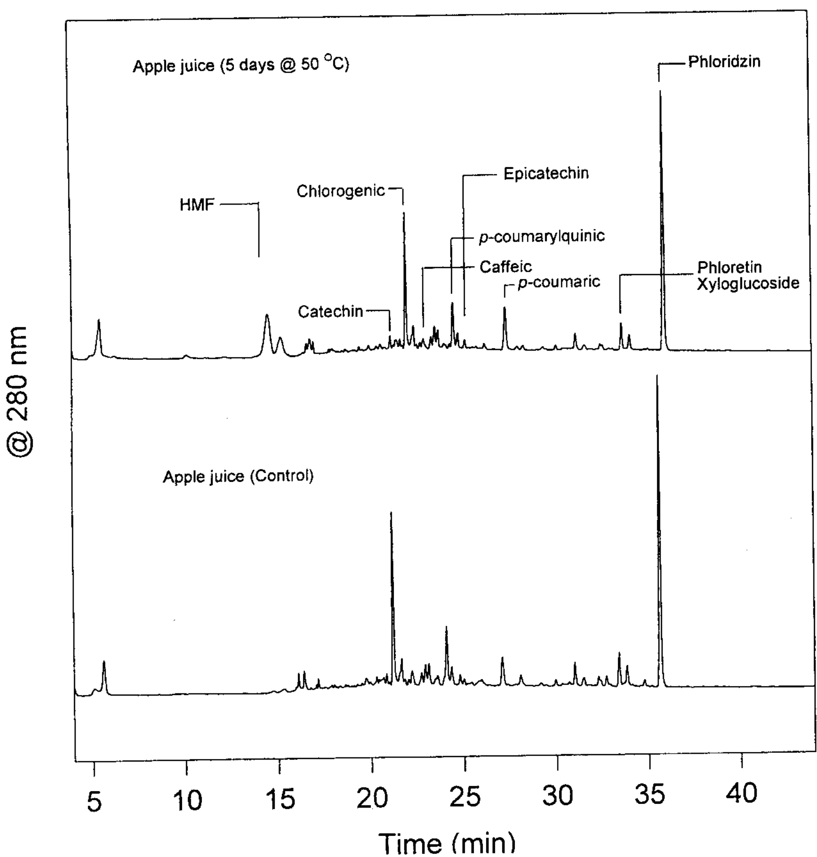


Fig. 8 HPLC separation of phenolics and HMF from apple juice.

juice. The HPLC apparatus was a Waters system (600E multisolvent delivery system, 717 plus autosampler, and the Millennium 2010 Chromatography Manager system). The HPLC column was a Supelcosil LC-18 column (150 × 4.6-mm ID, 3 μm). The solvent system used was a gradient of 10 mM KH_2PO_4 (eluent A) and 70% acetonitrile (eluent B), and the elution condition was based on a previous work by Kim and Lee (61).

For phenolics in fruit by-products such as apple seed, peel, cortex, and pomace, an HPLC method was also utilized. Apple waste is considered a potential source of specialty chemicals (58,62), and its quantitative polyphenol profile may be useful in apple cultivars for classification and identification. Chlorogenic acid and coumaroylquinic acids and phloridzin are known to be major phenolics in apple juice (53). However, in contrast to apple polyphenolics, HPLC with a 70% aqueous acetone extract of apple seeds showed that phloridzin alone accounts for ca. 75% of the total apple seed polyphenolics (62). Besides phloridzin, 13 other phenolics were identified by gradient HPLC/PDA on LiChrospher 100 RP-18 from apple seed (62). The HPLC technique was also able to provide polyphenol profiles in the peel and cortex of the apple to be used to characterize apple cultivars by multivariate statistical techniques (63). Phenolic compounds in the epidermis zone, parenchyma zone, core zone, and seeds of French cider apple varieties are also determined by HPLC (56). Three successive solvent extractions (hexane, methanol, aqueous acetone), binary HPLC gradient using (a) aqueous acetic acid, 2.5%, v/v, and (b) acetonitrile fol-

lowing thiolysis are applied to quantify the phenolic compounds. The HPLC analysis of the extracts after thiolysis allowed for the characterization of the procyanidins by their constitute units and their average degree of polymerization. Four main classes of phenolics in apple tissue could be characterized according to their class on the basis of their UV-visible spectra. Flavan-3-ols have a single symmetric band with maximum absorption at 278 nm, hydroxycinnamic acid derivatives show a maximum absorbance between 300 and 330 nm, and flavonols are characterized by an absorption band with a maximum above 340 nm, whereas dihydrochalcones present a single broad and asymmetric band with a maximum of 285 nm (56).

2. *Stone Fruits*

Peaches are rich in hydroxycinnamic acid derivatives, especially chlorogenic and neochlorogenic acids, flavan-3-ols, and flavonols. Peach phenolic compounds are extracted with methanol, separated into acidic and neutral fractions with C_{18} Sep-Paks, and then analyzed by HPLC (64). Relatively high concentrations of catechin, procyanidin B3, chlorogenic acid, and neochlorogenic acid were found in 15 varieties of peach fruits, while the caffeic acid content was relatively small. Binary-gradient HPLC methods using three different mobile phases were developed for low-molecular-weight phenolics, 3-flavanols, flavonol aglycones, and flavonol glycosides in several commercial juices and nectars, including peach, apricot, and other fruit juices (41). In this work, the presence of chemical marker compounds that could be useful in their characterization and differentiation were investigated. Myricetin is found only in peach, and apricot could be detected by the presence of two coumarins.

Two predominant phenolic compounds (neochlorogenic and chlorogenic acids) in prunes and prune juice can be analyzed by reversed-phase HPLC with diode array detection along with other phenolic compounds (65). Phenolic compounds were extracted from prunes with methanol and aqueous 80% methanol and analyzed by HPLC. Ternary-gradient elution (a) 50 mM $NaH_4H_2PO_4$, pH 2.6, (b) 80% acetonitrile/20% (a), and (c) 200 mM phosphoric acid, pH 1.5, was employed for an 80-min run time. Four wavelengths were monitored for quantitation: 280 nm for catechins and benzoic acids, 316 nm for hydroxycinnamates, 365 nm for flavonols, and 520 nm for anthocyanins. Phenolic analysis of pitted prune extract is presented in an HPLC chromatogram in Fig. 9, which is based on work done by Donovan and Waterhouse (65).

Phenolic compounds in olive fruits and olive oils are also often analyzed by HPLC (33,66,67). Olive fruit has long been used as a food and as a source of edible oil for frying and salad dressings. The phenolic content in olive fruits is known to contribute to the natural bitterness and the final color of fruits (66), and to provide natural antioxidant activity in olive oil (33,66,67). Also, the phenolic compounds in olives have a strong influence on the flavor of the oil and seem to be responsible for the high oxidation resistance of olive oil. The phenolic content in the flesh of olive fruits ranges between 1% and 3%, w/w, (66); oleuropein, vanillic, caffeic, *p*-coumaric acid, rutin, luteolin-7-glucoside, heterosidic ester of caffeic acid, and hydroxytyrosol (verbascoside) have been identified in olive fruits.

Olive and olive oil phenolics are extracted with ethyl acetate and analyzed by gradient HPLC (67) using (a) water, pH 2.0, and (b) acetonitrile on a Spherisorb ODS-2 column (Supelco). An isocratic RP-HPLC with an acetonitrile–water–acetic acid (20:90:0.18) mobile phase on a μBondapak C_{18} column (Waters) was also applied for major phenolic compounds of hydroalcoholic extracts (60% MeOH in water) of olive oils under electrochemical detection (33). The electrochemical detector offers greater sensitivity and stability at low concentration levels of these phenolics, but the gradual absorption of the phenolic compounds and their oxidation products on the electrode required frequent cleansing. The elution order is typical of reversed-phase chromatography followed by decreasing polarity: hydroxytyrosol > tyrosol > *p*-hydroxyphen-

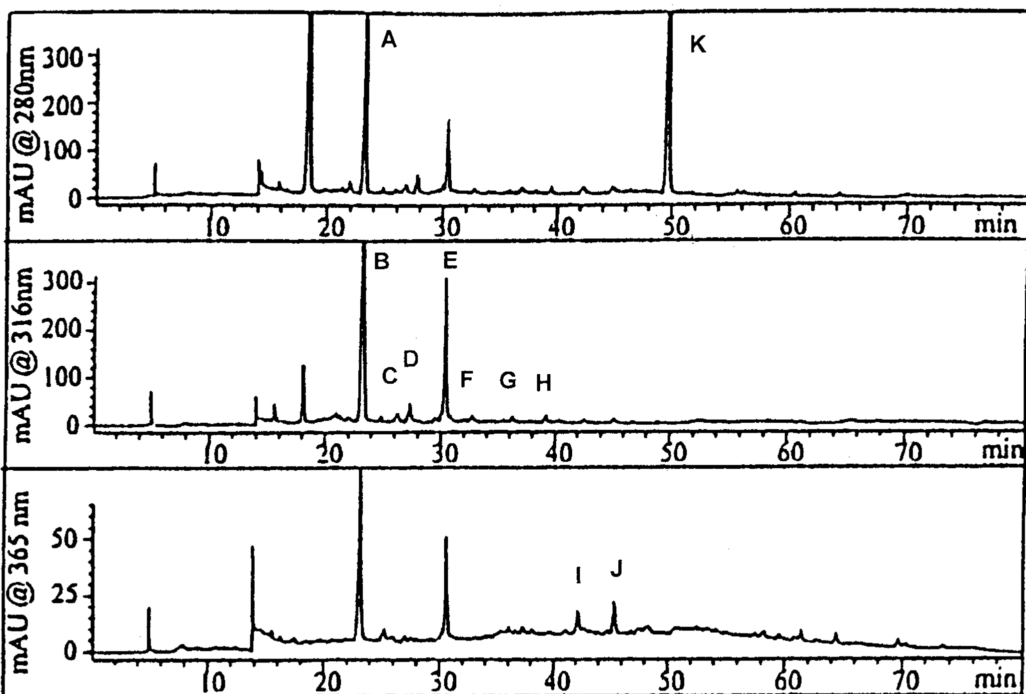


Fig. 9 HPLC chromatogram of pitted prune extract. © 1996, Society of Chemical Industry. Reproduced with permission. A. HMF; B. neochlorogenic acid; C. cinnamic; D. 3′-*p*-coumaroylquinic; E. chlorogenic; F. caffeic; G. cinnamate; H. coumaric; I. flavonol; J. rutin; K. sorbic acid.

ylacetic acid > homovanillic acid > and caffeic acid. Among the phenolic compounds identified previously in olive oils were hydroxytyrosol, tyrosol, caffeic, homovanillic, vanillic, *p*-hydroxyphenylacetic, *p*-hydroxybenzoic, *p*-coumaric, and syringic and protocatechuic acids. Four major phenolic compounds (caffeic, ferulic, gallic, and homovanillic acids) in the polar fraction of olive oil are also separated by RP-HPLC and determined using three different detection systems, including diode array, UV, and electrochemical detection (68). The variable-wavelength UV detector was found to be more suitable than the diode array detector for quantitative measurements. However, diode array detection was required for qualitative information on unknown components present in olive oil.

3. *Berry Fruits*

The HPLC analysis of flavonoids in red raspberries, blueberry, cranberry, and blackberry has been developed (69,70). The major flavonoids detected in *Vaccinium* spp., including blueberry, cranberry, and bilberry, were flavonol aglycones and flavonol glycosides with myricetin and quercetin (70). Flavonol aglycones (quercetin, myricetin, kaempferol) can be separated by isocratic elution using a ternary mobile phase consisting of 10% formic acid–acetonitrile–methanol, 65/10/25, for 35 min on a Partisil 5 ODS-3 column (250 × 4.6-mm ID) at a flow rate of 1 ml/min. Hydrolysis conditions for flavonol aglycones for different samples could be optimized using a different molarity (4 M–8 M) of hydrochloric acid and a heating period (30 min to

60 min) under reflux. Flavonol glycosides were also analyzed by ternary-gradient elution: (a) 1% aqueous acetic acid, (b) acetonitrile, (c) methanol on a YMC-Pack ODS-AM (250 × 4.6-mm ID, 5 μm) column with a total run time of 60 min (70). The YMC ODS-AM column is endcapped and is a very hydrophobic column (17% carbon loading). However, the Spherisorb ODS-1 column (Waters), which has a low carbon load (6% carbon loading) and no endcapping, was found to perform better than deactivated C_{18} columns with endcapping (69). Evidently, the hydroxy–silanol interactions in a C_{18} column with no endcapping play an important role in the separation of these components. HPLC conditions for flavonol glycosides and aglycones (69) are presented in Table 3.

For the analysis of flavonols in red raspberries, conventional materials were found to be ineffective in removing unwanted components from red raspberry extracts (69). Instead, small polyamide-6 columns were utilized effectively for the prefractionation of flavonoids prior to analysis (69). Two fractions, a fraction with methanol containing flavonol glycosides and aglycones and a second fraction of 0.5% NH_3/MeOH containing flavonol glucuronides, acylated flavonols, and ellagic acid, were obtained for HPLC. Ellagic acid content in small fruits, including strawberry, blackberry, blackcurrant, red raspberry, and cranberry, were found to be affected by environmental factors (71) as well as processing conditions (72). There were also great differences due to cultivars in red raspberry (72). Ellagic acid ($C_{14}H_6O_8$) is a dimeric derivative of gallic acid, which often exists as the bound form in plants (72), and has shown promising antimutagenic and anticarcinogenic activity in chemically induced cancer (73).

Flavonol glycosides were suggested as a suitable indicator of the adulteration of black currant products with red currants (74) and with blackberries (75). The black currant methanolic extract is purified using polyamide columns and analyzed for flavonoids by HPLC. Anthocyanins are removed by chromatography on Dowex 50W-X4, eluting with 70% methanol (74). Quercetin-3-0-gluccuronide was a suitable indicator to detect adulteration of blackcurrant products with blackberries (75).

For phenolic acids in bilberry juice, a reversed-phase HPLC method (16) using a linear-gradient elution of (a) 2% aqueous acetic acid and (b) acidified, aqueous acetonitrile on two C_{18} columns was able to separate the 12 phenolic acids and flavonoids (three flavonol glycosides and three flavonols) in ethyl acetate extract. Phenolics in blueberries were extracted, isolated, and

Table 3 HPLC Procedure for Flavonols in Berries

HPLC condition

Column: Spherisorb ODS-1 (250 × 4.6-mm ID, 5 μm)

Mobile phase: Eluent (A): acetonitrile; eluent (B): 1% acetic acid in water

Time (min)	0	5	35	40	47	57	62
% A	16	16	19	19	30	50	100
% B	84	84	81	81	70	50	0

Flow rate: 0.6 ml/min

Detection: Flavonols at UV 360 nm and ellagic acid at UV 260 nm

Injection volume: 10 μl

Sample preparation

Pour juice onto the polyamide-6 mini column, wash with water, elute flavonol glycosides with methanol, elute flavonol glucuronide, acylated flavonols, and elute ellagic acid with 0.5% NH_3 in MeOH. Evaporate MeOH to dry sample, redissolve in 4% H_3PO_4, filter (0.45 μm).

Source: Ref. 69.

quantified by reversed-phase HPLC (76). Chlorogenic acid was the major colorless phenolic of low-bush and high-bush blueberries.

4. *Grapes and Wines*

The phenolic compounds of grapes and wines are very important for their sensory effects as well as for their contribution to color. Grape juices and wines with higher polyphenolic concentration are more susceptible to oxidation, which can in turn lead to poor wine quality.

Reversed-phase HPLC is used for the analysis of the different groups of phenols, phenolic acids, hydroxycinnamic acids, flavonoids, and procyanidins in grapes and wines (22,46,47,77–80). However, due to the presence of a large quantities of various compounds, wine analysis is difficult. Thus, different sample preparation procedures, including fractionation and extraction, are often applied when various groups of phenolic compounds are studied together.

High-performance LC with reversed-phase silica-based columns has been the technique used by most investigators to analyze the phenolic composition of grape juices and wines. A gradient HPLC method with diode array detection, which was used for apple juice and pear juice phenolics (49,50), was able to quantitate 18 phenolic compounds, including phenolic acids and flavonol glycosides, from seedless grape juice (22). Procyanidins isolated from grape juice with Sephadex LH-20 (as shown in Fig. 7) were also determined by the same gradient elution. Bailey et al. (81) described the HPLC of cider and wine proanthocyanidin polymers using the Hamilton PRP-1 column, which is packed with acid-stable styrene-divinylbenzene copolymers. This polymeric HPLC column is known to outlast silica-based columns and is particularly useful for the analysis of anthocyanidins.

Different prepurification steps involving various solvent extractions (ethyl acetate or ethyl ether) at different pH values (pH 7.0 for neutral and pH 2.0 for phenolic acids), and solid-phase extraction using a C_{18} cartridge or PVPP (polyvinylpolypyrrolidone) have been necessary to extract the compounds of interest to obtain a clear chromatogram. The PVPP is particularly effective in adsorbing phenolic and polyphenolic compounds in white wines, supposedly by hydrogen bond formation. The use of PVPP in U.S. wine making is allowed under 21 CFR 173.50 and 27 CFR 240.1051 at levels up to 0.72 g/L. A maximum of 0.8 g/L is allowed within the European Community. Especially for phenolic analysis in both red grape and red wines, a fractionation technique using a C_{18} cartridge was often applied to analyze phenolic compounds other than anthocyanins using HPLC (17). The fractionation of grape phenolics into acidic and neutral groups was accomplished by passing deproteinated grape juice through a preconditioned C_{18} Sep-Pak cartridge (21,46,77). This technique can also provide selective extraction of phenolic compounds other than anthocyanins in red (Concord and de Chaunac) grapes (47) by preconditioning the C_{18} cartridge sequentially with 5 ml ethyl acetate, 5 ml methanol, and 5 ml 0.01 N HCl; phenolic compounds were then selectively eluted with 20 ml ethyl acetate. Anthocyanins were selectively fractionated with acidified methanol. Jaworski and Lee (21) and Oszmianski et al. (17) demonstrated the fractionation of phenolic acids from procyanidins and anthocyanins on a C_{18} cartridge in red wine. Most wine phenolics that have been fractionated by SPE could be analyzed by binary gradients.

However, due to the artifacts resulting from oxidation, hydrolysis of esters or ethers, or isomerization of phenolics during pretreatment of wines, as well as due to the low recovery rates of some phenolics, analysis of wine phenolics via direct injection of the filtered wine into the chromatographic column is often selected (80,82–84). For the red wine and musts (80), which were injected directly into the HPLC without sample preparation, a ternary-gradient system was often employed for phenolic compounds. Twenty-two phenolic compounds, including 10 anthocyanins, were analyzed from red wine. The separation of cinnamic acid derivatives (313 nm),

flavonoids (280 nm), and anthocyanins (520 nm) were monitored at three different wavelengths. A low pH of eluent (pH of 1.5) was necessary for the efficient separation of anthocyanins as well as for maximum sensitivity for detection at 520 nm of anthocyanins with no noticeable degradation of ODS bonded phase on the column. A more improved HPLC method with binary-gradient elution was able to quantify a relatively large number of phenolics, up to 31 phenolic compounds from wine and free-run juice (79). The binary gradient with direct HPLC injection (Table 4) allowed separation of 31 phenolic compounds, and the dominant phenolics in wine were hydroxycinnamates. Among them, *trans*-caftaric, *trans*-coutaric, and *cis*-coutaric were predominant. Flavonoids were present at low concentration, and benzoic acids were the minor compounds (79).

For white wines (85), a similar HPLC condition to that of Betes-Saura et al. (79) was employed with a Nucleosil C_{18} column (250 × 4.0-mm ID, 5 μm) with binary gradient using eluent (A): acidified water (pH 2.65) and eluent (B): 20% A with 80% acetonitrile applied for hydroxycinnamate derivatives esters (caffeoyl tartaric, *p*-coumaroyl tartaric, and feruloyl tartaric acid esters) and free hydroxycinnamic acids (caffeic, ferulic, and *p*-coumaric acids).

For the red wines (82–84), which were injected directly into the HPLC without sample preparation, a ternary-gradient system using aqueous acetic acid (1% and 5% or 6%), and acidified acetonitrile (acetonitrile–acetic acid–water, 30:5:6) was used for cinnamic acid derivatives, catechins, flavonols, flavonol glycosides, and proanthocyanidins. Due to the large number of peaks, the gradient was extended to 150 min for the resolution of many peaks of important phenolics. This direct injection method was able to separate phenolic acids and esters, catechins, proanthocyanidins, flavonols, flavonol glycosides, and other compounds (such as tyrosol, and *trans*-resveratrol) in wine in a single analysis. However, use of acetic acid did not permit the detector (PDA) to be used to record the UV spectra of phenolics below 240 nm (84).

Proanthocyanidins (condensed tannins), which are polymerization products of flavan-3-ols to oligomers, are commonly found in grapes and wine, and they attract the increasing attention of both pharmacists and physicians. They consist mostly of (+)-catechin and (−)-epicatechin units, linked by C-4–C-8 or C-4–C-6 bonds and sometimes esterified by gallic acid on the epicatechin moities. Proanthocyanidins play a very important role in the organoleptic properties of wines, such as astringency and bitterness, haze formation, oxidation and browning, color stability, and aging behavior (86). High-performance LC methods (22,30,46,87), including column

Table 4 Gradient HPLC Analysis for Phenolic Acids, *trans*/*cis*-Hydroxycinnamic Acids, Flavan-3-Ols, Flavonols, and Procyanidins

HPLC condition

Column: Nucleosil 120, C_{18} (250 × 4-mm ID, 5 μm)

Mobile phase: Eluent (A): acidified water (pH 2.64); eluent (B): acidified water–ACN (20:80, v/v)

Time	0	5	10	15	30	35	40	45
% A	100	98	96	90	80	70	0	100
% B	0	2	4	10	20	30	100	0

Flow rate: 1.5 ml/min

Column temperature: 40°C

Detection: UV 280 nm

Injection volume: 100 μl

Sample preparation

Use direct injection technique. Centrifuge the sample (20 min at 1800 g), filter (0.45 μm, PVDF), and concentrate (5 ml to 3 ml) before injection.

Source: Ref. 79.

chromatographies on Sephadex G-25 or LH-20, and solid-phase extraction on C_{18} cartridge, and normal-phase HPLC techniques, have been proposed to separate proanthocyanidins. Proanthocyanidins are separated using both isocratic and gradient elution on a C_{18} column.

Recently, the fractionation of grape and wine proanthocyanidins according to their degree of polymerization (monomers, oligomers, and polymers) into three fractions by different organic solvents on C_{18} cartridges was also developed (86). The fractionation of proanthocyanidins on C_{18} cartridges begins with an elution with ethyl acetate to isolate catechins and oligomeric proanthocyanidins and then with methanol to isolate polymeric proanthocyanidins (fraction 2). For the further separation of catechins from oligomeric proanthocyanidins, a sequential elution with diethyl ether (fraction 1) and then with methanol (fraction 2) from the cartridge was further needed. The degree of polymerization is the average number of flavan-3-ol units in the polymer, the mean degrees of polymerization for oligomeric and polymeric proanthocyanidins in red wine were 4.8 and 22.1, respectively (86). For the selective extraction of proanthocyanidins for preparative and industrial-scale HPLC, an ethyl acetate–water (ethyl acetate with 10% water) system was employed. Proanthocyanidins could not be extracted in the absence of water, and the increase in water content up to the saturation level resulted in substantial yield enhancement (87).

A dual-electrode liquid chromatography–electrochemistry (LCEC) system used in the detection and identification of flavanols and procyanidins in wines and grape seeds is a valuable tool (30). Voltammetric behavior of phenolic compounds by LCEC could provide information that cannot be obtained using HPLC with UV detection, for which the identification is usually based on a comparison of the retention time with that of standard compounds, especially for the identification of catechins and procyanidins with a small amount of sample available (30). Figure 10 shows the procyanidins commonly found in wines.

5. *Citrus Fruits*

The analysis of flavonoid constituents of *Citrus* species continues to gain attention not only for their remarkable taste properties but also for their therapeutic and pharmacological activities. Citrus contains specific flavanone glycosides that are not common in the plant kingdom. A great deal of effort has been spent on analytical methodology for the separation of these components in citrus products, for they are useful in determining the species of fruit from which a product was made. Analysis for these glycosidic compounds is suggested to be useful as taxonomic markers. Thus, precise quantitative data on the occurrence of flavonoids in citrus is needed for chemotaxonomic study. Furthermore, fruit juices, particularly orange juices, have always been prime targets for adulteration for economic gain. Flavonoid analysis to verify the authenticity of citrus products and to detect economic adulteration is one of the routine screening methods for quality in the citrus industry.

Flavonoid profiles by HPLC have also been applied to the testing of citrus jams for genuineness (88). Commercial jams produced with mixtures of orange, lemon, and grapefruit can easily be characterized by HPLC. The methanol extracts were analyzed on a reversed-phase column, LiCrochart RP-18 (Merck), with water–formic acid (19:1) and methanol as solvents. Linear-gradient elution started with 20% methanol and reached 80% methanol at 25 min. The flavonoid profiles appear to remain intact during jam manufacture and can be measured, without a cleanup step, through Amberlite XAD-2 resin, which is an essential step in the flavonoid analysis of other fruit jams, such as apricot and peach.

The major flavanone glycosides in citrus are 7-*O*-rutinosides and 7-*O*-neohesperidosides of eriodictyol, naringinin, hesperitin, and isosakuranetin. The structure of some flavanone glycosides are shown in Fig. 11. These flavonoid compounds in citrus greatly influence the quality of both the fresh fruit and processed products. Among these compounds, naringin and neohesperidin are important with regard to the bitterness of grapefruit juice. Accurate methods to measure bit-

Procyanidin B1

Procyanidin B2

Procyanidin B3

Procyanidin B4

Fig. 10 Structures of procyanidins. (From Ref. 30.)

ter substances in food and juice products are important so that processing parameters can be manipulated to control and limit bitterness in these products, to avoid a product with excessive bitterness. Hesperidin, a tasteless flavanone glycoside, is a significant component of the cloud in lemon and orange juice. Narirutin, another 7-*O*-glycoside of naringinin that is not bitter, differs from naringin only in the disaccharide attached to the parent flavanone.

The flavonoid pattern of orange juice is part of routine quality tests to determine if the juice has been blended or adulterated. Orange juice (*C. sinensis*) does not contain either naringin or neohesperidin, but grapefruit juice contains both. Thus, the naringin analysis by HPLC can be applied to detect orange juice adulteration with grapefruit juice. Also, naringin/neohesperidin ratios can be used to differentiate orange juice that may contain added grapefruit juices from orange juice that may include juices from other naringin-containing cultivars (89).

For the HPLC of flavanone glycosides in citrus, isocratic elution is often preferred because of the simplicity of the major flavanone glycosides occurring in citrus, and the flavanone glycosides are also present in citrus juice at fairly high levels. Furthermore, isocratic separations require no re-equilibration time between analyses and therefore are sometimes faster when only a few components are to be analyzed.

Most cases of isocratic HPLC reported were carried out on reversed-phase chromatography on silica-based ODS (C_{18} bonded phase) columns (1) for citrus juices. For mobile phases utilizing C_{18} columns, isocratic solvents of 80–82% aqueous acetic acid with acetonitrile were commonly used. A ternary mobile-phase system of water–acetonitrile–glacial acetic acid

Naringin
(Naringenin-7-β-neohesperidoside)

Narirutin
(Narigenin-7-β-rutinoside)

Neohesperidin
(Hesperetin-7-β-neohesperidoside)

Hesperidin
(Hesperetin-7-β-rutinoside)

Neoeriocitrin
(Eriodictyol-7-β-neohesperidoside)

Eriocitrin
(Eriodictyol-7-β-rutinoside)

Poncirin
(Isosakuranetin-7-β-neohesperidoside)

Didymin
(Isosakuranetin-7-β-rutinoside)

Fig. 11 Structures of flavanone glycosides. (From Ref. 39.)

(79.5 : 20 : 0.5, v/v) was most often applied for the separation of flavanone glycosides in citrus juices (89,90). Later, the elution strength of the mobile phase was changed by partially substituting the strong solvent tetrahydrofuran (THF) for acetonitrile (91–93). Using a quaternary mobile-phase system of (water–acetonitrile–THF–glacial acetic acid, 80 : 16 : 3 : 1, v/v), the separation was improved to remove interfering components from glycosides of eriodictol and to re-

Table 5 Isocratic HPLC Analysis for Flavanone Glycosides

HPLC condition
Column: Alltech, RP-18 UHS (250 × 4.6-mm ID, 5 μm)
Mobile phase: Water–acetonitrile–THF–glacial acetic acid (80:16:3:1)
Flow rate: 1.5 ml/min
Column temperature: Ambient
Detection: UV 280 nm
Injection volume: 20 μl

Sample preparation
Dilute citrus juice (5 ml) with dimethylformamide (DMF, 10 ml) and 50 mM ammonium oxalate (10 ml), and then steam-bath for 10 min at 90°C. Cooling, adjust the volume to 50 ml with water, centrifuge (10 min at 2500 g), and then filter (Acrodisc, 5 and 0.45 μm) the clarified juice. For standard preparation, prepare hesperidin (20 ppm) in DMF–water (2:1) solution and other flavanone glycosides (neohesperidin, eriocitrin, neoeriocitrin, naringin, and narirutin, 10 ppm) in the mobile phase.

Source: Refs. 91 and 92.

solve the six major flavanone glycosides (elution order: eriocitrin > neoeriocitrin > narirutin > hesperidin > naringin > neohesperidin) in citrus juices (91–93). Table 5 summarizes the isocratic HPLC procedure for flavanone glycosides in citrus products, which is based on previous work by Mouly et al. (91,92).

In particular, the nature of the organic modifier in the mobile phase could have a dramatic influence on the separation of phenolic compounds. There was an appreciable change in selectivity for the separation of hesperidin and naringin; the elution order was reversed between hesperidin and naringin with chromatography with THF substitution for acetonitrile. Widmer and Martin (94) were able to remove minor components interfering with the analysis of naringin and neohesperidin by utilizing lithium acetate or phosphate-buffered mobile phases at pH 5.0–6.3 and deactivated C_{18} columns. Extracts could be prepared from MeOH (95), dimethylformamide (DMF) (91–93,96), dimethyl sulphoxide (DMSO) (90), and DMSO/MeOH (1:1) (97,98). Often used was MeOH-DMSO (1:1) due to the insolubility of hesperidin and certain flavone compounds in methanol. Extraction with either MeOH or DMF did not present a general advantage over simple filtration for flavonoid recovery, although that of hesperidin was enhanced (39). Robards et al. (39) evaluated many variables, such as juice pH, temperature, and extraction solvents for the sample preparation. A juice product could be analyzed directly after dilution and/or filtration of the sample (89,94). In the preparation of standard flavanone glycoside solutions, a small amount of DMF was also added because of the low solubility of certain flavanone glycosides, notably hesperidin, and then made to volume with acetonitrile (89,94).

The quantitative recovery of flavanone glycosides can vary due to the adsorption of naringin and neohesperidin on the filter membrane; thus, the types of filter membrane, pore size, and sample amount filtered should be carefully considered, especially for a small amount of sample, less than 2 ml. The inorganic and cellulose acetate membranes showed little adsorption; the nylon, polysulfone, and versapore (acrylic on nylon) membranes showed significant adsorption (94). The use of a filter with a pore size less than 1.2 μm can reduce the recovery (89), but the use of a filter with a large pore size of 1.2 μm or more could increase column plugging, creating high pressure, and reducing the column life. An ammonium oxalate buffer was added to the orange juice to provide a constant pH range between 4.5 and 5 for better recovery (91), and the recovery of flavanone glycosides ranged from 95% to 100%, with a mean value of 99% (92).

The effective separation of all flavanone glycosides present from other components contained in citrus juice and fruit extracts within a reasonable period of time often requires gradient

elution with C_{18} columns due to the complex nature of the citrus juice matrix and differences in polarity between components. Under reversed-phase HPLC, the elution pattern for flavonoids containing equivalent substitution patterns is flavanone glycoside, followed by flavonol and flavone glycoside, and then the free aglycones in the same order (39). High-performance LC with gradient elution is effectively applied for the study of changes in phenolic compositions in developing lemons (97), antioxidative activity of phenolic compounds in citrus by-products (99), differentiating *Citrus* species (100), characterizing bioflavonoid in orange peel extracts (98), in new citrus hybrids (102), to verify the authenticity of citrus juices (103) and citrus jams (88), and to characterize the citrus juices (95,104). Such HPLC analysis for flavanone glycosides includes binary-gradient elutions with 0.01 M phosphoric acid (eluent A) and methanol (eluent B). A linear-gradient elution from 20% methanol to 100% methanol in a 55–60-min run is commonly used to separate flavanone glycosides as well as methoxy flavones (97,98,100,102). Twenty-five naturally occurring citrus flavonoids could be separated in a single run (100 min) on a LiChrospher 100 RP-18 column using the 0.01 M phosphoric acid–methanol gradient elution system (101). Various citrus tissue samples were separated with MeOH-DMSO (1 : 1, v/v) and further purified through a Sep-Pak C_{18} cartridge before HPLC analysis. Table 6 summarizes the chromatographic retention and UV maxima of some citrus flavonoids.

Table 6 Relative Retention by RP-HPLC and UV Maxima of Some Flavonoids

Common name	Systematic name	r^a	λ_{max} (nm)
Flavanone			
Eriocitrin	Eriodictyol-7-β-rutinoside	0.55	285
Neoeriocitrin	Eriodictyol-7-β-neohesperidoside	0.62	285
Narirutin	Naringenin-7-β-rutinoside	0.81	282
Naringin	Naringenin-7-β-neohesperidoside	0.90	284
Hesperidin	Hesperetin-7-β-rutinoside	1.00	285
Neohesperidin	Hesperetin-7-β-neohesperidoside	1.09	284
Neoponcirin	Isosakuranetin-7-β-rutinoside	1.70	284
Poncirin	Isosakuranetin-7-β-neohesperidoside	1.80	284
Isosakuranetin	5,7-dihydroxy-4′-methoxyflavanone	2.41	282
Flavonols			
Robinetin	3,7,3′,4′,5′-Pentahydroxyflavone	0.68	251, 318
Rutin	Quercetin-3-β-rutinoside	0.96	258, 360
Quercetin	3,3′,4′,5,7-Pentahydroxyflavone	1.76	256
Kaempferol	3,4′,5,7-Tetrahydroxyflavone	2.18	253, 266
Isorhamnetin	3,4′,5,7-Tetrahydroxy-3′-methoxyflavone	2.24	253
Rhamnetin	3,5,3′,4′-Tetrahydroxy-7-methoxyflavone	2.38	256
Flavones			
Isorhoifolin	Apigenin-7-β-rutinoside	1.16	267, 336
Rhoifolin	Apigenin-7-β-neohesperidoside	1.25	268, 336
Diosmin	Diosmetin-7-β-rutinoside	1.35	253, 268, 345
Neodiosmin	Diosmetin-7-β-neohesperidoside	1.44	255, 268, 345
Luteolin	3′,4′,5,7-Tetrahydroxyflavone	1.96	242, 256, 351
Apigenin	4′,5,7-Trihydroxyflavone	2.23	269, 335
Diosmetin	3′,5,7-Trihydroxy-4′-methoxyflavone	2.28	252, 268, 347
Sinensetin	3′,4′,5,6,7-Petamethoxyflavone	2.43	240, 265, 326
Acacetin	5,7-Dihydroxy-4′-methoxyflavone	2.56	269, 301, 329
Tangeretin	4′,5,6,7,8-Pentamethoxyflavone	2.65	271, 322

[a] Relative retention time vs. hesperidin, t_R = 32.09 min.
Source: Adapted from Ref. 101.

Table 7 Gradient HPLC for the Simultaneous Analysis of Flavanone Glycosides and Polymethoxylated Flavones

HPLC condition

Column: Alltima ODS (250 × 4-mm ID, 5 μm)

Mobile phase: Eluent (A): acetonitrile; eluent (B): water–acetic acid (96:4, v/v)

Time	0	12	43	44	49	50
% A	0	8	34	70	70	0
% B	100	92	66	30	30	100

Flow rate: 1.0 ml/min

Column temperature: Ambient

Detection: UV 280 nm for flavanone glycosides; UV 330 nm for polymethoxylated flavones

Injection volume: 20 μl

Sample preparation

Dilute citrus juice (25 ml) with dimethylformamide (20 ml), steam-bath for 10 min at 90°C. Cooling, adjust the volume to 50 ml with water, centrifuge (10 min at 2500 g), and then filter (Acrodisc, 5 and 0.45 μm) the clarified juice. For standards, prepare standards (narirutin, naringin, neohesperidin, didmin, and porcirin) with 30% DMF in water (10–80 ppm). Prepare hesperidin in 70% DMF in water (200 ppm). Prepare polymethoxylated flavones in methanol (25 ppm).

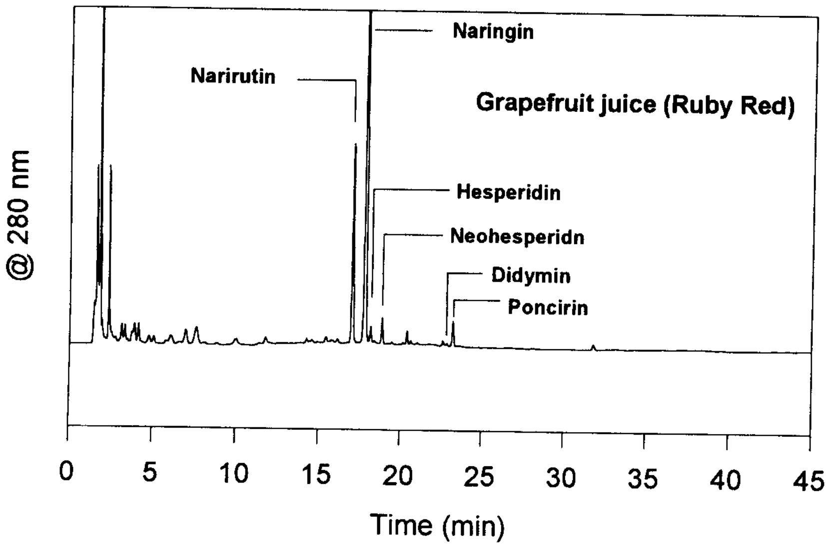
Source: Ref. 96.

Recently, an HPLC method using three gradient curve shapes (concave, linear, and convex) with (eluent A) acetonitrile and (eluent B) 4% acetic acid in water on a C_{18} column for the simultaneous separation of flavanone glycosides and polymethoxylated flavones (PMFs) in citrus juices was developed by Mouly et al. (96). A gradient HPLC procedure for citrus flavonoids by Mouly et al. (96) is summarized in Table 7. Better resolution was obtained using a concave gradient, and this technique allows for the characterization of polyphenolic profiles of various *Citrus sinensis* varieties, including flavanone glycosides and PMFs.

A relatively fast binary-gradient elution using acidified acetonitrile as an organic modifier (1% acetic acid in water and 1% acetic acid in acetonitrile) was also applied for flavanone glycosides by Perfetti et al. (104) and Lee et al. (95). A ternary-gradient elution using (eluent A) methanol, (eluent B) acetonitrile, and (eluent C) 0.5% acetic acid in water (101), or using (eluent A) 0.01 M KH_2PO_4 (pH 3.0–3.1), (eluent B) acetonitrile, and (eluent C) water, were also developed for the separation of complex mixtures of phenolic compounds in citrus products as well as for other fruit products (88,103).

High-performance LC with UV and fluorescence detection was also examined for use as a routine procedure for the analysis of citrus juice, particularly sweet orange. Recently, Robards et al. (39) established chromatographic profiles of the flavonoid fraction for several citrus juices, including sweet orange, grapefruit, lemon, lime, tangelo, and pummelo juices. Chromatograms were normalized using a Novapak C_{18} column with an aqueous acetonitrile mobile phase and UV detection at either 280 or 315 nm or fluorescence detection at 250 nm excitation and either 300- or 350-nm emission. For each juice, the fluorescence chromatogram was more complex than that obtained with UV detection.

Use of an acidified mobile phase has reduced tailing of flavanone glycosides on HPLC columns, most likely due to the presence of residual silanol group on the silica column packing. With improvement over the years in column packing and coating techniques, residual active sites on column packings were sufficiently eliminated for good peak shape. Figure 12 shows the chromatograms of flavanone glycosides and PMFs in citrus juices with a mobile phase consisting of water and acetonitrile as conducted in the author's lab. The HPLC chromatogram in Fig. 13 shows



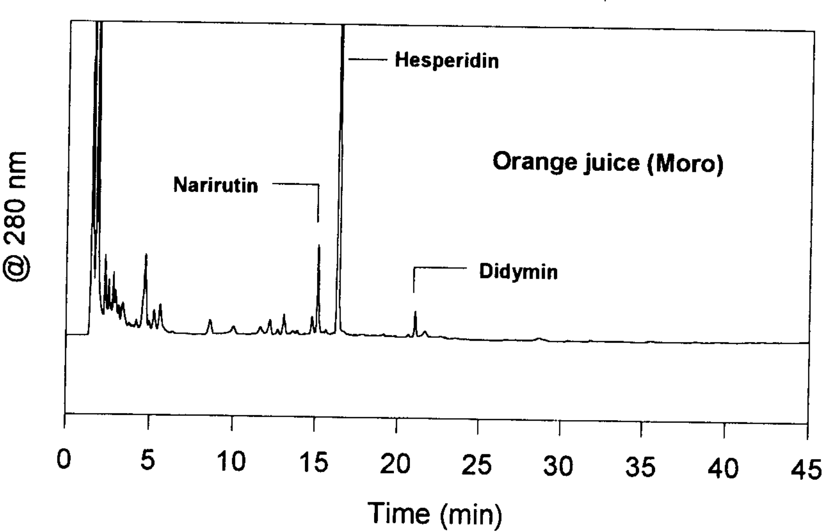


Fig. 12 HPLC separation of flavanone glycosides in grapefruit juice and orange juice.

orange juice spiked with didymin and polymethoxylated flavones (PMFs) (nobiletin, heptamethoxyflavone, and tangeretin). The HPLC used a Prodigy ODS (3) column (150 × 4.6-mm ID, 5 μm) from Phenomenex maintained at 25°C and eluted with a binary gradient with H_2O (eluent A) and acetonitrile (eluent B) at 1.2 ml/min. Elution was conducted with a stepwise gradient using 0–3 min, 10% B; 3–38 min, 42% B; 38–43 min, 42% B; and 43–55 min, 100% B. The juice sample was prepared by diluting with 40% acetonitrile (1:1), centrifuged (6500 rpm for 5 min), filtered through a cellulose acetate membrane filter (0.45 μm), and 5 μl injected. Detection was conducted at 280 nm for both compounds.

For hydroxycinnamic acid derivatives in citrus products, an HPLC method was also applied to determine the hydroxycinnamic acid esters with glucose or aldaric acids in the peel and pulp

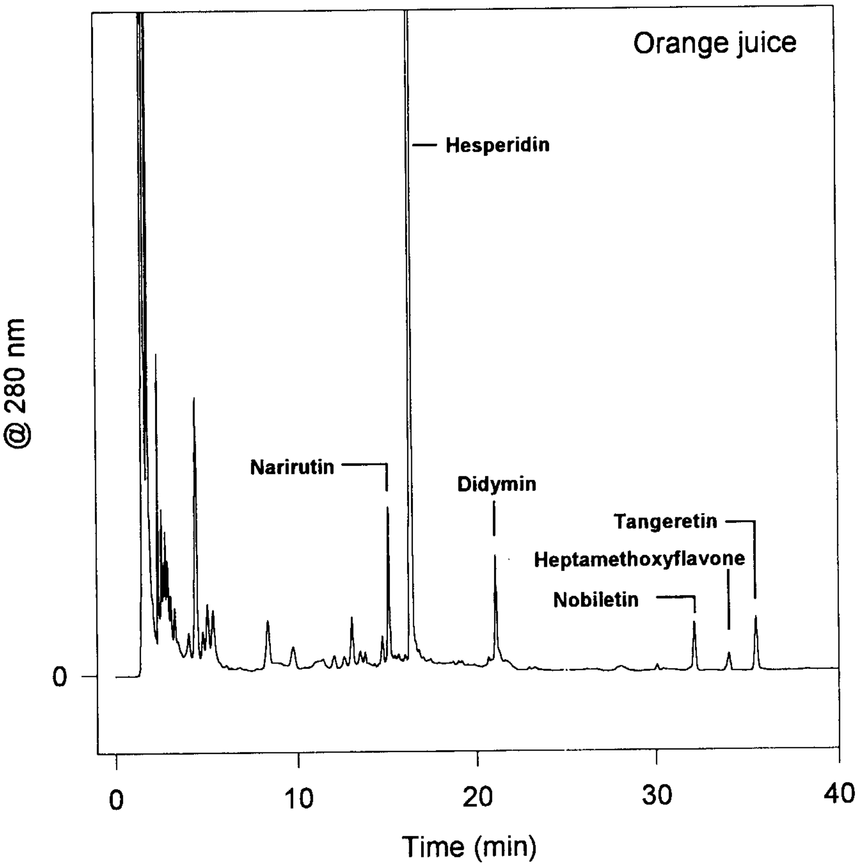


Fig. 13 HPLC separation of flavanone glycosides and polymethoxylated flavones (PMFs) in orange juice spiked with didymin and PMFs.

of orange, grapefruit, and lemon (105), and to separate cinnamic acid derivatives in blood orange juice (106). Qualitative and quantitative HPLC analyses were conducted on a Shandon ODS-Hypersil 5-μm column using gradient elution with 2% acetic acid in methanol (105). An optimization study of four cinnamics using a reversed-phase column with isocratic elution with various isocratic mobile phases was discussed (107). The best separation for sinapic, coumaric, ferulic, and caffeic acids was achieved by using THF as a modifier in the water or by using THF, methanol, and water for a ternary combination (107). The mobile phase consisting of 21% THF/ 79% water (with 2% HOAc) produced the best separation and allowed the most accurate quantitation of the four hydrocinnamic acids in orange juice.

For lemon juice, the flavonoid composition was characterized by HPLC with photodiode detection at 287 nm (108), the HPLC condition based on a procedure proposed by Kirksey et al. (103) for the detection of fruit juice adulteration. Hesperidin and eriocitrin were the characteristic flavonoids of lemon juice. Flavonoid content by HPLC was used to study the effects of processing and pulp removal on flavonoid composition in lemon juice. Eriocitrin is also used in distinguishing lemon juice from grapefruit and orange juices, which do not contain this flavonoid.

Polymethoxylated flavones are almost exclusively found in citrus. The structure of some polymethoxylated flavones are shown in Fig. 14. The polymethoxylated flavones are an interesting group of bioactive compounds that are concentrated on the fruit surfaces (peel) and are pres-

Fig. 14 Structures of polymethoxylated flavones. (Courtesy of Dr. W. Widmer, Florida Department of Citrus, Lake Alfred, FL.)

ent in processed juices and the edible fruit portions at very low levels. Analysis of the polymethoxylated flavones in citrus juice requires extraction and concentration to facilitate detection and quantitation. Sendra and co-workers (109) utilized a C_{18} SPE cartridge to isolate flavones from 30 ml of citrus juice. The cartridge was washed successively with H_2O (3 ml) and 25% acetonitrile (5 ml). Flavones were then eluted with 55% acetonitrile in H_2O (5 ml). Prior to analysis, the extract was concentrated and redissolved in 0.6 ml acetonitrile, filtered, and analyzed by gradient HPLC (92). Extracts were analyzed by reversed-phase on a microbore (2.1-mm ID) C_{18} column with H_2O–THF–ACN mobile phase. The THF concentration was kept constant at 2.5%, with gradient elution performed in a two-step linear gradient changing the acetonitrile concentration from 21.7% to 40.7%.

Polymethoxylated flavones are also characteristic of orange peel oils, and PMF determination has been used to ascertain the geographical origin of industrial peel oils (110). A normal-phase HPLC method for the determination of flavones in orange and mandarin oils was developed by Gaydou and co-workers (111). Using the normal-phase mode with a Licrosorb Si60

column and heptane–isopropanol (IPA) (60:40) as the mobile phase, six flavones (tangeretin, tetra-*O*-methyl-scutellarein, heptamethoxyflavone, nobiletin, hexa-*O*-methyl-quercetagetin, and sinensetin) were separated in 25 min (111).

For flavones in citrus peel oils, separations were accomplished with isocratic mobile phases of 38% and 40% acetonitrile in H_2O (1). The extracts of peel and cold-pressed peel oils were diluted in ethanol and analyzed by reversed-phase on various C_{18} columns with good results. For the dilute citrus oils, gradient elution was preferred, to prevent the accumulation of terpenes on the column. With normal-phase chromatography, the elution order is reversed; terpenes elute with the solvent front and are not a problem.

For the confirmation of PMFs in Valencia orange peel oil and juice, an HPLC method coupled with a thermospray mass spectrometry (HPLC-TSP-MS) detection system was utilized (112). A C_{18} column (μBondapak, 300 × 6-mm ID) was used with a mobile phase of H_2O–ACN (60:40, v/v) at a flow rate of 1.0 ml/min. Extract (20 μl) was injected into the HPLC-TSP-MS system, and positive-ion spectra from m/z 100 to 700 were recorded at 1360 ms. Mass spectrometric identification was done using positive chemical ionization (ICP). This technique allowed confirmation of the presence of eight flavones in the peel oils and seven flavones in the juice.

For coumarins and psoralens in citrus peel oil, most of the early work was done with normal-phase chromatography on silica columns (1) using isocratic elution with isooctane, heptane, and ethylacetate. The use of reversed-phase methods in citrus oils, fruits, vanilla flavorings, and alcoholic beverages is now more common than that of normal-phase methods. Normal- and reversed-phase modes were compared for their ability to resolve a number of coumarins (113,114). Albanese and Mussinan (113) reported both normal- and reversed-phase separation for 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP) in citrus oils. For normal-phase HPLC with the polyvinyl alcohol–coated silica column (PVA-SIL, 250 × 4.6-mm ID), the effects of the alcohol modifier on the separation was significant with this column packing material. At higher levels of modifier (such as hexanol or 2-propanol), the mobile phase becomes too polar and deactivates the column bed, resulting in a loss of separation. Using reversed-phase HPLC on a C_{18} or C_8 column with aqueous methanol or acetonitrile elution, these coumarins suffered unusual peak shape and broad peak shape, which may be due to the interaction of the oxygenated psoralen with the active sites of the column bed as well as the hydrophobic nature of the analytes (113). The resolution of neutral coumarins was better in the normal-phase mode using a silica gel column in a hexane–ethyl acetate solvent system than in the reversed-phase chromatography with aqueous acetonitrile or methanol (114). However, normal-phase and reversed-phase with an appropriate solvent system complemented one another for the resolution of complex mixtures of coumarins.

For coumarins in orange fruits (115), the HPLC used a Zorbax Rx C_8 (250-mm × 4.6-mm ID, 5 μm) column maintained at 25°C, and analysis was performed by binary-gradient elution using 0.1% HOAc in acetonitrile (eluent A) and 0.1% HOAc in H_2O (eluent B). In the author's lab, standard coumarins could be separated by isocratic elution on Zorbax Rx C_8 column with acetonitrile–0.1% HOAc in water (35:65) at 1.0 ml/min, as presented in previous work (1). The eluate from the column was passed to a UV detector (UV 330 nm) and then into a fluorescence detector (excitation at 340 nm, emission at 425 nm). As for the specificity, some of the coumarins do not have native fluorescence. Nine coumarins are separated under UV 330 nm, and three coumarins could not be detected with fluorescence detection. Detailed conditions for coumarin analysis in foods and absorption spectra of coumarins obtained by online diode array detector with HPLC were presented by Lee and Widmer (1). Since coumarins exhibit strong absorption in the ultraviolet region, absorption at approximately 313 nm has been used to estimate the dilution of cold-pressed lemon oil with distilled oil (12). Analysis of umbelliferone (7-hydroxycoumarin) and scopoletin (6-methoxy-7-hydroxycoumarin) in citrus fruits was performed using

an HPLC system of 10% acetonitrile in water, an RP8 Lichrospher 5-μm column, and fluorescence detection with ex. 335 nm and em. 455 nm (116).

Phlorin (phloroglucinol monoglucoside) was suggested as an outstanding phenolic indicator to detect pulpwash and peel extract in orange juice. Use of clarified orange peel extract or orange pulpwash as a substitute for orange juice is a cause for concern in the citrus industry. An HPLC method was developed based on organic acid analysis using isocratic 50 mM KH_2PO_4 elution at pH 2.6 on a 3-μm C_{18} column with a UV detector set at 214 nm. Phlorin was clearly resolved from other organic acids. The run time is 25 min, and retention time for phlorin is 20.2 min. Because orange peel extracts are a cheap source of soluble solids similar to those of orange juices, they can be used to adulterate juices in ways that are difficult to detect (117). However, phlorin is present in peel extracts and pulpwashes at much greater quantities than in the juices. In the quantitation, phlorin was estimated based on peak areas generated as compared to known amounts of citric acid and expressed as "citrate units." Phlorin was present in amounts from 200 to 600 units in orange peel extracts and averaged 133 units in pulpwash, compared to less than 5 units in juices (117).

6. *Other Tropical and Subtropical Fruits*

Avocado (*Persea americana Mill.*) is consumed primarily as a fresh fruit and contains a large number of phenolic acids. Phenolic acids of avocado were extracted by alkaline hydrolysis and 16 phenolic acids, including ferulic, caffeic, sinapic, *o*-, *m*-, and *p*-coumaric, syringic, vanillic, isovanillic, gallic, protocatechuic, hydroxybenzoic, and resorcilic acid, were identified by HPLC (4). Binary-gradient elution was performed with eluents (a) 5% aqueous acetic acid and (b) acetonitrile on an Alltech ODS adsorbosphere column. The order of elution of the phenolic acids followed polarity. Polarity is increased most by hydroxys at the 4-position, followed by those at the 3- and 2- positions. Methoxys and the acrylic groups reduce polarity and increase retention (4). Several commercial pineapple juices have been analyzed by RP-HPLC to establish their phenolic composition, providing the ability to characterize the phenolic profile of pineapple juices by the presence of sinapic acid and the absence of the flavonoids (41).

Both the free and the combined forms of phenolic acids in date fruit (*Phoenix dactylifera L.*) were analyzed by HPLC (19). The elution rate of phenolic acids increased with the degree of hydroxylation by isocratic elution using dioxane–acetic acid (15:85) on a μBondapak C_{18} column. Ferulic acid was the most abundant in the free form, and *p*-coumaric, vanillic, *p*-hydroxybenzoic, protocatechuic, and syringic acid were also identified in dates.

B. Vegetables

Vegetables are the main dietary sources of flavonols. In vegetables, quercetin glycosides predominate, but glycosides of kaempferol, luteolin, and apigenin are also present. Flavonols and flavones are flavonoids of particular importance, for they have been found to possess antioxidant and free-radical scavenging activity in foods (118). Figure 15 shows the common flavones and flavonols found in vegetables.

An HPLC separation method with diode array detector and mass spectrometric (MS) detection equipped with atmospheric pressure ionization (API) was developed to determine flavone, flavonol, and flavanone in various vegetables, including green bean, broccoli, brussels sprouts, celery, kale, leek, onion, parsley, pepper (green, yellow, and red), and tomato (118). The flavonoids were analyzed as aglycones after acid hydrolysis. The extraction and acid hydrolysis conditions are based on previous work by Hertog et al. (119). Quercetin is the overall major flavonol, followed by kaempferol. The flavones, apigenin and luteolin, were found only in limited foods,

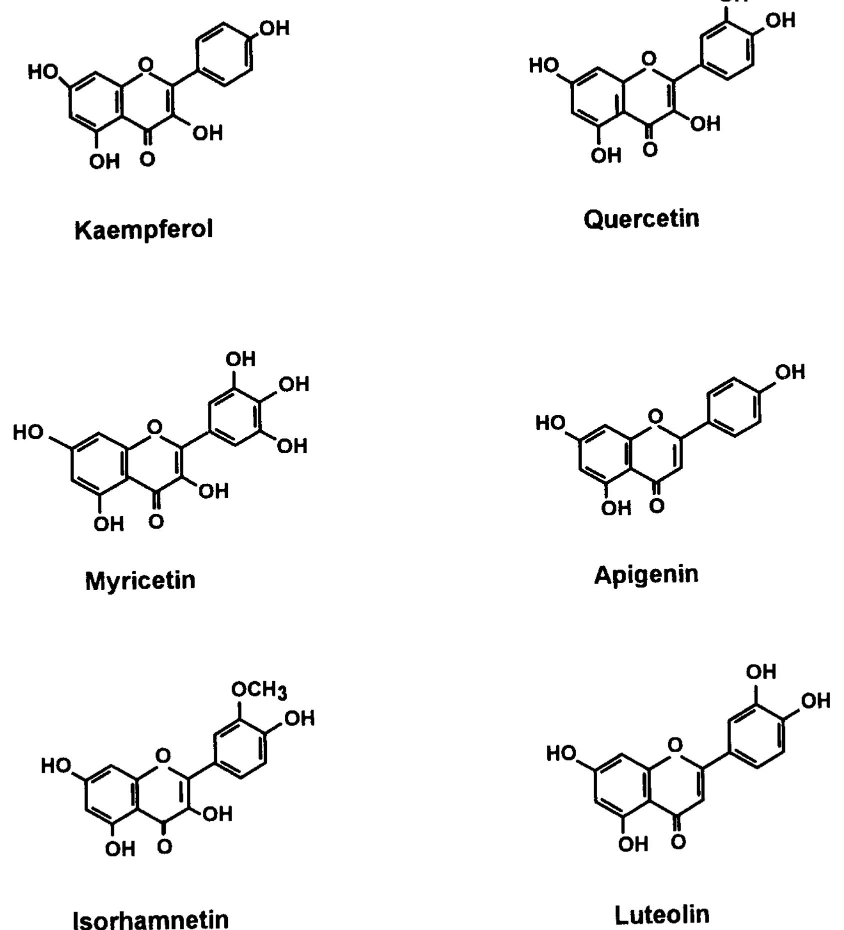


Fig. 15 Structures of common flavonols and flavones in vegetables. (From Ref. 118.)

such as in celery, sweet peppers (luteolin only), and parsley (apigenin only). The UV maxima and elution order for flavonol, flavone, and flavanone aglycones are as follows (118): myricetin (375 nm) > eriodictyol (289 nm) > quercetin (374 nm) > naringenin (292 nm), phloretin (287 nm) > luteolin (351 nm) and hesperitin (280 nm) > kaempferol (366 nm) > isorhamnetin (372 nm) > apigenin (341 nm). Quantitation of myricetin is difficult, due to its being less stable than other flavonoid aglycones, and phloretin was also degraded by the acid hydrolysis, due to the less stable open-ring dihydrochalcone structure (118). Precautions such as cooling of the autosampler, protecting vials form UV light, and freshly prepared standards are required, especially for quantitation of myricetin.

Hertog et al. (119) developed a fast HPLC method for the identification and quantification of five major flavonoid aglycones (quercetin, kaempferol, myricetin, luteolin, and apigenin) in freeze-dried vegetable and fruits. However, due to the inadequate resolution of quercetin and luteolin on RP-HPLC on Nova-Pak C_{18}, two different eluents of different solvent strength and viscosity were utilized. The conditions for hydrolysis and extraction were tested based on different conditions of hydrochloric acid concentration (1.2–2.0 M), reaction period (0.5–6 h), and meth-

anol concentration in the extraction solution, and optimized for samples containing various types of flavonoid glycosides.

Since flavonoid glycosides are more soluble in water and flavonoid aglycones are more soluble in methanol, extraction efficiency could depend on the water/methanol ratio; 50% aqueous methanol was found to be most efficient for extraction of flavonoids in freeze-dried vegetables and fruits (119). Their method was further applied to extract potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits, and hydrolyzed to their aglycones with HCl in 50% aqueous methanol (120). Subsequently, the resulting aglycones were quantified by RP-HPLC on a Nova-Pak C_{18} column (Waters) using acetonitrile–phosphate buffer (25:75, v/v, pH 2.4) as the mobile phase and UV detection at 370 nm. Seven flavonol glycosides were identified in cabbage leaf extract (121).

The HPLC procedure for the quantitative analysis of flavonoids of Hertog et al. (119) have been further refined by Crozier et al. (122) with an internal standard to monitor losses during sample preparation. Quantitative estimation of conjugated flavonoid content was obtained by using an HPLC procedure (122) to analyze the level of free flavonoids present in acid-hydrolyzed extracts from commercial fruits and vegetables (tomatoes, onions, lettuces, and celery). The cooking (microwaving and boiling) effect on vegetable flavonols was considerable, reducing the quercetin content significantly of both tomatoes and onions (122). The gradient HPLC procedure for free and conjugated flavonols and flavones in vegetables is summarized in Table 8.

High-performance LC with ^{1}H and ^{13}C NMR and FAB-MS techniques was utilized to isolate and identify the four esters of hydroxycinnamic acids from fresh and raw broccoli florets (123), and acylated flavonoid (patuletin, spinacetin, jaceidin) glycosides from spinach leaves (124). Development of suitable sample handling, extraction, and HPLC/diode array detector (DAD) based methods for the analysis of flavonoids and their conjugates in four varieties of onions were described (125). The predominant flavonoids in onions (white, brown, red, and pink varieties) were quercetin mono- and diglucosides, which accounted for approximately 80% of the total flavonol fraction.

C. Cereals and Legumes

High performance LC is commonly employed for separation and quantifying phenolic compounds in cereal grains (126), especially due to the existence of considerable evidence linking the presence of phenolic acids in grain to disease resistance and resistance to mold damage.

Table 8 Gradient HPLC Analysis of Flavonols and Flavones in Vegetables

HPLC condition
Column: Symmetry C18 (150 × 3.9-mm ID, 5 μm)
Mobile phase: Eluent (A): acetonitrile; eluent (B): water–trifluoroacetic acid, pH 2.5
Use 20-min gradient of 15–35% acetonitrile in water adjusted to pH 2.5 with TFA.
Flow rate: 1.0 ml/min
Column temperature: 40°C
Detection: UV 365 nm
Injection volume: 50 μl

Sample preparation
Extract lyophilized vegetables with 20 ml aqueous MeOH (60%) containing 125 μg kaempferol (int. std.) and 20 mM sodium diethyldithiocarbamate (antioxidant), add 5 ml of 6 M HCl, reflux at 90°C for 2 h. Take 100-μl aliquot, adjust volume to 250 μl with water, adjust to pH 2.5 with TFA, and filter (0.22 μm).

Source: Ref. 122.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is the major low-molecular-weight phenolic in barley and in many other common cereal grains. The HPLC analysis of ferulic acid can serve an excellent indicator of endosperm purity in the milling process and as a measure of bran contamination in wheat milling fractions. Also, it was shown to be useful in the related areas of wheat breeding and baking (10). Galletti et al. (38) developed an RP-HPLC method for determination of *p*-coumaric and vanillic acid together with phenols in wheat straw and lignin. Separation was based on a C_6 column with an isocratic elution (MeOH–0.1% $HClO_4$, 15:85) and a UV detector set at 280 nm and in series with ECD, which led to an increase in sensitivity.

Ferulic acid was also analyzed in an effort to develop protocols that enable the rapid screening of a wide range of barley samples in breeding or processing programs (126). Grain samples were acid hydrolyzed at 100°C with 0.2 N H_2SO_4 for 1 h and then digested for 60 min at 30°C by the use of enzyme (α-amylase) before analysis by HPLC. An isocratic procedure using a mobile phase of 0.01 M sodium citrate containing 13% MeOH, pH 5.4, and a C_{18} column with 5-μm Hypersil packing (Vydac) was employed. Ferulic acid was simultaneously detected at UV 280 nm with several phenolic acids, although UV 280 nm is not the optimum for ferulic acid. The HPLC procedure was highly correlated with the values obtained by absorbance measurement at 340 nm and fluorescence analysis for relative fluorescence intensities (126).

An HPLC method using a 90-min binary gradient with (a) acidified water, pH 2.4, and (b) acetonitrile on an Adsorbosphere C_{18}, 3-μl cartridge (Alltech) was also developed for phenolics in barley (127). Seven phenolic compounds, including vanillic acid, *p*-coumaric acid, ferulic acid, and their derivatives, were separated by HPLC after alkaline hydrolysis in order to evaluate the role of bound phenolic acids in their antioxidant activity in beer. In this method, cis and trans isomers of *p*-coumaric and ferulic acids are quantified by HPLC, although *cis-p*-coumaric acid was not well separated from its trans isomer in this analysis.

Column chromatography using XAD-2 and HPLC techniques was also applied to identify the antioxidative *C*-glycosyl flavonoid, isovitexin in rice hull (128). An HPLC method using a binary gradient (acidified water, pH 2.6/MeOH) on a Nucleosil C_{18} column was also applied to evaluate the bitterness and astringency of polyphenolic compounds in cocoa powder (129). Tannins, flavan-3-ol group [(+)-catechin, (−)-epicatechin, and (−)-epigallocatechin], and anthocyanins are responsible for the astringent taste and bitterness of cocoa. Thus, polyphenols, tannins and (−)-epicatechin were critical factors in defining the quality of cocoa used in chocolate manufacture (129).

Green bean phenolics could be fractionated into three groups (acidic fraction and two neutral fractions) using SPE on a C_{18} cartridge, as previously developed for wine phenolics (47). Figure 16 illustrates the fractionation of three phenolic groups from green beans. Neutral fraction A consists mainly of flavans and other polar phenolics, neutral fraction B is mainly flavonols and less polar phenolics, and the third group is acidic phenolics (130).

Due to the limited distribution of isoflavones in foods (genistein and daidzein are found in just a few botanical families), an HPLC method for isoflavones was limited to soybean foods. Coward et al. (131) described a gradient HPLC method including initial extraction using 80% aqueous methanol and defatted soybean extract for isoflavones in various foods derived from soybean, such as soy milk, tofu, miso, soy sauce, and tempeh. The antitumor activity of isoflavones in soybean foods was studied. Separation of 80% MeOH extract was achieved by HPLC on an Aquapore C_8 column (Brownlee) with a mobile phase consisting of a gradient of 0–46.4% ACN in 0.1% (v/v) aqueous TFA at a flow rate of 1.5 ml/min. The concentration of acetonitrile increased by 2.25% per min. The eluting components (daidzin > genistin > daidzein > genistein) were detected from their absorbance at UV 262 nm. Maximum absorbance occurred at 254 nm for daidzin, genistin, and genistein in 80% aqueous methanol, and at 250 nm for daidzein (131). Separation of both the isoflavone β-glucoside conjugates and aglucones can be done in a

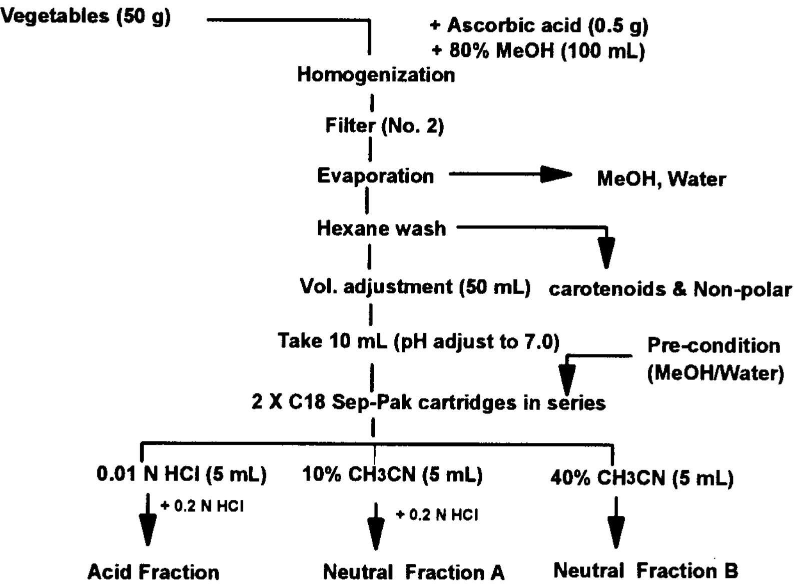


Fig. 16 Extraction procedure for phenolics in vegetables. (From Ref. 130.)

Table 9 Gradient HPLC Analysis of Isoflavones in Soybean Products

HPLC condition
Column: Brownlee aquapore C_8 (300 × 4.5-mm ID, 5 μm)
Mobile phase: Eluent (A): acetonitrile; eluent (B): water–trifluoroacetic acid (0.1%)
Use gradient of 0–46.4% acetonitrile (2.25%/min) in 0.1% aqueous TFA.
Flow rate: 1.5 ml/min
Detection: UV 262 nm
Sample preparation
Extract soybean foods with 80% aqueous MeOH (10 ml/g), containing 1.25 mg fluorescein (int. std.), by stirring 1 h at 60°C and centrifuge (10 min at 2500 g). Dry the supernatants, redissolve in 50% MeOH (5 ml), defatting by partitioning with hexane (4 × 20 ml). Evaporate the aqueous methanol phase to dryness, suspend in 80% MeOH (10 ml), and centrifuge (2 min at 14,000 g).

Source: Ref. 130.

single chromatographic run. The gradient HPLC analysis of isoflavones in soybean foods is summarized in Table 9.

Isoflavone derivatives along with nonvolatile components in soy sauce were also analyzed by gradient reversed-phase HPLC. This HPLC profile of soy sauce was further utilized by a pattern recognition program to understand the quality differences of soy sauces (132), and three isoflavone derivatives (esters of tartaric acid with daidzein, genistein, and 8-hydroxygenistein) were found to contribute significantly to the differentiation in fermented soy sauce. Fermented

soy sauce is now widely used in Asian countries and also in North America and Europe because of its unique appetizing flavor (132).

D. Beverages

The HPLC analysis of phenolic compounds in alcoholic and nonalcoholic beverages has been determined in many different ways. In nonalcoholic beverages such as tea, free gallic acid is the most important phenolic acid, and the amount of gallic acid increases during fermentation. The flavonols and flavonol glycosides in tea are one class of components responsible for the yellow-brown color in green tea. Green tea is a rich source of flavan-3-ols (catechins), and oxidation of tea flavanols during the manufacture of black tea leads to polymerization into orange-red theaflavins and orange-brown thearubigins. Analysis of flavanols and their polymerization products in tea is often considered as one basis in the determination of tea quality and for potential health properties of flavonoids (133,134).

Bailey and co-workers (9) separated the number of tea components, including gallic acid in a black tea liquor using a Hypersil ODS column (5 μm) and a diode array detector (280–460 nm). Separation was carried out by a linear gradient of 2% HOAc and acetonitrile, but only a single sample was analyzed without quantitation. Black and green teas also contain many flavonoids, including flavanols, flavonols, flavones, theaflavins, and thearubigins. Aglycones are present in small amounts, but the majority are present in the glycoside form. Finger et al. (135) previously published an excellent review on the chromatographic separation of tea constituents.

Flavanols are most abundent in fresh green tea leaves and may comprise up to 30% of the dry weight. The major flavanols present are (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and (−)-epigallocatechin gallate. The most efficient solvent for the extraction of flavanols was MeOH when 90% aqueous solutions of methanol, ethanol, and acetone were compared with water-saturated ethyl acetate (136). It was suggested that 1 hour of extraction using the Bolton extractor was the most suitable for determination of tea tannin and catechins (136). A reversed-phase isocratic method (135) using a C_{18} column for separation within 35 min of green tea flavanols or flavanols isolated from fermentation studies, utilizing HOAc-MeOH-DMF-H_2O (1:2:40:157) as the mobile phase. Gradient methods offer advantages with complex samples in being able to separate faster any components of widely differing polarity. Kuhr and Engelhardt (137) developed a rapid method for the separation of flavanols, caffeine, and phenolic acids in green and black teas from crude extracts that had been further cleaned up by SPE utilizing a C_{18} cartridge. Using aqueous HOAc and acetonitrile, their separation was accomplished in just 25 min. In black tea, the flavanols have for the most part been oxidatively polymerized to theaflavins and thearubrins. These can be isolated from tea by ethyl acetate extraction or can also be analyzed from whole tea infusions. Analysis of whole tea infusions using a small-particle C_{18} column with 1% aqueous HOAc and acetonitrile, gradient elution, and UV-VIS detection by diode array detector will separate both the polymerized flavanols and flavonol glycosides with good resolution (138). Copeland et al. (139) developed a method that enables the easy and inexpensive preparation of large quantities of (−)-epigallocatechin gallate, which is very expensive and unstable, from green tea. Flavanols are precipitated by caffeine treatment and further purified by solvent partition with ethyl hexanoate and propyl acetate. The separation of tea flavanols was achieved on a column packed with Hypersil ODS (Hichrom) with gradient elution using aqueous acetic acid and acetonitrile.

Flavan-3-ols are key precursors of the quality-determining black tea pigments known as theaflavins, theafulvins, and thearubigins (139). The stability of major theaflavins (theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3, 3′-digallate) during automated HPLC analysis was also evaluated due to the concern about the instability of aqueous extract of black

tea during overnight analysis (140). However, no evidence to avoid the use of an automated system for HPLC analysis of decaffeinated tea extracts was found. Also, the performance of acetic acid and citric acid as HPLC solvent modifiers was compared and shown to be identical (140).

Flavone glycosides in tea occur as *C*-glycosides and may be isolated from flavonols through hydrolysis of flavonol-*O*-glycosides. Flavone-*C*-glycosides are not hydrolyzed and may be isolated from hydrolyzed sugars and flavonol aglycones with SPE using polyamide and then separated by HPLC using conditions similar to analysis for the flavonol glycosides. However, flavones with an asymmetrical substitution pattern are susceptable to isomerization from Wessely–Moser rearrangement, and the amounts must be reported as a sum (135).

Flavonol glycosides in tea have been analyzed by both gradient and isocratic HPLC with good results (42,138). The flavonol glycosides present in tea are primarily O-linked glycosides, while the flavone glycosides occur mostly as C-linked glycosides. The flavonol glycosides are all derived from myricetin, quercetin, and kaempferol. The most important flavones are those derived from apigenin (vitexin and isovitexin). Extraction with methanol will remove both the flavone and flavonol glycosides from tea leaves. The flavonol glycosides can be further isolated from methanol extracts by CC with polyamide. Separation with C_{18} columns using aqueous HOAc and acetonitrile with isocratic (85:15) (42) or gradient elution (138) has provided good results. An advantage to gradient elution is that theaflavins and threarubigins may also be quantified in the same analysis. With the isocratic elution method (42), thirteen mono, di, and trisaccarrides were separated in 35 min. The procedure was then scaled up using a large-diameter column, and peaks were collected. Identities were confirmed by UV spectra (diode array detector), ^{1}H and ^{13}C NMR, and GC/MS analysis of the trimethylsilyl (TMS) derivatives of sugar residues after hydrolysis (42).

The major representatives of phenolic acid on coffee constituents are chlorogenic acid and its isomers (141). Chlorogenic acids comprise some groups of compounds formed mainly by quinic acid esterification with either caffeic, ferulic, or *p*-coumaric acids. Ramirez-Martinez (142) developed an RP-HPLC method for chlorogenic, three isochlorogenics, protocatechuic, and ferulic acid with flavanols from coffee pulp using an ODS/SIL-X column and a fixed wavelength at UV 280 nm. The HPLC separation was achieved in 36 min by combining linear-gradient elution with isocratic elution. Twelve coffee cultivars were analyzed, and chlorogenic acid content averaged about 42.2% of the total identified phenolic compounds. In the case of coffee, where phenolic compounds are so varied and some of them exiguous, quantitation of total chlorogenic acid could be facilitated by use of gel filtration chromatography (GFC). Trugo et al. (141) developed a high-performance gel filtration chromatography for simultaneous determination of total chlorogenic acid and caffeine in coffee samples by using a TSK-G 3000-SW (300 × 8-mm ID) column.

An HPLC method for chlorogenic acids with lactones in six different commercial brands of roasted coffee was developed by Schrader et al. (143). Hydroxycinnamic acid derivatives, including mono- and di-caffeoylquinic acids, corresponding lactones, and feruloylquinic acids were extracted from coffee with methanol at 80°C for 1 h under reflux. An HPLC method using step-gradient elution with 2% aqueous acetic acid (eluent A) and ACN (eluent B) for a 75-min run time was developed. Determination was carried out by HPLC with UV detection at 324 nm, and further confirmation was conducted by HPLC–thermospray (TSP)–MS and HPLC–diode array detection. Elution order for mono-caffeoylquinic acid (CQA) was 3-CQA, 5-CQA, followed by 4-CQA, which was different from the usual elution order of mono-CQA (Fig. 17). These results indicate that it is currently not possible to predict the elution order of different reversed-phase packings due to the different selectivity (143).

A reversed-phase HPLC procedure was proposed for the determination of seven phenolic acids in green coffee samples (144). The sample preparation technique involved extraction, alkaline hydrolysis, and liquid/liquid extraction. The chromatographic separation was achieved us-

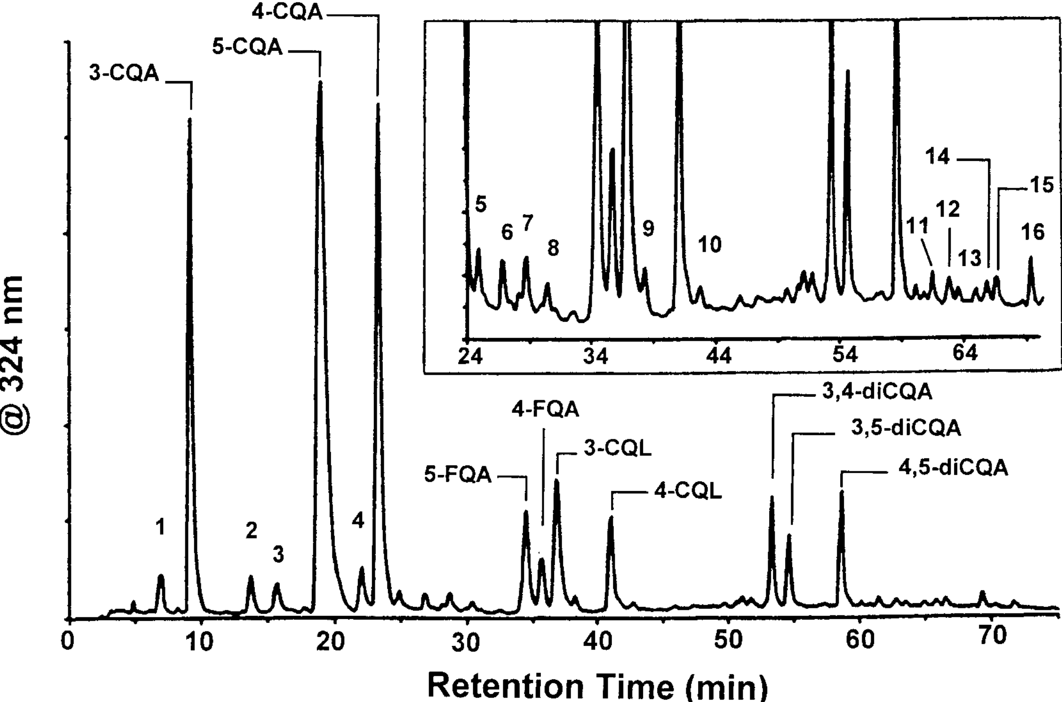


Fig. 17 HPLC separation of chlorogenic acids from roasted coffee. (From Ref. 143.) 1–15 = hydroxycinnamic acids; 7, 8 = coumaroylquinic acids; 16 = caffeoyltryptophan.

ing gradient elution with water–formic acid (19 : 1) and methanol on a Spherisorb ODS2 column. The method was reproducible (0.5% CV) and recovery values of caffeic acid from spiked green coffee samples were between 88.3% and 93.2%. These hydroxycinnamic acids by HPLC/PDA show the possibility for use as potential markers to assess the geographical origins of coffees.

Most of the applications for alcoholic beverages are included with wine, beer, and spirits such as brandy and whisky. Details of wine phenolic analyses are grouped with grapes in the fruit and fruit products section. Spirits are stored in oak barrels to acquire harmonious organoleptic characteristics. During maturation, certain coumarins are extracted from the wood, and they increase with the length of maturation. Salagoity-Auguste and co-workers (145) used RP-HPLC with a water–acetonitrile binary gradient for coumarins in wines and brandies stored in oak barrels. Coumarins were extracted by diethyl ether before injection. Esculetin, umbelliferone, scopoletin, and methyl umbelliferone were detected under dual detection by UV 313 nm absorption and fluorescence (Ex: 340/Em: 425 nm). These coumarins are known to be very flavor active and have an impact even though the concentrations found were less than 3 mg/L.

Puech and Moutounet (146) also applied binary gradient elution on a Micropak MCH-5 C_{18} column with (a) 5% HOAc in water and (b) ACN–water–HOAc (85 : 15 : 5) at 40°C to determine esculin, umbelliferone, scopoletin, and 4-methyl umbelliferone in different spirits of armagnac, brandy, calvados, cognac, whiskey, scotch whiskey, and rum. Spirits are injected directly, with no sample preparation. Scopoletin appeared to accumulate as the spirits aged. The scopoletin content of spirits after 14 years was 6.5 times higher than in spirits in new wood, but its content depends on the provenance of the wood, the age of the barrels, and the duration of maturation (146). The International Organization of the Flavor Industry (147) suggested the application of HPLC for the determination of coumarin in alcoholic beverages. However, the method is not specific for

coumarin and consequently may be seriously limited by coelution of other compounds that are also UV-active at 280 nm.

Beers are known to contain a wide variety of phenolic compounds, most of which originate from the raw materials of brewing, such as barley and hops. The use of HPLC with electrochemical detection, especially for the analysis of beer phenolic compounds, has received much attention (28–30). Reversed-phase HPLC with binary gradient using methanol and 3.5% HOAc in water, or isocratic elution with 15% MeOH with 0.1 M $NH_4C_2H_3O_2$ (pH 4.0) with electrochemical detection, was applied for major phenolic acids in beer (28–30). In the methanol gradient, as the methanol content increased, resolution between cinnamic acids (such as caffeic) and benzoic acids (vanillic) decreased. Increasing methanol content in the mobile phase will favor the solubility of cinnamic derivatives and will decrease their retention times more than those of benzoic acid derivatives. Thus, retention times of the cinnamic acid derivatives decreased faster than those for benzoic acid derivatives and consequently the separation between the two groups decreased (28).

The HPLC method using a combination coulometric-amperometric electrode detection system was suited to the direct analysis of beer samples and acetone extracts of barley samples, and was capable of the determination of proanthocyanidins and catechins at levels of 0.1–5.0 mg/L (148). This HPLC method using an acetic acid gradient (2.5–10%, v/v) with dual-electrode electrochemical detection offered improved sensitivity and selectivity compared to UV detection. In particular, the resolution between protocatechuic acid and prodelphinidin B3 was enhanced by switching the gradient from aqueous methanol to the acetic acid gradient. This method may be of particular interest to the brewer interested in selecting barleys with flavanol contents. Furthermore, sample preparation procedures were greatly simplified due to the high sensitivity of the electrochemical detector. An HPLC-ECD method was utilized for ferulic acid as a precursor of 4-vinyl guaiacol (148), which is an off-flavor to beer. Samples of beers were analyzed by direct-injection HPLC using an octadecyl silica column. Ferulic acid was detected by electrochemical detector, and response for detection was linear in the range 0.1–4.0 μg/L.

Hop flavonoids were also quantitatively characterized by HPLC-MS (149). Xanthohumol was the principal flavonoid and was accompanied by minor amounts of eight other flavonoids from nine hop (*Humulus lupulus*) varieties. Further applications of chromatographic separations on phenolic compounds in beers, spirits, and raw materials can be found from the previous work by McMurrough and Byrne (24).

E. Other Food Products

Flavonoid analyses are very useful as an adjunct in studies of the geographical origin of bee pollen and honey (150). A close correlation between phenolic acid patterns by HPLC and the botanical origin of honey has been found. Ellagic, *p*-hydroxybenzoic, syringic, *o*-coumaric, and gallic acids are characterized as the markers of the botanical origin (150). Ferreres and coworkers (151) also analyzed 10 flavonols, 6 flavones, and 2 flavanones from a honey extract by HPLC using aqueous methanol–water–formic acid (50:47:3, v/v). The extract was prepared by SPE through XAD-2 to remove the sugars and polar compounds. This was followed by cleanup with Sephadex LH-20 to remove the polymeric brown phenolics and phenolic acid derivitives. Analytical separation time was 30 min on a 100 × 4-mm, 5-μm, LiChrochart C_{18} column, but many of the components were poorly separated. The separation was further improved by an HPLC analysis procedure (57) using a multistep linear gradient with aqueous formic acid (5%) and methanol. They improve the gradient system as follows: isocratic for 15 min, 40% methanol at 20 min, 45% methanol at 30 min, 60% methanol at 50 min, and 80% methanol at 52 min. Separation of the flavonoid extract was improved, with poor separation occurring only with the kaempferol 3-OMe and isorhamnetin peaks. Extracts were also purified further, by passing the

sample through XAD-2 resin, and were subsequently extracted with Et_2O to remove polymeric components removed previously with Sephadex.

High-performance LC was applied to determine the 10 phenolic compounds in maple products (sap, concentrate, and syrup). Five different methods of extraction (lyophilization, ethyl acetate extraction, diethyl ether extraction, Sep-Pak extraction, Superclean column extraction) were developed for phenolic acid and furfural compounds (152). The best recovery was obtained with ethyl acetate extraction (87.6% of mean recovery) for all phenolic acids and furfural compounds. An HPLC separation was conducted by a gradient elution (47.5 min) using methanol (eluent A) and 0.2% aqueous TFA (eluent B) with a linear gradient of 2–40% solvent A. Ultraviolet diode array (UV 280 and 320 nm) and electrochemical detector were connected in series to provide spectral characteristics as well as to enhance the confirmation for identification. The HPLC-ECD analyses were also able to provide a dramatic increase (20–100 times) in the limits of detection of all phenolic compounds compared with those obtained by UV analyses. Highly significant differences were observed in phenolic profiles, depending on the harvest time and technological process used to manufacture maple syrup (152).

A rapid HPLC method for the simultaneous determination of the flavonoids hesperidin and naringin and their aglycones (hesperetin and naringenin) in soft drinks, marmalades, candies, yogurts, biscuits, and salad dressings was also developed (153). Samples were refluxed with 60% aqueous methanol at 90°C and chromatographed on a semimicro column and detected at 283 nm by UV. The detection limit for these phenolics was 0.5 μg/g.

Spices (rosemary, sage, thyme) contain considerable amounts of flavonols and flavones, mainly in the glycoside form (154). Thus, phenolic analyses in spices were often considered in order to determine the optimum time for plant collection to give maximum flavonoid contents and for health benefits (154,155). Flavonoids (naringin, luteolin, apigenin, and chrysoeriol) were extracted from spices using a percolation process at room temperature with solvents (MeOH and EtOAc), and HPLC analysis was carried out (155).

The high demand for authentic vanilla extract as a flavoring agent has resulted in frequent attempts at adulteration. An HPLC method for the quantitation of coumarins as an adulterant in a variety of vanilla flavorings, using a 10-μm μBondapak C_{18} column with MeOH-H_2O (40:60, v/v) as the mobile phase, was proposed (156). Phenolic analysis could be used further for the detection of mixtures of fruits in jams (157). The phenolics present in different commercial jams of apricot, plum, peach, strawberry, sour orange, apple, and pear have been compared and the characteristic compounds for each different jam identified for potential use as marker compounds.

High-performance LC techniques are often applied to various fruit juices and drinks (158–161) to evaluate the antioxidative activity, which is attributed largely to the phenolics, such as flavonoids and phenylpropanoids. Analysis of food phenolics is gaining popularity with the growing evidence of possible health-promoting benefits of phenolics in foods, such as antioxidative, antimicrobial, tumor-inhibiting, free-radical scavenging, and other clinically relevant activities.

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Pigments

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I. CAROTENOIDS

A. Introduction

1. Physical and Chemical Properties

Carotenoids cause the yellow, orange, and red colors of many plants and animals and are extensively used as nontoxic natural or nature-identical colorants in foodstuffs (1). The main representative of this group is β-carotene, which was first isolated from carrots (*Daucus carota*) by Wackenroder in 1831 (2). Carotenoids are isoprenoid polyenes formed by head-to-tail linkage of C_5 isoprene units, only in the center of the molecule there is a tail-to-tail linkage that makes the molecule symmetrical (3). Most carotenoids contain 40 carbon atoms (C_{40} carotenoids) and can be classified into *carotenes*, which are hydrocarbons (e.g., β-carotene, Fig. 1a) and their oxygenated derivates, the *xanthophylls* (e.g., zeaxanthin, Fig. 1b). Fruit xanthophylls are often acylated with fatty acids (4). Apocarotenoids are a manifold group, which enclose degraded forms with fewer than 40 carbon atoms in the skeleton, and higher carotenoids with 45 or 50 carbon atoms, respectively (5).

(a)

HO OH

(b)

Fig. 1 (a) Formula of β-carotene; (b) formula of zeaxanthin.

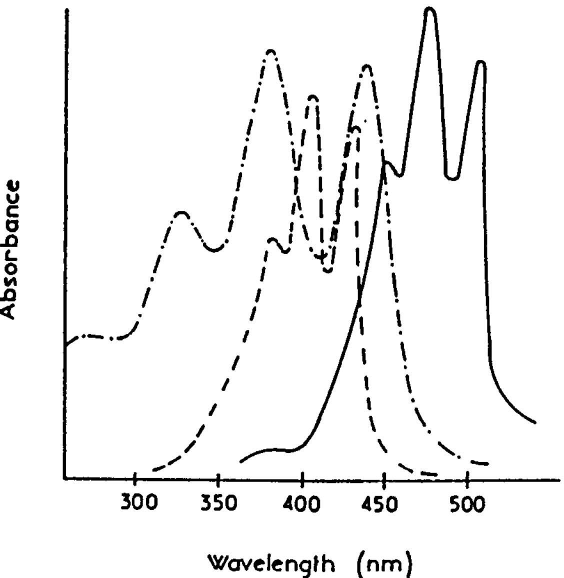

Fig. 2 Absorption spectra of some common carotenoids: (——) lycopene; (- - -) ξ-carotene (in petroleum ether); (-·-·-·-) persicaxanthin (in ethanol). (From Ref. 5.)

The characteristic absorption spectrum of each carotenoid is determined by a series of conjugated double bounds, the so-called "chromophore." Usually the spectrum shows three absorption bands, which are affected by the length of the chromophore, the nature of the double bound, and the taking out of conjunction of one double bond. Several absorption spectra of some common carotenoids are shown in Fig. 2. A change of solvent may, however, cause a shift of the absorption bands. Owing to the extensive double-bond system, carotenoids exist in many geometrical isomeric forms (Z or E isomers). In nature most carotenoids occur in the all-trans form (E isomers); cis isomers (Z isomers) are frequently present in small amounts (6). Cis isomers can be distinguished from trans isomers by a characteristic absorption band ("cis peak") that appears at 300–360 nm (7).

Carotenes are soluble in apolar solvents such as petroleum ether and hexane, while xanthophylls dissolve best in polar solvents such as ethanol and methanol (5). The stability of carotenoids is low; they are especially sensitive to light, oxygen, and peroxide, so special precautions have to be taken when handling them.

2. *Properties in Food*

Only higher plants, spore-bearing vascular plants, algae, and photosynthetic bacteria are able to synthesize de novo carotenoids (8). Carotenoids, which can be isolated from animal tissue (e.g., salmon (9), shrimps, egg yolk (10)), are originally synthesized in plants or bacteria. The distribution of carotenoids in fruits (11–13), vegetables (14,15), leaves (16,17), seeds and animals (18) has been extensively investigated, and good reviews are given by Goodwin (8) and Gross (5).

In natural tissues, carotenoids generally occur as complex mixtures of many substances; in orange juice, for instance, more than 50 carotenoids were identified. A survey of the carotenoid content in some fruits and vegetables is given in Refs. 19 and 20. Differences due to variety, site, and maturity must be considered.

3. *Arguments for Carotenoid Analysis*

Color is a main parameter affecting the quality of food (21). During processing of fruits many of the original carotenoids are degraded and color losses occur. The food industry has applied major effort to minimize these undesirable changes. For instance, Marty and Berset (22) have described methods to lower the degradation of β-carotene during extrusion cooking of vegetables. The increased application of carotenoids as foodstuff colorants is generated by regulations, which restrict the use of artificial colorants (23). Therefore the demand for natural food colors, like extracts of anatto, paprika, tagetes, tomato, and carrot, is constantly growing. Carotenoids are well-known natural pigments, and their application has been comprehensively discussed by Bauernfeind (24). The application of carotenoids as natural food colorants is widely permitted within the European Community (E 160 and E 161) and the United States (1). Demand for natural carotenoids is increasing within the fish industry, because salmonids (e.g., rainbow trout) often do not gain a satisfactory red pigmentation when they are bred in farms (25). Therefore they have to be fed with synthetic astaxantin, canthaxanthin, or natural compounds such as paprika and dried flowers (26).

Additionally there is an increasing interest in carotenoids because of their potentially beneficial health effects, such as provitamin A, anticarcinogenic, antiulcer, antiaging, and antioxidant properties and increased immune responses (27,28). Because the vitamin A potential of cis isomers is less than those of their all-trans counterparts, it is important to distinguish and quantify the various forms (29). According to reports and clinical studies carotenes may be important in the prevention of some forms of cancer (30). Since the National Cancer Institute has recommended an increased intake of food high in carotenoids (31), more detailed information about the carotenoid composition of foodstuffs is desirable.

B. Sample Preparation

Carotenoids are very instable, so precautions in handling these substances have to be observed. Since carotenoids should not be exposed to sunlight or ultraviolet light, the laboratory should be equipped with suitable blinds to produce diffuse, low-intensity daylight, and vessels containing carotenoids should be protected from light by wrapping them with aluminium foil (2). Heating should be avoided whenever possible; however, because it is necessary to remove the solvents used in extraction and purification, it is important to choose solvents with low boiling points. Moreover, carotenoids may be oxidized by oxygen or by peroxides, so operations such as evaporation should be carried out in an atmosphere of nitrogen. Because acids can initiate oxidative decompositions and isomerizations of carotenoids, they have to be eliminated from all stages of manipulation. Any failure may result in a low overall quantitative recovery, losses of labile carotenoids, conversion into other carotenoids, and/or the appearance of cis-trans artifacts.

1. *Extraction*

Carotenoids should be extracted from tissues as rapidly as possible. If an immediate extraction is not possible, samples should be stored below −18°C until required. For the extraction the exactly weighed sample and the solvent are transferred into a blender, where the sample is simultaneously grinded and extracted. Since fresh tissues contain a high percentage of water, and carotenoids are lipo-soluble, the first organic solvent must be miscible with water (e.g., acetone, ethanol, methanol). After one or two extraction steps, water-immiscible solvents (e.g., diethyl ether, benzene) can be applied. Dried materials may be also extracted with water-immiscible solvents, but carotenoid recovery is usually better if the tissue is first treated with a little water and then extracted with water-miscible solvents. Prior to the extraction of fruits the addition of antioxidants [e.g.,

butylated hydroxytoluene (BHT) (5,32)] and neutralizing agents [e.g., tris buffer (5), calcium or magnesium carbonate (33)] is recommended. The initial homogenate is filtered under vacuum through a funnel, and the residue is recovered for further extractions. The procedure is repeated until no more color can be extracted. Usually two to three such extractions are enough, but in special cases more extractions can be necessary [e.g., for grapes, six successive acetone extractions are recommended (35)]. The carotenoids are then transferred from the water-miscible solvent (e.g., acetone) to an appropriate water-immiscible solvent (e.g., diethyl ether, petroleum ether) by adding enough saturated NaCl solution (5). The two layers are allowed to separate, and the top layer, containing the carotenoids, is collected while the bottom layer is re-extracted several times until the top layer becomes colorless (34).

When only small quantities of sample material (0.5 g) are available, the tissue is ground in a mortar with anhydrous sodium sulphate and washed with silver sand. This powder can be extracted as described earlier (2). By using hexane/acetone/ethanol as solvents, Sadler et al. (34) have developed a rapid (15 min per extraction), reproducible method with a good recovery rate to extract lycopene and β-carotene from tomato and citrus products. A rapid method using only small volumes of solvents (isopropanol, hexan) for the extraction of β-carotene from milk was developed by Granelli and Helmersson (37) recently. Solid-phase extraction columns (Sep-Pak C_{18}) with additional magnesium oxide and diatomeaceous earth were used to fractionate mixtures containing different β-carotene isomers and chlorophylls (38). Minguez-Mosquera et al. (36) have used liquid-phase distribution with *N,N*-dimethylformamide and hexan or octadecyl (C_{18}) solid-phase extraction to obtain fat-free pigments from olive oil samples. Fisher and Rouseff (39) have cleaned up saponified orange juice with a C_{18} solid-phase extraction column prior to HPLC analysis. Supercritical CO_2 extraction of β-carotene from sweet potatoes increased the amount of carotenoids extracted, but it also resulted in a higher formation of cis isomers (40).

2. *Saponification*

Saponification is a purification procedure to remove unwanted lipids and chlorophylls. It has to be omitted when alkali-labile carotenoids (e.g., astaxanthin, fucoxanthin) or carotenoid esters are to be analyzed. To prevent the formation of artifacts produced by aldol condensation between acetone and carotenals, all traces of acetone have to be removed prior to saponification (41).

Saponification is carried out by adding sufficient potassium hydroxide (KOH) dissolved in methanol or ethanol to give an overall KOH concentration between 5% and 10% in the extract, which is then kept overnight at room temperature under nitrogen (42). Different alternative saponification methods are described, e.g., boiling the extract under a stream of nitrogen from 5 min (2) up to 30 min (4); keeping the extract at 60°C for 25 min (43), or at 40°C for 3 hours (5), respectively, at 4°C for 12 hours (37). It is evident that a lowering of the saponification temperature, which can help to minimize carotinoid degradation, makes longer reaction times necessary. If saponification is carried out at a higher temperature it has to be stopped by immediate chilling. Subsequently the cold alkaline solution is mixed with freshly distilled diethyl ether (1 : 1) in a separating funnel, to wash it free of alkali, dried, and evaporated in vacuum.

The two procedures of lipid extraction and saponification can be combined as a prolonged or hot alkaline alcoholic extraction, a method that is satisfactory for certain routine analysis (44).

3. *Removal of Sterols*

Sterols, which are very abundant in some fruits, can be removed by precipitating them in different solvents. The unsaponifiable matter is dissolved in a minimum volume of methanol, petroleum ether, or acetone. The precipitation is completed overnight at −20°C; following centrifugation, the sterol-free supernatant is used for analysis (5).

C. Analysis by High-Performance Liquid Chromatography (HPLC)

Because of advantages such as short analysis time, high resolution, good reproducibility, and little structural modification, HPLC has been widely applied to the study and analysis of carotenoids. Generally carotenoids are identified through their chromatographic behavior or by co-elution with authentic standards. Some flowers (e.g., chrysanthemum—flavoxanthin; nettles—neoxanthin) and fruits (e.g., mango—violaxanthin) contain mainly special carotinoids and so can be used to determine the identity (37). Additionally, the identity of carotenoids can be confirmed by their UV-VIS absorption spectra, which can be recorded by a stopped-flow scanning method or online with a photodiode array detector. Functional groups can also be detected when chromatograms are run before and after chemical tests. External or internal standards [e.g., β-apo-8′-carotenal (45), Sudan I (phenylazo—2-naphtalenol) (46)] are used to improve reproducibility. For the stationary phase, both normal-phase HPLC and reversed-phase HPLC are used, the elution being either isocratic or with a gradient; the latter is employed especially for complex extracts containing carotenoids of widely different polarities (1).

1. *Normal-Phase HPLC (NP-HPLC)*

A number of stationary phases and eluents commonly used for NP-HPLC are summarized in Table 1. The use of pressurized liquid chromatographic separation began with the work of Sweeney and Marsh (47), who separated β-carotene isomers on a mixture of magnesium hydroxid and calcium hydroxid. The efficiency of HPLC technique for the separation of complex mixtures of carotenoids was further demonstrated by Stewart and Wheaton (48). They developed a two-step procedure for the separation of the citrus peel carotenoids, fractionating the carotenes on magnesium oxide and the xanthophylls on zinc carbonate. Subsequently Stewart (49) established an HPLC procedure using a single magnesia column for the determination of α- and β-

Table 1 Stationary Phases and Eluents Used for the Separation of Carotinoids by NP-HPLC

Stationary phase	Mobile phase	Type of carotenoids	Ref.
$Mg(OH)_2 + Ca(OH)_2$	1.5% *p*-methylanisole/ petroleum ether	Isomers of β-carotene	47
MgO	A: acetone; B: hexane; g*	Carotene, cryptoxanthin	49
Silica	A: 20% *tert*-pentyl-alcohol/ hexane; B: hexane; g*	xanthophylls	50
Silica	A: acetone; B: hexane; g*	Carotenes, diols, cis-trans isomers, diastereoisomers	51
Silica	A: hexane; B: ethyl acetate; g*	Carotenoids, carotenoid esters	46
Silica	A: pentane; B: acetone/ pentane (80:20); g*	Carotenes, xanthophylls	58
$Ca(OH)_2$	Hexane/0.1–2.0% acetone	Isomers of β-carotene	52
Al_2O_3	Hexane	Carotene isomers	55
Spherisorb S—5 CN	Hexane/isopropylacetate/ acetone (48:17:7)	Astaxanthin	26
Spherisorb CN	Hexane/dichloromethane/ methanol/*N*-ethyldiisopropyl-amine (60:40:01:0.1)	Tunaxanthins	56
Sumipax OA-2000	Hexane/dichloromethane/ ethanol (54:10:0.1)	Stereoisomers of tunaxanthin	57

g* = gradient elution.

carotenes and β-cryptoxanthin. To follow the carotenoid changes in citrus juice, however, he used a two-step method where the carotenes and cryptoxanthin were analyzed on magnesium oxide and the xanthophylls were chromatographed on silica (50). An HPLC on silica was applied to the separation of carotenes diols, cis-trans isomers, and diastereoisomers and to the quantitative analysis of carotenoid fatty acid esters from unsaponified fruit samples (46). A typical separation of orange peel carotenoids with NP-HPLC on silica is shown in Fig. 3. The detection limit for β-carotene was found to be approximately 5 ng (51). For the separation of β-carotene isomers, NP-HPLC with calcium hydroxide columns is commonly utilized (52,53), the mobile phase usually containing small amounts of acetone in hexane (54). Vecchi et al. (55,56) managed to separate 11 cis isomers of β,β-carotene on alumina. The separation of all stereoisomers of tunaxanthin was achieved by employing a chiral column of Sumipax OA-2000 (57). Generally, insufficient separation of carotenes, increased formation of isomers (46), as well as poor suitability for gradient elution are the disadvantages of NP-HPLC.

2. *Reversed-Phase HPLC (RP-HPLC)*

Recently, reversed-phase partition chromatography has become the method of choice for both qualitative and quantitative analysis of carotenoids. The stationary phases commonly used are those with C_{18}-bonded chains (ODS); their performances are influenced by the extent of endcap-

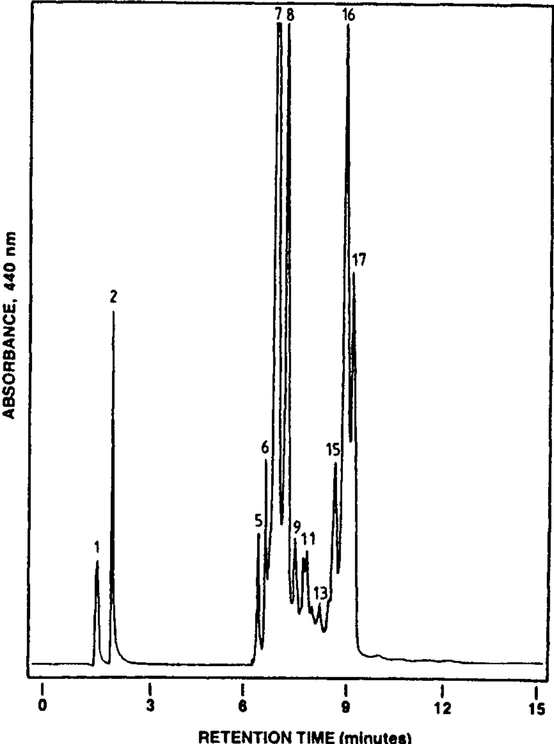

Fig. 3 Normal-phase HPLC separation of *Valencia* orange peel carotenoids. Peaks: 2 = α-cryptoxanthin esters; 5 = lutein diesters; 6 and 7 = violaxanthin diesters; 8 = luteoxanthin diesters; 15 and 16 = violaxanthin monoesters; 17 = luteoxanthin monoesters. The other peaks are not identified. (From Ref. 46.)

ping and the carbon loading. Bushway (59) compared various RP- and NP-HPLC methods and found that C_{18} columns provide the quickest and best separation of carotenoids. Polymeric C_{18} phases exhibited better selectivity for structurally similar carotenoids than did monomeric C_{18} phases, which represent the majority of the commercially available C_{18} columns (60). These findings were confirmed by Epler et al. (61), who evaluated 65 different HPLC columns under standardized conditions with respect to carotenoid separation and recovery. Numerous mobile-phase compositions have been employed for RP-HPLC of carotenoids, some commonly used solvents are listed in Table 2. Acetonitrile-based eluents are applied most frequently, with various organic modifiers (tetrahydrofuran, chloroform, ethyl acetate) added to improve the performance. Methanol-based eluents are also used, either with or without modifiers such as tetrahydrofuran, chloroform, water, and hexan. Epler et al. (61) observed that methanol-based solvents typically provided higher recoveries than acetonitrile-based solvents. Separating *Satsuma* carotenoids with RP-HPLC, Noga and Lenz (62) found satisfactory reproducibility of retention time and peak areas and limits of detection from 0.027 ppm for *trans*-violaxanthin to 0.073 ppm for α-carotene.

Table 2 Mobile Phases Used for Separation of Carotinoids by RP-HPLC

Mobile phase	Type of carotenoids	Application	Refs.
Methanol/chloroform (94:6)	β-carotene	Potato	40
Methanol/acetonitril/ethylacetate (80/10/10)	α-carotene, β-carotene	Kiwi fruit	12
Methanol/tetrahydrofuran/water (67/27/6)	Lycopene, β-carotene	Tomato; grapefruit	35
A: methanol; B: ethylacetate; g*	Carotenoids, carotenoid esters	Pepper, fruits	53, 66
A: methanol/water (47/25); B: ethylacetate; g*	Carotenes, xanthophylls	Kiwi fruit	12
A: methanol/water (9/1); B: acetone; g*	Carotenes, xanthophylls	Citrus	62
Acetone/water (70/30)	β-Carotene, xanthophylls	Grape	37
A: acetone/water (47/25); B: acetone/methanol (47/25); g*	Carotenes, xanthophylls	Carrot	63
A: acetone; B: water; g*	β-Carotene, xanthophylls	Grape, fruits, paprika	64, 67, 68
Acetonitrile/methanol/methylene chloride/hexane (55/22/11.5/11.5)	Cis-trans isomers of β-carotene	Vegetables	45
Acetonitrile/methylene chloride/ methanol (82/12/6)	Carotenes	Carrot	70
Acetonitrile/methanol/tetrahydrofuran (40/56/4)	Carotenes, xanthophylls	Vegetables, fruits	59
Acetonitrile/methanol/tetrahydrofuran (94/5/1)	Trans and cis β-carotenes	Vegetable oil	38
Acetonitrile/methanol/dichloromethan (80/14/6)	β-Carotene	Milk	39
Acetonitrile/chloroform (92/8)	α- and β-carotene	Carrot; tomato	35
A: acetonitrile/2-propanol (40/60); B: water; g*	Carotenoids, carotenoid esters, β-carotene	Paprika	65
A: acetonitrile/water (90/10); B: ethyl acetate; g*	Carotenes, xanthophylls	Tea	69

g* = gradient elution.

With a chromatographic system consisting of two Chrom-Spher PAH-glass columns, a mobile phase of acetonitrile–methanol–dichlormethane (80/14/6), and an injection volume of 10 μl milk extract, for β-carotene a linear working range from 0.67–4 μg/l, a quantification limit of about 30 ng/ml, and a repeatability of 5.3% R.S.D. was found (38). An effective separation of grape carotenoids has been achieved using an RP-C18 column and an acetone–water gradient (35). The concentration of α-carotene and β-carotene in various carrots was determined with an RP-HPLC-method using methanol–acetonitril–ethylacetate (80/10/10) as solvent; the separation is shown in Fig. 4 (63).

In general, RP-HPLC has been applied successfully to the qualitative and quantitative estimation of carotenes (64), xanthophylls, cis- and trans-carotenoids (54,60), and carotenoid fatty acid esters (53,65). Figure 5 shows an HPLC separation of (A) paprika extract, containing carotenoid esters, and (B) saponified paprika extract, containing the corresponding carotenoids (74a).

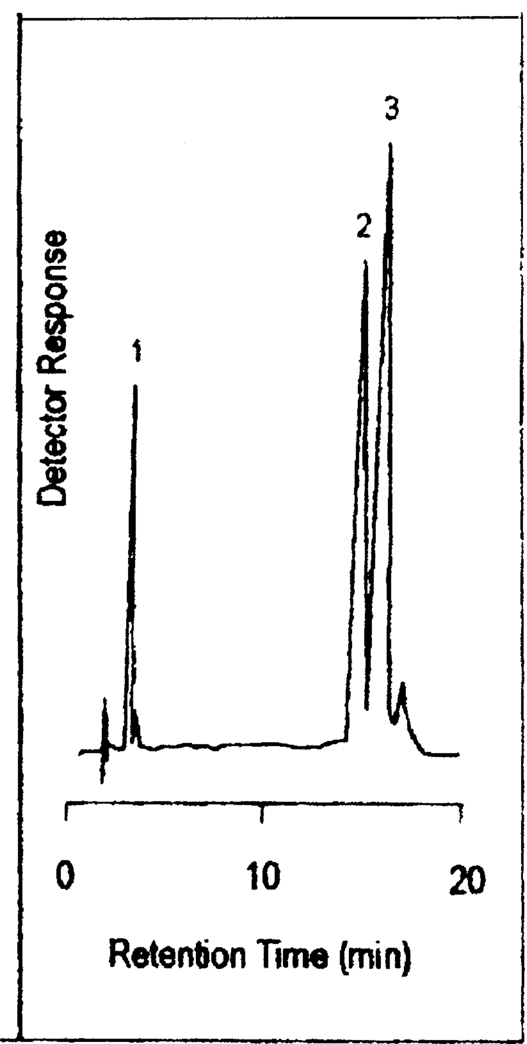

Fig. 4 RP-HPLC separation of the carotenoids in carrots. Peaks: 1 = Sudan I, 2 = α-carotene; 3 = β-carotene. (From Ref. 63.)

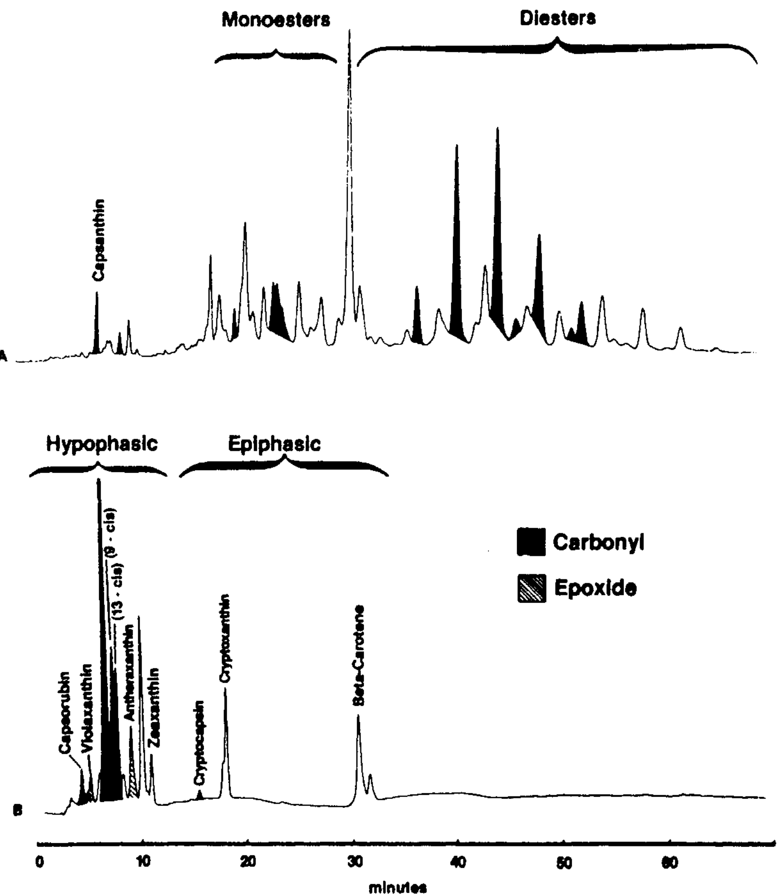

Fig. 5 RP-HPLC separation of (A) paprika extract and (B) saponified paprika extract on a Zorbax C_{18} column at 460 nm. The solid peaks represent carotenoids containing a ketone group, and the hash-marked peaks represent carotenoids containing an epoxide group. (From Ref. 74a.)

Reversed-phase ion-pair chromatography was employed to quantify the chlorophylls and carotenoids in olive oil (36) using the following eluents: (A) methanol/water/ion-pair reagent (0.05 M tetrabutylammonium and 1 M ammonium acetate), (B) acetone/methanol (1 : 1).

3. *Supercritical Fluid Chromatography (SFC)*

The use of supercritical fluid chromatography for carotene separation has been examined and optimized, especially in regard to temperature, pressure, and organic modifiers in the supercritical fluid (71). With an RP column it was possible to resolve an α-carotene–cis isomer from an all-trans carotene as well as two cis isomers of β-carotene from an all-trans β-carotene. As with HPLC, only polymeric C_{18} columns were able to resolve the cis isomers of α- and β-carotene from the all-trans isomers. Supercritical fluid chromatography offers the advantage not only of an efficient separation but also of fast analysis. Indeed, the use of SFC with ODS-based columns for the analysis of carotenoid pigments affords a threefold reduction of analysis time compared to HPLC (72). The elution order of carotenoids and their cis isomers was found to be the same as in RP-HPLC. The selectivity of the system could further be increased by adding modifiers (e.g.,

hexane, methanol, chloroform, methylene chloride) to the CO_2. The correlation found by comparing the selectivity of both SFC and RP-HPLC was very good, with a correlation coefficient of 0.975 (73).

4. *High-Performance Liquid Chromatography–Mass Spectrometry (HPLC-MS)*

High-performance LC-MS combines traditional HPLC separations with mass spectral identification in a single procedure. The complex carotenoid mixture is separated with RP-HPLC, and the ensuing chromatographic profile is monitored through the total ion current (TIC) response of the mass spectrometer. Usually mass spectra are collected in the electron impact (EI) mode using 40 eV at a source temperature of 240°C (74). Combining this data with the monitored UV-VIS spectra, the carotenoids can be identified and quantified. Taylor et al. (74) found a detection limit for carotenoids of approximately 50 ng. This method is relatively costly in regard to instrumentation and maintenance. These considerations are, however, compensated by its great potential for rapid carotenoid analysis and identification. The separation of asparagus carotenoids with HPLC-MS as well as typical EI mass spectra of some carotenoids were published by Taylor et al. (74).

D. Application: HPLC Analysis of Carotenoids and Carotenoid Esters in Red Bell Pepper (Ref. 66)

Red bell peppers without seeds are cut into small pieces and accurately weighed (2.00–3.00 g). Samples are homogenized with 50 ml methanol and 1 g $MgCO_3$ for 1 min. After filtration through a Bucher funnel under vacuum, the residue is repeatedly extracted with a total 100 ml of the extracting solvent (acetone/hexane, 52:20) until all the carotenoids have been extracted. The pooled filtrate is transferred to a separatory funnel, and a known amount of the internal standard canthaxanthin is added. An equal volume of distilled water is added, and the mixture is vigorously shaken and the phases allowed to separate for 5 min. The aqueous phase is filtered through a 0.4-μm nylon filter and used for HPLC analysis. The HPLC separation is carried out on a C_{18} column (3.9 $\times$ 150 mm) with a mobile phase of methanol and ethyl acetate in a linear gradient for 20 min. The detection wavelength is set at 475 nm, and the flow rate is 1.8 ml per min. Figure 6 shows the good separations of carotenoids and carotenoid esters that can be obtained with this method.

II. CHLOROPHYLLS

A. Introduction

1. *Physical and Chemical Properties*

Chlorophylls are the most widely distributed natural pigments, and every organism capable of photosynthesis, including all living plants, algae, and some photosyntetic bacteria, owes its green color to them. The word *chlorophyll* is derived from Greek and means “the green of the leaves.” In higher plants, chlorophylls are always nonconvalently linked to proteins and accompanied by carotenoid molecules. These chlorophyll-carotenoid proteins are found in organelles known as *chloroplasts*. The biological function of chloroplasts is to capture light energy that is subsequently used for the conversion of carbon dioxide to carbohydrates—the process is known as *photosynthesis*. Chlorophylls consist of 10 conjugated double bonds, so they absorb strongly in the blue and red regions of the spectrum, which causes a green color (Fig. 7).

In chemical terms, chlorophylls are porphyrins, such as the pigments of blood (hemoglobin) and muscles (myoglobin). The tetrapyrrole ring of chlorophylls is formed by four pyrrole residues joined together by methine groups; the four nitrogen atoms are coordinated with a cen-

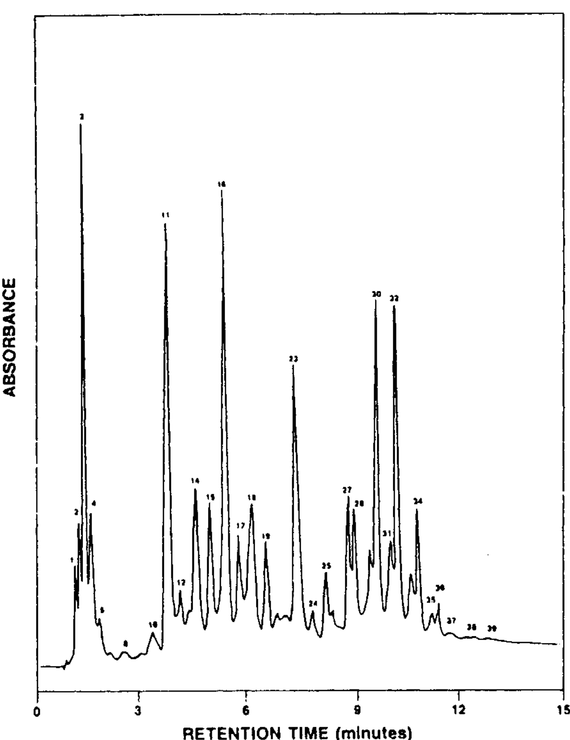

Fig. 6 HPLC determination of carotenoids and carotenoid esters in red bell pepper. Peaks: 2 = capsorubin; 3 = capsanthin; 11 = β-apo 8′-carotenal (int. std.); 12–15 and 18 = capsorubin monoester; 16 = capsanthin $C_{14:0}$; 17 and 19 = capsorubin monester; 23 = β-carotene; 25, 27, 28, 29, 31, 33, 36 = capsorubin diester; 30 = capsanthin $C_{12:0}$ and $C_{14:0}$; 32 = capsanthin $C_{14:0}$ and $C_{14:0}$; 34 = capsanthin $C_{14:0}$ and $C_{16:0}$; 35, 37, 38 = lutein diester. (From Ref. 66.)

tral magnesium atom (Fig. 8). Unlike hemes, which are iron complexes of porphyrins, chlorophylls contain an additional fifth isocyclic ring as well as a long hydrophobic side chain derived from propionic acid at C7, which is esterified to phytol, a C_{20} terpenoid alcohol (5). The chlorophyll molecule itself is relatively large, the porphyrin head being about 1.5 × 1.5 nm in size and the phytol chain being about 2 nm in overall length (75). The additional structural elements of chlorophylls vary widely; more than 50 structures are known already. The most widespread of them are chlorophyll *a* (chl. *a*) and chlorophyll *b* (chl. *b*), which were isolated for the first time by Sorby in 1873 (76). Chlorophyll *b* differs from chl. *a* only in having an aldehyd group in place of the methyl group at the C3 position. However, this small difference in the structure of the two chlorophylls causes significant differences in the absorption spectra and therefore also in their different green hues. As demonstrated in Fig. 7, the absorption maximum of chl. *b* is shifted toward the green region of the spectrum, so the color of chl. *b* is yellow-green and the color of chl. *a* is blue-green. Chlorophylls *c* (chls. *c*) comprises a group of different pigments (e.g., chl. c_1, c_2, c_3) that contain a free-acid function and therefore possess a higher polarity than the apolar chl. *a* and chl. *b*. They are most widely distributed in algae (77). Other pigments (e.g., chlorophyll *d*) and chlorophyll epimers (e.g., chlorophyll *a*′), which have been isolated and identified recently, also play an important role in photosynthesis (78). The chemical properties of chlorophylls have been reviewed by Jackson (79). In general, chlorophylls are relatively unstable; they are sensitive

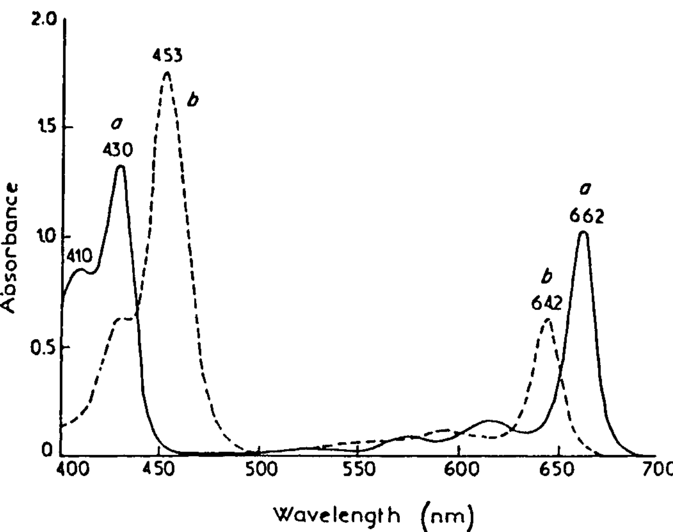

Fig. 7 Absorption spectra of chlorophylls *a* and *b* in diethyl ether. Chlorophyll *a* (———), chlorophyll *b* (— — —). (From Ref. 5.)

to light, heat, and oxygen as well as chemical degradation (80). The first step of chlorophyll degradation consists of dephytylation and/or demetallation to give their chlorophyllides (chlide) and/or pheophorbides (pheoide), respectively (Fig. 8). Depending on the plant species or the organ, the order of the degradation reactions varies. The phytyl ester is usually split off by an enzyme called chlorophyllase, forming the corresponding chlorophyllide with the free-acid function (81). Furthermore, under mild acid or alkali conditions, hydrolysis can happen whereby oxidation at C10 may occur. The central magnesium ion is removed immediately from the chlorophyll when diluted mineral acids are applied, forming pheophytin. Additionally, a demetalling enzyme, Mg-dechelatase, has been described in the literature (81). Pheophytins can be degraded to pheophorbide through hydrolysis or by chlorophyllase (5). Pheophytins and pheophorbids are yellow-brown colorants, so they impart an unattractive brown-green color to plants and food (82). Considering their solubility in liquids, these four substance groups can be classified into two groups. Chlorophylls and pheophytins are hydrophobic (lipophilic), since they contain the phytyl residue, whereas chlorophyllides and pheophorbides are hydrophilic.

2. *Properties in Food*

The distribution of chlorophylls among photosynthetic organisms has been listed by Jackson (79) and Scheer (80). Chlorophyll *a* is present in all organisms capable of oxygenic photosynthesis, in higher plants (green leaves and fruits), ferns, mosses, green algae, and prochlorophytes (the so-called "green line" (80)). Chlorophyll *a* is usually accompanied by chl. *b* in a ratio of 3 : 1. In some algae ("brown line," e.g., *Cryptophytes*, *Diatomes*, *Pheophytes*), chl. *a* is complemented by chls. *c*. The total chlorophyll concentration of fruits and vegetables has been reviewed by Gross (5) and Hermann (82). It can generally be assumed that the deeper the green color of a leaf or a fruit, the higher is its chlorophyll content. Green leaves of spinach, parsley, and green cabbage can contain up to 2000 mg chlorophyll per kg fresh weight, whereas, for instance, beans, peas, and cucumbers have about 100 mg per kg (82). In fruits and vegetables, the chlorophyll content, and often also the ratio chl. *a*/*b*, varies with the genus, species, cultivar, and environmental factors (5). Since chlorophylls generally disintegrate during fruit ripening, the content depends

Chlorophyllase

Chlorophyll

Chlorophyllide

Mg-dechelatase

Mg-dechelatase

Chlorophyllase

Pheophytin

Pheophorbide

	Chl *a*	Chl b
R	$-CH_3$	$-CHO$

Fig. 8 Formulas of chlorophyll *a* and *b* (chl. *a*: R: $-CH_3$; chl. *b*: R: $-CHO$) and degradation products. (From Ref. 81.)

Table 3 Average Chlorophyll Content in Fruits and Vegetables

	Chlorophyll ($mg\text{-}kg^{-1}$)		
	a	*b*	Ref.
Bean, green	76	35	85
Cabbage, green	1898	406	85
Cabbage, white	8	2	85
Cucumber	64	24	85
Parsley	890	288	85
Paprika, green	70	33	85
Pea, green	106	22	85
Spinach	946	202	90
Apple ("Golden Delicious," peel)	70	38	86
Grape ("Riesling")	11	4	91
Kiwi ("Bruno")	17	8	87
Pear ("Trévoux," peel, green)	31	13	88
Strawberry ("Tenira," green)	5	1	89
Tangerine ("Dancy," green)	249	52	5

highly on the developmental stage. But there are also some exceptions that retain a high chlorophyll content even in the ripe stage, e.g., certain apple, pear, and gooseberry cultivars. Table 3 shows the average chlorophyll content and the ratio chl. *a*/*b* of some fruits and vegetables. High amounts of chlorophylls can be found in the deep green peel of avocado (379 $mg\text{-}kg^{-1}$ (83)) and musk melon (345 $mg\text{-}kg^{-1}$ (84)). There are, however, pronounced differences between the chlorophyll content of the peel and of the pulp of these fruits.

During food processing and storage, chlorophylls undergo various alterations that cause dramatic color changes. Through blanching, preserving, heating, and storage, chl. *a* and chl. *b* are transformed to pheophytin *a* and *b*, respectively, which have a brown-green color. And if the preserved food has not been blanched, native enzymes (e.g., chlorophyllase) may also split off the phytol residue and degrade the resulting chlorphyllides. In canned food the central magnesium ion can be replaced with iron or tin ions, which may cause a grey-brown discoloration of the products. By contrast, the insertion of copper and zinc ions produces a green pigment with higher stability and greater tinctorial strength.

3. *Arguments for Chlorophyll Analysis—Regulations*

Because of their basic role in nature, chlorophylls have been the subject of many investigations. For a better understanding of photosynthesis it is necessary that chlorophylls and their derivatives be determined qualitatively and quantitatively. Investigations into their chemical structure, genesis, transformations, and functions in plants supply valuable information for plant physiological studies (92).

Color is an important attribute of food quality, so it can be used as a criterion for the classification of fruits and vegetables. For instance, with apples the fruit color is an important factor determining the selling price on the U.S. market, the preferred colors being red and yellow, with green fruits selling poorly (93). Numerous analytical methods have been established to quantify the color and to identify the native colorants of food (94,95). During the ripening of fruits, the green chlorophylls disappear and yellow pigments (e.g., carotenoids, flavonoids) become visible. However, when bananas are ripened at temperatures above 24°C they fail to develop a fully yel-

low peel, because the chlorophyll breakdown is suppressed. Those "green-ripe" bananas are perceived to be of poor quality and consequently fetch a low price (96). The physiology of degreening in maturing seeds is of great importance for the canola industry, because the presence of as little as 6% green seeds results in green, poor-selling oil. Generally the pigment content and the change in content during ripening can be used as an index of the physiological age of fruits (97). The chlorophyll concentration in apple peels is a potential indicator of maturity at harvest and helps to predict the quality (98). The senescence of leafy vegetables is characterized by a degradation of chlorophylls, accompanied by a marked loss of greenness. Both the pathway and the parameters influencing this undesirable alteration have been monitored, but further investigations are required (99).

During food processing (e.g., blanching, heat-drying, storage), the structural characteristics of natural pigments are often modified and the color of the product is usually changed. The determination of the chlorophyll content and its derivatives can therefore indicate the extent of color damage in processed food (100). During the traditional process of canning, most chlorophylls are converted to pheophytins and pyropheophytins due to the severe heat treatment. Since the bright green color of chlorophylls is more pleasing to the consumer than is the olive-brown color of pheophytins and pyropheophytins, great effort has been invested in developing better technologies. Recent studies have shown that in comparison to conventional preservation methods, aseptic technologies can reduce color losses (101). Furthermore, a correlation between the chlorophyll content and the undesirable "grassy" taste of black tea has been found. To minimize this off-flavor in black tea, the factors affecting chlorophyll content (e.g., nitrogen fertilization, plucking criteria) have been investigated (102). Using multiple regression analysis, a model for predicting the quality of manufactured black tea from the carotenoid and chlorophyll composition of fresh green tea leaves has been established (103). Chlorophylls and chlorophyll derivatives are also often used as food colorants to restore the color of freshly harvested crops. Since chlorophylls have been an integral part of vegetable foodstuff for animals and humans throughout history, they appear to be harmless. Unfortunately, their possible widespread application as a green food colorant is hampered by their inherent instability (75). Nevertheless, the substitution of synthetic pigments used in foods with natural ones would be an important step toward more healthful food. Mg-Chlorophyll (E 140) and Cu-chlorophyll (E 141) are both permitted as food additives, but Cu-chlorophyll has shown a better stability than Mg-chlorophyll with regard to the exposure to light, oxygen, temperature, sodium bisulfite, benzoyl peroxide, and acids (104). The commercial production of coppered chlorophyll has been described in detail by Humphrey (105). Other metal-substituted chlorophylls (e.g., iron(III), zinc(II) chlorophylls) are used for certain kinds of medications and have received further attention because of their potential use as electrode materials for photoelectron conversion (106).

B. Sample Preparation

Because chlorophylls are exceptionally labile pigments, proper care has to be exercised during extraction and analysis. All manipulations ought to be carried out rapidly in darkness or in dim light ($<1\ W/m^2$) to prevent photodestruction or photoisomerization. Green light (around 520 nm) is particularly suitable to working with photosynthetic pigments, since most of them absorb weakly in this region (81). High temperatures can destroy chlorophylls, so the extraction and purification steps should be carried out at low temperatures. The extended double bond system of pigments renders them susceptible to oxidative degradation; however, it is not necessary to work under oxygen-free conditions. To avoid increased oxidation, strong shaking of pigment solutions, which would favor the exchange between atmospheric oxygen and the chlorophylls, should be avoided. The native enzymes (e.g., chlorophyllase) should be inactivated during the purification

process. Finally, the concentration of acids has to be kept very low during the extraction process; otherwise the chelated metal in the cyclic tetrapyrroles may be lost, leading to the pheophytin or pheophorbide forms. If the plant tissue has a high native acid content, the acidity can be neutralized with sodium bicarbonate. Generally it is best to work as quickly as possible in a continuous way and to avoid lengthy storage before analysis (107).

For the determination of chlorophyll pigments it is preferable to use freshly grown plant material. If storage should become necessary, the tissue can be kept in the dark at a temperature of 0°C or below (92). A great variety of extraction methods have been used, and comprehensive discussions are given in the literature (5,108,109). The criterium for a good extraction procedure is whether it brings all pigments in solution and causes little or no alterations (108). Because of the instability of chlorophylls it is almost impossible to extract food material without the formation of alteration products. With the aim of inactivating enzymes, some researchers have immersed plant material in boiling water (blanching or scalding) before extraction. This causes isomerization of chlorophylls (e.g., chls. *a* and *b*), forming compounds that are diastereomeric at C13 (e.g., chl. *a'* and *b'*, (92)). Boiling also causes the breakdown of chlorophylls to pheophytins if the tissue is rather acidic. Under acidic conditions chl. *a* is generally less stable than chl. *b*, causing the formation of relatively large quantities of pheophytin *a* during the extraction of fruit samples (110). To avoid the conversion of chl. *a* to pheophytin *a*, the original acidity of the tissues must be neutralized with buffers such as sodium carbonate, calcium carbonate, and sodium hydrogenphosphate (92). For kiwi fruits, Fuke et al. (110) found that the addition of 2.0 g sodium carbonate per 50-g sample is sufficient. However, when the material is extracted under conditions that do not inactivate enzymes, chlorophylls will undergo hydrolysis and oxidation: Chlorophyllase will catalyze the hydrolysis of the phytyl group, resulting in the formation of chlorophyllides (see Fig. 9), and oxidative enzymes will promote the formation of 13-hydroxy-chlorophylls (111).

Commonly, the food material is disintegrated and chlorophylls are extracted in a blender. Alternatively, small portions may be ground in a mortar with the solvent and sand and then filtered or centrifuged. Up to 90% of the fresh weight of fresh plant tissue is water, so water-miscible solvents should be used for extraction. The most frequently used extraction solvents are acetone and methanol. They break the noncovalent linkage between pigments and proteins and bring the chlorophylls into solution (5). Generally, cold solvents are used (98), and the acetone or alcohol concentration in the extract should be maintained near 80% or 90% (92). Sometimes ammonium hydroxide (0.05 M) is added to acetone in order to prevent the loss of magnesium from the tetrapyrrole ring. However, acetone and methanol have disadvantages, because acetone is often a poor extractant for fresh plants and because alcohols such as methanol may cause some allomerization reactions (108). Solvent mixtures (e.g., acetone/methanol) were also used for extracting chlorophylls from diverse sources. The extraction of fresh plant material was improved with a mixture of methanol–petroleum ether (112). For the purification of chlorophylls and carotenoids from olive fruits, an extraction procedure with *N*,*N*-dimethylformamide and hexane was developed (113). All solvents must be stored in brown bottles and in darkness to minimize the formation of peroxides, which make pigments unstable (107).

To obtain clear solutions, the extracts are either filtered or centrifuged and the residues reextracted with the same solvents (108). Another possibility is to transfer the chlorophylls from the acetone phase into an ether phase by adding sodium chloride (114) or sodium sulfate (93). More recently acetone extracts were purified and fractionated with C_{18} solid-phase extraction cartridges, and dephytylated pigments were eluted with 70% acetone and phytylated pigments with 90% acetone (115). Often a concentrating of the pigment solution with a rota-evaporator under vacuum is necessary before analysis. Chlorophyll extracts should be analyzed as soon as possible, but if they have to be stored they must be kept in the cold and dark (107).

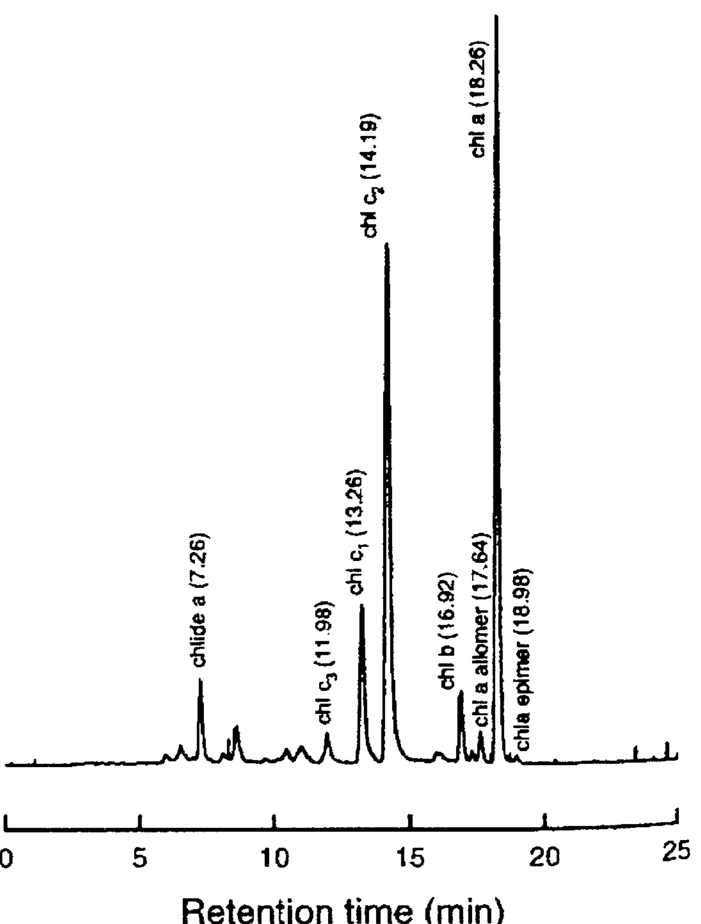

Fig. 9 Reversed-phase chromatogram of frozen pea extract. The C_{18}-column was subjected to an isocratic elution by acetone:water (70:17:13). The chlorophylls and pheophytins were detected by fluorescence, with the excitation wavelength at 413 nm and the emission wavelength at 669 nm. (From Ref. 107.)

C. Analysis by High-Performance Liquid Chromatography (HPLC)

In comparison with other analytical techniques, HPLC has significant advantages, and it is nowadays the method of choice for qualitative and quantitative analysis of chlorophylls in food. The use of HPLC offers several significant advantages, including better resolution, greater speed, and higher reproducibility and sensitivity (116,117). Furthermore, to date no degradation of samples has been reported when separating chlorophylls with HPLC. However, HPLC is handicapped by the high investments necessary and the high costs for each analysis. Another disadvantage is the nonvisualization of pigment progression because of stainless tubing (107).

Since chlorophylls have distinct spectroscopic properties, absorption or fluorescence spectrophotometry can be employed for their identification and quantification (118). Usually wavelengths between 430 and 440 nm and 645 and 660 nm, respectively (93,115), are used for spectrophotometric detection. With spectrophotometric detectors, detection limits of approximately 80 ng chlorophyll (119) and 1 ng chlorophyll (120), respectively, can be achieved. Fluorescence

detection is more sensitive and more specific than absorption measurement, thereby allowing the determination of picogram amounts of chlorophylls in the eluent (118). In general, the chlorophylls are excited at 430–440 nm and the emission is measured at 650–670 nm. Both normal-phase and reversed-phase methods have been developed for the analysis of chlorophylls and their derivatives. Assays performed with normal phases have sometimes not proved satisfactory, so the reversed-phase technique is now the most widely used method of routine analysis. The application of photodiode array detectors, which allow the simultaneous recording of chromatograms at various wavelengths, is preferable to single-wavelength detectors, which give little information. Also, fluorometric detection is a favorable possibility to detect and quanitify chlorophylls without any separation of carotenoids (Fig. 10). Furthermore, fluorescence spectra can be used to identify chlorophylls. The first application of HPLC to the analysis of chlorophylls was by Evans et al. in 1975 (116). They separated pheophytin *a* and *b* on Corasil II with a mobile phase consisting of ethyl acetate and light petroleum (1:5). Commonly used eluents for NP- and RP-HPLC as well as adsorbents and references are given in Table 4.

1. Normal-Phase HPLC (NP-HPLC)

Various researchers have employed NP-HPLC for the separation of chlorophylls (120,121). Silica gel was the predominantly used absorbent; different solvent mixtures were used for the pigment elution (Table 4). The separation mechanism of silica gel depends on polarity differences, so this support is highly efficient in resolving intermediate to nonpolar pigments with large polarity differences (e.g., chlorophylls); but it is less effective for polar pigments such as chlorophyllides (118). With NP-HPLC the simultaneous determination of chlorophylls and carotenoids in spinach (120,122) and algeal tissue was possible (119). However, it seems that RP-HPLC, especially when using C_{18} columns, is superior to NP-HPLC. Pigment degradation and long analysis times are two of the main drawbacks when silica is used as the column packing material (117). Furthermore, NP separation requires an additional extraction step to eliminate water from the extract and to transfer the pigments from the acetone solution into hexane. Water in the extract causes problems with the chromatographic resolution and leads to varying retention times (122).

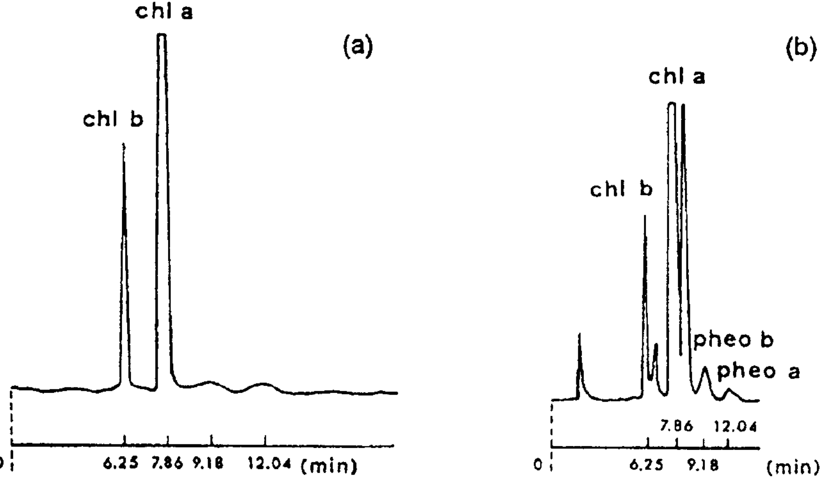

Fig. 10 Chromatogram of a pigment extract form a natural seawater sample on a polymeric column (Vydac 201 TP). Mobile phases: (a) methanol–pyridine (0.025 M, pH adjusted to 5.0 with acetic acid) (80:20, v/v); (b) acetontrile–acetone (70:30, v/v). (From Ref. 77.)

Table 4 HPLC Conditions for the Analysis of Chlorophylls

Column	Solvent	Detection (nm)	Pigments[a]	Application	Ref.
Normal phase					
Silica gel SS-05	1% Isopropanol in hexane	380	chl, pheo, car	Spinach	120
LiChrospher Si 100	0.8–2% 2-propanol in hexane	420, 430	chl	Clover	121
Partisil 10	Petroleum/acetone/dimethylsulfoxide/diethylamine (25/23.25/1.25/0.25)	440	chl, pheo, chlid, car	Phytoplankton	119
Silica gel Lober 60-B	A: heptane/ether/hexane (60/20/20) B: heptane/ether/hexane (60/20/15)	425	chl, car	Tabacco	134
Silica gel	0.5% 2-methylpropanol in hexane	440	chl	Apple	98
Silica gel	A: 1.7% 2-propanol in hexane B: 25% 2-propanol in hexane	658	chl, pheo, chlid, pheid	Spinach	122
Reversed phase					
μBondapak C18-Porasil B	A: 80–100% methanol in water B: 10–75% ether in methanol	440	chl, pheo, car	Spinach	128
μBondapak C18	A: 75% methanol in water B: ethyl acetate	654	chl, pheo	Spinach	93
μBondapak C18	Ethyl acetate/methanol/water (50/37.5/12.5)	654	chl, pheo	Rice grains	125
Hypersil ODS	A: 1.5% TBAA/water/methanol (10/10/80) B: 20% acetone in methanol	440 F 430/600	chl, pheid, car	Algae	135
Nova-Pak C18	Acetonitrile/chloroform/water (83/15/2)	440	chl, pheid, car	Bermuda grass	126
Chromasil C18	Acetonitrile/methanol/ethyl acetate (53/40/7)	430	chl, pheo, chlid, car	Pea, celery	125
Radial Pak RP	A: 80% methanol in water B: ethyl acetate	660	chl, pheo, chlid, pheid	Canola seeds	115
Zorbax ODS C18	Ethyl acetate/methanol/water (60/30/10)	436, 658	chl, pheo	Spinach	100
Zorbax ODS C18	A: acetonitrile/methanol (70/30) B: dichloromethane	437	chl, chlid, pheo, car	Beans	78
Hypersil ODS	A: 75% methanol in water B: ethyl acetate	450	chl, pheid, chlid, car	Tea leaves	136
Hypersil ODS	A: 75% methanol in water B: ethyl acetate	430	chl, pheo, car	Kiwi	137
Spherisorb ODS-2	A: methanol/acetonitrile/dichloromethane/hexane (15/75/5/5) B: methanol/acetonitrile/acetonitrile/dichloromethane/hexane (15/40/22.5/22.5)	430, 460	chl, car	Beans	127
Spherisorb ODS-2	A: water/ion-pair reagent/methanol (1/1/8) B: acetone/methanol (1/1) Ion-pair reagent: 0.05 M tetrabutylammonium acetate–1 M ammonium acetate in water	430	chl, chlid, pheo, pheid, car	Olives, olive oil	117

(*continued*)

Table 4 Continued

Column	Solvent	Detection (nm)	Pigments[a]	Application	Ref.
Vydac 201 TP	A: methanol/ion-pair reagent (80/20) B: acetonitrile/acetone (70/30) Ion-pair reagent: 0.025 M pyridine, pH 5.0	F 440/600	chl, chlid	Algae	77

[a] Abbreviations: chl = chlorophylls; chlid = chlorophyllids; pheo = pheophytins; pheid = pheophorbids; car = carotinoids; F = fluorescence.

The elimination of water can be achieved by drying the solvent using sodium sulfate of sodium chloride.

2. *Reversed-Phase HPLC (RP-HPLC)*

During the last few years RP-HPLC with chemically bonded nonpolar stationary phases (C_8 or C_{18}) has been widely applied to the study of chlorophylls and their derivatives (123–125). With this system, simple solvent mixtures such as methanol/water/ethyl acetate (95,100) can be used (Table 4). The retention mechanism is due mainly to the partitioning of the components between phases rather than to nonpolar interactions between solute molecules and carbon functional groups in the stationary phase. The elution order of chlorophylls depends on their polarity: the more polar a pigment is, the earlier it is eluted (115). The retention time increases in the following order: chlorophyllides *b* < chlorophyllides *a* < pheophorbides < chlorophyll *b* < chlorophyll *a* < pheophytin *b* < pheophytin *a* (122). The separation of polar pigments such as chlorophyllides and pheophorbides with RP-HPLC is less effective, but the resolution can be improved by the use of ion-suppression or ion-pairing techniques (117). A monomeric octadecylsilica column was capable of separating various algal chlorophylls when pyridinium acetate was used as the ion-pair agent. With a polymeric octadecylsilica column, a good separation of chlorophylls c_1 and c_2 was achieved when pyridinium was employed as counterion (77) (Fig. 9). Additionally polyethylene, which has a separation mechanism similar to that of bonded silica gel, has been employed as an efficient alternative support in the RP mode (118). Reversed-phase HPLC has been successfully applied to the determination of chlorophylls and their derivatives in a wide variety of foodstuffs, for example, in spinach (93,100,123), kiwis (129), green olives (117), olive oils (130), green tea leaves (131), canola seeds (115), green peas (125), celery leaves (125), turf Bermuda grass (126), and beans (127). In general, the duration of one analysis varies between 10 min (124) and 50 min (99); however, Eskins et al. (128) have published a method that required 270 min. The coefficient of variation for the chromatographic determination lies within the range of 0.6–1.8% (119); recovery values of 98.3% and 98.7% can be obtained for chls. *a* and *b*, respectively (127). Numerous papers (125,128) deal with the simultaneous determination of chlorophylls and carotenoids by means of RP-HPLC (Table 4). This offers the possibility of studying the essential pigments in fruits and vegetables within less than 30 min (127,129). For example, Schoefs et al. (78) have separated the photosynthetic pigments of bean leaves using RP-HPLC and a solvent gradient consisting of acetonitrile–methanol (70:30) and an increasing proportion of dichlormethan. Up to 100 μl of methanolic extract were injected; the detection was carried out at 430 nm, and the chromatographic run lasted 35 min. After each analysis the system is rinsed to eliminate any remaining contaminants that could lead to peak doublets.

A very promising method for the identification of single pigments is the online combination of HPLC and mass spectrometry. For instance, chls. *a* and *b* and the corresponding pheo-

phytins, chlorophyllides, pheophorbides, and pyropheophytins of fresh and heat-treated spinach tissue were separated with RP-HPLC and their molecular weights determined by positive-ion fast-atom bombardment mass spectroscopy (FAB-MS) (132). Plasma desorption mass spectroscopy (PD-MS) has been successfully used to analyze the formation of alteration products and to study the chlorophyll–protein complex (133).

D. Application: HPLC Analysis of Chlorophylls, Chlorophyll Derivatives, and Carotenoids in Spinach (Ref. 99)

Pigments are extracted by grinding 2.5 g of spinach leaves in 20 ml cold acetone and 2.5 ml distilled water with a mortar and pestle. Then the homogenate is filtered and rewashed with 80% cold acetone until the residue is colorless. In low light the solution is brought to a final volume of 50 ml. Prior to HPLC analysis the solution is passed through a 0.22-μm filter. Pigments are separated by a C_{18} column (4.6 × 250 mm) using two solvents: A: 80% methanol, 20% water; and B: ethyl acetate in a gradient. A chromatogram of pigments extracted from spinach leaves is shown in Figure 11.

III. ANTHOCYANINS

A. Introduction

1. Physical and Chemical Properties

Anthocyanins are the largest group of water-soluble pigments in nature. They are present in almost all higher plants and can be found in all parts of the plant, but their most obvious occurrence is in fruits and flowers, where they are responsible for the attractive red, violet, and blue colors (138). A special subgroup of anthocyanins are the yellow-colored 3-deoxyanalogs, such as apigeninidin, luteolinidin, and tricetinidin, which have been isolated from mosses and ferns as well as from members of the *Gramineae* and *Gesneriaceae* families of flowering plants (139). The term *anthocyanin* is derived from the Greek words for "flower" and "blue" and was introduced in

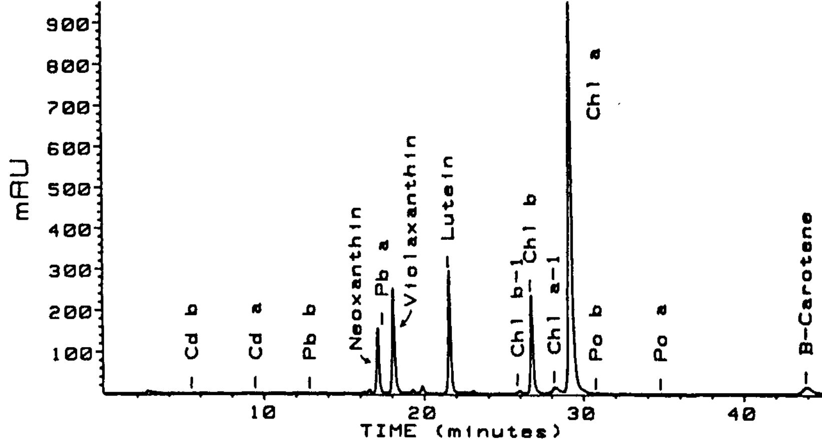

Fig. 11 Chromatogram of chlorophylls, chlorophyll derivatives, and carotenoids extracted from spinach leaves and analyzed by HPLC with a photodiode array detector. Chd = chlorophyllide, Pb = pheophorbide, Chl = chlorophyll, Po = pheophytin. (From Ref. 99.)

1835 by Marquart (5). Guiding contributions to the isolation, purification, and identification of anthocyanins were made by Willstätter and Everest (140).

The anthocyanidins are hydroxylated and methoxylated derivates of 2-phenylbenzopyrylium or flavylium salts; owing to their characteristic $C_6C_3C_6$ carbon skeleton, they are regarded as flavonoid compounds (141). Natural anthocyanic pigments (anthocyanins) are always glycosides, which will separate into the less stable aglycone forms (anthocyanidins) and sugars upon hydrolysis. Individual anthocyanins are characterized by the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, and the nature, number, and position of sugars attached to the molecule. So far, 17 naturally occurring anthocyanidins are known, but only six of them are of widespread occurrence and therefore commonly contribute to the pigmentation of plants (142). These common six anthocyanidines—cyanidin (cy), delphinidin (dp), malvidin (mv), pelargonidin (pg), peonidin (pn), and petunidin (pt)—are all C3, C5, C7, and C4′ hydroxylated derivatives (Fig. 12). Since each anthocyanidin may be glycosylated and acylated by various sugars and acids at different positions, the number of anthocyanins is approximately 15–20 times higher than the number of anthocyanidins. The sugars most commonly bonded to anthocyanidins are glucose, galactose, rhamnose, and arabinose and in some cases also di- and trisaccharides. Glycosylation frequently occurs at C3, C5, and C7, but glycosylation at C3′, C4′, and C5′ has also been observed. The most common anthocyanidin glycosides are 3-monosides, 3-biosides, 3,5-diglycosides, and 3,7-diglycosides. The sugar residues may be further acylated with organic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, malic acid, and acetic acid (142). Anthocyanins are reactive compounds and are either degraded or form polymeric pigments. Light absorbance measurements of solutions that contain linearly increasing anthocyanin concentrations do not follow Beer's law, but considerably higher absorbance values will be measured at higher concentrations. The exact character of this phenomenon, which is called self-association, is not completely understood; however, various studies by Hoshino (143) have provided strong evidence that vertical stacking of the anthocyanin quinoidal bases occurs that causes

Fig. 12 Formulas of the anthocyanidins (flavylium cations):

Name	R1	R2
Cyanidin (cy)	OH	H
Delphinidin (dp)	OH	OH
Malvidin (mv)	OCH_3	OCH_3
Pelargonidin (pg)	H	H
Peonidin (pn)	OCH_3	H
Petunidin (pt)	OCH_3	OH

a stronger chemical shift of the aromatic protons with increasing anthocyanin concentrations. A wide range of polymeric pigments can be formed by complexation of anthocyanins, with flavonoids as copigments. These new pigments exhibit an increased stability, and their absorption maximum is shifted toward higher wavelengths ("bathochromic shift"). The chemical structure of these anthocyanin/copigment complexes has been explained by two hypothesis: (a) horizontal (end-to-end) hydrogen bond stabilization of the quinoidal base; (b) vertical stacking. Recent results support the latter theory, which seems to be more realistic. To get an appreciable color augmentation and spectral shift, a copigmenting compound with a double bond in the 2,3-position of the flavonoid C ring seem to be necessary; flavones and flavonols proved to be superior to chalcones, aurones, flavanones, and catechins (139). Another possibility for forming polymeric pigments is the acetaldehyde-bridged anthocyanin–tannin condensation, which was first described by Timberlake and Bridle (144). Recently, new anthocyanin-derived pigments have been discovered in wine in the course of crossflow microfiltration. These pigments are formed by cycloaddition of the hydroxyl groups at C4 and C5 of the malvidin molecule and the double bond of the 4-vinylphenol, followed by subsequent oxidative polymerization, regenerating the flavylium cation form (145).

In aqueous media, anthocyanins undergo structural transformations that are pH-dependent (Fig. 13). It has been found (141) that four anthocyanin forms exist in equilibrium: the red flavylium cation (AH^+), the blue quinonoidal base (A), the colorless carbinol pseudobase (B), and the colorless chalcone (C). In strongly acidic media (pH values below 2), anthocyanins exist predominantly in their red-colored form as flavylium cations. At weakly acidic, neutral, and basic pH values, the carbinol and the quinonoidal base forms dominate the flavylium cation, so the color fades and shifts from red to blue (138). Besides the pH dependency, the anthocyanin equilibria are also affected by temperature, because the formation of chalcones (C) is an endothermic reaction. On heating an anthocyanin solution, the equilibria are driven toward the chalcone form and a resulting decrease in colored forms (AH^+, A) occurs (139). A blueing of the anthocyanic color is also achieved by the reaction of anthocyanins containing *ortho*-dihydroxy groups, with $AlCl_3$ at pH values 2–4 (bathochromic shift), and through the formation of anthocyanin–flavonoid complexes (copigmentation) (5).

Due to their long chromophore of eight conjugated double bonds, anthocyanins are intensely colored in an acid medium. Their absorption spectra are characterized by two separate bands, one in the visible region between 465 and 550 nm and the second, which is less intense, in the UV range between 270 and 280 nm (5). The absorption spectra in the visible region of pelargonidin, cyanidin, and delphinidin in 0.01% HCl/methanol are shown in Fig. 14. It must be noted that the absorption maxima and the corresponding intensities vary with the nature of the solvent; analytical measurements therefore have to be made under standardized conditions. The absorption characteristics of anthocyanin solutions are markedly affected by the structure of the pigment; for instance, an additional hydroxyl group will cause a relatively large bathochromic shift toward longer wavelengths (pelargonidin: A_{max} = 520 nm, delphinidin: A_{max} = 546 nm) and a change in color from scarlet red (pg) to blue-mauve (dp). In contrast, methylation has little or a negliable effect, while glycosylation at C3 produces an absorption maximum shorter by 15 nm; acylation of the glycosidic moiety with cinnamic acids causes a characteristic shoulder in the UV region at 310–335 nm (5).

All natural anthocyanins suffer from inherent instability, so they may be degraded to form colorless or brown-colored, often insoluble products. It is evident, that depending on the reaction type, different degradation products are formed; however, only little analytical data is available. For example, after reaction of anthocyanins with hydrogen peroxide, substances of the benzofuran type were detected (139). Studies with grape must like model solutions indicated that anthocyanins are degraded by coupled oxidation and that they form adducts with caffeoyltartaric acid

Fig. 13 Equilibrium distribution of four anthocyanin forms of malvidin-3-glucoside as a function of pH: The red flavylium cation (AH^+), the blue quinonoidal base (A), the colorless carbinol pseudobase (B), and the colorless chalcone (C). (From Ref. 138.)

quinones. The UV-VIS spectra of the colored condensation products suggest that they contain both caffeolytartaric acid and anthocyanin moieties (146). The major factors affecting the stability of anthocyanins are pH value, temperature, oxygen, hydrogen peroxide, and light, but other factors (enzymes, metal ions, ascorbic acid) can also increase the anthocyanin bleaching and degradation. In general, anthocyanins are most stable in acidic, oxygen-free media under cold and dark conditions.

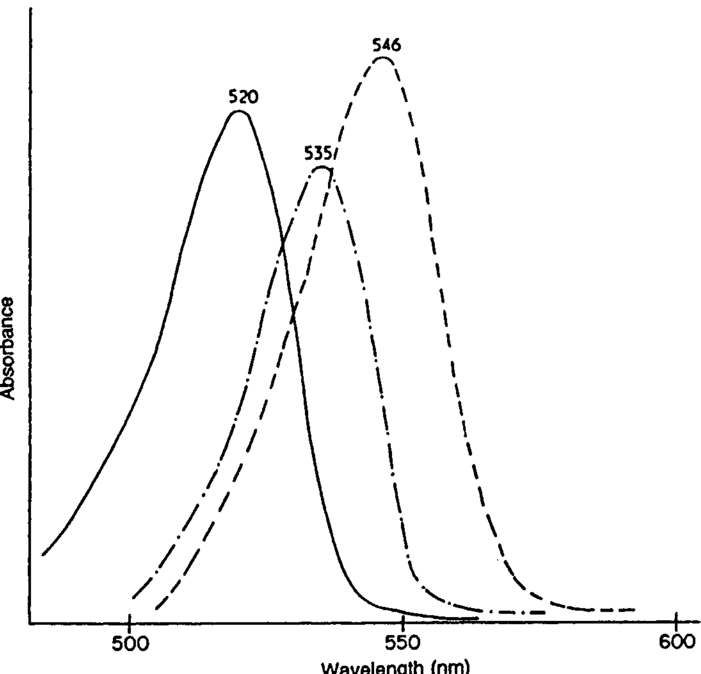

Fig. 14 Absorption spectra in the visible region of (———) pelargonidin; (-·-·-·-) cyanidine, and (- - - -) delphinidin in 0.01% HCl/methanol. (From Ref. 5.)

2. Properties in Food

Many fruits and vegetables owe their attractive blue, violet, or red colors to the presence of anthocyanins. In 70 species of 33 families of angiosperms, anthocyanins are found in membrane-bounded anthocyanoplasts located within the main cell vacuole (142). The fruits of the *Vitaceae* (grapes) and the *Rosaceae* (e.g., apple, cherry, plum, peach, strawberry, blackberry, raspberry) families are especially characterized by their anthocyanin content. Other families containing anthocyanic pigments include the *Ericaceae* (blueberry, cranberry), *Saxifragaceae* (red and black currant), *Caprifoliaceae* (elderberry), *Solanaceae* (e.g., tomatillo, huckleberry), *Gramineae* (e.g., maize, barley, rice), *Anacardiaceae* (e.g., mango) *Sapindaceae* (e.g., lychee), *Rutaceae* (e.g., blood orange), *Malvaceae* (e.g., roselle), *Fabaceae* (e.g., beancowpea, pea, soybean), *Apiaceae* (e.g., carrot), *Brassicaceae* (e.g., cabbage, radish), *Solanaceae* (tomato, eggplant), *Convolvulaceae* (e.g., sweet potato), and *Berberidaceae* (e.g., barberry), among others. Of course, many of the mentioned food plants also contain other pigments, such as carotenoids and chlorophylls. A large number of over 200 different anthocyanins can be found in these plants; but since only six anthocyanidins are common, this variation is due mainly to the glycosidic type (5). The color of most fruits is caused by a complex mixture of anthocyanins. For instance, Sapers et al. (147) found as many as 16 anthocyanins in highbush blueberry cultivars, and Wulf and Nagel (148) separated 21 anthocyanin components in the skins of *Vitis vinfera* cv. *Cabernet Sauvignon*. However, simple patterns of only one or two anthocyanins can also be detected, for example, in passion fruit and peaches. Cyanidin is the most common anthocyanidin in fruits (occurrence in 90% of the fruits examined), followed by delphinidin (35%), peonidin (30%), pelargonidin (20%), and petunidin and malvidin (each 15%) (141). These anthocyanins are also predominant in vegetables and grains (142). Glycosylation at the C3 position is very widespread; diglycosides are formed either by linkage of sugars to two different hydroxyls (generally C3 and C5 or C7) or by linkage of two

sugars to only one position (C3); triglycosides are not very numerous in fruits. Acylated anthocyanins are fairly often found in fruits (e.g., grapes (149), eggplant, and garden huckleberry) and in vegetables (e.g., cabbage, radish, mung bean, celery, chicory, potato); however, they occur rarely in grains (maize) (142). The total anthocyanin content of different plant tissues may display considerable differences. For example, in fruits it ranged from 0.25 mg per 100 g (pear) to 760 mg per 100 g (*Vaccinium elliotti*), red currant cultivars varied from 11.9 to 18.6 mg per 100 g, and cranberry cultivars varied from 46 to 172 mg per 100 g (141). The distribution and contents of anthocyanic pigments in fruits, vegetables, and grains have been comprehensively summarized by Gross (5), Macheix et al. (141), Mazza and Miniati (142), and Timberlake and Bridle (150), respectively. Examples showing the total anthocyanin content and their distribution in some fruits and vegetables are given in Table 5.

3. *Arguments for Anthocyanin Analysis—Regulations*

Color is an important attribute of most fruits and vegetables as well as their products. The red, violet, and blue colors of many plants are due to the presence of anthocyanins, so it is of interest to analyze them. Both quantitative and qualitative aspects are responsible for the color quality of the foodstuff. With the object of improving the chromatic characteristics of fruits, the evolution of anthocyanins in fruits and influencing factors (e.g., temperature, water) have been studied extensively (152,153). Unfortunately, anthocyanins degrade easily according to various reaction mechanisms, as has been reviewed by Markakis (154). During processing and storage of anthocyanin-

Table 5 Total Anthocyanin Content and Major Anthocyanins in Some Fruits

	Anthocyanin content (mg-kg)	Percentage of total anthocyanin distribution							
		cy3gl	cy3ar	cy3ga	cy3rt	cy3sm	cy3sp	cy3 (2xyru)	cy3 (2glru)
Apple (peel)	100–21,600	1–5	4–10	85–94	—	—	—	—	—
Billberry	4600	9	—	19	—	—	—	—	—
Blackberry	820–1800	68–100	—	—	0–32	—	—	—	—
Black currant (209)	2500–3900	17	—	—	35	—	1	—	—
Blueberry (151)	1600–5030	1–6	1–10	1–20	—	—	—	—	—
Cherry (sweet)	3500–4500	++	—	—	++	—	+	—	—
Cherry (tart)	288	3–19	—	—	11–27	—	1–16	—	25–77
Cranberry	460–1720	—	13–25	16–25	—	—	—	—	—
Elderberry (209)	4400–15,600	33–66	—	—	—	32–55	—	—	—
Grape (blue)	80–3880	1–6	—	—	—	—	—	—	—
Plum	19–53	37	—	—	45	—	—	—	—
Raspberry (red)	230–590	11–45	—	—	5–32	—	20–72	—	0–38
Red currant	119–186	2–10	—	—	8–17	10–31	4–9	28–73	0–28
Strawberry	127	0–50	—	—	—	—	—	—	—

Abbreviations: cy = cyanidin; dp = delphinidin; mv = malvidin, pg = pelargonidin, pe = peonidin, pt = petunidin; gl = glucosid, ar = arabinosid, ga = galactosid, xy = xylosid, rt = rutinosid, sm = sambubiosid, sp = sophorosid, xyru = xylosyrutinosid, glru = glucosylrutinosid; ++ = major pigment, + = minor pigment, tr = traces.
Source: Refs. 141 and 142.

containing food products such as juice, wine, jam, syrups, and dried and frozen fruits, the original color deteriorates and changes from natural red or purple to red-brown colors (155). Subsequently, problems often occur in fruit wines and juices, caused by the formation of haze and sediments (156). Numerous investigations have already shown that degradation and polymerization are influenced by oxygen, ascorbic acid, light, pH value, and temperature. Generally the anthocyanin degradation follows a first-order reaction kinetics (157). To minimize these undesirable modifications of sensory properties, further studies concerning the stability of anthocyanins should be carried out. However, in some products, e.g., red and port wines, a distinctive conversion of the original fruity red color to a well-developed, aged red wine color is desired (158).

Following the current trend away from synthetic colors, the use of anthocyanins as a natural food colorant will steadily increase (159). From the medical point of view, anthocyanic pigments are regarded as safe, since they have been consumed by men and animals for countless generations without apparent adverse effects to health. Furthermore, various beneficial therapeutic properties, particularly in ophthalmology and with some blood circulation disorders, are attributed to anthocyanins (160). Due to their polyphenolic nature, anthocyanins exhibit antioxidative activities, so it is suggested that they play an important role in the prevention of lipid peroxidation of cell membranes and carcinogenesis (161). However, their technical applicability is hampered by the inherent instability and pH dependence. Some of these disadvantages may be overcome by a recently discovered group of acylated B-ring substituted anthocyanins (162). Methods for the synthesis and derivatization of anthocyanins are reviewed by Iacobucci and Sweeny (139). The use of anthocyanins of natural origin for food and beverages is widely permitted within the

Table 5 Continued

Percentage of total anthocyanin distribution											
dp3ru	dp3gl	dp3ga	dp3ar	pg3gl	pt3gl	mv3gl	mv3ga	mv3ar	pn3ga	pn3ar	pn3gl
—	—	—	—	—	—	tr	—	—	—	—	—
—	9	19		—	—	22	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
30	13	—	—	—	—	—	—	—	—	—	—
6–19	1–9	6–20	4–15	—	0,5–9	1–21	8–32	5–14	—	—	—
—	—	—	—	—	—	—	—	—	—	+	+
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	23–39	11–21	—
—	—	—	—	—	—	—	—	—	—	—	—
—	5–17	—	—	—	6–12	36–43	—	—	—	—	5–13
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	50–100	—	—	—	—	—	—	—

European Community (E 163) and the United States. Numerous plants and plant parts (e.g., grapes, roselle, bilberry) have been suggested as potential commercial sources of anthocyanidins, but only those where the pigment is a by-product of another valuable product (e.g., juice, wine) are of economic interest.

Individual anthocyanin composition is distinctive for any given plant, so anthocyanin analysis is very useful in distinguishing between species. Chemotaxonomic differentiation is commonly based on qualitative differences (163), furthermore within one cultivar (e.g., grapes) even varieties can be discriminated by quantitative differences (164). The anthocyanic profiles of 11 different grape varieties obtained with RP-HPLC are shown in Fig. 15 (165). The characteristic differences in anthocyanin patterns have also been successfully applied to the detection of adulterations in products of cranberries (166), black currants (166), blackberries (167), and grapes (168).

B. Sample Preparation

During sample preparation and analysis some precautions have to be taken, since the anthocyanins are not stable in neutral or alkaline solutions, under high light intensities, or at high temperatures.

1. *Extraction*

Anthocyanins are decomposed in neutral or alkaline solutions, so extraction procedures generally involve the use of diluted acids in polar solvents. The most common extraction procedure involves the maceration or soaking of plant material in methanol containing a small amount (e.g., 1%) of HCl (169). Acidification with strong acids such as HCl serves to maintain a low pH value. It may, however, alter the native form of complex pigments by breaking associations with metals, copigments, and proteins (170). To obtain naturelike anthocyanins, the use of neutral solvents (e.g., 60% methanol, ethylen glycol, *n*-butanol, acetone) and mild organic acids (e.g., formic acid, acetic acid) has been recommended.

2. *Purification*

For the purification of crude anthocyanin extracts, various methods have been successfully applied. In the past, sample cleanup has been carried out through precipitation with basic lead acetate (171), paper chromatography (172), or solvent–solvent extraction (173). These methods are very time-consuming and cause considerable breakdown of anthocyanins. More recently, solid-phase extraction with insoluble polyvinylpyrrolidone (PVP) (174), Sephadex G-25 (175), Sephadex LH-20 (176), polyamid (177), ion-exchange resins (178), acid alumina (179), and octadecylsilane (180) have been used. The simple and rapid procedures are based on the principle that anthocyanins are bound to the adsorbents, whereas interfering substances (e.g., sugars, acids) are washed from the columns or cartridges. In general, anthocyanins are subsequently eluted from the adsorbent with acidified methanol (181).

C. Analysis by High-Performance Liquid Chromatography (HPLC)

Since the work of Manley and Shubiak (182), who were the first to apply HPLC to anthocyanin analysis, numerous HPLC techniques have been developed for the separation and quantification of anthocyanins and anthocyanidins. Nowadays HPLC has become the method of choice, because it offers the advantage that it is a rapid, sensitive, and quantitative method. For the peak identification and quantitative evaluation of chromatograms, the use of pure anthocyanin standards is recommended; however, only a limited, but constantly increasing, number of substances is avail-

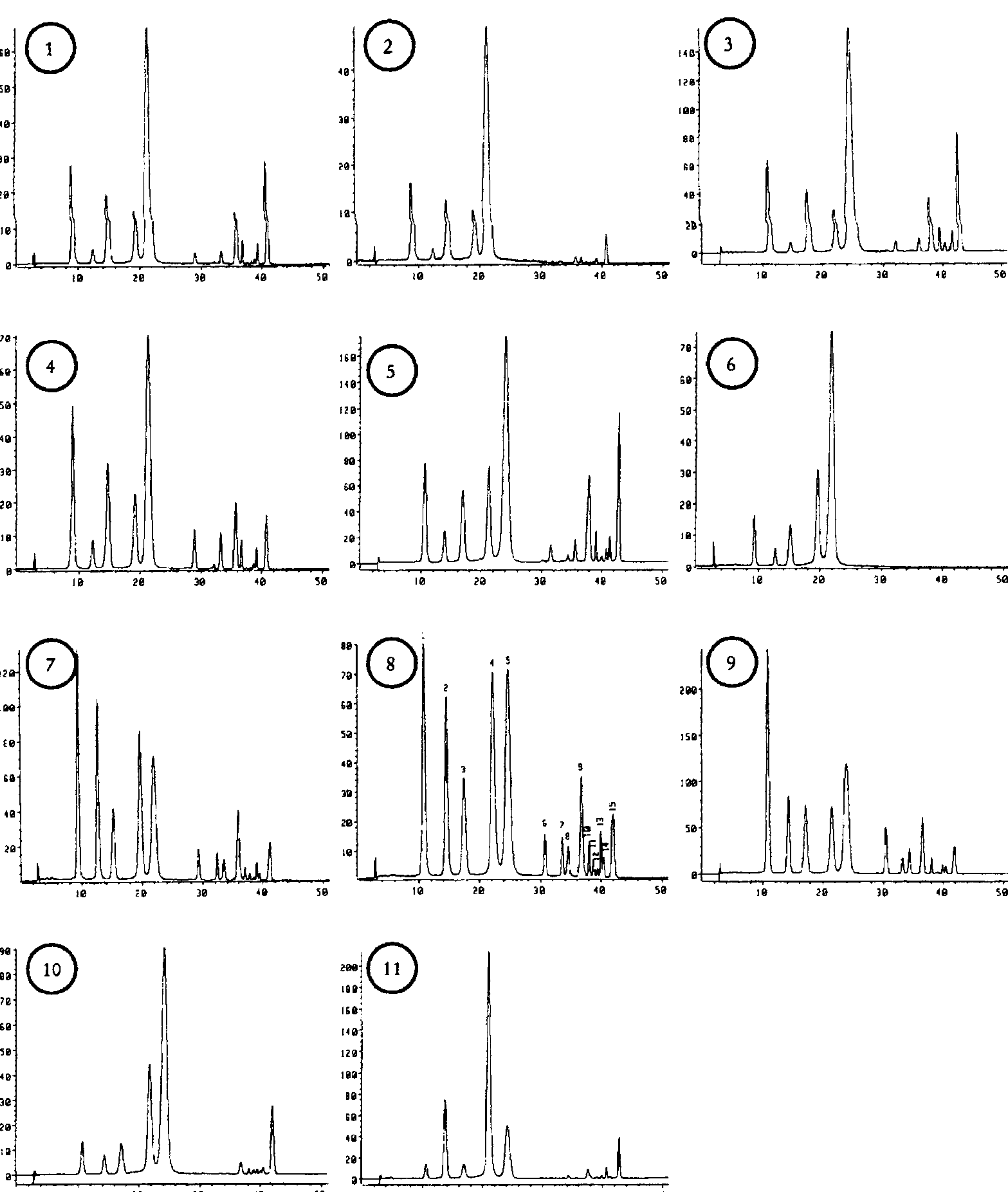

Fig. 15 RP-HPLC separation of anthocyanins of 11 different grape varieties: 1 = Zweigelt, 2 = Blaufränkisch, 3 = Blauer Portugieser, 4 = Blauburger, 5 = St. Laurent, 6 = Pinot Noir, 7 = Cabernet Sauvignon, 8 = Merlot, 9 = Cabernet Franc, 10 = Blauer Wildbacher, 11 = Trollinger. (From Ref. 165.)

able. If a special anthocyanin standard is not commercially available, the preparative isolation of pure anthocyanins is still necessary. A possible method for preparative anthocyanin separation is gradient-elution centrifugal partition chromatography, which was successfully applied to by-products of grapes and black currants (183). On the other hand, it is a common practice to use only one anthocyanin standard (e.g., malvidin 3-glucoside), and that all other anthocyanin peaks are quantified corresponding to this standard. Because there are considerable differences in the

quantitative absorbance values and the absorption maxima, this practice will give slightly incorrect results (184).

1. Reversed-Phase HPLC

The HPLC separation of anthocyanins has been carried out almost exclusively on silica columns bonded with octadecyl (C_{18}); occasionally, octyl (C_8) and hexyl (C_6) have also been used (181,185). The average particle diameter of HPLC packings is typically between 3 and 10 μm, with 5 μm being the most frequently used. In RP-HPLC, the anthocyanins elute according to their polarities (delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin) (181). The elution of pigments is usually achieved by gradient elution, but occasionally isocratic elution is also sufficient, for instance, with fruits that have a relatively simple anthocyanin pattern, such as mango (141), or for the separation of anthocyanin mixtures (186). Solvent systems for the HPLC analysis of anthocyanins always include an acid, to reach low pH values (<2.0), which ensure that most of the anthocyanins are present in the red flavylium cation form. Formic acid (up to 10%) is the most widely used. It is, however, quite corrosive (187), and perchloric acid (188) and trifluoroacetic acid (189) are equally aggressive. Commonly used alternatives are acetic acid (15%, (190)), phosphoric acid (3–4%, (191)) or phosphate buffer (180). It should, however, be considered that the extensive use of solvents more acidic than pH 2 could result in poor reproducibility and a short column lifetime due to the loss of bonded phases from the surface of the silica stationary-phase support. The employment of nonsilica, polymeric columns, which are stable from pH 1 to 13, allows the use of strong acidic solvents, thus causing sharper peaks because the anthocyanins are almost completely in their flavylium cation form (181). Commonly, methanol (192) and acetonitrile (193) are used as organic modifiers, but some researchers substituted acetone for methanol (194) and still obtained similar separations.

The eluting anthocyanins are monitored with UV-VIS or photodiode array detection systems (181). Usually the detection wavelength is set in the range between 510 and 545 nm. Depending on the sensitivity of the detector system and the anthocyanin pigment, a detection limit of 0.5–1.0 mg per liter anthocyanin can be assumed generally. The identification of anthocyanins is carried out by cochromatography of known, available standards or based on retention times, which are commonly compared with data from the literature. By scanning the absorption spectra online, photodiode array detection makes an additional structural characterization of pigments possible (180). By connecting the HPLC column to a mass spectrometer with a thermospray or electrospray interface, it is possible to identify the anthocyanins by their mass ions and fragmentation pattern (145,195). Chemically, anthocyanins can be identified by acid hydrolysis and subsequent determination of anthocyanidin and sugar moiety, or by mild alkaline hydrolysis and subsequent determination of the acyl component (172). Furthermore, the elucidation of structure, especially for acylated anthocyanins, can be confirmed by fast-atom bombardment mass spectrometry and NMR spectroscopy (196).

When samples contain high quantities of interfering material (e.g., sugars, colloids), a sample-cleanup procedure is recommended. A sample preparation with solid-phase extraction cartridges (C_{18}) was found to be quite useful and effective (180,193). Prior to HPLC analysis, all samples should be filtered through a 0.45-μm or smaller membrane filter.

During the last two decades, RP-HPLC analysis has been successfully used to study anthocyanins in numerous foodstuffs. Because of the great number of publications, only a short excerpt from those many applications and corresponding HPLC-methods can be given in Table 6. To demonstrate the potential of this technique, an RP-HPLC separation of black currant juice is shown in Fig. 16. Despite the high costs of acquisition and maintenance, RP-HPLC will likely remain the best-qualified method for anthocyanin analysis in the coming years, because it offers,

Table 6 Some RP-HPLC Methods and Their Applications in Anthocyanin Analysis

Mobile phase	Stationary phase	Sample	Anthocyanins	Ref.
Binary gradient A: formic acid/water (1/99); B: methanol	Superpac Pep-S (5 μm; 250 × 4 mm)	Grape	Anthocyanidins	201
Binary gradient A: formic acid/water (10/90); B: formic acid/water/methanol (10/44/45)	Novapak ODS (5 μm; 150 × 4.6 mm)	Grape	Anthocyanidin 3-glucoside, acylated anthocyanins	152
Binary gradient A: 5% formic acid; B: methanol	Micropak MCH-5 C_{18} (5 μm; 150 × 4 mm)	Red wine	Anthocyanidin 3-glucoside, acylated anthocyanins	202
Binary gradient A: formic acid/water (10/90); B: formic acid/water/methanol (10/40/50)	Supelcosil LC-18 (3 μm; 100 × 5 mm)	Cranberry (*V. oxycoccus*)	pn-glu, pn-gal, pn-ara, cy-glu, cy-gal, cy-ara	192
Binary gradient A: 4.5% formic acid; B: acetonitrile	μBondapak C_{18} (10 μm; 250 × 4.6 mm)	Grape	Anthocyanidin 3-glucoside, acylated anthocyanins	203
Binary gradient A: 10% formic acid; B: 10% formic acid/30% acetonitrile/60% water	RP-C_{18} (5 μm; 250 × 4 mm)	Chokeberry	cy-gal, cy-ara, cy-xyl	204
Binary gradient A: 10% formic acid; B: acetonitrile	HS-5-C_{18}	Blueberry	Anthocyanidin 3-glucoside, 3-galactosides, 3-arabinosides	151
Water/acetonitrile/formic acid (81/9/10)	LiChrospher RP-18	Black currant, strawberry, cherry, raspberry, elderberry	Anthocyanidin-glucosides, -galactosides, -arabinosides, -rutinosides	205
Binary gradient A: acetic acid/water (15/85); B: water/acetic acid/methanol (65/15/10)	LiChrosorb RP-18 (5 μm; 250 × 4 mm)	Grape	Anthocyanidin 3-glucoside, acylated anthocyanins	190
Water/acetic acid/methanol (43/10/19)	μBondapak C_{18} (10 μm; 250 × 4.6 mm)	Apple	cy-gal, cy-ara	206

(continued)

Table 6 Continued

Mobile phase	Stationary phase	Sample	Anthocyanins	Ref.
Binary gradient A: 15% acetic acid; B: acetonitrile	Supelcosil ODS (5 μm; 250 × 5 mm)	Cranberry, roselle, strawberry	Anthocyanidins	193
Binary gradient A: 15% acetic acid; B: acetonitrile	Supelcosil LC-18 (5 μm; 250 × 4.6 mm)	Raspberry	cy-soph, cy-glu, cy-glurut, pg-soph, cy-rut	156
Phosphoric acid/acetonitrile (80:20)	Dychrom C_{18}	Tart cherry	cy-soph, cy-glurut, cy-glu, cy-rut	191
Binary gradient A: 4% phosphoric acid; B: acetonitrile	PLRP-S (5 μm; 250 × 4.6 mm)	Cranberry, roselle, strawberry, blackberry, cherry, elderberry	Anthocyanidin-glucosides, -galactosides, -arabinosides, -sambubioside, -sophorosides	193
Binary gradient A: phosphate buffer, pH 1.8; B: methanol	LiChrosorb RP-18 (5 μm; 250 × 4 mm)	Grape, wine	Anthocyanidin 3-glucoside, acylated anthocyanins	180
Binary gradient A: 25% acetone/10% formic acid; B: 10% formic acid	HS-5-C_{18}	Grape leaves, stems, tendrils	Anthocyanidin-glucosides	207
Binary gradient A: 0.05 M phosphoric acid; B: tetrahydrofuran	Nucleosil-5-C_{18} (5 μm; 150 × 4.6 mm)	Elderberry	cy-samb, cy-glu, cy-sambglu	208
Binary gradient A: 20% methanol/0.0251 M perchloric acid; B: 80% methanol/0.0251 M perchloric acid	Sepharon-SGX.018 (150 × 3.2 mm)	Red wine	Anthocyanidin 3-glucoside, acylated anthocyanins	188

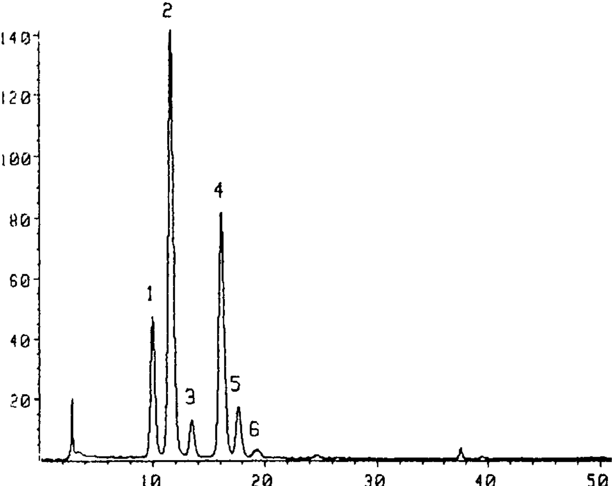

Fig. 16 RP-HPLC separation of black currant juice. Peaks: 1 = delphinidin 3-glucoside; 2 = delphinidin 3-rutinoside; 3 = cyanidin 3-glucoside; 4 = cyanidin 3-rutinoside; 5 = pelargonidin 3-glucoside (= int. std.); 6 = pelargonidin 3-rutinoside. (From Ref. 157.)

compared with other methods, shorter analysis time, better selectivity, higher versatility, and better sensitivity.

2. *Size-Exclusion HPLC*

The object of these methods is to separate the anthocyanin pigments or, more generally, the phenolic compounds as a function of their degree of condensation. A chromatographic separation is necessary, since techniques such as dialysis, ultrafiltration, and fractionated precipitation do not give satisfactory results. The application of two chromatographic techniques has been described in the literature: gel adsorption and gel filtration (197). Gel adsorption chromatography has been used mainly for the separation of procyanidins but also for the isolation of oligomeric anthocyanins. Best results were obtained with Biogel P4 (polyacrylamide) and water-insoluble PVP (polyvinylpyrrolidone) (198). However, it must be considered that fractionation is not based on the molecular weight but on the molecules' capability of being adsorbed on the gel surface (197,199). For gel filtration chromatography, there exists a wide variety of gels of different types and pore size. Commonly used gels for the separation of phenolic compounds are Sephadex G-25 and Sephadex LH-20 (200). The substances are fractionated more or less according to their molecular size. However, strong adsorption on the gel occurs, so separation depends to a great extent on the interaction between the molecule and the gel. By using gel filtration media of the Sephadex G type in an HPLC system, a fast separation of grape polyphenols by molecular size into two fractions could be achieved with good reproducibility. Furthermore, heating the column (e.g., 60°C) reduced the adsorption of molecules on the gel surface (197).

3. *Future Developments*

Since the determination of polymeric anthocyanins is still an open problem, new analytical methods such as capillary electrophoresis and SFC will perhaps make the analysis of these polymeric pigments possible. Due to advantages such as speed and selectivity, it can be assumed that HPLC will stay the method of choice for the analysis of monomeric anthocyanins in the coming years.

Better separations of complex anthocyanin mixtures can be expected through the use of micro-HPLC and newly developed stationary phases. The quantification of individual anthocyanins will be improved by an increased application of pure anthocyanin standards. By direct combination of HPLC with MS, a new avenue in online characterization and identification of anthocyanins is opened, albeit limited by the high costs (201).

D. Applications in Food Analysis

The content and distribution of anthocyanins in fruits and vegetables has been the object of numerous investigations during the last century (139). Chemical analyses have made it possible to follow the changes in anthocyanin composition that occur during the ripening, processing, and storage of fruits (153,188) and to determine factors that affect the stability of these pigments (138). Chemotaxonomic studies have discovered quantitative and qualitative differences in the distribution of individual anthocyanins between cultivars and varieties (193,202). These valuable findings have been applied to the characterization and verification of cultivars (151,191). Usually, varieties differ only in the relative amount of anthocyanins present, and since these quantities are determined not only by genetics but also by environmental factors, extensive statistical calculations are necessary for the classification of varieties (164). The distinctive anthocyanin profiles have also been a useful tool for the detection of adulterations in fruit juice products that are rich in anthocyanin pigments (181). In red wine, HPLC analyses of anthocyanin profiles have also been used for geographical and varietal classifications (202).

Application: Separation of Red Wine or Fruit Juice Anthocyanins by RP-HPLC (Ref. 180) Prior to HPLC analysis, the wine or juice sample is transferred quantitatively onto a C_{18} solid-phase extraction cartridge that has been preconditioned with 2 ml methanol and 2 ml water. The loaded cartridge is washed with 2 ml water, and then the cartridge is dried with vacuum. The anthocyanins are slowly eluted with 1 ml methanol containing 0.1% HCl. Subsequently, 10 μl of this methanolic anthocyanin extract are injected into the HPLC system and separated with a LiChrospher 100 RP (5 μm, 250 × 4 mm) analytical column, which is protected by a short (4 × 4 mm) precolumn filled with the same material. The elution of anthocyanins is carried out with a gradient of phosphate buffer, pH 1.8, and methanol, the flow rate is set at 0.8 ml per minute, and the column temperature is set at 40°C. The eluting pigments are monitored with a photodiode array detector set at 525 nm. Figure 17 shows a typical chromatogram of the red wine variety "Zweigelt."

IV. BETALAINS

A. Introduction

1. *Physical and Chemical Properties*

Betalain, a term introduced by Mabry and Dreiding in 1968 (210), includes two classes of water-soluble plant pigments: the red-violet betacyanins and the yellow betaxanthins (211). For a long time the chemistry of these pigments was obscure and they were confusingly named "nitrogenous anthocyanins." But since 1957 the successful elucidation of their structure has begun. Wyler and Dreiding (212) and Schmidt and Schönleben (213) simultaneously isolated and characterized betanin, the red-violet glucoside from the *Beta vulgaris* root. The betaxanthins were subsequently discovered in 1964, when indicaxanthin was isolated from prickly pear and its structure fully clarified (214). Further investigations confirmed the assumption that betacyanins and betaxanthins are chemically and genetically closely related substances. The general formula of betalains represents the condensation of a primary or secondary amime with betalamic acid, and its struc-

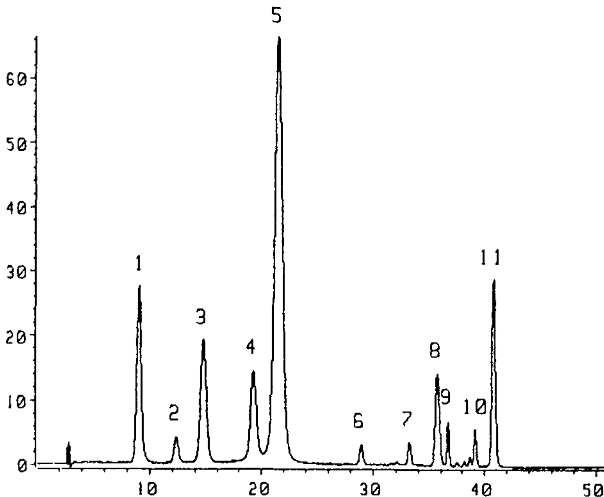

Fig. 17 Separation of red wine anthocyanins cv. "*Zweigelt*" by RP-HPLC. Peaks: 1 = delphinidin 3-glucoside; 2 = cyanidin 3-glucoside; 3 = petunidin 3-glucoside; 4 = peonidin 3-glucoside; 5 = malvidin 3-glucoside; 6 = delphinidin 3-acetylglucoside; 7 = petunidin 3-acetylglucoside; 8 = peonidin 3-acetylglucoside and malvidin 3-acetylglucoside; 9 = delphinidin 3-cumarylglucoside; 10 = petunidin 3-cumarylglucoside; 11 = peonidin 3-cumarylglucoside and malvidin 3-cumarylglucoside. (From Ref. 180.)

Fig. 18 The betalain chromophore and its structural transformations. (From Ref. 75.)

ture can be described as a pentasubstituted 1,7-diazaheptamethin system (215). This betalaimic chromophore can undergo structural transformations, which are presented in Fig. 18. The distinction between betaxanthins and betacyanins lies in the different substitution on the dihydropyridine moiety by specific R and R′ groups. While in yellow betaxanthins the conjugation system of 1,7-diazaheptamethin is not extended and the absorption maximum is at approximately 480 nm, the conjugated chromophore is extended in betacyanin by R and R′ groups comprising a substituted aromatic ring (cyclodopa), which causes an absorption maximum of approximately 540 nm (3).

Currently over 50 betacyanins have been identified, but most of them are based on the two isomeric aglycones betanidin and isobetanidin (the C15 epimer of betanidin), whose chemical structures are shown in Fig. 19. According to the formula for betanidin, there are two asymmet-

Fig. 19 Formula of betacyanins: R, R′ = OH (betanidin, isobetanidin); R, R′ = *O*-glucose (betanin, isobetanin); R, R′ = 2′-glucuronic acid-glucose (amaranthin, isoamaranthin); R, R′ = *O*-glucose-6-sulfate (prebetanin, isoprebetanin). (From Ref. 215.)

ric carbon atoms present in the molecule (C2 and C15); betanidins are therefore optically active. In acetate buffers betanidin has a specific optical rotation of $[\alpha]_{680}$: +530 ± 50°, betanin (pH 5.0) of $[\alpha]_{680}$: +310°, and isobetanin of $[\alpha]_{680}$: −840 ± 60° (216). Isomerization will occur under acidic conditions and/or the application of heat, giving rise to the iso-compounds (215). In nature all betacyanins are glycosylated, glucose, sophorose, and rhamnose being the most common glycosyl moieties (217). The most prominent betacyanin is betanin, the 5-*O*-β-glucoside of betanidin, which is the major pigment of red beets, *Beta vulgaris* (218). Amaranthin, the pigment of Amaranth (*Amaranthus tricolor*), is also a betacyanin-like betanin; the basic structure is extended by a glucuronic acid molecule linked to the 2 position of the glucose. Acylation of betacyanins occurs quite frequently; to date the structures of over 40 acylated derivatives have been elucidated. Numerous acylating moieties have been identified; the most common of them are sulphuric, malonic, 3-hydroxy-3-methylglutaric, citric, *p*-coumaric, ferulic, caffeic, and sinapic acids (211).

In betaxanthins, the betalain chromophore is substituted with either an amine or an amino acid (Fig. 20). Some of the occurring substitution patterns for R′ are glutamine (vulgaxanthin-I; R = H), glutamic acid (vulgaxanthin-II, R = H), methionine sulphoxide (miraxanthin-I, R = H), aspartic acid (miraxanthin-II, R = H), tyramine (miraxanthin-III, R = H), L-DOPA (dopaxanthin, R = H), and histidin (muscaaurin-VII, R = H). The R group is usually a hydrogen; except in indicaxanthin and portulaxanthin, the R and R′ groups form a prolin or hydroxyproline moiety (211). Betaxanthins usually occur as aglycones; like betacyanins, they are optically active.

Both betacyanins and betaxanthins are water soluble and exist as internal salt (zwitterions) in the vacuoles of plant cells. They are insoluble in organic solvents and migrate in electrophoresis as anions, even at pH values as low as 2.4 (217). As with other natural pigments, betalains have a limited stability; they are very sensitive to factors such as heat, oxygen, pH value, and light (219). Von Elbe et al. (220) showed that the thermally induced degradation of betanin in model solutions followed first-order reaction kinetics, and the half-life of betanin solutions at 50°C and pH 5.0 was 312 ± 30 min. These results were essentially confirmed by Saguy (221), who found that the heat-induced degradation of red beet pigments followed first-order reaction kinetics, with activation energies of 19.2 ± 0.5 and 16.3 ± 0.6 kcal per mole for betanin and vulgaxanthin-I, respectively. Additionally, the stability, especially the thermal stability of betalains, is strongly

Fig. 20 Formula of betaxanthins: R = NH_2 (vulgaxanthin I); R = OH (vulgaxanthin II). (From Ref. 211.)

influenced by the pH value. Savolainen and Kuusi (222) established that both betacyanins and betaxanthins were most stable at pH values between 5 and 6 and less stable at lower pH values. Betalains were found to be highly sensitive to oxygen; indeed, even trace levels of residual oxygen increased the pigment degradation (223). In the presence of sufficient oxygen, betanin loss followed first-order reaction kinetics, with an activation energy of 20.4 kcal per mol for heated betanin solutions. But in the absence of oxygen, betanin stability was greatly enhanced and degradation occurred according to a 0.5 reaction rate order (223). Finally, betalains are also subject to photochemical degradation. It could be observed that betanin degradation is strongly increased upon exposure to daylight (224) or to fluorescent lighting (219). On the other hand, the stability of betanin was improved by sequestrants such as EDTA and citric acid (221), which caused an increase in half-life values by 1.5 (223). Schwartz and von Elbe (225) identified the degradation products of betanin in solution upon heating as betalamic acid and cyclodopa 5-*O*-glucoside. These two degradation products are also formed under mild alkaline conditions (226). An important aspect to note is that the degradation reaction requires water. Thus, when water is unavailable or limited, betanin is very stable. Another important fact is that the degradation reaction is partially reversible (215).

Because betalains are very sensitive substances, special precautions, similar to those mentioned for carotenoids, have to be taken in handling them.

2. *Properties in Food*

The distribution of betalains in plants is restricted to the order *Centrospermae* (synonym: Caryophyllales), wherein only ten betalain-producing families have been identified. Betalain pigment accumulations in cell vacuoles of the flowers, fruits, leaves, stalks, and/or roots are responsible for the attractive yellow, red, or violet colors of these plant parts (227). Only a few betalain-containing plants are used for human nutrition, in essence these are red and yellow beet (*Beta vulgaris L.*), prickly pear (*Opuntia ficus indica*), *Basella rubra*, pokeberry (*Phytolacca decandra L.*), and amaranth (*A. tricolor* and *A. caudatus*). Additionally, special betalains (e.g., muscapurpurin, muscaaurins I to VII) have been found in mushrooms; they are, however, usually poisonous (221). The main source of betalains in food are beet roots, which have a reported betacyanin level

of 35–223 mg per 100 g fresh weight and a betaxanthin level of 33–91 mg per 100 g fresh weight (228). Among cultivars the colorant properties showed considerable diversity; besides large differences in the betalain content, qualitative discrepancies could be observed (224). While red beets (*Beta vulgaris L.*) contain both red (betacyanins) and yellow pigments (betaxanthins), the yellow beet (*Beta vulgaris L.* cv. *Burpee's Golden*) has only yellow pigments. The main red pigment in the red beet is betanin, which accounts for 75% to 95% of the total betacyanin content. The dominant yellow pigment is vulgaxanthin-I, which is also the major pigment of the yellow beet (224). Other betalains occurring in beets are the betacyanin derivates isobetanin, isobetanidin, prebetanin, and isoprebetanin and the betaxanthin derivate vulgaxanthin-II. The color of prickly pear, which is usually consumed either as fresh fruit or as juice, is produced mainly by the betaxanthin derivate indicaxanthin (211,230). Recently the betalains of the fruit *Basella rubra*, which is used as a vegetable in tropical areas and also as a raw material for cosmetic dyes, could be identified as gomphrenin-I (15S-betanidin 6-*O*-β-glucoside), gomphrenin-II (15S-betanidin 6-*O*-[6′-*O*-(4-coumaroyl)-β-glucoside), small amounts of the respective R forms (isogomphrenin-I and II), and gomphrenin-III (15S-betanidin 6-*O*-[6′-*O*-feruloyl-β-glucoside]) (231). Considerable amounts of the red-violet betacyanin derivates amaranthin and isoamaranthin are accumulated in mature leaves of some amaranth species (*A. tricolor* and *A. caudatus*), so these plants, which are already grown as a grain crop and as a vegetable, are a potential source for the production of natural food colorants (232).

3. *Arguments for Betalain Analysis—Regulations*

Color is an important quality characteristic, so it is of interest in determining the corresponding pigments and studying their stability (220). Betalains are the pigments responsible for the color of certain foodstuffs (e.g., beets, prickly pear). To improve the visual color of betalain-containing food (e.g., canned red beets), it is necessary to carry out extensive investigations, for instance, concerning the relationship between the variety and content and/or distribution of pigments (224), the relationship between environmental factors and pigment accumulation in plants, and color changes during processing and storage (233).

The documented use of betalain pigments as food colorants dates back at least one century, when inferior red wines were colored with betalain-containing juices (e.g., red beet juice). This common practice was, however, soon prohibited, and the application of betalain colorants was widely replaced by artificial dyes, which displayed better stability, at a lower price, and with higher purity. But in recent years the interest in natural food colorants has been renewed, mainly because of consumers' concerns about the safety of some artificial colorants, which may be hazardous to human health (234). As a result, the number of permitted artificial dyes has been markedly reduced, and new efforts had to be made to develop natural food colorants (235). However, current legislation restricts the application of betalain colorants to concentrates or powders (E 162) obtained from aqueous extracts of beets (211).

Since only 0.1% to 0.3% of the fresh weight of beet roots are betalains, effective extraction procedures are required. While pressing yielded a pigment recovery of only 45% to 50%, the use of a continuous diffusion-extraction procedure increased the yield of betacyanin and betaxanthin to 90% and 80%, respectively (236). Commercial beet colorants contain 0.4% to 1.0% pigment (expressed as betanin), which are accompanied by various plant metabolites, such as sugars (80%), proteins, and amine bases, choline, glutamine, and geosmin, which are together responsible for the typical, undesirable taste and smell (211). To improve the color stability and also to remove the off-flavor, commercial beet colorants were purified by ion-exchange and gel filtration chromatography (237) or by fermentation (238). But the application of the resulting products is still prohibited (211). More recently, microbiologically purified beet colorants were also used in

various ways for pharmaceutical purposes (239). If betalains are to become a more competitive and viable alternative to synthetic colorants in the near future, further investigations into their biochemistry and genetics are necessary.

B. Sample Preparation

1. *Extraction*

Before the extraction of betalains can take place, the plant material (e.g., beet roots) should be washed, blanched, and cut into small slices; occasionally they are also lyophylized and powdered (222). Subsequently the tissue is either pressed, to produce a pigment-containing juice, or homogenized with extraction solvents. Betalains are highly soluble in water, so they are usually extracted with water (240) or water-based solvents, such as water/0.1% HCl (241), and 0.1 M McIlvaine's citric–phosphate buffer solution, pH 5.0 (241). In some cases celite was added to the mixture to improve the clarification procedure. Other authors employed alcohol-containing solvents for the betalain extraction, such as ethanol/water (1 : 1) (234) and 60% and 80% aqueous methanol (242). The initial homogenate is clarified by either filtration or centrifugation; the filter cake may be re-extracted until no more pigments can be extracted. Both the clarified homogenate and the plant juice (e.g., beet juice) can be used directly for HPLC analysis, though an additional dilution step may be necessary. The juice and homogenate contain, however, many interfering substances, which may lead to bad separation and erroneous results. To avoid such faults, additional purification steps (e.g., gel filtration, ion chromatography) are occasionally carried out (243).

2. *Purification*

The purification of crude betalain extracts is usually accomplished by chromatographic or electrophoretic methods. These procedures not only purify but also separate the pigments, thereby allowing the quantitation of total and individual betalains simultaneously (211). Numerous researchers employed a two-step purification procedure: The first step is either ion chromatography with cationic-exchange resins such as Dowex 50W-X2, H^+ form (244), or gel filtration with Sephadex G-25, for instance (245). The second purification step is carried out by chromatography on polyamid (244) and/or polyvinylpyrrolidone (e.g., Polyclar AT (219,246)). Prior to cation-exchange or gel filtration chromatography the pH value of the crude extract has to be adjusted with hydrochloric acid to approximately 3.0 (245). Piatelli et al. (244) developed a purification procedure that was also suitable for the separation of complex betalain mixtures. This method is based on nonionic absorption of betacyanins and betaxanthins onto cation-exchange resins (Dowex 50W-X2, H^+ form) and subsequent elution of the pigments with water. The purified pigment mixture was then separated on polyamid, using a citrate buffer (pH 4.0) as eluent. These chromatographic methods still play an essential role in the identification of unknown betalains, because they allow the preparative isolation of pure pigments required for structure elucidation (242).

C. Analysis by High-Performance Liquid Chromatography (HPLC)

The first successful attempt to use HPLC for the analysis of betalains was carried out by Vincent and Scholz (247). With the help of paired-ion chromatography (PIC) on an RP column (μBondapak C_{18}) they separated betacyanins and betaxanthins of red beets. The ion-pairing approach was adopted in order to exploit the highly ionic character of these pigments and the durability of the C_{18} columns. Using methanol–water at pH 7.5 and tetrabutylammonium as the PIC reagent,

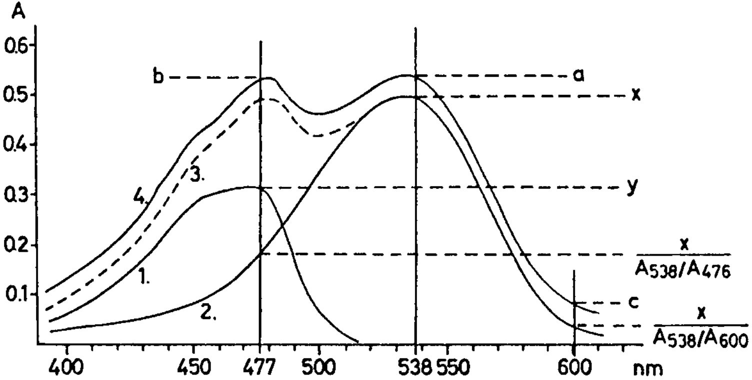

Fig. 21 Calculation of vulgaxanthin-I and betanin's proportion of the absorption of beet root juice. Curve 1: Vulgaxanthin's proportion of the absorption. Curve 2: Betanin's proportion of the absorption. Curve 3: Betanin and vulgaxanthin without impurities. Curve 4: Fresh beet juice, including impurities. (From Ref. 216.)

nonpolar unprotonated complexes of the solute molecules were generated. Two wavelength settings (476 and 538 nm) were used to monitor selectively the yellow and red pigments, although the red betacyanins do absorb to a certain degree at 476 nm, making 476 nm the wavelength of choice for a comprehensive screening of betalains (Fig. 21). Due to their chemical structure, yellow betaxanthins were more polar and therefore eluted earlier than the betacyanins. For quantitative analyses the HPLC system was calibrated with a purified betanin standard, and a good linearity over a work range of 0.005–0.03% (w/v) betacyanin could be obtained. The relative standard deviation among replicate samples was about 2.0% (247). Huang and von Elbe (245) separated and purified betanin and amaranthin from amaranth leaves using a combination of gel filtration and PIC. In spite of these good results, the majority of HPLC investigations concerning betalains was carried out without the addition of ion-pair reagents. RP-C_8 (248) or RP-C_{18} (249) columns were employed almost exclusively as the stationary phase, while the mobile phase commonly consisted of an acid or a buffer (e.g., phosphate buffer, acetic acid), which ensures a low pH value (pH ~ 3.0), and an organic modifier (e.g., methanol, acetonitrile) (250). The conditions of some selected HPLC analyses are compiled in Table 7. Generally the HPLC separation of betalains can be accomplished within approximately 20 min; a separation of betacyanins and betaxanthins of fermented red beet root extract is shown in Fig. 22. The quantitative estimation of betacyanins is often based on the absorptivity value $E^{1cm}_{1\%} = 1120$ for betanin. However, to achieve more exact results, Schwartz and von Elbe (249) recommended the use of individual absorptivity values (e.g., betanidin hydrochloride $E^{1cm}_{1\%} = 1275$). With the aid of HPLC methods, some little-investigated betalains could be detected and valuable information concerning the stability and degradation of various betacyanins and betaxanthins could also be gathered (223,249). Due to its speed, high resolving power, good sensitivity, and robustness, HPLC has become the method of choice for quantitative analyses of individual and total betalains (211).

3. *Future Developments*

Further improvements of HPLC separations can be expected through the use of newly developed columns (e.g., microcolumn) and stationary phases (e.g., microbore material), which lower the required sample volume and the consumption of solvents. Online characterization and identification of eluted pigments will be improved through the employment of more sophisticated de-

Table 7 Selected Experimental Conditions for HPLC Analysis of Betalains

Column	Solvent	Detection (nm)	Betalain	Application	Refs.
μBondapak C_{18} (10 μm)	A: methanol/phosphate buffer, pH 2.75 (18/82); B: methanol; gradient	535	betcyan	Red beets	234, 249
LiChrosorb RP-8	A: water/acetic acid (98/2); B: water/acetic acid/methanol (70/10/20); gradient	436	betxanth	Yellow beets	248
LiChrosorb RP-18 (5 μm; 250 × 4 mm)	A: 1.5% phosphoric acid in water; B: phosphoric acid/acetic acid/acetonitrile/water (1.5/20/25/53.5); gradient	480	betcyan, betxanth	*Aizoaceae*, *Cactaceae*	250
μBondapak C_{18} (300 × 4 mm)	A: 0.005 M PIC reagent A in water; B: 0.005 M PIC reagent B in methanol; gradient	476, 538	betcyan, betxanth	Red beets	247
μBondapak C_{18}	A: 8% acetonitrile in 5 mM PIC A solution; B: 12% acetonitrile in 5 mM PIC A solution; gradient	546, 436, 313	amarbet	Amaranth	245
μBondapak C_{18}	Methanol/phosphate buffer, pH 3.0 (1:5)	535	betanin	Red beets	219
Nucleosil C_{18}	A: 1.5% phosporic acid in water; B: 1.5% phosporic acid, 20% ethanol, 25% acetonitrile in water; gradient	540	betcyan	*Basella*, *Gomphrena*	231, 242

tectors, such as electrochemical and mass selective detectors. The use of spectroscopic methods like MS and NMR for the unquestionable identification of betalains will continue to increase (242). More recent techniques, such as supercritical fluid chromatography and capillary electrophoresis, will give new possibilities to betalain research in the future.

D. Applications in Food Analysis

The special pigments of *Centrospermae* have attracted considerable interest during the past 40 years; the pigment composition of red beet has especially been the topic of many investigations. Initially the betalains of red beet were separated and isolated using paper electrophoresis (244) and column chromatography (216); their chemical structure was elucidated by means of chemical tests and spectroscopic methods (214). The traditional methods for the quantitative determination of betacyanins and betaxanthins in beet root were spectrophotometry, mainly the Nilsson method (216). More recently, HPLC has become the method of choice for the separation and quantification of beet pigments (247).

Beet roots are an interesting source for the production of natural food colorants, so numerous studies concerning the colorant content and stability of different beet root varieties have been undertaken (224). Several researchers have studied the influence of pH value, temperature, oxygen, and light on betacyanins and betaxanthins by HPLC (215). Furthermore they have calculated the corresponding degradation rates and isolated and identified the main degradation products (223,225). The extraction of pigments from beet root is often incomplete, and so new techniques

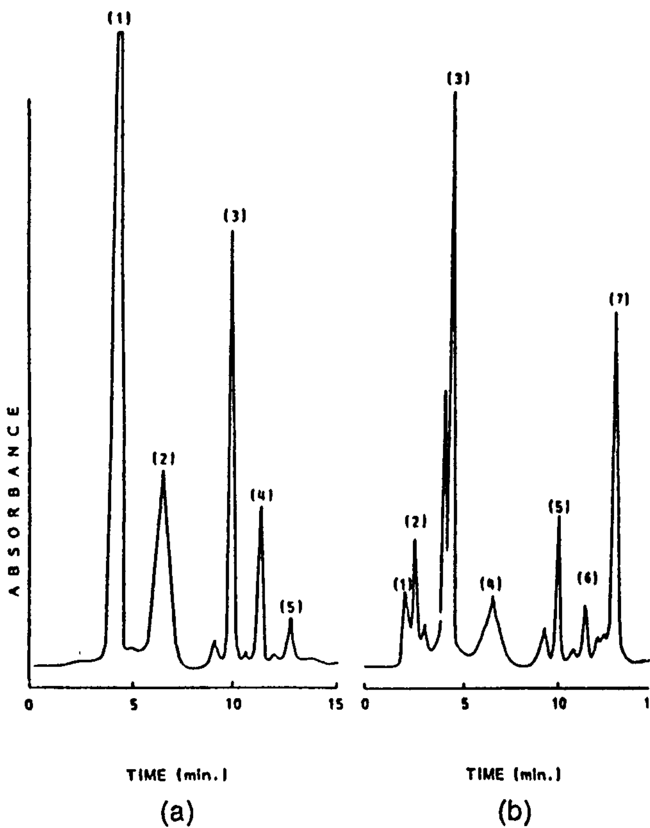

Fig. 22 HPLC separation of betalains in fermented red beet root extract. (a) Detection of betacyanins at 538 nm. Peaks: 1 = betanin, 2 = isobetanin, 3 = betanidin, 4 = isobetanidin, 5 = prebetanin. (b) Detection of betaxanthins at 477 nm. Peaks: 1 = vulgaxanthin I, 2 = vulgaxanthin II, 3 = betanin, 4 = isobetanin, 5 = betanidin, 6 = isobetanidin, 7 = prebetanin. (From Ref. 239.)

have been tested to increase pigment recovery (229). Photometric analysis showed, however, that the resulting extract could contain at most only 0.4–1.0% pigment (expressed as betanin). Chromatographic methods were successfully applied to increase the betalain concentration in the colorant and to remove interfering metabolites, which impart an undesirable smell and taste to the pigment extract (238).

Application: Determination of Red Beet Betacyanin Pigments by HPLC (Ref. 249) One hundred grams of fresh beets from five randomly selected roots are used for analysis. The tissue is blended for 1 min with 100 ml ethanol/water mixture (50:50, v/v) under a stream of nitrogen to lessen oxidative enzymatic reactions. The blender walls are washed with 100 ml of water, and the mixture is blended for an additional 5 min under nitrogen. Fifty grams of this homogenate are removed, filtered over a 10-g bed of celite, and washed with 300–400 ml of water until the tissue–celite mixture is colorless. The filtrate is quantitatively transferred to a 500-ml volumetric flash and brought to volume. An adequate dilution (1:5) of this sample is used for HPLC analysis.

After filtration through a 0.45-μm filter, 20 μl of the sample are injected and separated on a reversed-phase column (μBondapak C_{18}, 10 μm). A linear gradient is used, starting from 100% solvent A = methanol/0.05 M KH_2PO_4 (18:82, v/v) adjusted to pH 2.75 with phosphoric acid

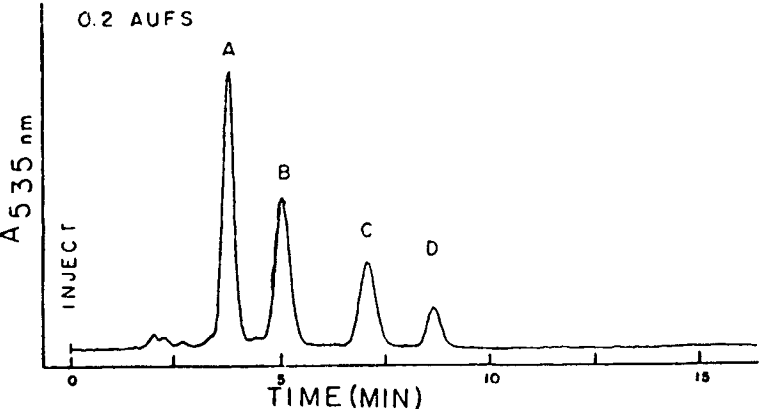

Fig. 23 HPLC separation of betacyanin pigments on a μBondapak C_{18} column. Peaks: A = betanin, B = isobetanin, C = betanidin, D = isobetanidin. (From Ref. 249.)

and ending with 80% solvent A and 20% solvent B = methanol. The chromatogram is monitored at 535 nm; it takes less than 15 min to run. A typical example of an HPLC separation of betacyanins is shown in Fig. 23.

V. MYOGLOBIN

A. Introduction

1. Physical and Chemical Properties

Myoglobin, a globular heme containing protein that is situated in muscle, is mainly responsible for meat color (3). Additional contributors to the color of meat are the blood pigments hemoglobin and cytochrome. The proportion of hemoglobin in the meat color depends mostly on the degree of bleeding out of the meat; in well-bled pieces from cattle and pig it will be less than 6% and 8%, respectively (251). The myoglobin molecule consists of a prosthetic group, the heme, and a polypeptide chain, the globin, which is composed of 153 amino acids (252). Due to species-specific changes in the amino acid sequence, the molecular weight of myoglobin varies from 16,900 to 17,850 daltons (253).

The heme prosthetic group is responsible for oxygen binding and confers an intense red or brown color to the protein. The reactive heme group consists of a central iron atom and a large planar ring, the porphyrin (protoporphyrin IX), whose structure is that of four pyrrole rings linked together by methene bridges, as shown in Fig. 24 (254). The bonding of the iron to the tetrapyrrollic ring structure satisfies four of the six coordination positions of the atom. The fifth is coordinated to the imidazole residue of histidine within the protein structure, and the remaining, sixth, position is capable of binding high-field ligands like O_2, CO, NO, CN, and N_3 (255). The central iron may be found in the oxidation states II or III, whereby at high pH values the iron of the heme is predominately in the ferrous state, while low pH values accelerate the conversion of ferrous iron into the ferric state. Unlike oxygen, which can bind only to the ferrous form of heme pigments, CO, NO, CN, and N_3 can bind to either form of the heme pigments, and Cl^- and H_2O can bind only to the ferric form (256). The most functional property of myoglobin is its ability to bind molecular oxygen (O_2) reversibly, in this way creating an intracellular reservoir of oxygen and facilitating the diffusion of oxygen from the plasma membrane to the mitochondria where the

Fig. 24 Structure of heme (Fe^{2+} protoporphyrin). (From Ref. 261.)

oxygen is consumed (257). In addition to enabling the heme group to perform its physiological function as an oxygen carrier, the globin converts the insoluble free heme into a water-soluble complex and protects the heme from oxidation; therefore the oxidation of the iron can take place only if the protein is denaturated. The heme moiety in myoglobin is not a fixed prosthetic group, so reversible dissociation into heme and apoproteins may occur. While the affinity of the heme for the protein is very high at neutral pH values (equilibrium constant: 10^{12}–10^{15} M), it is considerably lower at more acidic pH values (254). Myoglobin is quite reactive and can undergo several reactions, such as auto-oxidation, reaction with nitrite, and denaturation, all of which are of great importance to the colors of fresh and processed meats (258). The different myoglobin forms that can be formed during meat processing are shown in Fig. 25 (259). Fresh meat color contains mainly oxymyoglobin, the reduced form of myoglobin, which imparts a cherry red color to the meat. However, oxymyoglobin is relatively unstable during storage and processing, so it is easily oxidized to the brown metmyoglobin. Also, formation of metmyoglobin is coupled with lipid oxidation in meats. Myoglobin can also react with nitric oxid (NO) formed from nitrite or nitrate, and is then converted to the pinkish red nitric oxide myoglobin, the typical colorant of cured meat (260).

2. *Properties in Food*

Meat color is mainly a result of the concentration of meat pigments, the chemical state of these pigments, and physical characteristics such as fat deposition and diverse surface properties. Dependent upon the species, breed, sex, age, type of muscle, training, and nature of nutrition, the myoglobin content of meat can vary greatly. A general rule says that the more intensively a muscle is used, the higher is its myoglobin content; for example, the constantly operating muscle of the diaphragm has more myoglobin than the only occasionally and less intensively used *M. long. dorsi* (261). The average myoglobin content of this muscle is also a good illustration of the differences between species; for instance, in pig, cattle, and whale it contains 0.10%, 0.50%, and 0.90% myoglobin, respectively (262). As mentioned earlier, the iron atom of the heme can exist in either the ferrous or the ferric state, and it can form complexes with certain ligands, all of which greatly affects the color of the meat. The color of fresh meat is defined by the relative amounts of three derivatives of myoglobin: myoglobin (Mb, Fe^{2+}), oxymyoglobin (oxymyoglobin, Fe^{2+}), and metmyoglobin (metmyoglobin, Fe^{3+}). The absorption spectra of these three myoglobin deriva-

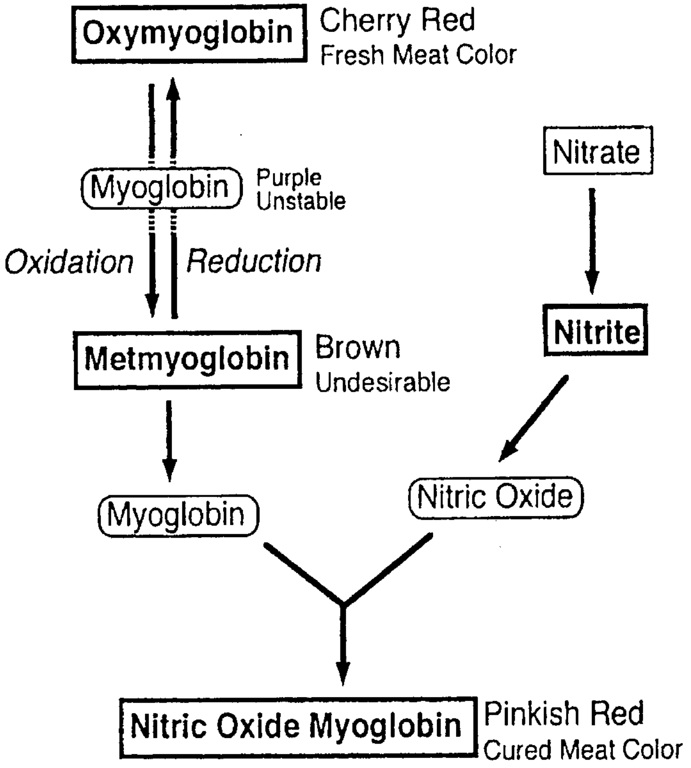

Fig. 25 Derivative changes of myoglobin during meat processing. (From Ref. 259.)

tives are shown in Fig. 26. Myoglobin is the purple pigment of deep red muscles; however, it is unstable at higher oxygen content (A_{max} = 555 nm). Upon exposure to air, the reduced myoglobin (Fe^{2+}) combines with oxygen to form the bright red oxymyoglobin (A_{max} = 542 nm), which is synonymous with freshness and considered attractive by the consumer. On the other side, oxidation of myoglobin leads to the formation of the oxidized form (Fe^{3+}), metmyoglobin (A_{max} = 505 nm and 635 nm), which is brown or grey and rather unattractive (255). These three forms of myoglobin are in a state of equilibrium in meat muscles, and they are constantly interconverted within a dynamic cycle. The formation of the attractive oxymyoglobin is favored by a high oxygen tension ($pO_2 > 4$ torr); the undesired oxidation toward metmyoglobin is predominant when the oxygen tension is approximately 4 torr, and an oxygen tension below 4 torr leads to the predominant formation of myoglobin. In fresh meat, reducing substances such as NAD^+ and FAD^+ are endogenously produced, and they are responsible for the constant reduction of the brown-grey metmyoglobin to the purple myoglobin, which means the dynamic cycle can continue as long as sufficient oxygen is present (257). The heating of meat samples, however, causes considerable destruction of the iron–porphin complex; therefore, mainly the denaturated heme proteins are present in cooked meats. The dark red color of cured meat is achieved by the reaction of myoglobin with nitric oxide, a process that forms nitric oxide myoglobin (MbNO). After heat denaturation this pigment is converted to the more stable nitrosyl-hemechrom, which is pink (258). The chemistry of meat pigments has been the subject of numerous investigations, and comprehensive reviews are given by various authors (255,257,258).

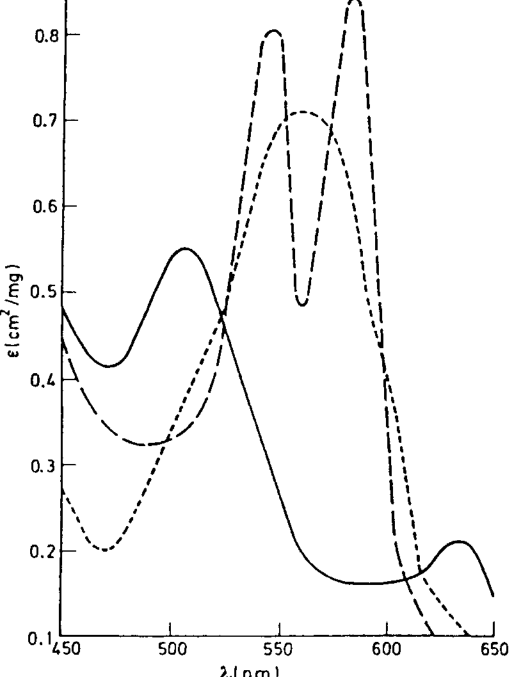

Fig. 26 Absorption spectra of oxymyoglobin (— — —), myoglobin (- - - -), and metmyoglobin (———). (From Ref. 3.)

3. *Arguments for Myoglobin Analysis—Regulations*

The red color of meat is probably the most important sensory property affecting the consumer's choice. It is well known that the consumer considers the bright red color of oxymyoglobin in fresh meat desirable, while the brown color of metmyoglobin is considered undesirable (263). Studies concerning the factors influencing the stability of myoglobin have shown that this pigment is very susceptible to heat, acid denaturation, and autoxidation at freezing temperatures; additionally it has been detected that the stability of myoglobin from different animals varies considerably (264). Since the formation of metmyoglobin is the major cause of surface discoloration in fresh meat, a better knowledge of the reactions involved in metmyoglobin formation might help to optimize the color of meat products. A traditional method to achieve a more stable and more attractive meat color is to cure it, but because of the toxicity of nitrites and related substances, the resulting chemical changes of the myoglobin have been investigated extensively and consequently the permitted amount of nitrite has been reduced considerably (254). An interesting alternative may be the use of specific bacteria, mainly lactobacilli, to reduce metmyoglobin concentrations in meat (265,266). The often-observed problem that the color of cooked cured meats rapidly fades on exposure to air and light has also been the object of numerous investigations (267). Another argument for myoglobin analysis is the possibility of determining the origin of meat and meat components in meat products (e.g., sausages, burgers). This very important and challenging task in meat hygiene and meat control is based on the fact that myoglobin has a species-specific microheterogeneity. Therefore it has been possible to detect nonpermitted or nondeclared meats by myoglobin analysis employing analytical methods such as serology (267), electrophoresis (268), and MS (253). With the intention of determining the degree of bleeding out of the meat (269) or to detect an eventual addition of blood to the meat (270), various analyt-

ical methods have been developed to differentiate between myoglobin and hemoglobin. Finally it has been shown that the analysis of free porphyrins, which result from the decomposition of myoglobin, is a suitable method for the determination of meat spoilage (271).

B. Sample Preparation

1. Extraction

Due to its hydrophilicity, myoglobin can be extracted from meat with deionized (272) and/or distilled water (273) or aqueous buffers. Commonly used buffer solutions are 0.075 M or 0.5 M Tris/HCl buffer, pH 7.9 or 8.9 (273,274), and 0.04 M, 0.05 M, or 0.067 M phosphate buffer, pH 6.8, 7.5, or 6.5 (275,276), respectively. The colored heme moiety, which is set free after dissociation of the heme-protein molecule, is usually extracted with organic solvents such as ethanol and acetone (277). A common procedure for the extraction of myoglobin is to trim off the visible fat from the meat, cut the piece into small slices, and homogenize the slices together with one of the solvents just mentioned. To achieve an effective extraction of the meat pigments, the homogenate is stirred for 2 or 3 h at 37°C. Afterwards the homogenate is centrifuged and/or filtered and the resulting supernatant, beneath the lipid layer, is collected (276). To minimize myoglobin decomposition, the extraction is carried out at low temperatures (e.g., 4°C); the addition of potassium azide is recommended to prevent bacterial growth (277). The crude meat extract can be used either directly for myoglobin analysis or be subjected to further purification and concentration procedures.

2. Purification

Three analytical procedures are usually applied to the purification of myoglobin from crude meat extracts.

1. *Fractionating with ammonium sulfate between 55% (w/v) and 90% saturation*: Prior to the fractionation the extract is adjusted to a pH of 7.0, and then solid ammonium sulfate is added to give a 55% and 90% saturation. The last precipitate is redissolved in distilled water and dialyzed against distilled water (278). In a similar procedure, myoglobin and hemoglobin are separated by precipitating the hemoglobin in an 85% ammonium sulfate solution (270). Hayden (267) successfully removed hemoglobin and sarcoplasmic proteins from myoglobin with 20% lead subacetate, potassium phosphate, and 70% saturated ammonium sulfate.
2. *Dialysis*: Sugden and Saschenbrecker (279) dialyzed the centrifuged meat–buffer homogenate against nine volumes of water to precipitate actomyosin and myosin. A two-step procedure consisting of a first dialysis against distilled water and a second dialysis against a species-specific buffer was developed by Renerre (273).
3. *Column chromatography*: Various column supports have been reported for the purification of myoglobin from crude meat extracts; gel filtration media such as Sephadex G-25, G-25, and G-75 have been widely used (278,280). Satterlee and Zachariah (281) purified metmyoglobin from cytochrome C and nonheme proteins by chromatography on DEAE cellulose. A chromatographic column containing hydroxylapatite was successfully used for the concentration of myoglobin in an extract from heated meat products (282). Myoglobin from turkey was isolated and purified by applying a three-step chromatographic procedure starting with Sephadex G-75, followed by CM cellulose and DEAE cellulose. Due to their low myoglobin content, extracts of some meat samples (e.g., heated meat, sausages) have to be concentrated prior to myoglobin analysis; a simple and commonly used procedure for this is ultrafiltration (268).

C. Analysis—High-Performance Liquid Chromatography (HPLC)

High-performance LC was first used for the determination of myoglobin by Powell et al. in 1984 (283). Human muscle, urine, and serum myoglobin was injected directly in an anion–exchange column (AX-300) and eluted isocratically with 0.022 M tris buffer, pH 7.5. Serum and muscle myoglobin gave two peaks in the chromatograms, while usually only one peak could be detected in urine. The minimum detection level was 2 mg per L, and the mean recovery was 94% to 95%. Due to the omission of a sample pretreatment, this method is very simple and rapid; the HPLC run can be carried out in less than 10 min. Ghrist et al. (284) reported differences in the retention times of HPLC separations of myoglobin and hemoglobin when they used a zirconia-stabilized silica-particle packing stabilized with diol bonding. When the aim of the HPLC analysis is the quantification of myoglobin, the heme group of the protein should be converted to the same state of oxidation prior to analysis. Oellingrath and Slinde (285) transformed the myoglobin to its cyanoferric form by adding a small amount of potassium ferricyanide or potassium cyanide to the extract. They tested three HPLC columns for their suitability for the analysis of meat pigments in ground beef extract. The best separation of myoglobin and hemoglobin could be achieved with a hydrophobic interaction column (Bio-Gel TSK Phenyl-5-PW), which was operated with a linear gradient of 1.7 M ammonium sulfate, 0.1 M sodium phosphate buffer, pH 7.0 (eluent A), and 0.1 M sodium phosphate buffer, pH 7.0 (eluent B). Figure 27 shows the chromatograms of a standard solution (myoglobin and hemoglobin) and of a ground beef extract. The time of analysis was shorter with the anion-exchange column (DAED Microanalyzer MA 7P) than with the hy-

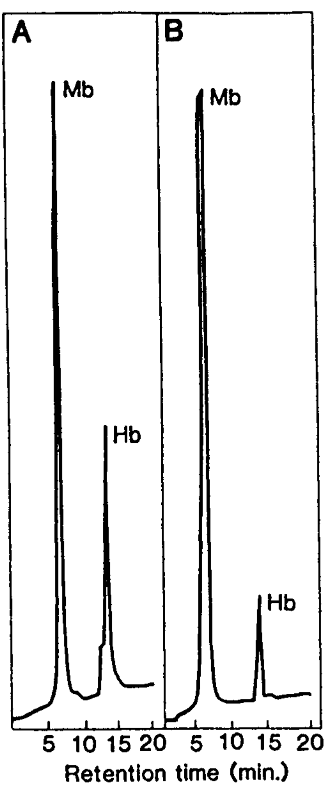

Fig. 27 HPLC separation of myoglobin and hemoglobin on Bio-Gel TSK Phenyl-5-PW. (A) Standard solution. (B) Ground beef extract. (From Ref. 286.)

drophobic interaction column, the retention times for myoglobin being 0.3 min and 7.8 min, respectively (286). The percentage recoveries for both myoglobin and hemoglobin ranged from about 90% to about 110%. Gel filtration chromatography on a Bio-Sil TSK-SW column was not suitable for the separation of heme proteins. However, size exclusion HPLC with a Spherogel TSK-3000 SW was successfully applied to the separation of bovine and porcine muscle proteins, including myoglobin (272). One good resolved myoglobin peak could be separated from porcine sarcoplasmic extracts by means of RP-HPLC using trifluoroacetic acid and acetonitrile as eluents (287). In general HPLC is well reproducible, sensitive, and rapid and has a great potential as a routine method for the determination of myoglobin in meat samples.

The potential of electrospray mass spectrometry to identify heme pigments from pig, beef, sheep, and horse was demonstrated by Taylor et al. (253). They obtained species-specific spectra corresponding to the molecular weights of the globin portions. Electrospray mass spectrometry is favored by its speed and sensitivity, but it is hampered by high costs. The online combination of HPLC and mass spectrometry is also a very promising method for the selective and rapid determination of myoglobin from protein mixtures. McLuckey et al. (288) separated numerous proteins, including myoglobin, down to 2.5 pmol per component using a combination of ion-spray liquid chromatography and ion-trap mass spectrometry. High-performance capillary electrophoresis also has great potential for the fast separation of charged molecules. Testing the influence of column temperature on the electrophoretic behavior of myoglobin, Rush et al. (289) were able to separate Fe^{3+} myoglobin from Fe^{2+} myoglobin.

D. Applications in Food Analysis

Due to its fundamental physiological function as an oxygen carrier and its importance to the meat's color, myoglobin has been the object of numerous investigations since the beginning of the eighteenth century. Spectrophotometry has been the classical method for determining the relative and the absolute pigment concentrations of myoglobin derivatives in fresh whole and comminuted meats and fish (279). Column chromatography is a valuable method for the isolation and purification of heme proteins from various sources, e.g., beef, pig, sheep, and turkey meats. Additionally a gel chromatographic method using Sephadex G-50 was described for the quantitative analysis of myoglobin in muscles of rat, pig, sheep, and ox (275). SDS electrophoretic methods are capable of separating myoglobin and hemoglobin. With polyacrylamide gelelectrophoresis (PAGE) and isoelectric focusing, myoglobin could be separated into a species-specific series of myoglobin bands and can be used for the identification of animal species and for the quality control of meat products (274). HPLC techniques such as size-exclusion (272), anion-exchange (283), hydrophobic interaction (285) and reversed-phase HPLC (287) were applied to separate sarcoplasmic proteins and to determine myoglobin. New analytical methods such as HPLC-MS and high-performance capillary electrophoresis are very promising, and their suitability for myoglobin analysis will be studied within the coming years.

Application: Myoglobin Determination by HPLC (Ref. 283) Ten mg of skeletal muscle is homogenized in 1.0 ml distilled water and centrifuged at 10,000 g for 2 min. Ten μl of the supernatant is injected directly into an anion-exchange column (AX-300, 6.5 μm, 250 × 4 mm). Myoglobin is eluted with a 22 mM tris buffer, pH 7.5; two peaks are monitored at 405 nm. One HPLC run can usually be accomplished within less than 10 min.

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21
HPLC Analysis of Organic Bases

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I. INTRODUCTION

This chapter deals with a not very well-defined class of compounds, generically called organic bases. According to the Brønsted–Lowry definition a base is any molecule or ion that accepts protons. The definition given by Lewis is even more general, since it calls a base any substance donor of an electron pair. This theory is useful in recognizing organic bases such as amines as having basic properties.

Many kinds of organic bases are found in food, both of vegetal and animal origin. Here we classify this complex group of compounds in the following rather arbitrary way:

1. Biogenic amines and alkylamines
2. Heterocyclic aromatic amines
3. Unsulfonated aromatic amines
4. Purine and pyrimidine bases
5. Methylxanthines
6. Various alkaloids

In particular we will discuss in general what has been published mainly since the 1980s to the present day about HPLC analysis of organic bases in food and beverages. Generally the most recent papers (those that appeared after 1992–93) are described in the text, while most of the methods discussed in the previous edition are presented in tabular form. The tables report, when available, the method used, the experimental conditions, the detection limit (DL), the kind of samples considered, and the amount found in the different foods investigated, as well as the essential steps concerning the extraction and the sample treatment before the analysis.

II. BIOGENIC AMINES

A. General Information

Biogenic amines (BAs) are organic bases of low molecular weight occurring naturally in food and beverages. Their amount increases during microbial fermentation or spoilage (1–11) as a result

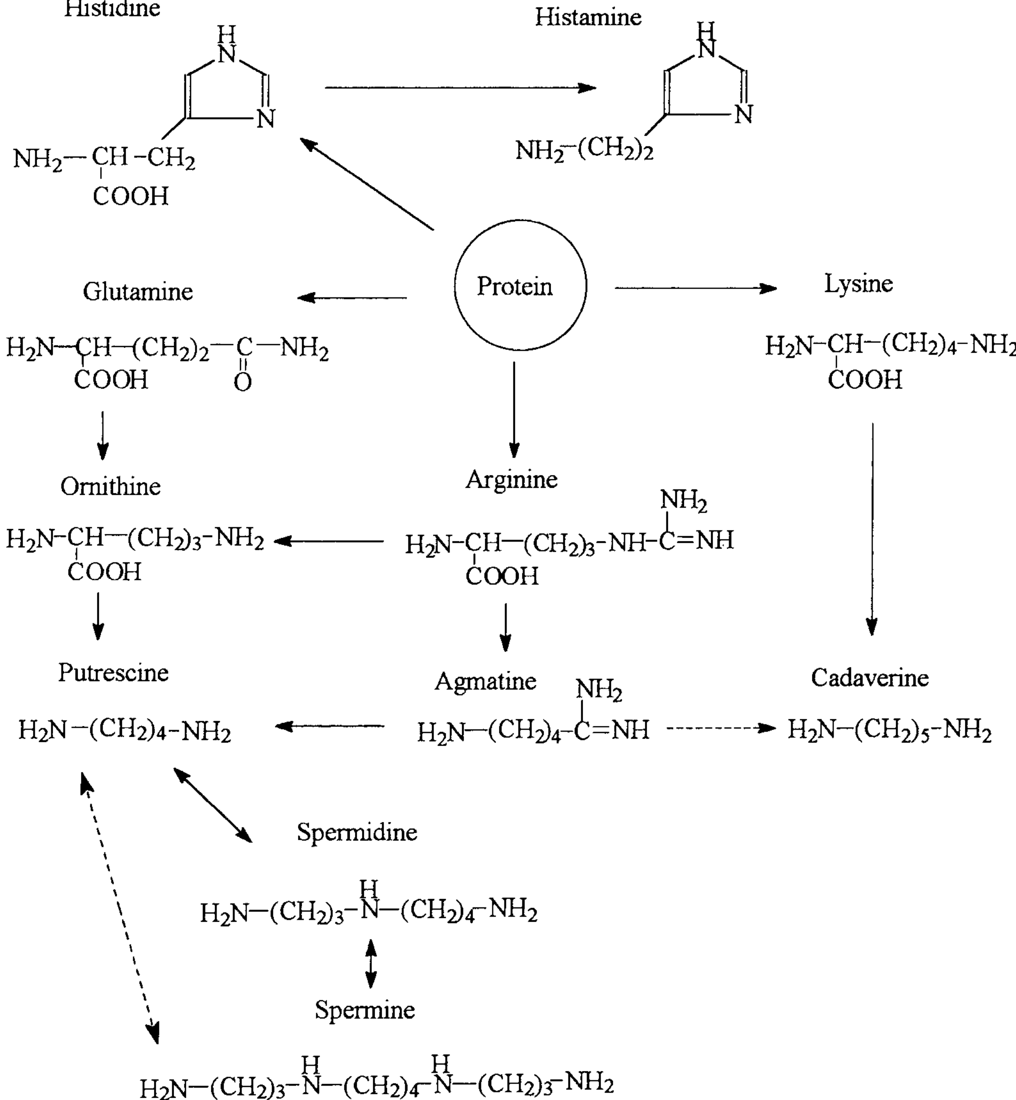

Fig. 1 Biological pathways for the formation of biogenic amines.

of amino acid decarboxylation reactions catalyzed by exo- or endogenous enzymes specific for each amino acid (AA) and that have pyridoxal-5-phosphate as a cofactor (12) (Fig. 1).

Although many of these active substances serve normal roles in mammalian physiology, they can also cause toxic effects when consumed in large amounts (13). Toxicological problems resulting from the ingestion of foods containing relatively high levels of BAs have been reviewed (14,15). It is well known that the ingestion of BAs, especially tyramine (Tyr), may provoke hypertensive crises in patients treated with monoaminooxidase inhibitor (MAOI) drugs (16). Histamine (His) may induce pseudoallergic reactions in humans, and the uptake of large amounts can result in headache, hypo- or hypertension, or anaphylactic shock syndromes (17–19). Histamine is the causative agent of histaminic intoxication (scombroid poisoning) (20); this is partly due to

the inhibition of monoaminooxidase (MAOs) also occurring in tissues and body fluids. The U.S. Department of Agriculture has proclaimed an upper safe limit for His of 50 mg/100 g of food.

Other BAs, such as putrescine (Put), cadaverine (Cad), tryptamine (Try), β-phenylethylamine (Phe), spermine (Spm), and spermidine (Spd), have been described as potentiators that enhance the toxicity of His (21,22); likewise, Tyr and Phe are thought to precipitate migraine attacks in susceptible subjects (23).

The amount and ratios of BAs in food can serve as quality indicators via the "biogenic amine index" (BAI), introduced by Karmas (24) and calculated from the most abundant BAs in fish and food according to the following equation:

$$\text{BAI} = \frac{c\text{His} + c\text{Put} + c\text{Cad}}{1 + c\text{Spd} + c\text{Spm}}$$

where His = histamine, Put = putrescine, Cad = cadaverine, Spd = spermidine, Spm = spermine, and c = concentration in $\text{mg}\cdot\text{kg}^{-1}$.

Thus, in order to avoid excessive uptake, it is very important to determine the BAs of interest in food and beverages. In addition, polyamines may produce carcinogenic nitrosamines in the presence of nitrite (25,26); this is another reason for preventing the accumulation of BAs in cured products.

B. Analysis of Biogenic Amines

A comprehensive work on BAs, covering their origin, their presence in food, their importance, their metabolism, and their determination, has been written by Askar and Treptow (1).

According to the literature, the analysis of BAs can be performed by thin-layer chromatography (TLC) (27), ion-exchange chromatography (IC) (28), gas chromatography (GC) with packed (29) or capillary columns (30–32), and enzymatic (33) and radioimmunological (34) methods.

A fast and simple analytical method for amine determination is still not available, but reversed-phase HPLC is usually considered the most suitable technique for this analytical purpose.

Since BAs occurring in food do not exhibit satisfactory absorbance or fluorescence in the visible or ultraviolet range, chemical derivatization, either pre- (35–37) or postcolumn (38), is usually used for their detection in HPLC. The most frequently employed reagents for precolumn derivatization are fluorescamine, aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (39, 40), 9-fluorenylmethyl chloroformate (FMOC) (41–43), 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsylchloride, DBS) (44), *N*-acetylcysteine (NAC) (45,46), and 5-dimethylamino-1-naphthalene-1-sulfonyl chloride (dansylchloride, DNS) (47,48), phthalaldehyde (PA), and *ortho*-phthaldialdehyde (OPA) (49–51), together with thiols such as 3-mercaptopropionic acid (MPA) (37) and 2-mercaptoethanol (ME) (35,49).

1. *Meat, Fish, and Cheese*

Biogenic amines can be found in processed meat products as a consequence of microbial activity related to the fermentation involved in their processing, but amines can be also found in poor-quality raw materials as a consequence of microbial contamination. Therefore the BA content in cooked meat (not fermented) products might serve as a useful indicator of the hygienic quality of the meat employed for its elaboration. However, this relationship for ripened meat products is rather complex, since the ability to produce BAs of the fermentative microflora need to be well known before limits can be set. According to the few studies performed on BAs in meat products,

their concentration varies among the type and origin of meat products. Histamine and Tyr are the BAs most often studied (6,52). The content of others amines, such as Put, Cad, and Try, especially in ripened products, has also been the object of some works (53,54), but studies on cooked meat are still scarce. In general, BA concentration levels reported for meat products are much higher than those reported for fish products, which have been more investigated, and legal limits or at least tolerable maximum contents of His have been established for them (e.g., FDA; EU).

Beside BAs, low-molecular-weight alkylamines, commonly used as indicators of food quality, can also be present in fish muscle. Tri- and dimethylamine (TMA and DMA) are produced by bacterial reduction of the osmoregulatory substance trimethylamine oxide (TMAO) in fresh marine fish and by enzymatic reduction in frozen storage of gadoid fish (cod, cusk, hake, pollack), with concurrent formation of formaldehyde.

All the analytical methods for BA determination in proteic foods involve two main steps: (a) amine extraction from the matrix, including purification of the raw extract, and (b) determination of the BAs, the most critical first step in terms of obtaining an adequate recovery. The extraction of amines from a solid matrix can be carried out with water so that only the free amines are extracted (55), or in acid medium, generally HCl (56–57), $HClO_4$ (58,54), or trichloroacetic acid (TCA) (59–62) so that amines linked to other matrix components can also be extracted.

Several organic solvents, such as MeOH (63,64), ACN-$HClO_4$ (65), and CH_2Cl_2-$HClO_4$ (66) have been used too. However, an extract purification step may follow, because the needed degree of purity is a function of the final analytical technique. The purification treatment of the extract can be divided into two main groups: (a) column chromatography with alumina and (b) ion-exchange resins.

Moret et al. (67,68) studied all the parameters that influence amine recovery under conditions where a liquid–liquid purification step with an organic solvent follows the acid extraction, prior to derivatization with DBS and RP-HPLC analysis. The optimized methods of sample preparation for different foods, including cheese, meat, and fish, are given. The same research group (69) optimized the extraction conditions for Phe, Put, Cad, His, Tyr, Spe, and Spd. Food samples were first mixed with TCA and centrifuged and then basified and extracted with BuOH/$CHCl_3$ (1:1). The BAs were then derivatized with DNS and separated on a Spherisorb 3S TG column with an ACN-H_2O gradient. The method was applied to samples of tuna, salmon, and salami.

Saito et al. (70) developed a method for the determination of eight BAs in fish. After a cleanup procedure, including acidification with $HClO_4$ and cation-exchange HPLC, the samples were subjected to on-column derivatization and HPLC separation on an Ashipak ODP-50 column. The eluent was an ACN–borate buffer containing *o*-phthaldehyde (PA) and *N*-acetyl-L-cysteine (NAC); the fluorimetric detection was performed at 430 nm. The method was applied to canned fish such as tuna, salmon, crab, and clams.

A method for the determination of 15 BAs in different foodstuffs, namely, Swiss cheese, salami, milk, beer, and wine, was developed by Petridis and Steinhart; it is based on an automated precolumn derivatization of BAs [after acidification with TCA and solid-phase extraction (SPE) on Amberlite resin] with OPA/ME and separation on a Spherisorb ODS-2 column with gradient elution (ACN–MeOH–phosphate buffer) and fluorescence detection (71).

Hernández-Jover et al. (72) derived an improved analytical method from the HPLC procedure setup they developed in 1995 (73) for the determination of BAs in fish. The method consists of the extensive extraction with $HClO_4$, ion-pair (with sodium octanesulfonate) RP-HPLC separation, postcolumn derivatization with PA/ME, and spectrofluorimetric detection. Determination limits were up to 1.5 mg/kg. In particular, His, Tyr, Phe, Ser (serotonine), Cre (creatinine), Try, Oct (octopamine), Dop (dopamine), Cad, Put, Agm (agmatine), Spm, and Spd were studied in pork and beef meat, fresh, cooked, or ripened. Tyramine, His, Put, Cad, and Try levels were

higher in the ripened meat than in the fresh. Octopamine and Dop were not found in any samples, Phe was detected in just the ripened products, and Spm and Spd have been found as naturally occurring amines in meat (12,74,75). Much data is reported for different samples.

Krause et al. (44) extensively studied the derivatization with DBS, proposed by Chang et al. (76), since DBS reacts with both primary and secondary amines and amino acids at room temperature, leading to stable products detectable in the visible region with high sensitivity. In particular they developed a method for the analysis of BAs, together with the AA, in cheese, seasonings, rice wine, and ham. The deproteinized samples (by ultrafiltration or acidification with TCA) were derivatized with an automated precolumn method and analyzed by RP-HPLC-UV. The DBS derivatization was successively applied to study the occurrence of BAs and free AA in raw-milk cheese (detection at 436 nm). They found that the proportions of free AA and BAs relative to the total amount fell into three broad groups that were independent of the type and ripeness of the cheese (77).

Bockhardt et al. (78) derived a manual extraction and automated [4-(4-dimethylaminophenylazo)benzensulfonyl] derivatization procedure from the method previously proposed by Krause et al. (44). Reversed-phase HPLC was carried out on a Spherisorb ODS-2 column at 50°C with gradient elution and detection at 436 nm, reaching detection limits (DLs) of 0.3–0.8 pmol.

Valle et al. (79) extracted ammonia and BAs (methylamine, Try, Put, His, Tyr, Spm, Spd) from fish with 0.2 M $HClO_4$ and labeled with DNS at 60°C for 5 min in the dark. The evaporated organic phase was redissolved in ACN and analyzed by HPLC on a Kromasil C18 column with UV detection (at 254 nm). Quantitation was performed by using 1,3-diaminopropane dihydrochloride as internal standard (IS).

Hwang et al. developed a rapid and sensitive HPLC-UV method for the analysis of nine derivatized BAs, with benzoyl chloride as the derivatization agent. The reaction is faster than with tosyl chloride and leads to stable products with shorter elution times than do dansyl derivatives. The amines were previously extracted after acidification with TCA. The method was applied to detect BAs in fried marlin fillet, implicated in a food poisoning incident (in Taipei City in 1996) and indicated that a high level of His (84.1 mg/100 g) was present in the sample (80).

2. *Vegetable and Fruit Juices*

In general, there is a low risk of high amounts of BAs in vegetables and root crops, where BAs are produced as a result of lactic acid fermentation (81,82). Furthermore, the His contents reported in sauerkraut (83,84), cucumbers (85), and green table olives (86) are far below the level of 100 mg per 100 g that has been associated with outbreaks of food poisoning. Nevertheless, a safe evaluation of the products of natural lactic fermentation should take into account other amines, because Tyr, Put, and Cad can also be produced during sauerkraut fermentation (87). Moreover, certain strains of lactobacilli and cocci have been associated with the presence of these compounds in fermented products (82) and synthetic broth (88).

Hornero-Méndez and Garrido-Fernandéz (19) proposed a method for determining BAs in fermented vegetables. The BAs are extracted using the Hui and Taylor (89) method (slightly modified to adapt it to the matrices considered) and then derivatized with DNS and analyzed by HPLC-DAD. Putrescine, Cad, and Tyr were found to be present in the olive samples considered (Fig. 2).

Kirschbaum et al. (90) reported that also 2-naphtylolycarbonylchloride (NOC) can be successfully employed for automated precolumn derivatization and fluorescence detection of BAs in food, drink, and seasonings, with a DL of approximately 49–113 μg/kg (except for His: 74 μg/kg). Remarkably, in citrus juices high amounts of Put were determined, whereas in grape

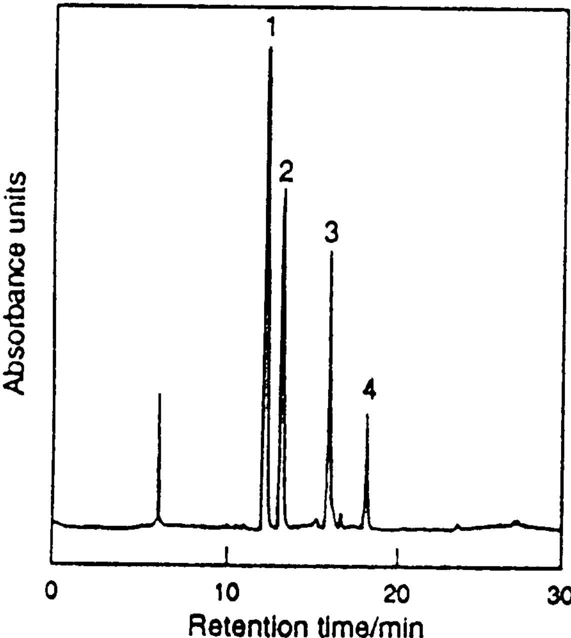

Fig. 2 Reversed-phase HPLC chromatogram of the dansyl derivatives of amines from a sample of spoiled table olives. Peak identities: 1, putrescine; 2, cadaverine; 3, 1,7-diaminoheptane (IS); 4, tyramine. (From Ref. 19.)

juice the measured amounts were much lower. Sauerkraut juice presents a typical example of the drastic increase of BAs in the course of lactic fermentation: Put was 230 mg/L; His, Cad, Tyr: 20–50 mg/L. In frozen salmon relatively high amounts of Tyr and Cad were measured. In the wines considered very low amounts of BAs, namely, Put and Tym, were detected, with the exception of Rioja red wine (Spain). The highest amounts of Put, His, and Tyr were detected in vinegar made from red wines and sherry.

3. *Cocoa Powder*

Steinert et al. (91) developed a sensitive LC determination of hydrophobic primary and secondary amines by derivatization to highly fluorescent thiazoles. Thioureas formed by addition of amines to the nonfluorescent 5-isothiocyanato-1,3-dioxo-2-*p*-tolyl-2,3-dihydro-1*H*-benz[de] isoquinoline are oxidized to the corresponding stable and highly fluorescent thiazoles, with large Stoke's shift. Liquid chromatographic separation of the thiazoles was achieved both by normal and reverse phase, and DLs ranged from 2 to 8 fmol. An application to the determination of Phe in cocoa powder is given: the concentration found, 1.4 ± 0.2 mg/kg, agrees with data from the literature (92).

4. *Wine and Beer*

Since their discovery in 1954 by Tarantola (93), more than 30 amines have been identified in wine. Among the most common are: butylamine, Cad, Try, ethanolamine, 1,3-diaminopropane,

DMA, ethylamine, hexylamine, His, indole, iso- and *n*-propylamine, methylamine, 2- and 3-methylbutylamine, morpholine, iso- and *n*-pentylamine, Phe, Put, pyrrolidine, 2-pyrrolidone, Ser, and Tyr (94–103). In wines, amines occur as salts, and their concentration has been reported to range from a few to about 50 mg/L, depending on the quality of the wine (50). They are odorless, but with the pH prevailing in the mouth, amines are released and their flavor can be tasted. The physiological influence on the human organism of the presence of BAs in wines has been discussed (104,105), and in alcoholic beverages it is important to take into account the synergetic effect that seems to exist between amines, ethanol (EtOH), and acethaldeyde (106).

Several countries have fixed the maximum allowed concentration for some of the BAs in wine; e.g., Switzerland recommends 4 mg/L for His, Netherlands 5 mg/L, Germany 2 mg/L, and France 8 mg/L. High-performance LC techniques are largely used to determine BAs content in wines, with ion exchange (96,107) or RP columns; with pre- (100,108–110) or post- (10) column derivative formation or without derivatization (98,4,111,112); with different detection means, mainly UV (50,110,113,114) or fluorescence (97,100,50,108,109). Nevertheless, if BAs are to be determined at low levels with no interference from other compounds, e.g., AA, previous cleanup and preconcentration steps are required.

The main part of the works relating to the determination of BAs in wines concern the major ones, such as His and Tyr; few studies have been performed on amines such as Spd and Spm. These amines, even though they appear less toxic than His and Tyr (115,116), nevertheless exert many functions at the cellular level. For instance, by their polycationic long-chain structure they can interact with DNA, RNA, proteins, and the membrane phospholipides (117,118), and they are also strongly implied in cellular growth phenomena. The presence of these polyamines in grapes and the role that they play have been studied for some years (119).

The most important advantage of OPA over other derivatization reagents is that it quickly reacts with amines and enables the BAs to be detected at femtomole levels. The drawback is that OPA reacts only with primary amines and leads to poorly stable compounds (120). Better results are obtained with DNS that also reacts with secondary amines and gives stable compounds that can be treated in subsequent concentration steps without using an automated system (103,121,5).

Also, wine is a very complex matrix and so cleanup or preconcentration steps must be undertaken prior to chromatography. Many analytical methods include these steps, mainly using liquid–liquid extraction (LLE), both before and after derivatization (49,100,103,5,122,123). Nowadays SPE is preferred, and studies have been carried out using this method before and after derivatization (37,48,51).

The method commonly proposed is based on cation-exchange extraction followed by derivatization of the fraction of interest with OPA (46,55,98,99,114,124–126) (when isolating BAs in wines). Solid-phase extraction has been performed with several stationary phases based on anionic (113) or cationic (37,39) exchangers or octadecylsilane groups (38), as well as a combination of both (51).

Many of the recent studies on the BAs in wines are from the research group of Busto. In 1994 this group developed a method suitable for the determination of 19 BAs in wine (48) that involves the removal of phenolic compounds with polyvinylpirrolidone (PVP), derivatization with DNS, SPE extraction with C_{18} cartridges, concentration, and HPLC-UV analysis reaching DLs between 50 and 150 μg/L. The method was applied to five different red and white wines from Catalonia.

Another method (37) that involves cleanup of wine by cationic exchange and a preconcentration step under controlled vacuum, before derivatization of the amines with PA and RP-HPLC elution with fluorimetric detection, (DL 25–50 μg/L), was used to identify and quantify BAs in several red wines of Tarragona region.

In Ref. 51, cleanup treatments with PVP, SAX (strong-anion exchanger), and C_{18} cartridges are compared; in a second step, C_{18} cartridges were used simultaneously to clean up and concentrate the analytes after adding sodium octanesulfonate, sodium decanesulfonate, or sodium dodecanesulfonate as ion-pair reagents. Amines, precolumn derivatized with OPA/ME, were separated by RP-HPLC and detected by fluorimetry with an average sensitivity of the order of 20–90 μg/L. Fifteen amines were determined in red wines from the Tarragona region by this method.

An automatic precolumn derivatization with AQC followed by SPE with strong-cation exchanger (SCX) cartridges was used to detect eight undesirable amines in red wines from the Tarragona region (40). Busto et al. (127) also developed an on-column (45,46) derivatization procedure with OPA/NAC. The derivatization agent is present in the chromatographic eluent, and the column is C_{18} polymeric based, to be resistant to alkaline medium. Two different SPE cartridges, an anionic SAX and a C_{18}, were tested for the sample cleanup, prior to the derivatization. The method was employed for determining eight BAs (methylamine, His, ethylamine, Tyr, Put, Cad, Phe, and 3-methylbutylamine) by fluorimetric detection in Tarragona red wines (DLs between 100 and 300 μg/L). Histamine, 3-methylbutylamine, ethylamine, and Put were found in all the samples; none showed the presence of Tyr, isoamylamine, or Phe.

To determine a small amount of His in sparkling wine, Tarrach proposed a method based on SPE with an Amberlite GC-50 column, derivatization of the eluate with PA, and HPLC analysis on a Supershere 100 RP-18 column (gradient elution with ACN–acetate buffer). Fluorimetric detection at 450 nm allowed DLs of 0.05–2.00 mg/L (128).

Bauza et al. (43) studied the presence of BAs, including the polyamines, in 54 red wines, 15 rose wines, and 15 white wines from the Vallée du Rhône (France), using FMOC as the derivatization agent. The FMOC reacts quantitatively at ambient temperature within a few minutes with primary and secondary amino groups, forming stable carbamates, thus allowing the direct analysis in wines of the five polyamines (Put, Cad, Agm, Spm, Spd) simultaneously with other BAs (His, Phe, Tyr) and their AA precursors (129–132). In these wines, Cad, Spm, and Spd were found to be present in small quantities, while only Agm and Put appear at levels significantly higher than 1 mg/L. The presence of Put is strongly correlated with the presence of Tyr and His. Levels of polyamines, His, and Tyr are higher in red wines than in the other types of wines.

Following the method developed by Pfeiffer to detect nine BAs, wine was applied to a Bond-Elut SCX SPE column and the eluted portion was derivatized with PA. The reaction mixture was analyzed on a LiChrospher 100-RP-18-5 column with gradient elution (MeOH–phosphate buffer) and fluorimetric detection at 440 nm: the DL was about 50 μg/L (133).

The levels of BAs in beers have been evaluated in some studies (15). Since beer is generally consumed in larger quantity than wine, it has been suggested that beer might be more of hazard to the consumer (134). Histamine has been found at concentration levels of 2.6–4.7 mg/L in Swedish beer, 3.2–15 mg/L in Danish beer, 7.3 mg/L in Dutch beer, 6.7 mg/L in German beer, and 20 mg/L in French beer. Tyramine levels have also been surveyed in beers from several countries and appear to occur at higher levels than His (135). Tyramine has also been found in nonalcoholic beers.

Buiatti et al. (136) investigated the content of eight BAs (His, Tyr, Try, Phe, Put, Cad, Spm, Spd) in alcoholic and nonalcoholic beers. Liquid–liquid extraction was performed with BuOH:$CHCl_3$ (1:1); the analytes were derivatized by DNS and analyzed according to Moret et al. (5). Sixteen different types of beer were analyzed; just six amines were found. In particular, His and Spm were not present in detectable amounts, whereas, despite the absence of any reference in the literature to Spd content in beer, this amine was found in all the samples. Nonalcoholic beers did not show significantly lower amounts of BAs than alcoholic beers.

Table 1 reports information about the papers dealing with BA analysis published between 1980 and 1994–95 (137–162).

Table 1 Biogenic Amines

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
His, Try, Tyr, Phe, Put, Cad with 1,6-diaminohexane (DAH) and tryptophan	RP-HPLC separation of PA derivatives, with coulometric array detection (16 porous graphite working electrodes at increasing potentials).	ODS HR80 PTFE as stationary phase. Mobile phase: acetate, ACN, THF (pH = 6.5) at different ratios and in gradient elution. Potential between 0 and 1200 mV (increments of 80 mV at each electrode) vs. solid-state Pd reference electrode.	His: 16 μg/L Tryptophan: 21 μg/L Try: 18 μg/L Tyr: 16 μg/L Phe: 22 μg/L Put: 25 μg/L Cad: 21 μg/L Dah: 12 μg/L	Gewürztraminer, Barolo, and Port wines	0.20-μm filtration and derivatization reaction with PA.	137
Tyr, Put, Cad, Try, Phe	RP-HPLC with precolumn derivatization with FMOC and fluorimetric detection at 315 nm (λ_{ex} = 265 nm).	RP as stationary phase. Mobile phases: sodium acetate (pH = 4.4) and ACN in gradient elution.	About 1 mg/L	Wine, cheese, fish	Acidification, borate buffer addition, FMOC derivatization, addition of a mixture of heptylamine, ACN, and HCl to remove the excess of the reagent.	41
Cad, His, Phe, Put, Spm, Spd, Tyr, Try, 5-hydroxy-Try	Ion-pair HPLC with post-column derivatization with PA in MeOH, boric acid, KOH, Brij-35 solution, ME. Fluorescence detection at 445 nm (λ_{ex} = 340 nm).	Novapak C-18 as stationary phase. Mobile phase: acetate, sodium octane sulfonate (pH = 4.5) with MeOH or MeOH–ACN in gradient elution.	0.30–0.65 mg/L	Beer, hops, malt, wort	$HClO_4$ extraction.	38
Cad, His, Put, Spm	IC with pulsed amperometric detector (Au working electrode, stainless steel counter electrode, and Ag/AgCl reference electrode).	IonPac CS10 cation exchange and IonPac CG10 guard column. Mobile phase: ACN, $HClO_4$, $NaClO_4$ in gradient elution.	Put: 5 ng; Cad and His: 12 ng; Spm: 25 ng.	Fish	Homogenization, TCA–HCl–heptane extraction, centrifugation (5000 g, 15 min). Re-extraction with TCA acid, cleaning up on LC-18 SPE cartridge and elution with HCl.	138
BAs	RP-HPLC with fluorimetric detection at 455 nm (λ_{ex} = 340 nm) of PA.	Nucleosil 100 7C18 as stationary phase. Mobile phase: hexanesulfonic acid, H_2PO_4, ACN in gradient elution.	0.5 mg/kg	Fermented sausages	Homogenization with $HClO_4$, centrifugation (9000 g, 10 min, 0°C), filtration, dilution 1/10 v/v.	139

(continued)

Table 1 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Agm, Put, Spm, Spd	HPLC with postcolumn derivatization with PA. Fluorimetric detection.	Polyaminepak (strong cation-exchange resin) column. Mobile phase: sodium citrate, ACN.	—	Vegetables	Homogenization with $HClO_4$, cooling ($T = 0°C$), centrifugation.	140
His, Tyr, Put, Phe, Cad, isoamylamine	HPLC with precolumn derivatization (PA) and fluorescence detection at 440 nm ($\lambda_{ex} = 230$ nm).	Nucleosil 100-5C18 column. Mobile phase: ACN–acetic acid in gradient elution.	His, Tyr: 1.0 mg/L; Put, Phe, Cad, isoamylamine: 0.5 mg/L	Wine	Derivatization (heptylamine as the internal standard) with PA in borate buffer, pH = 10.4.	50
BAs	RP-HPLC-UV at 254 nm of DNS derivatives.	Spherisorb ODS-2 column as stationary phase. Mobile phase: ammonium acetate, ACN in gradient elution.	1–5 mg/kg	Minced meat, cheese, tuna fish, dry sausages	Homogenization with $HClO_4$, centrifugation, filtration, re-extraction. $HClO_4$ addition, incubation at 40°C with NaOH, $NaHCO_3$, and DNS in acetone for 45 min. NH_4OH addition, 30 min digestion, ACN, centrifugation, filtration.	54
Cad, His, Phe, Put, Spd, Spm, Tyr, Try	RP-HPLC-UV detection at 254 nm of DNS derivatives; 1-7-diaminoheptane as IS.	Spherisorb 3S TG RP as stationary phase. Mobile phase: ACN, water, phosphate buffer solution, pH = 7, gradient elution.	Ranging between 0.02 ng (Put and Tyr) and 0.08 ng (Try)	Ground cheese (parmesan). Found amount, mg/100 g: Cad 0.01 Spm 0.01 His 0.45 Tyr 4.20 Phe, Put, Spm >0.10	Homogenization (HCl, diaminoheptane), centrifugation (14,000 g, 20 min, 4°C), re-extraction of aqueous residuum, Na_2CO_3 addition (pH > 12), extraction (BuOH, $CHCl_3$), HCl addition, evaporation to dryness, HCl dissolution, derivatization (DNS, $NaHCO_3$), evaporation, ACN dissolution.	5
Cad, His, Phe, Put, Tyr, Try, isoamylamine, methylamine,	HPLC with fluorimetric detection of PA derivatives (445 nm, excitation 356 nm).	RP-18 as stationary phase. Mobile phase: ACN, octan-2-ol, Na_2HPO_4 in gradient elution.	—	Wines	SO_2 addition, precolumn derivatization with PA/ME.	109

ethylamine						
His, Phe, Tyr, Try	Ion-interaction HPLC and spectrophotometric detection at different λ.	Spherisorb ODS-2 as stationary phase. Mobile phase: aqueous solution of octylammonium salicylate and octylammonium *o*-phosphate.	His 900 ppb Phe 500 ppb Tyr 400 ppb Try 20 ppb	Red wines. Found amounts: Phe 72 mg/L Try 4 mg/L	Dilution (1/10 v/v), 0.20-μm filtration.	113
Agm, Cad, His, Phe, Put, Spd, Spm, Tyr, Try	RP-HPLC-UV at 254 nm.	Lichrospher 100 RP-18 column. Mobile phase: water–MeOH mixture in isocratic or gradient elution.	—	Canned fish: tuna, bonito, mackerel, squid, anchovy	Homogenization (TCA), filtration, addition of NaOH and benzoyl chloride, addition of saturated NaCl, extraction (ethyl ether), evaporation, MeOH addition.	141
His, Phe, Spm, Spd, Try with alkylamines and benzylamine	RP-HPLC method. PRISMA Chemometric optimization method of IMGE (isoselective multisolvent gradient elution).	Novapak C18 as stationary phase. Mobile phase: multisolvent (THF, ACN, MeOH, H_2O) and ammonium acetate.	—	Animal feed, spoiled foodstuff	$HClO_4$ dissolution, centrifugation, NaOH and saturated $NaHCO_3$ addition, DNS reaction (acetone as the solvent).	142
BAs	HPLC analysis with three-component mobile phase optimization through PRISMA model.	Novapak C18 as stationary phase, $T = 37°C$. Mobile phase: mixture of THF, MeOH, ACN, and ammonium acetate.	—	Fish	Dissolution in $HClO_4$, centrifugation, addition of NaOH and $NaHCO_3$, DNS reaction (in acetone), digestion, NH_4OH addition.	143
Cad, Phe, Put, His, Tyr	HPLC with UV at 440 and 570 nm or fluorimetric detection at 485 nm (λ_{ex} = 254 nm). IC.	HPLC method: Hypersil ODS as stationary phase, T = 35°C. Mobile phase: EtOH, ACN, buffer at pH = 8. IC: column DC-6A resin (Sarasin), $T = 65°C$. Gradient elution with NaCl in citrate buffer (pH = 5.65)–EtOH–Brij.	UV detection: Tyr: 5 mg/L His 1 mg/L Fluorescence: His 5 mg/L Tyr 20 mg/L	Cheese	Extraction, DNS derivatization for HPLC analysis. Homogenization with TCA for IC.	118

(continued)

Table 1 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
BAs and metabolites	HPLC with ED, vitreous carbon electrode, potential range 0.72–0.85 V.	—	—	Wine, beer	Deproteinization by ultrafiltration, cleaning with Amberlite CG-50, $HClO_4$ as eluent.	144
Tyr, Phe	Ion-interaction RP-HPLC-UV at 230 nm.	Spherisorb ODS2 as stationary phase. Mobile phase: octylammonium salicylate or octylammonium *o*-phosphate, pH = 6.4.	—	Chocolate	Homogenization with ice cold $HClO_4$ and EDTA, extraction, centrifugation.	145
Cad, His, Phe, isoamylamine, Put, Spm, Spd, Tyr, Try	HPLC with UV (254 nm) and fluorescence detection at 490 nm (λ_{ex} = 360 nm) of DNS derivatives.	Lichrospher RP-18 as stationary phase. Mobile phase: EtOH, ACN, TRIS, acetic acid buffer (pH = 8).	0.5 mg/L in liquids, 5.0 mg/L in solids	Wine, cheese, meat	Addition of 1,7-diamino-heptane (IS), extraction with ACN/$HClO_4$. DNS reaction (in the presence of ethylamine). Extraction in ethylacetate. Evaporation to dryness. Dissolution in ACN.	146
BAs	HPLC with fluorimetric detection at 455 nm (excitation 345 nm) of the PA derivatives.	Shim-Pak CLC-ODS stationary phase, $T = 50°C$. Mobile phase: Na–hexane sulfonate, $NaClO_4$, methanol, pH = 3.0.	—	Herring	Dried herring homogenized with TCA, centrifuged, diluted.	61
BAs	HPLC with postcolumn reaction with ninhydrin (125°C).	LC-5000 amino acid analyzer with column of BTC2710 resin, $T = 65°C$. Elution with citrate buffer solution (pH 5.45–5.70) over 65 min.	1 mg/L and 0.1 mg/L after preconcentration	Wines	Direct analysis.	149
Tyr	HPLC with amperometric (glassy carbon electrode, at 0.7 V vs. Ag/AgCl reference electrode) and fluorimetric detection at 305 nm (λ_{ex} = 225 nm).	LiChrosorb RP-Select B stationary phase. Mobile phase: phosphate buffer pH = 7.5, EDTA, and ACN.	—	Camembert cheese	$HClO_4$ and CH_2Cl_2 addition, homogenization. NaOH addition, dilution, 0.45-μm filtration.	66

Cad, His, Put, Spm, Spd, Tyr	Normal-phase HPLC. Fluorimetric detection at 470 nm (excitation 335 nm) of DNS derivatives.	Ultrasphere Si-column as stationary phase. Mobile phase: ethylacetate–hexane–ethanolamine.	0.1 mg/kg	Fish, fish products	Homogenization with TCA, heating at 60°C (15 min), filtration, addition of NaOH (pH = 9), extraction in BuOH and HCl. DNS derivatization (40°C, $NaHCO_3$, acetone). Extraction of dansyl derivatives into benzene, Na_2SO_4, filtration, evaporation to dryness, dissolution in benzene.	147
Adrenaline, noradrenaline, Spm, Spd, Tyr	Automated ion-exchange method.	—	—	Mild and old cheese	TCA, $HClO_4$, and MeOH extraction. Comparison of recovery yields.	28
Cad, His, Put, Tyr, Spm, Spd	HPLC analysis, $T = 40°C$. Diode-array detector: 254 nm.	Hypersil ODS as stationary phase. Mobile phase: acetic acid, ACN, MeOH in gradient elution.	Cad 0.2 mg/kg His 0.3 mg/kg Put 0.2 mg/kg Tyr 0.3 mg/kg Spd 0.3 mg/kg Spm 0.5 mg/kg	Poultry broiler chicken: monitoring of microbial spoilage.	Homogenization with $HClO_4$, chilling, filtration, extraction with $HClO_4$, KOH addition (pH = 5.5), dilution, $T = 4°C$, overnight digestion, filtration. Cleaning up with Amberlite CG-50 for Spd and Spm analysis.	148
His, Tyr, and AA precursors histidine, tryptophan	HPLC-UV at 220 nm.	μ-Bondapack C18 as stationary phase. Mobile phase: butane, pentane, hexane, octane, or camphor–sulphonate (Na salts) in MeOH-H_2O-ACN or ACN-H_2O, pH = 3.0.	—	Cheese	Homogenization with HCl, cleaning up, multistep extraction.	57

(continued)

Table 1 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
His, Phe, Tyr, Try and AA precursors histidine, 2-phenylalanine, tyrosine, tryptophan	Ion-pair HPLC-UV at 215 and 260 nm.	CP Spher C18 Chrompak as stationary phase, $T = 40°C$. Mobile phase: heptane–sulfonate–KH_2PO_4–H_3PO_4–MeOH (or ACN).	10 mg/kg	Gouda cheese	Extraction in TCA, cooling at 3°C, centrifugation, removal of fatty layer, dilution.	59
Cad, His, Put, and AA precursors arginine, histidine, lysine	Ion-pair HPLC-UV at 254 nm.	Octylsilane column. Mobile phase: TBA–phosphate in MeOH-ACN in linear gradient elution.	4 mg/L for each AA and 2 mg/L for each BA.	Fish, meat	Extraction with MeOH, three elutions through pH 7–buffered Amberlite IRC-50 Plus cation-exchange column, two elutions through pH 4.25–buffered column of the same material, elution with MeOH-HCl, evaporation at 65°C, addition of saturated $NaHCO_3$-MeOH, derivatization with DNS.	63
Cad, His, Put, Tyr, Try	HPLC, with postcolumn derivatization (ninhydrin, hydrindantin) and thermosensitized reaction ($T = 145°C$). Spectrophotometric detection at 546 nm.	Nucleosil C18 as stationary phase. Mobile phase: ninhydrin, hydrindantin, dimethylsulfoxide, acetate buffer (pH = 5) and Na dodecylsulfate in different ratios.	2 mg/kg in cheese for each of the BAs; 0.8 mg/kg in sauerkraut; 0.3 mg/kg in wine.	Cheese, tuna fish, wine, sauerkraut, chocolate	Homogenization with trisodium citrate, digestion, addition of TCA, centrifugation (10,000 g, 4°C), filtration (0.45 μm), dilution.	10
Cad, His, Phe, Put, Tyr	HPLC and fluorimetric detection, identification with field-desorption MS.	Spherisorb ODS (5 μm) stationary phase.	—	Cheese, yogurt, chocolate, fish, beverages	Water extraction, centrifugation, HCl extraction, centrifugation, Zerolit 236 column (Na^+ form) passage, HCl elution, evaporation to dryness, treatment with fluorescamine.	55

BAs	HPLC analysis with fluorimetric detection at 450 nm (excitation 340 nm) of PA derivatives.	Ultrasphere ODS stationary phase. Mobile phase: Na acetate (pH = 6.6), THF, MeOH in gradient elution.	—	Wine	Shaking with PVP, filtration up to 0.45 μm.	100
Cad, His, Put	HPLC and fluorescence detector.	—	—	Putrefaction of fish products, raw fish	—	150
BAs	Three-step gradient elution from a cation-exchange column. Spectrophotometric detection with ninhydrin at 570 nm.	—	<1 mg/L	Fresh, matured, and spoiled pork and beef; amount found: <10 ppm in fresh meat	Acid buffer solution extraction.	151
BAs	Cation-exchange chromatography.	—	—	Ground beef, minced beef, as indication of freshness and quality	HCl extraction.	152
BAs	Automated IC.	—	—	Fresh and processed meat, meat products	Comparison between TCA, $HClO_4$, and MeOH extraction.	153
Ethanolamine, His, Tyr, Phe, Put, Cad, isoamylamine	HPLC and fluorescence detection.	RP-18 Ultrasphere as stationary phase. Mobile phase: MeOH, Na acetate, THF in nonlinear gradient elution.	—	Wine	No pretreatment.	108
His, Phe, Put	HPLC of DNS-amines and detection at 254 nm.	Ultrasphere ODS as stationary phase. Mobile phase: MeOH, ACN, *o*-phosphoric acid in gradient elution.	2–5 mg/100 g of sample	Tuna, swiss cheese	Extraction in MeOH, multiple extraction in BuOH-HCl, evaporation, DNS reaction.	89

(continued)

Table 1 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Agm, Cad, His, Phe, ethanolamine, Put, Spm, Spd, Tyr, Try	RP-HPLC and fluorimetric detection of PA derivatives (λ_{ex} = 340 nm, λ_{em} = 440 nm), of the DNS derivatives (λ_{ex} = 360 nm, λ_{em} = 460 nm), and of fluorescamine derivatives (λ_{ex} = 395 nm, λ_{em} = 460 nm).	μBondapak C18 column. Mobile phase: MeOH, ACN, acetic acid, and buffer in different concentration ratios in isocratic and gradient elution.	pmol injected in 10 ml: Agm 0.5; Cad 6.0; ethanolamine 13.0; His, Tyr, Try 5.0; Phe 7.5; Put 5.5	Red must and Villard Noir wine	Derivatization with DNS, fluorescamine, and PA in presence of ME. Extraction in ethyl acetate.	99
	IC	—	—	Cheese, fish, meat	—	154
Cad, Put	HPLC.	—	—	Vinegar, fruit juice	—	155
His, Put, Tyr	Automated IC.	—	—	Cheese, fish	—	156
Cad, His, Put	HPLC.	—	—	Fish, sardine, tuna	—	157
His, Put, Spm, Spd	HPLC.	—	—	—	—	158
His, Tyr	Ion-pair HPLC of PA derivatives and fluorimetric detection at 425 nm (λ_{ex} = 338 nm).	Nova-Pak C-18 stationary phase. Mobile phase: MeOH, hydrogen-phosphate, heptanesulfonic acid, pH = 3.0.	1–5 mg/L in cheese	Dairy products, infant formulae, yogurt, cheese; found: in cheese 25–223 mg/L	Homogenization with MeOH at T = 60°C, cooling, MeOH addition, filtration.	159
BAs, di- and trimethylamine	HPLC with UV detection at 207 nm.	IMP-HPX-72-0 Bio Rad column in OH form. Mobile phase: NaOH.	—	Spoilage assessment of seafood, cod, mackerel	$HClO_4$ homogenization, KOH to pH = 7, filtration 0.22 μm.	160
Alkylamines and BAs	HPLC of PA derivatives and fluorimetric detection at 425 (λ_{ex} = 335 nm).	Fine-SIL C18 column. Mobile phase: MeOH-NaH_2PO_4 in different ratios, in isocratic and gradient elution.	1 μM/ml of extract	Fresh tea shoots, made tea	Steamed, frozen, dried, pulverized, extracted in water (T = 50°C), filtered, derivatized with PA/ME in borate buffer solution (pH = 10).	161
BAs, urea, NH_3, DMA, TMA	HPLC-UV detection at 208 and 214 nm.	Bio-Rad HPX-72-0 column. Mobile phase: NaOH.	—	Seafood	—	162

III. HETEROCYCLIC AROMATIC AMINES (HAA) AND AZAARENES (PANH)

A. General Information

Even though diet has for a long time been associated with varying cancer rates in human population, the causes of the observed variation in cancer patterns have not yet been adequately explained (163). Various types of carcinogens are present in foods as minor components, and much effort has been expended on the identification and measurement of these compounds in food. Some of them occur naturally as a result of the action of microorganisms; others (found in fish and meat products) are believed to be formed during cooking by pyrolysis of amino acids and proteins (at temperatures over 150°C). Their formation has been explained by condensation reactions of creatine or creatinine with amino acids and sugars or by their thermal decomposition products in meats. These mutagens are identified as heterocyclic aromatic amines (HAAs), and they are present at concentration levels of nanograms per gram.

As early as 1964, polycyclic aromatic hydrocarbons (PAHs) known to be carcinogenic, such as benzo[*a*]pyrene, were detected in broiled meat (164). A number of papers have been published about PAHs found in smoked and thermally treated foods as a result of pyrolysis or incomplete combustion of organic matter (165–169). Less information is available on the nitrogen analogs, the basic azaarenes polycyclic aromatic nitrogen-containing hydrocarbons (PANHs), but they have been shown to be present in association with PAHs in various samples that contain nitrogen, such as processed food (170–172).

The introduction in 1975 of the Ames test (173) provided a rapid tool for isolating potential carcinogens in food on the basis of their mutagenic activity. Over the past 10 years a number of potent bacterial mutagens, all belonging to the class of HAAs, have been purified from pyrolyzed amino acids and proteins in protein-rich foods such as beef, chicken, and fish, cooked by typical household methods (174–178), as well as in beef extracts (179). The overlap of mutagenicity and carcinogenicity, although controversial, is now widely accepted (180). Bacterial mutagenicity assays have shown most of these compounds to be powerful mutagens, and several of them induce cancer at multiple sites in rodents and may be potential human carcinogens (181). Figure 3 reports the structures of the most common HAAs.

B. Analysis

The assessment of mutagenic activity in cooked foods requires tedious extraction work in order to isolate and quantify the responsible chemicals at the nanogram level. Efforts have been made to develop a rapid and efficient method to obtain chromatograms free of interfering material. Coextracted matrix components influence analyte detection limits more than does absolute detector sensitivity (182). The sample workup therefore is the most critical part of the analysis (183). Solid-phase extraction with different coupled columns provides an improvement (176) over LLE and the use of large columns filled with XAD resin. The determination of PAHs and PANHs in food has been carried out by different chromatographic techniques, including LC with fluorescence (164,171,184) and/or UV detection (185,171) and GC with FID (168,186) or MS detection (187).

Recommended analytical procedures for the determination of PAHs are documented or proposed in several European and U.S. guidelines, including EPA methods 8310 and 610 (188,189), the ISO method (190), and the German Standard (DIN) method (191) in environmental samples; no specific methods are given for food samples. All of these methods specify RP chromatography using C_{18} bonded phases in combination with either fixed or wavelength-programmed UV and fluorescence detection techniques.

IQ: 2-amino-3-methyl imidazo[4,5-*f*] quinoline	**MeIQx**: 2-amino-3,8-dimethyl imidazo [4,5-*f*]quinoxaline	**MeIQ**: 2-amino-3,4-dimethyl imidazo[4,5-*f*]quinoline
4,8-DiMeIQx: 2-amino-3,4,8-trimethylimidazo-[*f*] quinoxaline	**Trp-P-1:** 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole	**Trp-P-2:** 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole
AαC: 2-amino-9H-pyrido[2,3-*b*]indole	**MeAαC**: 2-amino-3-methyl-9H-pyrido[2,3-*b*] indole	**Glu-P-1:** amino-6-methyl dipyrido-[1,2-*a*:3',2'-*d*]imidazole
PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine	**harman**: 1-methyl-9H-pyrido[4,3-b]indole	**norharman**. 9H-pyrido[4,3-b]indole
DMIP: 2-amino-1,6-dimethylazo[4,5-*b*] pyridine	**1,5,6- TMIP**: 1,5,6-trimethyilimidazopyridine	**3,5,6-TMIP**: 3,5,6-trimethyilimidazopyridine

Fig. 3 Molecular structures of some HAAs.

Ultimately major efforts to develop coupled chromatographic techniques have been performed to alleviate the problem of manual sample pretreatment and to enhance sensitivity and selectivity in the analysis of PAHs in foodstuffs (192) and environmental samples (193–195). Liquid chromatography/MS (196,197), GC/MS (175), HPLC with UV absorbance, fluorescence (177) (see Fig. 4), or electrochemical (ED) detection (179), and ELISA immunoassay (198) have been successfully used for the determination of HAAs.

Galceran et al. (199) developed an SPE-HPLC method for the determination in beef extract of five mutagenic amines, namely: Trp-*P*-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole),

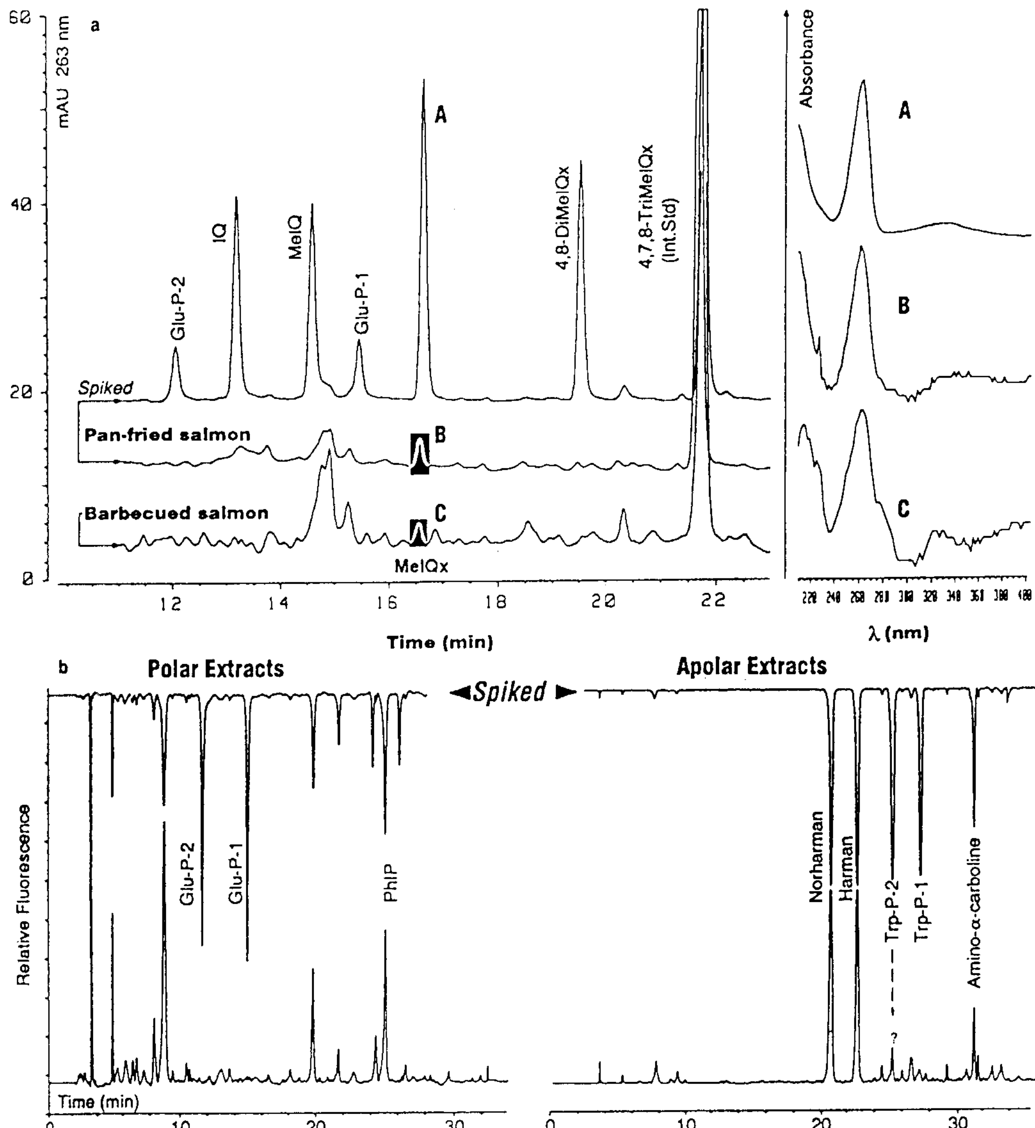

Fig. 4 (**a**) Polar extracts of fish pan fried and barbecued for 9 min. Pan frying at 200°C produced more MeIQx (peak B) than barbecuing for the same time at 270°C (peak C). MeIQx peak C (<1 ng/g) illustrates the DL of the method. Online recorded UV spectra from MeIQx peaks are shown at the right. (**b**) Polar and apolar extracts of fish barbecued per side at 270°C. These samples contained detectable PhIP in the polar extract (left) and AαC, norharman, and harman in the apolar extract (right). (From Ref. 177.)

Trp-*P*-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), AαC (2-amino-9*H*-pyrido[2,3-*b*]indole), MeAαC (2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole), and PhIP (2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine) and two β-carbolines comutagens: harman (1-methyl-9*H*-pyrido[4,3-*b*]indole) and norharman (9*H*-pyrido[4,3-*b*]indole). The optimized SPE procedure for isolation and preconcentration comprises the use of diatomaceous earth, propylsulfonyl silica gel, and C_{18} cartridges to separate selectively the imidazopyridine and indolpyridine derivatives from those of

quinoxaline and quinoline. The analysis was carried out on a C18 column in isocratic conditions, and the detection was performed by ED and fluorescence. Harman and norharman were found in the beef extract at levels of 110 and 53 ng/g, and Trp-P-2 and AαC were tentatively identified.

Knize et al. (200) reported some preliminary results for a new SPE technique for the analysis of aromatic mutagen amines in foods for which no method was still developed, together with results obtained by the SPE-PRS method developed by Gross. The underdevelopment method should also be able to extract trimethyilimidazopyridine (TMIP) and DMIP (2-amino-1,6-dimethylazo[4,5-*b*]pyridine) by using an acid/ion-exchange scheme (SCX method). The extracts were analyzed by HPLC with UV or fluorescence detection. The content of resultant HAAs depended on the kind of meat and on the cooking conditions.

The analytical methods presented in literature for the HPLC determination of HAAs in food are summarized in Table 2.

IV. UNSULFONATED AROMATIC AMINES

A. General Information

A study in the 1970s estimated that the annual individual consumption of food in the United States was 645 kg, 5.5 g of which was synthetic colors. According to a similar evaluation, in the U.K. the amount of food containing added color represents nearly half of the total diet and the average amount of synthetic colors consumed is around 10 g per person per year (203–205). The kind and the amount of food and beverages with added color in amounts up to 120 mg per kg of product are surprisingly high. Besides the possible toxicity of the dye itself, synthetic food colors often contain toxic impurities.

The vast majority of synthetic dyestuffs used in industry are based on azo chemistry. Unsulfonated aromatic amines can be contained as impurities in the reagents. Most are diazotized and coupled with an intermediate such as R-salt, sulfanilic acid, or Shaeffer's salt during the color manufacturing process, but low levels (ng/g range) of unreacted free amines can still be found in most food dyes. Aromatic amines may also be formed in side processes during the chemical synthesis of the dyes. Thus aromatic amines such as 1- or 2-naphthylamine (1- and 2-NA), aniline, and benzidine (Bz), together with other toxic species like 4-aminobiphenyl and aromatic polycyclic hydrocarbons, can be present in many dyes. Moreover, these dyestuffs can split off aromatic amines in chemically reducing media (e.g., reductive stripping of azo dyes) or in human organs by enzymatic reduction [the azo bound found in several food dyes can be cleaved in the gut by azo-reductase, an enzyme present in human intestinal flora (206)].

Moreover, admitted colors can undergo degradation reactions that lead to the formation of aromatic amines, in particular aniline, together with naphtionic and sulfanilic acids. These processes were shown to be particularly favored by light and by the presence of ascorbic acid and can therefore easily develop in bottled soft drinks that are displayed in bright sunlight, as, for example, in shop windows. The problem is particularly important in countries that experience strong sunlight (207).

Carcinogenity and toxicity must be checked therefore not only for dyes but also for the impurities therein contained and their degradation products. A number of these nonsulfonated aromatic amines (NAAS) have been found to be carcinogenic to humans and animals.

In 1985 the FDA published specifications for free amines in FD&C Yellow No. 6 (or Sunset Yellow, 1-(4′-sulfo-1′-phenylazo-)2-naphthol-6-sulfonic acid disodium salt), and FD&C Yellow No. 5 (or tartrazine, 4,5-dihydro5-oxo-1-(4-sulfophenyl)-4-[(4-sulfophenyl)azo]-1*H*-pyrazole-3-carboxylic acid trisodium salt) and expressed concern about amines in other colors (208).

Table 2 Mutagenic Heterocyclic Amines

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
HAAs	HPLC with: (i) UV detection at 263 nm; (ii) ED at 950 mV; (iii) fluorimetric detection at 450 nm (λ_{ex} = 360 nm).	LiChrosorb RP-Select B column. Mobile phase: NH_4 (acetate) (pH = 4.5), MeOH, ACN in isocratic and gradient elution.	—	Food flavors, meat extract	Cleaning up using the Gross method (94). The spray-dried product was Soxhlet extracted, placed on Kieselgur, and extracted with ethyl ether. Cleaning up by affinity chromatography on Cu–phthalocyanine complex.	201
Quinoline and quinoxaline derivatives	HPLC with diode array and fluorimetric detection.	TSK-gel ODS80 and Supelguard LC-8-DB precolumn. Mobile phase: triethylamine, ACN, phosphoric acid (pH = 3.2 and 3.6) in gradient elution.	1 ng/g	Pan-broiled meat, fish, salmon; quinoxaline up to 4.7 ng/g of fried fish pan broiled 200°C; 4.6 ng/g oven cooked at 200°C; and <1 ng/g barbecued at 270°C (indole derivatives: maximum amount in barbecued at 270°C)	SPE in Extrelut column-CH_2Cl_2 filled and Bond-Elut PRS cartridge. Sequential and selective extraction with HCl, MeOH, H_2O, NH_3, ammonium acetate.	177 (see Fig. 4)
HAAs	RP-HPLC-ED (0.9 V with vitreous carbon electrode vs. Ag/AgCl).	SynChropak SCD-100 column (short alkyl-chain ligand, endcapped). Mobile phase: ACN-NH_4 (acetate) buffer (pH = 5.5).	80–475 pg	Food products	—	202
10 HAAs	HPLC-ED at + 1000 V.	TSK-Gel ODS 80T 50 mM NH_4 (acetate)–ACN at various pH.	—	Beef extract	Samples purified by the method of Gross (176).	179
HAAs	HPLC-MS.	Supelco LC-CN or Supelco LC-18-DB column. Mobile phase: NH_4 (acetate) (pH = 6.8)–ACN–MeOH in gradient elution.	Low ppb level	Cooked beef products, beef extract, fried beef	MeOH extraction, cleaning on XAD-2, ethylacetate elution.	196

The content of NAAS in synthetic food dyestuff is limited to 0.01% by European color additive specifications (209).

B. Analysis

Methods for the analysis of aromatic amines in synthetic azo dyes and in food and beverages colored with these dyes have been developed. High-performance LC or GC methods are generally employed, often with the help of derivatization reaction and fluorimetric detection, with HPLC generally regarded as the best technique for the determination of aromatic amines.

A method frequently used to determine aromatic amines in water-soluble dyes involves their extraction with chloroform, followed by diazotization of amines and coupling of diazonium salts with a reagent R-salt (disodium-3-hydroxy-naphthalene-2,7-disulfonate) or pyrazolone T (4,5-dihydro-5-oxo-1-(4-sulfophenyl-)1*H*-pyrazole-3-carboxylic acid). The separated products are detected by UV-VIS spectrophotometry or fluorescence (210–212).

The most recent paper on this topic has been published by Lu and Huang (213). The method consists of an online enrichment of the aromatic amines on a carboxymethyl-bonded silica precolumn and an HPLC-UV (at 254 nm) analysis. The mobile phase, ACN–acetate buffer (pH 4.66) (40:60, v/v), was used to desorb the analytes and for the subsequent separation. The method was applied to the determination of several compounds (4-aminoazobenzene (4-AAB), benzidine (Bz), 3,3′-methylbenzidine (DMBz), 4-aminobiphenyl (4-ABP), 3,3′-dichlorobenzidine (DCBz), and 2-naphthylamine (2-NA) together with some substituted naphthalens and phenols) in aqueous solution of four food dyes: Direct Blue 6, Amaranth, Sunset Yellow FCF, and D&C Orange No. 4. Detection limits ranged between 0.6 and 1.6 μg/g. Most part of the methods developed for this kind of determination are reported in Table 3.

V. PURINES AND PYRIMIDINES

A. General Information

Pyrimidines and purines are nitrogen-containing organic bases. They are part of the building blocks (nucleotides) of DNA and RNA, together with a pentose (ribose or desoxiribose, respectively) and phosphoric acid. The nucleic acids, polinucleotides of high molecular weight, are the prosthetic group of the nucleoproteins, one of the main constituents of the cell nucleus.

Cytosine (2-hydroxy-6-amino-pyrimidine), uracil (2,6-dihydroxypyrimidine), and thymine (5-methyluracil) are pyrimidine derivatives; adenine (6-aminopurine), guanine (2-amino-6-hydroxypurine), and xanthine (2,6-dihydroxypurine) are purine derivatives (Fig. 5).

Pyrimidines and purines derivatives act as bases and can be acquired through the diet. In particular, organ meats such as liver are a rich source of DNA and RNA. Most dietary purines are oxidized by enzymes to uric acid in the intestinal mucosa; that is their excretory product in humans. The desease known as gout is related to high levels of uric acid in serum and the result of deposition of urate salts in various tissues.

An important role is played by adenosine triphosphate (ATP), involved in energy exchange: relatively large amounts of free energy are released when ATP is hydrolyzed. A consequence of the loss of ATP in muscle postmortem is its conversion to hypoxanthine. Some 5′-mononucleotides, intermediates in the production of hypoxanthine and with the ribose component hydroxylated at position 6, are flavor enhancers in muscle foods. Compounds of this kind are, for example, inosine 5′-monophosphate (IMP) and guanosine 5′-monophosphate (GMP). The ATP is first converted to ADP and then to AMP by a disproportionation reaction. The AMP is then deaminated to IMP. The IMP can degrade to inosine and eventually to hypoxanthine. Hypoxanthine

Table 3 Unsulfonated Aromatic Amines

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Aniline, 1-NA, 2-NA, 2-ABP, 4-ABP	Ion-pair HPLC with spectrophotometric detection at 426, 512, and 625 nm.	Supelco LC-18 column. Mobile phase: MeOH, TBA-I in gradient elution.	<0.3 ng/ml	Soft drinks, hard candies. Aniline was found in orange beverage up to 12.6 ng/ml, 1-NA up to 8.25 ng/ml, and 2-NA up to 1.12 ng/ml in grape product. In hard candies aniline was found up to 9.2 ng/g and 1-NA up to 10.6 ng/g.	Preconcentration on C18 cartridge column, elution with MeOH, H_2O. Dilution. pH adjustment to 2.1–2.6 with H_2SO_4. $Na_2S_2O_4$ addition. pH adjustment to 8.5 with NaOH. NaCl addition, $CHCl_3$ extraction, H_2SO_4 addition, diazotization and coupling reaction with disodium-2-naphthol-3,6-disulfonate. Dissolution in MeOH.	214
1-NA, 2-NA	Ion-pair HPLC and spectrophotometric detection at 522 nm.	Supelco LC-18 as stationary phase. Mobile phase: MeOH and TBA phosphate.	8 ng/g	Commercial samples of amaranth food color. Found: up to 435 μg/g of 1-NA and 214 μg/g of 2-NA (5% in the free state).	Na_2SO_4 addition, NaOH, NaCl, and $CHCl_3$ addition, diazotization, coupling reaction with 3-hydroxynaphtalene-2,7-disulfonic acid and ditionite.	215
Aniline, *p*-cresidine	HPLC with diode array detection at 254 and 546 nm.	Bio-Sil C18 column equilibrated with ammonium acetate and ACN. Mobile phase: H_2O, ammonium acetate, ACN in linear gradient elution.	5 μg/L	FD&C Red No. 40. *p*-Cresidine was found in 26 samples: amount 0–2115 ppb (average 40 ppb), aniline 5–169 ppb.	NaOH addition to water solution, multiple-step extraction on Extrelut QE columns with $CHCl_3$, H_2SO_4 addition, evaporation. H_2SO_4 addition, diazotization, and coupling reaction with R-salt or pyrazolone for aniline.	216
Dichloroaniline, 2,4-dimethylaniline	HPLC-ED. Vitreous working electrode and Ag/AgCl reference electrode. Applied voltage 0.9–1.0 V.	μ-Bondapak C-18 stationary phase. Mobile phase: phosphate buffer (pH = 6.0) and ACN.	—	Fatty foods and food packaging, vegetable oils	Dissolution in hexane, extraction with HCl, hexane, ACN.	217

(continued)

Table 3 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Aniline, Bz, 4-ABP, 4-AAB	HPLC with spectrophotometric detection at 254 and 510 nm.	Microsorb C18 column. Mobile phase: H_2O–ACN–ammonium acetate in gradient elution.	Bz2.3 $\mu g/L$ Aniline 34.2 $\mu g/L$ 4-ABP 4.6 $\mu g/L$ 4-AAB 4.2 $\mu g/L$	In food color FD&C Yellow No. 6. Found in 34 certified samples: aniline 5–422 ppb (average 98), 4-ABP 0.2–23.0 ppb (average 3.5 ppb), 4-AAB 0.5–1098.0 ppb (average 4.0 ppb). Bz: absent.	Two extraction methods employed: (a) NaCl and NaOH addition, $CHCl_3$ extraction, H_2SO_4 addition, $CHCl_3$ removed. (b) Warm water and NaOH addition. Extraction in dry Extrelut QE column. $CHCl_3$ extracts treated as in method (a). Diazotization and coupling reaction with R-salt or pyrazolone-T.	212
Bz, aniline, 4-ABP, 4-AAB	HPLC and spectrophotometric detection at 254 and 510 nm.	Novapak C18 stationary phase.	—	In 25 commercial samples of food color FD&C Yellow No. 5. Bz was found in five samples under 1 ng/g. All the samples contained measurable levels of aniline (mean 67 ng/g, maximum amount 487 ng/g).	Aqueous alkaline solution extracted into $CHCl_3$, solvent evaporation, extract dissolution in diluted acid. Diazotization and coupling reaction with R-salt.	211
Aniline, 1-NA, 2-BPA, 4-BPA, *p*-cresidine	HPLC and spectrophotometric detection at 512 nm.	Gradient mobile phase: H_2O-MeOH.	—	Commercial samples of tartrazine, Sunset Yellow FCF and Allura Red. Maximum content: aniline 51.9 ppm, 1-NA 5.0 ppm, 2-ABP 2.0 ppm, 4-ABP 210 ppm.	Na_2SO_4 addition, extraction with $CHCl_3$, diazotization, and coupling with R salt.	218
Aromatic amines (*p*- and *m*-cresidine) in Food Red 17	RP-HPLC-UV at 290 nm.	Develosil ODS HG-5 operating at 40°C. Mobile phase: 0.01 M ammonium acetate–MeOH (3:2).	*p*-cresidine: 0.05 $\mu g/g$; *m*-cresidine: 0.1 $\mu g/g$	Food Red No. 40 (CI food red 17).	Sample of dyes dissolved in 0.01 M NaOH, solution extracted with $CHCl_3$. Extract washed with H_2O, treated with H_2SO_4 0.015% and evaporated to dryness. Residue dissolved in aq. 0.3% NaH_2PO4-MeOH (1:1).	219

pyrimidines		purines	
cytosine	2-hydroxy-6-aminopyrimidine	adenine	6-aminopurine
uracil	2,6-dihydroxypyrimidine	guanine	2-amino-6-hydroxypurine
thymine	5-methyluracil	xanthine	2,6-dihydroxypurine
		hypoxanthine	6- hydroxypurine

Fig. 5 Structure of pyrimidine and purine and names of the related bases.

has a bitter flavor, and it has been suggested as a cause of off-flavors in stored fish and in unheated irradiated beef.

The determination of purine and pyrimidines derivatives and their breakdown products can give useful information about the degree of food spoilage or food degradation undertaken in treatments such as storing and heating.

B. Analysis

Several techniques have been developed for the determination of purine and pyrimidine derivatives in food sample and in particular for hypoxanthine quantification: biosensors (220–223) and electrochemical methods making use of immobilized enzyme electrode (224–227), electrochemical enzymatic-based FIA methods (228,229), enzyme reaction with fluorimetric detection (230), radioimmunoassay (231), colorimetric methods (232), capillary electrophoresis (233), and TLC (234). Many HPLC methods have also been developed and are reported in Table 4 (235–247); the most recent ones are described next.

Liu et al. (248) developed an HPLC-UV (260 nm) method to detect cytosine, thymine, adenine, theophylline (TP), and caffeine (CF) in tea. The mobile phase consisted of ACN/H_2O/1 M KH_2PO_4/1 M acetic acid (2:96.9:1:0.1), and the stationary phase was a Spherisorb C18 operated at 35°C.

Gosch and Montag (249) determined and compared the concentration of purines and pyrimidines in different coffees. The freeze-dried extract was dissolved in water, hydrolized with TFA/formic acid at 235°C, and applied to an RP18 SPE cartridge. The eluted sample (with methanol) was analyzed by HPLC on a LiChrosphere 100 RP-18. The mobile phase was a phosphate buffer containing *N,N*-dimethyloctylamine, and the detection was spectrophotometric at 269 nm. Adenine, guanine, hypoxanthine, xanthine, AMP, GMP, IMP, UMP, and uric acid levels were tabulated for arabica and robusta coffees from various sources.

An analytical method for the determination of three purines (guanine, adenine, hypoxanthine) and creatinine in commercial yeast extract and meat flavorings was developed by Ruther and Balthes (250). The hydrolyzed samples (with HCl) were evaporated, redissolved in 0.1 M HCl, and applied to a column containing cation-exchange resin AG 50 W X4. The eluate was analyzed on a Eurosphere 100 C_{18} column at 20°C, with an ACN/H_3PO_4 mobile phase at pH 2.3, containing sodium heptanesulphonate (ion-pair reagent). The presence of meat extract is recognizable from the content of creatinine and contributes to the adenine content, while hydrolyzed vegetable protein contains neither purines nor creatinine.

Table 4 Purines and Pirimidines

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Hypoxanthine	Amperometric enzymatic method (at 0.7 V, Pt vs. Ag) and HPLC.	HPLC: LC-8 column, mobile phase 0.01 M KH_2PO_4, pH = 4.5.	—	Cooked and raw fish	—	235
Hypoxanthine together with ADP and breakdown products	RP-HPLC-UV at 254 nm.	Column: μ-Bondapak C18. $T = 30°C$. Mobile phase: KH_2PO_4 0.04 M, K_2HPO_4 0.06 M buffer.	—	Fish	$HClO_4$ homogenization, centrifugation, pH adjustment to pH 6.5–6.8, filtration.	236
Hypoxanthine and inosine	HPLC.	—	—	Fish	—	237
Hypoxanthine	HPLC.	—	—	Refrigerated fish	—	238
Guanosine, adenine, deoxyguanosine purine nucleosides, adenosine, deoxyadenosine	HPLC-UV at 254 nm and TLC.	Separation on dextran-based gels Sephadex G10, G25 and LH20, Sephasorb HP Ultrafine columns. Mobile phase: acetate pH = 4.0. HPLC: Zorbax C8 and Versapack C18 columns. TLC: cellulose F254.	—	Beer and wort	—	239
Purine derivatives, inosine, nucleic acids	HPLC-UV at 254 nm.	μ-Bondapak C_{18} and LiChrosorb RP-8 columns. Mobile phase: KH_2PO_4 (pH = 6.0).	—	Food	Enzymatic hydrolysis for purine bases and inosine HPLC determination.	240
Purine bases	IC-UV at 260 nm.	T program 45–70°C. Elution with citrate buffers pH = 3.0, 4.25, 5.45.	—	Foods rich in carbohydrates	Hydrolysis with TFA and formic acids (15 min, 240°C), CH_2Cl_2 extraction.	241
Purine bases	IC.	Computer programs for calculating the relative compositions of nucleotides, nucleic acids, purines.	—	Food	TFA and formic acid hydrolysis.	242

Purine bases	HPLC-UV. The ratio of the absorbance at 245 and 254 nm is used to calculate adenosine content.	Column Bondapak C18 or Zorbax ODS. Mobile phase: phosphate buffer (pH = 3.5) and THF in different ratios.	—	Chocolate	Defatting extraction.	243
Orotic acid, uric acid, uridine, allantoin, cytidine, hypoxanthine, xanthine, guanine, pseudopurines, pyrimidines	HPLC-UV at 254 nm.	μ-Bondapak C-18 column. Mobile phase: $NH_4H_2PO_4$, pH = 6. Beckman M82 and Hamilton HP-B-80 columns. Elution with citrate buffer at different gradient elution, pH, and *T*.	—	Milk	—	244
Purine bases	Automated cation-exchange amino acid analyzer. Detection at 260 nm.	—	200 mg	Protein-rich foods, liver	Hydrolyzation with TFA and formic acid (90 min) at 240°C, under pressure in a PTFE-coated vessel. Cooling, addition of 6-chloropurine as the internal standard, 0.2-μm filtration.	245
Purine derivatives, nucleotides	HPLC.	Column: RP-8.	—	Malt wort, beer	—	246
Hypoxanthine	HPLC-UV at 254 nm.	Mobile phase: 0.01 M potassium phosphate buffer, pH = 4.5	10 ng	Fish	The sample was extracted with $HClO_4$.	247

VI. METHYLXANTHINES

A. General Information

As a category of alkaloids with distinctive bioactivity, methylxanthine derivatives have in recent years received increasing attention in the food and pharmaceutical industries. The most important compounds of this class, characterized by a bitter flavor, are caffeine CF, theobromine TB, and theophylline TP (Fig. 6).

Caffeine is a naturally occurring substance found in the leaves, seeds, or fruits of more than 60 plants. These include coffee and cocoa beans, kola nuts, tea leaves, guarana (*Paulinia cupana*) and Paraguay tea. Thus it is present naturally in many beverages, such as coffee, tea, and cola drinks, or is added in small amounts (up to 200 ppm) in some soft drinks and in foods such as chocolate. Caffeine is obtained by solvent or supercritical fluid extraction from green coffee beans, mainly during the preparation of decaffeinated coffee.

Caffeine is a mild stimulant and can have adverse effects on humans if ingested in large amounts. In particular, the effects of stimulation of the central nervous system (251,268,269) include nervousness, restlessness, and insomnia. However, people differ greatly in their innate sensitivity to CF; some sensitive individuals may experience mild, temporary effects, including headache, irritability, and nervousness when their daily intake is quickly and substantially altered. Caffeine does not accumulate in the bloodstream or body and is normally excreted within several hours following consumption. During the past decade, extensive research on CF in relation to cardiovascular disease, fibrocystic breast disease, reproductive function, behavior in children, birth defects, and cancer has identified no significant health hazard from normal CF consumption. In a Federal Register notice published in May 1987, the FDA came to a confident position on its safety.

Presently, CF is used extensively as a flavoring agent in the preparation of cola and power beverages (252). Depending on the strength and the preparation method, the CF content in coffee ranges from about 60 to 150 mg per cup. In the pharmaceutical field it is employed as an analgesic; in dermatology it is used to revitalize the skin.

Theobromine is an alkaloid found in the cocoa shell, tea (only in very small amounts), and kola nuts, but it is not found in coffee. In cocoa, its concentration is generally about seven times as great as CF. Although CF is relatively scarce in cocoa, it is mainly because of TB that cocoa is "stimulating." TB is considerably weaker than CF and TP, having about one-tenth the stimulating effect of either. Its diuretic properties are well known (253), however, and it is also used in other applications in the pharmaceutical field.

Caffeine (CF)	**Theobromine (TB)**	**Theophylline (TP)**
1,3,7-trimethylxanthine	3,7-dimethylxanthine	1,3-dimethylxanthine

Fig. 6 Molecular structures of the methylxanthines.

Theophylline is found in coffee and in tea in very small amounts, but it has a stronger effect on the heart and breathing than does CF. Along with CF, TP is used as a medicine to treat emphysema and bronchitis (254,255). Caffeine and TP inhibit cAMP phosphodiesterase. In their presence the effects of cAMP, and thus the stimulatory effects of the hormones that lead to its production, are prolonged and intensified.

B. Analysis

Although a great variety of analytical techniques have been applied to the simultaneous determination of methylxanthines in various matrices, HPLC is the one most frequently used nowadays. Most of the methods are based on reversed-phase HPLC, using ACN, MeOH, or THF in acetate or phosphate buffer as mobile phase and UV spectrophotometric detection (256–270). Some RP-HPLC methods were proposed in combination with solid-surface room-temperature phosphorimetric detection (271), mass spectrometry (272), or amperometric (273) detection. The separation can also be achieved by RP ion-pair or ion-interaction HPLC (274–277) or micellar HPLC (278). In contrast, in recent years few normal-phase HPLC methods (279) were reported (see Table 5).

An RP-HPLC separation of CF together with catechins in green tea was developed by Goto et al. (280) and carried out on a Develosil ODS-HG column with a gradient water–ACN–phosphoric acid elution. The DLs were about 0.2 ng for the nine analytes.

High-performance gel filtration chromatography was used to separate trigonelline, chlorogenic acid (CGA), and CF in green coffee by De Maria et al. (281). The method has the advantage, with respect to RP-HPLC, of an aqueous eluent.

The presence of CF in honey seems to be an excellent base investigation in order to assess product quality. A method for its determination in orange honey was developed by Defilippi et al. (282). The honey samples are dissolved in water, filtered (0.45 μm), and injected in the HPLC. The column was a LiChrocart 250-4 RP-18 and the mobile phase was a triethylammonium phosphate–water solution. The orange honeys analyzed contained CF in amounts between 1.89 and 8.47 mg/kg.

Kiehne and Engelhardt (283) developed HPLC-MS with thermospray interface (ammonium acetate solution for buffer ionization) to determine flavonols, flavonol *O*-glycoside, flavone *C*-glycosides, CF, TB, theogalline, and theanine from green tea.

Moriyasu et al. (267) developed a method to detect CF, TB, and TP in foodstuffs. Solid food was dialyzed for 1 hour against hot water, chewing gum was extracted in hot water, and liquid samples were diluted with phosphoric acid. The extracts were passed in a BakerBond SFE cartridge. The eluate (with ammonium–MeOH–15% NaCl solution) was analyzed by HPLC-UV (275 nm) on a Cosmosil 5C 18-AR column, with ACN–phosphate buffer eluent. The DL was 10 μg/g.

Nakazato et al. (284) determined the CF and geniposidic acid content in leaves of *Eucommia ulmoides* used in health foods and beverages. The samples (dried leaves or granules) were refluxed with water, filtered, acidified, and cleaned up on a Mega Bond Elut C18 cartridge linked to an Elut SAX cartridge. The eluate (with MeOH–Tris hydrochloride buffer) was applied to a Inertsil ODS-2 column at 40°C and eluted with an ACN–phosphate solution mixture. Detection was performed at 240 nm, reaching a DL of 10 μg/g.

Yang et al. (285) determined CF, TB, and TP in tea, together with vitamin C. The water solution of boiled tea leaves was treated with 0.5 M saturated lead acetate solution and 0.01 M HCl. The precipitate was filtered and removed. The solution was applied to a Hypersil ODS column with the temperature set at 40°C, and detection was performed at 230 nm.

Table 5 Methylxanthines

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
CF together with food additives	Capillary zone electrophoresis (CZE) and HPLC with UV detection at 214 nm.	*CZE*: fused-silica capillary (60 cm × 75 mm) column. Mobile phase: sodium phosphate buffer, pH 11. Running voltage 15 kV. *HPLC*: NovaPak C18 column and mobile phase of phosphate buffer and ACN.	0.1–2.0 mg/L	Foodstuffs and beverages	*Beverages*: sonication and water-dilution. *Powders*: water solution and 0.2-μm filtration.	294
CF, TB, TP	Semimicro HPLC-UV at 207 nm.	Column: Inertsil ODS-2, $T = 40°C$. Mobile phase: MeOH, water, phosphate buffer at pH = 3 (12/33/5, v:v:v).	0.1–2.5 mg/ml	Foods	Extraction of pulverized samples with water and EtOH by sonication. Evaporation to dryness. Dissolution in phosphate buffer. Cleaning up on Sep-Pak C18 cartridge.	270
CF and additives	HPLC-UV at 230 nm.	Column: μ-Bondapak C18. Mobile phase: 20 mM ammonium acetate and MeOH.	10 ng	Beverages	NH_3 addition and filtration.	295
CF, TB, TP	HPLC-UV at 274 nm.	Column: LiChrospher 100 RP-18. Mobile phase: 5.0 mM octylamine phosphate, pH = 6.4.	CF 0.4 mg/L, TB 0.15 mg/L, TP 0.3 mg/L	Found amount (mg/L): *CF* Decaffeinated tea 6 Decaffeinated coffee 137 Diet cola 158 Tea 433 Espresso coffee 1760 *TB* Diet cola 1.7 Other beverages 17–26	Water dilution and 0.2-μm filtration.	280

CF	HPLC-UV at 254 nm.	Column: Ultrasphere ODS. Mobile phase: H_2O–MeOH mixture.	0.02 mg/ml	Beverages	Addition of benzylalcohol in H_2O–MeOH.	296
CF, flavonols, theogallin, gallic acid	HPLC method.	Column: Hypersil ODS with a Nucleosil C18 guard-column. Mobile phase: H_2O, acetic acid and ACN in gradient elution.	—	Tea	Boiling in H_2O, filtration, addition of ACN or MeOH at $T = 40°C$. Cleaning up on RP-18 cartridges and elution with ACN.	297
CF	RP-anion-exchange HPLC with UV, electrochemical, and fluorescence detection.	—	—	Plants and plant extracts	—	298
CF, colors preservatives, sweeteners	HPLC method.	Column: Altex Ultrasphere-TM-ODS. Mobile phase: ammonium acetate–MeOH.	—	Beverages and foods	—	300
CF	High-performance gel filtration chromatography with UV at 280 nm.	Column: TSK-G 3000 SW with a LKB guard column. Mobile phase: 0.05% NaN_3 in water.	30 μg/ml	Green and roasted coffee, instant coffee	Coffee beans extracted with H_2O, $T = 80°C$. The extracts as well as the instant coffee samples filtered, cleared (Carrez solution), centrifuged. The supernatant dissolved in H_2O.	299
CF, TB, TP	HPLC-UV at 272 nm and at 248 nm for TB (1st derivative), at 278 nm for TP (2nd derivative), and at 275 nm for CF (zero order).	Column: C-8 Lichrocart. Mobile phase: 0.01 M sodium acetate, ACN, acetic acid pH = 4.0.	5 μg/ml	Tea, coffee, cream chocolate	MeOH extraction.	268

(continued)

Table 5 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
CF, TP, sweeteners, and additives	HPLC-UV.	Column: Superspher RP-Select B. Mobile phase: KH_2PO_4-ACN (pH = 4.2–4.4 for sorbic and benzoic acids).	4 mg/L	Lemonades, fruit juices, dairy products, yogurt, salad dressing	Dilution, addition of $K_4Fe(CN)_6$ and $ZnSO_4$, filtration for beverages and milk shakes. For dessert, powders, yogurts, salad dressing: digestion with water, extraction on C_{18} cartridge, and elution with KH_2PO_4-ACN mixture.	301
CF	Hydrophobic HPLC-UV at 214 nm.	Column: Fractogel TSK HW-40, $T = 35°$ and 40°C. Mobile phase: KH_2PO_4, Na_2HPO_4 in different ratios and pH = 4.3 or 6.9.	CF: 3 ng/ml	Cola beverages	—	302
CF, TB, TP, preservatives, and antioxidants	HPLC-UV.	Column: LiChrospher 60 RP-Select B.	—	Coffee, tea, cocoa products	Alkaline treatment with MgO. Extraction in acetate buffer solution and oxalic acid, EtOH, propan-2-ol, and ACN.	304
CF	GLC with FID detection and HPLC-UV at 274 nm.	*GC*: 3% OV 17 Chromosorb WHP column, T program from 120° to 220°C, N_2 as carrier gas. *HPLC*: μ-Bondapak C_{18} column. Mobile phase: H_2O–ACN–acetic acid in gradient elution.	0.2 mg (GLC); 0.3 mg (HPLC)	Coffee tablets and beverages, chocolate, coffee	Water and NH_3 addition, Soxhlet extraction with $CHCl_3$ and isopropane, filtration, evaporation, dilu tion with $CHCl_3$. For HPLC analysis distillation and dissolution in the mobile phase.	303
CF, water-soluble vitamins and sodium benzoate	HPLC-UV at 210 nm.	Column: Nucleosil C18, $T = 40°C$. Mobile phase: H_2O, ACN, triethylamine, heptanesulfonate (pH = 2.8).	60 μg/ml	Beverages	Addition of acetanilide (IS) and dilution with the mo bile phase.	305
CF	HPLC-UV at 275 nm.	LiChrosorb Si 60 column. Mobile phase: water, CH_2Cl_2, EtOH.	1 μg/ml	Coffee, black tea, green tea	Water extraction and cleaning up on Extrelut column.	306

CF	Ion-pair HPLC-UV at 280 nm.	Conventional and micro-bore C18 column. Mobile phase: MeOH and TMA-Br in different ratios.	—	Coffee-flavored foods, low-calorie products, coffee-cream–filled pralines, wafers	—	307
CF, food additives	RP-HPLC-UV at 254 nm.	Column: μBondapak C18. Mobile phase: MeOH–acetic acid–H_2O–acetate buffer in different ratios.	10 ng	Ready-to-serve beverages, ice candy, ice cream, fruit squash, tomato sauce	Direct analysis after water extraction.	308
CF	HPLC-UV at 270 nm.	μBondapak C18 column (with a precolumn of PVP). Mobile phase: 30% MeOH (pH = 3.5).	—	Tea extracts	—	309
CF, TB, TP	HPLC-UV at 273 nm.	Spherisorb 800 or LiChrosorb RP-18 as the stationary phase. Mobile phase: MeOH, CH_2Cl_2, 0.01 M acetate, ACN.	—	Coffee, tea, mate, cola, and nonalcoholic beverages	Aqueous, NH_3 addition, and $CHCl_3$ extraction.	283
CF	HPLC-UV at 214 nm and 254 nm.	Column packed with Cyclobond I, β-cyclodextrin bonded to irregular silica gel. Mobile phase: MeOH–1% TEA acetate, pH = 4.5.	—	Nonalcoholic beverages, sweetener Equal, diet Pepsi, diet 7-Up, diet soft drinks, diet Coke	—	310
Separation of CF from trigonelline	HPLC-UV at 264 nm and TLC-UV at 254 nm.	*HPLC*: Baker octadecyl column and elution with $CHCl_3$ TLC on Silica gel GF254, with aqueous 92% MeOH as the mobile phase.	—	Coffee	Addition of MgO and water, HCl extraction, filtration, dilution.	311
CF, TB, TP	HPLC-UV at 280 nm.	Column: Spherisorb ODS and RSil C18 HL precolumn. Mobile phase: 0.02 M NaH_2PO_4 (adjusted to pH = 7 with NaOH) in MeOH.	—	Guarana (*Paulinia cupana* var. sorbilis) seeds. Found amounts: CF 3.6–5.8%, TB 0.03–0.17%, and TP 0.02–0.06%.	Seeds are air-dried, roasted, and ground. Boiling-water extraction under reflux, filtration, dilution.	312
CF	HPLC-UV at 214 nm.	Column: Partisil-10SCX. Mobile phase: containing 0.1 M $(NH_4)H_2PO_4$.	50 ng	Soft drinks	Decarbonation, filtration.	317

(continued)

Table 5 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
CF and additives	HPLC-UV at 214 nm.	Column: μBondapak C18. Mobile phase: H_3PO_4, triethylamine, water, pH = 4.3.	0.05 mg/ml	Cola beverages	Water dilution and degassing.	318
CF	SFC directly coupled with supercritical fluid extraction and HPLC-UV (272 nm).	SIL C18 column. Mobile phase: H_2O-MeOH.	—	Roasted coffee beans	Grounding, H_2O addition, extraction on cartridge. SFC (T = 48°C) extract with CO_2 extract trapped on activated charcoal cartridge. Elution with MeOH.	314
CF	HPLC-UV and GC with FID and MS detection.	*HPLC*: column Nucleosil C-18; mobile phase of 10% MeOH (pH = 3.2). *GC*: column 1% of OV-17 on Supelcoport.	—	Orange, citrus, lemon. No CF found in orange juice.	Extraction of frozen samples in 80% EtOH, filtration, adjustment to pH = 2.0, centrifugation. Adjustment of the supernatant solution to pH = 9.0, BuOH extraction, azeotropic distillation of the combined butanolic phases.	313
CF, TB, TP	HPLC-UV at 280 nm.	Column: FLC-ODS. Mobile phase: water and acetonitrile (1:15) mixture.	—	Tea, coffee, cocoa, chocolate	Extraction with H_2O and $CHCl_3$ from tea and coffee and with CCl_4 from cocoa and chocolate.	315
CF, TB, TP	Microbore HPLC-UV at 280 nm.	Column: Whatman Micro-B of ODS-3. Mobile phase: H_2O–MeOH–acetic acid.	CF 150 pg/ml, TB 100 pg/ml, TP 100 pg/ml	Cocoa	Aqueous extraction.	261
CF	HPLC-UV at 254 nm.	Column: RP MCH-10. Mobile phase: acetic acid 1% and ACN (4:1).	50 mg/ml	Tea	Addition of MgO and H_2O, boiling 1 h. Cooling, filtration, addition of 8-chlorotheophyllin (IS), dilution.	316
CF, TB, TP	HPLC-UV at 275 nm.	Column: LiChrosorb RP-8. Mobile phase: H_2O-MeOH, phosphate buffer 0.2 M, pH = 5.0 (36:9:5, v/v/v) at T = 45°C.	CF 10 μg/g; TB, TP 5 μg/g	Cocoa, chocolate, coffee, tea, cola beverage	Cleaning up on a Sep Pak C-18 cartridge.	260
CF	HPLC-UV at 254 nm.	Column: μBondapak C-18 equipped with a Bondapak C18/Corasil guard column. Mobile phase: acetic acid, H_2O, propan-2-ol (10:87:3, v/v/v).	—	Beverages	Water dissolution, 0.45-μm filtration.	319

Maier et al. (286) compared four methods for the analysis of CF. The DIN 10777 Part 2 (1986) involves: extraction from dilute NH_3 solution, purification of the extract on two silica gel columns (one containing NaOH to retain impurities and the other containing H_2SO_4) retaining CF, elution with CH_2Cl_2, and UV determination at 276 nm. The ISO 10095 (1992), regarded as the reference method, uses extraction with hot water in the presence of MgO, purification of the extract on a phenyl cartridge, and HPLC analysis on a C_{18} column at 40°C, with a H_2O-CH_3OH (30:70, v/v) mobile phase and detection at 280 nm. The revised DIN 10777 Part 2 (1994) is similar to ISO 10095, but with modifications to reduce the time taken. The rapid method of Stennert and Maier (287) was a further simplification of the revised DIN method. The UV and the three HPLC methods were compared for precision, recovery, and DL. The best overall results were given by the DIN 10777 Part 2 (1994) method. The samples considered were two commercial roasted coffees, one with caffeine and the other decaffeinated, and two variety of beans, Arabica and Robusta, roasted and ground.

Shi et al. (288) determined CF in soft drinks, coffee, and tea by HPLC-UV (260 nm). The eluent was a CH_3OH–phosphate buffer at 35°C and the column was a Spherisorb C18. The DL was at the level of micrograms per liter.

According to the British Standard, BS 7820: Part 1: 1995 [ISO 10727:1995] (289), ground tea or black tea or instant tea of known dry matter is mixed with MgO and water and heated to 90°C. The resulting mixture is cooled and filtered. The filtrate is diluted and analyzed by HPLC on a C18 column, with detection at 254–280 nm. For a Partisphere C_{18} column, a mobile phase of CH_3OH/H_2O (3:7, v/v) at 40°C is suitable.

VII. ALKALOIDS

A. General Information

The term *alkaloids* can be considered as more pharmaceutical and medical than chemical, since alkaloids come from a variety of otherwise-unrelated organic compounds. Alkaloids can be classified in terms of their biological activity, chemical structure (nucleus containing nitrogen), or biosynthetic pathway (the way they are produced in the plant).

In fact they constitute a heterogeneous group of compounds containing nitrogen in a heterocyclic ring reacting with acids to form salts. They are characterized by biological activity in low doses, and their bitter taste is often accompanied by some toxicity. They are normally white solids (nicotine, a brown liquid, is an exception) characterized by an intense bitterness and giving a precipitate with heavy metal iodides. They are secondary plant metabolites, common to about 15–20% of all vascular plants, and in plants they may exist in the free state, as salts or as *N*-oxides. The basic units in the plant biogenesis of the true alkaloids are amino acids: AA are decarboxylated to amines and then react with amine oxides to form aldehydes; Mannich-type condensation of the aldehyde and amine groups creates their characteristic heterocyclic ring. The non-nitrogen-containing rings or side chains are derived from terpene units and/or acetate, while methionine is responsible for the addition of methyl groups to nitrogen atoms.

B. Analysis

Current multiresidue methods for alkaloids typically involve extraction with a polar solvent such as ethanol, evaporation to remove the solvent, and a series of acid/base liquid–liquid partition steps for cleanup. An alternative method is extraction with aqueous acid followed by alkalinization and extraction into organic solvent or by the use of SPE columns. Direct solvent extraction

has also been used for rapid toxicological screening. After the extraction alkaloids are separated and detected by TLC, GC/NPD, GC/MS, or HPLC (316–318) and ion-pair HPLC (319,320).

VIII. SOLANUM-TYPE GLYCOALKALOIDS

A. General Information

Glycosides are ethers that join a noncarbohydrate moiety, the aglycone, by a ester bond to a carbohydrate moiety. In solanum-type glycoalkaloids, the aglycone is a C_{27}-steroid base. Steroid alkaloids have a fairly complex nitrogen-containing nucleus. The two most important classes of steroid alkaloids are the *Solanum* and the *Veratrum* types. One example of a *Solanum* alkaloid is the solanidine (derived from cholestane) that is the aglycone yielding (with two different trisaccharides) two important glycoalkaloids: solanine and chaconine (Fig. 7A, B).

Glycoalkaloids are natural toxins, occurring in all parts of plants of the *Solanum* species, comprising potatoes, tomatoes, various nightshades, and Jerusalem cherries. They were widely introduced to the world diet about 400 years ago with the dissemination of the potato from the Andes. They are toxic to humans; the lethal dose is considered to be 3–6 mg per kg body mass. The glycoalkaloids influence the quality of the potatoes due to their toxicity and cause a bitter taste above 14 mg/100 g and a burning sensation to mouth and throat above 20 mg/100 g. Many factors influence the production of glycoalkaloids in potatoes, for example, storage, damage, greening. Production of *Solanum*-type glycoalkaloids is favored by the same conditions that promote the development of chlorophyll (i.e., the concentration is highest in potato sprouts and green potato skins and in tomato vines and green tomatoes). These alkaloids are not destroyed by cooking or drying at high temperatures.

The glycoalkaloids are more poisonous than the steroid alkaloid aglycones. Humans and all classes of livestock are susceptible to poisoning by *Solanum*-type glycoalkaloids. Luckily, glycoalkaloids are poorly absorbed in the gastrointestinal tract of mammals, since an appreciable amount of *Solanum*-type glycoalkaloids are hydrolyzed in the gut of mammals to the less toxic aglycones, metabolites that are rapidly excreted. Because exposure to these poisons is generally by ingestion, it takes a relatively large amount to cause death (lethal dose = 3–6 mg/kg body mass). In particular, *Solanum*-type glycoalkaloids are gastrointestinal tract irritants and inhibit cholinesterase. By blocking nerve transmission, they affect the nervous system; when consumed in large doses convulsions, paralysis, coma, and death due to respiratory paralysis may occur. Total toxins are present in normal potatoes at a level of 15 mg per 200 g potato (75 ppm), which is less than a tenfold safety margin from the measurably toxic, daily dose level for humans.

Tomatine, belonging to the group of solanum steroid alkaloids and having tomatidine as aglycone, is an antifungal steroidlike molecule that is one of the natural toxins in tomatoes. Tomatine is present at 36 mg per 100 g tomato.

The solanum alkaloids are of interest as a starting material for the synthesis of steroid hormones, and show interesting pharmaceutical and fungicidal activity.

B. Analysis

Chromatographic analysis of glycoalkaloids can be performed in a number of ways (321,322). The intact compounds can be analyzed by GC after derivatization (323). After hydrolysis the aglycone skeleton can be examined by GC without the need of derivatization (324). For routine determinations of the glycoalkaloids present in potato tubers, HPLC is probably the method of choice. Column "acidity" caused by active silanol sites on the packing surface strongly influences the chromatographic separation of *Solanum* alkaloids. In fact, basic compounds react with

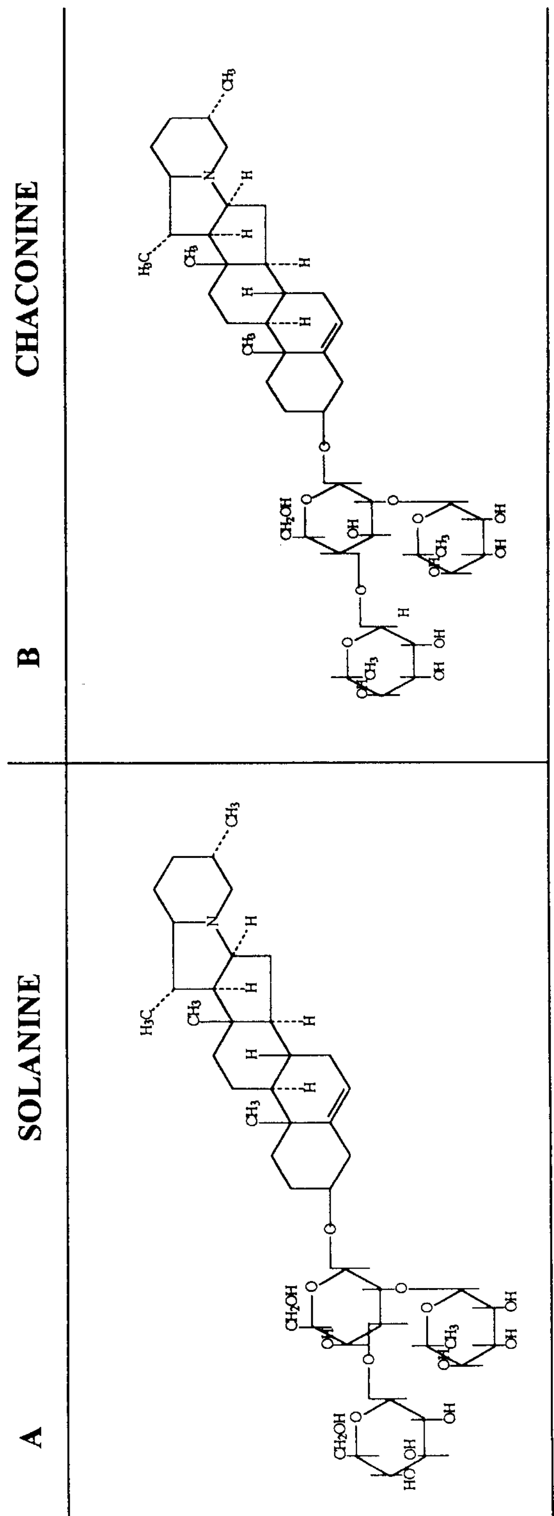

Fig. 7 Structure of the glykoalkaloids solanine and chaconine (aglycone: solanidine).

silanols in a cation-exchange mode, creating a second retention mechanism to the primary hydrophobic mode. The mixed-mode retention frequently causes tailing and excessive elution time with basic samples. Friedman and Levin (326) carried out a comparative study of the behavior of ten stationary phases in the analysis of these compounds (326) (Fig. 8). The methods commonly use eluents containing a relatively high amount of buffer (325–327) or employ a less acidic column with a reduced amount of silanol groups (328).

Analytical methods concerning glycoalkaloid analysis are summarized in Table 6.

IX. CAPSAICINOIDS

A. General Information

The capsaicinoids are cytotoxic alkaloids from various species of *Capsicum* (pepper, paprika) of the Solanaceae. They cause pain, irritation, and inflammation due to substance depletion from sensory (afferent) nerve fibers. Compared to other spicy substances, such as mustard oil (zingerone and allyl isothiocyanate), black pepper (piperine), and ginger (gingerol), they are unique, in that capsaicinoids cause a long-lasting selective desensitization to the irritant pain, as a result of repeated doses of a low concentration or a single high-concentration dose. The result is an increasing ability to tolerate hotter foods.

Pure capsaicin (C), the predominant capsaicinoid, also known as *N*-vanillyl-8-methyl-6-(*E*)-noneamide (Fig. 9A), is a whitish powder soluble in alcohol and fats but insoluble in cold water, which is why drinking water to alleviate the burning does not work. Capsaicin is the most pungent of the group of capsaicinoids isolated from peppers. It has been found to work as an anticoagulant, thus possibly helping prevent heart attacks or strokes caused by blood clot. Small amounts of C can produce numbing of the skin and have a slight anti-inflammatory effect.

The second most common capsaicinoid is dihydrocapsaicin (DC). Capsaicin and DC together make up 80–90% of the capsaicinoids found in the fruit. The minor capsaicinoids include nordihydrocapsaicin (NDC) [dihydrocapsaicin with $(CH_2)_5$ instead of $(CH_2)_6$], homocapsaicin (HC) [capsaicin with $(CH_2)_5$ instead of $(CH_2)_4$], and homodihydrocapsaicin (HDC) [dihydrocapsaicin with $(CH_2)_7$ instead of $(CH_2)_6$]. The pungencies of these five pure compounds in Scoville Units (SU) are as follows:

Compound	Pungency (× 100,000 SU)
Capsaicin (C)	160
Dihydrocapsaicin (DC)	160
Nordihydrocapsaicin (NDC)	91
Homocapsaicin (HC)	86
Homodihydrocapsaicin (HDC)	86

B. Analysis

Since the effect they produce is actually an irritation to the nerve endings responsible for heat and pain sensation in the mouth, standardization of the level of heat is essential. Methods for their analysis include organoleptic and spectrophotometric methods. Historically the organoleptic one has been preferred by the food industry since it is a direct measure of levels of heat. Nowadays a variety of GC (after derivatization) and HPLC with UV or fluorescence detection methods have been proposed.

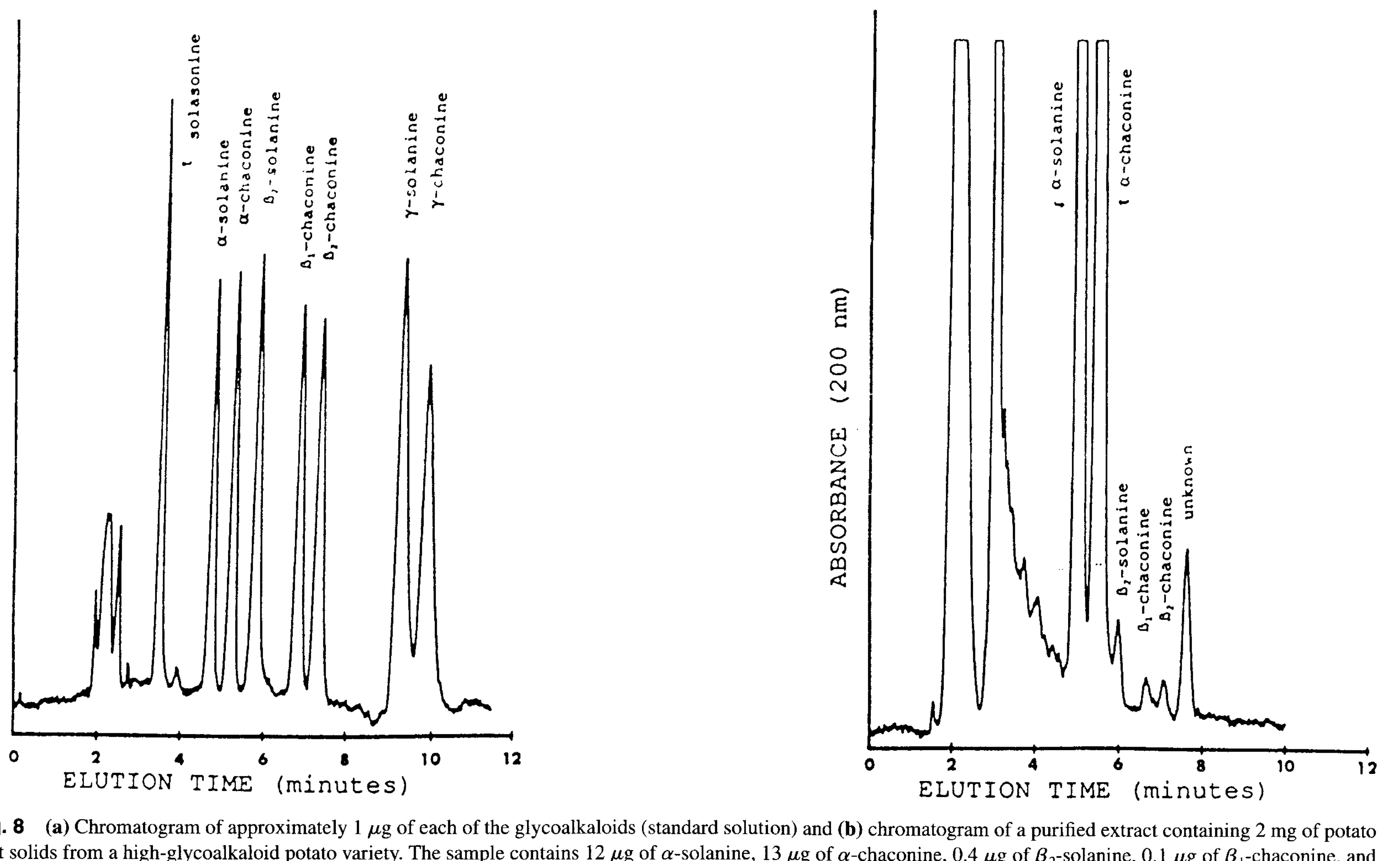

Fig. 8 (**a**) Chromatogram of approximately 1 μg of each of the glycoalkaloids (standard solution) and (**b**) chromatogram of a purified extract containing 2 mg of potato root solids from a high-glycoalkaloid potato variety. The sample contains 12 μg of α-solanine, 13 μg of α-chaconine, 0.4 μg of β_2-solanine, 0.1 μg of β_1-chaconine, and 0.2 μg of β_2-chaconine. (From Ref. 326.)

Table 6 Solanum Alkaloids

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
α-Chaconine, α-solanine	RP-HPLC-UV at 200 nm and TLC.	C_{18} Perkin Elmer coupled cartridge. 5 mM sodium lauryl sulfate, 5 mM sodium sulfate decahydrate ACN–H_2O (50:50 v/v), pH 4.5 H_2SO_4.	—	Found amounts (sum of two alkaloids, mg/100 g): Freeze-dried french potatoes 4.4 Skins 3.1–20.3 Potato chips 2.5–11 Potato pancake powder 4.5–6.5	Extraction (adapted from Bushway 1986: (369)) freeze-dried sample powder stirred in THF-H_2O-ACN. Extract centrifuged, supernatant collected and concentrated in a rotary evaporator, with added HCl, suspension centrifuged and pH adjusted with NH_4OH to pH 10–11. Water bath at 70°C, 30 min. The precipitate collected and boiled in MeOH. Hot suspension filtered and evaporated. ACN-H_2O added.	321
α-Solanine, α-chaconine, β_2-solanine, β_1-chaconine, β_2-chaconine	RP-HPLC-UV at 200 nm.	RP C_{18} column. Mobile phase: ACN and ammonium phosphate pH = 3.5 in different ratios.	—	Potatoes, found amounts (μg/mg): α-solanine 6.0 α-chaconine 6.5 β_2-solanine 0.2 β_1-chaconine 0.05 β_2-chaconine 0.1	THF, H_2O, ACN, acetic acid addition. Centrifugation, filtration, evaporation to 4 ml, centrifugation, heptanesulfonic acid washing, filtration. Cleaning up with SPE.	325
α-Chaconine, α-solanine	HPLC-UV at 208 nm.	Nucleosil 5-NH_2. Mobile phase: ACN–20 mM $KH_2(PO_4)_3$ (75:25, v/v).	—	Potato products and potato starch.	Extraction in MeOH, purification on Sep-Pak C18 or Sep-Pak NH_2 cartridges.	327
α-Chaconine, α-solanine	HPLC-UV at 202 nm.	RoSil C8 column with mobile phase: ACN/diammonium phosphate buffer or Nucleosil 5 C18 with mobile phase: ACN/H_2O (60:40, v/v).	—	Potato tubers; found: 72 μg/mg of α-solanine and 46 μg/mg of α-chaconine.	Sodium 1-heptanesulfonate and acetic acid extraction, filtration. Elution through a C18 SepPak cartridge, conditioned with MeOH, sodium 1-heptanesulfonate. Elution with water-ACN mixture to remove interferents and glycoalkaloids elution with ACN and diammonium phosphate buffer.	328
α-Chaconine, α-solanine	HPLC-UV at 215 nm.	Carbohydrate column. Mobile phase: THF–H_2O–ACN (50:30:20, v/v/v).	0.4 μg/ml	Potato tubers; amount found: α-chaconine 4–23 mg/100 g, α-solanine 2–13 mg/100g.	Addition of MeOH, $CHCl_3$. Filtration and concentration. HCl addition, sonication, centrifugation, addition to the supernatant of concentrated NH_4OH. 70°C H_2O bath (30 min) and refrigeration overnight. Dissolution in THF, H_2O, ACN.	329

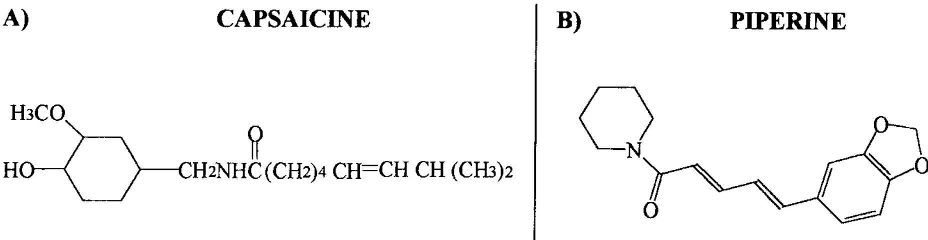

Fig. 9 Molecular structures of capsaicine and piperine.

Peusch et al. (330) developed a method for the determination of C, DHC, and NC by RP-HPLC and fluorimetric detection at 320 nm (λ_{ex} = 280 nm), using as external standard C or nonanoic acid vanillylamide. The method was applied to low-pungency paprika and oleoresins.

The British Standard method (331) of test for spices and condiments gives a procedure for the determination of total capsaicinoid content of chilies and chili oleoresins by HPLC. The powdered or ground sample is mixed with an equal volume of acid-washed sand and Soxhlet extracted for 6–8 h with MeOH. The filtered extract, diluted with MeOH, is analyzed by HPLC on a C18 column, with an ACN–H_2O–glacial acetic acid mobile phase and detection at 280 nm. Oleoresins are diluted with MeOH and analyzed in a similar manner.

Sakamoto et al. (332) applied an HPLC method to analyze capsaicinoids and their phenolic intermediates in *Capsicum annuum* and to characterize their biosynthetic status. Descended fruit is extracted with 80% EtOH, the extract is evaporated to dryness, and the residue is dissolved in DMSO and analyzed on an Inertsil ODS-2 column with gradient elution (ACN–aq. TFA) and detection at 280 nm. The method was used to study changes in capsaicin content during ripening.

X. PIPERINE

A. General Information

Commercially available black pepper comes from the entire unripe fruit of a *Piper nigrum* L. plant and is valued for its pungent taste and aroma. Besides its "hot" flavor, there are many folk medicine remedies using black pepper. It has antiallergic activity. Piperine strongly inhibits hepatic arylhydrocarbonhydroxylase and UDP-glucuronyl transferase activities. Some proven uses for black pepper extracts are as an insecticide and as a tumor inhibitor. To research claims such as these, it is necessary to isolate the components of pepper.

Black pepper is made of several substances, among them 5–9% is the alkaloid trans,trans-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid, otherwise known as piperine (Fig. 9B). Piperine, together with other closely related nitrogen-containing compounds, such as piperidine, chavicine, and piperettine, are responsible for the sharp, biting taste and pungency. In addition, pigments, resins, sugars, and fixed oils may also be found in the nonvolatile ether extract.

Piperine in solution is rapidly isomerized by exposure to light to iso-piperine, chavicine and iso-chavicine. These compounds, which may occur in low concentrations in pepper, have little taste, so any assessment of the quality of pepper, based on the determination of the piperine content, must be capable of separating piperine from the related isomers.

B. Analysis

Since piperine (Pi) is universally accepted as the predominant pungent principle in pepper, the quality of pepper and oleoresin is dependent largely on its content, and so methods for estimat-

ing piperine are required. Methods include: Kjeldahl nitrogen determination; adaptation of the chromotrophic acid test for formaldehyde; colorimetric methods using nitric acid, sulfuric acid, aromatic aldehyde, and phosphoric acid and based on alkaline hydrolysis; reaction with *p*-nitrophenyl diazonium fluoborate; volumetric analysis; and spectrophotometric analysis.

Since the 1970s, HPLC methods for the quantitative determination of piperine have been developed (333–335), and they are now in the most common use for their sensitivity, rapidity, and specificity (336–352).

Table 7 lists the methods present in the literature for the determination of the alkaloids from *Capsicum* and pepper.

XI. *CHINCONA* ALKALOIDS

A. General Information

The principal *Chincona* alkaloids, quinine (Qn), quinidine (Qd), cinchonine (Cn), and cinchonidine (Cd), occur naturally in the bark of the cinchona tree, which is indigenous to the high eastern slopes of the Andes. In 1639, the bark extract of the cinchona tree was shown to be effective against malaria, a result that led the Dutch to cultivate these trees extensively in the Dutch East Indies. In 1820, pure quinine was isolated for the first time, and the elucidation of its chemical structure became a classic problem of the last quarter of the 19th century. The *Chincona* alkaloids are used in the pharmaceutical, food, and drink industries. The spread of resistance to the synthetic drug chloroquine in the parasite *Plasmodium falciparum* has re-established Qn as an antimalarial drug (Fig. 10). Quinidine is widely used as an antiarrhytmic drug; Qn is a major source of bitter flavoring in the food industry. The FDA limits the addition of Qn to 83 mg/L.

B. Analysis

The quantitative analysis of Chincona bark by the classical methods of titrimetry, gravimetry, and polarimetry has been performed for many years in order to ascertain its commercial value. These compounds have been analyzed by GC, GC-MS, TLC, and mainly HPLC (353–361) with UV or electrochemical detection. Ion-pair HPLC was also used with UV or fluorescence detection by Jeuring et al. (357). Photoreactions of Qn in aqueous citric acid solution have been studied by Laurie et al. (358). After isolation of the components by HPLC and TLC, different spectroscopic techniques (MS, NMR, IR) were used to identify the photoproducts.

There are no very recent papers dealing with *Chincona* alkaloid determination in the literature. Table 8 reports some of the most significant works in this field.

XII. ERGOT ALKALOIDS

A. General Information

Ergot is the sclerotium (the form the plant assumes to pass the winter) of the fungus *Claviceps purpurea*, which is parasitic on cereals. Rye grass is by far the most widespread species parasitized, though wheat, barley, and other cultivated grains and wild grasses can also be affected.

Ergot naturally produces a wide range of chemical compounds, collectively known as the *ergot alkaloids* and including ergotamine, ergosine and β-ergosine, ergonine, ergovaline, ergostine, ergotine and β-ergotine, ergocornine, ergocristine, and ergocryptine and β-ergocryptine. These compounds all have some degree of toxicity and psychoactivity. Epidemics of ergot poisoning have occurred, and still occur, when contaminated grain is used for food preparation (these

Table 7 Alkaloids of Spicy Foods

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Piperine	RP-HPLC-UV at 345 nm. Phenazine as the IS.	Column: C_8 (250 × 7.0 mm, 5 μm). Mobile phase: H_2O-ACN-THF (63:30:7, v/v/v), flow 2.0 ml/min.	—	Found amounts (%): White pepper 2.20–4.35, black pepper 2.10–5.03	EtOH addition, boiling for 45 min.	336
NDC, C, DHC, and minor capsaicinoids	HPLC with UV (280 nm) and fluorimetric detection (λ_{ex} 220 nm, λ_{em} 320 nm). DMBMO (dimethoxybenzyl-methyloctamide) as IS.	Column: Supelco C18 stainless steel column, (250 × 4.6 mm, 5 μm). Mobile phase: MeOH-H_2O (3:2, v/v).	—	Oleoresin and hot sauces	*Oleoresin*: MeOH addition, shaking, centrifugation, and injection of the filtered supernatant. *Tincture*: MeOH addition, filtration. SPE on silica gel activated with hexane and elution with MeOH.	337
Capsaicinoids, gingerols	RP-HPLC-UV at 280 nm. *N*-(4-hydroxy-3-methoxy benzyl) octanamide as ES.	Column: Spherisorb ODS-2. Mobile phase: ACN/H_2O 1% acetic acid (1:1, v/v), for determination of capsaicinoids; (13:1, v/v) for determination of gingerols).	—	Chilies and ginger	Ground dried chilies or dried ginger: Soxhlet extraction with MeOH, concentration, filtration. Dissolution: *Capsicum* oleoresin in MeOH-THF; ginger oleoresin in MeOH.	338
Vanillyloctanamide, vanillyldecanamide, NDC, DHC, C, HC, HDC	HPLC-UV at 280 nm.	Column: C18 Rad-Pak column, 5 μm. Mobile phase: MeOH 63%, H_2O 37%, flow rate 3.5 ml/min.	—	*Capsicum* fruit and *Capsicum* oleoresins	Ground dehydrated *Capsicum* or fresh *Capsicum*: dissolution in ACN, dilution with distilled H_2O, SPE on C_{18} Sep-Pak cartridge, conditioned with ACN and distilled water. Elution with ACN containing 1% of acetic acid.	339
C, NDH, Pi, DHC	HPLC-UV at 280 nm.	C_{18} stationary phase. Mobile phase: sodium pentansulfonate and acetic acid in MeOH-H_2O.	25 mg/L	Black pepper, *Capsicum* fruit oleoresins. Quality control procedure to determinate SU of chilies and discover if samples have been treated with "synthetic" C (*N*-vanillyl-	Spices are ground to pass through U.S. 20 mesh (oleoresins warmed). Refluxing for 1 h with acetone, then dilution with the mobile phase.	340

(*continued*)

Table 7 Continued

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
				n-nonamide).		
Capsaicinoids	RP-HPLC with UV (280 or 232 nm) and fluorescence detection (λ_{ex} = 280 nm, λ_{em} = 360 nm).	MikroPak MCH-10 column. Mobile phase: MeOH-H_2O containing acetic acid and $AgNO_3$.	—	—	Extraction from ground pepper by soxhleting with CH_2Cl_2.	341
Piperine	HPLC-UV detection at 343 nm.	C_{18} stationary phase. Mobile phase: H_2O-MeOH (23:77, v/v).	—	*Piper nigrum*	EtOH extraction.	342
C, NDC, DHC	HPLC-UV at 280 nm, *N*-vanillyl-*n*-nonamide as ES.	Column: μBondapack C18, 10 μm. Mobile phase: 1% acetic acid in ACN-H_2O (60:40, v/v).	C 0.5 mg, DHC 0.5 mg, NDC 0.6 mg	Ground red pepper: NDC 0.004–0.030% C 0.047–0.277% DHC 0.017–0.152%	25 g of ground red pepper: extraction by heating at 65–75°C for 5 h in 200 ml of MeOH 95%. Filtered surnatant is injected on HPLC.	343
C, DC, NC, DHC, HC	HPLC with UV (at 280 nm) and fluorimetric detection with λ_{ex} at 280 nm, λ_{em} at 316 nm.	Column: 5-μm Ultrasphere ODS. Mobile phase: 0.05 M $AgNO_3$ in MeOH-H_2O (60:40, v/v), pH 2.5 with acetic acid.	500 μg/L	Animal feed, oleoresins; found: 2.27% as total capsaicinoids	*Oleoresins*: sample dissolved in hexane, LLE with ACN, evaporation under vacuum at 30°C, dissolution in MeOH. *Animal feed*: Multistep LLE with MeOH/0.1 N HCl (8:1), CH_2Cl_2, benzene, acetone, MeOH, H_2O.	344
C, DHC	RP-HPLC-UV at 280 nm.	RP-C_{18} column, gradient elution (MeOH-H_2O).	—	—	Extraction of the capsaicinoids from *Capsicum* juices by lypophilization and subsequent LLE by three different solvents.	345
Capsaicinoids, piperine	HPLC-MS and field desorption MS.	—	—	Identification of components of *Capsicum* oleoresins and black	—	346

				pepper.		
Piperine, chavicine, iso-chavicine, isopiperine	Microscale NP-LC-UV at 252 nm (anthraquinone as the IS).	Column: polyphenol RSil 10 μm, 250 × 0.32 mm. Mobile phase: hexane-THF (40:60, v/v) at $T = 50$°C.	—	Ground pepper; found: 3–5% w/w of piperine in black and white pepper; 0% in cayenne pepper; 25–40% w/w in pepper extracts.	Samples, added at mobile phase, are stirred for 30 min in a vessel protected from light, and the supernatant is injected.	347
Pi, piperettine, piperlongumine, piperyline, piperanine, piperettyline	HPLC-UV at 340 nm.	Column: C_{18} Altex 5 μm (250 × 4.6 mm). Mobile phase: 0.19% Na pentane sulfonate solution in MeOH–H_2O–acetic acid (67:32:1, v/v/v).	170 mg/L	*Piper nigrum*; found: (% dry basis): 3.05–5.00 of piperine, 0.21–0.64 of piperettine.	Ground pepper heating and acetone refluxing, dilution with aqueous MeOH.	348
Piperine	RP-HPLC with UV dual-wavelength detection at 280 and 343 nm or 254 and 364 nm, *N*-(4-hydroxy-3-methoxybenzyl)octanamide as the IS.	Column: Spherisorb ODS2, 5 μm (250 × 4.6 mm). Mobile phase: aqueous 1% acetic acid/ACN (1:1) at 1.5 ml/min.	—	Pepper and pepper oleoresins	EtOH extraction.	349
Capsaicins and piperine	HPLC with UV and electrochemical detection.	Column: Altex Ultrasphere ODS or Varian MCH-5. Mobile phase: 45% ACN in 0.3% phosphate buffer at pH 4.0.	C 0.06 mg/L	Spices and spicy food	MeOH extraction, filtration.	350
Piperine	Spectrophotometry at 345 nm and HPLC-UV at 280 or 345 nm.	Column: μBondapak CN (300 × 3.9 mm). Mobile phase: H_2O-MeOH (1:1).	— —	Found amounts of Pi: black pepper 3.24–5.05%, green pepper 48.3–55.2%, white pepper 41.4–47.2%.	Ground pepper is extracted in Soxhlet apparatus with diethylether for 20 h, dried under vacuum, and redissolved in MeOH.	351
Piperine	HPLC.	—		Pepper and pepper extracts	—	352

Table 8 *Cinchona* Alkaloids

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Qn, Qd, Cn, Cd, DQd, DQn, DCn, DCd, quinamine, cinchonamine, corynantheal	HPLC-UV at 220 nm.	Column: LichroSorb RP-8 Select B. Mobile phase: 15% ACN in phosphate buffer, pH = 3.0.	— — —	Bark of *Cinchona* species; found amounts (% of original dry sample): Cn 0.25%, Cd 1.23%, Qd 0.34%, Qn 1.20%	Bark samples dried at 80°C to constant mass. Bark grounded is mixed with $Ca(OH)_2$ and 5% NaOH for 90 min, then extracted in Soxhlet apparatus for 7 h with toluene. Extract was evaporated to dryness under a stream of He at room temperature and diluted with mobile phase.	359
Cinchona alkaloids	TSP-LC-MS.	—		Plant cell cultures	—	360
Qn, DQn, saccharine, benzoate	RP-HPLC-UV at 254 nm.	Column: μBondapack C_{18}. Mobile phase: CH_3OH–ACN–H_2O–acetic acid (20:10:69:1, v/v/v/v).		Drinks	—	361

[a] DQn = dihydroquinine, DQd = dihydroquinidine, DCn = dihydrocinchonine, DCd = didhydrocinchonidine.

Fig. 10 Molecular structure of quinine.

Fig. 11 Molecular structure of ergotamine.

alkaloids are not broken down by heat). The symptoms include mental disturbance and intensely painful peripheral vasoconstriction leading ultimately to gangrene of the extremities.

Ergotamine (Fig. 11) has medical uses; for example it is frequently prescribed (often in combination with caffeine) as a therapy for migraine headaches.

Ergot alkaloids are molecules based on a complex aromatic acid, and chemically they fall into two major categories, according to whether they possess an amine side chain or an amino acid side chain. Compounds with an amine side chain include lysergic acid diethylamide (LSD), methylsergide, and ergometrine. Compounds with an amino acid side chain include ergotamine, which acts on α-adrenoceptors, dihydroergotamine, and a semisynthetic compound, bromocriptine, which acts selectively on dopamine receptors. Ergometrine, which has a simple aliphatic side chain, acts selectively on the smooth muscle of the uterus. Many ergot alkaloids are agonists or antagonists at 5-HT receptors.

B. Analysis

Current chromatographic methods for these alkaloids are based on TLC or electrophoresis on silica gel plates and RP-HPLC (362–368). The most significative HPLC methods presented in the literature are summarized in Table 9. An example is shown in Fig. 12.

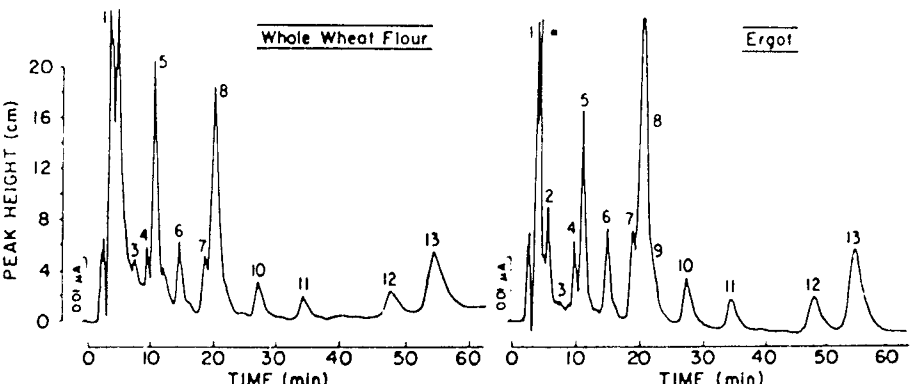

Fig. 12 LC of extract of whole wheat flour (at left, 625 mg equivalent injected) estimated by fluorescence to contain 3.2 μg/kg of ergosine (peak 4), 18.1 μg/kg of ergotamine (peak 5), 6.1 μg/kg of ergocornine (peak 6), 6.2 μg/kg of α-ergokryptine (peak 7), and 25.8 μg/kg of ergocristine (peak 8). At right, extract of wheat ergot with fluorescence detection under the same conditions. Peaks 1, 2, 9, 10, 11, 12, 13 are ergometrine, ergometrinine, ergosinine, ergotaminine, ergocorninine, α-ergokriptinine, ergocristinine, respectively. Peak 3 is unidentified. (From Ref. 362.)

Table 9 Ergot Alkaloids

Analytes	Analytical method	Experimental conditions	DL	Applications	Sample treatment	Ref.
Six ergot alkaloids	LC with fluorimetric detection ($\lambda_{ex} = 235$ nm, $\lambda_{em} = 254$ nm).	Lichrosorb RP-8 5 μm as the stationary phase. Mobile phase: ACN and $(NH_4)_2CO_3$ in different ratios.	0.5 ng, except for ergometrine 0.1 ng.	Found in flour (μg/kg): Ergosine 10.8–14.2 Ergotamine 1.4–36.9 Ergocornine 9.1 α-Ergokryptine 10.3 Ergocristine 2.7–62.2 Ergometrine 0.27–10.4	Extraction with a mixture of CH_2Cl_2, ethylacetate, MeOH, and NH_4OH. Decantation, filtration, evaporation to dryness. Ether–MeOH dissolution, HCl extraction. Acid layers washed with hexane, basified, LLE in CH_2Cl_2. Organic extract evaporated, dissolved in MeOH, and filtered.	362 (see Fig. 12)
Ergot alkaloids	HPLC-UV (at 330, 280, 254 nm), fluorimetric ($\lambda_{em} = 408$ nm, $\lambda_{ex} = 254$ nm), amperometric detection (vitreous carbon electrode at 1.2 V vs. Ag/AgCl ref. electrode).	C_{18} column. Mobile phase: phosphate buffer and 1-octanesulfonic acid.	UV 1–20 ng/ml, fluorescence 5–500 ng/ml; amperometric 5–50 ng/ml.	Orange and vegetable juices, milk, cola beverages	*For orange juices and vegetables*: juice extraction using Analytichem C18 solid phase (SPE). *For milk samples*: protein precipitation with HCL, vortex mixing, centrifugation, 1-octanesulfonic acid extraction. *For cola*: MeOH addition, vortex mixing, filtration.	365
Five ergot alkaloids	HPLC-UV at 280 nm.	Hitachi gel n. 3111:0 Column. Eluent: *n*-hexane, EtOH, triethylamine.	around 1 μg.	Ergot	Ether defatting. Multiple extraction with 1,2-dichloroethane, aqueous solution pH = 10, tartaric acid, H_2SO_4, NaOH, $CHCl_3$. Evaporation at 40°C.	366

Eleven ergot alkaloids	HPLC with fluorimetric detection: λ_{ex} = 327 nm, λ_{em} = 398 nm.	Column: Hypersil ODS 3 mm, 250 × 4.6 mm. Mobile phase: ACN–water (47:53) containing 0.1 g of ammonium carbonate.	Less than 20 ppb of each alkaloid.	Cereals and cereal products; found a total content below the legal limits (0.05% w/w) in both middle European and Canadian product	CH_2Cl_2–ethyl acetate–MeOH–aqueous NH_3 (50:25:5:1): extraction, evaporation, redissolution in toluene–MeOH (49:1), Extrelut column pretreated with tartaric acid, cleaning up by hexane: isopropilic ether (1:1), elution with CH_2Cl_2 after basification with gaseous NH_3. Eluate evaporation and redissolution in mobile phase, filtration.	367
Fourteen ergot alkaloids	HPLC with fluorimetric detection (λ_{ex} = 327 nm, λ_{em} = 398 nm); TLC with colorimetric detection with *p*-dimethylamino-benzaldehyde and HCl; GC-CIMS with methane as the reactant gas, mass range: 100 and 600 amu.	*HPLC*: column Shandon Hypersil ODS 3 μm, 250 × 4.6 mm; mobile phase: ACN-H_2O (47:53, v/v), containing 0.1 g/L of ammonium carbonate. *TLC*: plate Kieselgel 60, 15 cm, thickness 0.5 mm; mobile phase: MeOH CH_2Cl_2 (1:9). *GC/MS*: column DB-1. T program: from 180 to 300°C.	Standard solution: less than 20 ppb; samples: ranging from 25 to 100 ppb.	Bread, brown bread, corn flakes, confectionery products, all bran-product cereals, linseeds, sesame seeds, infant formula	Products are ground and, when necessary, defatted, then extracted with CH_2Cl_2–ethylacetate–MeOH-conc. aq. NH_3 (50:25:5:1). Extracts filtered are evaporated, dissolved in hexane, and cleaned with an Extrelut modified method.	368

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22
Determination of *N*-Nitroso Compounds in Foods and Beverages Using HPLC

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I. INTRODUCTION

N-Nitroso compounds (NOC), commonly known as nitrosamines, are a group of organic compounds of diverse chemical structure all containing the functional group N—NO. These compounds are usually formed by the interaction of nitrous acid or other nitrosating agents (e.g., NO_2 gas) with secondary, tertiary, or even quaternary amines (1,2). Although NOC have been known for over 100 years, it is only during the past 30–35 years that these compounds have aroused a great deal of interest and concern (3,4). This has happened mainly for two reasons. First, many NOC have been shown to be potent carcinogens inducing cancer in a wide range of animal species (3). Second, traces to fairly high levels of certain NOC have been shown to be formed in foods, especially those preserved with nitrite (5–7). Until 1990, approximately 25 NOC have been reported to be present in foods and beverages, and of these at least 10 are carcinogenic in experimental animals. Details of these findings can be obtained in recent reviews on this topic (7–10).

Because of this concern, a variety of methods have been developed for the determination of NOC in foods. These include thin-layer chromatographic (TLC) (11,12), gas chromatographic (GC) (13–15), GC–mass spectrometric (GC-MS) (15–17), and high-performance liquid chromatographic (HPLC) (18–20) techniques. The purpose of this article is to review various HPLC methods developed for this purpose. Unfortunately, however, only limited advances have been made in this area, mainly because of the lack of sensitive and specific detectors. Most published methods for NOC reported to date are GC-based techniques. Therefore, this review will be a brief one and will emphasize the most recent HPLC developments.

II. CHEMISTRY OF NITROSAMINES

A detailed discussion of the chemistry of NOC is beyond the scope of this chapter. The reader is advised to consult other reviews (21–24) for details.

Broadly, NOC are divided into two groups: (a) *N*-nitrosamines and (b) *N*-nitrosamides. In *N*-nitrosamines, the two remaining substituent groups on the >N—N=O atom can be either alkyl or aryl, or they can form the part of a heterocyclic ring (as in *N*-nitrosopyrrolidine). On the other hand, in *N*-nitrosamides, more correctly called *N*-acyl-*N*-nitroso compounds, one of these

Fig. 1 Chemical formulas of some *N*-nitroso compounds: (a) *N*-nitrosodimethylamine (NDMA), (b) *N*-nitrosodibenzylamine (NDBzA), (c) *N*-nitrosopyrrolidine (NPYR), (d) *N*-nitrosoprolin (NPRO), (e) *N*-nitroso-4-hydroxyproline (NHPRO), (f) *N*-nitroso-2-(hydroxymethyl)-thiazolidine-4-carboxylic acid, (g) *N*-nitroso-D-fructose-L-histidine, (h) *N*-nitroso-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid.

substituent groups usually contain an acyl or substituted acyl functional group. Chemical formulas of several common *N*-nitrosamines and *N*-nitrosamides are shown in Figs. 1 and 2.

The low-molecular-weight dialkyl or cyclic *N*-nitrosamines are pale yellowish liquid and steam volatile. Conversely, the high-molecular-weight ones or those containing a polar group (e.g., NPRO) are either high-boiling liquid (steam nonvolatile) or solid at room temperature. For analytical purposes, *N*-nitrosamines are roughly divided into two groups, namely, volatile and nonvolatile *N*-nitrosamines, based on their steam volatility. The ease of steam volatility for the former group of compounds offers an excellent way to isolate these compounds from the bulk of

Fig. 2 Representative chemical formulas of various types of *N*-nitrosamides: (a) *N*-nitroso-*N*-methylacetamide, (b) *N*-nitrosobenzthiazuron, (c) *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG), (d) *N*-nitrosocarbaryl, (e) *N*-methyl-*N*-nitrosocyanamide, (f) *N*-nitroso-*N*-methyl-p-toluenesulfonamide.

the food matrix. Some of the important physical properties of NOC that are likely to be detected in foods can be obtained from previous reviews on this topic (25,26).

The UV spectra of NOC usually display two absorption bands in aqueous solution: one in the 220–235-nm region and the other at 330–375 nm. The first maximum, being stronger, is used for detection purposes in many HPLC methods.

Another property that is useful in selecting the proper chromatographic conditions (column, mobile phase, etc.) is water solubility or distribution coefficient between a polar (e.g., acetonitrile) and a nonpolar (e.g., *n*-heptane) solvent. Data on water solubility and UV absorption maxima for a large number of NOC can be obtained from Druckrey et al. (25). Eisenbrand et al. (26) reported the distribution coefficients between acetonitrile and *n*-heptane for several NOC. Those for *N*-nitroso-dioctylamine, NDMA, and *N*-nitrosomethyl-2-hydroxyethylamine were reported to be 0.5, 17.3, and 32.0, respectively, in this system. This would suggest that in a normal-phase system, using a silica column, these compounds would elute in the same order in which they are mentioned, but the elution order would be reversed in a reversed-phase (C_{18} column) system.

In general, dialkylnitrosamines are extremely stable compounds, especially in neutral and alkaline aqueous solution. Extensive resonance stabilization due to *n*- and π-electron interactions, as shown in the following equation for NDMA, are believed to be responsible for this extraordinary stability (27):

These compounds, however, undergo slow denitrosation in acidic solution that is usually catalyzed by various nucleophiles (e.g., I^+, SCN^+, Br^+, Cl^+) (23). On the other hand, the *N*-nitrosamides are highly unstable and decompose easily by acid- and base-catalyzed pathways (28). In

aqueous solution, especially at pH > 7, *N*-nitrosamides decompose by an addition–elimination pathway, involving nucleophilic catalysis, to produce diazohydroxides and diazoalkanes, which are powerful alkylating agents (28).

Under acid-induced catalysis, the *N*-nitrosamides can undergo denitrosation (mainly at pH < 2) or deamination (at pH > 2), which also generates diazohydroxides. The mechanism of acid/base-catalyzed hydrolysis of these compounds has been discussed in detail by Berry et al. (28) and Chow (29). *N*-Nitrosamides are also very unstable to heat. However, in contrast to *N*-nitrosamines, which decompose to secondary amines and nitric oxide at high temperature, the *N*-nitrosamides undergo rearrangement, yielding mainly nitrogen and diazonium ions but very little nitrogen oxide (27–29).

N-Nitrosamines undergo efficient photodecomposition, especially in acidic (pH < 4) aqueous solution. Polo and Chow (27) have proposed the following mechanism for photodecomposition of NDMA:

$(H_3C)_2N{=}N{-}O{\cdots}H^+$ (protonated)

n-π* exitation (~340 nm) → $[(H_3C)_2N(H){\cdots}NO]$ → $(H_3C)_2NH^{+\bullet}$ + $NO^{\bullet}$ → $H_2C{=}\overset{+}{N}HCH_3$ + NHO

π-π* exitation (~230 nm) → $(H_3C)_2N{-}NO$ + :OH ⇌ $(H_3C)_2\overset{+}{N}H_2$ + HNO_2

As can be seen from this equation, under π-π^* excitation the NDMA–acid complex photohydrolyzes to the parent dimethylamine and nitrous acid, which can be detected by Griess reagent, and this reaction is utilized for the post-HPLC-photohydrolysis detection of NOC in a method to be discussed later. Also, by incorporating a nitrous acid scavenger (e.g., urea, hydrazoic acid, ascorbic acid, sulfamic acid) in the photohydrolysis system, the unit can be used for safe destruction of NOC (27). Under n–π^* excitation, NDMA releases nitric oxide and dimethyl aminium radicals, which in aqueous solution decay, respectively, to hyponitrous acid and formylidinemethylamine. The thermal dissociation of NDMA is believed to follow the same pathway as the photolysis, leading to the formation of dimethyl aminium radical and nitric oxide. With long-wavelength UV light (>340 nm), the *N*-nitrosamides also photolytically decompose to amidyl and nitric oxide radicals, but further reactions of the transient amidyl radicals are quite complex. The topic has been reviewed by Chow (29).

Another property of NOC that has been utilized for their detection is their facile oxidation or reduction to the corresponding nitramines or hydrazines, respectively. *N*-Nitrosamines are easily oxidized to nitramines upon treatment with a strong oxidizing agent, such as pertrifluoroacetic acid or a mixture of nitric acid and ammonium persulfate (30). *N*-Nitrosamines can be reduced to hydrazines by treatment with a variety of reducing agents (e.g., Zn dust in acetic acid, sodium amalgam, $LiAlH_4$) (31). On treatment with a stronger reducing agent (e.g., Zn dust and dilute HCl), *N*-nitrosomethylphenylamine undergoes fission at the N—N linkage to form ammonia and

methylphenylamine (32). Nitrosamines can also be reduced electrochemically to the corresponding hydrazines (33,34).

Aromatic *N*-nitrosamines on treatment with acid can undergo the well-known Fisher–Hepp rearrangement (32). For example, *N*-nitroso-*N*-methylaniline (NMA) on treatment with concentrated HCl in ethanol rearranges to *N*-methyl-4-nitrosoaniline. It has been mentioned that *N*-nitrosamines are slowly denitrosated by treatment, especially on heating, with inorganic acids. Such denitrosation, however, proceeds smoothly and much faster in anhydrous medium, e.g., upon treatment with HBr in glacial acetic acid (35). Analysis of the resulting secondary amines or the nitrous acid provides the basis for several analytical methods to be discussed later.

Since this review deals mainly with the analysis of preformed NOC in foods, the chemistry of formation of these compounds is not dealt with in this chapter. It might just suffice to say that the formation of NOC in foods is dependent on a variety of factors, such as the nature (basicity) and concentration of the precursor amines, that of the nitrosating agents, the presence or absence of catalysts or inhibitors, pH, and the temperature of the processing or cooking conditions. The details have been discussed in recent reviews (5,6,9,10).

III. TOXICITY OF NITROSAMINES

The first evidence that NDMA, the simplest member of the NOC, could be toxic to humans came from the observation that industrial workers exposed to NDMA developed cirrhosis of the liver (36). Later studies by Magee and co-workers established that NDMA was indeed a potent hepatotoxic agent in laboratory animals (37) and, furthermore, that it was a potent carcinogen (38). Since then, more than 300 NOC have been tested for carcinogenicity, and about 90% of them have been shown to be carcinogenic, inducing tumors in a wide range of animal species, including the primates (33,39,40). It should be emphasized, however, that most of these experiments were carried out with relatively high doses of NOC. There is no evidence to indicate that food or beverages containing low levels of NOC have induced cancer in man. The daily intake of volatile NOC is estimated to range from 10 to 100 μg (40a).

Both the carcinogenic potency and the target organ seem to vary with the chemical structure of the NOC, the species of the animal being tested, and their age, sex, and nutritional status. Besides being carcinogenic, many NOC are mutagenic; some are teratogenic. A full discussion of these aspects can be found in several excellent reviews on this topic (39,40).

N-Nitrosamines, themselves, are believed to be noncarcinogenic. They require metabolic activation before being converted to reactive alkylating agents (carbonium ions or diazoalkanes), which are the ultimate carcinogens (33,39,40). The *N*-nitrosamides, however, are direct-acting carcinogens requiring no metabolic activation. For the same reason, the *N*-nitrosamines are mutagenic only after metabolic activation, which is a a-*C*-hydroxylation by cytochrome P450 dependent monoxygenases, whereas the *N*-nitrosamides are direct-acting mutagens. Since the biological activity and the relative abundance of various NOC in foods vary widely, analysts should take these factors into consideration while developing analytical methods for these compounds.

IV. SAMPLE PREPARATION AND CLEANUP TECHNIQUES

The main purpose of these procedures is to separate the NOC from the bulk of the food matrix and to prepare a concentrated extract that would permit detection of low ppb levels of these compounds in a particular food without interference from any coextracted food material. A summary of the various procedures developed for this purpose is discussed next.

A. Volatile *N*-Nitrosamines

As mentioned earlier, some form of distillation (steam, vacuum, or atmospheric) is used to isolate the volatile *N*-nitrosamines from foods or beverages. Basically, such sample preparation techniques consist of the following steps: (a) distillation of the sample from an appropriate medium (usually from aqueous acidic or basic solution), (b) extraction of the aqueous distillate with dichloromethane (DCM), and (c) concentration of the DCM extract using a Kuderna-Danish (K-D) concentrator to a small volume (0.5–1 ml). Furthermore, special precautions are needed to prevent or minimize artifactual formation of *N*-nitrosamines that can occur during workup of the sample. This is usually controlled by adding a nitrite scavenger (e.g., ascorbic acid, sulfamic acid, sodium azide) and monitored by incorporating an easily nitrosatable secondary amine (e.g., morpholine or 2,6-dimethylmorpholine) to the sample at the start of the analysis. Any increase in the concentration of *N*-nitrosomorpholine (NMOR) or *N*-nitroso-2,6-dimethylmorpholine over that present in the control sample (analyzed without the addition of the previously mentioned marker amines) is taken as a measure of the artifactual formation. There should be negligible or no such increase. Details of these methods can be obtained from a monograph recently published by the International Agency for Research on Cancer (IARC) (15).

In some of the recent methods, the lengthy distillation step has been omitted. Instead, the food or beverage being analyzed is extracted directly with DCM, and the DCM extract is treated with liquid N_2 to remove fats. The defatted extract is then concentrated using a K-D concentrator (41). Alternatively, in some methods an aliquot of the sample is mixed with Celite, the mixture is packed in a chromatography column, the column is eluted with DCM, and the DCM extract is concentrated as before (15). These methods are much faster than the distillation methods and, therefore, are useful for rapid screening purposes. However, only limited information is available on the applicability of such extracts to HPLC analysis. Most of the published methods dealing with determination of volatile *N*-nitrosamines in foods and beverages have employed either aqueous (42,43) or mineral oil distillation (44) for cleanup.

B. Nonvolatile Nitrosamines

In contrast to the methods for volatile *N*-nitrosamines, there appears to be no single sample preparation and cleanup technique that is applicable to the determination of all nonvolatile NOC in foods. This is mainly because the physical and chemical properties of these compounds vary widely, and, therefore, different approaches are needed for the different classes of nonvolatile NOC. Most methods, however, include the following basic steps: (a) extraction with a suitable solvent, (b) a defatting step to remove fats and lipids, (c) cleanup, and (d) concentration of the final extract to a small volume. Furthermore, special precautions are needed for the workup of *N*-nitrosamides, for they are highly unstable, especially under alkaline pHs. As with the volatile *N*-nitrosamines, a suitable nitrite scavenger should be added to the extracting solvent to minimize artifactual formation. In this regard, sodium azide is preferable to other nitrite scavengers, because it is effective over a wide pH range, including pH $\cong$ 4, at which *N*-nitrosamides are most stable (21,22).

Table 1 (second column) summarizes the salient features of the cleanup procedure reported for the determination of nonvolatile NOC in foods. For brevity, the extraction and cleanup procedures as well as the respective chromatographic conditions and other details of various methods have been included in this table. The HPLC conditions and detectors are discussed later.

There are alternative cleanup methods (54), which might also be useful for such purposes, but they have not yet been applied to HPLC analysis. More recently, rapid Celite column extraction methods were described. Pensabene and Fiddler (55) reported the determination of 10

Table 1 Summary of a Few Selected HPLC Methods Reported for the Determination of Nonvolatile NOC in Foods and Beverages

Nature of sample analyzed	Extraction and cleanup procedure used	Derivatization technique	HPLC conditions			Percentage recoveries	Ref.
			Column	Mobile phase	Detector		
Nonpolar nonvolatile NOC in foods and beverages	1. Extraction with CH_3CN 2. Partitioning with isooctane	None	300 × 4 mm μ-Porasil (Waters Associates)	1–5% acetone in 2,2,4-trimethylpentane	TEA	50–90	45
NPRO in raw bacon	1. Extraction with water 2. Cleanup on anion-exchange resin	None	12 × 0.25 in. μBondapak C_{18} (Waters Assoc.)	1% Na_2HPO_4 in water	Photohydrolysis followed by detection of nitrite with Griess reagent	85–90	46
NSAR and NPRO in bacon and other cured meats	1. Extraction with basic CH_3CN 2. Cleanup on acidic alumina	None	250 × 2.1 mm LiChrosorb Si60 (5 μm)	Dichloromethane : acetone : ethanol : diethylamine acetate buffer : water (20:20:4:4:1)	TEA	69–86	47
NThZ, NHMThZ, NMeThZ, and NHMFuThZ in bacon	1. Extraction with CH_2Cl_2 2. Cleanup on three cyanopropyl Bond Eluts in series	None	250 × 4.6 mm Spherisorb ODS 2 (5 μm)	Aceton : 0.05 M triethylamin/0.15 M phosphoric acid (gradient from 10:90 to 60:40)	TEA	35–59	47a
N-Nitroso-*N*′,*N*′-dimethyl piperaziniumiodide in cured meats	1. Extraction with water containing sodium azide 2. Ion-pair extraction into an organic solvent	None	300 × 3.9 mm (10 μm) Bondapak CN (Waters Assoc.)	10 mm camphorsulfonic acid in ethanol	TEA	83	48
NSAR, NPRO, NHPRO, and several *N*-nitroso dipeptides in cured meats	1. Extraction with ethyl acetate from aqueous suspension containing H_3P0_4 and ammonium sulfamate 2. Cleanup on Bond-Elut Aminopropyl column	None	250 × 4.5 mm Microbondapak CN	*n*-Hexane : ethanol : acetic acid (87:12:1)	TEA	55–80	49, 50

(*continued*)

Table 1 Continued

Nature of sample analyzed	Extraction and cleanup procedure used	Derivatization technique	HPLC conditions			Percentage recoveries	Ref.
			Column	Mobile phase	Detector		
NSAR and NPRO in ham	1. Extraction with 1 M HCl containing sulfamic acid 2. Removal of fats by partitioning with *n*-hexane 3. Cleanup on anion-exchange resin	Methylation with BF_3^- methanol	250 × 4 mm Cyano (5 μm) (Alltech Assoc.)	*n*-Hexane : dichloromethane : isopropanol (70 : 29.75 : 0.25) at 90°C	TEA	70–100	51
NMU in fried bacon	1. Extraction with ethyl acetate from aqueous suspension 2. Flash evaporation and transfer of extract to methanol/KCl-HCl buffer containing sulfamic acid (pH 2) 3. Removal of fats by partitioning with *n*-hexane 4. Reextraction of NMU from aqueous layer with dichloromethane	None	250 × 4.6 mm Lichrosorb Si 60 (5 μm) (Alltech/Applied Science)	20% Acetone in *n*-hexane	HI-catalyzed chemical denitrosation-TEA	90–100	52
NHMThZ and NHMTCA in bacon and smoked meats	1. Extraction with CH_3CN or CH_3OH in the presence of sulfamic acid and 0.5 N H_2SO_4 2. Removal of fats by partitioning with *n*-hexane 3. Separation of the two compounds by passing through acidic alumina sample preparation cartridge	Only HM-NTCA is esterified with CH_2N_2	250 × 4.6 mm Lichrosorb Si 60 (5 μm) (Alltech/Applied Science)	5–20% Acetone in *n*-hexane using linear programming	TEA	70–100	53

NHMThZ and NHMTCA in bacon, smoked meats, smoked poultry products, and smoked cheeses	1. Extraction with CH_3CN or CH_3OH in the presence of sulfamic acid and 0.5 N H_2SO_4 2. Removal of fats by partitioning with *n*-hexane 3. Separation of the two compounds by passing through acidic alumina sample preparation cartridge	Only HMNTCA is esterified with CH_2N_2	250 × 4.6 mm Lichrosorb Si 60 (5 μm) (Alltech/Applied Science)	5–20% Acetone in *n*-hexane using linear programming	TEA	70–100	53a
NTHβCCA and NMTHβCCA in smoked meat	1. Extraction with CH_3CN or CH_3OH in the presence of sulfamic acid and 0.5 N H_2SO_4 2. Removal of fats by partitioning with *n*-hexane 3. Neutralization with ammonia 4. Extraction with ethyl acetate 5. Cleanup on acidic alumina	None	150 × 4.6 mm Supelco ABZ (5 μm)	10–50% CH_3CN in 10 mM trifluoroacetic acid	TEA	Not given	53b

NPRO = *N*-nitrosoproline; NSAR = *N*-nitrososarcosine; NHPRO = *N*-nitroso-4-hydroxyproline; NMU = *N*-nitroso-*N*-methylurea; NThZ = *N*-nitroso-thiazolidine; NMeThZ = *N*-nitroso-2-methyl-thiazolidine; NHMThZ = *N*-nitroso-2-(hydroxymethyl)thiazolidine; NHMFuThZ = *N*-nitroso-2-(5-hydroxymethylfuryl)-thiazolidine; NHMTCA = *N*-nitroso-2-(hydroxymethyl)-thiazolidine-4-carboxylic acid; NTHβCCA = *N*-nitroso-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid; NMTHβCCA = *N*-nitroso-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid.

Source: Adapted from Refs. 20 and 54.

nonvolatile *N*-nitrosamino acids in cured meats. Again, the method was applied only to GC–thermal energy analysis (GC-TEA). It is possible that extracts prepared by this method (55) might also be suitable for HPLC-TEA analysis. Lillard and Hotchkiss (55a) extracted *N*-nitrosodiphenylamine (NDPhA) from apples treated with diphenylamine for scald. The frozen and blended apples were mixed with Celite and extractes with *n*-hexane. A silical gel SPE followed. Analysis were performed using HPLC-UV photolysis-chemiluminescence.

C. Polar Acidic Nitrosamines and *N*-Nitrosamides

Only limited research has been carried out in this area. Cleanup methods for the determination of NHPRO and NHMTCA, both belonging to the polar acidic group of compounds, are listed in Table 1. There is a need for the development of such methods for *N*-nitroso sugar amino acids, which might be formed in foods by the nitrosation of sugar amino acids commonly known as Amadori compounds (56). Reyes et al. (56a) synthesized the two structurally similar *N*-nitroso Amadori compounds 1-Deoxy-1-(*N*-nitroso,*N*-glycinyl)-D-fructose and 1-Deoxy-1-(*N*-nitroso, *N*-alaninyl)-D-fructose and separated them by RP-HPLC. They pointed out the need for the improvement of the solvent trapping system when water is used in the HPLC-TEA system, because the eluent CH_3CN/CH_3OH/0.01 M oxalic acid (50:50:0.75 v/v/v) gave very noisy peaks. Similarly, very little work, other than that for NMU determination mentioned in Table 1, has been carried out. Extraction and cleanup methods for the determination of *N*-nitrosoglycocholic acid, *N*-nitrosocimetidine, and *N*-nitrosotrimethylurea in gastric juice and urine have been reported (57,58). Perhaps these methods can be adapted for the determination of these nitrosamides in foods and beverages.

D. Derivatization Techniques

Theoretically, there should be no need for derivatization for HPLC analysis of NOC. With the choice of proper stationary and mobile phases, one should be able to analyze most NOC by HPLC, provided sensitive and specific detectors are available for this purpose. The TEA is a highly specific and sensitive detector commonly used for the detection of NOC (18,45). It is, however, quite expensive and not available in many laboratories. This prompted Verhagen and Strating (44) to develop a derivatization technique for the determination of NDMA in malt and beer using a conventional fluorescence detector. The NDMA was first denitrosated to dimethylamine (DMA) by treatment with HBr:acetic acid, and the liberated amine was derivatized with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to yield a highly fluorescent derivative. Cox (42), on the other hand, reduced NDMA to DMA electrochemically, and then prepared the 2,4-dinitrophenyl derivative and analyzed it using a UV (230 nm) detector. The highly fluorescent 1-dimethyl-aminonaphthalene-5-sulfonyl (DANSYL) derivative of DMA can also be prepared and analyzed (59). The major drawback of these techniques is that they measure NDMA indirectly. Since DMA occurs naturally in many foods and beverages, it must be removed completely from the food extracts before applying the foregoing derivatization techniques. Also, any compound that can release DMA upon denitrosation or electrochemical reduction would give false-positive results for NDMA.

Sen and Kubacki (54) have reviewed the derivatization techniques for the GC determination of nonvolatile NOC in foods. These include trimethylsilylation (for acidic as well as hydroxylated NOC), esterification with CH_2N_2 or BF_3/CH_3OH, and *O*-methylation using CH_3I/NaH. Only those applied to HPLC analysis are briefly discussed here.

Again, only very little information is available in the literature in this area. Kubacki et al. (51) esterified *N*-nitrosamino acids and *N*-nitrosodipeptides with BF_3/CH_3OH before analyzing

by HPLC-TEA. They applied the technique to the determination of NSAR and NPRO in ham. Sen et al. (53) similarly esterified NHMTCA with CH_2N_2 before analyzing by HPLC-TEA. Both groups of researchers encountered problems in analyzing underivatized *N*-nitrosamino acids by HPLC-TEA (due to the incompatibility of TEA with aqueous mobile phase or phases containing ion-suppressing acids). Therefore, prior esterification permitted HPLC-TEA analysis of these compounds using normal-phase systems (see Table 1 for conditions). A confirmation method for NHMTHZ has been reported that is based on derivatization of the compound to its *O*-methyl ether and analysis by HPLC-TEA (60).

V. CRITERIA FOR SELECTION OF STATIONARY AND MOBILE PHASES

In general, there is a wide variety of chromatographic modes (types) that can be employed for the HPLC determination of food components, but only a few have been used for the determination of NOC. These include partition/adsorption on silica gel, liquid–liquid partition on polar-bonded phase (e.g., cyano, amino) or nonpolar hydrophobic-bonded phase (e.g., reversed-phase), and anion-exchange chromatography. Macrae (61) discussed the theories behind the various modes of chromatography.

The choice of the proper stationary and mobile phases for the foregoing purpose would depend on several factors, such as the nature (polarity, stability in mobile phase) of the NOC analyzed and the availability/compatibility of the detector used. For example, if only a TEA is available as a detector, the use of an ion-exchange or a reversed-phase system is ruled out, because both require aqueous mobile phase for proper operation. Moisture in the mobile phase causes freeze-up of the cold traps in the TEA and also results in noisy response due to interference during chemiluminescence detection. Similarly, if one is using, as the detector the newly developed HI-catalyzed denitrosation-TEA (62) or the photolytic cleavage-TEA (58), a reversed-phase system using aqueous mobile phase would be the method of choice. These detectors, however, have not been demonstrated to work in the normal-phase system. The use of an electrochemical detector will also be incompatible with an organic solvent as the mobile phase.

A. Volatile *N*-Nitrosamines

A review of the literature indicates that most researchers prefer the normal-phase system for HPLC separation of the volatile *N*-nitrosamines. The use of a silica or a μBondapak NH_2 column as the stationary phase and a mixture of organic solvents of low polarity as the mobile phase usually gives a good separation of all the volatile *N*-nitrosamines commonly detected in foods (18,63). The relative retention times of various *N*-nitrosamines often changes from system to system, and this property can be used for confirmatory purposes. Furthermore, HPLC and GC techniques can complement each other. Since separations on GC (mainly based on vapor pressure or solubility) and on HPLC (mainly based on polarity) arise from different principles, a positive result for a particular volatile *N*-nitrosamine by both techniques can be taken as a good evidence of the presence of the compound (64), although confirmation by GC–mass spectrometry is recommended. This is best exemplified in Fig. 3, in which the presence of NDMA, NPYR, and *N*-nitrosopiperidine (NPIP) was demonstrated by both GC-TEA and HPLC-TEA analyses (63). Again, the difference in the relative elution orders of the three compounds in the two systems should be noted. The HPLC technique, however, has one advantage over the GC mode. Many thermolabile NOC (e.g., *N*-nitrosodiphenylamine, NDPHA; *N*-ethylaniline, NEA) decompose in the GC injector or on GC column. These compounds are preferably analyzed by HPLC (18). Isaaq et al. (65) used both β-cyclodextrin bonded silica gel and C_{18} reversed-phase columns for

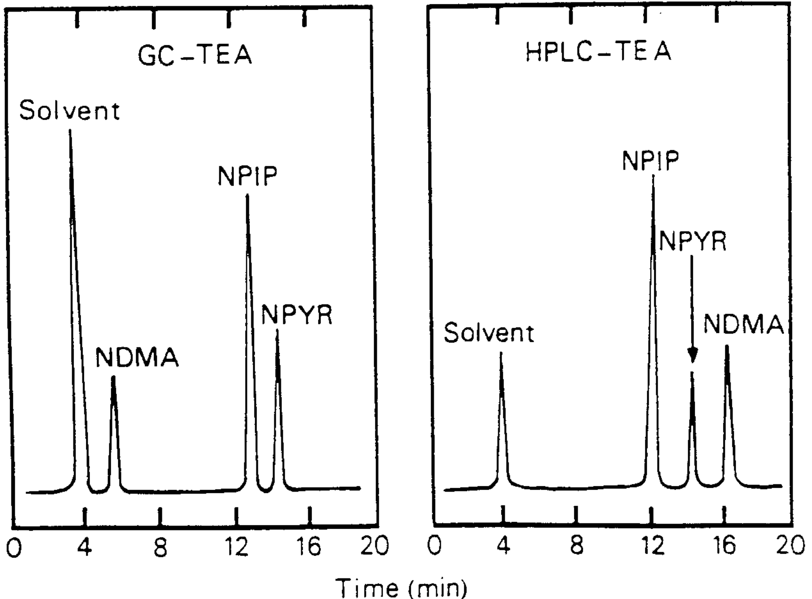

Fig. 3 Comparison of GC-TEA and HPLC-TEA chromatograms of a Russian dried meat extract. (For conditions, see Ref. 63.) (Reproduced from Ref. 63 with permission of the IARC.)

HPLC determination of acyclic *N*-nitrosamines. Both gave acceptable results, but the former gave a much superior resolution of the respective E and Z isomers.

B. Nonvolatile Nitrosamines

Of the various nonvolatile NOC, the *N*-nitrosamino acids have received the most attention. These compounds can be analyzed by HPLC either in the underivatized free-acid form or after esterification. Some of the chromatographic conditions used for this purpose are presented in Table 1. The free acids are best analyzed using either a C_{18} reversed-phase or an anion-exchange column, although some researchers have successfully used silica and cyano (in the presence of acetic acid as an ion-suppressing agent) columns for this purpose (Table 1). The methyl esters are best analyzed on a cyano or a silica column. The details of the chromatographic conditions can be obtained from Table 1 or the respective references.

Under certain chromatographic conditions, the *N*-nitrosamino acids are separated into the respective *syn* and *anti* conformers. If one is interested in determining the relative concentrations of the two conformers, HPLC on an α-cyclodextrin-bonded silica gel column using acetonitrile : triethylammonium acetate buffer as the mobile phase might be used (66). Earlier, Sen (14) reported complete resolution of NSAR and NPRO conformers using the system described in Table 1 (47). The technique was used for studying heat-induced decarboxylation of NSAR and NPRO in bacon. More recently, Conboy and Hotchkiss (58) used a C_{18} reversed-phase system for the HPLC determination of NSAR, NPRO, and three other *N*-nitrosamino acids. The *syn* and *anti* conformers were, however, not resolved in that system, which might be an advantage for the purpose of quantitation.

There are only limited references in the literature regarding HPLC determination of nonpolar nonvolatile NOC in foods. In one of these studies, Fine et al. (45) used a μ-Porasil column and 1–5% acetone in iso-octane as the mobile phase for the HPLC-TEA determination of

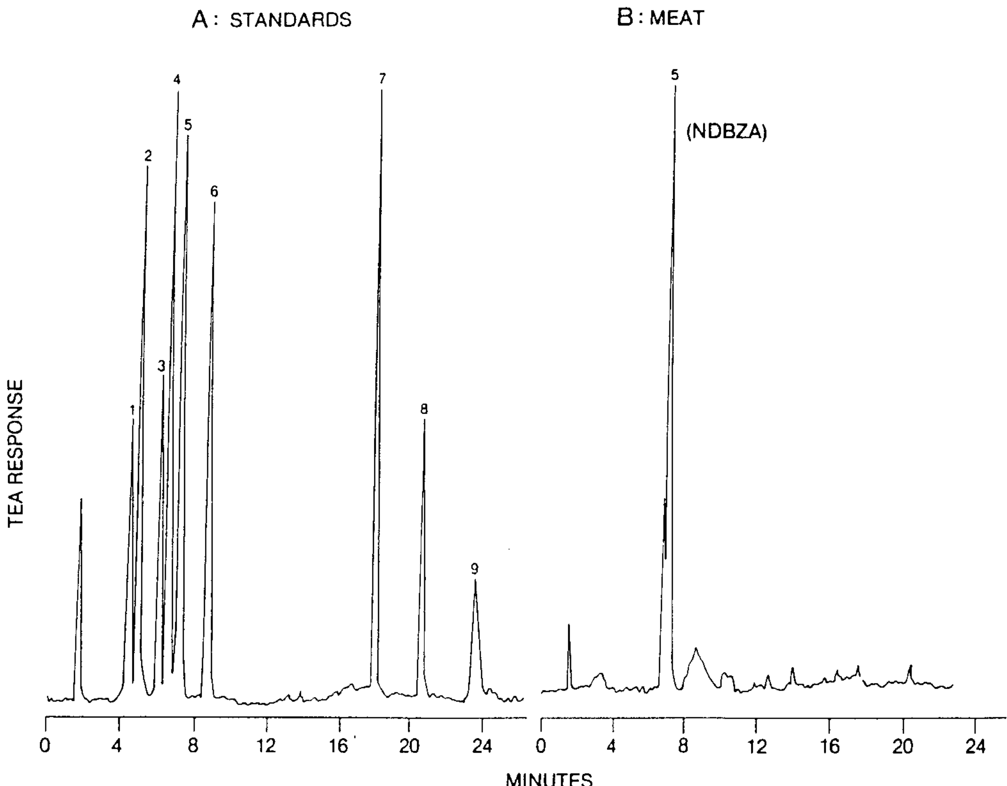

Fig. 4 HPLC-TEA chromatograms for various *N*-nitrosamines (≅20 ng each). (A) (1) NDPhA, (2) *N*-nitrosoethylphenylamine (NEPhA), (3) *N*-nitrosodicyclohexylamine, (4) NMA, (5) NDBzA, (6) *N*-nitrosodi-*n*-propylamine (NDPA), (7) *N*-nitrosobutyl-4-hydroxybutylamine, (8) dinitrosopentamethylenetetramine, (9) NDELA. (B) A meat extract showing the presence of NDBzA and traces of *N*-nitrosodi-*n*-butylamine (NDBA) (unresolved small peak preceding that of NDBzA). Conditions: Column, 250 mm × 4.1 mm (ID) packed with LiChrosorb Si 100 (5 μm); mobile phase programming, 1% acetone in *n*-hexane initially, then linearly programmed to 40% acetone in *n*-hexane in 15 min, and then held 10 min. (Reproduced with permission from Ref. 67. Copyright 1988, Institute of Food Technologists.)

NDPHA, *N*-nitrosoatrazine, *N*-nitrosobenzylphenylamine, *N*-nitrosocarbazole, and *N*-nitrosocarbaryl in foods. In another study, Sen et al. (67) used a normal-phase system for the determination of NDBZA in cured pork products packaged in elastic rubber nettings (Fig. 4). As can be seen from the figure, appropriate solvent programming allowed the simultaneous determination of six nonpolar and three polar NOC.

The hydroxylated *N*-nitrosamines form another major group of nonvolatile NOC that can be divided roughly into two subgroups: (a) those with moderate polarity containing 1–2 hydroxyl groups, and (b) those of highly polar nature containing >2 hydroxyl groups or containing both hydroxyl and acidic functional groups (e.g., *N*-nitroso sugar amino acids). *N*-Nitrosohydroxypyrrolidine (NHPYR), *N*-nitrosodiethanolamine (NDELA), and NHMTHZ, all containing no more than 2 hydroxyl groups, can be analyzed either on a silica (53,64), amino (68), or C_{18} reversed-phase (62) column using appropriate mobile phases. Roeper (69) reported HPLC-UV determination of 13 *N*-nitroso sugar amino acids on a C_{18} reversed-phase column using the mobile phase water:acetonitrile (9:1) containing 50 mM tetrabutyl ammonium phosphate (pH 7.5)

as an ion pair. The system gave good resolution of the E and Z isomers of the compounds analyzed, but the method was used only for the analysis of the standards. Scanlan and Reyes (70) attempted HPLC determination of *N*-nitroso-D-fructosylglycine and *N*-nitroso-D-fructosyl-L-alanine using an Alltech carbohydrate column as the stationary phase and a mixture of acetonitrile:methanol:0.01 M oxalic acid (50:50:0.75 v/v/v) as the mobile phase. Two different detectors (UV and TEA) were evaluated. As expected, the UV detector gave a fairly good chromatogram, but the TEA gave a noisy response due to interference by moisture in the solvent. Further research is needed in this area.

C. Nitrosamides

Several *N*-nitrosamide standards have been chromatographed using C_{18} reversed-phase systems (58,62,71), but only limited information is available on the HPLC determination of these compounds in foods or biological materials. For compounds containing acidic (e.g., *N*-nitrosoglycocholic acid) or hydroxyl groups, a reversed-phase system should be the method of choice (57), although a normal-phase system has been successfully used for the determination of several *N*-nitrosamides, including streptozotocin [a *N*-nitrosamide containing a glucose moiety] (72).

D. Microbore HPLC

Recent advances in chromatography have made it possible to employ microbore HPLC for the determination of NOC. Its main advantage is that it uses a very low mobile-phase flow (20–100 μl/min). This might make the TEA compatible with a reversed-phase system. Massey et al. (73), in fact, have successfully used reversed-phase chromatography for the HPLC-TEA determination of *N*-nitroso-*N*′,*N*′-dimethylpiperazinium iodide. A 500-mm × 1-mm microbore ODS column and a mobile phase consisting of 0.1 M ammonium heptane–sulfonate in methanol:water (70:30) (flow rate 20 μl/min) was used for the HPLC separation. In another study, Rühl and Reusch (74) used a microbore Spherisorb 3 SW column for HPLC-TEA determination of volatile *N*-nitrosamines. The mobile phase was a mixture of 2-propanol and *n*-hexane (2.5:97.5). Further application of such techniques for the determination of various polar NOC, especially *N*-nitrosamides, in foods is desirable.

VI. DETECTION SYSTEM

A variety of detectors have been used for the HPLC determination of NOC in foods. These include UV, fluorescence, electrochemical, TEA, and various postcolumn denitrosation detectors. To be applicable for the low-ppb detection of these compounds in foods, an HPLC detector should meet the following criteria: high sensitivity and specificity, responsive to all classes of NOC, linearity over a fair range of concentration, compatibility with both normal- and reversed-phase mobile phases, and minimal interference from changes in solvent composition, thereby making it amenable to solvent programming. As will be seen from the following discussion, none of the detectors currently available meet all these criteria.

A. Ultraviolet Detectors

Since most NOC absorb strongly in the UV region (220–235 nm and 330–375 nm), UV detectors should be very useful for the determination of NOC in foods. However, the detector is quite nonspecific, and there is a possibility of false-positive results from other UV-absorbing com-

pounds present in food extracts. For this reason, the use of such detectors has been limited to the analysis of standards only (65,66,69,70). A UV detector should still be useful for analyzing very clean extracts or studying *N*-nitrosamine formation in model systems.

B. Fluorescence Detectors

Although fluorescence detectors are more specific and sensitive than UV detectors, their use for the detection of NOC has been limited due to the fact that very few of these compounds are fluorescent. These compounds must first be converted to suitable fluorescent derivatives before analysis. As mentioned earlier, Verhagen and Strating (44) developed such a method for the determination of NDMA in malt and beer. The results obtained by this technique compared well with those obtained by GC-TEA analysis of underivatized NDMA. You et al. (74a) developed a precolumn flourescence derivatization with 9-phenantrenecarbonyl chloride. Eight standards were separated on a RP C18 column with a CH_3OH–H_2O–triethylamine mixture as eluent. These fluorescence techniques are, however, quite lengthy and laborious, and there is the danger of false-positive results from amine impurities in reagents or naturally occurring amines in foods.

C. Electrochemical Detectors

Electrochemical detectors can be used for the determination of both inorganic and organic compounds that are capable of oxidation or reduction at an electrode surface (61). Since most NOC can be reduced or oxidized, such detectors give highly sensitive response to these compounds. Samuelsson and Osteryoung (34) and Vohra and Harrington (75) have studied the voltammetric behavior of several *N*-nitrosamines that are likely to be present in foods. In acidic solution, electrochemical reduction of *N*-nitrosamines was shown to involve four electrons with pH dependence, whereas that in basic media involved two electrons and was pH independent (34). As little as 0.8 ng *N*-nitrosodi-*n*-propylamine (NDPA) was detectable by differential pulse-mode polarography (75). Again, most of the applications have been limited to the detection of standards. Further applications of these techniques to food analysis would be desirable.

D. Thermal Energy Analyzer (TEA)

As mentioned earlier, of all the detectors, TEA is the most widely used for both the GC and HPLC determination of NOC in foods. It was developed by Fine et al. (76) in the early 1970s, first as a GC detector (77) and later interfaced with an HPLC unit (18). Basically, it is a chemiluminescence detector and based on the detection of NO radicals produced from the pyrolysis of NOC in the hot pyrolyzer tube of the TEA. After pyrolysis, the NO radicals are allowed to react with ozone to form excited NO_2 molecules that afterwards return to the ground state with the emission of light in the near-infrared region (600–3000 nm). The light in the 600–800-nm range is amplified and measured for quantitation (Fig. 5). In the GC mode, various pyrolysis products, other than NO, are adsorbed either on a Tenax cartridge or in suitable cold traps (−140 to −160°C); only the NO molecules are allowed to enter the chemiluminescence detection chamber. This makes it highly selective for the determination of NOC or compounds that produce NO upon pyrolysis.

In the HPLC mode, most solvent vapors and pyrolysis products are similarly trapped in a series of two cold traps (dry ice + ethanol). This has been found to be quite efficient in removing most organic solvent vapors, but is unworkable with aqueous mobile phases because of freeze-up and blockage of the cold traps. Also, traces of moisture entering the detection chamber interfere with the chemiluminescence detection. Furthermore, mobile phases containing in-

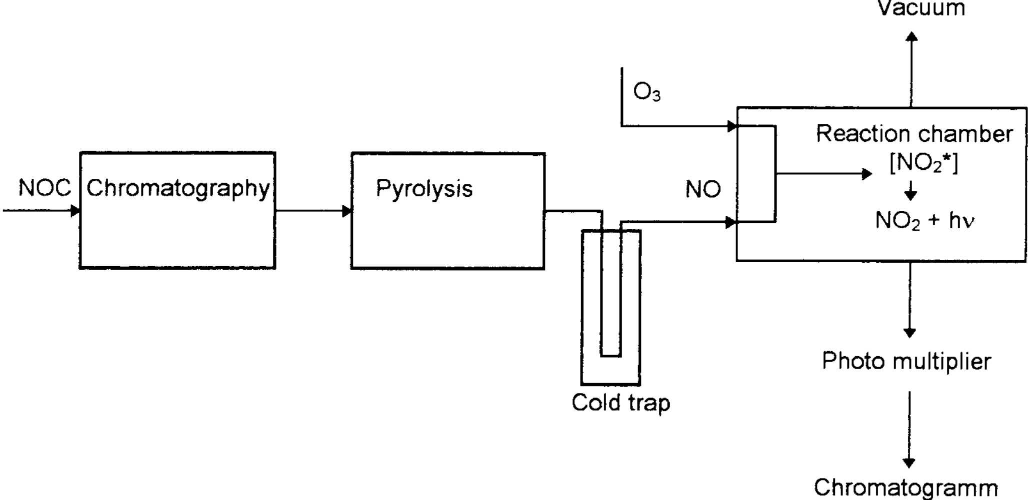

Fig. 5 Diagram of a TEA detector. (Adapted from Ref. 77a.)

organic buffers are incompatible with the detector because of possible coating of the catalyst in the pyrolyzer tube, thus affecting sensitivity. These factors make TEA quite restrictive as an HPLC detector.

Another drawback of the TEA (both in the GC and HPLC modes) is that *N*-nitrosamides give extremely poor yields (≈1%) of NO upon pyrolysis. When heated, these compounds, instead of splitting into NO and the parent amides, rearrange to yield diazoalkenes and nitrogen. Recently reported modifications of the TEA have somewhat improved this deficiency in the GC mode, but the corresponding improvements in the HPLC mode are yet to be worked out (78).

In spite of these drawbacks, TEA is an extremely useful detector for the determination of NOC in foods. It is both highly selective and sensitive, and the response is linear over a wide range (2–3 orders of magnitude) of concentrations. Although a few other compounds (e.g., nitrite, *C*-nitro compounds, some olefines) give positive response to TEA, these compounds can be removed either by first treating the food with nitrite scavengers or taking the food extract through suitable cleanups. Several examples of application of the technique have already been mentioned in Table 1; others are discussed later.

E. Postcolumn Denitrosation Detectors

A variety of methods have been reported for the HPLC determination of NOC based on post-column denitrosation and detection of the liberated nitric oxide radical or resulting nitrite ion. These methods differ in both the denitrosation procedures and the final determinative techniques. Table 2 gives a brief outline of these methods. Since many of them have already been covered in previous reviews (20,54), only a few selected ones (c, d, g, and h) are discussed here.

In method (c), the NOC after HPLC separation was photolyzed by a UV lamp (254 ± 10 nm), and the charged nitrite species was determined amperometrically (79). The denitrosation reaction was found to be dependent on the wavelength of the UV light, lamp intensity, exposure time, and pH of the solution. The effluent from the HPLC column was passed through a capillary PTFE tubing coiled around a 40-W mercury lamp. The electrochemical detector used permitted either single- or dual-mode detection corresponding, respectively, to detection limits of 60 pg and 20 pg for NDMA. The method was applied to the determination of NDMA in beer and of

Table 2 Various Postcolumn Denitrosation Techniques Reported for the HPLC Determination of NOC

Denitrosation principle and common name of the technique	Preferred mobile phase for HPLC separation	Initial (ultimate) species detected	Detection method	Comments	Refs.
1. Methods based on UV photolysis					
(a) Photohydrolysis	Aqueous buffer	$NO^{\bullet}$ (NO_2^-)	Colorimetric (with Griess or Bratton–Marshall reagent)	Applicable to most NOC	57, 57a
(b) Photoconductivity	Deionized aqueous solution containing organic modifier	$NO^{\bullet}$ (NO_2^-)	Photoconductivity cell	Thus far applied to volatile *N*-nitrosamines only	43
(c) Photolysis-electrochemical detection	Aqueous phosphate buffer	$NO^{\bullet}$ (NO_2^-)	Amperometric	Applicable to most NOC	79
(d) Photolysis-TEA	Aqueous solution containing organic modifier	$NO^{\bullet}$	Chemiluminescence	Applicable to most NOC	58
2. Methods based on acidic hydrolysis of N—NO bond					
(e) Fluorescence detection	20% CH_3CN in water	NO_2^-	Fluorescence (reduction of Ce^{4+} to Ce^{3+} by NO_2^-)	Applicable onty to *N*-nitrosamides	80
(f) Colorimetric detection	5–80% *n*-Propanol in water	NO_2^-	Colorimetric (with Griess or Bratton–Marshall reagent)	Applicable only to nitrosamides; fully automated	71
3. Methods based on HX (X = Br or I)-catalyzed denitrosation					
(g) HX denitrosation-TEA	5–80% Acetone in *n*-hexane	NOX ($NO^{\bullet}$)	Chemiluminescence	Applicable to most NOC (not compatible with reversed-phase)	72
(h) HI denitrosation-TEA	Aqueous solution containing organic modifier	NOI ($NO^{\bullet}$)	Chemiluminescence	Applicable to most NOC (not compatible with normal-phase)	62

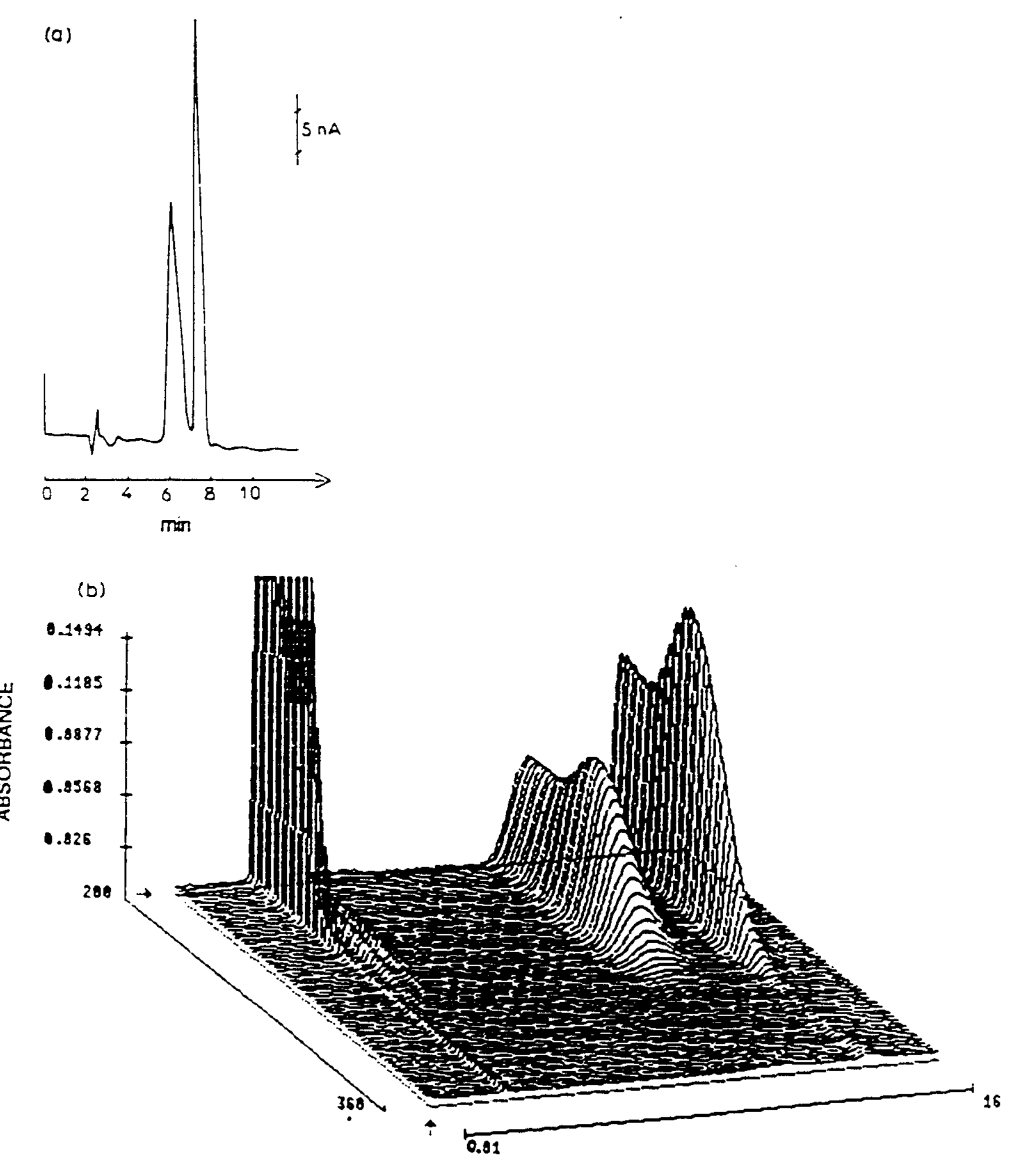

Fig. 6 HPLC analysis of *N*-nitrosofructosylmethionine: (a) electrochemical detection, (b) detection by UV photodiode array. (For conditions, see Ref. 79.) (Reproduced from Ref. 79 with permission from Elsevier Science Publisher.)

N-nitroso-fructosyl methionine, a nitroso sugar amino acid, standard. The latter gave two peaks, probably corresponding to its E and Z isomers, and each exhibited the same UV features as those of the standard when analyzed by a photodiode array detector (Fig. 6).

Method (d) differs from the foregoing in both the design of the UV photolysis apparatus and the detection method. Here the effluent from the HPLC column containing the separated NOC is introduced into a glass coil and then irradiated with UV light (200-W mercury vapor lamp). Helium gas is also introduced into the glass coil to purge the generated nitrogen oxide, which is then rapidly separated from the solvent (by passing through a series of cold traps) and let into the TEA for chemiluminescence detection. In essence, the method is similar to normal

HPLC-TEA but differs in two important aspects. First, the cleavage is done photolytically, thus making it amenable to the determination of nitrosamides, which give poor yields of NO under the normal TEA pyrolytic conditions. Second, by omitting the TEA furnace, this detector allows one to use aqueous mobile phases necessary for reversed-phase or ion-exchange chromatography. However, the composition (ratio of the organic modifier to water) of the mobile phase seemed to have a marked effect on the response of various NOC tested. The response was linear between 1 and 100 ng NPRO injected, but fell off at over 100 ng. A typical chromatogram obtained from the analysis of a mixture of NOC standards is shown in Fig. 7. The technique has not yet been applied to the determination of NOC in foods.

Two postcolumn denitrosation techniques have been reported in which HBr or HI was used as the denitrosating agent. In method (g), the effluent from the HPLC column was introduced into a heated flask containing refluxing ethyl acetate and small amounts of HBr or a mixture of concentrated H_2SO_4 and solid KI. The flask was connected to the TEA through a series of cooled condenser, a cold trap, and a solid KOH trap. Argon, as carrier gas, was bubbled into the solvent in the flask that carried the generated NO into the TEA. Since *N*-nitrosamides are denitrosated more easily than *N*-nitrosamines, the use of HI as the denitrosating agent gave a full mole-to-mole response but gave only approximately one-tenth the theoretical response with *N*-nitrosamines. HBr should be used for the determination of the latter group of compounds.

The preceding method worked well for the determination of several *N*-nitrosamides, including streptozotocin. Because of the high dead volume of the system, however, considerable peak broadening occurred, especially for the late-eluting peaks. The technique was applied to the determination of NMU in fried bacon (detection limit 10 ppb) (Table 1).

In the other method (h), the effluent from the HPLC column was mixed with a mixture of concentrated H_2SO_4, glacial acetic acid, and KI solution, and the mixture was passed through a Teflon reaction coil kept immersed in a 70–80°C water bath. As before, the carrier gas, introduced after denitrosation, carried the liberated NO through a series of cold traps into the TEA. The system worked well with aqueous mobile phases but not with normal-phase solvents. Response for NPRO was linear from 3.5 to 900 ng injection, with a coefficient of variation of 3–5%. The temperature of the reaction coil seemed to be critical for the determination of *N*-nitrosamines, which are difficult to denitrosate with HI. While a temperature of 23°C was adequate for the denitrosation of NPRO and several *N*-nitrosoureas, a much higher temperature (up to 70°C) was required for comparable response from NDMA or NPYR.

Method (d), based on UV photolysis–TEA detection, seemed to give positive response to alkyl nitrites and *C*-nitroso compounds. In this respect, it is less specific than the preceding two HX-catalyzed denitrosation techniques, which give either negligible or no response for such compounds. Data on the actual use of the methods mentioned in Table 2 for the determination of NOC in foods is, however, limited.

The usefulness of these methods would depend largely on the chromatographic resolution of the system employed. For example, cured smoked meats may contain as many as 8–10 *N*-nitrosamino acids and other NOC. Therefore, it would be imperative that the chromatographic system used for the analysis of cured smoked meats by any of the foregoing denitrosation methods give acceptable resolution of these *N*-nitrosamino acids and other NOC (e.g., NDMA, NPIP, NHPYR) that might be present. Future research should be directed toward improving the chromatographic aspects.

VII. HPLC–MASS SPECTROMETRY (HPLC-MS)

Mass spectrometry provides a valuable tool for the characterization and quantitation of trace levels of toxic chemicals in food. In this regard, GC-MS has been more widely used than the HPLC-

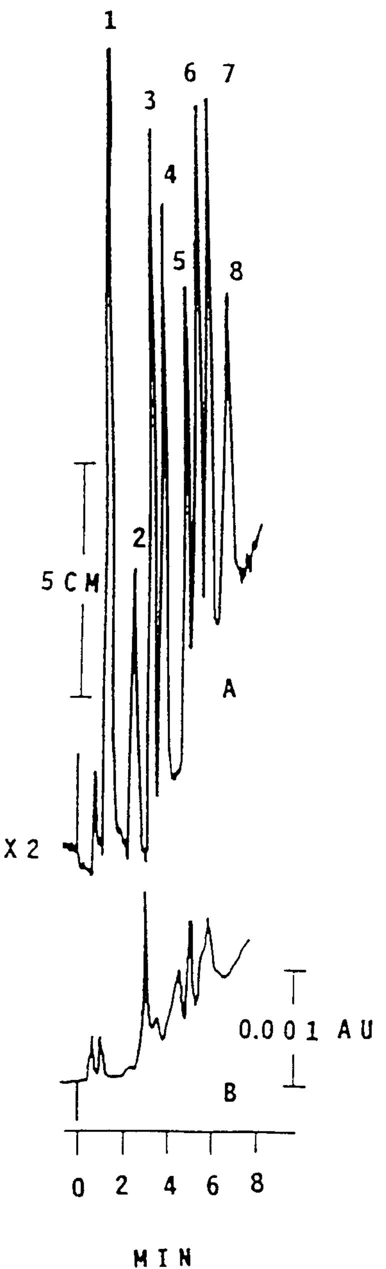

Fig. 7 HPLC analysis of several NOC. (A) UV photolysis, TEA detector. (B) UV (254 nm) detector (58). (1) NHPRO, (2) NSAR, (3) NMU, (4) NPRO, (5) *N*-nitrosothiazolidine-4-carboxylic acid, (6) *N*-nitroso-*N*-ethylurea, (7) MNNG, (8) *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid. (Reproduced from Ref. 58 with permission from the Royal Society of Chemistry, Cambridge, United Kingdom.)

MS techniques, mainly because of its ease of operation, the commercial availability of advanced instrumentation, and the high resolving power of capillary column GC that allows one to carry out specific high-resolution measurement at extremely low levels. The GC-MS techniques are, however, not applicable to all compounds, especially the thermolabile compounds, such as *N*-nitrosamides, or the nonvolatile NOC. Furthermore, direct HPLC-MS analysis of nonvolatile NOC eliminates the need for derivatization.

Although the potential benefits of the HPLC-MS techniques are obvious, the main difficulties lie in interfacing an HPLC system with a MS. Recent progress made in this area has been reviewed (81,82). Various interfacing techniques found to be promising include: (a) moving belt, (b) direct liquid introduction with normal as well as microbore column involving extremely low (10–50 μl/min) mobile-phase flow (83), (c) thermospray, and (d) various forms of atmospheric pressure ionization, including HPLC/MS/MS (81). Eerola et al. (83a) applied HPLC-MS to the determination of NDMA, *N*-nitrosodiethylamine (NDEA), NPYR, and NPIP in dry sausages. Compounds were separated on a Spherisorb ODS 2 column with gradient elution. An atmospheric pressure chemical ionization (APCI) interface operated in the positive-ion mode. While NDMA was detected in the single ion monitoring (SIM) mode, other NOC were detected by tandem MS. Some of these developments are of recent origin, so the necessary instrumentation is quite expensive and unavailable in most laboratories. This may explain the paucity of data in the literature on the application of such techniques to the determination NOC in foods.

Beattie et al. (84) described an HPLC-MS technique for the determination of several NOC, mainly *N*-nitrosamino acids. A moving polyimide belt interface and chemical ionization (ammonia as the ionizing medium) were used for this purpose. Most compounds produced $(M+H)^+$ and $(M+NH_4)^+$ peaks; the base peaks for the *N*-nitrosamino acids were found to result from the loss of both COOH and NO fragments. While the technique provided useful structural information, sensitivity was poor and deemed unsuitable for trace analysis. More recently, Sen et al. (85) used reversed-phase HPLC-thermospray MS technique for the detection of two *N*-nitroso-tetrahydro-β-carboline compounds in one sample of nitrosated Chinese pickled vegetables and one nitrosated cheese. The HPLC-thermospray MS technique has also been used to determine NDPhA in hazardous industrial wastes (86); the technique might be adapted to the analysis of foods. An HPLC-$h\nu$-MS method for the simultaneous detection and confirmation of *N*-nitrosodialkylamines was given by Volmer et al. (86a). They used online photolysis to generate structurally significant photolysis products, followed by electrospray ionization run in the positive-ion mode for the detection of *N*-nitrosodialkylamine acid complexes and in the negative-ion mode for the detection of nitrite and nitrate, respectively. For the detection of NDMA in beer they detected $[NDMAH]^+$ in the SIM mode (Fig. 8).

VIII. APPLICATION OF HPLC TO THE DETERMINATION OF NITROSAMINES IN FOODS

Various HPLC methods that have been reported for the determination of NOC in foods can be divided into three categories: (a) those relating to the development of methodology, (b) those actually using such methods for food analysis, and (c) those of a confirmatory nature in which an HPLC method has been used to confirm GC-TEA findings. Weston in New Zealand used HPLC-TEA for the determination of NDMA in milk powder (87) and malt and beer (88). Dennis et al. (89) and Sen et al. (90) also used HPLC-TEA methods for the determination of *N*-nitrosamino acids, NMA, and other NOC in Icelandic smoked mutton. More recently, Sen et al. (85) used both HPLC-TEA and an HPLC-postcolumn chemical denitrosation technique (method h in Table 2) for detecting the presence of two *N*-nitroso-tetrahydro-β-carbolines in several nitrosated samples

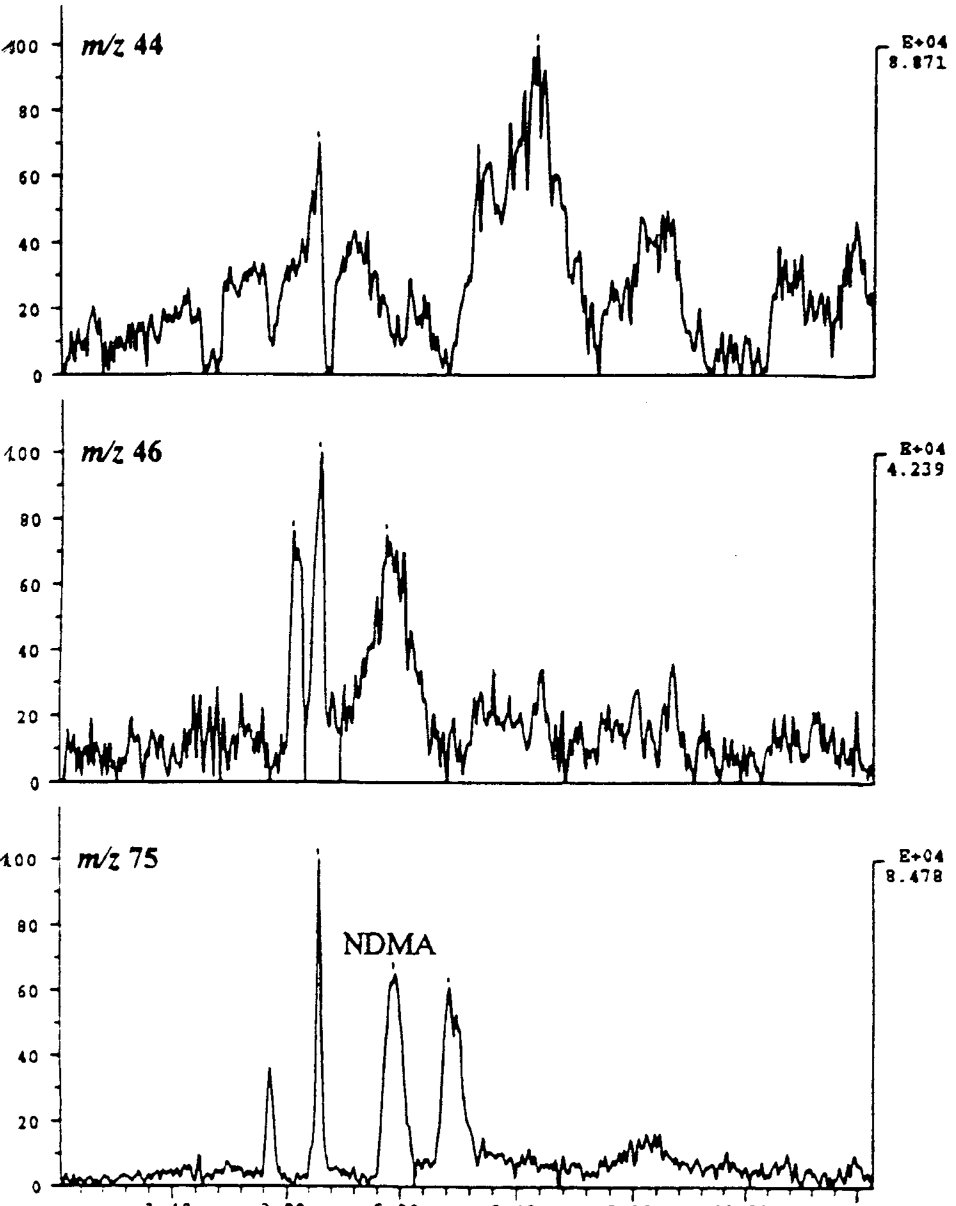

Fig. 8 HPLC-$h\nu$-UV Analysis of NDMA in beer. Extracted ion current profile, in the SIM mode for NDMA-acid complex (*m/z* 75) and that of its dissociative photolysis products, $[(CH_3)_2NH_2]^+$ (*m/z* 46) and $[(CH_3)(CH_2)NH]^+$ (*m/z* 44). (Reprinted with permission from Ref. 86a. Copyright 1996, American Chemical Society.)

of Chinese and Japanese pickled vegetables, soy sauce, and cheese. Several researchers have used HPLC-TEA for confirming initial GC-TEA findings. These include (a) NDMA, NPYR, and NPIP in various dried meats (63), (b) NPYR in instant coffee (91), (c) NDBA in dried fish (92), (d) volatile *N*-nitrosamines in various dried fish and meat and Chinese pickled vegetables (93), (e) NMOR in butter and margarine (94), and NDMA in Scotch whisky and beer (95). There were

good agreements between the GC-TEA and HPLC-TEA data in the last two studies, thus providing additional support to the validity of these findings.

IX. CONCLUSION

Although there are many advantages in using HPLC methods for the determination of various NOC, especially the *N*-nitrosamides and nonvolatile *N*-nitrosamines, in foods only limited application of such techniques have been reported. Some of the newer methods of detection, such as the postcolumn denitrosation techniques, thermospray MS, and atmospheric pressure ionization MS (HPLC/MS/MS), appear highly promising. Future studies should be concentrated on greater use of these techniques and on improving the chromatographic aspects so as to obtain good resolution of various NOC that are likely to occur in foods. There appears to be a need for a simpler extraction-and-cleanup procedure and for a general HPLC method for the rapid screening of foods for the presence of a large number of NOC. This will greatly facilitate the search for hitherto-unidentified NOC in foods, and help provide useful data on the total average daily intake of such compounds by humans through foods and beverages.

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23

Residues of Growth Promoters

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I. INTRODUCTION

The European Union (EU) prohibits the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists (1). In particular, the member states must prohibit (a) the administering to a farm or aquaculture animal, by any means whatsoever, of substances having a thyrostatic, oestrogenic, androgenic, or gestagenic action and of beta-agonists; (b) the holding, except under official control, of animals referred to in (a) on a farm, the placing on the market or slaughter for human consumption of farm animals that contain the substances referred to in (a), or in which the presence of such substances has been established, unless proof can be given that the animals in question have been treated for the exceptional cases, mentioned later; (c) the placing on the market for human consumption of aquaculture animals to which substances referred to in (a) have been administered and of processed products derived from such animals; (d) the placing on the market of meat of the animals referred to in (b); (e) the processing of the meat referred to in (d).

For therapeutic purposes and under the control of a responsible veterinarian, the administration to farm animals of 17β-estradiol, testosterone, and progesterone and derivatives that readily yield the parent compound on hydrolysis after absorption at the site of application may be authorized by the individual EU member states. Also, for therapeutic purposes, the administration of authorized veterinary medicinal products containing (i) allyl trenbolone, administered orally, or beta-agonists to *equidae* and pets, provided they are used in accordance with the manufacturer's instructions, (ii) beta-agonists, in the form of an injection to induce tocolysis in cows when calving may be authorized. Again, these veterinary medicinal products must be administered by a veterinarian under his direct responsibility.

Certain zootechnical treatments constitute the second exception to the general prohibition of medicinal products having an estrogenic, androgenic, or gestagenic action. In particular, EU member states may allow the synchronization of estrus and the preparation of donors and recipients for the implantation of embryos. With regard to aquaculture animals, young fish may be treated for the first three months, for the purpose of sex inversion, with veterinary medicinal products that have an androgenic action.

It is immediately clear that a sound analytical methodology is indispensable for monitoring compliance with the directive. The organization of the monitoring of Council Directive 96/22/EC is laid down by Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products (2). It contains, among others, the sampling

strategy and the instructions for sampling levels and frequency, which are at the base of the so-called national surveillance plans, which each member state has to implement. More detailed rules on official sampling for the monitoring are laid down by Commission Decision 97/747/EC of 27 October 1997 (3).

Veterinary drugs in general are used for both therapeutic and prophylactic purposes. A third application is the use of certain compounds or mixtures of compounds with a view to better breeding efficiency. To these belong, among others, the steroids and other substances that have similar pharmacological activity and are used to improve the efficiency of protein conversion. The better this conversion, the faster the animal grows and the earlier it can be slaughtered. These are obvious economic reasons that do not take into account possible harmful effects for the consumer of the products derived from the carcass of the slaughtered animal.

Another group of compounds are the thyrostats, which are sometimes called antihormones because they inhibit the activity of the thyroid gland.

A new class of compounds found its way into animal breeding about a decade ago. The beta-agonists, sometimes referred to as repartitioners, are licensed only for respiratory diseases in cattle and horses. They are being used, however, because they increase the ratio of lean tissue to fat.

Finally, the corticosteroids, of which dexamethasone is probably the most frequently used representative, are also being used, apparently with varying success. There is still no unanimity as to their mode of action.

An important feature of thyrostats, beta-agonists, and corticosteroids is that they are in general orally active. This means that they can be given via the fodder or drinking water, leaving no trace of percutaneous administration, which is one of the annoying indications when orally inactive steroids are injected.

Only two active substances, belonging to the compounds currently but illegally used as growth promoters, have been assigned a maximum residue limit (MRL) (4). Dexamethasone concentrations in muscle, liver, and kidney of bovine, porcine, and *equidae* species may not exceed 0.75 μg/kg, 2 μg/kg, and 0.75 μg/kg respectively. In bovine milk 0.3 μg/kg is the upper limit. Similarly, provisional MRLs, expiring on July 1, 2000, have been set for clenbuterol in bovine liver, kidney, muscle, and milk and in liver, kidney, and muscle of Equidae. It is explicitly mentioned for the former animal species that clenbuterol can be used only for tocolysis in parturient cows and for the latter species only for tocolysis and the treatment of respiratory aliments.

The U.S. Food and Drug Administration policy is based on the conclusions of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In its Thirty-Second Report (1988), it concluded, on the basis of its safety assessment of residues of estradiol-17β, progesterone, and testosterone, and in view of the difficulty of determining the levels of residues attributable to the use of these hormones as growth promoters in cattle, that it was unnecessary to establish an acceptable residue level. As to trenbolone acetate (TBA), a synthetic steroid with anabolic properties, JECFA concluded that its safety assessment could be based on establishing the no-hormonal-effect level. It therefore recommended a maximum residue level of 2 μg/kg for β-trenbolone in meat and of 10 μg/kg for α-trenbolone in liver on the basis of a daily intake by a 70-kg person of 500 g of meat. β-Trenbolone is the major metabolite in muscle. For zeranol, an acceptable residue level of 10 μg/kg for bovine liver and 2 μg/kg for bovine muscle was established.

II. GENERAL ANALYTICAL APPROACH

Within the European Union, enlarged in 1995 with three new member states, the need for a standardized and uniform methodology for the detection of residues has been recognized. The rou-

tine analytical procedures authorized for detecting residues of substances having a hormonal or thyrostatic action shall be immunoassay, thin-layer chromatography, liquid chromatography, gas chromatography, mass spectrometry, spectrometry, or any other method that fulfills comparable criteria to those laid down for related methods. Samples for analysis shall be taken in accordance with the following rules:

1. The sample must be representative and of sufficient size to allow adequate analysis and to allow a repeat analysis and any confirmatory analysis.
2. The sample must be marked in such a way that identification remains possible at all times.
3. The sampling procedure, packaging, preservation, transport, and storage of the samples must be such as to maintain their integrity and not prejudice the result of the examination. Unauthorized access to the samples must be prevented.

From the annex of Commission Decision 93/256/EEC (5), a few interesting definitions can be taken, which are reproduced or summarized next.

Routine methods of analysis are defined as methods of analysis to be used by member states to implement national plans for the control of residues in food-producing animals and their products in compliance with Council Directive 96/23 (2). Routine methods must have been validated by operational laboratories and must fulfill the relevant criteria set out in the annex of Commission Decision 93/256 (5). They may be used for screening and/or confirmatory purposes.

Screening methods are methods that are used to detect the presence of an analyte or class of analytes at the level of interest. These methods have a high sample thoughput and are used to sift large numbers of samples for potential positives. They are aimed at avoiding false-negative results.

Confirmatory methods are methods that provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest. These methods are aimed at preventing false-positive results as well as having an acceptable probability of false-negative results.

Reference material is a material of which one or several properties have been confirmed by a validated method so that it can be used to calibrate an apparatus or to verify a method of measurement.

A positive result is one that proves the presence of the analyte in the sample according to the analytical procedure when the general criteria and the criteria specified for the individual detection method are fulfilled. For substances with a zero tolerance, the result of the analysis is "positive" if the identity of the analyte in the sample is proven unambiguously. For substances with an established MRL, the result of the analysis is "positive" if the experimentally determined content of the analyte in the sample (after applying any correction for recovery) is greater than the established maximum residue limit, which takes into account the acceptable probability of obtaining false-positive or false-negative results.

A negative result is the result of an analysis regarded as "negative" according to the analytical procedure when the general criteria and the criteria specified for the individual detection method are fulfilled in the case of appropriate reference materials and blank determinations and (a) in the case of substances for which there is a zero tolerance, the identity of the analyte has not been proven unambiguously or (b) in the case of substances with an established maximum residue limit, the measured content

of the analyte in the sample is below the level specified for a positive result. A negative result does not prove in case (a) that the analyte is absent from the sample or in case (b) that the true content of the analyte is below the maximum residue limit.

The general requirements as to the methods used for the detection of residues, as laid down in Commission Decision 93/256, are summarized in the following text.

A. Screening Methods

Fixed requirements cannot be set for screening methods. The most important aspect of performance is that the incidence of false-negative results at the level of interest be minimal.

1. Specificity

This must be defined.

2. Accuracy and Precision

Quantification may not be necessary. Depending on whether the use of a substance is prohibited or authorized, a screening method may be qualitative or quantitative. False-positive results are acceptable, but false-negative results at the level of interest should be minimal.

3. Limits of Detection

These should be appropriate for the purpose. For substances with an established maximum residue limit, the limit of detection must be sufficiently low to detect residues at this level. For substances that are not authorized for use in food-producing animals, the limit of detection should be as low as possible.

4. Practicability

A high sample throughput capability is desirable, and costs should be low.

B. Confirmatory Methods

1. Specificity

As far as possible, confirmatory methods must provide unambiguous information on the chemical structure of the analyte. When more than one compound gives the same response, then the method cannot discriminate between these compounds. Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods.

If a single technique lacks sufficient specificity, the desired specificity may be achieved by analytical procedures consisting of suitable combinations of cleanup, chromatographic separation(s), and spectrometric or immunochemical detection, e.g., gas chromatography/mass spectrometry (GC-MS), liquid chromatography/mass spectrometry (LC-MS), immunoaffinity chromatography (IAC)/GC-MS, GC-IR or LC/immunography (IMG). An immunogram is a graphical plot of immunochemical response versus retention time or elution volume as obtained from chromatographic separation with (in general, offline) immunochemical detection of the components of the sample extract.

2. Accuracy

In the case of repeated analysis of a reference material, the guideline ranges for the deviation of the mean experimentally determined content (after applying any correction for recovery) from the true value are as follows:

True content (mass fraction)	Range
<1 μg/kg	−50% to +20%
1–10 μg/kg	−30% to +10%
>10 μg/kg	−20% to +10%

3. Precision

In the case of repeated analysis of a reference material under reproducibility conditions, typical values for the interlaboratory coefficient of variation (CV), calculated according to the Horwitz equation, $(CV(\%) = 2^{(1 - 0.5 \log C)}$, where C is the content expressed as a power of 10, are as follows:

Content (mass fraction)	CV
1 μg/kg	45%
10 μg/kg	32%
100 μg/kg	23%
1 mg/kg	16%

For analyses carried out under repeatability conditions, the intralaboratory CV would typically be between one-half and two-thirds of these values. The limit of detection, limit of determination, and sensitivity should be adequate for the purpose.

4. Practicability

Speed and cost are of less importance, than are the screening methods. For confirmatory methods, most aspects of practicability are of minor significance compared with the other criteria defined in this decision. It is usually sufficient that the required reagents and equipment are available.

5. Calibration Curves

If the method depends on a calibration curve, then the following information must be given:

1. The mathematical formula that describes the calibration curve
2. Acceptable ranges within which the parameters of the calibration curve may vary from day to day
3. The working range of the calibration curve

Whenever possible, suitable internal standards and reference materials should be used for the quality control of calibration curves of confirmatory methods, and details of the variance of the variables, which is valid at least for the working range of the calibration curve, should be given.

6. *Susceptibility to Interference*

For all experimental conditions that could in practice be subject to fluctuation (e.g., stability of reagents, composition of the sample, pH, temperature), any variations that could affect the analytical result should be indicated. The method description shall include some means for overcoming any foreseeable interference. If necessary, alternative detection principles suited for confirmation shall be described.

If cochromatography is carried out, then only one peak should be obtained, with the enhanced peak height (or area) being equivalent to the amount of added analyte. With GC or LC, the peak width at half maximum height should be within the range 90–110% of the original width, and the retention times should be identical within a margin of 5%. For TLC methods, only the spot presumed to be due to the analyte should be intensified; a new spot should not appear, and the visual appearance should not change.

It is of prime importance that any interference that might arise from matrix components be investigated.

7. *Relationship Between Permitted Residue Levels and Analytical Limits*

For substances that are not authorized for use in food-producing animals, the limit of detection of the analytical method must be sufficiently low that residue levels that would be expected after illegal use will be detected with at least 95% probability. For substances with an established maximum residue limit, the limit of determination of the method plus three times the standard deviation that the method produces for a sample at the maximum residue limit shall not exceed the established maximum residue limit.

For substances with an established maximum residue limit, the method should be validated at that limit and at one-half and twice the limit.

Criteria for the identification and quantification of residues are also taken up in Commission Decision 93/256 (5). Specific criteria for identification by means of GC or LC using nonspecific detection are established. The analyte should elute at the retention time that is typical for the corresponding standard analyte under the same experimental conditions. The nearest peak maximum in the chromatogram should be separated from the designated analyte peak by at least one full width at 10% of the maximum height. For additional information, cochromatography may be used and chromatography using at least two columns of different polarity may be used.

For identification by liquid chromatography coupled to spectrometric detection, e.g., with diode array detection (DAD), additional criteria are applicable. The absorption maxima in the spectrum of the analyte should be at the same wavelengths as those of the standard analyte within a margin determined by the resolution of the detection system. For diode array detection this is typically within ± 2 nm. The spectrum of the analyte above 220 nm should not be visually different from the spectrum of the standard analyte for those parts of the two spectra with a relative absorbance of no less than 10%. This criterion is met when the same maxima are present and at no observed point is the difference between the two spectra more than 10% of the absorbance of the standard analyte. For confirmatory purposes, if the method is not used in combination with other methods, cochromatography in the LC step is mandatory.

There are not yet specific criteria set for LC-MS. However, it is clear that the MS criteria of Commission Decision 93/256 fully apply to any method where low-resolution MS is used as the detection system. For use in screening methods, the intensity of at least the most abundant di-

agnostic ion must be measured. For use in confirmatory methods, the intensities of preferably at least four diagnostic ions should be measured. If the compound does not yield four diagnostic ions with the method used, then the identification of the analyte should be based on the results of at least two independent LC-MS methods with different derivatives (although not very likely in LC) and/or ionization techniques, each producing two or three diagnostic ions. The molecular ion should preferably be one of the diagnostic ions selected. The relative abundances of all diagnostic ions monitored from the analyte should match those of the standard analyte, preferably within a margin of $\pm 10\%$ (electron impact mode) or $\pm 20\%$ (chemical ionization mode).

Nowadays these criteria are under heavy discussion. It is clear that they are not in line with the potential of most of modern analytical techniques. At the time of the editing of this chapter, a revised version was in preparation.

In none of the papers taken up in this review is reference made to the EU criteria. On the other hand, there is an increasing trend towards thorough validation of the methodology. The application of the EU criteria for identification, as well for screening purposes as for confirmation analysis, is provisionally a matter for the field laboratories involved in monitoring programs for the illicit use of growth promoters in animal husbandry.

The vast majority of the papers published concerning the detection of growth promoters are related to matrices such as urine, feces, injection sites, and plasma. Interest in detecting and measuring residue concentrations in edible parts of the animals arose less than a decade ago. Hormone residue analysis in foods was comprehensively reviewed by Ryan in 1975 (6) and by Shepherd in 1991 (7). A review of sample pretreatment methodology in drug residue analysis was presented by Haagsma (8).

Analytical strategies for the screening of veterinary drugs and their residues in edible products were comprehensively reviewed by Aerts et al. (9). A general review was dedicated by Van Ginkel et al. (10) to liquid chromatographic purification and detection of anabolic compounds and by Jansen et al. (11) from the same group to high-performance liquid chromatographic separation and detection methods for anabolic compounds. The influence of the matrix and of the method applied to the detection of anabolic residues in biological samples was studied by Smets et al. (12). Several authors have made considerable contributions to the liquid chromatographic separation and detection of steroid hormones, at the nonresidue concentration level, such as illegal preparations of reference compounds (13–16). The same applies to beta-agonists. Methods for the determination of beta-agonists in biological matrices have been reviewed by Boyd et al. (21). Several authors have made contributions to the analysis of beta-agonists.

In this review, attention is focused on detection and quantification methods of residues of anabolic steroids, corticosteroids, and beta-agonists, which make use of HPLC.

III. SURVEY OF GROWTH PROMOTERS USED IN ANIMAL BREEDING

Hormones used in animal breeding can be classified according to scheme of Hoffmann and Karg (22). Table 1 shows their original classification augmented with compounds introduced later, based on data obtained from 9 years of practical experience with the analysis of injection sites and collected at Belgian slaughterhouses (23). The chemical structures of the most common compounds are shown in Fig. 1. The chemical structure of the corticosteroids, most often encountered in illicit preparations for animal breeding but also as residues of a treatment, are shown in Fig. 2. The chemical structures of a number of closely related beta-agonistic drugs are shown in Fig. 3. The list of those compounds encountered in illegal preparations or animal feed is growing continuously. Depending on the nature of the R_3 substituent, they can be divided into two major classes: substituted anilines and substituted phenols.

Table 1 Classification of Hormones Used in Animal Breeding

	Pharmacological activity	
Basic classification	Estrogenic	Nonestrogenic
Endogenous steroids	17β-Estradiol	Progesterone Testosterone
Exogenous steroids	17β-Estradiol (esters) Ethinylestradiol	Testosterone (esters) Trenbolone (acetate) Methyltestosterone Nortestosterone (esters) Boldenone Methylboldenone Stanozolol Chlorotestosterone (acetate) Fluoxymesterone Norethandrolone Methandiol Ethylestrenol Medroxyprogesterone acetate Chlormadinone acetate Megestrol acetate Algestone acetophenide 17α-Hydroxyprogesterone acetate 17α-Hydroxyprogesterone caproate Delmadinone acetate Norethisterone Norgestrel
Nonsteroidal structures	Diethylstilbestrol Hexestrol Dienestrol Zeranol	—

Fig. 1 Chemical structures of 17β-estradiol (I), progesterone (II), testosterone (III), ethinylestradiol (IV), trenbolone (V), methyltestosterone (VI), nortestosterone (VII), boldenone (VIII), methylboldenone (IX), stanozolol (X), chlorotestosterone (XI), fluoxymesterone (XII), medroxyprogesterone acetate (XIII), chlormadinone acetate (XIV), megestrol acetate (XV), algestone acetophenide (XVI), 17α-hydroxyprogesterone acetate (XVII), 17α-hydroxyprogesterone caproate (XVIII), delmadinone acetate (XIX), norethisterone (XX), diethylstilbestrol (XXI), dienestrol (XXII), hexestrol (XXIII), and zeranol (XXIV).

III

IV

V

VI

VII

VIII

IX

X

XI

XII

Fig. 1 *(continued)*

XIII XIV

XV XVI

XVII XVIII

XIX XX

XXI XXII

Fig. 1 *(continued)*

XXIII

XXIV

Fig. 1 *(continued)*

XXV

XXVI

XXVII

XXVIII

XXIX

XXX

Fig. 2 Chemical structures of dexamethasone (XXV), betamethasone (XXVI), flumethasone (XXVII), prednisolone (XXVIII), methylprednisolone (XXIV), and triamcinolone (XXX).

Beta-agonist	R_1	R_2	R_3	R_4	R_5
Clenbuterol	H	Cl	NH_2	Cl	CH_3
Mabuterol	H	CF_3	NH_2	Cl	CH_3
Mapenterol	H	CF_3	NH_2	Cl	CH_2-CH_3
Cimaterol	H	CN	NH_2	H	H
Salbutamol	H	H	OH	CH_2OH	CH_3
Terbutaline	H	OH	H	OH	CH_3
Carbuterol	H	H	OH	$NHCONH_2$	CH_3
Tulobuterol	Cl	H	H	H	CH_3

Fig. 3 Chemical structure of a number of closely related beta-agonistic drugs.

IV. SAMPLE PREPARATION

Sample preparation usually begins with a homogenization with an appropriate aqueous solution or with water, followed by a centrifugation step. This treatment may yield a crude extract that is appropriate as such for immunochemical detection. In most instances, however, the high degree of impurity of this type of extracts does not allow direct analysis by chromatography, coupled to one or another type of spectrometry as the detection system. This is particularly the case for concentrations below the microgram-per-kilogram (ppb) range. The specificity of the final detection principle may mitigate this requirement to a certain extent.

An alternative approach to mechanical homogenization is enzymatic digestion of the tissue by means of proteolytic enzymes such as subtilisin (24). The mechanically obtained homogenate, as well as the enzymatic digestate, are then extracted with an organic solvent or a mixture of organic solvents. After evaporation of the organic phase, the residue may be taken up in an appropriate buffer solution to enable enzymatic cleavage of conjugates. The most common preparation for this purpose is the juice of the snail *Helix pomatia*, which has sulfatase and glucuronidase activity.

A purification step that may be joined to the organic-phase extraction is acid–base partitioning, which may be applied to easily ionized substances.

Formerly, the method of choice for a more or less thorough purification of the crude extract was column chromatography. The sample extract was applied on the top of a glass column filled with an adsorptive material such as silica, alumina, Celite, or carbon. An organic solvent or a mixture of solvents was allowed to migrate through the adsorbent bed, during which an efficient separation of the analytes and the bulk of interferences was supposed to take place.

About a decade ago a valuable alternative to conventional column chromatography became available. Disposable columns containing a fixed amount of adsorptive material allow a simple, fairly inexpensive, and easy-to-perform purification of the crude extract. The technique is usually referred to as solid-phase extraction (SPE). Straight-phase (silica, cyano, amino) as well as reversed-phase (C_{18}, C_8) materials are available in various container sizes (1, 5, 10 ml). The classical principles of analyte–stationary phase interactions are valid, so the eluent composition will allow the optimization of separation efficiency. Such SPE columns are low-resolution chromatographic systems. They offer a rough cleanup of the crude extract, which might not be sufficient for some detection methods, such as low-resolution mass spectrometry. Therefore, many methods appeal to HPLC with automated fraction collection. Fractions containing the analyte of interest are evaporated to dryness, yielding a residue that in most cases is suitable for gas chromatographic detection after suitable derivatization. Because of the relative insensitivity of the UV detector, online monitoring of the HPLC effluent is rarely performed. There are a few applications of electrochemical detection.

A relatively new technique that has been launched for the determination of veterinary drug residue is matrix solid-phase dispersion (MSPD) as proposed by Barker et al. (25). Basically, MSPD consists of grinding the tissue thoroughly with C_{18} packing material to ensure extended contact of the residues with the extracting solvent. The mixture is put in a 10-ml syringe-barrel column and eluted with appropriate solvents after washing out interfering substances with other eluents.

Probably the most contemporary innovation in sample cleanup is the technique of immunoaffinity chromatography (IAC). A small column is filled with a gel to which antibodies against a particular compound or some of its metabolites are immobilized by covalent binding. When an extract containing those compounds is passed through the column, these are retained by the specific antigen/antibody reaction. Impurities are washed off with appropriate solutions, after which desorption takes place by reversible denaturation of the antibodies with an elution solvent, which contains high concentrations of methanol or ethanol. Such columns can be regenerated and reused for up to 100 times. Several different antibodies can be bound in one column to provide a multianalyte capability. The main disadvantage, despite the possibility of the repeated use of the column, is that substantial amounts of antibody are required. A review of the applicability and limitations of IAC in multiresidue analysis of growth promoters has been published by Van Ginkel (26). A major problem associated with all immunochemical methods is the availability of the antibody. First the immunogenic response is not guaranteed and failures are common. Furthermore, an animal may take from 6 months to 3 years to respond satisfactorily to the antigen.

V. SEPARATION TECHNIQUES

Despite the relative importance of these cleanup techniques, HPLC still holds a central position in the residue analysis for growth promoters, be it as a fractionation step prior to the final determination by gas chromatography, coupled to mass spectrometry as the detection principle, or as the final separation step, coupled either to mass spectrometry or to conventional detection systems such as UV-DAD and electrochemistry.

Most chromatographic methods seek a compromise between the length of sample preparation and the required specificity of analyte detection. Because, except for dexamethasone and clenbuterol, there are no maximum residue limits for growth promoters, the emphasis in most of the published methods is laid on specificity rather than on accuracy and precision. The use of

isotope-labeled internal standards is limited because of the unavailability of most deuterated homologs of the analytes. Details on general chromatographic separation procedures are abundantly available in the literature.

VI. APPLICATIONS IN FOOD ANALYSIS

In this section only references published in the last 10 years (since 1989) will be reviewed.

The target matrices for the detection of residues of growth promoters in edible products of animal origin are mainly muscle tissue, liver, kidney, fat, and to a lesser extent milk and eggs. All these materials have a highly complex composition that is subject to variations, depending on animal species, tissue type, and the nutritional regime of the animal. In most cases the parent compound—the molecule that has been administered—is considered the target molecule and therefore is traced. Very little is known about the chemical form in which residues are deposited in the animal tissues. A well-known exception is 19-nortestosterone. The pharmacologically active form in which it is administered has the hydroxyl group at position 17 in the beta-configuration. Part of the dose is metabolically converted into the 17α configuration, which is the predominant form in urine. Another one is trenbolone, which occurs in the α-configuration in the liver and in the β-configuration in muscle tissue.

The applications of liquid chromatography to food analysis are summarized in Table 2 (methods used for anabolics) and Table 3 (methods used for beta-agonists).

There is only a limited amount of methodology for corticosteroids in animal tissues. The first method ever published concerns the determination of dexamethasone in bovine tissues by coupled-column normal-phase high-performance liquid chromatography and capillary gas chromatography combined with mass spectrometry (73). Crude extracts are obtained by means of a three-phase liquid–liquid extraction scheme. The resulting residue is subjected to coupled-column normal-phase HPLC, which serves to isolate the drug for the purpose of screening and quantification. A sample is injected onto the first column of the system, a phenyl column, from which a heart cut is diverted to a short silica column. The contents of this column are backflushed onto a cyanopropyl column, which isolates dexamethasone. Quantification is performed by UV response at 239 nm. Identity confirmation was carried out by GC-MS analysis of the TMS-enol-TMS derivatives. Shearan et al. (74) published a method for the determination of dexamethasone in tissues by HPLC. Following extraction with ethyl acetate (muscle, kidney, liver) or a diethyl ether (fat) and cleanup of the tissue extract, the drug residue is isolated using C_{18}-SPE. Determination is carried out by RP-HPLC, with UV detection at 254 nm.

The determination of dexamethasone in bovine, porcine, and ovine liver and muscle has been reported by Mallinson et al. (75). In liver the procedure consists of extraction with ethyl acetate from an alkaline homogenate. After centrifugation the organic supernatant ondergoes silica gel SPE. Muscle tissue is subjected to an acetonitrile/hexane partitioning step. Analysis of the crude extracts is performed by reversed-phase HPLC on Lichrosorb RP-18. Detection is achieved by UV absorption at 239 nm. For confirmation, GC-MS of the 11,17-diketo derivative of dexamethasone is carried out. Arts et al. (76) make use of a radioimmunoassay and confirm the positive urine and liver samples with LC-MS. Bagnati et al. (77) use multiple reaction monitoring HPLC-MS with an ion spray source to detect corticosteroids in urine and tissues.

Different LC methods for the determination of corticosteroids in biological matrices have been compared by Stolker et al. (78). A review of the HPLC analysis of corticosteroids, with no special attention to food as the matrix, has been published by Volin (79). Mixtures of reference standards of corticosteroids are separated by Gonzalo-Lumburas et al. (80).

Table 2 Chromatographic Methods Used for Anabolics

Analyte	Sample type	Sample preparation	Sample cleanup: Stationary phase	Sample cleanup: Mobile phase	Sample cleanup: Detection	Separation	Detection	Ref.
Melengestrol acetate	Bovine tissues	Homogenization in water, extraction in CH_3CN, liquid–liquid partition	Spherisorb phenyl 3 μm; silica 5 μm	$CH_2Cl_2/CH_3OH/H_2O$ (100:5:0.1)	UV, 254 nm	HPLC	UV, 287 nm	27
Anabolics	Meat	Enzymatic digestion	Immunoaffinity chromatography (IAC)			GC	MS of di-HFB derivatives	28
19-nortestosterone	Tissues (meat, liver, kidney)	Enzymatic digestion, XAD-2 column chromatography, deconjugation (liver, kidney), online IAC	Chromospher C_{18}, 5 μm	CH_3CN/H_2O (35:65)	UV, 247 nm	GC	MS of TMS derivatives	29
Anabolics	Fatty tissues	Extraction in hexane, CH_3OH–acetate buffer pH 5.2 partition, back-extraction in CH_2Cl_2	C_{18}	CH_3OH	—	HPTLC	H_2SO_4/CH_3OH	30
Medroxyprogesterone acetate	Kidney, fat	Extraction in petroleum ether (40–60°C) SPE (silica)	Lichrospher Diol 5 μm	Petroleum ether/ethylacetate (90/10)	—	—	RIA[a]	31
Melengestrol acetate	Beef fat	Extraction in hexane, liquid–liquid partition	Silica TLC	Hexane	UV, 254 nm	GC	MS	32
Anabolics	Muscle tissue	Extraction in *t*-butylmethylether SPE (RP18)	Nucleosil C_{18}, 5 μm	Tris acetate (pH 7.2)/CH_3CN (gradient)	UV, 340 nm	—	RIA	33
Methyl-testosterone	Trout	Extraction in $CHCl_3/CH_3OH$ (2:1), liquid–liquid partition	Lipidex-5000 Silica SPE	— —	— —	HPLC	Fluorescence (enzyme reaction)	34
Diethylstilbestrol	Animal tissue	Extraction in *t*-butylmethylether, 1 N NaOH partition	C_{18}	CH_3OH/H_2O (80:20)	—	HPLC	Electrochemical	35
Nortestosterone, methyltestosterone	Meat	Enzymatic digestion, extraction in diethylether, SPE (C_{18})	Lichrospher RP 18, 5 μm	CH_3OH/H_2O (65/35)	—	—	RIA	36
Anabolic steroids	Animal tissues	Homogenization in CH_3OH, SPE (Carbopack B), SPE (Amberlite CQ-400 I)	C_{18}, 5 μm	CH_3CN/0.01 M, KH_2PO_4 (48/52)	UV, 242 nm	—	Electrochemical	37
Anabolic steroids	Muscle tissue	Enzymatic digestion, extraction in ether, SPE on C_{18}	RP-18, 5 μm	CH_3OH/H_2O (65:35)	—	GC	MS HFB[b] and/or TMS derivatives	38

(continued)

Table 3 Methods Used for Beta-Agonists

Analyte	Sample type	Sample preparation	HPLC			Separation	Detection	Ref.
			Stationary phase	Mobile phase	Detection			
Clenbuterol	Liver, muscle, feedstuffs	Enzymatic digestion, liquid–liquid extraction	Silica	NH_3/CH_3OH (0.25/99.75)	—	GC	MS of TMS[a] derivatives	47
Clenbuterol	Animal tissues	Enzymatic digestion, SPE (Chem Elut)	Lichrosorb RP-8	NaAc 0.01 M/acetonitrile (30/70)	—	—	Colorimetry HPTLC	48
Beta-agonists	Cattle tissue, feed	Homogenization in aqueous solution, SPE (C_{18}), IAC	—	—	—	GC	MS of TMS derivatives	49
Beta-agonists	Liver	Enzymatic digestion, liquid–liquid extraction, SPE (alumina)	—	—	—	GC	MS of TMS derivatives	50
Clenbuterol, cimaterol	Animal tissues, feedstuffs	Enzymatic digestion, extraction in dilute acid, SPE (Chem Elut)	Nova-Pak C_{18}	buffer pH 3.5/acetonitrile (53/47)	—	—	Colorimetry	51
Clenbuterol, cimaterol	Liver	Homogenization in aqueous solution, SPE (C_{18})	RP-Select B	NaAc 0.01 M/acetonitrile (30/70)	—	—	Colorimetry	52
Salbutamol	Liver	Homogenization in dilute acid, IAC	RP-Bondapack C_{18}	—	—	—	Fluorescence	53
Clenbuterol	Meat	Enzymatic digestion, IAC	—	—	—	—	ELISA[b]	54
Clenbuterol	Liver	Homogenization in aqueous solution, IAC	—	—	—	GC	MS of TMS derivatives	55
Clenbuterol	Bovine liver	Homogenization in aqueous solution, preincubation with immobilized antibody	—	—	—	—	EIA[c]	56
Clenbuterol	Liver	Matrix solid-phase dispersion (MSPD) with C_{18}	—	—	—	—	EIA	57
Salbutamol	Liver	Enzymatic digestion, SPE (C_{18})	—	—	—	—	EIA	58
Clenbuterol	Tissues	Enzymatic digestion, liquid–liquid extraction	—	—	—	—	EIA	59
Clenbuterol	Meat	Enzymatic digestion, IAC	—	—	—	—	ELISA	60
Clenbuterol, salbutamol	Tissue samples	Homogenization in aqueous solution, heat deproteinization, liquid–liquid extraction, IAC	—	—	—	—	Chemiluminescence IA	61

(continued)

Table 3 Continued

Analyte	Sample type	Sample preparation	HPLC			Separation	Detection	Ref.
			Stationary phase	Mobile phase	Detection			
Clenbuterol, salbutamol	Swine muscle	Homogenization in acetonitrile, liquid–liquid extraction, SPE (C_{18})	NovaPak C_{18}	Acetonitrile/water (gradient)	UV (DAD)	—	—	62
Ractopamine	Swine, cattle, turkey feeds	Liquid–liquid extraction, SPE (silica)	Partisil 5 ODS-3	Ammonium phosphate buffer/acetonitrile 77/23	—	—	Coulometry	63
Ractopamine	Swine, turkey tissue	Homogenization in methanol, liquid–liquid extraction, SPE (silica)	LC-18-DB	Ammonium phosphate buffer/acetonitrile 80/20	—	—	Coulometry	64
Beta-2-agonists	Milk replacer	Precipitation of proteins, SPE (C_{18})	Spheri-5 RP-8	Methanol/ammonium acetate 0.05 M 60/40	UV	—	MS	65
Clenbuterol	Retina	Sonication-aided extraction in phosphate buffer, SPE (C_{18}), liquid–liquid extraction	Ultrabase C8	Acetonitrile/ammonium phosphate pH 2.8 25/75	—	—	UV	66
Ractopamine	Swine serum	Dilution in phosphate buffer, SPE (cation exchange)	LC-18-DB	Ammonium phosphate/acetonitrile 79/21.5	—	—	Coulometry	67
Beta-agonists	Liver	Homogenization in aqueous solution, deconjugation, SPE (SCX)	C_{18}	Acetonitrile/ammonium acetate (gradient)	—	—	MS-MS	68
Clenbuterol	Bovine hair	Dissolution in alkaline medium, liquid–liquid extraction	NovaPak C_{18}	Methanol/ammonium acetate 0.1 M (60/40)	—	—	Coulometry (MS)	69
Beta-agonists	Bovine retina	Sonication-aided extraction in dilute acid, SPE (C_{18})	NovaPak C_{18}	Acetonitrile/ammonium acetate 0.01 M (90/10)	—	—	APCI-MS[d]	70
Clenbuterol	Beef liver, minced beef	Homogenization in dilute acid solution, SPE (cation exchange), IAC	Super-ODS	Ammonium acetate 14.7 mM/acetonitrile/H_2O (gradient)	—	—	Electrospray MS	71
Clenbuterol	Bovine retina	Sonication-aided extraction in dilute acid solution, liquid–liquid extraction	Lichrospher 100, RP-18e	Methanol/water (34:66) + 1% formic acid	—	—	Amperometry	72

[a] TMS: trimethylsilyl
[b] ELISA: enzyme-linked immunosorbent assay
[c] EIA: enzyme immunoassay
[d] APCI-MS: atmospheric pressure chemical ionization mass spectrometry

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Determination of Anions and Cations in Food by HPLC

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I. INTRODUCTION

The determination of inorganic anions and cations in food is of obvious importance from a health-related viewpoint. With respect to cations, the alkali metals Na^+ and K^+ are essential to maintenance of a proper electrolyte balance, and excessive levels of Na^+ have been linked to high blood pressure. The alkaline earths Mg^{2+} and Ca^{2+} are important for bone growth, and concentrations of these cations plus Na^+ and K^+ in infant formulas are regulated by the U.S. government. Environmental hazards such as arsenic in seafood and transition metals such as Cu^{2+} and Ni^{2+} in vegetables are of importance. Certain anions are likewise routinely determined analytically. Halogenated anions, such as bromate (BrO_3^-) added to improve the strength of flour and reduce fermentation time as well as I^- in table salt, are measured in the presence of chloride. Nitrate (NO_3^-) and nitrite (NO_2^-) are commonly quantified in meat products, since nitrite is added as a preservative and color enhancer. Nitrite is also of concern since it can react with secondary amines to form carcinogenic nitrosamines. The determination of sulfite (SO_3^-) is of utmost importance since it is commonly added to a wide variety of foods, such as produce, seafood, and bakery products, to act as an antioxidant or antimicrobial growth factor. Sulfate (SO_4^{2-}) and phosphate (HPO_4^{2-}) are also sometimes assayed.

A variety of analytical methods have been utilized for the analysis of food samples for ions. Atomic spectroscopy is commonly employed for the determination of cations. In particular, atomic absorption spectroscopy (AAS) has been used when one or two cations are of specific interest. Matrix effects, such as phosphate in the determination of Ca^{2+}, can be a problem, and standard addition methods may be required. Simple flame emission will permit the easy determination of alkali metals, while inductively coupled plasma (ICP) is well suited for the simultaneous determination of many metals at the trace level. However, the ICP instrument is still quite expensive. A few cations, such as Na^+, K^+, and Ca^{2+}, can be determined using ion-selective electrodes (ISE). In addition, some anions, such as the halides (F^-, Cl^-, Br^-, I^-), and nitrate can be monitored; however, interferences from other anions are likely. Long-term stability and response are also of concern for some electrodes (1).

Liquid chromatography, in particular, ion chromatography (IC), offers an attractive alternative methodology for the determination of anions and cations (2,3). Using relatively inexpen-

sive equipment, both anions and cations can be determined simply by changing the ion-exchange column. Usually IC operating conditions can be optimized to permit the measurement of a trace anion of interest in the presence of a large concentration of another anion not of interest, e.g., chloride. Sample matrix effects are also minimized. In particular, IC has evolved to be probably the most important analytical technique for the determination of anions. A good overview of IC, including food applications, has been published (4). Therefore, what follows is only a basic background in ion-exchange chromatography and IC instrumentation, to help explain the methodologies used in the cited research articles describing food applications.

II. ION-EXCHANGE CHROMATOGRAPHY

A. Ion-Exchange Equilibria

Ion-exchange chromatography refers to the separation of substances by their differential migration on an ion-exchange column. Ions (cations or anions) are separated from one another on the basis of electrostatic equilibria interactions with functional groups on the column packing. The strength of the interaction is a characteristic of each type of ion. The most common functional groups or active sites on a resin (R) for cation-exchange chromatography are the sulfonic acid group, $RSO_3^-H^+$, and the carboxylic acid group, $RCOO^-H^+$. Anionic exchangers contain quaternary ammonium groups, $RN(CH_3)_3^+OH^-$, or primary amine groups, $RNH_3^+OH^-$. Most IC of inorganic cations or anions employs strong ion-exchange columns (either RSO_3^- or $RN(CH_3)_3^+$).

A variety of column packings based on polymers or silica are in common use. Crosslinked polystyrene-divinylbenzene polymers are popular; however, acrylate materials, such as polymethylmethacrylate, have also been used as supports. Surface-agglomerated resins have been developed to form low-capacity resins. To make such an anion-exchange resin, a sulfonated cation-exchange resin is contacted with colloidal latex anion-exchange particles, which electrostatically stick to the cationic microspheres. This pellicular-type ion-exchange resin provides not only rapid analyte mass transfer, because of the small size of the latex colloidal beads, but better pressure stability and resistance to swelling compared to traditional polymeric packings. Appropriately silanized and modified silica particles have shown excellent utility as ion-exchange column packings. Dynamic ion exchangers have been prepared using reverse-phase column packings, such as C-18 silica and polystyrene-divinylbenzene polymers, and ion-interaction reagents, such as tetrabutylammonium ion and heptanesulfonic acid hydrophobically retained on the microspheres.

As an example of ion-exchange equilibria, consider a column packed with a sulfonic acid ion exchanger that has been brought into contact with an aqueous solution containing a cation, M^{x+}. An exchange equilibrium is set up that can be described by

$$xRSO_3^-H^+(s) + M^{x+}(aq) = (RSO_3^+)^-_xM^{x+}(s) + xH^+(aq) \tag{1}$$

where $RSO_3^-H^+$ represents one of many sulfonic acid groups on the cation exchanger. Initially, when the cation-containing sample is at the top of the column, the concentration of the cation is highest in the aqueous phase, and the equilibrium shown in reaction (1) shifts to the right, placing the cation onto the exchange material. If at this time a solution of dilute hydrochloric acid is placed in contact with the exchanger, the equilibrium will shift to the left, releasing the cation, M^{x+}, back into solution. An equilibrium constant, K_{ex}, describing this exchange reaction for a singly charged cation is as follows:

$$K_{ex} = \frac{[RSO_3^-M^+](s)[H^+](aq)}{[RSO_3^-H^+](s)[M^+](aq)} \tag{2}$$

Rearranging Eq. (2) yields

$$\frac{[RSO_3^-M^+](s)}{[M^+](aq)} = \frac{K_{ex}[RSO_3^-H^+](s)}{[H^+](aq)} \tag{3}$$

During elution, experimental conditions are such that the aqueous concentration of the hydrogen ion solution is much larger than the concentration of the cation, M^+. Also, the number of exchange sites relative to the number of M^+ cations being retained is very large, and the previous equation can be reduced to

$$K = \frac{[RSO_3^-M^+](s)}{[M^+](aq)} \tag{4}$$

The constant K_{ex}, where K is analogous to the partition coefficient of an analyte between two phases, describes the affinity of the cation for the ion-exchange material relative to another ion (here H^+). If K_{ex} is large, there is a strong tendency for the material to retain the cation; if K_{ex} is small, the reverse is true. By selecting a common reference ion, such as H^+, distribution ratios for a variety of cations on a particular ion-exchange material can be compared. Such experiments show that polyvalent ions are much more strongly held than singly charged species. Within a given charge group, differences in the retention of ions are related to hydration energy or the degree to which the ion interacts with water. For simple inorganic ions, this effect depends on size; the smaller ions, such as F^- and Li^+, have a strong interaction with water and are poorly retained by the resin (5). For a typical sulfonated cation exchanger, the values for K_{ex} decrease in the order (6): $Tl^+ > Ag^+ > Cs^+ > Rb^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+$. For divalent cations, the retention order is $Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > UO_2^{2+}$. For anions on a quaternary ammonium-based anion exchanger, K_{ex} decreases as follows (6): $SO_4^{2-} > CO_4^{2-} > I^- > NO_3^- > Br^- > Cl^- > HCO_2^- > CH_3CO_2^- > OH^- > F^-$.

From an experimental point of view, the K_{ex} value depends on:

1. Capacity of the ion-exchange resin (mEq of exchange sites/g resin)
2. Ionic strength (sum of the concentration and $(\text{charge})^2$ product of each ion in the mobile phase) of the mobile phase.
3. pH and/or any complexation effects of the mobile phase

If the capacity is low, the retention of the sample ions will likewise be small. As the ionic strength increases, the retention of the sample ion will decrease. It has been shown that a linear inverse relationship exists between the logarithm of the analyte retention factor for an inorganic ion and the logarithm of the eluent concentration. The slope of the line is the ratio of the charge of the eluent ion to the charge of the analyte ion. If the pH is such that the sample component, e.g., carboxylic acid, is highly charged, the ion-exchange retention will be reduced. If complexation of the sample ion, e.g., a transition metal, with a complexing agent like EDTA occurs, retention by cation exchange will be reduced and retention by anion exchange more favored.

B. Ion Chromatography

One recent book, entitled *Ion Chromatography* (6A), provides a particularly comprehensive and detailed overview of the subject. This section provides a short summary of the basic concepts and modes important to IC; the reader is referred to Ref. 6A for more information.

1. Conductivity Detection

Historically, ion-exchange chromatography was applied to the separation of analytes, such as transition metals and amino acids, that could be easily detected using some postcolumn reaction

scheme. Other inorganic cations, such as alkali metals, as well as organic cations and both inorganic and organic anions do not form colored complexes with most of the commonly used color-forming reagents. Thus, although ion-exchange resins have a well-known ability to provide excellent separation of ions, a universal but sensitive detector like the UV-VIS detector for reverse-phase chromatography would be desirable.

The one physical property that all ionic solutions possess is conductance, and it is generally the conductivity of the column effluent that is monitored in ion chromatography. However, since the eluents used to elute the analyte ions from the column are in themselves ionic and thus conductive, distinguishing the difference in conductivity between the analyte and the eluent can be difficult.

Small et al. (7) and later Stevens et al. (8) described a novel ion-exchange procedure using conductivity detection, which was applicable to both cations and anions. The method uses a suppression system to reduce the conductivity of the eluent. The instrumental configuration is shown in Fig. 1. The analytical column is packed with a strong anion-exchange resin, with the exchange sites occupied by, or equilibrated with, carbonate ions. The suppression system can be a strong

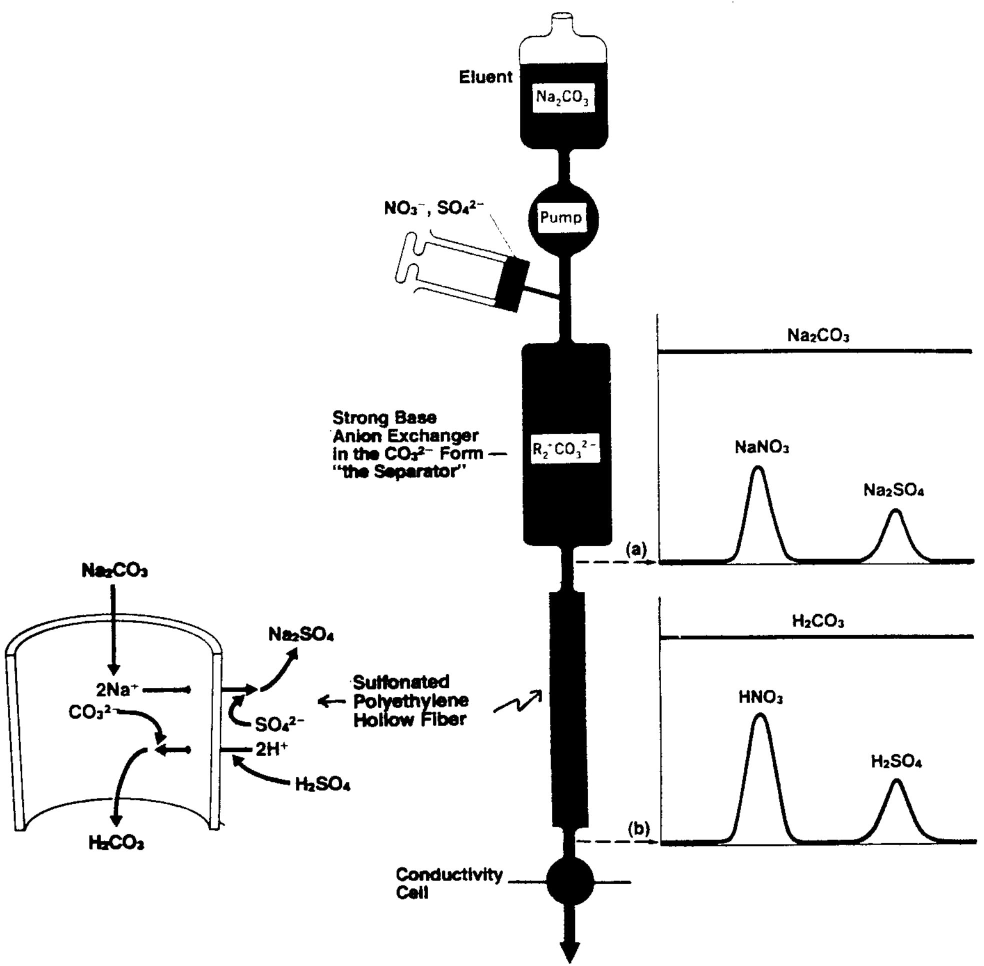


Fig. 1 Block diagram of anion-suppressed ion chromatography using a hollow fiber suppressor.

cation-exchange hollow fiber, with the exchange sites equilibrated with sulfuric acid. A solution of a sodium carbonate is continuously pumped through the chromatographic system. As the separated sample ions (e.g., halides X^-) elute from the analytical column, they enter the suppression system, where two important reactions occur. The sodium ions are removed by the cation-exchange sites of the hollow fiber and replaced by protons. The protons react with the carbonate to form carbonic acid, which is neutral. These reactions occur continuously. The halide ions can then easily be detected on a background of poorly conducting carbonic acid. An analogous scheme for cation analysis can be envisioned in which dilute acid serves as the eluent, and an anion-exchange hollow fiber used as the suppressor is washed with sodium hydroxide. Membrane suppressors having a lower dead volume in the 40-μl range are now state of the art and have replaced the hollow fibers; however, the chemistry is still the same. Recently, electrically polarized ion-exchange beds pumped with water can produce either OH^- or H^+ electrolyte of steady and controllable concentration for, respectively, anion-exchange and cation-exchange chromatography (8A). The advantage is that water can be used as the pumped phase, avoiding off-line eluent preparation. Generally, only analytical columns of standard dimensions (2- or 4-mm ID × 10 cm) are employed. However, microcolumn ion chromatography involving a packed fused-silica capillary with a hollow-fiber suppressor has been applied to the determination of anions in fruit juices (9). With the advent of suppressed-column ion chromatography, the analytical utility of ion-exchange separations included the alkali and alkaline earth metals, organic cations, and a large number of both inorganic and organic anions. Still, transition metals were not amenable to suppressed ion chromatography, for these metals result in hydroxide precipitation in the suppression device.

In 1979, Gjerde et al. reported that if ion-exchange resins of very low capacity (0.07–0.04 mEq) were used, conductivity detection could be employed without the use of a suppression system (10–12). Only low-ionic-strength eluents were required to elute the analyte ions, and the small background could be compensated electronically. A variety of separations, including anions and organic cations, as well as the alkali and alkaline earth metals and some transition-metal ions or rare-earth ions have been reported using this type of nonsuppressed ion chromatography. Typical eluents for anion separations have included benzoate, biphthalate, and *o*-sulfobenzoic acid. For separations of the alkali metals, nitric acid serves well as an eluent; for the alkaline earth and transition metals, a stronger counterion, such as ethylenediammonium nitrate, is required for their separation and elution.

Recently a new method for suppressed ion chromatography has become commercially available (12A). Basically, small-volume cartridges packed with either cation-exchange beads in the H^+ form (for anion analysis) or anion-exchange beads in the OH^- form (for cation analysis) are the suppression devices. Two suppressor cartridges are used so that while one is involved in the eluent conductivity suppression, the other is switched out using a valve for regeneration through the electrolysis of water. The cartridges are impregnated with a dye so that visual inspection can be used to ensure that the suppressor cartridge has been completely recharged. Compatibility of the suppression module with a wide variety of HPLC instrumentation and columns is the primary advantage.

2. *Indirect Detection Chromatography*

In 1982, Small and Miller devised a single-column system to allow for the separation and detection of ions using a spectrophotometric detector. The method is based on the concept that spectrophotometers may be used to monitor the many transparent ionic species commonly thought not to be amenable to this type of detection (13). Consider an ion-exchange column, for example, a cation exchanger, that has been equilibrated with an eluent, E^+Cl^-. There is a specific concen-

tration of E^+ in the effluent and an equivalent concentration of Cl^-. When a sample ion, S^+, elutes from the column, two things occur simultaneously. There is a net increase in S^+ concentration accompanied by a net decrease in E^+, or counterion, concentration. Since the anion (Cl^-) concentration is fixed, electroneutrality dictates that the equivalent increase in sample ion concentration exactly match the decrease in E^+. A sample cation can be quantified by detecting the decrease in E^+, which will be proportional to the sample concentration increase. An analogous argument can be made for the detection of anions with the eluent E being negatively charged. Elimination of system or extra peaks due to the displacement process can be done by a careful pH match between the sample and the eluent. Such a detection method, as previously mentioned, is termed an *indirect* method. In indirect photometric chromatography (IPC), the counterion (either E^+ or E^-) possesses the property of absorbance at a wavelength where the sample ions (S^+ or S^-) do not.

The magnitude of the analyte detection limit is proportional to the concentration of the UV-absorbing eluent but inversely related to the transfer ratio (the number of molecules of detectable ions displaced by each analyte molecule) and the dynamic reserve (the ratio of background absorbance to noise) (13A). Theoretically, a low concentration of a singly charged eluent with a high molar absorptivity (or other detectable property) is desirable for a low detection limit. Practically, better detection limits for analyte ions with a high affinity for the stationary phase can be obtained by using a low concentration of a multicharged eluent ion, such as Ce^{3+} (13B) or naphthalenetrisulfonate (13C).

Besides UV-VIS, most of the other detectors commonly used in HPLC have been used for indirect detection. Indirect refractive-index detection has been used for the determination of anions in wine (14). Indirect fluorometric chromatography (IFC) and indirect electrochemical chromatography (IEC) are alternative approaches (15,16). Either method can be applied to IPC separations where interferants in the sample matrix cause overlapping peaks in the chromatogram. In addition, IFC or IEC can also be employed if one or more of the analyte ions have absorption properties at the same wavelength as the counterion, resulting in obscured analyte-ion peaks. Complex mixtures are difficult to analyze by IC with indirect detection because gradient programming is not easily carried out. However, indirect detection is useful when IC is not commonly practiced and only a conventional HPLC instrument is available.

3. *Ion-Exclusion Chromatography*

Ion-exclusion chromatography (IEC) is an effective technique for the separation of nonionic substances such as sugars using an ion-exchange column. The retention mechanism is based on the partitioning of the sample between the mobile phase held within the resin beads and the mobile phase outside between the resin particles. Hydrophobic interaction of the organic part of a solute with the resin can also occur. Ions are excluded from the resin pores by electrostatic repulsion and pass through the column unretained. A typical example is the separation of aliphatic carboxylic acids on a cation-exchange column using 10 mM HCl. Both suppressed and suppressorless detection schemes can be used (3). In the membrane-suppressed mode, the H^+ ions are replaced by the tetrabutyl ion, reducing the relative conductance by a factor of 4. In the nonsuppressed method, better detection limits can be obtained using benzoic acid. In the field of food analysis, weak acids such as sulfurous, nitrous, and ascorbic can be determined by ion-exclusion chromatography.

III. HPLC APPLICATIONS TO FOOD

Applications of all the techniques already mentioned for the determination of anions and cations in food, including beverages, will be shown. At least two review articles have been written in the

chromatography journals. One tends to focus on sulfite, nitrite, and nitrate (16A). The second article (16B) provides a recent comprehensive review of IC methods for inorganic ions in food. In addition, a good summary of the different sample preparation methods is included. However, most of the references to IC methods for food are tabulated and little detail is provided in text form. This section will avoid tabulation and instead provide a brief description of each article in text form.

A. Anions

The determination of halogen-, nitrogen-, and sulfur-containing anions is of major concern in the food industry. To a lesser extent, phosphate and related compounds are of interest. Certainly, various combinations of these simple, inorganic anions have also been determined by ion chromatography for specific food applications. A final section describing the separation of inorganic and organic anions together in a mixture will be provided.

1. Halogens

Few methods were found for Cl^- in food, although this is a common anion determined by IC for other sample types. One such was a low-temperature ashing procedure for organic materials to facilitate the determination of Cl^- by IC. Pretreatment with potassium carbonate was important to achieve recoveries of chloride of at least 90%. Determination of Cl^- in corn starch and potato starch by IC was shown [16C].

The determination of bromate, BrO_3^-, in bread has been one of the more common applications involving halogen-type anions. Because bromate is an oxidizing agent and a potential carcinogen, determination of any residue is important from a health point of view. Suppressed-anion chromatography involved retention of BrO_3^- on a concentrator column while the interferants Cl^-, PO_4^{3-}, and SO_4^{2-} were passed through. The bromate was then eluted off using Na_2CO_3, with recoveries at the 95% level and detection limits of 1–2 mg/L (17). Similar methods that involved eliminating the interference of Cl^- by using a silver-coated resin have also been reported (18,19). After anion exchange using a pH 3.4 $NaClO_4$ eluent, bromate was allowed to react with iodide to form the tri-iodide ion that could be detected at 250 nm (20). A detection limit of 0.05 mg/L was possible. The determination of residual bromate in baked goods by IC with inductively coupled plasma–mass spectrometry (ICP-MS) detection (20A) offers the advantage of positive qualitative identification. Both bromide and bromate were determined separately by suppressed ion chromatography (21) using the same column but with different mobile phases (0.002 M Na_2CO_3, and 0.0035 M $Na_2B_4O_7$, respectively). The detection limit using the standard addition method was 0.5 mg/L. At less than 40 μg/g added bromate, any residue present in the bread is in the form of bromide. At the 100-μg/g level, about 3.5% bromate remains. A more general method for the determination of ascorbate, bromate, and bromide in bread improvers has been reported using unsuppressed ion chromatography, with chlorosulfonate as the mobile phase and UV detection at 205 nm (22). Sample preparation involved simply sonication in water, centrifugation, and filtration. Interference by Cl^- was not a problem, as shown in Fig. 2. Sulfate was also determined by conductivity detection. Soya meal feed mix samples were analyzed for all four ions simultaneously.

The effect of interfering ions on the determination of total bromine in foods was investigated by using a 5-cm precolumn–25-cm anion-exchange analytical column combination with a carbonate–bicarbonate mobile phase and detection at 193 nm. The determination limit was 0.5 μg/g of Br; recoveries for various foods were 90–102% (22A). Determination of bromide ions in food by unsuppressed IC with ultraviolet detection after microwave digestion in a sealed PTFE vessel was also examined (22B). Determination of bromide residues in rice by ion chro-

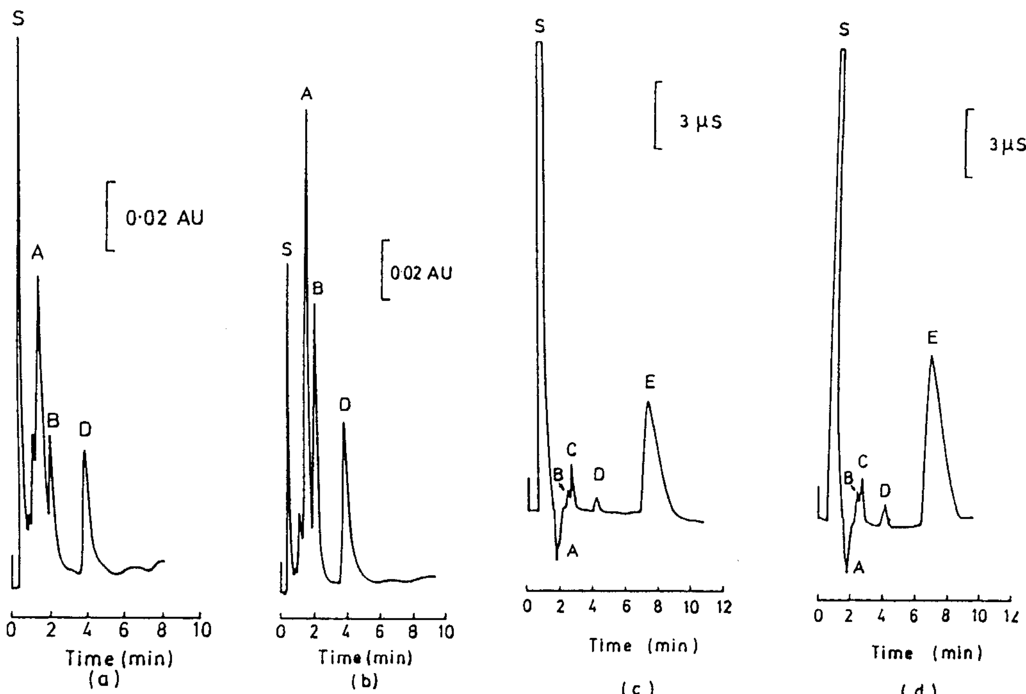


Fig. 2 Determination of ascorbate (A), bromate (B), chloride (C), bromide (D), and sulfate (E) in bread improver extract (a, c) and spiked bread improver (b, d) with UV absorption (a, b) and conductivity (c, d) detection (S = solvent). Conditions: column, TSK IC anion PW (50 × 4.6 mm); mobile phase, 7 mM chloromethanesulfonate, pH 5.5, flow rate, 4.5 ml/min; chart speed, 5 mm/min; injection volume, 10 μl; detector sensitivities, 0.2 AUFS or 25 μSFSD; sample concentrations, 200–500 mg/L. (Reprinted by permission from Ref. 22.)

matography with conductivity detection using an anion-exchange column equipped with a guard column was also performed with a carbonate–bicarbonate buffer. A calibration curve was linear from 0.1–14 μg/ml, with detection limits of 1.25 mg/kg. The recovery was 94.9%, with a relative standard deviation (RSD) of 5.75% (22C).

Methylbromide has been used to fumigate various dried foods, such as cereals, nuts, and mushrooms. Inorganic bromide can be found as a residual. Total bromine in crop samples has also been determined by conversion to bromide and then IC using a biphthalate mobile phase and UV detection (23). Detection limits were about 1 mg/L, with recoveries at the 70–90% level.

The determination of iodide in salt (24) and iodine as iodide in milk-based products (25) is straightforward, since ion-pair chromatography and UV detection at 226 nm is possible. Detection limits are in the 3–5-ng range. Using a hexadecyltrimethylammonium chloride/acetonitrile mobile phase with a C-18 column, the retention of iodide was about 25 minutes. This was necessary to prevent the overlap of matrix components in the milk and chocolate samples. For these samples, combustion in a modified Shoeninger flask using hydrazine and hydroxide was necessary. The detection of iodide in dairy products and table salt after solid-phase cartridge cleanup by ion chromatography with amperometric detection using an Ag working electrode has also been reported using a silica anion-exchange column and 5.5 mM–KH_2PO_4 as the mobile phase. The detection limits were 25 μg/L, and the calibration curve was rectilinear from 2 to 100 ng I^- (25A). Iodide and bromide in milk powder and salts were determined by ion chromatography us-

ing postcolumn detection with chloramine T at 500 nm (26). Detection limits were about 20 pg for iodide or 15 ng for bromide. Unsuppressed ion chromatography using methanesulfonate as the mobile phase has allowed UV detection at 214 nm. After alkaline ashing and neutralization of butter, iodide (0.2 g) could be determined in such a sample (27). Free iodide in food-coloring agents was determined by suppressed ion chromatography on a carbon ceramic column with conductivity detection and 12.5 mM H_2SO_4 as the scavenger (27A). Table salt was acidified before analysis by ion-pair chromatography and ion-exchange chromatography, with detection at 205 and 210 nm (27B). Working ranges were 0.2–1.0 mg/L of IO_3^- by ion-pair chromatography and 0.3–2.5 mg/L of IO_3^- by ion-exchange chromatography. For samples of whey powders, cod, and shellfish, they were ashed with $NaCO_3^-$-$ZnSO_4$ before ion-pair chromatography and amperometric or UV detection of I^- at 226 nm. A simplified method of inorganic I^- detection has also been described.

A method for simultaneous determination of IO_3^-, BrO_3^-, ClO_3^-, Br^-, and I^- was applied to drinking water, soup, and human urine. The samples were subject to HPLC on a gel permeation column with elution by 50 mM malonic acid–37.5 mM tetramethylammonium hydroxide and inductively coupled plasma–mass spectrometry (ICP-MS) detection. The ICP-MS response did not depend on the chemical form of the element, and detection limits were 25 pg, 0.8 ng, and 36 ng for I, Br, and Cl species, respectively (27C). The halogens I^-, Br^-, Cl^-, and F^- were separated using pyrohydrolysis for preparation and inductively coupled plasma–mass spectrometry and IC for measurement. The results obtained by the proposed method and the certified values were in good agreement (27D).

2. *Nitrogen*

Ion chromatography was used to determine total nitrogen in food after Kjeldahl digestion. The HPLC separation of ammonium ion in the presence of substantial sulfate involves the use of a polystyrene-divinylbenzene cation-exchange column with dilute nitric acid as the eluent and indirect conductivity detection. The use of IC improves the speed of analysis compared with the conventional method of titration (27E).

The determination of nitrogen-type anions in food products has focused on nitrate and nitrite, either separately or together. A discussion of methods first for nitrate and then for nitrite will be presented in the next two paragraphs. The methods indicating the separation of both nitrate and nitrite primarily in vegetable and meat samples will be summarized in the last two paragraphs of this section.

Nitrate and bromide have been separated on an amino silica column by weak ion exchange, with a phosphate mobile phase adjusted to pH 3.5 (28). After some sample pretreatment involving homogenization, precipitation of proteins with Carrez solution, and centrifugation, nitrate in cheese, whey, salad, and vegetables could be determined at the 2–5-mg/L level. Using UV detection at 210 nm, a detection limit of 0.05 mg/L in tap water was possible. Bromide was found at the 50-mg/L level in flour and rice samples. Nitrate has also been determined in vegetables by strong anion-exchange chromatography at 35°C, again using a phosphate buffer mobile phase and detection at 210 nm (29). Nitrate in milk was quantified using a weak anion (DEAE) exchange column (30). Nitrate and chloride were separated by single ion chromatography using conductivity detection, with detection limits of 2 and 0.2 mg/L, respectively (31). Two different types of eluents were chosen, depending on whether the nitrate was found at high (2000 mg/L) or low (250 mg/L) levels in various vegetable samples. Nitrate has also been determined in beer or wort after cleanup using a Sep-Pak C-18 cartridge by ion chromatography, with conductivity detection in the low mg/L range (32). A comparison of HPLC with UV-VIS detection and the reducing Cd column with spectrophotometry method for the determination of nitrite in cured meat products

was performed. Ultraviolet detection limits at 215 nm for the HPLC method were 0.03 mg/L for nitrite and 0.9 mg/L for nitrate. The chromatographic method was found to be more precise, gave better recoveries, and was quicker than the spectrophotometric method (32A). A comparison between IC with conductivity detection and a spectrophotometric method for the determination of nitrate in 76 raw and cooked pork meat products was also made. The IC results compared well with those of a standard colorimetric method; however, agreement between the methods was better for raw products than for cooked products (32B). A comparison of HPLC with detection at 220 nm and the Cd-Griess colorimetric method was made for nitrate in meats. Recoveries were 80% for the HPLC method but only 31% by the Cd-Griess method (32C).

Ion chromatography with electrochemical or luminescence detection provides important selectivity for the determination of nitrite. With respect to meat products, the determination at the 100-μg/kg level for the electrochemical method, as compared to 5-mg/kg for spectrophotometric methods, was found (33). Nitrite levels were also determined in refrigerated and frozen spinach by IC with electrochemical detection. Determination levels around 20 ng/ml were performed with good accuracy, and although freezing did not alter the nitrite levels, refrigeration caused an increase 4–8 days later (33A). Recently, ion-exclusion chromatography (IEC) with electrochemical detection has been found to be an effective approach for the determination of nitrite in cured meat. Sample extraction times were only 1 minute, and ascorbate did not interfere. Using an applied voltage of 1.0 V, a detection limit of 1 ng/ml was possible. Recoveries of 50 mg/L NO_2^- added to hot dogs were 103 $\pm$ 4% (34). A reverse-phase HPLC method for nitrite involves derivatization in acid prior to the separation step (35). The tetrazolophthalazine product could be monitored either by UV at 228 nm or fluorescence, providing detection limits at the 0.25- and 0.06-ng/mL levels of nitrite nitrogen. The determination of nitrite in fish, ham, wieners, tomato, and spinach food products was possible, even though the latter two foods had very low amounts. Rapid and sensitive nitrite determination in cured meats and baby food was performed by flow injection or HPLC with chemiluminescence detection. A C18 column was used to retain the organic sample matrix and allow the nitrite to pass through for the postcolumn reaction with KI in the presence of acid. Chemiluminescence detection of the NO formed was done with a thermal energy analyzer. The detection limit was 0.1 ng, the RSD was 0.2–4%, and the recoveries were 92–108% (35A). The simultaneous HPLC determination of nitrite and formaldehyde after derivatization with hydralazine was applied to food samples. The derivatives were separated with a C18 column and detected fluorometrically, with detection limits of 0.1 ng/g for nitrite and 0.5 ng/g for formaldehyde (35B).

Many ion chromatography methods published describe the determination of both nitrate and nitrite in food products. The primary advantage of ion chromatography is that prior reduction of nitrate to nitrite by cadmium for indirect determination of nitrate is not required. With a separation time of 10 min on a standard strong anion-exchange silica column, detection limits at 220 nm were at the 0.7-ng level. Samples were blended with water, treated with Carrez solution, centrifuged, and filtered before analysis. The distribution of nitrate in carrot tissue and the effect of processing on the levels of both ions in carrots were monitored (36). A similar approach using a polymeric column has been proposed for both nitrate and nitrite in meat products (37). After sample extraction and solid-phase cartridge cleanup, determination of nitrate and nitrite in cured meat by HPLC with UV detection at 214 nm gave results of 1–40 mg/kg of nitrite and 1–162 mg/kg of nitrate, which compared well with the colorimetric method and took less time (15 min compared to 90 min) (37A). The ion chromatography (IC) method for nitrate and nitrite with UV detection at 220 nm was applied to media and cell extracts with good success (38). Glutamine and acetate did not interfere. It was concluded that the reduction methods using an enzyme or hydrazine for nitrate may prove to be an advantage only when a large number of samples must be assayed per day in a batch mode. A suppressed ion chromatography technique for nitrate, chlo-

ride, and phosphate in meat products has been published (39). If the chloride:nitrite ratio was over 40%, interference from chloride was a problem, and prior precipitation using Ag(I) was required. Single column IC of nitrite and nitrate in ham using sodium perchlorate as the mobile phase and UV detection has been reported (40). The high capacity of the column (1 mEq/g) permitted adequate separation from chloride with low detection limits of 0.02 and 0.1 ng of nitrite and nitrate, respectively, without preconcentration. A novel method for the separation of nitrate by ion-interaction chromatography was developed by Iskandarani and Pietrzyk (41). Tetraphenylammonium ion is adsorbed to a nonpolar poly(styrene-divinylbenzene) column to set up a dynamic ion exchanger. Ratios of 1:200 and 300:1 nitrite:nitrate mixtures could be separated without peak overlap. A variety of foods, such as apple juice, corn, bacon, and beer, were assayed for both nitrite and nitrate. This same method was extended to meat products (42).

Procedures for nitrate and nitrite determination in vegetable extracts were assessed. Interference by SO_4^{-2}, PO_4^{-3}, and Cl^- was observed in HPLC with conductivity detection, and the method was slow (12–15 min per sample). The HPLC with UV detection was 10 times more sensitive (about 4 ug/L) and showed no interference (Fig. 3). When compared to automated spectrophotometry, HPLC with UV detection was found to be the method of choice for the determination of trace nitrate and nitrite; however, for medium-to-large samples of vegetables, automated spectrophotometry was preferred due to its greater speed (42A). After HPLC with UV

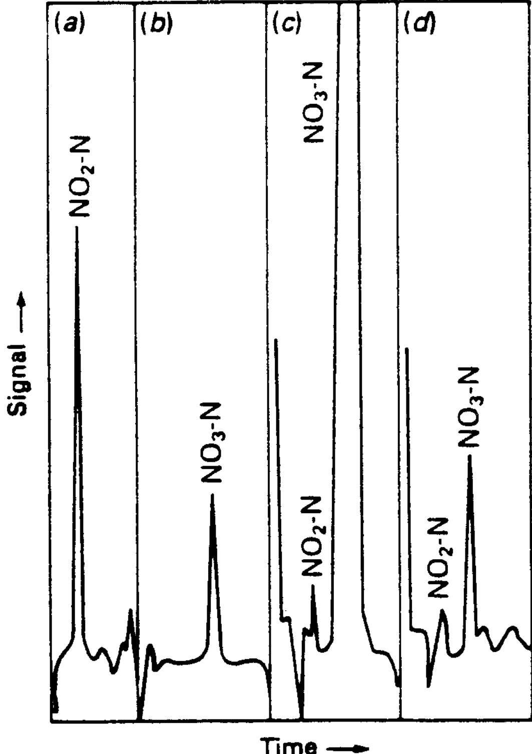


Fig. 3 Separation of nitrite and nitrate using a Waters IC-PAK anion-exchange column with a 0.025-mol/dm^3 KOH eluent and UV detection at 214 nm. (a) Nitrite standard (0.25 mg/L); (b) nitrate standard (0.25 mg/L); (c) beet root extract (1 + 9 dilution); (d) carrot extract (1 + 9 dilution). (Reprinted by permission from Ref. 42A.)

detection at 210 nm, nitrate recoveries were 98–100.5% from tap water but only 69–87% from lettuce and 63–65% from apple leaves. Nitrite was detected only in apple leaves, with 70–77% recoveries (42B). Preparation of samples for determination of nitrite and nitrate in foods by the respective methods spectrophotometry after derivatization at 540 nm and ion chromatography with UV detection has also been discussed. Respective recoveries for nitrate and nitrite of 90–102% and 84–101% were found (42C). The simultaneous determination of nitrate and nitrite in foods containing Cl^- has been applied to more than 700 food products using first a solid-phase extraction cartridge before separation on a polymeric anion-exchange column, with detection at 214 nm (42D). Response was linear from 0.5 to 100 μg/ml.

3. *Sulfur*

Although most sulfur analysis of food involves the determination of sulfite (SO_3^-), a few methods for total sulfur in food have been published. The species S_x is used as a protective fungicide for a variety of fruits, vegetables, and grains. A reverse-phase HPLC method with amperometric detection at a dropping Hg electrode was developed, with a limit of detection of 5 ng (43). Total sulfur in rice, flour, bamboo leaves, and powdered fish was determined by ion chromatography after Schoeninger flask combustion of sulfur to sulfate (44). Chlorine, sulfur, and phosphorus after oxidation with sodium peroxide fusion were determined by IC as chloride, phosphate, and sulfate. Residual NaOH formed during the oxidation step was neutralized using electrodialysis sampling pretreatment. Detection limits were 0.005% for chlorine and 0.008% for sulfur and phosphorus (44A).

The importance of the determination of sulfite is evidenced by the fact that four sulfating agents are accepted for use in food: sulfur dioxide, sulfite, bisulfite, and metabisulfite. Sulfite in foods was determined by ion chromatography after a purge-and-trap technique (45). Sulfur dioxide was produced by the sample and then absorbed by NaOH to form SO_3^{2-}, again in a clean matrix prior to the separation step. Detection limits were 3 μg/g, with recoveries on the order of 73–103%. A similar system using suppressed ion chromatography was compared to the classical, Monier–Williams titration procedure (46). Several advantages, such as a lower detection limit (1 vs. 50 mg/L) and better accuracy in the presence of volatile carboxylic acids, were concluded using the ion chromatography method. This modified Monier–Williams method was adopted by the Association of Official Analytical Chemists (47). It was modified to improve the recovery of sulfite from shrimp by replacing the acid distillation step with either an alkaline–formaldehyde or water–methylene chloride extraction step (48). Sulfur dioxide in foods such as beer, jam, brussel sprouts, mustard, sugar, and shrimp was analyzed by IC, with indirect photometric detection at 280 nm with phthalate as the mobile phase. No interference from other volatile compounds was observed, and recoveries of sulfite were over 80% (48A). Sulfite after derivatization with *N*-(9-acridinyl)maleimide for 30 min at 50°C was determined in wine by reversed-phase HPLC, with fluorimetric detection at 445 nm (48B). Linear calibration curves from 0.5 to 500 μM were generated.

In addition to IC, many of the methods for the determination of free and total sulfites in food have been accomplished using ion-exclusion chromatography (IEC) with electrochemical detection. Extraction of a variety of food, such as pears, potatoes, and bell peppers, at pH 2 yielded free sulfite, whereas at pH 8.9 a sample representing total sulfite could be obtained. Detectability of 0.1 mg/L SO_2, representing an order of magnitude better than that by standard IC, was achieved by IEC with electrochemical detection (49,50). This method has been extended in three other publications (51–53) to consider more types of foods, such as grapes, and comparisons to the Monier–Williams procedure have been made. A representative chromatogram is shown in Fig. 4. Amperometric detection of sulfite was performed after ion chromatography

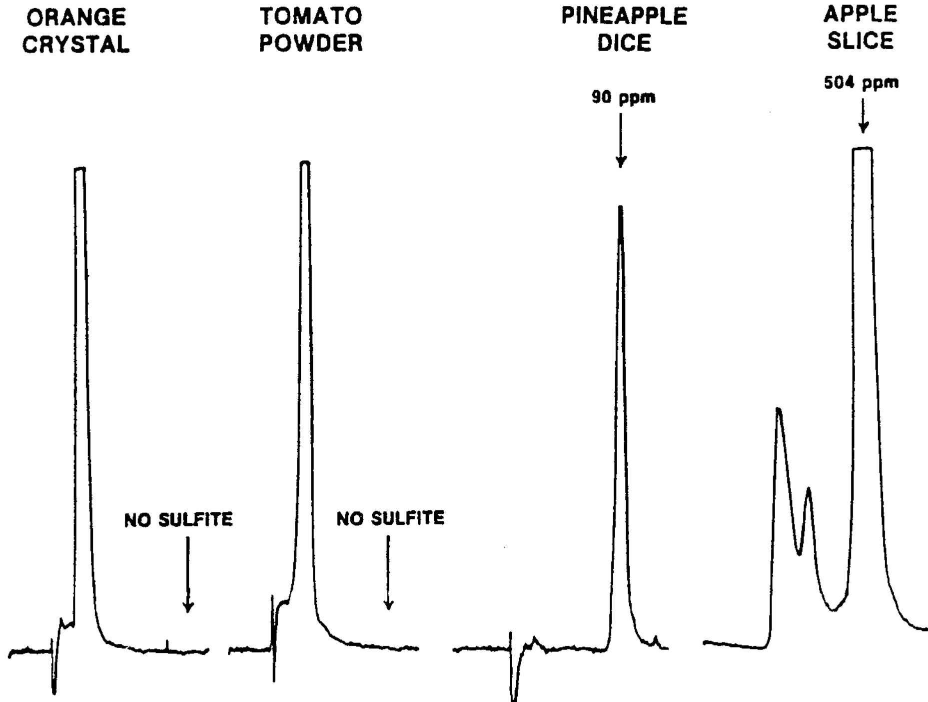


Fig. 4 Determination of sulfite by ion exclusion chromatography with electrochemical detection in dehydrated foods. Sulfite peak retention time about 2 min. Conditions: Column, Brownlee Polypore H (sulfonated polystyrene-divinylbenzene, 4.6 × 100 mm); mobile phase, 6 mM H_2SO_4; flow rate, 1 ml/min; sample size, 20 μl. Platinum electrode: +0.4 V vs. Ag/AgCl electrode. (Reprinted by permission from Ref. 52.)

using a head space sampling technique (54). Fresh sausages were analyzed for free and total sulfites by an anion-exclusion column and electrochemical detection with a glassy carbon electrode operated at 1150 mV. Mean recovery was 86.4% (54A). Sausages, mushrooms, artichokes, and tomato sauce were analyzed for sulfites by IC. An instrumental setup including an anion-exchange polymethacrylate column with sodium borate/gluconate of pH 8.5 as the mobile phase and a conductivity detector gave a 0.5-μg/ml detection limit and an average recovery of 90.4% (54B). Ion-exclusion chromatography with electrochemical detection was used to determine sulfite in corn starch, lemon juice, wine, dehydrated seafood, and instant mashed potatoes. Samples were alkali-extracted to release SO_2, and then analyzed by IEC on a sulfonated polystyrene-divinylbezene column. The detection limit was 0.01 mg/L using a Pt working electrode set at 0.6 V (54C). Total sulfite in cellulosics (thickening agents) was determined by IEC with electrochemical detection. Results were compared with those obtained by Monier–Williams distillation, which was found to give a low recovery and poor reproducibility (54D). Similar conclusions were found in another comparison study of IEC with electrochemical detection and the Monier–Williams method for total sulfite in food (54E). Sulfite in peach and orange juice samples was also determined by IEC at a Pt modified glassy carbon electrode in the pulsed amperometric detection mode (54F). Analytical data including a linear calibration curve from 0.5 to 250 μg/ml with a detection limit of 0.25–0.50 μg/ml was found. Potatoes, urine, lemon juice, and cola was analyzed by "heart-cut" column-switching techniques for sulfite. Recoveries were between 85 and 112% (54G).

Emphasis on sulfite stabilization to improve the reliability of data has also been an important issue. Sulfite upon reaction with aldehydes or ketones will form hydroxysulfonic acid. Formaldehyde is commonly used, and Dasgupta et al. (55) found the stability of the resultant sulfite product to be best in a pH 4 buffer. A study of different stabilizers for sulfite has confirmed this result (56). The formaldehyde stabilization method has been applied after sample distillation to the ion chromatography of sulfite in various foods, such as jam, wine, and onions (57). Reverse-phase ion-pair HPLC of hydroxymethylsulfonate was carried out on a C-18 column using tetrabutylammonium hydroxide and a postcolumn derivatization method for the sulfite derivative involving 3-carboxyl-4-nitrothiophenolate ion and detection at 412 nm (58). Recovery levels of 5–10 mg/L SO_2, representing better than 90% of the SO_2 in the original sample, were possible. Similarly, ion-pair HPLC was used to determine fresh and reversibly bound sulfite in wine and other foods, with the same postcolumn reaction but detection at 450 nm. The average recovery was 100% (58A). The same HPLC method with colorimetric detection was applied to grapes and grape products, with recoveries in the 5–20-mg/L level near 100%; the results obtained were similar to those of the official AOAC method (58B).

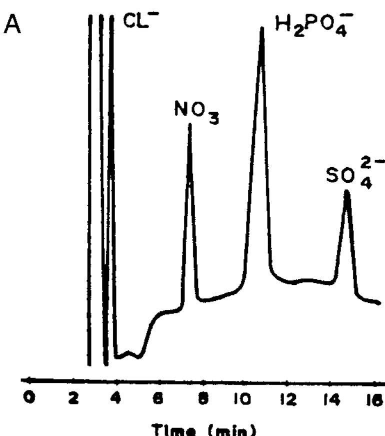
Sulfite and ascorbic acids were simultaneously analyzed by pulsed amperometry combined with IEC in beer samples. For best results, a standard amperometric cell was operated in a pulsed mode and cleaning cycles were continually applied (58C). For the mean recovery of sulfite and ascorbic acid, beer samples were analyzed on an anion-exchange column protected by a cation guard column. Electrochemical detection limits were 0.2 and 0.5 mg/L of sulfite and ascorbic acid, respectively (58D). Sulphite and ascorbic acid were determined in beer by the use of pulsed amperometry combined with IEC. Recoveries were 99–118% for 8 mg/L ascorbic acid and 95–105% for 2 mg/L sulfite (58E). High-performance LC with fluorescence detection at 390 nm and postcolumn derivatization was used for the analysis of aldehyde bisulfites in beer. This analysis was 20–100 times more sensitive than HPLC with electrochemical detection (58F).

Sulfate determination in food has also been carried out by IC. Sulfate in brine and serum and iodide in mineral water and fruit juice were analyzed by IC with alumina columns combined with common anion-exchange columns. Alumina was found to be highly selective in the preconcentration of SO_2 and was well suited for online column coupling. Alumina was found to be a suitable complement to existing ion-exchange stationary phases, rather than a replacement (58G). Tea was analyzed for oxalate and sulfate, with recoveries of 98.7 and 97.8% by single-column IC (58H). Human breast milk was analyzed by IC for amounts for free sulfate and total sulfoesters. The increase of sulfate after acid hydrolysis was a measure of the sulfoesters (58I). Sulfite and sulfate were also determined, with little effect from other inorganic and organic anions, by IC with a cetylpyridinium-coated C-18 reverse-phase column and a weakly basic phthalate eluent (58J). Sulfate and malate in plant extracts were also determined simultaneously using IC with tandem conductivity and UV detectors. Nitrite was also quantified in large backgrounds of chloride using this method (58K).

4. *Phosphorus*

The determination of total phosphate as well as other ion chromatography (IC) applications for anions such as Cl^-, NO_3^-, and SO_4^{2-} in vegetable juice have been briefly reviewed with sample preparation information (59). Phosphate was determined in flour using a borate–gluconate complex with conductivity detection. Alternatively, phosphate in potato process water was determined using a C18 column with tetrabutylammonium ion to form a dynamic ion exchanger and refractive index detection (59). Phosphate has also been determined in milk by ion-interaction HPLC using heptylammonium salicylate with a C18 column and detection at 254 nm (59A). Naturally occurring orthophosphate in various raw and processed foods, such as meats, eggs, fruits,

vegetables, and aquatic products, was determined by ion chromatography with suppressed conductivity detection. Creatine phosphate, AMP, and cyclic AMP were all well separated from orthophosphate, and recovery was 91–112.3% (59B). Water-soluble inorganic phosphates were determined in fresh vegetables by IC. An anion-exchange polymethacrylate column with borate/gluconate as the eluent and conductivity detection was used (59C); typical chromatograms are shown in Fig. 5. Polyphosphate in foods such as processed cheese and sausage has been determined by HPLC (59D). A comparison of polyphosphate analysis by IC and by modified end-group titration was performed. This has been an area of interest in the phosphate manufacturing



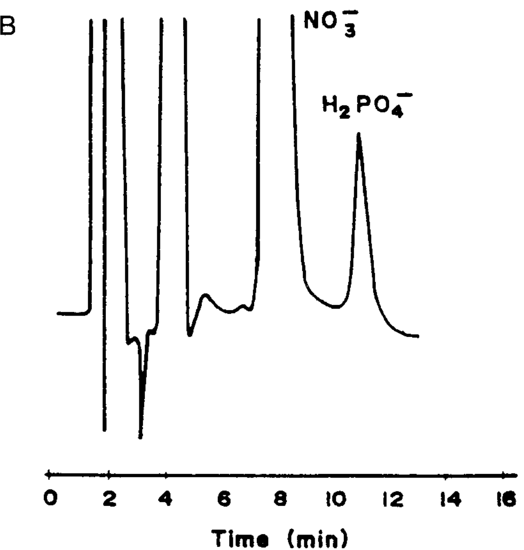


Fig. 5 (A) Chromatogram of a tomato extract. (B) Chromatogram of a chard sample with a high nitrate concentration (4500 μg/g). Separations were done using a Waters IC–PAK anion-exchange column with a sodium borate/gluconate, pH 8.5, eluent and conductivity detection. (Reprinted by permission from Ref. 59C.)

and food-processing industries (59E). The identity of an unknown peak in shrimp treated with tripolyphosphate was identified by ICP-MS (20A).

Separation of mixtures of other anions as well as phosphate is a common application for IC. Ion chromatography with suppressed conductivity detection has been used for the separation of Cl^-, NO_3^-, HPO_4^{-2}, and SO_4^{-2} in liquor-vodka production (59F). A mixture of anions such as $H_2PO_4^-$, Cl^-, and SO_4^{2-} have been separated in fruit juice using tetrabutylammonium salicylate as the mobile phase and a C18 column (60). Grape, pear, and apple juice, condensed milk, caramel liquid, honey, lemon syrup, and cane and dietetic vegetable sugars were also analyzed for HPO_4^{-2}, Cl^-, and SO_4^{2-} (60A). Intra- and interrun coefficients of variation were 2.9, 4.1, and 2.4% and 1.6, 2.9, and 1.9% for Cl^-, HPO_4^{-2}, and SO_4^{-2} respectively in a 10-min analysis. Determination of the anions chloride, phosphate, and sulfate in beer by ion chromatography was done as part of an interlaboratory study involving 15 locations (60B). The same anions with the addition of nitrate were determined in alcoholic vinegars by IC with suppressed conductivity detection (60C).

5. *Anions and Organic Acids*

Organic acids and inorganic anions in tea were simultaneously determined by IC using an anion-exchange column and a phthalate mobile phase. The method is simple and sensitive, with detection limits of 0.044–0.19 mg/L (60D). Phosphate and other components have also been determined using column-switching IC. Acetate, sorbate, and benzoate were separated on column A and saccharin, phosphate, and citrate were separated on column B using an NaOH eluent and suppressed conductivity detection (60E). A gradient IC method for organic acids in fruit juices using an NaOH eluent and suppressed conductivity detection was also used for a mixture of chloride, nitrate, lactic, and acetic acids (60F). The main carboxylic acids in wine, along with chloride, nitrate, and sulfate, were determined by IC with a phthalate eluent and conductivity detection (Fig. 6). Detection limits were in the 0.3–5-mg/L range, and a good comparison was made for the IC method with other methods, such as spectrophotometry and gas chromatography (60G). Nine organic acids and six inorganic anions were determined in all phases of beer production by IC and suppressed conductivity detection, with detection limits at 0.2–1 mg/L (60H). A multivariate technique was applied to wine analysis for sugars, organic acids, anions, and the transition metals Pb and Cd using IC, HPLC, and, AAS (60I).

B. Cations

The determination of alkali and alkaline earth methods as well as the transition metals by ion chromatography is important to the food industry. Although other methods are available, such as atomic spectroscopy, the simplicity and versatility of ion chromatography are still important for cation analysis. Sodium, potassium, magnesium, and calcium probably form the most common group of cations determined in foods by ion chromatography.

1. *Alkali and Alkaline Earth Metals*

Ion chromatography is well suited for the determination of the alkali metals, particularly if ammonium ion is also of interest. Using suppressed chromatography, sodium and potassium were determined in canned corn using a 5 mM HCl mobile phase (61). Sample preparation was simply grinding or homogenization, dilution with the 0.005 M HCl eluent, and filtration. Ammonium ion, since it elutes between the sodium and potassium ions, will often show up as well. A comparison of sodium assays in canned tomato juice by four different techniques (IC, AAS, ISE, and the AOAC method) showed results were closest between IC and the AOAC method. Sodium in

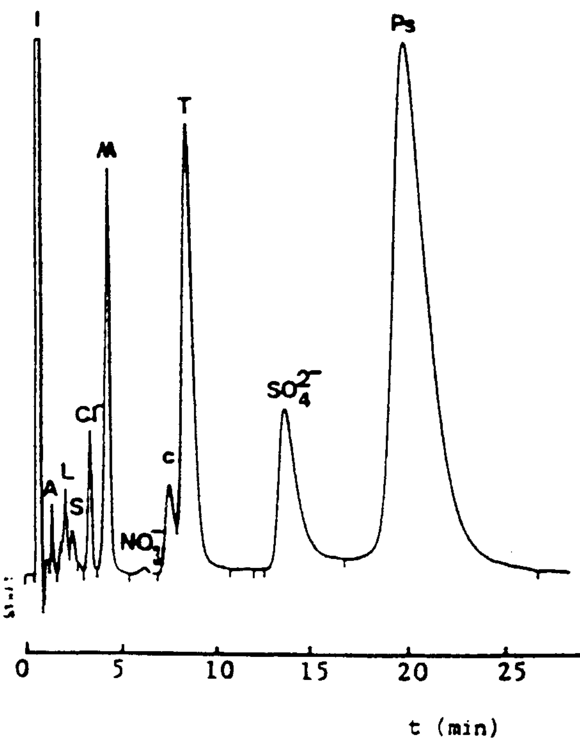


Fig. 6 Chromatogram of a Muscatel sample. Peak identification: I = injection peak, A = acetate, L = lactate, S = succinate, Cl^-, M = malate, NO_3^-, C = citrate, T =tartrate, SO_4^{2-}, PS = system peak. Separation was done using a Shimpack IC-AI anion-exchange column with a 0.975 mM phthalic acid (pH 4.15) at 1.5 ml/min (39°C) and conductivity detection. (Reprinted by permission from Ref. 60G.)

Table 1 Comparative Results of Sodium Analyses in Canned Vegetables

Sample	Sodium content (mg/100 g)			
	Ion chromatography	Atomic absorption	Ion-selective electrode	AOAC
Tomato juice	339	289–304	330–325	335
Tomato juice	322	248–154	284–289	318
Tomato juice	319	212–280	287–297	321
Tomato	204	123–152	171–172	175
Corn	244	208–198	255–217	200
NBS standard (bovine liver)	264	255/243	208/206	243 ± 13

Source: Reprinted with permission from Ref. 61.

canned beef was similarly assayed by IC, AAS, ISE, and flame emission (61). The determination of sodium in tonic water has also been made by ion-exchange chromatography using Ce(III) with indirect electrochemical detection (62). The monovalent ions Na^+, NH_4^+, and K^+ were determined in dilute cooking red wine using a microbore ion-exchange column and indirect photometric detection after postcolumn ion replacement (63). After the eluent was converted to water with

standard OH^--loaded anion-suppressor column, the separated cation hydroxide components were passed through a naphthalenesulfonate-loaded anion-exchange column. Replacement of the OH^- by naphthalene-sulfonate permitted UV detection of the analytes. Because of the miniaturized system, picomole detection limits were possible. Using a bile salt–coated stationary phase, IC was used to detect alkali metal ions. A C-18 column coated with taurodeoxycholate was used with 5 mM $CuSO_4$ as the mobile phase to convert the bile salt to its Cu^{2+} form. The alkali metals were detected as negative peaks at 200 nm by indirect photometric detection (IPD). Separation of Na^+, K^+, Rb^+, and Cs^+ was possible in 11 min; however, in other experiments Li^+ coeluted with Na^+ and NH_4^+ co-eluted with K^+. The method was applied to Na^+ and K^+ determination in wines, waters, and isotonic beverages (63A). A microcolumn ion chromatography system consisting of an effused silica column (50 mm × 0.35 mm) packed with porous silica gel was applied to the separation of inorganic monovalent cations such as lithium, sodium, ammonium, potassium, rubidium, and cesium. Cations in whisky and ketchup samples were separated within 10 min using benzyltrimethylammonium chloride as the mobile phase and indirectly detected at 208 nm, with detection limits at the 3.3–12-μM level (63B). Sodium, potassium, and ammonium ions in various foods, such as milk, sugar, vinegar, and tomato puree, were determined by ion chromatography. Recoveries were good, with detection limits of 0.1, 0.3, and 0.1 μg/ml for Na^+, K^+, and NH_4^+, respectively (63C). After solid-phase extraction, canned whole-kernel maize and tomato soups were analyzed for sodium content using high-performance cation-exchange chromatography with a 0.05 M citric acid mobile phase and nonsuppressed conductivity detection. The correlation between the claimed and measured Na contents was good (63D).

Alkali and alkaline earth metals can be determined together by isocratic or gradient IC. Indirect photometric detection with ion-exchange chromatography using Ce(III) showed that the simultaneous determination of Na, K, Mg, and Ca ions in diluted milk products was possible (64). Separation was complete in 17 min, and no interference from the sample matrix was noted (Fig. 7). A comparison plot of the IPC results and those obtained by AAS showed a correlation coefficient of 0.981. Fruit juices were analyzed by isocratic IC for the determination of cations such as Na^+, Ca^{2+}, Mg^{2+}, and K^+ as well as ammonium ion (64A). A comparison of the IC methods to atomic absorption spectroscopy was favorable. The determination of magnesium and calcium in oatmeal using an ethylenediamine eluent and conductivity detection was also shown in the previously cited application article (61). High-performance chelation ion chromatography involving dye-coated resins has been used to determine barium and strontium in samples of water and milk powder. Detection limits were 0.03 mg/dm^3 (64B). The detection of alkali, alkaline earth, and heavy-metal ions after separation by IC was compared between conductivity and indirect UV detection at 208 nm. A comparison of eluents containing organic complexing acids and copper sulfate was also made with IPD. Conductivity detection was found to be more sensitive from the 0.5–8-μg/L range (64C).

Ion chromatography for both anions and cations in various food samples has recently become of interest. Wine and sake were analyzed on anion-exchange and cation-exchange columns connected in series with anion and cation suppressors connected in parallel using a suppressor-switching technique. The cations Na^+, NH_4^+, K^+, Mg^+, and Ca^{+2} were separated in less than 30 min, and anions were separated in less than 40 min (64D). Juices and extracts of vegetables, fruits, and plants were also analyzed for elemental content (total chloride, bromide, phosphate, sulfate, and a variety of transition metals) by IC. The major focus was a sample pretreatment by UV photolysis study that was simple and not subject to contamination by added reagents (64E).

Using existing single-column IC equipment with the addition of a switching valve, anion and cation columns in series, and three different eluents, the simultaneous detection of fluoride, chloride, nitrite, bromide, nitrate, phosphate, sulfate, sodium, ammonium, potassium, rubidium, magnesium, calcium, and barium is possible in 20–30 min (64F). A representative chromato-

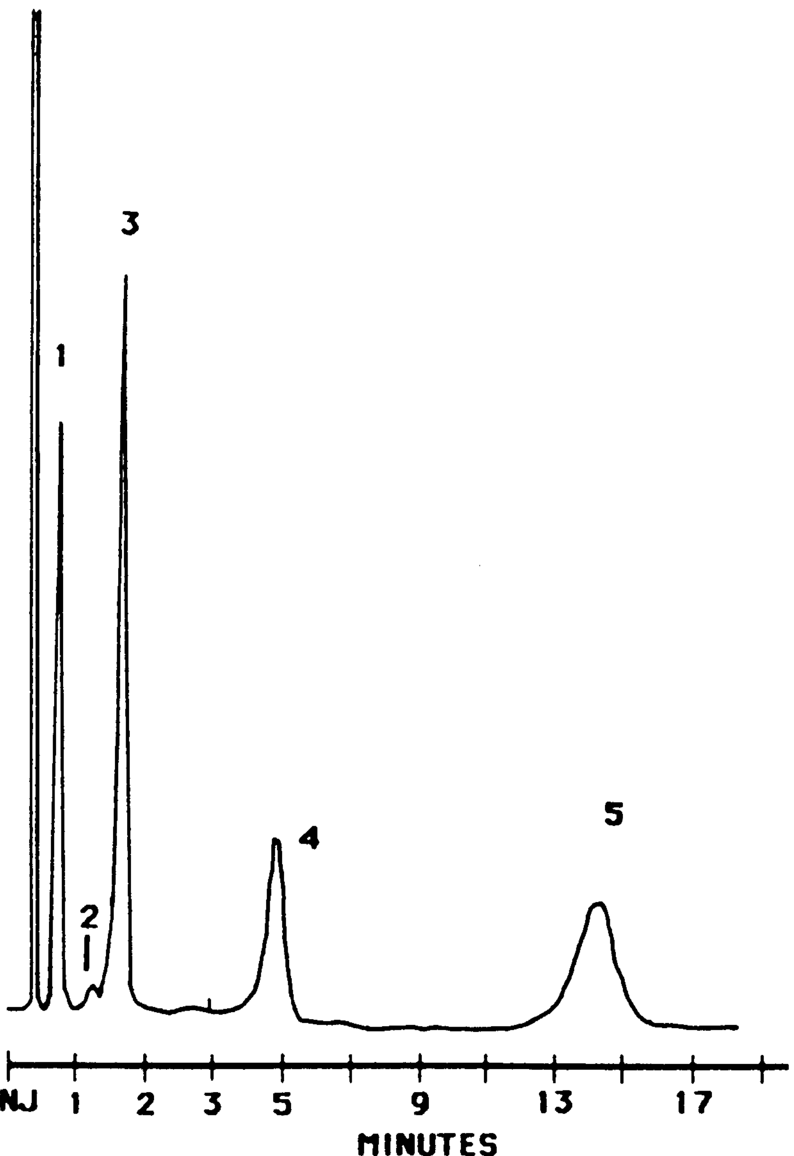


Fig. 7 Separation of cations in a whole milk sample: (1) sodium, (2) ammonium, (3) potassium, (4) magnesium, (5) calcium ions. Column: Interaction ion 210 (100 × 3.2-mm ID); eluent, 0.05 mM Ce(III); flow rate, 1.0 ml/min; sample volume, 20 μl. Peaks were changed positive by reversing detector output leads to the integrator. Chart speed of integrator was automatically switched from 1.0 to 0.5 cm/min at 3.0 min. (Reprinted by permission from Ref. 64.)

gram is shown in Fig. 8. In chromatogram (A), the resolution of the anions is favored because of the higher pH of the eluent; in chromatogram (B), resolution of the anions takes longer because of the lower pH, but ammonium ion can be separated; in chromatogram (C), the addition of ethylenediamine to the eluent decreases the cation retention, permitting the separation of calcium and magnesium. The simultaneous detection of organic acids, inorganic anions, and cations in beverages was possible with a mixed-bed stationary phase of anion and cation exchangers (64G).

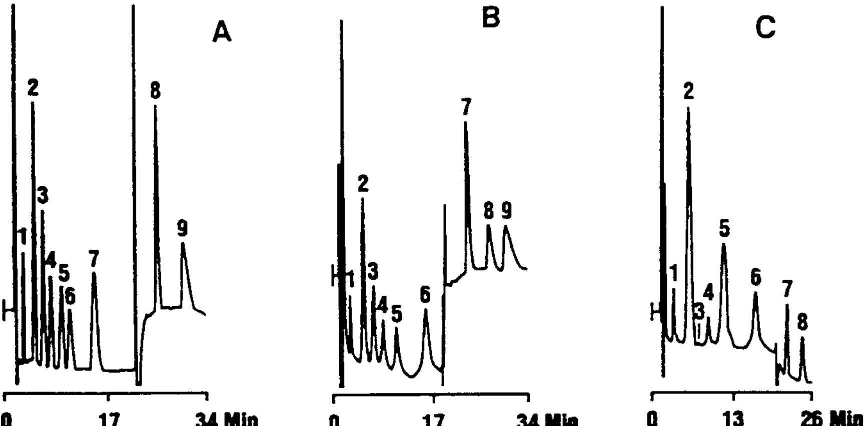


Fig. 8 Chromatograms of standard ions using various eluents. (A) 5 mM lithium *p*-hydroxybenzoate, pH 7.8. Peaks: 1 = fluoride, 2 = chloride, 3 = nitrite, 4 = bromide, 5 = nitrate, 6 = phosphate, 7 = sulfate, 8 = sodium, 9 = potassium. (B) 4 mM lithium hydrogen phthalate, pH 4.5. Peaks: 1–5 as in (A), 6 = sulfate, 7 = sodium, 8 = ammonium, 9 = potassium. (C) 4 mM lithium hydrogen phthalate—1 mM ethylene diamine, pH 4.5. Peaks: 1–5 as in (A), 6 = sulfate, 7 = magnesium, 8 = calcium. Column: Alltech Universal Anion column (150 × 4.6-mm ID) and Wescan Cation/R column (100 × 3.2-mm ID). Mobile-phase flow rate: 1.0 ml/min. Detector: conductivity. (Reprinted by permission from Ref. 64F.)

2. *Transition Metals*

The determination of transition metals such as zinc(II) and iron(II) in canned green beans was carried out by suppressed ion chromatography using an oxalic acid : citric acid mobile phase (61). In organic mixtures simulating beverages, iron(II) was speciated using HPLC with electrochemical detection, and total iron was measured by flame AAS. Species of Fe in white wine were also investigated by this method (64H). The separation of aluminum and iron(II/III) in apple products by a low-capacity cation-exchange column using a sulfosalicylic acid : ethylenediamine mobile phase with postcolumn detection by pyridylazoresorcinol (PAR) has been reported (65). Copper and Zn were analyzed in soybean flour extract by HPLC coupled with inductively coupled plasma optical emission spectrometry (65A). The ion-pair reverse-phase separation of dithiocarbamate metal complexes has been used for the determination of Cu(II) and Ni(II) in legumes (66). At an optimum detection wavelength of 320 nm, 0.2–0.5 ng of each metal could be detected. Normal-phase HPLC of dithiozone or diethyldithiocarbamate metal complexes extracted into chloroform permitted the determination of Cu(II), Ni(II), and Pb(II) in kale samples at the 2–5-mg/L level or in fish at the 1-mg/L level. A wet ashing procedure using nitric acid–hydrogen peroxide was required. For both types of samples the amount of Mn(II) was found to be at the 15–20-mg/L level (67).

3. *Nonmetals*

A few miscellaneous nonmetals have been determined in food samples. Boron has been determined as a cationic species using HPLC with fluorescence detection (68). A water-soluble organoarsenic compound, arsenobetaine, has been assayed in shark by ICP after ion-exchange chromatography (69). Inorganic arsenic, monomethylarsenic, and dimethyl arsenic were determined by cation-exchange chromatography using a 0.5 M NaCl, H_2O, and 20% NH_3 step gradi-

ent. Detection at the low-μg/g level in mollusks using arsine generation and AAS was carried out (70). The determination of As in a liquid health supplement by IC with ICP atomic emission spectroscopy has been reported (20A).

IV. CAPILLARY ELECTROPHORESIS FOR IONS IN FOOD

Because capillary electrophoresis (CE) continues to grow in importance as an alternative to HPLC for charged analytes, a short review of CE as applied to the determination of small ions in food seems appropriate. Capillary electrophoresis is a relatively new analytical technique that entails the application of a high voltage (up to 30 kV) across a narrow-bore fused-silica capillary on the order of 60 cm long. The sample anions are swept by the electro-osmotic flow of cations toward the negative cathode and often are detected by spectrophotometry (direct or indirect) inside a bare region of the capillary. A rapid separation of up to 15 anions in only 3 min has been shown (71). It has been shown that naphthalenedisulfonate is an effective reagent in terms of mobility match, permitting indirect photometric detection (IPD) of organic acids as well as inorganic anions (71A). A CE instrument with conductivity detection is also commercially available, and numerous applications for ions have been demonstrated (71B). Recently in our laboratory, covalently bonded capillaries have been spliced to the CE conductivity sensor, permitting either inorganic anions or inorganic cations to be separated with the same running electrolyte but with just a switch in the applied voltage polarity (71C). The separation of inorganic cations with IPD by CE has also been reported (72). The prime advantages of CE are very high resolution and small sample volumes (on the order of 10 nl). Therefore, detection limits are excellent compared to IC based on a picomole comparison, but generally poorer based on concentration.

Capillary electrophoresis with IPD at 254 nm using chromate was applied to the determination of anions in water and soft drink samples. The detection limits were under 1 mg/L (73). Orange juice and orange pulp were analyzed for chloride and sulfate by CE with IPD and for nitrate by CE, with direct detection at 214 nm (74). Capillary electrophoresis with IPD at 254 nm using chromate was used to determine nitrate and nitrite in vegetables. Detection limits were very good at about 0.03 mg/L (75). After solid-phase extraction, nitrite and nitrate in food were also determined by CE, with direct detection at 210 nm. Thiocyanate was the internal standard; recoveries ranged from 91–104% (76). Recently, capillary electrophoresis was used to determine sulfite in food and beverages. Samples were first subjected to Monier–Williams distillation, and the SO_2 formed was oxidized to sulfate with peroxide (77). Nitrate and sulfate were separated; detection was by IPD with chromate. Yogurt, mineral water, beer, and red wine were analyzed for the cations Na, K, Ca, and Mg by CE with IPD using *p*-aminopyridine as the visualizing agent (78).

It is likely that CE will continue to become popular for food analyses in which a rapid profile of both inorganic and organic ions is desired. For example, the assay of beer for common inorganic anions such as Cl^-, SO_4^{2-}, NO_3^-, and HPO_4^{2-} as well as simple carboxylic acids due to hop acids has been demonstrated (71) and is shown in Fig. 9. Chloride, sulfate, oxalate, formate, malate, citrate, succinate, pyruvate, acetate, lactate, phosphate, and pyroglutamate ions were determined in beer using CE with IPD and 2,6-pyridinedicarboxylic acid as the background electrolyte (79). The effect of temperature on the salt balance of milk was also studied by CE with IPD at 254 nm. The results showed pasteurization and sterilization decreased the Ca, phosphate, and citrate content (80). Chloride, bromide, sulfate, thiosulfate, oxalate, molybdate, bicarbonate, citrate, acetate, propionate, borate, and butyrate were also separated under the conditions described previously (75). Other applications using CE, particularly for the determination of both inorganic and organic ions in a variety of foods, will undoubtedly be published in the future.

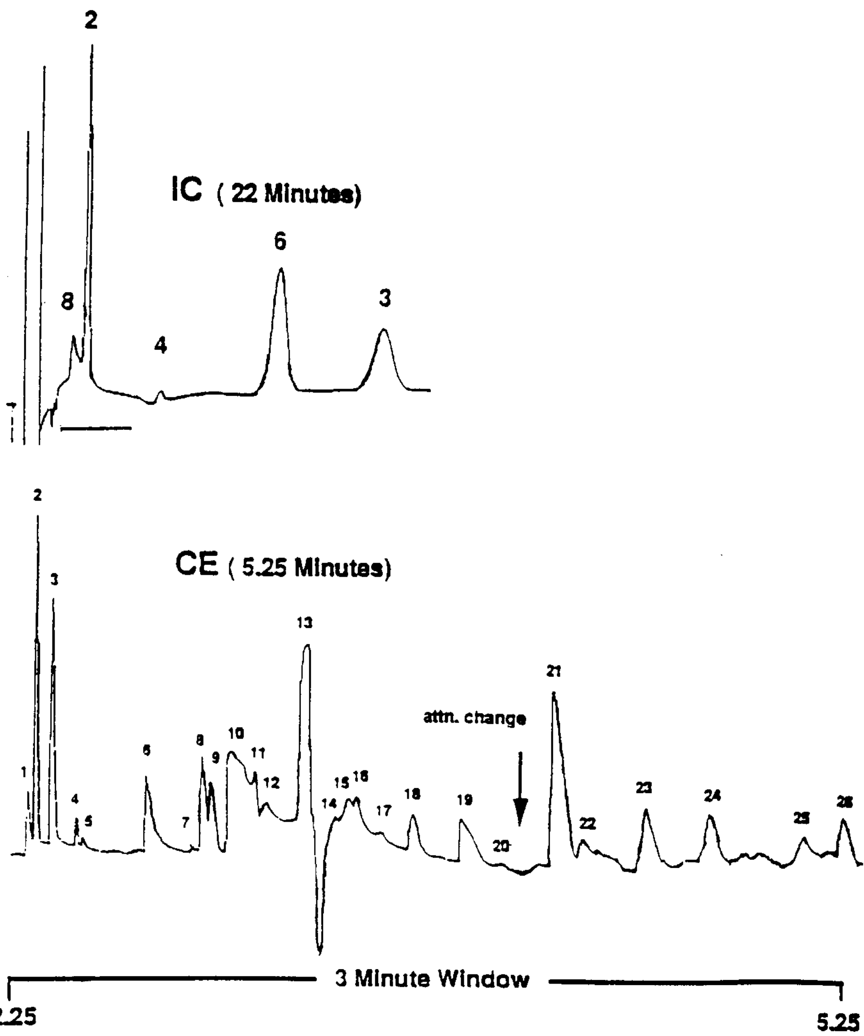


Fig. 9 Comparison of ionic information obtained by IC and CE from injection of "light" beer sample (1/10 diluted). Conditions: IC Water IC-PAK Anion HC column with borate–gluconate eluent. CE conditions: Waters NICE-PAK system, 20 kV. Peak ID: 2, chloride; 3, sulfate; 4, nitrate; 6, phosphate; 8, carbonate. Peaks 9–20 represent weak carboxylic acids; peaks due to hop acids may be found in the 21–26 range. (Reprinted by permission from Ref. 71.)

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Journal of Chromatography A, 789 (1997) 529–548

JOURNAL OF CHROMATOGRAPHY A

Review

State-of-the-art ion chromatographic determination of inorganic ions in food

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Abstract

A review of the applications of ion chromatography (IC) to the determination of inorganic ions in food is presented. The most promising sample preparation techniques, such as accelerated solvent extraction, supercritical fluid extraction, solid-phase extraction, UV photolysis, microwave-oven digestion and pyrohydrolysis are discussed. Among the various inorganic anions, nitrogen, sulphur and phosphorus species and halides are widely determined in foods and to a lesser extent only, cyanide, carbonate, arsenic and selenium species are considered. IC determination of inorganic cations deals with ammonium ion, alkali, alkaline-earth, heavy and transition metals particularly and only a small amount of literature is found on the other ones, like aluminium and plantinum. A particular advantage of IC over traditional techniques is the simultaneous determination of several species. © 1997 Elsevier Science B.V.

Keywords: Reviews; Food analysis; Inorganic cations; Inorganic anions

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1. Introduction

Ion chromatography is now a routine technique in food analysis and the number of ion-chromatography (IC) standard methods is growing very rapidly [2–4,42,43,101]. This review deals with the ion chromatographic literature of practical use, related to the determination of inorganic species in foods, thus in many cases we have not referred to the papers that describe the feasibility of ion chromatography in food analysis without taking into consideration real samples.

Tap or mineral water analysis was not included in the present review as these categories are generally included in the environmental ones.

Adequate sample preparation has growing importance because it allows the full exploitation of the potential of ion chromatography, so it is essential to overcome the traditional sample preparation techniques in food analysis, which very often result in solutions that are easily contaminated from the high quantity of reagents involved or prone to causing IC column contamination.

The most widespread detection technique in IC is still conductimetry coupled with post-column chemical suppression: this technique has been recently simplified by the introduction of self- regenerating devices [76] that electrolytically hydrolyse water in the eluent stream, in order to produce the ions necessary to the regeneration of the suppressor, thus avoiding the need for a separate regenerant device. The amperometric detector is also well established in food analysis because of its use in carbohydrate determination, but its applicability has been extended to the inorganic species [27,29,47,108] too. Other detectors have been used in IC determination of inorganic species, with or without post-column derivatization techniques, such as UV–Vis for transition metals determination, inductively coupled plasma (ICP), ICP atomic emission spectrometry (AES), etc.

The anion-exchange columns are been improved in terms of selectivity; speed [87], capacity [31,32] and solvent compatibility. Use of macroporous substrates with chemically grafted ion-exchange sites can reduce the organic anion interference in inorganic anion determination or allow their determination in complex matrices. But improvements in ion-exchange columns were particularly relevant in cation analysis, where the introduction of cation-exchange columns with carboxylic acid based ion-exchange sites [30,48,56,75] allow the separation of alkali and alkaline-earth metals in a few minutes with a simple eluent system. A new column for transition metals determination was introduced that is suitable for use with gradient conditions and, if using temperature control, the analysis can be shortened to less than 10 min [79].

The use of gradient elution in IC for resolving complex matrices is going to be a routine technique [10,28,100] and the interest of food analysts was enhanced by the introduction of a new dedicated

column with higher selectivity and shorter retention times [26,87].

References have been assembled by analyte and are presented in Tables 1–9 . Any single row represents a single determination of different species. Species are listed in order of elution. References inside the same table are ordered by date: the last published is the first in the order.

2. Modern sample preparation techniques

In consideration of the few opportunities of injecting food samples directly, or by simple dilution, into the analytical system, the sample preparation plays a vital role in exploiting the potential of ion chromatography. The traditional sample preparation techniques for inorganic species determination in foods generally involve digestion steps like wet digestion, dry ashing or alkaline fusion that require a high quantity of reagents to remove the strong interfering and/or chelating effect of the organic matrix. This kind of sample preparation often results in solutions that are easily contaminated from the reagents involved or prone to causing IC column contamination. In addition conventional sample preparation procedures are time consuming and generate a lot of waste.

Unfortunately it is not possible to use a simple sample treatment technique in food analysis due to the multiplicity of matrix and type and concentration range of the species to be determined.

The modern sample preparation techniques hereby listed are experienced with organic species, and fewer references are reported for inorganic ones. The most promising techniques in order to obtain solutions apt to the direct IC injection seem to be the following:

2.1. Accelerated solvent extraction (ASE)

The accelerated solvent extraction technique employs basically the principles of traditional solvent extraction (whose entire experience can be directly used) but at higher temperature and pressure where solvents show better extraction properties. The high temperature increases the rate of extraction and high pressure elevates the boiling point of the solvent. This results in better extraction efficiency along with short extraction time and low solvent requirements. The time required for extraction is practically independent of the mass of sample.

The efficiency of extraction is mainly dependent on temperature as it influences physical properties of the sample and its interaction with the liquid phase. The extraction is influenced by the surface tension of the solvent and its penetration into the sample (i.e. its viscosity) and by the diffusion rate and solubility of the analytes: all parameters that are normally improved by the temperature increase. The effect of high pressure is to maintain the liquid phase of solvent beyond its boiling point and also to help penetration of the solvent in the sample matrix. The temperature is normally kept in the range between 50–200°C and the pressure is maintained at 1200–3000 psi.

2.2. Supercritical fluid extraction (SFE)

The supercritical fluid extraction uses the principles of traditional liquid–solid extraction. Supercritical fluids are substances above their critical temperature and pressure and they provide an unusual combination of properties. Supercritical fluids diffuse through solids like gases, but dissolve analytes like liquids, so the extraction rate is enhanced. The most commonly used supercritical fluid is carbon dioxide, which can be used as it is or modified with some percent (1–10%) of an organic solvent.

The solvent strength of any supercritical fluid can be adjusted depending on the pressure and temperature at which the fluid is used (i.e. its density), so allowing a single supercritical fluid to substitute for a variety of conventional solvents.

In supercritical fluid extraction, a pump is used to supply the extraction fluid to the extraction vessel, which is heated to maintain it at a temperature above the critical point. A restrictor device is placed at the end of the system to maintain pressure. During the extraction, the soluble analytes are partitioned from the bulk sample matrix into the supercritical fluid, then swept through the flow restrictor into a collection device.

Supercritical fluid extraction is extensively used for separating organic species from foods (like fats, etc.) and is used for the separation of inorganic

Table 1
Ion chromatographic determination of nitrogen species in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
NO_3^-	Fruits juice	Dionex OmniPac PAX-500	NaOH–ethanol–methanol	Conductivity	[85]
NO_3^-	Beverages, carbonated	Dionex IonPac AS11	NaOH	Conductivity	[28]
NO_3^-	Rice flour Tea leaves	Dionex IonPac AS12A	Na_2CO_3–$NaHCO_3$	Conductivity	[14]
NO_3^-	Wine	Shimadzu Shim-pack IC-AI	Phthalic acid	Conductivity	[63]
NO_3^-	Milk	Dionex IonPac AS11	NaOH	Conductivity	[39]
NO_3^-	Carrot juice Vegetables	Metrohm Metrosep Anion Dual 1	Na_2CO_3–$NaHCO_3$	Conductivity	[23]
NO_2^- NO_3^-	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid	UV–Vis	[60]
NO_3^-	Pork meat products	Dionex IonPac AS4	$Na_4B_2O_7$	Conductivity	[8]
NO_2^- NO_3^-	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[102]
NO_3^-	Tea Vegetables	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity/UV–Vis	[12]
NO_2^- NO_3^-	Cereal based baby foods	Waters IC-PAK Anion	KH_2PO_4-Na_2HPO_4	UV–Vis	[68]
NO_2^-	Spinach	Hamilton PRP-X100	Phthalic acid–10% acetone	Coulometry	[11]
NO_2^- NO_3^-	Coffee Sake, Japanese Wine	Mixed-bed laboratory packed with Yokogawa ICS-A23 and Yokogawa CH1	Oxalic acid	Conductivity	[25]
NO_3^-	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[66]
NO_3^-	Beer Wort	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[58]
NO_3^-	Wine vinegar	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[33]
NO_2^- NO_3^-	Fruits, syruped canned Infant foods Jams Marmalades Meats Meats, cured Pork, chopped Vegetables, canned	Waters IC-PAK Anion	KH_2PO_4–Na_2HPO_4	UV–Vis	[88]
NO_2^- NO_3^-	Meats, Meats cured	Dionex IonPac AS4A	$Na_4B_2O_7$	Conductivity	[34]
NO_3^-	Vegetables	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[67]
NO_3^-	Milk Popcorn, buttered Salad dressing	Waters IC-PAK Anion	Sodium octanesulphonate	Conductivity	[51]
NO_3^-	Beer Wort	Dionex OmniPac PAX-500	NaOH–ethanol	Conductivity	[10]
NO_2^- NO_3^-	Food extracts	Alltech Universal Anion	Lithium 4-hydroxybenzoate or Li-hydrogenphthalate or Li-hydrogenphthalate–ethylendiamine	Conductivity	[84]
NO_3^-	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[98]

Table 1 (*continued*)

Species	Matrix	Column	Eluent	Detector	Ref.
NO_2^-	Meats, cured	Wescan Anion exclusion/HS	H_2SO_4	Amperometry	[54]
NO_3^-	Vegetables	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[16]
NO_3^-	Fruits, canned liquor	Dionex IonPac AS5	Na_2CO_3–$NaHCO_3$	Conductivity	[44]
NO_2^- NO_3^-	Fruits juice Meats, cured Vegetables	Merck LiChrosorb RP	*n*-Octylamine–H_3PO_4	UV–Vis	[97]
NO_3^-	Vegetables	Laboratory packed BAKC Ion exchanger	Potassium gluconate–boric acid or potassium phthalate	Conductivity	[73]
NO_3^-	Vegetable juice	Waters IC-PAK Anion	Potassium hydrogenphthalate	Conductivity	[20]
NO_2^-	Beer	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[80]

species too. Unfortunately the selection of supercritical fluids and modifiers is largely empirical because very little analyte solubility data exists for modified supercritical fluids. Interactions between supercritical fluid, target analytes and sorptive sites on the bulk matrix are poorly understood.

2.3. Solid-phase extraction (SPE)

Solid-phase extraction has become very popular over the past ten years. The technique offers many improvements over liquid–liquid extraction and permits both interferent's removal and analytes concentration at the same time.

As the sample solution passes through the sorbent bed, analytes concentrate on its surface, while the other sample components pass through the bed. The most common starting material for sorbent bed is silica, because it is reactive enough to permit its surface to be modified by chemical reaction and yet stable enough to allow its use with a wide range of sample. Polymer based sorbent beds are becoming very popular, thus offering a wide range of selective properties for extraction.

The extraction is performed in four steps: conditioning (the functional groups of the sorbent bed are solvated in order to make them to interact with the samples), retention (the analytes are adsorbed to the bed surface), rinsing (undesired species are removed) and elution (the analytes are desorbed and collected for IC injection).

The extraction conditions are mainly affected by pH, matrix ionic strength, polarity of the elution solvent, flow-rate and physico-chemical characteristics of the sorbent bed.

2.4. UV photolysis (UVP)

In the preparation of food samples, UV photolysis has shown distinct advantages over traditional dissolution techniques owing to the very low blank values. In Table 10 the reaction mechanisms involved are shown and it is evident that the organic matrix is degraded indirectly via OH radicals and not directly through UV radiation.

It can be seen from the above radical formation reaction that the H_2O quantity is sufficient for producing HO*, however for food samples, where organic matrix is very high, addition of H_2O_2 will accelerate the radical formation resulting into shortening the oxidation time.

Usually high-pressure mercury lamps with a high intensity, as well as a great radiant flux are used. Inserting reflecting surfaces between the sample tubes and the external liquid cooling system to have multiple reflection of UV radiations in the sample enhances the high intensity. An experimental difficulty in the use of these lamps is their high heat development, which can lead to high evaporation losses of the samples, so UV digesters must have cooling systems, which permit oxidation without any losses.

The UV digestion of any sample is directly proportional to the UV intensity and irradiation time. It is inversely proportional to the organic substance concentration. The digestion is also directly proportional to the temperature of the sample; this needs

Table 2
Ion chromatographic determination of sulphur species in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
SO_4^{2-}	Tea	Shimadzu Shim-pack IC-AI	Potassium hydrogenphthalate–phthalic acid	Conductivity	[24]
SO_4^{2-}	Beverages, carbonated	Dionex IonPac AS11	NaOH	Conductivity	[28]
SO_3^{2-}	Beverages Foods	Dionex IonPac ICE-AS1	H_2SO_4	Pulsed amperometry	[27]
SO_4^{2-}	Rice flour Tea leaves	Dionex IonPac AS12A	Na_2CO_3–$NaHCO_3$	Conductivity	[14]
SO_4^{2-}	Wine	Shimadzu Shim-pack IC-AI	Phthalic acid	Conductivity	[63]
SO_3^{2-} SO_4^{2-}	Wine, red	Laboratory packed Shiseido Capcell Pak C18, cetyl pyridinium bromide-coated	Phthalate–triethanol-amine–5% methanol	UV–Vis	[61]
SO_4^{2-} $S_2O_3^{2-}$ $S_2O_6^{2-}$	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzene disulphonic acid	UV–Vis	[60]
SO_4^{2-}	Milk	Dionex IonPac AS11	NaOH	Conductivity	[39]
SO_4^{2-}	Tea	Shimadzu Shim-pack IC-AI	Phthalic acid–Tris	Conductivity	[35]
SO_4^{2-}	Carrot juice Vegetables	Metrohm Metrosep Anion Dual 1	Na_2CO_3–$NaHCO_3$	Conductivity	[23]
SO_4^{2-}	Beer	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[13]
S-SO_4^{2-}	Liver, bovine	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[70]
SO_4^{2-}	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[102]
SO_4^{2-}	Tea	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[99]
SO_4^{2-}	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[66]
SO_4^{2-}	Beer Wort	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[58]
SO_4^{2-}	Wine vinegar	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[33]
SO_3^{2-}	Artichokes, canned Fruits juice Mushrooms Sausages Shrimps Tomato sauce Vegetables, canned	Waters IC-PAK Anion	Sodium borate–gluconate	Conductivity	[83]
SO_4^{2-}	Tea	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[19]
S-SO_4^{2-}	Rapeseed	Waters IC-PAK Anion	Sodium borate–gluconate	Conductivity	[107]
SO_3^{2-}	Cola soft drink Corn starch Lemon juice Potatoes, instant mashed Wheat starch Wine	Dionex IonPac AS4A or Dionex IonPac AS2	Na_2CO_3–$NaHCO_3$–HCOH	Conductivity	[103]

Table 2 (*continued*)

Species	Matrix	Column	Eluent	Detector	Ref.
SO_4^{2-}	Milk Popcorn, buttered Salad dressing	Waters IC-PAK Anion	Sodium octanesulphonate	Conductivity	[51]
SO_4^{2-}	Beer Wort	Dionex OmniPac PAX-500	NaOH–methanol–ethanol	Conductivity	[10]
SO_4^{2-}	Liver Animal tissues	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$–4.5% acetonitrile	Conductivity	[81]
SO_3^{2-}	Cellulosic thixotropic materials	BioRad Aminex HPX-87H ABI Polypore H	H_2SO_4	d.c. Amperometry	[78]
SO_3^{2-}	Beer	Dionex IonPac ICE-AS1	H_2SO_4–acetonitrile	d.c. Amperometry	[106]
SO_4^{2-}	Food extracts	Alltech Universal Anion	Lithium 4-hydroxybenzoate or lithium–hydrogenphthalate or lithium–hydrogenphthalate–ethylendiamine	Conductivity	[84]
SO_3^{2-}	Beer	Two Dionex IonPac AS2 in series Hamilton PRP-1+ Dionex IonPac AS2	Na_2CO_3–$NaHCO_3$–formaldehyde	Conductivity	[104]
SO_4^{2-}	Milk, human breast	Dionex IonPac AS2	Na_2CO_3–$NaHCO_3$	Conductivity	[59]
SO_4^{2-}	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity/ UV–Vis	[98]
SO_3^{2-}	Corn starch Lemon juice Potato, instant mashed Seafood, dehydrated Wine cooler	Wescan Anion exclusion/ HS or Waters or BioRad or Brownlee Polypore H	Sulphuric acid	Amperometry	[53]
SO_3^{2-}	Grapes	Wescan Anion exclusion/ HS	Sulphuric acid	Amperometry	[55]
SO_4^{2-}	Citrus leaves Liver, bovine Tomato leaves	Dionex IonPac AS3	Na_2CO_3–$NaHCO_3$	Conductivity	[40]
SO_4^{2-}	Apple Beef Cake Flour Milk, powder Oil	Dionex IonPac AS4A	NaOH–Na_2CO_3	Conductivity	[92]
SO_4^{2-}	Fruits, canned liquor	Dionex IonPac AS5	Na_2CO_3–$NaHCO_3$	Conductivity	[44]
SO_4^{2-}	Vegetables juice	Waters IC-PAK Anion	Potassium–hydrogenphthalate	Conductivity	[20]
SO_4^{2-}	Colour additives	Dionex IonPac AS1	Na_2CO_3–$NaHCO_3$	Conductivity	[36]

Table 3
Ion chromatographic determination of phosphorus species in foodstuffs (PolyP=Polyphosphates)

Species	Matrix	Column	Eluent	Detector	Ref.
$H_2PO_4^-$	Tea	Shimadzu Shim-pack IC-AI	Potassium–hydrogenphthalate–phthalic acid	Conductivity	[24]
PolyP	Foods	Dionex IonPac AS11	NaOH	Conductivity	[5]
PO_4^{3-}	Cola soft drink	Waters IC-PAK Anion	Sodium gluconate–borate–H_3BO_3-*n*-butanol–acetonitrile–glycerol	Conductivity	[6]
PO_4^{3-}	Fruits juice	Dionex OmniPac PAX-500	NaOH–ethanol–methanol	Conductivity	[85]
PO_4^{3-}	Carrot juice Vegetables	Metrohm Metrosep Anion Dual 1	Na_2CO_3–$NaHCO_3$	Conductivity	[23]
PO_4^{3-}	Cola beverages Soft drinks	Dionex IonPac AS4	Na_2CO_3–NaOH	Conductivity	[113]
$H_2PO_4^-$	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzene-disulphonic acid	UV–Vis	[60]
PO_4^{3-}	Milk	Dionex IonPac AS11	NaOH	Conductivity	[39]
P-PO_4^{3-}	Rice flour Tea leaves	Dionex IonPac AS12A	Na_2CO_3–$NaHCO_3$	Conductivity	[14]
PO_4^{3-}	Beer	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[13]
$H_2PO_4^-$	Tea	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[99]
P-PO_4^{3-}	Liver, bovine	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[70]
PO_4^{3-}	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[102]
PO_4^{3-}	Vegetables	Waters IC-PAK Anion	Sodium borate–gluconate	Conductivity	[82]
PO_4^{3-}	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[66]
PO_4^{3-}	Beer Wort	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[58]
PolyP	Shrimp, processed	Dionex IonPac AS7	HNO_3	ICP-AES or UV–Vis	[41]
PO_4^{3-}	Wine vinegar	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[33]
HPO_4^{2-}	Tea	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[19]
PO_4^{3-}	Pea Tomato, cherry	Laboratory packed with BioRad AG MP-1	Borate–NH_4Cl	UV–Vis	[89]
PO_4^{3-}	Beer Wort	Dionex OmniPac PAX-500	NaOH–ethanol	Conductivity	[10]
PO_4^{3-}	Food extracts	Alltech Universal Anion	Lithium 4-hydroxybenzoate	Conductivity	[84]
$H_2PO_4^-$	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[98]
PO_4^{3-}	Beer	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[80]
PO_4^{3-}	Fruits, canned liquor	Dionex IonPac AS5	Na_2CO_3–$NaHCO_3$	Conductivity	[44]
PO_4^{3-}	Flour	Waters IC-PAK Anion	Sodium borate–gluconate	Conductivity	[20]
PO_4^{3-}	Potato process water	Waters Resolve C_{18}	Tetrabutylammonium phosphate–formic acid–methanol	Refractive Index	[20]
PO_4^{3-}	Colour additives	Dionex IonPac AS1	Na_2CO_3–$NaHCO_3$	Conductivity	[36]

optimisation keeping in view the volatilisation limits of the analytes.

Appreciable results have been obtained in the trace determination of metals in foods (honey, wine, olive oil, and milk) or plant materials [14].

2.5. Microwave-oven digestion (MOD)

Microwave-oven digestion consists in keeping the sample and acids in a polytetrafluoroethylene (PTFE) vessel and heating the contents using microwave radiations. The PTFE vessels are transparent to microwaves and the sample directly absorbs electromagnetic energy in MHz–GHz wavelength range which transmits its energy to the polar molecules present in the sample (water, acids, etc.) forcing them to vibrate at high frequency. This results in high sample temperatures without the vessel being heated.

Table 4
Ion chromatographic determination of halides in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
Cl^-	Tea	Shimadzu Shim-pack IC-AI	Potassium hydrogenphthalate–phthalic acid	Conductivity	[24]
F^-	Beverages	Shimadzu Shim-pack SCR-102H	*p*-Toluenesulphonic acid	Conductivity	[38]
I^-	Food, colouring agents	Carbon BI 01	Tetrabutylammonium hydroxide–Na_2CO_3–acetonitrile	Conductivity	[71]
Cl^-	Wine	Dionex OmniPac PAX-100	NaOH–acetonitrile	Conductivity	[52]
Cl^-	Fruits juice	Dionex OmniPac PAX-500	NaOH–ethanol–methanol	Conductivity	[85]
F^- Br^- Cl^-	Citrus leaves Milk, non-fat powder Orchard leaves Oyster tissue Tomato leaves	Dionex IonPac AS9	Na_2CO_3–$NaHCO_3$	Conductivity	[93]
Cl^-	Beverages, carbonated	Dionex IonPac AS11	NaOH	Conductivity	[28]
Cl^- Br^-	Rice flour	Dionex IonPac AS12A	Na_2CO_3–$NaHCO_3$	Conductivity	[14]
Cl^-	Cola beverages Soft drinks	Dionex IonPac AS4+Dionex IonPac AS2	Na_2CO_3–NaOH	Conductivity	[113]
Cl^-	Wine	Shimadzu Shim-pack IC-AI	Phthalic acid	Conductivity	[63]
F^- Cl^-	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzene-disulphonic acid	UV–Vis	[60]
Cl^-	Milk	Dionex IonPac AS11	NaOH	Conductivity	[39]
Cl^-	Carrot juice Vegetables	Metrohm Metrosep Anion Dual 1	Na_2CO_3–$NaHCO_3$	Conductivity	[23]
I^-	Milk products Soy-based infant formula	Dionex IonPac AS11	HNO_3	d.c. Amperometry	[29]
F^- Br^- Cl^-	Coffee Sake, Japanese Wine	Mixed bed laboratory packed with Yokogawa ICS-A23 and Yokogawa CH1	Oxalic acid	Conductivity	[25]
Cl^-	Beer	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[13]
Cl^-	Liver, bovine	Dionex IonPac AS4A SC	Na_2CO_3–$NaHCO_3$	Conductivity	[70]
F^- Cl^-	Tea	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[99]
Cl^-	Spinach Vegetables	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[66]
Br^-	Rice	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[105]
F^- Cl^-	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[102]
Cl^-	Beer Wort	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[58]
Cl^-	Wine vinegar	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[33]
Br^-	Bakery products	Dionex IonPac AS10	NaOH	ICP-MS	[41]

(Continued on p. 538)

Table 4 (*continued*)

Species	Matrix	Column	Eluent	Detector	Ref.
Br^-	Bread Meats Soups Vegetables Tomato juice	Tosoh TSKgel IC-Anion PWXL	KH_2PO_4–K_2HPO_4	UV–Vis	[62]
Cl^-	Tea	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[19]
Cl^- Br^- I^-	Milk Popcorn, buttered Salad dressing	Waters IC-PAK Anion	Sodium octanesulphonate	Conductivity	[51]
F^-	Milk Popcorn, buttered Salad dressing	Waters IC-PAK Ion exclusion	Octanesulphonic acid	Conductivity	[51]
F^- Br^- Cl^-	Beer Wort	Dionex OmniPac PAX-500	NaOH–methanol–ethanol	Conductivity	[10]
F^- Br^- Cl^-	Food extracts	Alltech Universal Anion	Lithium 4-hydroxybenzoate or lithium hydrogenphthalate or lithium hydrogenphthalate–ethylendiamine	Conductivity	[84]
I^-	Cod Milk Shell fish Whey powders	Merck LiChrosorb RP	KH_2PO_4–Na_2HPO_4–cetyltrimethylammonium bromide	UV–Vis/amperometry	[95]
Cl^-	Spinach	Dionex IonPac AS4A	Na_2CO_3–$NaHCO_3$	Conductivity	[98]
F^-	Apple Beef Cake Flour Milk, powder Oil	Dionex IonPac AS4A	NaOH–Na_2CO_3	Conductivity	[92]
F^- Cl^- Br^-	Fruits, canned liquor	Dionex IonPac AS5	Na_2CO_3–$NaHCO_3$	Conductivity	[44]
Cl^-	Agar Cellulose powder Corn starch Gelatin Potato starch	Laboratory packed with Dowex AG1	Na_2CO_3–$NaHCO_3$	Conductivity	[69]
Cl^-	Vegetables	Laboratory packed BAKC Ion exchanger	Potassium gluconic acid–boric acid or Potassium phthalate	Conductivity	[73]
Cl^-	Vegetable juice	Waters IC-PAK Anion	Potassium hydrogenphthalate	Conductivity	[20]
Cl^-	Beer	Dionex IonPac AS4	Na_2CO_3–$NaHCO_3$	Conductivity	[80]
Cl^- Br^-	Certifiable colours	Dionex IonPac AS1	Na_2CO_3–$NaHCO_3$	Conductivity	[36]

Table 5
Ion chromatographic determination of oxyhalides in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
ClO_2^- ClO_3^-	Vegetables rinse water	Dionex IonPac AS12A	Na_2CO_3–$NaHCO_3$	Conductivity/ amperometry	[7]
BrO_3^-	Bakery products	Dionex IonPac AS10	NaOH	ICP-MS	[41]
IO_3^-	Milk Popcorn, buttered Salad dressing	Waters IC-PAK Anion	Sodium octanesulphonate	Conductivity	[51]
IO_3^-	Table salt	Merck LiChrosorb RP	NaCl–hexadecyltri-methylammonium chloride	UV–Vis	[95]
IO_3^-	Table salt	Macherey-Nagel Nucleosil 10-NH_2	NaCl–HCl	UV–Vis	[95]
BrO_3^-	Bread Dough conditioners	Waters IC-PAK Anion	Benzoic acid	Conductivity	[20]
BrO_3^-	Bread	Dionex IonPac AS 1	Sodium tetraborate	Conductivity	[72]

The vessel always remains at a lower temperature than the sample thereby resulting in negligible contamination or absorption of the sample analytes by the vessel.

The digestion is automatically controlled by a pressure and a temperature sensor, at the normal limits of 200°C (at a maximum pressure of 100–150 p.s.i.) and 1200 p.s.i. (at a maximum temperature of 50°C) (1 p.s.i.=6894.76 pa). These limits are imposed by the physical structure of the apparatus and the chemically inert microwave transparent materials used.

Recently an offshoot of MOD has been established using microwaves in conventional solvent extraction technique, known as microwave-assisted solvent extraction (MASE). It may be mentioned here that the molecules with higher dielectric constant absorb more energy from microwaves and attain higher temperatures. The differential temperature between solvent and sample pushes the analyte from sample to the solvent.

Many papers deal with the microwave digestion of food samples [62,64,77]; good results have been obtained in the trace determination of transition metals.

2.6. Pyrohydrolysis (PH)

Pyrohydrolysis is a technique which uses decomposition of the matrix by superheated water vapours. The apparatus is very simple and consists of a round bottom flask, a quartz delivery tube passing through a small furnace and a condenser; it can be assembled in any analytical chemistry laboratory. The sample is kept in a quartz or platinum or alumina boat inside the delivery tube in the zone of the furnace. Extra pure water is heated and the vapours are pushed using an inert gas through the sample and are condensed. The flowing superheated water vapour extracts volatile and semivolatile substances from the sample, which after condensation are available for analytical determination. The furnace surrounding the sample helps in further heating the water vapours and enhances the volatilisation of analytes.

This technique is normally used for the determination of halogens, borates, nitrates, sulphates, etc. in various food matrices [40,93].

3. Inorganic anions

3.1. Nitrogen species

Nitrite and nitrate: Nitrates are naturally present in many foods, noticeably vegetables where their content varies to a great extent because of the wide-

Table 6
Ion chromatographic determination of other anionic species in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
CrO_4^{2-}	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid	UV–Vis	[60]
MnO_4^-	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid	UV–Vis	[60]
PO_3F^{2-}	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid	UV–Vis	[60]
SO_3F^-	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid	UV–Vis	[60]
SeO_3^{2-} SeO_4^{2-}	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid	UV–Vis	[60]
CO_3^{2-}	Beverages	Shimadzu Shim-pack SCR 102H	*p*-Toluenesulphonic acid	Conductivity	[38]
CN^-	Flaxseed	Waters IC-PAK Anion	H_3BO_3–NaOH-Na_2CO_3– methanol–ethylendiamine	Amperometry	[17]
CN^-	Apricot	TSKgel IC-Anion PW	2-Dimethylaminoethanol– formic acid–$Na_4B_2O_7$– triethylenetetramine-N,N,N′,N″,N‴,N‴– hexaacetic acid hexasodium salt	Conductivity	[37]
AsO_2^- AsO_4^{3-}	Liquid health food supplement	Dionex IonPac AS4A	NaOH	ICP-AES	[41]
Total Se	Egg proteins	Laboratory packed with Pharmacia Q-Sepharose Fast Flow	Bis Tris-propane–HCl–NaCl	UV–Vis/FIA–AAS	[46]
Total Se	Milk, goat proteins	Laboratory packed with Pharmacia Q-Sepharose Fast Flow	Imidazole–urea–2-mercaptoethanol–NaCl	UV–Vis/MH-AAS	[21]
$PtCl_6^{2-}$	Bean pod Corn Tobacco	Dionex IonPac AS4A	HCl–$NaClO_4$	UV–Vis	[49]
Total Se	Milk, cow proteins	Laboratory packed with Pharmacia Q-Sepharose Fast Flow	Imidazole–urea–2- mercaptoethanol–NaCl	UV–Vis/MH-AAS	[22]
CN^-	Chopped fruits	Waters IC-PAK Anion	H_3BO_3–NaOH–Na_2CO_3– methanol–ethylendiamine	Amperometry	[18]
HCO_3^- CO_3^{2-}	Food additives	Dionex ICE-AS1	Octanesulphonic acid	Conductivity	[57]

spread nitrogenous fertilisers' use. Nitrate must be monitored, particularly in infant food preparation, because the reduction in the intestine of nitrate to nitrite can induce metahemoglobinemia. In addition, it must be reminded that nitrate, although not very toxic, in the adult digestive tract is reduced under physiological conditions to nitrite, which reacts with secondary and tertiary amines forming high carcinogenic nitrosamines.

Vegetables are not the only source of nitrate intake, the potassium and sodium salts of both nitrate and nitrite are commonly used in food industry, in curing meat for fixing the color, for inhibiting the microbial growth and for obtaining the characteristic flavour.

The nitrate content is important also for monitoring the food adulteration in dairy industry.

The determination of nitrite and nitrate is usually performed at the same time and mostly by using a bicarbonate/carbonate eluent and suppressed conductivity detection alone or coupled with absorbance in the case of complex matrices.

3.2. Sulphur species

Sulphite, sulphate: Sulphites are commonly used

Table 7
Ion chromatographic determination of I and II group cations and ammonia in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
I group NH_4^+ II group	Beverages, carbonated	Dionex IonPac CS12	Methanesulphonic acid	Conductivity	[28]
I group NH_4^+ II group	Fruit, juice and puree	Dionex IonPac CS12A	Methanesulphonic acid	Conductivity	[100]
I group NH_4^+	Ketchup Whisky	Laboratory packed with Nomura Chemical Develosil porous silica gel	Benzyltrimethylammonium chloride–acetonitrile	UV–Vis	[65]
Ca^{2+} Mg^{2+}	Orange juice Potato chips	Hamilton PRP X-100	2,5–Dihydroxy-1,4-benzene-disulphonic acid	UV–Vis	[60]
I group NH_4^+	Beverages	Mixed bed laboratory packed with Yokogawa ICS-A23 and Yokogawa CH1	Oxalic acid	Conductivity	[25]
I group II group	Tea	Dionex IonPac CS3	HCl–2,3-diaminopropionic acid	Conductivity	[99]
I group NH_4^+	Spinach	Dionex IonPac CS1	HCl	Conductivity	[102]
Ca^{2+} Sr^{2+} Ba^{2+}	Milk, skimmed powder	Laboratory packed Dionex PS-DVB 120 Å +Phthalein purple (*o*-cresolphthalein-3′,3″-bis-methyleneiminodiacetic acid)	KNO_3–lactic acid	UV–Vis	[50]
I group NH_4^+ II group	Bread crumbs Cheese Parsley Peanut butter Pretzels	Waters IC-PAK Cation M/D	EDTA–HNO_3	Conductivity	[64]
I group II group	Tea	Dionex IonPac CS3	HCl–2,3-diaminopropionic acid	Conductivity	[19]
NH_4^+	Food simulants	Dionex IonPac CS10	HCl–2,3-diaminopropionic acid	Conductivity	[9]
K^+	Spinach Vegetables	Dionex IonPac CS1	HCl	Conductivity	[67]
I group II group	Bakery products Cream Diary products Soups, dried	Metrohm Super Sep Cation	EDTA	Conductivity	[96]
Na^+ K^+ NH_4^+ Rb^+	Food extracts	Wescan CATION-R	Lithium hydrogenphthalate or lithium 4-hydroxybenzoate	Conductivity	[84]
Mg^{2+} Ca^{2+} Ba^{2+}	Food extracts	Wescan CATION-R	Lithium hydrogenphthalate–ethylendiamine	Conductivity	[84]
I group N-NH_4^+	Grain	Waters IC-PAK Cation	HNO_3	Conductivity	[45]
Na^+ K^+ N-NH_4^+	Grain	Protein Pak SP-5PW	HNO_3–acetone	Conductivity	[45]
I group NH_4^+ II group	Foods	Metrohm Supersep 125 IC-Cation	Citric acid–pyridine-2,6-dicarboxylic acid	Conductivity	[109]
I group	Wine	Biotronik BT IV KA	HNO_3	Conductivity	[91]
I group	Wine	Dionex IonPac CS1	HCl	Conductivity	[91]
I group NH_4^+ II group	Molasses	Dionex IonPac CS3	HCl–2,3-diaminopropionic acid	Conductivity	[86]

(Continued on p. 542)

Table 7 (*continued*)

Species	Matrix	Column	Eluent	Detector	Ref.
Mg^{2+} Ca^{2+}	Fruit juice	Macherey-Nagel Nucleosil 5SA	Oxalic acid–ethylenediamine–acetone	Conductivity	[90]
K^{+}	Beer Fruit juice Milk Vegetable juice Wine	Dionex IonPac CS1	HCl	Conductivity	[97]
I group NH_4^+	Cheese	Waters IC-PAK Cation	HNO_3	Conductivity	[20]
Mg^{2+} Ca^{2+}	Wort	Waters IC-PAK Cation	Citric acid–ethylenediamine	Conductivity	[20]
II group	Porridge oats Wine, red	Macherey-Nagel Nucleosil SA10	Tartaric acid	UV–Vis	[110]
I group NH_4^+	Beer	Dionex IonPac CS1	HCl	Conductivity	[80]
II group	Beer	Dionex IonPac CS1	HCl–*m*-phenylenediamine	Conductivity	[80]

in the food industry as a preserving agent; only recently has there been focus on the health problems associated with their widespread use. Moreover, the sulphite content is controlled by national and international regulations where sulphite data must be provided for each component of food formulation.

Most of the IC papers concerning sulphur compounds analysis are related to the sulphite determination as previously reviewed [74]; a major improvement was the introduction of pulsed amperometric detection. Sulphate concentration can be affected by the technological use of sulphites. In consideration of the presence of sulphur bound to organic molecules too, sulphate is the anion usually determined as final product of total oxidation of sulphur compounds, when it is necessary to quantitate the total amount of sulphur species. The determination of sulphite if stabilised in a suitable way and sulphate can be performed at the same time.

Sulphate can be simultaneously determined together with other inorganic species of sulphur such as thiosulphate and dithionate.

3.3. Phosphorus species

Phosphate, polyphosphate: Phosphorus compounds are present in most vegetable and animal foods; some of them, such as proteins and phospholipids, are important indicators of metabolic activity. Phosphorus is important for skeletal integrity, that directly depends on calcium/magnesium/phosphorus ratio. A phosphorus excess leads to tissue ossification.

Inorganic phosphates are extensively used as fertilisers so the same considerations that apply for nitrogen species are also valid for phosphorus content in vegetables. Phosphate determination in foods is very important: for example its concentration in milk affects almost all aspects of cheese manufacturing, in soft drinks formulation where it acts as acidifier and flavour, etc.

Polyphosphates are widely used as additives: in meat-based products for reducing water loss during the commercial life of the food, but they can be also used to increase the uptake of water for economic fraud; in fruit juices as flavour and color preservatives; in the dairy industry as additive for cheese, etc.

Total phosphorus content is one of the parameters used to define product quality and genuineness. The most common way of determining phosphate is its separation with bicarbonate/carbonate eluent followed by suppressed conductivity detection. The gradient IC introduction caused polyphosphate analysis to move from traditional absorbance detection following a post-column reagent addition step to suppressed conductivity.

3.4. Halides

In the most common analytical conditions, ion

Table 8
Ion chromatographic determination of transition and heavy metals in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
Pb^{2+} Cd^{2+}	Rice flour Tea leaves	Dionex IonPac CS10	H_2SO_4–HCl–KCl	UV–Vis	[14]
Fe^{3+} Cu^{2+} Ni^{2+} Zn^{2+} Co^{2+}	Rice flour Tea leaves	Dionex IonPac CS5	Pyridine-2,6-dicarboxylic acid CH_3COOH–CH_3COONa	UV–Vis	[14]
Cu^{2+} Zn^{2+} Mn^{2+}	Soybean, defatted flour	Macherey-Nagel Nucleogel Pharmacia Superdex	Tris(hydroxymethyl)amino methane–HCl	ICP-OES	[94]
Cd^{2+} Pb^{2+} Ni^{2+} Cu^{2+} Co^{2+} Fe^{3+} Cr^{3+}	Orange juices Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid–EDTA	UV–Vis	[60]
Cd^{2+}	Oyster tissue	Shimadzu Shim-pack IC–Cl	Lactic acid	UV–Vis	[112]
Pb^{2+}	Wine	Dionex IonPac CS5	Oxalic acid	UV–Vis	[15]
Mn^{2+}	Foods	Metrohm Supersep 125 IC-Cation	Citric acid–pyridine-2,6- dicarboxylic acid	Conductivity	[109]
Zn^{2+}	Wort	Waters IC-PAK Cation	Citric acid–ethylendiamine	Conductivity	[20]
Fe^{3+} Fe^{2+}	Apples Lentils	Dionex IonPac CS2	Sodium sulphosalicylate– ethylenediamine	UV–Vis	[111]
Fe^{3+} Cu^{2+} Pb^{2+} Zn^{2+} Ni^{2+} Co^{2+} Cd^{2+} Fe^{2+} Mn^{2+}	Porridge oats Wine, red	Macherey-Nagel Nucleosil SA10	Tartaric acid	UV–Vis	[110]
Fe^{3+} Cu^{2+} Ni^{2+} Zn^{2+} Co^{2+} Pb^{2+} Fe^{2+}	Porridge oats Wine, red	Dionex IonPac CS2	Tartaric acid	UV–Vis	[110]

Table 9
Ion chromatographic determination of other metals in foodstuffs

Species	Matrix	Column	Eluent	Detector	Ref.
Al^{3+}	Orange juice Potato chips	Hamilton PRP X-100	2,5-Dihydroxy-1,4-benzenedisulphonic acid–EDTA	UV–Vis	[60]
Al^{3+} Fe^{2+}	Apples Lentils	Dionex IonPac CS2	Sodium sulphosalicylate– ethylenediamine	UV–Vis	[111]

Table 10
Reaction mechanisms involved in UV photolysis

● Radical formation:	$H_2O+h\upsilon \rightarrow H^*+HO^*$ $H_2O_2+h\upsilon \rightarrow 2HO^*$
● H_2O_2 decomposition:	$HO^*+H_2O_2 \rightarrow H_2O+HO_2^*$ $HO_2^*+H_2O_2 \rightarrow O_2+H_2O+HO^*$
● Termination reactions:	$HO^*+e^- \rightarrow OH^-$ $HO^*+H^* \rightarrow H_2O$ $2\ HO^* \rightarrow H_2O_2$
● Oxidation reactions:	$HO^*+CH_3\text{-}CH_2\text{-}OH \rightarrow H_2O+CH_3\text{-}C^*H\text{-}OH$ $HO^*+CH_3\text{-}C^*H\text{-}OH \rightarrow H_2O+CH_3\text{-}CHO$

chromatography allows the simultaneous determination of fluoride, chloride and bromide with suppressed conductivity detection, while iodide is usually determined separately with amperometric detection. For some oxy-halogenated species a good alternative is absorbance detection.

3.4.1. Fluoride

Fluoride is an important factor for skeletal bone integrity and dental health. A correct daily intake is necessary, but excess consumption causes symptoms of acute and chronic fluoride toxicity (fluorosis). The principal source of fluoride intake is water, nevertheless other foods, such as tea and fish can be a source of fluoride. High values of fluoride in milk from cows indicate environmental pollution. In dairy products, such as cream and cheese, fluoride content increases and reaches about three folds the original milk concentration. A relevant quantity of fluoride in meat may be caused by the presence of finely ground osseous material.

3.4.2. Chloride

Chloride is one of the most common inorganic anions in foods. Its content is usually related to the sodium presence and it is very important for metabolic acid–base equilibrium. In the food industry it is commonly added in the form of NaCl as a preserving agent or to enhance the sapidity of the product. Chloride concentrations can vary over a wide range, and its determination is helpful in the definition of the quality. Excess in chloride content creates a series of adverse effects not only on human health but also on technological process steps such as production and storage.

3.4.3. Bromide

The bromide content in foods is related to disinfecting with methyl bromide, some plants such as carrot, tomato, celery and melon accumulate bromide, and its determination can be used as a marker of methyl bromide treatment.

High bromide values in soft drinks can derive from the addition of brominated vegetable oils.

The narrow tolerance limits make the bromide determination very important to avoid risk for human health.

3.4.4. Iodide

Trace iodide is necessary for normal physical and mental development. Common sources of iodide include iodised table salt and seafood, but also other foods, such as eggs and milk, contain iodide. Iodophors used as disinfectants are a possible external source of iodide in dairy products. An incorrect dietary balance can lead to thyroid disorders.

These considerations have led to a concern over both high and low iodide levels in diet and a nutritional labelling requirement for iodide/iodine content.

3.4.5. Oxyhalides

Bromate is a potential carcinogenic agent thus its determination is important at low levels. There is some concern about the health effects on residual bromate in bakery products, because bromate salts are used as dough conditioners in the baking industry. The majority of bromate is reduced to bromide during the baking process; however residual bromate has been found in some baked goods.

Iodate is added to table salt as integrator of iodine

in the diet. Its importance has been previously pointed out under the iodide paragraph.

3.5. Other species

3.5.1. Cyanide

Cyanide is a very toxic compound, concentrations as low as few ppms are dangerous for human health. Cyanide is naturally present in some vegetables, such as cassava, sorghum and in fruit seeds. Its presence in fruit is also related to ethylene biosynthesis, i.e. post-harvesting storage and ripening degree. Cyanide is closely associated with membrane structure and can be released by technological processes involving fruit seed presence. The amperometric detection provides the higher sensitivity for cyanide, even if there is actually no literature in food analysis.

3.5.2. Carbonate

Carbonate is naturally present in fermented beverages or added to the soft drinks. It is usually determined when it is important to evaluate its buffering capacity. In consideration of its weak acidity the separation technique of choice is ion exclusion coupled with conductivity detection.

3.5.3. Arsenic species

Arsenic species are toxic compounds and their presence must be detected at very low levels. The indiscriminate use of arsenical compounds in agriculture at the beginning of this century caused an actual pollution of soil that became an important source of arsenic in foods. Other main sources of arsenic contamination are industrial pollution and the use of water with high arsenic content during the food preparation process.

The use of ion chromatography for arsenic determination provides important toxicity information as various species have widely different toxic effects. In the case of a sophisticated detection technique, such as ICP-AES, not being available, a good way of detecting both arsenite and arsenate is the use of amperometric detection coupled with suppressed conductivity.

3.5.4. Selenium species

Selenium is a fundamental micro-element in nutrition and its deficiency in diet can lead to typical diseases, such as Keshan disease. Normally selenium is present in trace amounts in proteins, as it substitutes sulphur in aminoacids.

Intensive studies have been done about different concentrations of selenium in foods. An important source of selenium species can also be the waters used during the food processing.

The use of ion chromatography for selenium determination provides important information about oxidation states, because selenite and selenate can be determined in the same analysis.

4. Inorganic cations

Inorganic cations are naturally present in foods and their concentrations can vary in a wide range. Industrial food production processes and modifications occurring during the commercial life of the product or metabolic modifications can change the normal content of different cations.

Ion chromatography allows the simultaneous determination of I and II group cations plus ammonium ion with conductivity detection, while heavy and transition metals are more commonly determined by post-column derivatization technique followed by absorbance detection.

4.1. I and II group cations and ammonium

Group I and II metals are mainly monitored for mass balance purposes. Most of them are essential nutrients whose improper levels can lead to health diseases, so sodium and potassium contents are important for nutritional labelling.

Ammonium ion is an indicator of the food quality and it is considered in this group because of its chromatographic behaviour, in fact it normally elutes in the same chromatographic conditions used for I group cations elution.

Magnesium is very common in food and it is mostly supplied from vegetables as a part of the chlorophyll molecule. There is a strict correlation among magnesium, calcium and phosphorus related to skeletal bone integrity that enhances the importance of the magnesium determination.

Calcium is widespread in food, mostly in complexed form; thus its concentration as ion determin-

able by ion chromatography is often related to the technological process to which the food and/or sample preparation undergoes.

4.2. Heavy and transition metals

During the last decades, evidence has grown on essentially, bioavailability, mobility, retention, accumulation and toxicity of heavy and transition metals in foods.

Normally they are present at trace levels in complex matrices, thus requiring high sensitivity and specificity of the analytical technique chosen. The importance of speciation between ionic forms, such as Cr (VI)/Cr (III) or Fe (III)/Fe (II), due to health concerns associated to some of them, increased the use of ion chromatographic analysis.

4.3. Other metals

There are only a few examples of ion chromatographic analysis of ionic species not considered before.

4.3.1. Aluminium

The use of aluminium and its derivatives, for example in food packaging and agriculture, made its determination at low levels more important, because of its toxicity.

Aluminium is determined by cation-exchange followed by a post-column derivatization technique and absorbance detection.

4.3.2. Platinum

The wide use of catalysts for the purification of car exhaust gases and the increase of its concentration in the environment caused its growing importance. The only example, included in Table 6, is related to its determination as a chloro-complex by anion-exchange separation and absorbance detection.

5. Conclusions

New developments in ion-exchange columns with gradient capabilities, higher capacities coupled with high efficiency, different selectivity, solvent compatibility and major diffusion of ion chromatographic detection techniques other than conductivity require an update of the most recent and comprehensive review on the subject [74]. Furthermore the recent interest about a promising analytical technique for ionic substances determination, such as capillary zone electrophoresis (CZE), has reduced the attention devoted to an 'established' technique like ion chromatography.

Ion chromatography reliability and versatility was revealed as the winning card in analysing complex matrix typical of food samples, with high selectivity, sensitivity and reproducibility; by now CZE has been not able to establish itself in food analysis [1], where its cumbersome analysis set-up and erratic behaviour complicate the intrinsic instrumental simplicity.

This review also gives evidence that the increased characterisation requirements of components, contaminants or additives, generates the need of ion chromatographic methods for the determination of certain less common ions in food samples. Testing for the possibility of analysing them is actually in progress.

The state-of-the-art ion chromatography will refocus the attention of food chemists on their analytical requirements, because nowadays ion chromatography cannot be merely considered the chloride, nitrate, and sulphate determination technique.

The completion of the present review with the state-of-the-art ion chromatographic determination of organic ions in food will follow in a short time.

Acknowledgements

The authors would like to thank Dr. Rosa Draisci of Istituto Superiore di Sanità, Rome, Italy. Dr. D. Jensen of Dionex G.m.b.H., Idstein, Germany and the Literature Service of CNR Area di Ricerca di Bologna for their help in finding some rare literature, and Dr. G. Cazzulani for his valuable assistance.

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Journal of Chromatography A, 789 (1997) 169–180

JOURNAL OF
CHROMATOGRAPHY A

Review

Capillary electrophoresis of inorganic anions and its comparison with ion chromatography

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Abstract

Determination of inorganic anions by capillary electrophoresis is critically compared with ion chromatographic determinations on the basis of recent literature in the field. After a very brief summary of the theoretical background, the selection and optimization of the running electrolyte system are discussed, especially in connection with modification of the electroosmotic flow. Preconcentration techniques are surveyed, as are the approaches to the sample introduction and analyte detection. The principal analytical parameters of the determinations are evaluated and illustrated on selected applications described in the literature. © 1997 Elsevier Science B.V.

Keywords: Reviews; Inorganic anions

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1. Introduction

In contrast to determination of organic analytes, where the use of high-performance separations is almost inevitable in complex matrices, inorganic analysis has powerful tools in highly selective and sensitive spectroscopic methods and thus a separation step is often unnecessary. This is especially true for determination of inorganic cations; for this reason, we do not deal with their separations in this review in spite of the fact that the pertinent literature is quite extensive. There has always been a much more limited selection of methods for the determination of inorganic anions. In addition to spectrophotometric procedures, some useful practical determinations are offered by ion-selective electrode

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PII S0021-9673(97)00830-3

potentiometry [1,2]; however, all these methods are usually not sufficiently sensitive for trace analyses and are mostly capable of determining only a single analyte. Therefore, high-performance separations now find extensive use in analyses for inorganic anions.

Traditionally, classical ion-exchange chromatography has long been used to preseparate and/or preconcentrate inorganic ions for their subsequent spectroscopic or electrochemical determination. The development of HPLC has made it possible to carry out multianalyte trace analyses for inorganic ions using not only ion-exchange principles but also employing ion-pairing and complexing agents in the most common HPLC mode, i.e. reversed-phase liquid chromatography (RPLC). The ion-exchange technique called ion chromatography was introduced [3] in 1975, has become highly successful commercially and is now widely used in such important fields like the quality control of all kinds of water.

Ion chromatography (IC) is now a well-established method and has been recommended officially for a number of analyses, e.g. by the Environmental Protection Agency (EPA) in the USA for determination of nitrate and nitrite in potable water [4]. The enormous recent development of various techniques of capillary electrophoresis (CE) has also brought about its application to inorganic ions [5] which is rapidly gaining practical importance. The name, capillary ion electrophoresis (CIE), has been coined. Both IC and CE have been treated in monographs and reviews (see, e.g. Refs. [6–15]).

The relative properties of HPLC and CE are now often discussed and are schematically summarized in Table 1. The following conclusions can be drawn from Table 1:

- CE is generally cheaper and faster than HPLC;
- the small samples accepted by CE make analyses of rare materials easy but cause difficulties when the technique is to be used for preparative purposes;
- CE exhibits a better resolution than HPLC but analyte identification is more difficult;
- CE procedures are usually more easily and rapidly developed and optimized than those in HPLC but the range of possibilities and the number of tunable parameters are more limited;
- the peak capacity in CE tends to be smaller than that in HPLC, especially when gradient elution is employed in HPLC;
- CE has problems with long-term stability of the migration times;
- the on-capillary detection in CE has the advantage of simplicity, a small volume and a simple geometry of the detection space, but the detection sensitivity and application possibilities of detection techniques are limited compared to HPLC;
- CE is more environmentally friendly than HPLC, as the use of organic solvents is limited.

Nevertheless, it can be expected that applications of CE to determination of inorganic anions will further grow and will partially replace IC in practical laboratories. Finally, it should be emphasized that chromatographic and electrophoretic techniques have a common basis in the concept of differential migration (see, e.g. recent work in Ref. [16]) and must be considered as complementary approaches rather than as competitors.

Table 1
Typical operational parameters of IC and CE

Parameter	IC	CE
Sample	1–50 μl	<1–5 nl
Volume flow-rate	0.1–2.0 ml/min	0.1–2.0 μl/min
Peak volume	ca. 500 μl	1–10 nl
Peak elution time	ca. 30 s	1–5 s
Time of analysis	10–40 min	5–15 min
Cost of column or capillary	ca. 300 USD	ca. 10–15 USD
Cost of mobile phase or running buffer per a series of analysis	ca. 15–25 USD	ca. 0.5–2.0 USD
No. of theoretical plates	ca. 10^4	ca. 10^5
LOD (UV detection)	ca. 10^{-7} to 10^{-9} mol/l	ca. 10^{-5} to 10^{-7} mol/l
Reproducibility of the retention parameters	<2.0%	<5%
Precision and accuracy for quantitation	ca. 2.0%	ca. 2.0–5%

2. Basic properties of CE in separations of inorganic ions

As follows from the general theory of CE (see, e.g. Refs. [11,13]), the retention behaviour of inorganic ions and its prediction are much simpler than in chromatography. The separation in CE is based on differences in the electrophoretic mobilities of the solutes. Ions migrate at an apparent velocity ν_{app} given by the sum of the electroosmotic flow velocity ν_{eo} and the electrophoretic velocity, ν_{ep}. The electroosmotic flow (EOF) in fused-silica capillaries with the injection side at the anode is directed toward the cathode; hence, anions move to the positive electrode against the electroosmotic flow. The apparent velocity of an ion is related to its apparent electrophoretic mobility, $\mu_{app(ion)}$, by Eq. (1).

$$\mu_{app(ion)} = \nu_{app}/E \quad (1)$$

where E is the electric field intensity.

The electrophoretic mobility of an ion, $\mu_{ep(ion)}$, can be calculated from the migration time of the ion, t_m, the applied voltage, V, the length of the capillary from the injection port to the detector, L_D, the total length of the capillary, L_T, and the mobility corresponding to the EOF, μ_{eo}, calculated from the migration time of an uncharged compound ($t_{m(eo)}$),

$$\mu_{app(ion)} = L_D L_T / V t_m = \mu_{ep(ion)} + \mu_{eo} \quad (2)$$

The electrophoretic mobilities depend on the ionic strength of the running electrolyte. According to the Kohlrausch law, they are inversely proportional to the square root of the electrolyte concentration and, as the ionic strength decreases, the electrophoretic mobilities approach limiting values that can be predicted from the limiting ionic equivalent conductances, λ_{equiv}°,

$$\mu^{\circ}_{ep(ion)} = \lambda^{\circ}_{equiv}/F, \quad (3)$$

where F is the Faraday constant (96 487 A s equivalent^{-1}).

The limiting ionic conductances and thus also the limiting electrophoretic mobilities of ions can be calculated from the hydrated ionic radii (the Stokes radii of the hydrated species), r_i (that are tabulated, e.g. in Ref. [17]),

$$\mu^{\circ}_{ep(ion)} = \mu^{\circ}_{equiv}/F = (10^7 z_i e)/(6\pi\eta r_i), \quad (4)$$

where z_i is the valence number of the ion, determining the direction of the electrophoretic mobility vector, e is the electron charge (1.60×210^{-19} C), π is the Ludolf number and η is the dynamic viscosity of the electrolyte (poise).

Eqs. (2)–(4) yield the following dependence for the apparent electrophoretic mobility of an ion, $\mu_{app(ion)}$,

$$\mu_{app(ion)} = (10^7 z_i e)/(6\pi\eta r_i) + L_D L_T / V t_{m(eo)} \quad (5)$$

Eq. (5) has two limitations: first, the mobilities depend on dissociation of weak acids or bases and, second, the effective charges of ions in an electrolyte are different from the nominal charges (except for the case of infinite dilution, i.e., for the limiting electrophoretic mobilities $\mu^{\circ}_{ep(ion)}$).

Nevertheless, a good agreement has been obtained between the experimentally measured and calculated values $\mu_{ep(ion)}$ for anions using the data given in Table 2 [13]. A value of 8.45×10^{-12} for the coefficient $(10^7 z_i e)/(6\pi\eta r_i)$ corresponds well to the value found by curve fitting (7.78×10^{-12}).

Table 2
Apparent mobilities $\mu_{app(ion)}$ and hydrated ionic radii r_i for selected anions [13]

Anion	μ_{app}	r_i ($\times10^{-8}$ cm)
Bromide	0.0010690	1.0505
Chloride	0.0010569	1.0750
Iodide	0.0010340	1.0679
Nitrite	0.0010250	1.1423
Nitrate	0.0010010	1.1488
Azide	0.0009683	1.1886
Chlorate	0.0009269	1.2656
Fluoride	0.0008888	1.4806
Formate	0.0008710	1.5021
Chlorite	0.0008293	1.5772
Bicarbonate	0.0007669	1.8430
Ethanesulfonate	0.0007252	2.0711
Acetate	0.0007046	2.0054
Propionate	0.0006744	2.2910
Propanesulfonate	0.0006641	2.2110
Butyrate	0.0006396	2.5150
Benzoate	0.0006018	2.5317

Values of $\mu_{app(ion)}$ were calculated from Eq. (2) using migration times of anions, L_D=52 cm, L_T=60 cm and V=30 kV. Hydrated ionic radii were calculated from limiting ionic conductance taken from Ref. [17] using Eq. (4).

3. Optimization of separation

Great attention has been paid to the selection and optimization of the running electrolyte composition. To attain a good peak shape and an optimal separation of anions, the electrolyte anion (carrier anion) should have a mobility similar to the mobilities of the analytes. If possible, the EOF should have the same direction as the migration of the analytes to shorten the analysis. If indirect UV detection is used, the carrier anion should strongly absorb in the UV region. The most common carrier anions for analyses of inorganic anions in combination with indirect UV detection are chromate and pyromellitic acid. Their dissociation and thus their electrophoretic mobilities are influenced by the pH. The separation of anions with lower mobilities can be optimized by decreasing the pH.

As follows from Eq. (4), the electrophoretic mobility of analytes can be changed by changing their charge-to-mass ratio. For weak acids this can be done by changing the pH in the vicinity of the analyte pK_a.

However, most inorganic acids have pK_a values below 2 and thus the change in the pH to modify the selectivity is not practical. At low pH values the EOF is too low in a silica capillary to carry inorganic anions towards the detector (cathode) and the current generated at a pH lower than 2 makes the analysis difficult if not impossible because of a high Joule heat.

The effect of the pH on the separation of weak acids can be demonstrated on an example of phosphate present at a high concentration (more than 800 μg/l) beside a low concentration of fluoride (1 μg/l). At a pH of 7, protonation of hydrogenphosphate results in its slower migration and leads to an improved separation from fluoride [18].

An addition of a solvent, e.g. 1-butanol, results in selectivity changes. This can be explained by changes in the relative hydration of anions and, as a consequence, an improved separation, e.g. of iodide and chloride [19]. Organic solvents added to the running electrolyte decrease the EOF; they increase the viscosity and decrease the pK_a of silanol groups on the capillary walls and make the migration times more reproducible with a less noisy baseline [20]. The temperature also affects the selectivity, as it influences both the mobilities and the EOF through a change in the solution viscosity.

While cations can be analyzed directly in CE, the elution of anions in bare-silica capillaries requires modulation of the electroosmotic flow. The EOF can be modified by cationic additives, by changing the concentration of the running electrolyte, or by a chemical modification of the capillary walls.

Cationic surfactants are mostly added to modify the EOF in analyses of anions. At concentrations below the critical micelle concentration, CMC, hemicelles are formed at the capillary wall that reverse the EOF. If the anions interact with the monomeric surfactant present in the electrolyte, then their electrophoretic mobilities are influenced through ion association. For a monovalent anion, the ion association constant, K_{IA}, is defined by

$$K_{IA} = [AB]/[A^-][B^+] \tag{6}$$

where [AB], $[A^-]$ and $[B^+]$ are the equilibrium concentrations of the associate, the anion and the free cationic surfactant. As the surfactant is present in an excess over the analytes, $[B^+]$ can be replaced by the bulk surfactant concentration, c_{sf}.

An equation derived by Kaneta et al. [21] relates $\mu_{app(ion)}$ to K_{IA} and c_{sf},

$$1/\mu_{app(ion)} = Kc_{sf}/\mu_{ep} + 1/\mu_{ep} \tag{7}$$

If the surfactant concentration exceeds the CMC value, the partitioning into the micelles occurs and Eq. (7) changes into

$$1/\mu_{app(ion)} = (1 + k')/k'\,\mu_{mc} + \mu_{app(CMC)} \tag{8}$$

where $\mu_{app(CMC)}$ is the anion mobility at the CMC, μ_{mc} is the mobility of the micelles and k' is the capacity factor, $\mu_{app(ion)}/(\mu_{app(ion)} + \mu_{mc})$.

The most common cationic surfactants used are, e.g. tetrabutylammonium (TBA), dodecyltrimethylammonium (DTA), tetradecyltrimethylammonium (TTA), cetyltrimethylammonium (CTA) bromides or hydroxides, hexadimethrine (HDM) and hexamethonium (HM) hydroxide (patented, together with other running electrolyte components, by Waters) (e.g. Ref. [19], or their binary mixtures, e.g. Ref. [22]). They are added to the running electrolyte in concentrations below the CMC and their association

with inorganic anions is small as proved by the elution order of the anions which corresponds to their molar conductivities (with the exception of iodide that is retarded even below the CMC). Although the use of surfactants at concentrations above the CMC, e.g. in micellar electrokinetic chromatography (MEKC), is not very frequent in the analysis of inorganic anions, the different selectivity obtained in MEKC in comparison with CZE can be useful in special applications [21].

Various EOF modifiers were compared [23]. The size of the alkyl ammonium ion affects not only the EOF, but also determines the optimum concentration range of the surfactant which decreases with increasing ion size. When using high-molecular-mass additives, e.g. HDM, a good efficiency can be obtained even at very low surfactant concentrations (0.0001%). This is important for two reasons: first, hydrophobic alkyl ammonium ions have a limited solubility in the presence of chromophores and, second, there is a danger of formation of insoluble pairs between the alkylammonium ions and the components of both the electrolyte and of the sample. A dependence of the EOF on the concentration of EOF modifiers is shown in Fig. 1 [23]. The optimal separation conditions (except for the modifier concentration) and the analytical parameters are similar for all the EOF modifiers tested, indicating that the influence of these conditions is small provided that the EOF is reversed; HM does not reverse the EOF, only decreases it. When combining HM with the fully dissociated chromophore pyromellitic acid, stable complexes are formed that are adsorbed onto the silanol groups at the capillary walls.

Weak acid anions are more strongly affected by

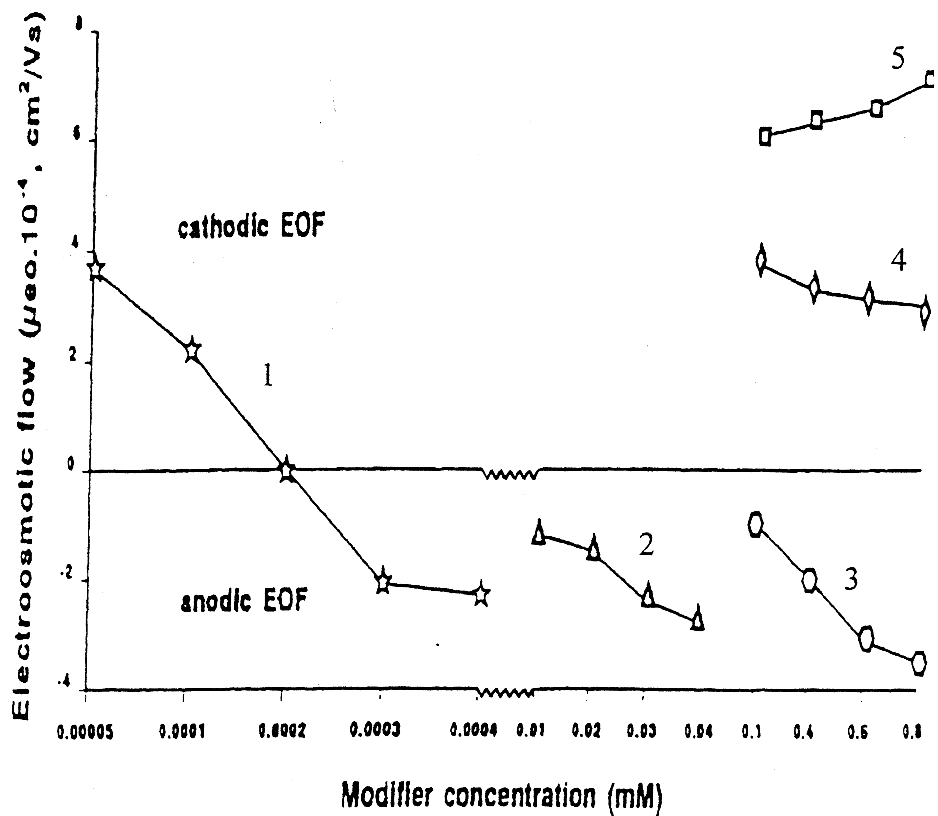


Fig. 1. Dependence of the EOF on the concentration of various modifiers [23]. 1, HDM; 2, CTA; 3, TBA; 4, HM; 5, TTA.

the EOF modifier concentration than strong acid anions. Higher concentrations lead to longer migration times due to the formation of equilibrium ion pairs. This difference in the selectivity allows complex samples to be analyzed by properly selecting the EOF modifier concentration and thus increasing the weak acid anion migration times. An addition of organic solvents increases the solubility of quaternary ions.

Another possibility for EOF modification is the coating of the capillary walls with cationic polymers, e.g. poly(1,1-dimethyl-3,5-dimethylene)piperidinium and -pyrrolidinium chloride [24] or a reactive polyamide with various functional groups [25]. A net positive charge is then formed at the capillary walls. In the former case, the mobility of anions is strongly influenced by organic modifiers (methanol or acetonitrile). The migration of ions is affected by the running electrolyte properties, such as solvation and structural conformation. Ion-exchange is the main interaction, but hydrophobic interactions also play a role. In the latter case, the elution order is the same as in CE with EOF reversal. A good run-to-run, but a poor column-to-column reproducibility is, however, obtained.

The use of EOF modifiers complicates the analysis of those anions that move so slowly that they do not require EOF reversal. Reducing the EOF, e.g. by coating the capillary walls with silane, is sufficient for attaining sufficiently short analysis times for some anions [26]. These capillaries are especially useful with direct UV detection, e.g. of bromide and iodide.

The EOF can also be modified by using capillaries made of materials other than fused silica. For example, an untreated polypropylene hollow fibre was used for the CE analysis of anions [27]. Theoretically these capillaries have no surface charge, but practically there is a small negative charge and thus a small EOF is observed. Fast separations are possible but the efficiency is poorer. Advantages of polypropylene capillaries are their good stability at high pH, a low cost and no hysteresis effect. Fast moving anions can be separated directly without any modification to the electrolyte or the capillary wall. The capillaries are transparent to visible and near UV radiation.

4. Detection modes

Detection in CE must take place directly on the separation column, because the elution profile outside the electric field rapidly changes from plug to parabolic, similar to IC, and the separation efficiency decreases drastically.

Direct UV detection is both selective and sensitive and is often employed in the analysis of cations after their complexation, but its application to the analysis of inorganic anions is limited to a few of them which absorb the UV radiation, e.g. to nitrate, sulfide, nitrite, iodide, bromide and thiocyanate. For example, a detection limit of 10 μg/l was attained for sulfide in waste water using direct UV detection at 229 nm [28]. The running electrolyte contained sodium sulfate and an EOF modifier in the OH form.

Indirect UV detection is most common. It is nearly universal and requires no modification to the instrument. Detection limits in a sub-mg/l region have been obtained with running electrolyte containing chromate, with a time of analysis of 3 min [29]. A disadvantage of chromate lies in its aging that causes changes in the analyte migration times, e.g. for fluoride [30]. *p*-Aminobenzoate was found to be optimal for simultaneous determination of low mobility organic and high mobility inorganic anions. The separation was facilitated by an addition of barium salts [31].

Anionic chromophores (benzoate, anisate) and cationic buffers (Tris, ethanolamine) were tested for simultaneous detection of nonabsorbing anions and cations [32]. An equation was derived for the dependence of the difference in the absorbance, ΔA.

So far the best separation of anions with indirect UV detection has been attained in the IonPhor PMA electrolyte buffer consisting of 2.5 m*M* pyromellitic acid, 6.5 m*M* NaOH, 0.75 m*M* hexamethonium hydroxide and 1.6 m*M* triethanolamine, with a pH of 7.7 (e.g. Ref. [19]).

A CE method with indirect UV detection was validated for eight anions and two electrolyte systems: pyromellitic acid + hexamethonium hydroxide and chromate + TTAB [33]. The detection limits were between 1 and 3 mg/l, the repeatability and reproducibility of the measurement differed for different compounds and were, with the exception of

fluoride and phosphate, 6 and 5–10%, respectively. Linear calibration curves were obtained in a concentration range between 1 and 10 mg/l.

A disadvantage of indirect detection is a high background absorbance and thus a high noise and a limited linear dynamic range. CE with indirect UV detection exhibits a lower concentration sensitivity than IC due to mass loading limitations, despite its superior mass sensitivity.

Direct fluorescence detection is not applicable to inorganic anions as they cannot be suitably derivatized. Indirect fluorescence detection, though possible, has no advantage over indirect UV detection.

Conductivity detection (CD) is a nearly universal bulk property detection mode for small ions and, similar to IC, both non-suppressed and suppressed CD is used. There are more options for the selection of the running electrolyte in combination with CD. The co-ion must have a substantially different conductivity. In non-suppressed CD, low mobility buffers with higher ionic strengths provide an extended linearity and improve preconcentration by sample stacking.

In comparison with indirect UV detection, CD is 10 times more sensitive [34]. The linear dynamic range extends over three concentration decades and the reproducibilities of the migration time, peak area and height are better than 0.15, 1.985 and 1.255%, respectively. A determination of anions in drinking water with CD is shown in Fig. 2 [34].

A borate buffer (2 m*M*, pH 9.2) combined with suppressed conductivity detection provided good peak shapes due to a close match of the borate mobility with those of the separated anions and fulfilled the principal condition of suppressed CD, i.e. could be suppressed to form a weakly conducting species. Additives, such as barium ions, decreased the EOF and the migration velocity of high-mobility anions, so that they could be analyzed simultaneously with organic anions [35]. Detection limits in a range of 1–10 μg/l were reported with suppressed conductivity detection [36]. A similar sensitivity has been attained for inorganic anions in a large-diameter plastic capillary (300 μm) and conductivity detection [37].

Other detection modes can be used, e.g. direct combination of CE with mass spectrometry via an ion spray–sheath flow interface [38] or potentiometry with ion-selective microelectrode [39], but their application has so far been less common.

A combination of various detection modes, e.g. CD-UV on-line, substantially enlarges sample matrix information.

5. Preconcentration techniques for inorganic anions

Sample preconcentration is usually necessary for the analysis of highly dilute solutions of anions, e.g. for the analysis of anions in deionized water or their determination in the presence of a large excess of a matrix component.

Isotachophoretic enrichment by electrostacking at the sample–buffer interface is often used. Isotachophoresis can be carried out off-line, on-line or directly in the analytical CE instrument. The sample matrix can assist the stacking process by functioning as the leading or terminating electrolyte. The co-ion of the running electrolyte has to be chosen so that the analyte mobilities are between those of the ions of the electrolyte and the matrix. Limits of detection lower than 50 nmol/l have been attained in the simultaneous analysis of inorganic and organic anions in rain water when enriching by sample stacking with a dynamic injection (the injection volume was 300 nl, corresponding to a 10% filling of the capillary [31]). This preconcentration method permits determination of inorganic anions in the presence of a fluoride matrix up to an analyte:matrix ratio of $1{:}6\times10^6$ [40]. A mathematical treatment of electrostacking can be found in Ref. [41].

Preconcentration with the electrokinetic injection can be used for non-ionic matrices. With long injection times, the ionic components are preconcentrated at the expense of the interferents. The EOF has the direction opposite to the migration of the analytes. The matrix effects caused by ionic components can be decreased by suppressing their dissociation by a pH change, thus enriching the analytes by up to two orders of magnitude. The choice of the amount injected is influenced by the analyte mobility, the electroosmotic flow and the sample and buffer ionic strengths.

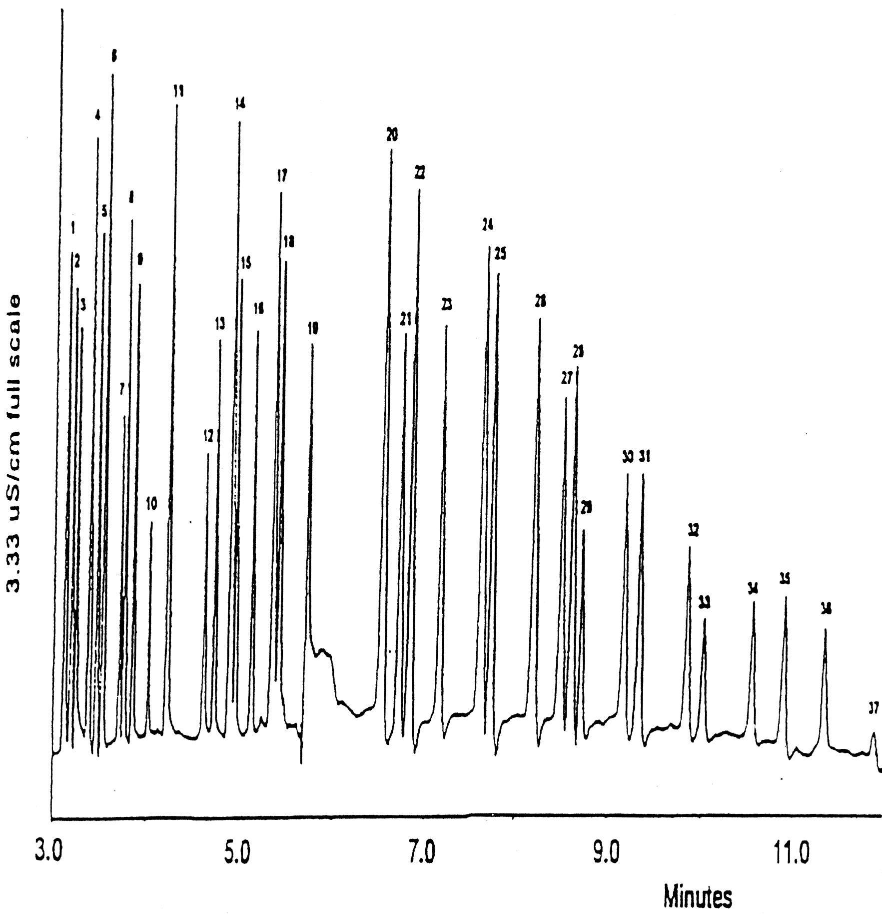


Fig. 2. Determination of inorganic and organic anions by CE with direct conductivity detection [34]. The electrolyte: 50 m*M* CHES (2-N-cyclohexylaminoethane-sulfonate), 20 m*M* LiOH and 0.03% Triton X-100; 60 cm×50 μm I.D. capillary; 25 kV; injection at 25 mbar for 12 s; the EOF was modified by preflushing the capillary with a 1 m*M* CTAB solution. Anions (concentrations in mg/l): 1, bromide (4); 2, chloride (2); 3, hexacyanoferrate (7); 4, nitrite (4); 5, nitrate (4); 6, sulfate (4); 7, azide (2); 8, oxalate (3); 9, molybdate (5); 10, tungstate (6); 11, 1,2,4,5-benzenetetracarboxylic acid (7); 12, fluoride (1); 13, tartrate (5); 14, selenite (10); 15, phosphate (4); 16, citraconate (methylmaleate) (5); 17, glutarate (10); 18, phthalate (10); 19, carbonate (4); 20, acetate (10); 21, chloroacetate (10); 22, ethanesulfonate (20); 23, dichloroacetate (15); 24, propionate (15); 25, propanesulfonate (20); 26, crotonate (15); 27, butanesulfonate (20); 28, butyrate (15); 29, toluenesulfonate (15); 30, pentanesulfonate (20); 31, valerate (15); 32, hexanesulfonate (20); 33, caproate (15); 34, heptanesulfonate (20); 35, morpholineethanesulfonate (35); 36, octanesulfonate (20); 37, *d*-gluconate (40).

The detection limits and the analyte-to-matrix ratios for inorganic and organic anion impurities in boric acid, obtained using hydrostatic or electrokinetic injection and with sample stacking (the capillary is filled with the sample up to the detector, a voltage is applied to preconcentrate the sample anions at the sample–buffer interface, a reversed EOF is used to remove the matrix components and then the CE analysis is carried out), are compared in Table 3 [40].

The effect of the preconcentration time and of the voltage on the sensitivity of a CE determination of trace inorganic and organic anions in matrix-free pure water has been studied [42] and compared with IC: IC yields similar or better detection limits (nl/ml level), but requires trace enrichment times much

Table 3
Comparison of limits of detections (LOD) and analyte-to-matrix ratios (ATMR) for inorganic and organic anion impurities in boric acid obtained using hydrostatic and electrokinetic injection and sample stacking [40]

Anion	Hydrostatic injection		Electrokinetic injection		Sample stacking	
	LOD (μmol/l)	ATMR	LOD (μmol/l)	ATMR	LOD (μmol/l)	ATMR
Bromide	8	$1:1.1\times10^5$	0.3	$1:2.7\times10^6$	0.07	$1:1.2\times10^7$
Chloride	7	$1:1.3\times10^5$	0.5	$1:1.7\times10^6$	0.04	$1:2.2\times10^7$
Sulfate	5	$1:1.8\times10^5$	0.2	$1:3.8\times10^6$	0.02	$1:3.5\times10^7$
Nitrate	7	$1:1.3\times10^5$	0.3	$1:2.8\times10^6$	0.04	$1:2.2\times10^7$
Oxalate	5	$1:1.8\times10^5$	0.4	$1:2.1\times10^6$	0.03	$1:2.8\times10^7$
Chlorate	7	$1:1.3\times10^5$	0.4	$1:2.2\times10^6$	0.05	$1:1.5\times10^7$
Malonate	5	$1:1.8\times10^5$	0.4	$1:2.0\times10^6$	0.05	$1:1.7\times10^7$
Fluoride	10	$1:0.9\times10^5$	0.7	$1:1.2\times10^6$	0.89	$1:9.0\times10^7$

LOD is defined as three times the signal-to-noise ratio.

longer than CE (ca. 5–10 min, compared with 45 s in CE). Using a 45-s electromigration injection, CE detection limits of 0.5 μg/l of anions in deionized water have been obtained (Fig. 3) [42]. The detection of cations is even more sensitive [43].

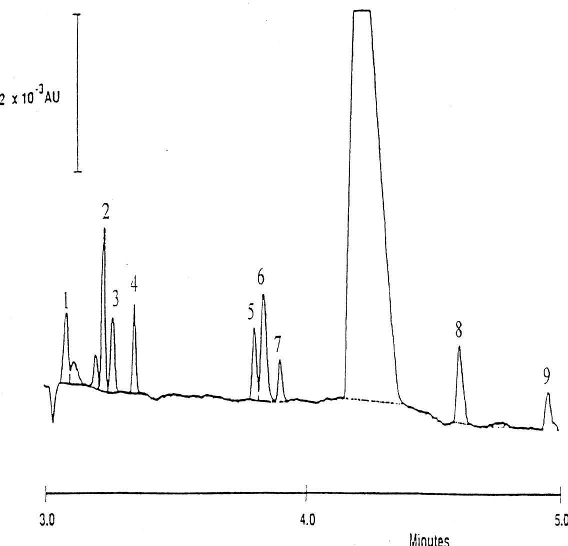


Fig. 3. Trace determination of some inorganic and organic anions in pure water, after an electrophoretic enrichment at 5 kV for 45 s with an addition of 75 μ*M* octanesulfonate to the sample [42]. The electrolyte: 10 m*M* sodium chromate and 0.5 m*M* OFM-BT (a surfactant used as the EOF modifier), adjusted to pH 8 with sulfuric acid; 15 kV; 60 cm×75 μm I.D. capillary, distance to detector, 52 cm; UV photometric detection at 254 nm. Anions (concentrations in mg/l): 1, chloride (3.5); 2, sulfate (4.8); 3, nitrate (6.2); 4, oxalate (5); 5, fluoride (1.9); 6, formate (5); 7, phosphate (3.2); 8, acetate (5); 9, propionate (5).

The reproducibility of the electrokinetic injection is poorer than that of the dynamic pressure injection and strongly depends on the ionic strength. Internal standards are usually added to improve the accuracy and precision [44]. For a stacking injection, a mathematical model has been developed to account for the increase in the migration time with increasing sample injection time; a good agreement with the theory has been found [45].

6. Comparison of CE and IC in selected applications

As pointed out above, IC is a well-established method for the analysis of inorganic anions and has become the method of choice in many application areas. Many techniques are available using single-column [46] or dual-column systems with various detection modes. IC can be used both for analytical and preparative purposes. Large sample volumes, up to 1300 μl, can be injected to determine trace anions and cations and to attain detection limits of 10–400 ng/l. For determinations at a μg/l to mg/l level, a sample size of 10–50 μl is sufficient. Preconcentration is necessary for lower concentrations (an additional column, a sample pump, an extra valve and an extra time are the disadvantages of this approach [47]). With an IEC column and isocratic

elution, it is impossible to separate both inorganic and organic ions.

As pointed out in Section 1, CE and IC are complementary techniques and their use in tandem is advantageous in many cases. For example, CE and IC analyses of anions in residues of explosives were compared (Fig. 4) [48]. The differences in the separation modes of CE and IC help to determine the nature of the sample and the use of the two methods in tandem makes it possible to decrease the interference by other ions and to confirm the peak identity. IC with a greater capacity allows for screening of a variety of explosive residues. Analogously, a combination of CE and IC helps to solve the problems of peak confirmation in complicated matrices, e.g. in atmospheric aerosols (Fig. 5, Ref. [49]). Two IC

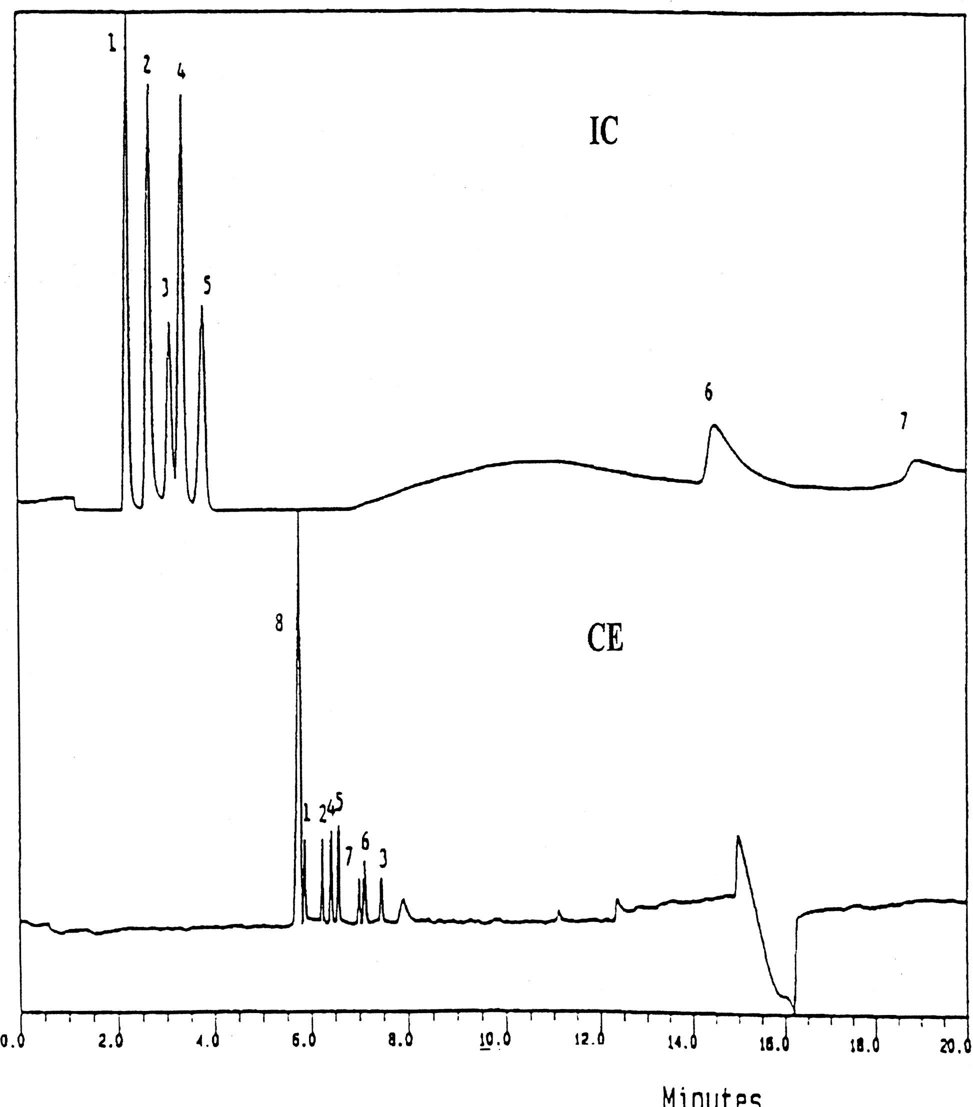
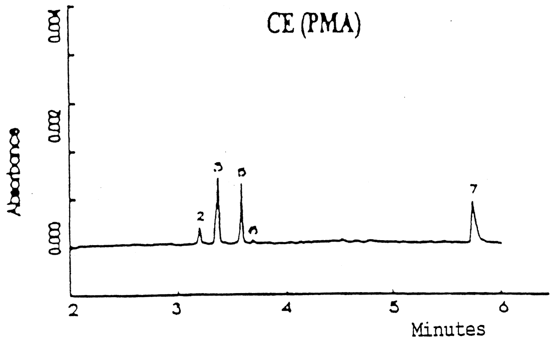
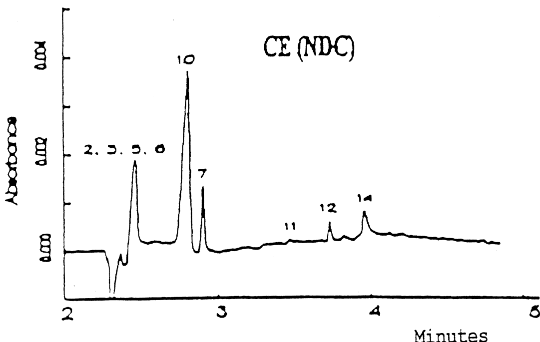


Fig. 4. Analysis of an anion standard solution by IC (a) and CE (b) [48]. IC conditions: a Vydac 302IC4.6 column, a flow-rate of 2.5 ml/min, an injection volume of 25 μl, an isophthalic acid mobile phase, UV detection at 280 nm. CE conditions: an electrolyte of potassium dichromate, sodium tetraborate, boric acid and the DETA (diethylenetriamine) EOF modifier, pH 7.8; 65 cm×75 μm I.D. capillary; 20 kV; indirect UV detection at 280 nm. Anions: 1, chloride; 2, nitrite; 3, chlorate; 4, nitrate; 5, sulfate; 6, thiocyanate; 7, perchlorate; 8, bromide.





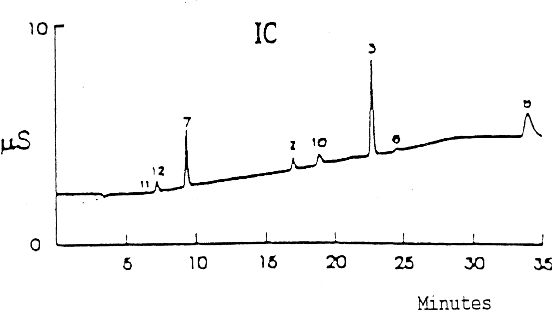


Fig. 5. An analysis of a coarse atmospheric aerosol extract by CE and IC [49]. CE conditions: a 57 cm×75 μm I.D. capillary, distance to detector, 50 cm. Electrolyte: 2.25 m*M* PMA (pyromellitic acid), 0.75 m*M* HMOH (hexamethonium hydroxide), 6.50 m*M* NaOH and 1.60 m*M* TEA (triethanolamine), pH 7.7 or 2.0 m*M* NDC (2,6-naphthalenedicarboxylic acid), 0.5 m*M* TTAB (tetradecyltrimethylammonium bromide) and 5.0 m*M* NaOH, pH 10.9; 30 kV (PMA) or 20 kV (NDC); pressure injection for 10 s; indirect UV detection at 254 nm (PMA) or 280 nm (NDC). IC conditions: an IonPac-AS10 column with an IonPac-AG10 guard precolumn; conductivity detection using an anion self-regenerating suppressor (ASRS-I) in the recycle mode. Analytes: 2, chloride; 3, sulfate; 5, nitrate; 6, oxalate; 7, formate; 10, hydrocarbonate or carbonate; 11, acetate; 12, propionate; 14, benzoate.

columns with automated column switching or CE combined with IC can separate anions in aqueous soil extracts and process solutions [30]. The IC detection limits were found to be lower than in CE (0.2 μg/ml for IC compared with 2 μg/ml for CE), but, due to a higher separation efficiency, the peaks were resolved better in CE than in IC and the quantitation was easier.

A CE determination of fluoride in rain water was compared with IC and ISE potentiometry; the IC response was related to the total concentration, whereas CE and ISE responded to free fluoride [50]. The fluoride concentrations obtained by CE and ISE were systematically lower than those obtained by IC due to the fluoride complexation with aluminium. The detection limits for IC and ISE were similar (0.2 and 0.3 μmol/l) and somewhat lower than those for CE (0.6 μmol/l). CE was evaluated as an alternative method to the EPA ion chromatographic method for the determination of anions in water and a better resolution and a shorter analysis time were found for CE [51].

A strong adsorption of dyes observed in IC columns was not encountered in CE when analyzing chloride, sulfate and phosphate in sulfonated dyes [52]. Proteins need not be removed prior to CE analysis and do not interfere with low-molecular-mass ions as is the case in IC [53].

The IC analysis of polyphosphates and polycarboxylates (builders in detergents) is complicated by strong affinities of these compounds toward ion exchangers [54]. CE can readily be used for this purpose (a LOD of 2×10^{-5}–5×10^{-5} *M*).

The applications of CE analysis to inorganic anions are already numerous and are rapidly growing. The application ranges include, e.g. clinical chemistry [53], the pulp and paper industry [55], environmental samples [49], waste waters from processing plants [56], process control, industrial applications [43,57–59], explosive residue analysis [48], biological samples [60], or drugs and intermediates [61,62].

7. Conclusion

As can be seen from the above examples, the relative advantages and drawbacks of IC and CE in

analyses for inorganic (an)ions basically correspond to the brief general survey given in Section 1. It can be expected that the future development in the field will primarily be based on judicious combinations of chromatography and electrophoresis and in their coupling with more sophisticated detection techniques.

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Journal of Chromatography A, 789 (1997) 181–194

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Review

Separation and determination of inorganic anions by reversed-phase high-performance liquid chromatography

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Abstract

An overview and discussion is given of literature methods published after 1989 devoted to the ion-interaction chromatographic determination of inorganic anions. Seventy references are quoted. Ion-interaction chromatography makes use of commercial reversed-phase stationary phase and conventional high-performance liquid chromatography instrumentation. The basis of the technique, the modification of the stationary phase surface, the choice of the ion-interaction reagent as well as the dependence of retention on the different variables involved are discussed. Examples of application in the fields of environmental, clinical and food chemistry are presented. The experimental conditions of stationary phase, of mobile phase composition as well as detection mode, detection limit and application are also summarized in tables. © 1997 Elsevier Science B.V.

Keywords: Reviews; Ion-interaction chromatography; Inorganic anions

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1. Introduction

This review is devoted to the ion-interaction chromatographic determination of inorganic anions.

Ion-interaction chromatography is a powerful technique, which permits the separation of inorganic anions on commercial reversed stationary phases and conventional high-performance liquid chromatography (HPLC) instrumentations.

The mobile phase is an aqueous or hydro–organic solution of a suitable ion-interaction reagent.

Ion-interaction methods offer, with respect to ion

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PII S0021-9673(97)00971-0

chromatography, advantages of lower cost in relation to both instrumentation and columns and can be advantageously employed in laboratories where only conventional HPLC systems are available. On the other hand, resolution and sensitivity are comparable to those obtained in ion chromatography, assuming that a suitable ion-interaction reagent is chosen.

Literature methods for the determination of inorganic anions based on both ion chromatography and ion-interaction chromatography published before 1984 have been exhaustively reviewed by Haddad and Heckenberg [1]; and a review by Marina et al. published in 1989 was devoted to HPLC applications in the analysis of inorganic species [2].

In the present review, the more recent papers concerning the ion- interaction determination of inorganic anions are considered.

For more immediate information and comparison, methods and experimental conditions are summarized in Tables 1 and 2. The packing material, the mobile phase composition, the kind of detection, the detection limit and (if given) the possible interferences and examples of application are reported [3–53].

2. Basis of ion-interaction methods

Discussion is still open about the mechanisms involved in the ion- interaction chromatographic processes and the analogies or differences compared with ion chromatography or ion-pair chromatography.

Snyder et al. [54] suggested the following definition to distinguish between ion-exchange and ion-pair chromatography: (i) ion-exchange chromatography is the technique that makes use of cationic or anionic stationary phase with an aqueous solution for the mobile phase and (ii) ion-pair reversed-phase chromatography is that technique which uses a reversed-phase column with an aqueous–organic mixture for the mobile phase, to which an ion-pairing is added.

This definition does not consider the mechanisms involved and does not take anyway into consideration the technique which uses a reversed-phase packing material and an aqueous solution of the ion-interaction reagent as the mobile phase.

The ion-interaction (or ion-pairing) reagent is essentially a salt formed by a lipophilic cationic species (alkylammonium, tetraalkylammonium, cetyltrimethylammonium, etc.) and an anion which can be both organic and inorganic.

According to many authors, the ion-pairing is added to the mobile phase to form, with the ionic analyte, an ion-pair which due to its increased lipophilicity can be retained on the reversed-phase stationary phase surface.

According to other hypotheses [55–57] the ion-pairing (or the ion-interaction reagent) added to mobile phase, when flowing in isocratic conditions, induces a dynamic modification of the surface of the reversed-phase packing material. The modification of the original packing material might proceed through a first step in which the lipophilic cation is retained onto the surface of the stationary phase through adsorption forces. Through electrostatic forces the anion is bound as well with the formation of an electrical double layer. A new moiety is adsorbed onto the surface (or on part of it), which modifies the original properties of the stationary phase and makes it able to retain both cationic (as, for examples, amines, triazines . . .) and anionic (both organic and inorganic) species. Retention can occur by three possible routes: (i) ion pairs are formed between the anions (or the cations) of the analyte and the cations (or anions) of the ion-interaction reagent and these ion-pairs are adsorbed onto the stationary phase [58,59], (ii) the analyte is retained through an ion-pair complex formed with an amphiphilic ion previously adsorbed onto the surface of the hydrophobic material [60] and (iii) the analyte is retained through ion-exchange reactions with the already adsorbed ion-pair reagent [61–65].

A general trend, even if not universally used, is to report as ion-pair chromatography the methods in which ion-pairs are formed before retention and are retained onto the unmodified reversed-phase surface, while those reported as ion-interaction chromatographic methods are the methods where the surface modification is assumed to take place.

Very likely, ion-exchange and adsorption mechanisms coexist in determining retention. This possibility is also supported by the elution sequences observed for the anionic analytes. The sequence generally does not follow the elution sequence found

Table 1
Experimental conditions of ion-interaction methods based on modified C_{18} and C_8 RP stationary phases

Ref.	Anions	Stationary phase	Mobile phase	Method	Detection and interferences	LD	Applications
[3]	NO_2^-, NO_3^-, Br^-, I^-, VO_3^-, SO_4^{2-}, CrO_4^{2-}, WO_4^{2-}, MoO_4^{2-}	Silasorb 300 ODS (100×4.0 mm I.D.), irregular particles, 10 µm (Lachema, Brno, Czechoslovakia).	1–2 m*M* TBA hydroxide aqueous solution adjusted to pH 3–7 with phosphate (50 m*M*) or Tris buffer.	Dynamic modification, (study about the influence of organic counter ion, mobile phase pH and ionic strength).	UV at 210 nm.	–	–
[4]	IO_3^-, NO_2^-, Br^-, NO_3^-, I^-	Spherisorb ODS, (250×5.0 mm I.D.), 5 µm (HPLC Technology).	0.018 *M* Phosphate buffered ACN–water (35:65 v/v) micellar mobile-phase containing $1\cdot10^{-2}$ *M* (>CMC $=9.9\cdot10^{-4}$ *M*), HDTMA chloride.	Dynamic modification.	UV at 205 nm.	µg/l NO_2^- 200 Br^- 250 NO_3^- 150 I^- 100	Nitrite and nitrate in domestic water.
[5]	NO_3^-, ClO_3^-, Cl^-, Br^-, F^-, IO_3^-, BrO_3^-, I^-	Zorbax C_{18} (150×4.6 mm I.D.), spherical particles, 6 µm (DuPont) and PRP-1, (150×4.1 mm ID), 10 µm (Hamilton).	Several mobile phases containing 1,10-phenantroline $[Fe(phen)_3]^{2+}$ salts, with different organic modifier (ACN or MeOH) percentage, pH, buffer composition, ionic strength, type and concentration of counter-anion.	Dynamic modification	Indirect UV–Vis at 510 nm	About 1 ng	–
[6]	Cl^-, NO_3^-	SB-Octyl-50 (C_8) open tubular capillary column (10 m×50 µm I.D. and 0.5 µm thickness) (Lee Scientific), precoated with cetylpyridinium chloride 50 m*M* in 2% MeOH.	Sodium salicylate aqueous solution $2.5\cdot10^{-6}$ *M*.	Permanent coating.	Indirect fluorescence (λ_{Em}: 325 nm)	Order of 0.5 µg/l.	–
[7]	Cl^-, CNO^-, SO_4^{2-}, $S_2O_3^{2-}$, SCN^-	Supelcosil LC-18 (150×4.6 mm I.D.), 5 µm; Supelcosil LC-18DB (150×4.6 mm I.D.), 5 µm (Supelco); PLRP-S (150×4.6 mm I.D.), 5 µm (Polymer Labs.); PRP-1 (150×4.1 mm I.D.), 5 µm (Hamilton). Coating: 0.5 m*M* cetylpyridinium chloride in 18 or 23% ACN–water mixture (low and high capacity column).	Mobile phases with different solvent percentage, pH, buffer (TEA, THAM, TMA) and eluent acid (1,3,5-benzenetricarboxylic acid, 5-sulphosalicylic, phthalic acid, salicylic acid) are tested.	Permanent coating.	Indirect UV at 254 nm.	About 50 µg/l	Samples from gold process effluents
[8]	PO_4^{3-}, Cl^-, NO_3^-, NO_2^-, Br^-, SO_4^{2-}, I^-, $S_2O_3^{2-}$	Resolve C_{18} Radial-Pak cartridge (10 cm×8 mm), 5 µm.	0.4 m*M* TBA, 0.4 m*M* salicylic acid water solution at pH 4.62.	Comparison between precoating and dynamic modification.	Direct and indirect UV at 288 nm and conductometric. Interference between NO_3^- and Br^- is studied.	µg/l Cl^- 120 NO_3^- 740 NO_2^- 280 Br^- 1240	–
[9]	NO_3^-, BrO_3^-, IO_3^-, F^-, Cl^-, PO_4^{3-}, SO_4^{2-}, ClO_3^-, CN^-, S^{2-}, I^-, Br^-, $S_2O_3^{2-}$, NO_2^-, SCN^-	PLRP-S (150×4.8 mm I.D.), 5 µm (Polymer Labs.); ODS Hypersil (150×4.8 mm I.D.), 5 µm (Shandon).	3.3 m*M* Citric acid, (a) 0.16 m*M* HDTMA hydroxide in ACN–water 3:7, pH 11 or (b) 4.1 m*M*, pH 5.5.	Dynamic modification	Direct and indirect UV at 220 nm and electrochemical (glassy carbon working electrode applied *V*=0.85).	(a) 50–500 µg/l: IO_3^-, NO_3^-; (b) 0.5–5 mg/l: F^-, Cl^-, PO_4^{3-} SO_4^{2-}, ClO_3^-; (c) <50 µg/l: NO_2^-, $S_2O_3^{2-}$, S^{2-}, I^-, SCN^-	–

(Continued on p. 184)

Table 1. Continued

Ref.	Anions	Stationary phase	Mobile phase	Method	Detection and interferences	LD	Applications
[10]	Cl^-, Br^-, NO_3^-, SO_4^{2-}, ClO_3^-	Develosil ODS-3K (100×0.32 mm I.D.), 3 μm (Nomura Chemical, Seto, Japan). Coating: 3 m*M* CTA bromide in MeOH–water (50: 50, v/v).	1 m*M* Salicylate (sodium salicylate–salicylic acid, 90:10) water solution–ACN (95:5, v/v).	Permanent coating.	Indirect UV at 230 nm.	0.7–1.9 mg/l.	Cl^-, NO_3^-, SO_4^{2-} in tap water.
[11]	IO_3^-, PO_4^{3-}, $P_2O_6^{4-}$, Cl^-, CN^-, BrO_3^-, SO_3^{2-}, SO_4^{2-}, HCO_3^-, NO_2^- PO_3^{3-}, NO_3^-, PO_2^{3-}, ClO_3^-, S^{2-}, I^-, $S_2O_3^{2-}$, SCN^-	Separon SIC 18 (150×1.0 mm I.D.), 5 μm.	0.4 mm CTA bromide, 4 m*M* citric acid water solution–MeOH (70:30, v/v) at pH 7.1.	Dynamic modification	UV at 210 nm, direct and indirect conductometric and amperometric (carbon fibre array working, stainless-steel counter and Ag/AgCl reference electrode for NO_3^-, $S_2O_3^{2-}$, SO_3^{2-}, CN^-, SCN^-, IO_3^-, PO_2^{3-}, PO_3^{3-}, $P_2O_6^{4-}$, 2–1.5 V)	mg/l IO_3^- 11.6 PO_4^{3-} 6.7 $P_2O_6^{4-}$ 1.8 Cl^- 2.8 BrO_3^- 17.9 SO_3^{2-} 4.0 SO_4^{2-} 17.4 NO_2^- 3.7 PO_3^{3-} 6.7 NO_3^- 7.6 PO_2^{3-} 53.0 ClO_3^- 17.6 S^{2-} 2.2 I^- 7.0 $S_2O_3^{2-}$ 5.3 SCN^- 3.9 CN^- 5.3	–
[12]	S^{2-}	μBondapak C_{18} (150×3.9 mm I.D.), 10 μm (Waters).	ACN–water (40:60, v/v) with 0.5% glacial acetic acid and 5 m*M* low UV PIC B5 (pentanesulfonic acid).	Pre-column derivatization with N,N-dimethyl-*p*-phenylendiamine of S^{2-}; RP-ion pair.	Vis at 664 nm. Cl^-, Br^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, CO_3^{2-}, CN^- do not interfere at 10 000 fold excess.	μg/l S^{2-} 0.8	–
[13]	NO_2^-, Br^-, Cl^-, NO_3^-, SO_4^{2-}	Partisil 10 ODS-3 (250×4.6 mm I.D.) (Whatman).	0.5–16 m*M* TBA iodide and potassium hydrogenphthalate (0.5–2.0 m*M*), 4<pH<6.5.	Dynamic modification. (method optimization through a three factor composite experimental design).	Conductivity.	–	–
[14]	Br^-, $H_2PO_4^-$, NO_2^-, NO_3^-, Cl^-, I^-, SO_4^{2-}	μBondapak C_{18} (250×4.6 mm I.D.) 10 μm (Waters).	50 mg Papaveraldine perchlorate added to 850 ml of 7 m*M* sodium citrate buffer, 1.75 g TBA hydroxide and ACN 150 ml (pH 3.5).	Dynamic modification.	Indirect UV detection at 325 nm.	mg/l Br^- 3.2 $H_2PO_4^-$ 9.5 NO_2^- 2.7 NO_3^- 2.5 Cl^- 1.4 I^- 4.8 SO_4^{2-} 3.8	Water analysis; assay of blood bromide.
[15]	NO_2^-, SO_4^{2-}, Br^-, NO_3^-	TSK gel ODS-80T_M (50×4.6 mm I.D.), 5 μm (Tosoh), coated with 0.01 *M* cetylpyridinium chloride in 10% MeOH.	1 m*M* Citrate–2.5% methanol adjusted to pH 6.5 with diluted NaOH solution.	Permanent modification.	UV at 210 nm	–	Human serum, saliva and urine.
[16]	IO_3^-, BrO_3^-, Br^-, NO_2^-, NO_3^-, I^-, SCN^-	Zorbax ODS (250×4.6 mm I.D.), 5 μm (DuPont).	ACN–water (35:65, v/v), 2 m*M* cetylpyridinium bromide (>CMC $1\cdot10^{-3}$) and 10 m*M* phosphate buffer.	Dynamic modification.	UV at 222 nm.	ng IO_3^- 25 BrO_3^- 30 Br^- 35 NO_2^- 8 NO_3^- 19	NO_2^-/NO_3^- in human saliva, iodide in commercial salt.

Table 1. Continued

Ref.	Anions	Stationary phase	Mobile phase	Method	Detection and interferences	LD	Applications
						I^- 16 SCN^- 20 (loop volume not given)	
[17]	F^-, Br^-, Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}	LiChrosorb RP-18 (125×4.5 mm I.D.), 7 μm (Merck), coated by crystal violet or methyl green or methylene blue basic (pH 9) 1 m*M* aqueous solution.	3 m*M* *p*-Hydroxybenzoic acid aqueous solution, pH 6.0 (methylene blue) or pH 8.0 (methyl green and crystal violet).	Permanent coating	Conductivity.	μg/l F^-, Br^- 100 Cl^- 50 NO_2^- 100 NO_3^- 200 PO_4^{3-} 500 SO_4^{2-} 300	–
[18]	CrO_4^{2-}, $Cr_2O_7^{2-}$, SCN^-, ClO_4^-	LiChrospher C_{18}, 10 μm (Merck).	MeOH–phosphate buffer (7.0 m*M* Na_2HPO_4–7.2 m*M* NaH_2PO_4), (30:70, v/v), pH 6.8 with TBA hydrogensulfide 3.0 m*M*.	Dynamic modification. Interferences: $S_2O_3^{2-}$ at 1 fold excess level; $Fe(CN)_6^{4-}$, $Fe(CN)_6^{3-}$ at 100 fold excess level	Photolytic-electrochemical detection, glassy carbon working electrode at +1.15 V (vs. Ag/AgCl) and +1.00 V for CrO_4^{2-}.	μg/l CrO_4^{2-} 500 $Cr_2O_7^{2-}$ 300 SCN^- 70 ClO_4^- 300	–
[19]	Cl^-, Br^-, NO_2^-, NO_3^-, SO_4^{2-}	RP-Partisil 10 ODS-3 (250×4.6 mm I.D.) (Whatman)	8 m*M* TBA iodide, 1 m*M* potassium hydrogenphthalate water solution at pH 6.	Dynamic modification	UV at 222 nm.	0.1–0.2 mg/l.	Analysis of atmospheric precipitation and aerosols.
[20]	NO_2^-, NO_3^-	Spherisorb ODS-2 (250×4.6 mm I.D.) 5 μm (Phase Separations).	Aqueous solution of 5.0 m*M* octylamine at pH 6.40 for *ortho*phosphoric acid or for salicylic acid.	Dynamic modification	NO_2^- 5 μg/l, in the presence of chloride 0.60 *M*. Interference by high (0.60 *M*) chloride concentration is studied.	UV at 230 nm	Lagoon water.
[21]	Cl^-, Br^-, NO_3^-, SO_4^{2-}, BF_4^-, NO_2^-, ClO_3^-, I^-, K^+, Na^+	TSK gel ODS-80T$_M$ CTR (100×4.6 mm I.D.), 5 μm (Tosoh) column and SIL-C_{18}/5B (150×4.6 mm I.D.), 5 μm (Yokogawa)	Properties of different alkyl-ammonium ions as IIR are tested. Phthalate, naphthalene-1,5-disulphonic acid and naphthalene-2,6-dicarboxylic acid as absorbing eluent ions are examined. Eluents are prepared by mixing the eluent ion, the hydroxide of the IIR, CH_3OH and water.	Dynamic modification	Indirect photometric detection at 300 nm.	Several ppbs	River, tap, rain and sea waters
[22]	NO_2^-, NO_3^-, IO_3^-, Br^-, $S_2O_3^{2-}$, I^-, SCN^-	Capcellpak C_{18} (150×4.6 mm I.D.), 5 μm (Shisheido). TSK gel ODS-80T$_M$, (150×4.6 mm), 5 μm (Tosoh). Coating with CTA 1 m*M* in water–MeOH (80:20, v/v).	0.1 *M* NaCl aqueous solution, 5 m*M* sodium phosphate buffer (pH 5.8).	Permanent precoating	UV at 225 nm and amperometric (AMP) (glassy carbon working electrode (+1.0 V) vs. Ag/AgCl). Interference free from Cl^-, SO_4^{2-}, Br^-.	μg/l UV NO_2^- 4 NO_3^- 8 amperometric NO_2^- 2	Artificial sea water.

(Continued on p. 186)

Table 1. Continued

Ref.	Anions	Stationary phase	Mobile phase	Method	Detection and interferences	LD	Applications
[23]	I^-, SCN^-, NO_2^-, $S_2O_3^{2-}$	Capcellpack C_{18} (AG 120) (150×4.6 mm I.D.), 5 μm (Shiseido), precoated with silicone polymer, equilibrated with 1 m*M* CTA chloride in water–MeOH (80:20, v/v).	0.1 *M* NaCl, 5 m*M* sodium phosphate buffer (pH 5.8).	Permanent precoating	UV at 225 nm and amperometric (AMP) (glassy carbon working electrode (+1.0 V) vs. Ag/AgCl)	μg/l UV NO_2^- 4 $S_2O_3^{2-}$ 60 amperometric NO_2^- 20 $S_2O_3^{2-}$ 100	Determination of anionic-impurities in inorganic analytical-reagent grade chemicals.
[24]	NO_2^-, NO_3^-	Spherisorb ODS-2 (250×4.6 mm I.D.), 5 μm (Phase Separations).	Several aqueous mobile phases at pH 6.40 for *ortho*phosphoric acid with different ion-interaction reagents (IIR) (alkylamines), and IIR concentration were studied.	Dynamic modification	UV at 230 nm.	–	–
[25]	N_3^-	ODS-2 column 250×4.6 mm I.D., 5 μm Spherisorb (Phase Separations).	Aqueous solution of 5.0 m*M* octylamine at pH 6.40 for *ortho*phosphoric acid.	Dynamic modification.	UV at 230 nm. Interference free from Cl^-, CO_3^{2-}, $S2^-$, NO_3^-, I^-, SCN^-, Br^-, F^-.	30 μg/l	Azide in tap water.
[26]	NO_2^-, S^{2-}, I^-, $S_2O_3^{2-}$, SCN^- oxidizable anions	Shim-Pack CLC-C_8 (150×6 mm I.D.), 5 μm.	MeOH–phosphate buffer (15:85, v/v), (pH 5), 3.0 m*M* TBAOH and 0.1 m*M* EDTA.	Dynamic modification.	Amperometric, glassy carbon electrode $V=+1.0$ V (vs. Ag/AgCl). For 0.4 mg/l NO_2^-, S^{2-}, I^- and 40 mg/l $S_2O_3^{2-}$ no interference by 1000 fold excess of SO_4^{2-}, SiO_4^{4-}, CO_3^{2-}, PO_4^{3-}, BrO_3^-, NO_3^-, MoO_4^{2-}, Cl^-; 500 fold excess of oxalate, Br^- and 100 fold of SO_3^{2-}.	μg/l NO_2^- 0.8 S^{2-} 1.1 I^- 3.6 $S_2O_3^{2-}$ 99 SCN^- 104	Lake water.
[27]	NO_2^-, NO_3^-, I^-, Cl^-, SO_4^{2-}	Spherisorb ODS-2 (250×4.6 mm I.D.), 5 μm (Phase Separations).	Aqueous solution of 5.0 m*M* octyl-amine at pH 6.4 for *ortho*phosphoric acid or for salicylic acid.	Dynamic modification	UV at 230 nm (direct) and at 254 nm (indirect); Conductivity (Cl^-, SO_4^{2-}). Interference free from sea water.	μg/l. NO_2^- 20 NO_3^- 47 I^- 6 Cl^- 8 SO_4^{2-} 25	Lagoon water
[28]	NO_2^-, NO_3^-	PR-18 (250×4.0 mm I.D.), 5 μm (ZOCh, Poland).	2.0 m*M* Nonylammonium phosphate, pH 6.5.	Dynamic modification.	UV at 205 nm and 190 nm in presence of Cl^-.	μg/l NO_2^- 2.0 NO_3^- 1.5	–
[29]	I^-, SCN^-	Develosil ODS-5 (150×0.35 mm I.D.), 5 μm (Nomura Chemical, Seto, Japan) microcolumn and L-Column ODS (250×4.6 mm I.D.). Columns are coated with zwitterionic surfatants (CHAPS, CHAPSO, Zwittergent 3–14).	Pure water	Electrostatic ion chromatography (EIC)	Spectrophotometric at 220 nm.	μ*M* I^- 0.3 SCN^- 1.2	I^- and SCN^- in human saliva.

Table 1. Continued

Ref.	Anions	Stationary phase	Mobile phase	Method	Detection and interferences	LD	Applications
[30]	SO_4^{2-}	Spherisorb ODS-2 column (250×4.6 mm I.D.), 5 μm (Phase Separations)	Aqueous solution of 5.0 m*M* octylamine salicylate at pH 6.40.	Dynamic modification. Method validation. Proficiency tests.	Conductivity	–	Lagoon water
[31]	Br^-, SCN^-	ODS-L column (250×4.6 mm I.D.) (Chemical Inspection and Testing Institute Tokyo, Japan), coated with strong/strong positive/negative charged zwitterionic surfactant reagents (CHAPS) micelles.	Pure water.	Electrostatic ion chromatography (EIC).	ICP-AES (230 nm) conductivity.	–	–
[32]	N_3^-	Spherisorb ODS-2 column (250×4.6 mm I.D.), 5 μm (Phase Separations)	Different aqueous solutions of alkylamines at different concentration and pH are tested. Chemometric optimisation.	Dynamic modification.	UV at 230 nm	–	–
[33]	NO_2^-, SCN^-, N_3^-, BrO_3^-	Spherisorb ODS-2, (250×4.6 mm I.D.), 5 μm (Phase Separations).	Aqueous solution of 5.0 m*M* octylamine at different pH (*ortho*phosphoric acid) ranging between 3.0 and 8.0	Dynamic modification.	UV at 230 nm.	–	–
[34]	$S_2O_3^{2-}$, I^-, SCN^-, NO_3^-	Microcolumn (150×0.35 mm I.D.) packed with Develosil ODS-5, 5 μm (Nomura Chemical) micelle-coated by taurine-conjugated bile salts (sodium taurodeoxicholate and sodium taurocholate).	5 m*M* Cu(II) sulfate aqueous solution (pH=4.5).	Micellar coating.	Indirect photometric detection at 210 nm.	–	–
[35]	NO_3^-, NO_2^-	ODS-2 (25×4.5 mm I.D.), 10 μm (Spherisorb).	Water–MeOH (80:20) containing 1 m*M* TBA.	Dynamic modification.	Spectrophotometric at 214 nm. Interference free from SO_4^{2-} and PO_4^{3-}.	ng NO_3^- 0.32 NO_2^- 0.26	Natural waters
[36]	Cl^-, Br^-, I^-, SCN^-, SO_4^{2-}, NO_3^-, NO_2^-	ODS-L column (250×4.6 mm I.D.) (Chemical Inspection and Testing Institute) coated with zwitterionic surfactants (CHAPS, CHAPSO, Zwittergent 3–14) (see Refs. [30,37,39]).	Pure water	Electrostatic ion chromatography (EIC)	Conductimetric and photodiode array UV–Vis	–	–
[37]	Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, CrO_4^{2-}, MoO_4^{2-}	Nucleosil 5 C_{18} (250×4.0 mm I.D.) (Macherey–Nagel).	1 m*M* TBA +0.5 m*M* DCTA in ACN–water (10:90, v/v) pH 6.2.	Ion-pair of inorganic anions and cations by on-column derivatization with chelating agents.	UV at 210 nm	mg/l Cl^- 200 NO_2^- 200 NO_3^- 7.6 SO_4^{2-} 8.5 CrO_4^{2-} 850 MoO_4^{2-} 35	–
[38]	SeO_3^{2-}, SeO_4^{2-}	Teknokroma ODS-1 (250×4.6 mm I.D.), 5 μm.	5.0 m*M* TBA phosphate in MeOH–water (5:95, v/v).	Dynamic modification. Comparison with ion-exchange.	ICP-MS	μg/l SeO_3^{2-} as Se 50 SeO_4^{2-} as Se 80	River, tap, well waters
[39]	F^-, Cl^-, NO_2^-, Br^-, NO_3^-	Spherisorb ODS (50×4.6 mm I.D.), 3 μm, coated with 0.1 m*M* CTA bromide.	0.5 m*M* Potassium chromate water solution at pH 7.0	Permanent coating.	Indirect UV–Vis at 328 nm.	mg/l F^- 0.24 Cl^- 0.55 NO_2^- 0.72 Br^- 0.83 NO_3^- 0.97	NO_3^- In tap-water. Separation together of organic acids.

(Continued on p. 188)

Table 1. Continued

Ref.	Anions	Stationary phase	Mobile phase	Method	Detection and interferences	LD		Applications
[40]	SO_4^{2-}	Waters NovaPak C_{18} (150×3.9 mm I.D.), 4 μm, precoated with 1,12-diaminododecane 5 m*M* in water–MeOH (90:10, v/v), at pH 4.20 (H_2SO_4).	30 μ*M* Potassium hexacyanoferrate water solution.	Permanent coating.	Indirect UV at 205 nm. Alogenides, NO_3^-, NO_2^-, PO_4^{3-}, HCO_3^- do not interfere at concentrations up to 64:1. Interference by some organic acids was studied.	 SO_4^{2-}	mg/l. 0.1	Water analysis (tap, well, surface, mineral, waste).
[41]	Cl^-, NO_3^-, Br^-	ODS-L column (250×4.6 mm I.D.) (Chemical Inspection and Testing Institute), coated with CHAPS or Zwittergent 3–14 micelles.	Pure water	Electrostatic ion chromatography (EIC).	Conductivity	–		–
[42]	NO_2^-, NO_3^-, I^-, Br^-, Cl^-, SO_4^{2-}	Spherisorb ODS-2 column (250×4.6 mm I.D.), 5 μm (Phase Separations).	Aqueous solution of 5.0 m*M* octylamine at pH 6.40 for orthophosphoric acid or for salicylic acid.	Dynamic modification	UV at 230 nm	–		Lagoon water.
[43]	Cl^-, NO_3^-, Br^-	ODS-L column (250×4.6 mm I.D.) (Chemical Inspection and Testing Institute), coated with CHAPS or Zwittergent 3–14 micelles.	Pure water (WMP-IC:water mobile phase ion chromatography).	Electrostatic ion chromatography (EIC).	Conductivity	–		–
[44]	AsO_3^{3-} and AsO_4^{3-} with organo-As species	C_{18} LiChrospher (250 mm), 5 μm (Hewlett–Packard).	6 m*M* TBA hydroxide at pH 7.3.	Dynamic modification.	Simultaneous UV at 200 nm and amperometric (vitreous carbon working electrode, voltage range: $V = -1.5 - +2.5$ (Ag/AgCl ref. electrode).	 AsO_4^{3-}	μg/l. 56.2	Arsenic species in urine.
[45]	Br^-, NO_2^-, NO_3^-, Cl^-, SO_4^{2-}	Nova-Pak C_{18} (150×3.9 mm I.D.), 4 μm, coated with 0.01 *M* *n*-cetylpyridinium chloride in water–MeOH (90:10, v/v).	Aqueous solution of potassium hydrogenphthalate 0.4 m*M*, pH 4.9.	Potassium coating.	UV at 254 nm.	 Br^- NO_2^- NO_3^- Cl^- SO_4^{2-}	mg/l 0.10 0.09 0.29 0.05 0.10	Determination of inorganic anions in wines together with carboxylic acids.
[46]	SO_4^{2-}, Cl^-, Br^-	ODS-L column (250×4.6 mm I.D.) (Chemical Inspection and Testing Institute) coated with zwittergent 3–14 surfactant micelles.	Pure water.	Electrostatic ion chromatography (EIC).	UV–Vis and conductivity.			Br^- in sea water.

Table 2
Experimental conditions of ion-interaction methods based on modified stationary phases other than C_{18} and C_8

Ref.	Anions	Stationary phase	Mobile phase	Method	Detection and interference	LD	Application
[47]	F^-, HCO_3^-, Cl^-, NO_3^-, Br^-, SO_4^{2-}	Bischoff Hyperchrome SC column (125×4.6 mm I.D.) packed with Hamilton PRP-1, 5 μm, coated with 0.5 g/l methyl green (pH=9.0).	$6.0 \cdot 10^{-3}$ *M* 4-hydroxybenzoic acid (eluent A) or 2,4-dihydroxybenzoic acid (eluent B).	Permanent coating.	Indirect UV detection at 311 nm.	Ranging between 20 and 1500 μg/l with eluent B and between 100 and 1000 μg/l with eluent A.	Tap water.
[48]	F^-, Cl^-, NO_2^-, HPO_4^{2-}, $H_2AsO_4^-$, NO_3^-, ClO_3^-, SO_4^{2-}, CrO_4^{2-}, BF_4^-	Hamilton PRP-1 (150×4.1 mm I.D.), spherical particles, 10 μm.	0.1 m*M* Ru(II) complex with 1,10-phenanthroline or 2,2′-bipyridine ($Ru(phen)_3^{2+}$, $Ru(bpy)_3^{2+}$)+0.1 m*M* succinate buffer at pH 6.1.	Dynamic modification.	Direct and indirect Vis at 445 nm and fluorescence detection (phen: λ_{Ex} 465 nm, λ_{Em} 600; bpy: λ_{Ex} 460 nm, λ_{Em} 580).	–	–
[49]	F^-, Cl^-, NO_2^-, Br^-, NO_3^-, SO_4^{2-}, HPO_4^{2-}, $H_2AsO_4^-$, $HAsO_4^{2-}$, $H_2BO_3^-$, HS^-, HCO_3^-, CO_3^{2-}	Hamilton PRP-1 (150×4.1 mm I.D.), 5 μm column coated with 0.2 m*M* CTA bromide.	1 m*M* Potassium phthalate+Tris 1 m*M* in 24% methanol at different pH values.	Permanent coating.	Indirect UV at different wavelengths, ranging between 282 nm and 254 nm.	–	–
[50]	$S_2O_3^{2-}$, SO_3^{2-}	PRP-1 Hamilton (150×4.1 mm I.D.), 5 μm and PLPR-S (120×4 mm I.D.), 8 μm (Polymer Labs.).	0.001 *M* Na_2CO_3+0.002 *M* of TBA hydroxide in water–ACN (85:15, v/v), pH=11.	Dynamic modification.	UV at 231 nm and UV diode-array detection.	0.2 mg/l of $S_2O_3^{2-}$ and 0.4 μg/l of SO_3^{2-}.	Commercial products of sodium sulphide.
[51]	F^-, Cl^-, Br^-, NO_3^-	Macroporous polystyrene–divinylbenzene RP-1 (Hamilton) column, (150×4.6 mm), 10 μm.	Different amounts of $Ru(phen)_3(ClO_4)_2$ (0.1–0.01 m*M*) and of sodium tartrate (0.02–0.1 m*M*) in water are tested.	Dynamic functionalization.	Indirect fluorimetric detection; λ_{Ex}=447, λ_{Em}=261 nm.	μg/l F^- 5 Cl^- 5 Br^- 10 NO_3^- 10	–
[52]	Selenite, seleniate	Hamilton PRP-1 (150×4.6 mm I.D.), 5 μm.	3% methanol+5 m*M* tetrabutylammonium phosphate pH=7.6	Dynamic functionalization.	ICP-MS	22–74 pg (as Se)	Selenium species in urine.
[53]	F^-, Cl^-, Br^-, NO_3^-	Macroporous polystyrene–divinylbenzene RP-1 (Hamilton) column, (150×4.6 mm I.D.), 10 μm.	0.01 m*M* $Ru(phen)_3(ClO_4)_2$+0.02 sodium tartrate (0.01–0.1 m*M*) water solution.	Dynamic functionalization.	Indirect fluorimetric detection; λ_{Ex}=447, λ_{Em}=261 nm.	μg/l F^- 3 Cl^- 2 Br^- 5 NO_3^- 5	

in ion chromatography, is different for the different ion-interaction reagents and, in addition, can also vary for the same ion-interaction reagent, as a function of different experimental conditions, as, for example, the mobile phase pH [6] or the ionic strength [3].

3. Modification of the reversed-phase stationary phase; choice of the ion-interaction reagent; detection

The modification process can be essentially performed through two different ways:

1. a dynamic process in which the mobile phase contains (or sometimes only consists of) the ion-interaction reagent. When eluting in isocratic conditions, the mobile phase itself modifies the surface, or
2. through a process, referred to as permanent coating, in which the modifier agent is firstly adsorbed onto the stationary phase (often dynamically, by passing it through the column for a certain time); then the column is rinsed and used with a different mobile phase.

Once modified, no great difference can be found in the behaviour of permanently- and dynamically-modified columns, apart from the composition of the mobile phase which, as said, is generally different from the modifier agent when using the permanently-coated stationary phases and in contrast contains (and often consists of) the ion-interaction reagent itself for the dynamically-coated.

The modifications essentially concern C_{18} and C_8 packing material, sometimes in capillary microcolumns [9,13]. Examples are also reported of modifications induced on other packing materials, such as cyano-, diol- or polymeric support. The experimental conditions of these methods are reported in Table 2 [47–53].

Comparisons between the performances obtained in ion-interaction chromatography by the use of different original packings and their possible modifications have been studied [8,10,12]. So, for example, in the modification with iron(II)-1,10-phenanthroline as the ion-interaction reagent, a polymeric base gave results more advantageous with respect to a C_{18} base regarding: (i) retention reversibility of the modifier on the surface, (ii) stability in relation to pH variation, (iii) greater efficiency, (iv) longer column life, (v) higher modifier load [8]. In contrast, when using ion-interaction reagents formed by salicylic, 1,2,4-benzenetricarboxylic, 1,3,5-benzenetricarboxylic or 5-sulphosalicylic acid with tris(hydroxymethyl)aminomethane and tetramethylammonium hydroxide and tetraethyammonium hydroxide as counter ions, the bonded C_{18} silica columns were more efficient than the polystyrene–divinylbenzene (PS–DVB) ones. Furthermore the PS–DVB phases were slower to equilibrate when elution conditions were changed [10]. With a methanol–water mixture of hexadecyltrimethylammonium hydroxide and citric acid [12] as the ion-interaction reagent, comparable retentions were obtained for inorganic anions with polymeric and octadecylsilica stationary phases, while organic anions displayed significant differences in the two systems.

So, no general rule showing advantages for a stationary phase composition can be drawn out, because retention, resolution and sensitivity depend on a number of factors, whose effect is difficult to predict.

According to Haddad and Heckenberg [1] the ''ability'' in developing a new ion-interaction method is ''to tailor the mobile phase composition to suit the particular solute ion being studied'' or to separate the particular mixture.

In principle each salt consisting of a lipophilic cation can be used as the ion-interaction reagent.

Generally, in dynamic coating the ion-interaction reagent is usually characterized by relatively low hydrophobicity, as, for example, are salts of alkylammonium (with alkyl chain length generally ranging between 4 and 10) and tetraalkylammonium (with alkyl chain lengths generally ranging between 1 and 4).

In permanent coating, for the surface modification process, more lipophilic ion-interaction reagents are used, as, for example, salts of cetyltrimethylammonium. For the mobile phase, less hydrophobic reagents (as, for example, tetraalkylammonium salts) are then employed.

Concerning the anion of the salt, both inorganic (*ortho*phosphate, chloride, hydroxide . . .) and organic (salicylate, tartrate . . .) anions are used. Re-

garding their choice, the compatibility with the kind of detection used must be considered.

When conductivity is employed and the background conductivity of the eluent must be minimized, methods which make use of pure water or of very diluted solutions as the mobile phase are highly appreciable.

For UV detection, on the other hand, the eluent must be characterized by the lowest absorptivity at the detection wavelength for direct detection or by the highest for the indirect one. Eluents like phosphate or hydroxide should be avoided when conductometric detection is used but are very suitable for UV detection [13]. Salicylate ions are suitable for conductometric detection and for indirect UV detection. For indirect UV detection papaveraldine perchlorate [17] has also been used. Since some anions (nitrate, nitrite, bromide, bromate, iodide, iodate, periodate, thiocyanate and thiosulfate) absorb between 195 and 220 nm, detection selectivity with respect, for example, to chloride even if present at very higher concentration can be advantageously used, as, for example, in the determination of bromide in common salt [18] and sea-water [42], and of nitrite and nitrate in sea-water [21]. Amperometric detection has been selectively used for oxidable anions [14,19,22,26,44].

4. Dependence of retention on different variables

There are many variables which must be considered in planning the mobile phase composition and which must be optimized to control retention. They include the organic modifier content, the concentration and type of the ion-interaction reagent (regarding both the lipophilic cation and the counter anion), the pH value and the ionic strength. The effect of the variables on retention is often non-linear and interdependent and therefore it is difficult to predict, taking also into account that the mechanisms which govern retention are still not completely understood.

A good optimization of the variables could be obtained through chemometric treatment of experimental design [16,24,32] which optimizes sensitivity and resolution of analytes in a mixture in the lowest total analysis time.

The dependence of retention on the different variables involved has been studied by different authors [3,5,6,8,10,14,16,39–41]: besides the packing material of the stationary phase, retention is greatly affected by the chemical properties of the ion-interaction reagent, the presence and the concentration of the organic modifier in the mobile phase, the concentration of the ion-interaction reagent, the ionic strength of the mobile phase, and the temperature.

Concerning the effect of the organic modifier contained in the mobile phase, it was shown that, as expected, retention decreases when the concentration of the organic modifier increases. This effect is due to (besides the increased eluotropic strength of the solvent) desorption effects exerted by the organic solvent towards the moiety adsorbed onto the surface and to competition equilibria taking place between the solvent and the modifier. The adjustment of organic modifier concentration in the mobile phase can therefore regulate the amount of the ion-interaction reagent adsorbed and by consequence the retention [11,66,67].

Many examples of methods which make use of aqueous solutions of the ion-interaction reagent can be found (Table 1), that offer advantages of low cost and environment protection.

Systematic studies performed in the pH range between 3 and 8 permitted investigation of the effect exerted on the retention by the pH value of the mobile phase. It was shown that retention of both organic and inorganic anions always decreased with pH increase. The behaviour which holds for both strong and weak anions is likely related to the effect that mobile phase pH plays on the moiety already adsorbed onto the stationary phase surface [33,66]. A pH increase induces a decrease of concentration of the cationic form adsorbed and forming the primary electrical layer. Consequently the number of the total active sites available for retention also decreases. Regarding sensitivity, this, as expected, is practically constant in the whole pH range for the anions of strong acids, while increases with pH increase for the weak ones.

The effect of the counter-anion is also very important [5]. The retention dependence on chloride

concentration of nitrate and nitrite ions, when chloride concentration is high as in sea-water can be ascribed to a substitution of the original phosphate counter-anion with chloride ions [21].

The dependence of retention on ion-interaction concentration as well as the effect of mobile phase ionic strength has been also studied [3,14,67]. The anion of the salt seems to compete for solute for the active site on the stationary phase or to exert an electrostatic shielding, that affects not only retention (which generally decreases with ionic strength increase) but also selectivity and elution order [3,67].

Examples are also reported of micellar chromatography, in which the modifier agents in the mobile phase are used at concentrations greater than their critical micellar concentration [4,18].

Also worth noting is a relatively new technique, known as electrostatic ion chromatography, based on the modification of the reversed-phase octadecylsilica and in which the modifier agent is a zwitterion surfactant immobilized on it; the mobile phase is pure water. The analytes are inorganic ions which are eluted as ion-pairs. Sometimes specific ions are added to the mobile phase in order to perform the exclusive partitioning of the analytes as specific ion-pairs [29,31,35,41,43,46]. The analytes are released from the Stern layer to the diffuse layer. Due to the zwitterion which coats the stationary phase, the analytes are forced into a state of simultaneous electrostatic attraction and repulsion in the column, under a "ion-pairing-like" form. Detection sensitivity is enhanced by the low background deriving from using pure water as the mobile phase.

As a general consideration, it can be said that no general rule can be given about the choice of the optimal experimental conditions for developing a new ion-interaction chromatographic method. They strictly depend on the particular application. On the other hand, the dependence of retention on so many experimental conditions makes the technique very versatile for solving many separation and resolution problems. In addition, the technique can be advantageous in overcoming matrix interference, in particular towards more lipophilic components. For the same reasons, no general comparison can be made between the performance of ion chromatography or ion-interaction methods.

5. Examples of applications

Ion-interaction methods can be also advantageously used in the determination of metals through formation of anionic complex species. Different complexones (nitrilotriacetic acid, 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, diethylenetriaminopentaacetic acid, ethylenedioxybis(ethylenenitrilotetraacetic acid, [N-(2-hydroxyethyl)-diaminoethylene]-N,N,N-triacetic acid, and triethylenetetraaminohexaacetic acid) and direct and indirect UV detection have been used for determination of a number of metals [37]. For the determination of selenium the formation of selenotrisulfide has been employed [68] and metal cyano complexes have been formed for the analysis of precious metals in gold processing solutions [69].

Concerning the application of ion-interaction methods in the determination of inorganic anions in real samples, a sensitive application concerns the determination of impurities in analytical grade reagents [23]. Many examples can be found in the fields of environmental, clinical and food chemistry.

Typical inorganic anions have been determined in tap [4,5,13,25,38–40] and surface waters [26,38–40], sea or lagoon waters [21,26,37,38,41,70], atmospheric precipitation as rain, snow, aerosols in correlation with temperature, urban and rural sites [7].

Examples of applications in clinical chemistry are the determination of nitrite and nitrate in human saliva [18], of bromide in blood [17] and of arseniate in urine [44].

Food chemistry applications are the determination of inorganic and organic anions in wines [45] and in fruit juices [6] and of iodide in commercial salt [18].

6. Abbreviations

ODS	octadecylsilica
TBA	tetrabutylammonium
ACN	acetonitrile
CMC	critical micellar concentration
EIC	electrostatic ion chromatography
I.D.	internal diameter
MeOH	methanol
HDTMA	hexadecyltrimethylammonium

TEA	tetraethylammonium
THAM	tris(hydroxymethyl)aminomethane
TMA	tetramethylammonium
CTA	cetyltrimethylammonium
CHAPSO	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CHAP	3-[(3-cholamidoproyl)dimethylammonio]-1-propanesulfonate
Tris	tris-(hydroxymethyl)aminomethane

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Simultaneous separation and detection of anions and cations in ion chromatography

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An overview of new possibilities for the simultaneous determination of anions and cations by ion chromatography is presented. A short comparison of the characteristics of separation and detection methods for simultaneous anion–cation determinations of ions is given and applications are discussed. **©2001 Elsevier Science B.V. All rights reserved.**

Keywords: Ion chromatography; Simultaneous determination of anions and cations; Zwitterion ion exchanger

1. Introduction

The main goal of ion chromatography (IC) as an analytical technique is to provide complete information about the ionic composition of the analyzed sample. For obvious reasons, it is often necessary to use two different sets of IC conditions for the separation and determination of cationic and anionic species. Thus, data on the ionic composition of a sample can be obtained either by two parallel analyses on two different ion chromatographs (which is expensive), or by two consecutive analyses of the same sample with one instrument but with different columns and under different elution conditions. In the second case the change of chromatographic column followed by equilibration with new eluent substantially increases the duration of the analysis and may make the procedure unsuitable for the determination of analyte ions of low stability. The ideal case is simultaneous determination of cations and anions by IC from a single injected sample. Numerous attempts to realize this goal have been undertaken, even from the very early days of IC, and these methods have been reviewed in 1994 [1]. Consideration of recent advantages in this dynamically developing field of IC is the subject of the present review.

2. Classification of approaches to simultaneous IC determination of anions and cations

In accordance with the definition given in [1], methods interpreted as being suitable for the simultaneous determination of anions and cations in IC are those in which a single injection of a restricted volume of the analyzed sample is made into the injection port (usually, an injection valve) followed by chromatographic separation. This definition accommodates methods which use variations in the nature of the mobile and stationary phases, changes in the direction of flow and recycling of the mobile phase, and the use of auxiliary devices within the closed chromatographic system. This broad definition includes the combination of different IC mechanisms and a classification scheme for the various methods is presented in Table 1. As with any multi-component determination in IC, this particular analytical task can be successfully approached either with the use of a highly selective detector combined with a less efficient separation system, or using a universal detector combined with a highly efficient separation system. Organic cations and anions like tetraalkylammonium and carboxylates can also be separated on the basis of differences in hydrophobicity using reversed-phase HPLC, but these methods are beyond the scope of this review.

3. Multi-column systems

As mentioned above, the IC determination of the total ionic composition of the sample usually involves two chromatographic runs with separately injected volumes under conditions (columns,

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0165-9936/01/$ – see front matter
PII: S0165-9936(01)00072-3
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Table 1
Classification of methods for simultaneous determination of anions and cations by IC

<table>
<tr><td colspan="4">SEPARATION</td></tr>
<tr><td colspan="4">Multicolumn techniques</td></tr>
<tr><td colspan="2">Multi-eluent systems</td><td colspan="2">Single eluent systems</td></tr>
<tr><td colspan="2">Parallel column</td><td>In-series columns</td><td>Column switching</td></tr>
<tr><td colspan="4">Single column techniques</td></tr>
<tr><td colspan="4">Ion-exclusion/cation-exchange chromatography</td></tr>
<tr><td colspan="4">Separation on zwitteric ion-exchange columns</td></tr>
<tr><td>Mixed- bead columns</td><td>Agglomerated columns</td><td>Immobilized zwitterionic molecules</td><td>Mixed layers</td></tr>
<tr><td colspan="4">Anion exchange chromatography of anions and negatively charged metal complexes</td></tr>
<tr><td colspan="4">DETECTION</td></tr>
<tr><td colspan="2">Single detector system</td><td colspan="2">Multi-detector system</td></tr>
</table>

mobile phases) which are different for the separation of anions and cations. The first attempts at simultaneous determination of anions and cations were therefore directed towards a combination of two ion chromatographs in one unit. This can be accomplished using columns in parallel or in series or with a system for switching between columns.

3.1. Parallel columns

The use of a joint injection valve is the main practical difference between a chromatographic unit with parallel columns and two separate IC instruments. The most recent paper using this approach was published in 1993 [2], when a fully automated IC system comprising four ion chromatographs (three gradient and one isocratic), four micromembrane suppressors, four guard columns, four conductivity detectors and two PCs was used. This was applied in the Canadian Technological Center for Environmental Protection to the simultaneous determination of 10 inorganic and organic anions and 10 cations (alkali metal ions or alkaline earth metal ions) in atmospheric aerosol. Automation of the analysis via a single loading system permitted isocratic anion analysis, anion gradient (with and without preconcentration) and cation gradient methods with chemical suppression and conductimetric detection applied in an unattended regime for 48 h. The obvious drawbacks of all parallel IC systems and, in particular, the above-mentioned system are the high cost of analysis and maintenance as well as their low reliability. These drawbacks have reduced the attractiveness of this approach over recent years.

3.2. In-series anion and cation exchange columns

A step forward in simplification of multi-column systems involves the use of anion exchange and cation exchange columns connected in series. Such systems can be readily organized inside a standard isocratic ion chromatograph. In this approach a single eluent is used for separation of anions and cations and ideally the eluted analytes are detected with a single detection unit. In this way Takeuchi et al. [3] separated a mixture of Na^+, NH_4^+, K^+, Cl^-, Rb^+, Cs^+, Mg^{2+}, NO_3^- and Ca^{2+} with dual Develosil ODS-UG5 columns (50×0.32 mm) connected in series, one of which was modified with sodium dodecylsulfate (SDS) and the other with cetyltrimethylammonium bromide

(CTAB). The use of 10 m*M* copper sulfate solution as eluent allowed indirect photometric detection of both anions and cations at 200 nm or conductimetric detection if preferred. However, the stability of the ion exchangers obtained by dynamic modification of reversed-phase stationary phases in the absence of SDS and CTAB in the eluent has to be checked carefully.

In many cases the detection of the eluted ions cannot be performed with a single detector and additional devices are required. For example, the simultaneous determination of 11 cations and anions in tap water was achieved in 10 min by using a Dionex HPIC-CS3 column followed by a detector selective for monovalent cations in series with a Dionex HPIC-AS4A column followed by a detector selective for singly charged inorganic and organic anions [4]. Two ion-selective electrodes (ISE) served as selective detectors.

A more complex scheme was proposed for the simultaneous determination of organic and inorganic anions and cations in wine and sake [5]. The separation was performed on an anion exchange column Yokogawa SAM3-075 (75×4.9 mm) and a cation exchange column Yokogawa ICS-C25 (125×4.6 mm) connected in series with 1.5 m*M* oxalic acid as eluent. The detection system consisted of an anion suppressor and a cation suppressor connected in parallel, combined with a switching valve to allow each detector to be selected as required. An evident drawback of the proposed scheme is its inability to detect oppositely charged ions simultaneously.

A general difficulty associated with the use of chromatographic systems with columns in series is a problem of high back pressure at the top of the first column and the necessity to have a pressure-resistant detector flow cell if it is placed between two columns. Therefore, the use of short columns of 5–10 cm length or columns packed with relatively coarse particles of size greater than 10 μm is recommended.

3.3. Switching columns

The introduction of switching valves into IC systems allows not only an improvement in flexibility of detection as shown above but also an increase in the separation ability of the chromatographic column [1]. Deguchi et al. [6] proposed an interesting approach which they called flow gradient ion chromatography. Their system consisted of two columns (50×4 mm), one containing 10 μm Hitachi IC 2710 anion exchange resin and the other Hitachi IC 2720 cation exchange resin, connected in series with a junction between them for a second mobile phase and two pumps with a flow gradient capability which delivered the same mobile phase of 2.5 m*M* benzoic acid and 1.5 m*M* Tris. By varying the flow rates of the two pumps but keeping the total flow rate constant at 1 ml/min, retention of both anions and cations could be manipulated so that they were well separated. This new method was applied to the simultaneous separation and determination of 8–52 μg/ml Li^+, Na^+, NH_4^+, K^+ and 51–89 μg/ml chloride, nitrite, bromide and nitrate in test solution. Reproducibility for retention times and peak areas was 0.13–0.34% and 0.13–1.61% (n=5), respectively.

There have been only a few published papers on the use of multi-column systems for simultaneous determination of anions and cations since 1993, probably due to the drawbacks mentioned earlier.

4. Single-column systems

Single-column systems are established using different types of column packing which generally contain both positively and negatively charged sites in the form of mixed particles of different ion exchangers or specially designed zwitterionic ion exchangers. The classification and application of zwitterionic ion exchangers in liquid chromatography has been recently reviewed [7]. However, in accordance with the classification of methods of simultaneous IC determination of anions and cations given in Table 1, the following modes can be identified.

4.1. Mixed bed columns

Packing of a column by a mixture of particles of cation and anion exchange materials represents a further simplification of the 'column in series technique' because of the replacement of the two columns by a single column containing a mixed bed stationary phase. A short mixed bed column (100×4.6 mm) packed with a 1:1 mixture of anion exchange resin (Yokogawa ICS-A23) and cation exchange resin (Yokogawa ICS-C25) was used for the IC separation and determination of $H_2PO_4^-$, Cl^-, Na^+, K^+, NO_3^-, SO_4^{2-}, Mg^{2+} and Ca^{2+} in tap water using 1.5 m*M* pyromellitic acid as elu-

ent and conductivity detection [8]. The advantage of this approach is the use of a single column and hence the elimination of additional broadening of chromatographic peaks arising from extra connectors, tubing and frits as in the case of two columns in series. An interesting aspect of the retention of cations on a similar mixed bed column has been noted [9]. Here, ion exchange with elution by H^+ was the primary retention mechanism, but there was a clear influence on retention of cations caused by the anion exchange groups present in the column. This secondary mechanism was particularly evident for monovalent cations. On the other hand, anions showed the same elution behavior on the mixed bed column as on a monofunctional anion exchange column.

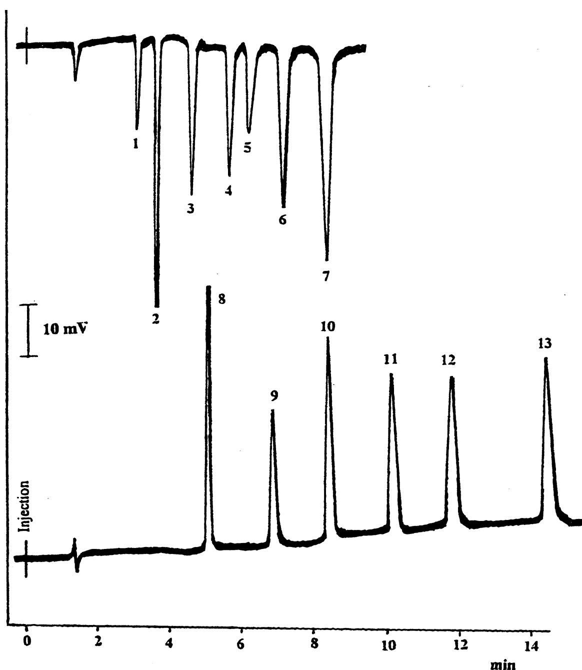

Fig. 1. Simultaneous separation and detection of anions and cations on a latex agglomerate column. Column: Dionex HPIC-CS5 cation exchange column (250×2 mm) with precolumn HPIC-CG5 (50×4 mm); eluent 0.5 m*M* copper sulfate, pH 5.62; flow rate 0.5 ml/min; sample volume 20 µl containing 0.1 m*M* of each ion; detection: two potentiometric detectors equipped with different ion-selective electrodes in series. Peaks: (1) chloroacetate, (2) chloride, (3) nitrite, (4) benzoate, (5) cyanate, (6) bromide, (7) nitrate, (8) sodium, (9) ammonium, (10) potassium, (11) rubidium, (12) cesium, (13) thallium. Reprinted with permission from [10].

The particles of the two different resins may have a different density, size, and porosity which could present a problem for their homogeneous packing by slurry methods.

4.2. Agglomerated ion exchangers

The above-mentioned problem has been solved in agglomerated ion exchangers, manufactured by Dionex (Sunnyvale, CA, USA), in which positively charged latex microbeads are electrostatically retained as a monolayer at the surface of sulfonated poly(styrene–divinylbezene) particles, producing an anion exchanger. Agglomerated cation exchangers can be prepared by an additional coating of the agglomerated anion exchanger with a further layer of sulfonated latex microbeads. All of these agglomerated ion exchangers exhibit zwitterionic ion exchange properties [7] that allow them to be used successfully for the simultaneous determination of anions and cations. The simultaneous determination of up to 13 ions was demonstrated using the cation exchange column Ionpac-CS5 (Fig. 1) [10]. It is clear from the figure that a lack of resolution of oppositely charged ions and overlapping chromatographic peaks, which would be observed with conductivity detection, could be eliminated by their selective detection with two ISEs in series. The authors noted that the ISEs used for this work gave a stable response for 1 week.

4.3. Mixed layer zwitterionic ion exchangers

Negatively charged sulfopolysaccharides, such as heparin, dextran sulfate, and chondroitin sulfate, can be used instead of latex microbeads for the modification of anion exchangers [11,12]. This type of modification of the surface should provide a more homogeneous distribution of functional groups at the surface with better mass transfer characteristics for the separation of anions and cations. However, it was found that the properties of these zwitterionic ion exchangers are sensitive to the charge density and molecular weight of the polymeric chains of the coating material, and the porous structure of the ion exchanger used as support material [11]. It was proposed that large polymers (dextran sulfate of MW ∼75 000) do not penetrate into the pores and form a kind of net around the core of the anion exchanger (TSK-Gel IC-Anion SW, pore diameter 14 nm). On the other hand, smaller

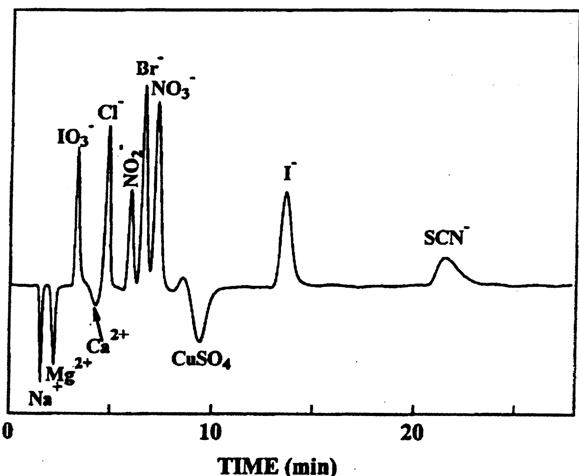

Fig. 2. Simultaneous separation of a model mixture of ions using anion exchange column coated with sulfopolysaccharide. Column: TSK-Gel IC-Anion SW (100×0.32 mm) dynamically modified with heparin; eluent 1 m*M* copper sulfate; flow rate 4.2 µl / min; sample volume 0.2 µl; detection UV 200 nm. Reprinted with permission from [11].

molecules, such as dextran sulfate with MW ~5100 or heparin with MW ~2200, can easily occupy the pore volume of the resin and so form a polyzwitterionic structure which is selective for simultaneous separation of anions and cations. The simultaneous separation of three cations and eight anions was achieved for the latter ion exchanger (Fig. 2) using 1 m*M* copper sulfate as eluent and photometric detection of all 11 analytes using a single wavelength.

4.4. Ion exchangers with immobilized zwitterionic ion exchange molecules

Close proximity of the oppositely charged functional groups can be achieved in ion exchangers with covalently bonded zwitterionic molecules in which two or three methylene chains separate the carboxylate or sulfonate and quaternary ammonium groups [13–15]. This arrangement results in the establishment of a combination of repulsion and attraction electrostatic forces and such stationary phases have been used for the separation of seven or eight anions and cations [13,14].

There are still only a few published applications of sorbents with covalently bonded zwitterionic molecules for simultaneous determination of anions and cations. This is related to the difficulties associated with synthesis of these sorbents [13], especially obtaining an equal concentration of positively charged quaternary ammonium $-NR_4^+$ groups and negatively charged $-SO_3^-$ groups [13]. For this reason a very intensive study has been undertaken in recent years on electrostatic ion chromatography (EIC) [16], which involves the use of reversed-phase hydrophobic sorbents with dynamic coatings of zwitterionic surfactants. An additional feature of EIC is that the separation of ions can be performed with pure water as the eluent and this provides very sensitive conductivity detection without the need for suppressors. The main separation forms in pure water are ion pairs between anion and cations from the injected sample [16]. After redistribution and formation of new ion pairs in the chromatographic column the total number of possible peaks before detection is increased drastically (the exact value is equal to the number of anions multiplied by the number of cations included in the sample). Complete separation and quantification in such a system is difficult

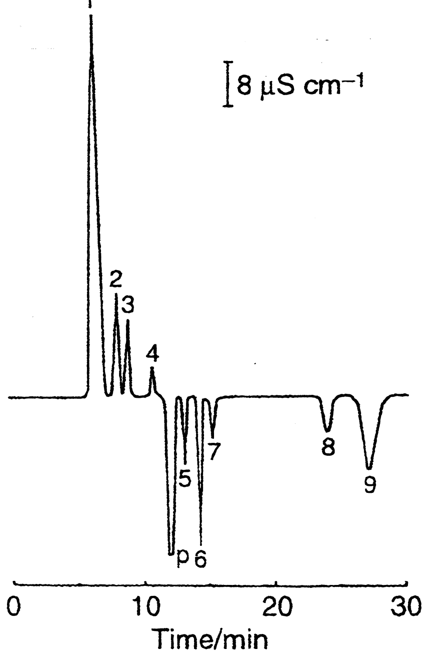

Fig. 3. IEC / CEC separation of anions and cations. Column: TSK-Gel OA-Pak A (300×7.8 mm, 5 µm); eluent 5 m*M* malic acid–methanol (95:5); flow rate 1.2 ml / min; sample volume 25 µl; detection conductivity. Peaks: (1) sulfate, (2) chloride, (3) nitrate, (4) fluoride, (5) sodium, (6) ammonium, (7) potassium, (8) magnesium, (9) calcium. Reprinted with permission from [19].

Table 2
Anion exchange chromatography of inorganic anions and negatively charged metal complex anions with different reagents

Anion exchange column	Eluent	Studied ions (as injected in a column)	Detector	Ref.
TSK guardgel QAE-SW 250×4.6 mm, 5 μm	0.15 m*M* 1,2,4,5-benzenetetracarboxylic (pyromellitic) acid	PO_4^{3-}, Cl^-, NO_2^-, Br^-, NO_3^-, I^-, SCN^-, SO_4^{2-}, $S_2O_3^{2-}$, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}	Cond.	[20]
TSK guardgel QAE-SW 150×4.6 mm, 5 μm	0.5 m*M* 1,2,4-benzenetricarboxylic (trimellitic) acid–0.25 m*M* EDTA, pH 6.0	HCO_3^-, Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, Mg^{2+}, Ca^{2+}	UV, 270 nm	[21]
Vydac 302 IC, 250×4.6mm, 5 μm	0.75–2.0 m*M* CDTA, pH 5.8–methanol	Cl^-, NO_3^-, SO_4^{2-}, Fe^{3+}, Cr^{3+}, Y^{3+}, La^{3+}, Nd^{3+}, Gd^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, Sr^{2+}, Zn^{2+}, Mo(VI)	UV, 210/195 nm	[22]
Hamilton PRPx100 (dimensions not given)	0.375 m*M* 2,5-dihydroxy-1,4-benzenedi-sulfonic acid–0.125 m*M* EDTA, pH 6.5	F^-, Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, $S_2O_6^{2-}$, SO_3F^-, SeO_3^{2-}, SeO_4^{2-}, CrO_4^{2-b}, $PO_3F_2^-$, $H_2PO_4^-$, MnO_4^-, Ca^{2+}, Cd^{2+}, Mg^{2+}, Pb^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, Al^{3+}, Fe^{3+}, Cr^{3+}	UV, 335 nm	[23]
Dionex AG9 50×4.0 mm and Dionex AS9 250×4.0 mm, 15 μm	3.5 m*M* Na_2CO_3, pH 9.75	BrO_3^-, SeO_3^{2-}, SeO_4^{2-}, $HAsO_4^{2-}$, WO_4^{2-}, MoO_4^{2-}, CrO_4^{2-}, Cl^-, NO_3^-, SO_4^{2-}, Pb^{2+a}, Ni^{2+a}, Cd^{2+a}, Cu^{2+a}	Cond.	[24]
Waters IC-Pak C anion, 50×4.6 mm and IC-Pak pre-column 50×4.6 mm	1 m*M* 2,6-pyridinedicarboxylic acid, pH 6.0	HCO_3^-, $H_2PO_4^-$, Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, Mg^{2+}, Ca^{2+}	UV, 290 nm	[25]

[a] Metal ions were complexed with EDTA before injection in a column.
[b] Direct UV detection at 370 nm.

and it has been found more practical to perform EIC separations in eluents containing dilute solutions of electrolytes, especially those which can be suppressed (such as $NaHCO_3$).

4.5. Ion exclusion chromatography / cation exchange chromatography (IEC/CEC)

This novel variant of IC has become increasingly popular for the simultaneous separation of anions and cations. Tanaka et al. [17] found that a polymethacrylate-based weakly acidic cation exchange resin (Tosoh TSK gel OA-Pak A in the H^+ form) could be used to retain cations by a cation exchange mechanism and anions by an ion exclusion mechanism. The optimum separation (Fig. 3) of three or four anions (SO_4^{2-}, NO_3^-, Cl^-, F^- or NO_2^-) and five cations (Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) was achieved with eluents comprising dilute solutions of organic acids (tartaric, malonic, malic, sulfosalicylic acid) at pH 2.8–3.0, containing methanol and 18-crown-6 ether for the regulation of separation selectivity of metal cations [17–19]. The practical implementation of IEC/CEC has been realized in a portable ion analyzer Shimadzu PIA-1000, which was successfully used for acid rain monitoring in different countries of East Asia. However, the future of this method is not completely clear as all applications are connected with the use of only one type of chromatographic column, Tosoh TSK gel OA-Pak A.

4.6. Anion exchange chromatography of anions and negatively charged complexes of cations

Alkaline earth and transition metal ions can be converted to negatively charged complexes by reaction with different ligands and can be separated in the same mixture with inorganic anions using anion exchange methods. This makes possible the use of standard IC equipment for their quantitative determination and this approach has proved popular (see recent applications in Table 2).

There are two main directions evident from published research. The first is the possibility of increasing the sensitivity of the photometric detection using complexing reagents like pyromellitic [20], trimellitic [21] and dipicolinic acid [25]. These have been studied as binary complexing eluents in combination with EDTA or CDTA as a single-component eluents (Table 2). The second research direction is to increase the range of analyte ions

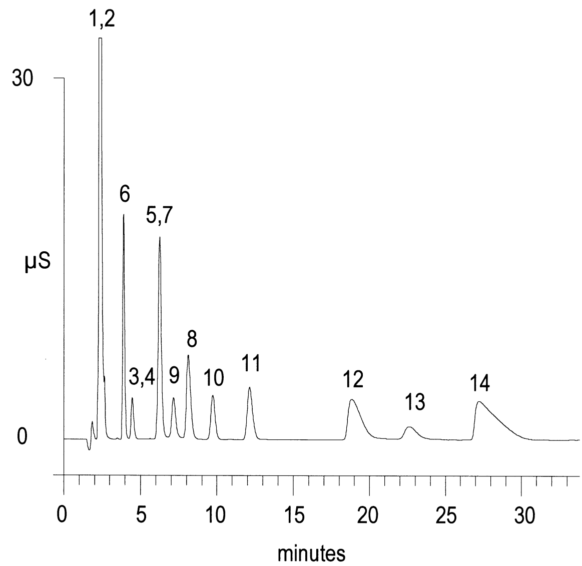

Fig. 4. Anion exchange chromatography of a model mixture of anions, negatively charged complexes and oxoanions of metals. Column: Dionex AS9 anion exchange column (250×4 mm) with precolumn HPIC-AG5 (50×4 mm); eluent $[HCO_3^-]+[CO_3^{2-}]$=3.5 m*M*, pH 9.75; flow rate 1.0 ml/min; sample volume 50 μl; detection: conductimetric with suppressor AMMS-II. Peaks: (1) BrO_3^-, (2) Cl^-, (3) Pb^{2+}, (4) Cd^{2+}, (5) Ni^{2+}, (6) NO_3^-, (7) SeO_3^{2-}, (8) Cu^{2+}, (9) $HAsO_4^{2-}$, (10) SO_4^{2-}, (11) SeO_4^{2-}, (12) WO_4^{2-}, (13) MoO_4^{2-}, (14) CrO_4^{2-}. Reprinted with permission from [24].

suitable for this approach. In this regard, Sarzanini et al. [24] studied retention behavior of a group of analytes and separated not only simple anions and complexes of transition metals with EDTA, but also a number of oxoanions of metals (Fig. 4, Table 2). An impediment to the wide practical application of this approach is its unsuitability for the determination of alkali metal ions, which are not retained by anion exchangers. The use of multi-element spectroscopic detection could be a possible solution [1].

5. Simultaneous detection of anions and cations

The question of which type of detection system is most suitable for the simultaneous determination of oppositely charged ions remains open and presents somewhat of a dilemma in this area of IC. On the one hand, if the developed separation system provides high resolution of chromatographic peaks, a simple detection system such as conductimetry or

Table 3
Comparison of the most successful IC systems for the simultaneous determination of anions and cations

Chromatographic system	Total number separated ions (anions[a] / cations)	Limits of detection (μ*M*)	Time (min)	RSD[b] (%)	Ref.
Multi-column modes					
Parallel columns	20 (10/10)	0.2–2.20	15–20	6–20	[2]
In-series columns	13 (5/6)	0.15–4.0	10	No data	[4]
Single-column modes					
IEC / CEC with conductivity detection	9 (4/5)	0.4–5.0	30	0.1–0.3, n=11	[18,19]
Agglomerated ion exchanger with two ISE potentiometric detection	13 (5/6)	0.15–4.3	15	1.4–1.8, n=10	[10]
Mixed bed of sorbents–conductivity detection	8 (4/4)	0.63–9.3		0.2–10.8; n=5	[8,9]
IC on bonded zwitterionic molecules with conductivity detection	7 (3/4)	0.8–1.2	8	No data	[14,15]
Mixed layers sorbent–indirect UV detection	11 (8/3)	No data	25	0.3–0.5; n=5	[12]
Anionic IC of anions and negatively charged complexes	13 (6/7)	0.3–5.3	30	<1; n=8	[20]

[a]The data presented only for inorganic anions.
[b]Peak heights or peak squares.

indirect photometry is sufficient for the determination. The use of a single universal detector is preferable from the point of view of simplicity, cost and absence of extra broadening of chromatographic peaks. However, it is a difficult task to identify the optimal eluent composition providing suitable quality of separation and also sensitive detection for all the separated ionic forms having very different properties.

From a second viewpoint, low selectivity and efficiency of separation can be compensated by the use of a second selective detection unit such as ICP–OES [1], two ISEs in series (Fig. 1) [5,10], switching of the suppressor system for conductivity detection [6], etc. Sometimes, selective detection facilitates the simultaneous determination of anions and cations without any separation (for example, the FIA determination of calcium and chloride ions in natural waters [26]). Therefore, both detection and separation must be considered for a balanced comparison of the different approaches to simultaneous determination of anions and cations (Table 3).

6. Applications

More than 90% of all papers on simultaneous determination of anions and cations by IC are devoted to the development of a simple, fast and reliable system for monitoring of the quality of waters (seawater [10], rainwater [17,19–21], river water [10,17,20,21,24], atmospheric aerosols [2,18], tap water [8,10,22,23], etc.). However, none of these systems (Table 3) completely satisfies the demands of environmental monitoring, except in the case of the very complex system using parallel separation of anions and cations described in [2]. In accordance with US EPA method 300 'The determination of inorganic anions in water by ion chromatography', at least seven anions (F^-, Cl^-, PO_4^{3-}, SO_4^{2-}, Br^-, NO_3^-, NO_2^-) must be determined together with cations, which are assumed to be Na^+, NH_4^+, K^+, Mg^{2+} and Ca^{2+}, to evaluate the real quality of natural waters. Obviously, the IEC/CEC system is the most advanced for this analysis and offers the determination of four anions from the above list and all five cations (Fig. 3) [19].

Other applications include analysis of wine [5,9], juice [9,23], potato chips [23] and soil [23]. Relatively little attention has been paid to applications related to the determination of negatively and positively charged ionic forms of transition metals of great interest for metal speciation investigations, except for speciation of Cr(III)–Cr(VI) which has been studied extensively.

7. Conclusions

A clear trend to simplify IC systems used for the simultaneous determination of anions and cations

can be identified. New single-column separation chemistries like IEC/CEC or systems using immobilized zwitterionic molecules or sorbents with oppositely charged layers of polymer have been developed for this purpose. These have been used to separate the main ions occurring in natural waters and have been applied in practice, e.g. in portable ion analyzers for the monitoring of the concentration level of nine ions in rainwater. The absence of commercially available columns for simultaneous determination of alkali metal cations and inorganic anions will stimulate further work on the design and development of zwitterionic exchangers. The high popularity of IC of anions and negatively charged metal complexes should also be noted and this popularity arises from the fact that this approach is a simple adaptation of standard chromatographic equipment and separation conditions to enable simultaneous determination of anions and cations.

Further improvement of a detection system has also been achieved. Potentiometric detection with two ISEs has been found to be a very useful and promising detection technique for determination of monovalent cations and anions and this approach can compensate for poor separation performance of the chromatographic column.

Finally, the existence of other analytical methods for the simultaneous determination of anions and cations including FIA [26], bidirectional isotachophoresis [27] and capillary electrophoresis [28] should be mentioned and will undoubtedly promote further investigations in this area.

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Sample treatment techniques and methodologies for ion chromatography

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Sample pretreatment is necessary when the analytical method cannot provide good separation and quantification due to interferences from sample matrix components. In this paper we discuss two important approaches to dealing with troublesome sample matrices in ion chromatography – choose a column or column combination that can manage the problematic matrix or modify the sample matrix to eliminate the problem. **©2001 Elsevier Science B.V. All rights reserved.**

Keywords: Ion chromatography; Sample preparation; Ion chromatography–mass spectrometry

1. Introduction

One of the most important aspects of developing a rugged IC method is the ability to recognize when undesirable chromatographic effects are derived from sample matrix interferences rather than from hardware problems. Sample matrix effects can include shortened retention times, poor peak efficiency, poor resolution, poor reproducibility, irregular baseline and fouling of the electrode when using an electrochemical detector.

When retention times progressively shorten, injection to injection, the cause may be that the sample matrix contains one or more highly retained ions. These ions may be organic or inorganic and may be retained by adsorption as well as by ion exchange, depending on their structures. Since these ions are not easily eluted by the eluent they effectively reduce column capacity. For example, polyvalent species such as polyphenols can cause shortened retention times on anion exchange columns. Polyphenols can be selectively removed from sample matrices using polyvinylpyrrolidone resin as described in Section 4. Transition metals and their complexes can also be highly retained. The metals are retained as cations but some are also anionic complexes such as $FeCl_4^-$. As cations, transition metals can be removed from sample matrices using a cation exchange resin. Iminodiacetate resin can selectively chelate transition metals in the presence of alkali and alkaline earth metals.

Poor peak efficiency can result from the presence of high concentrations of matrix ions. One example is seen as the poor peak shapes that result in cation analysis when a sample of very low pH is injected onto a weak acid-type cation exchange column. The sample matrix protonates some of the cation exchange sites on the stationary phase, lowering charge density and capacity, resulting in low peak efficiency. When a matrix ion is present at a concentration that overloads the ion exchange column, retention times for eluting ions can shorten. Loss of resolution between the matrix ion and a slightly earlier eluting analyte may be seen. Later-eluting analyte ions can be subject to elution on the tail of a large matrix ion, making peak integration difficult. In either case, matrix elimination of the matrix ion, a change in column or a change in chromatography conditions is needed.

Poor reproducibility in peak area and low peak response can be caused by fouling of the detector electrode, when using pulsed or direct current amperometric detection. Matrix components such as fats, proteins and surfactants can foul the surface of the working electrode resulting in reduced signal. Fouling of the analytical system, including connecting tubing and sample loop, with iron, barium or calcium can cause low recovery and poor reproducibility for analytes including sulfate and phosphate. All of these types of matrix species can be removed from samples using techniques described in Section 4.

Some effects described above can be dealt with directly by proper choice of analytical column; others are best handled by using sample pretreatment chemistries either on-line or in disposable cartridges. Various sample pretreatment chemistries have been used for matrix elimination with or with-

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0165-9936/01/$ – see front matter
PII: S0165-9936(01)00069-3
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out simultaneous analyte concentration [1–3]. The sample types include soil and peat extracts [4–9], oil field waters [10,11], biological matrices [12–17], pulp and paper [18], water and wastewater [19–26], and foods [27–29]. In this work we examine the options for dealing with difficult sample types. The options include the use of higher capacity analytical columns, various column coupling techniques and the use of sample pretreatment chemistries that are specifically designed for ionic analytes.

2. Column selection

A common problem in ion chromatography is poor resolution of an analyte ion from matrix ions that are present in relatively high concentrations, the so-called high–low problem. There are several factors that improve the ability to quantify a trace level analyte in the presence of a high concentration of another ion. Column capacity, selectivity and efficiency are the three most important parameters. Higher capacity columns have higher loading capacity but may have longer analysis times. Selectivity provides better peak spacing to handle overloaded peaks. Peak efficiency has been improved in recent years by the introduction of 5-μm polymeric substrate particles for IC columns.

In a typical high–low problem, the goal is to quantify an analyte at 50 μg/l in the presence of a close-eluting matrix ion present at several hundred mg/l. One approach is matrix elimination but recent developments in ion exchange column technology [30–32] have produced high capacity analytical columns that can manage the matrix challenge. Since no sample pretreatment is necessary in these cases, the analytical method is simplified.

2.1. Anion analysis

Higher capacity columns for IC such as the IonPac® AS9-HC, AS11-HC, AS15 and AS16 (all Dionex Corp.) have 170–290 μEq/column, 2–5× the capacity of columns used for routine anion exchange applications. Common inorganic anions such as chloride and sulfate show higher retention on the higher capacity columns, as expected. The higher capacity also means that the column has a higher loading capacity so that peak shapes are maintained at higher analyte and matrix concentrations. One application that benefits from a higher capacity analytical column is the determination of bromate in drinking water [33]. In this work, a bromate:chloride ratio of 1:10 000 was managed by column capacity alone. For comparison, the lower capacity IonPac AS9-SC column, with a capacity of

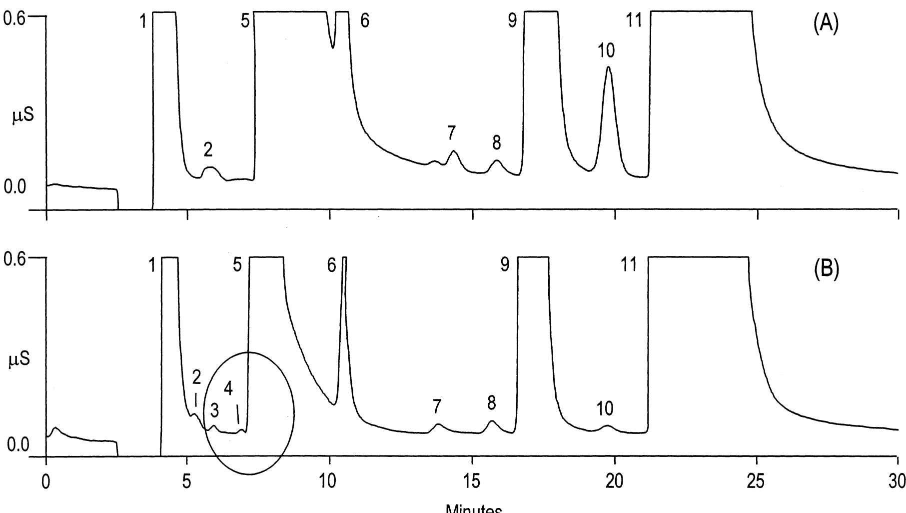

Fig. 1. Trace bromate determination using matrix elimination and preconcentration. See text for details. Peaks: 1, fluoride 1.0 mg/l; 2, unknown; 3, chlorite, 0.01 mg/l; 4, bromate 0.005 mg/l; 5, chloride, 200 mg/l; 6, nitrite 0.1 mg/l; 7, bromide, 0.01 mg/l; 8, chlorate, 0.01 mg/l; 9, nitrate, 10.0 mg/l; 10, *o*-phosphate, 0.1 mg/l; 11, sulfate, 200 mg/l. Sample B was pretreated with OnGuard II Ag, H cartridges. Samples also contained 200 mg/l bicarbonate.

30 μEq, can manage a bromate:chloride ratio of about 1:1000. The bromate:chloride ratio can be improved to 1:40 000 if sample pretreatment is used to remove the chloride in conjunction with the high capacity columns (Fig. 1). Fig. 1B shows the separation of 5 μg/l bromate in the presence of 200 mg/l chloride, a ratio of 1:40 000, using the IonPac® AS9-HC (250×4-mm i.d.) analytical column and sample pretreatment as discussed in Section 4. The eluent was 9.0 m*M* sodium carbonate flowing at 1 ml/min. The analytical column had an ion exchange capacity of 190 μEq. The injection volume was 500 ul. Peaks were detected by conductivity using the ASRS®-I suppressor in the external water mode.

Since the resolution of carbonate from chloride is high on the AS9-HC column, it can also be used to quantify chloride and sulfate in the presence of high carbonate without sample pretreatment. Ratios of 1:250 are easily achieved.

2.2. Cation analysis

The optimization of column capacity and selectivity is very important in cation analysis. The IonPac CS15 (Dionex Corp.) column has high selectivity for ammonium and potassium and can therefore be used in a coupled column configuration to remove potassium through a switching valve. The IonPac CS16 (Dionex Corp.) column is specifically designed for high–low applications of cations. It uses higher capacity and optimized selectivity to accomplish these separations. Due to the higher capacity, it can also separate cations in matrices containing up to about 125 m*M* H^+ without sample pretreatment. Lower pH samples can be treated using anion exchange resin to raise the pH, as discussed in Section 4. By comparison, the lower capacity IonPac CS12A (Dionex Corp.) column requires sample pretreatment when the sample hydronium ion concentration is greater than 50 m*M*.

2.3. Carbohydrate analysis

Samples that contain a high ratio of hydrophobic amino acids to carbohydrate can be problematic when using the amperometric detector for analysis of the monosaccharides. The monosaccharide content of a glycoprotein is often determined after acid hydrolysis using an anion exchange separation at high pH coupled with pulsed amperometric detection [34]. Hydrophobic amino acids such as lysine can foul the gold working electrode surface causing a low response for close-eluting monosaccharides. An AminoTrap guard column (Dionex Corp.) with a high selectivity for amino acids, specifically lysine, can be placed immediately before the CarboPac PA10 (Dionex Corp.) carbohydrate analytical column in order to increase the retention time of the lysine in relation to galactosamine, the nearest-eluting monosaccharide. The selectivity and capacity of the AminoTrap and the CarboPac PA10 are matched for this application. In this method, the lysine elutes after the monosaccharides and can be cleaned from the analytical column as part of the mobile phase gradient.

2.4. Borate

Borate can be removed from sample matrices using a high capacity, cis-diol-based resin as in BorateTrap columns (50×4-mm i.d., Dionex Corp.). For example, borate can be removed from high borate matrices for the determination of transition metals by ICP-MS (private communication). A lower capacity version of this material is used as a borate concentrator for the analysis of sub-ppb borate in ultra pure water [35]. The formation of the borate–diol complex occurs at neutral to high pH where the borate is in the tetrahedral form. Therefore the sample pH must be adjusted into this range for efficient trapping of borate.

3. Coupled techniques

These techniques use a switching or diverter valve to direct portions of the sample matrix to waste as the sample components are separated.

3.1. ICE-IC

Kaiser [32] has described the determination of common inorganic anions in concentrated weak acids including hydrofluoric acid (HF) by coupling two different separation modes (Fig. 2). This technique involves the coupling of an ion exclusion (ICE) column to an anion exchange (IC) concentrator column with a switching or diverter valve. Acids with low pK_a values such as sulfuric acid have little to no retention by ion exclusion since they are completely ionized. They elute early from an ion exclusion column. Weaker acids such

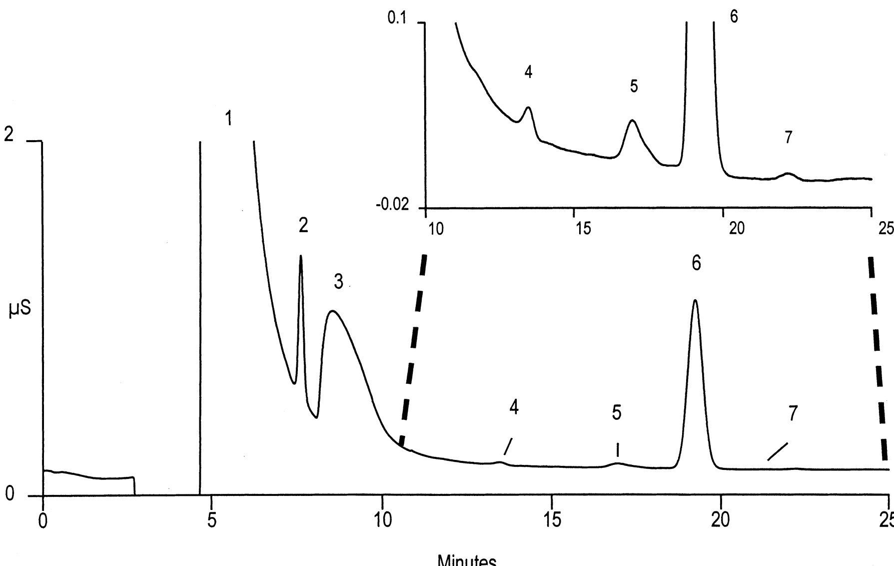

Fig. 2. Anion exchange separation of trace anions in HF following fluoride removal using ion exclusion. See text for details. Peaks: 1, fluoride; 2, chloride, 7.9 µg/l; 3, carbonate; 4, nitrate, 0.89 mg/l; 5, unidentified; 6, sulfate 10.1 mg/l; 7, phosphate, 2.4 µg/l.

as HF are retained on an ion exclusion column. In this method the anions that elute early from the ion exclusion column, i.e. sulfate, are trapped on an anion exchange concentrator column and the later-eluting weak acids are diverted to waste as they elute. The anions of strong acids therefore can be separated from weak acids and collected for later analysis. The retained weak acids such as HF are diverted to waste after the early-eluting peaks pass onto a concentrator column so that the large fluoride peak does not obscure the other peaks in the anion exchange chromatogram.

In Fig. 2, the columns were IonPac ICE-AS6 (250×9-mm i.d.), AG9-HC (concentrator, 50×4-mm i.d.) and AG9-HC/AS9-HC (analytical, 250×2-mm i.d.). The ion exclusion sample treatment eluent was deionized water and the flow rate was 0.55 ml/min. The sample volume was 750 µl. The ion exchange eluent was 8.0 m*M* sodium carbonate and 1.5 m*M* sodium hydroxide. The flow rate on the 2-mm analytical column was 0.25 ml/min. Detection was by suppressed conductivity using the ASRS®-I electrolytically regenerated suppressor in the external water mode.

The ion exclusion process is based on Donnan exclusion. In order for Donnan exclusion to operate in this application, the resin must be in the acid form. It is usually necessary the wash the ion exclusion column with acid between injections in order to remove alkali, alkaline earth and transition metal cations that are retained on the ion exclusion resin from the sample matrix. This washing process is usually automated into the analytical method for good reproducibility.

3.2. IC-IC

Kaiser [31] used on-line sample preparation to determine trace anions in solvents, including isopropanol, acetone and *N*-methylpyrrolidone. A large solvent injection can interfere with both the ion exchange separation and the conductivity detection by causing large disturbances in the baseline. In this application the anions were concentrated on a AG9-HC guard column and then the solvent was sent to waste before it could enter the analytical column. After the solvent was cleared from the AG9-HC concentrator column, the AG9-HC concentrator was switched in line with the AS9-HC analytical column for the separation. The method detection limits for chloride, sulfate, phosphate and nitrate are reported in the sub µg/l range.

4. Off-line sample pretreatment cartridges

Off-line sample pretreatment in ion chromatography most often involves matrix elimination using

small, disposable cartridges containing 1–2.5 g of highly selective, functionalized resin. Samples are usually applied to the cartridges using a disposable syringe. The effluent is injected into the ion chromatograph. In the matrix elimination mode, the resin phases are non-selective for the analytes; the matrix ions (interferences) are bound to the phase while the analytes are unretained. The devices can also be used for pH adjustment and counterion replacement.

4.1. Precipitation of chloride and sulfate

Matrix removal of chloride and sulfate is based on the precipitation of these anions with counterions from a sulfonated resin. Fully sulfonated cation exchange resin is available with a variety of counterions. The most commonly used counterions are Ag^+, Ba^{2+} and H^+, for matrix elimination of chloride, sulfate and general cations, respectively.

The functionalization of the styrene–divinylbenzene resin is accomplished by sulfonation with sulfuric acid or chlorosulfonic acid. On a wet basis, fully sulfonated resin typically has a cation exchange capacity of 2.2–2.5 mEq/g. In terms of chloride removal, this means that a wet gram of resin can precipitate chloride from 12–14 ml of 0.17 *M* (1%) sodium chloride.

The percentage of crosslinking in the resin is determined by the ratio of divinylbenzene to styrene in the polymerization reaction. A higher crosslinking yields a resin with higher selectivity for divalent cations relative to monovalent cations as well as a resin with less susceptibility to shrinking and swelling during use. Resins used for sample preparation usually have crosslinking in the 10–16% range.

The silver form of sulfonated resin is primarily used to precipitate halides and thus provides matrix elimination of those ions. This reaction is most effective for monovalent ions that have pK_{sp} values greater than 8 with silver. Since silver hydroxide readily forms at basic pH values, samples must be below pH 8 for effective removal of halides. Silver-form resin is not very effective for removing phosphate since trivalent phosphate is present at high pH. For trace analysis of phosphate, however, recovery through silver-form resin should be verified using a standard addition technique.

In order to avoid contamination of the analytical column and suppressor with silver ions, the silver-form cartridges are usually followed by a second cartridge in the hydronium ion (acid) form. Any free silver ions are exchanged onto the hydronium ion resin because of the much higher selectivity of sulfonated resin for silver ion than for hydronium ion. The hydronium-form resin bed usually takes the form of an additional cartridge [22]. New, two-layer disposable cartridges have a layer of hydronium ion-form resin at the outlet with a layer of silver-form resin above it for an all-in-one treatment.

The chemistry of barium precipitation has been discussed in an earlier publication [3]. There are two important points to note regarding the use of barium-form sample resin to remove sulfate. Barium is available for precipitation with sulfate only if it is not in use as the counterion for the resin. This means that there must be sufficient cation content in the sample matrix to exchange onto the resin and to displace the barium ion. This requires approx. 200 mg/l Na^+ or 100 mg/l Ca^{2+}. Since barium is a divalent cation, this means that the most effective displacing matrix is one that contains other divalent cations such as calcium.

In [3] we describe a method for spiking calcium into the sample in order to insure efficient removal of sulfate when the matrix is low in cation content (Fig. 3). The cartridge device contains three layers of resin: barium form, silver form and hydronium ion form, in that order, inlet to outlet, to remove sulfate and chloride (Fig. 3). The sample is spiked with calcium chloride to insure removal of sulfate. The hydronium ion-form resin traps any re-dissolved silver ion. The equations governing this system are

$$\begin{gathered} R-(SO_3^-)_2Ba^{2+} + Ca^{2+} = R-(SO_3^-)_2Ca^{2+} + Ba^{2+} \\ Ba^{2+} + SO_4^{2-} \rightarrow BaSO_4\downarrow \end{gathered} \tag{1}$$

$$R-SO_3^-Ag^+ + Na^+Cl^- \rightarrow R-(SO_3^-)Na^+ + AgCl\downarrow \tag{2}$$

$$R-SO_3^-H^+ + X^+ = R-SO_3^-X^+ + H^+ \tag{3}$$

where R=resin, X=silver or other cation.

Another important point is that barium sulfate is soluble at acidic pH. This means that this precipitation reaction is not very effective for removing sulfate from sulfuric acid.

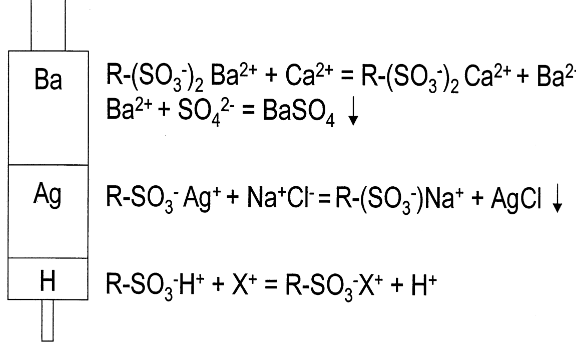

Fig. 3. Schematic of the three-layer OnGuard II Ba/Ag/H sample pretreatment cartridge.

4.2. Adsorption-based matrix elimination

Polymeric reversed phase resins are synthesized from divinylbenzene with styrene, methylstyrene or other styrenic monomers. Divinylbenzene is the major component and provides crosslinking. These resins are macroporous, and the surface area is usually in excess of 300 m^2/g. This surface area provides the adsorptive surface for retention of hydrophobic species. These resins can be used for matrix elimination of surfactants, weak carboxylic acids, fats, proteins, etc.

Hydrophobic matrix components such as fats can foul electrochemical detector electrodes by blocking the active metal surface and inhibiting interaction between the analytes and the metal surface of the electrode. This problem is usually see as fairly rapid loss in peak area, injection to injection.

The determination of iodide in milk (2% milkfat) by ion chromatography coupled with pulsed amperometric detection on a silver electrode is an application that benefits from matrix elimination of fats. The pulsed amperometric waveform improves reproducibility by electrochemically cleaning the working electrode on each pulse. In addition, the fats are removed from the sample using a disposable cartridge containing a polymeric reversed phase resin (OnGuard® II RP, Dionex Corp.). When 50 μl of 0.1 mg/l iodide was added to 200 μl of prepared milk, the recovery was 100%. The iodide peak area and retention time RSDs were 1.4% and 0.4% respectively [28].

4.3. Removal of weak acids

Samples containing high concentrations of weak acids in the matrix can be treated to remove the weak acids. Weak acids can be adsorbed onto polymeric reversed phase resins if the sample pH is low enough for the acids to be protonated and the sample matrix does not contain organic solvent. In order to facilitate this process, samples of undetermined pH can be passed through a hydronium-form cartridge to acidify the sample prior to use of a reversed phase cartridge for removal of weak acids. For example 50% of acetic acid (100 mg/l) can be removed by the reversed phase resin at pH 2.5 while only 19% is removed at pH 4.5 using OnGuard® II H and OnGuard II RP (Dionex Corp, Sunnyvale, CA, USA) cartridges in series.

4.4. Trapping of phenols and azo dyes

Phenols and azo dyes can be removed from sample matrices by adsorption onto polymeric reversed phase resin. However, these species can be removed with much higher capacity (per gram of resin) and selectivity using a polyvinylpyrrolidone (PVP) phase. Species such as humic and tannic acids contain many phenolic groups and are retained on a PVP phase with high specificity. This type of sample pretreatment cartridge facilitates the determination of anions and cations in ground waters that contain humic acids without fouling of the analytical column [4–9]. Another common application for this type of phase is the determination of anions in inks since many inks contain dyes bearing azo groups. These azo dyes are selectively retained by the polyvinylpyrrolidone polymer.

4.5. Concentration/elimination of transition metals

Cationic transition metals are easily retained on a sulfonic acid-functionalized resin such as OnGuard II H. However, they can be selectively concentrated or removed from a matrix by using an iminodiacetate resin cartridge, OnGuard II C, even in the presence of high amounts of sodium [36–39]. This type of resin chelates transition metals at $pH > 4$ and releases the transition metals at $pH < 2$. These metals include Ti(IV), V(IV) and (V), In(III), Y(III), Cd(II), Mn(II), Fe(II) and (III), Co(II), Pb(II), Ni(II), Al(III), Cu(II), Zn(II), Ag(I) and most lanthanides. If EDTA or other strong chelators are present in the sample matrix then exhaustive digestion by EPA method 200.8 is necessary. When competing chelators are present in the sample matrix or

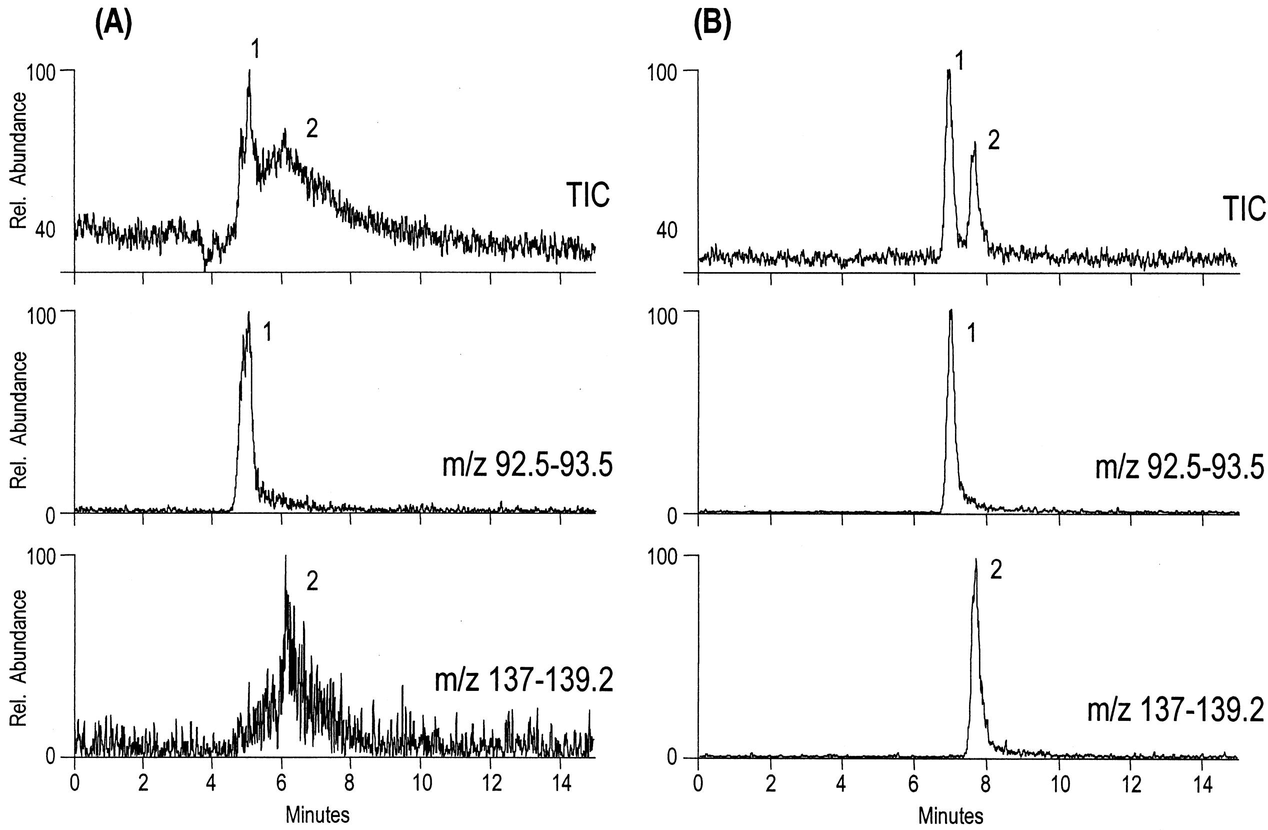

Fig. 4. IC-MS of chloroacetic acid and bromoacetic acid in a matrix containing 1000 mg/l chloride, without (A) and with (B) sample pretreatment. Peaks: 1, chloroacetic acid, 8 mg/l; 2, bromoacetic acid, 7 mg/l; TIC, total ion chromatogram, *m/z* 92.5–93.5, extracted ion for chloroacetic acid, *m/z* 137–139.2, extracted ion for bromoacetic acid. B: After sample is passed through OnGuard II Ag/H cartridge.

if the sample pH is below 4, the sample band is not efficiently treated and the colored metals can be seen to smear along the length of the cartridge. However, using optimum elution conditions, transition metals such as cadmium, copper, cobalt, iron, lead, titanium, vanadium and zinc can be concentrated and recovered at 99–117% at the 50 µg/l level.

4.6. Matrix elimination for IC-ESI/MS

Sample pretreatment is useful when a mass spectrometer with atmospheric pressure electrospray ionization (ESI/MS) is used as the detector for ion chromatography. High ionic strength matrices are known to suppress analyte ionization and cause poor reproducibility in ESI/MS [40]. We investigated the use of off-line sample pretreatment to remove chloride in order to improve sensitivity in the determination of chloroacetic acid and bromoacetic acid (Fig. 4).

The chromatographic system comprised a GP50 gradient pump, LC30 chromatography module, an ED40 conductivity detector and a ThermoFinnigan AQA® mass spectrometer (all Dionex Corp.). Data were collected using Chromeleon 6.2 software (Dionex Corp.). Haloacetic acid separations were accomplished on an IonPac® AS16 column (250×4-mm i.d.) using 10 m*M* sodium hydroxide eluent flowing at 0.25 ml/min. An ASRS® Ultra suppressor (2-mm, Dionex) in the external water mode provided background conductivity suppression. The AQA mass spectrometer was operated in the negative electrospray mode. The electrospray probe was set at 275°C and −2.5 kV. The source voltage was 10 V. Chloroacetic acid and bromoacetic acid were obtained from Aldrich and prepared at 8 mg/l and 7 mg/l respectively in water. The injec-

tion loop was 5 μl. Sample pretreatment was accomplished using OnGuard II Ag/H dual layer cartridges for the removal of chloride.

Fig. 4A shows the total ion current (TIC) and the extracted chromatograms of chloroacetic acid at masses 92.5–93.5 and bromoacetic acid at masses 137–139.2 when 0.16% (1000 mg/l) chloride is present. The chloride coelutes with bromoacetic acid under these conditions and causes band spreading of that peak. There is also loss in sensitivity for chloroacetic acid of about 50%. Fig. 4B shows the same three traces in the presence of 0.16% sodium chloride after sample pretreatment to remove the chloride. The recovery of these acids through the OnGuard II Ag/H dual layer cartridge was 94%.

5. Summary

It is important to be able to recognize the chromatographic problems that are sample-related as opposed to system hardware-related. A variety of techniques are available to deal with chromatography problems caused by sample matrix. The use of high capacity analytical columns should be the first consideration. Column selectivity can be used in a variety of coupled column arrangements to remove matrix components on-line. On-line trap columns or off-line sample preparation cartridges or barrels can be used to remove common foulants and interferents including chloride, sulfate, hydronium ion, surfactants, fats, transition metals, phenols, azo dyes, amino acids, borate, etc.

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Journal of Chromatography A, 789 (1997) 85–126

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Review

Inductively coupled plasma mass spectrometric detection for chromatography and capillary electrophoresis

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Abstract

Inductively coupled plasma mass spectrometry (ICP-MS) is now a well established detection technique for liquid chromatography, gas chromatography, supercritical fluid chromatography and capillary electrophoresis. A review of the literature with particular regard to ICP-MS as a chromatographic and capillary electrophoretic detector is presented. The various modes of chromatography and capillary electrophoresis are discussed and practical descriptions for hyphenating the techniques with the ICP mass spectrometer are given. Sample introduction systems and data acquisition methods are reviewed along with the numerous applications of ICP-MS as a chromatographic detector. In addition, alternative plasma sources, such as the atmospheric and reduced pressure helium microwave-induced plasmas for chromatographic detection are described. © 1997 Elsevier Science B.V.

Keywords: Reviews; Inductively coupled plasma mass spectrometry; Detection, LC; Detection, GC; Detection, electrophoresis; Detection, SFC

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PII S0021-9673(97)00970-9

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1. Introduction

Inductively coupled plasma mass spectrometry is now recognised as a useful and powerful technique for the detection of trace elements in chromatographic eluents. The analysis of both metals and non-metals in a wide variety of samples may be achieved using this sensitive and selective method of detection. The use of inductively coupled plasma (ICP) MS as a chromatographic detector was first described in the late 1980s and, since that time, the versatility of the detector has been realised for many chromatographic applications.

Compared to other methods of detection, ICP-MS offers unique advantages, including element specificity, a wide linear dynamic range, low detection limits and the ability to perform isotope dilution analysis. The use of chromatography hyphenated with ICP-MS has been reviewed by Byrdy and Caruso [1,2] Heitkemper et al. [3] and Bloxham et al. [4] for the analysis of environmental samples. Other reviews have mentioned the use of ICP-MS for various chromatographic applications [5–13] including metal ion analysis [10], gas chromatography [7], ion chromatography [12], human nutrition and toxicology [9] and selenium in environmental matrices [11].

Many papers describing ICP-MS as a chromatographic detection method have been specifically aimed towards speciation analyses. The determination of the chemical form of an element in a sample is particularly advantageous to the analyst, especially where risk assessment is required. Direct aspiration of a liquid sample into the ICP-MS immediately atomises and ionises the elements present so that information regarding the chemical form cannot be obtained. By separating the various species using a chromatographic method and analysing these species by ICP-MS, toxic and innocuous forms of an element may be separated. The fate and mobility of certain compounds in biological and environmental systems may also be monitored, for instance for tracing the metabolism of drugs in the human body or the breakdown of organometallic compounds in aqueous systems. Reviews concerning elemental speciation are also available [14–24].

The use of various chromatographic separation methods is reviewed in this work for both multielement and elemental speciation analyses. The different chromatographic techniques reported in the literature are discussed individually and the use of capillary electrophoresis, with directly coupled ICP-MS, as a separation technique is also described. This review is intended to provide instruction and insight regarding the potential for coupling chromatography with ICP-MS. The use of other spectrometric tech-

niques such as ICP atomic emission (AES) and atomic absorption spectrometry (AAS) as chromatographic detectors are not discussed.

2. Liquid chromatography

2.1. Interfacing liquid chromatography with ICP-MS

Liquid flow-rates used in most liquid chromatography techniques are of the order of 1 ml min^{-1} which are comparable to conventional liquid flow-rates for direct aspiration of solutions into the ICP. Conventional pneumatic nebulisation with cross-flow and concentric nebulisers may, therefore, be used along with single or double-pass spray chambers. To provide a connection between the LC column outlet and the ICP-MS sample introduction system, a transfer line must be constructed. This is commonly a relatively simple task and a length of polyether ether ketone (PEEK) or PTFE inert tubing may be used. The length and inner diameter of the transfer line must be kept to a practical minimum, commonly 20–50 cm, to ensure that peak broadening is not observed.

Use of a conventional sample introduction system with pneumatic nebulisation and spray chamber are inefficient and only 1–3% of the sample entering the nebuliser is actually transported to the plasma. It is apparent that if this sample transport efficiency is increased, the sensitivity of the technique should improve and lower detection limits should be possible. Nebulisers with higher transport efficiencies have been described for LC–ICP-MS. An increase in the amount of solvent reaching the plasma results in higher reflected powers and this causes plasma instability, possibly detrimental to the RF generator. This problem may be solved by desolvating the sample aerosol before it reaches the plasma and is commonly achieved using a cooled spray chamber which serves to condense the solvent. Membrane dryers and Peltier condensers have also been utilised for this purpose [25] and are able to desolvate approximately 89% of the liquid sample when coupled to the front of the nebuliser/spray chamber arrangement [25].

Many variations of the conventional pneumatic nebuliser/spray chamber sample introduction system have been described in the literature for improved sensitivity with LC–ICP-MS. The use of hydraulic high pressure nebulisation (HHPN) has been shown to increase sensitivity for many elements when compared to conventional pneumatic nebulisation [26].

Thermospray has also been applied as an LC–ICP-MS interface [27,28] and involves forcing the chromatographic eluent through an electrically heated capillary at flow-rates of about 2 ml min^{-1}. Heating serves to desolvate the droplets before aspiration into the plasma.

An oscillating capillary nebuliser has also been shown to improve detection limits when compared to a concentric glass nebuliser [29]. The nebuliser is essentially two fused-silica capillary tubes, mounted concentrically. The nebuliser gas flows through the outer tube while the liquid sample flows through the inner tube. This nebuliser may be operated at flows as low as 1 $\mu l\ min^{-1}$ and may be interfaced with macrobore, microbore and capillary LC columns [29].

An ultrasonic nebuliser (USN) may also improve sample transport, and efficiencies are generally in the region of 10–30%. Commercial USNs have in-built desolvation systems which remove most of the solvent. The nebuliser shows excellent improvements in sensitivity.

The direct injection nebuliser was developed by Shum et al. [30–32] specifically for LC–ICP-MS interfacing. The nebuliser is positioned inside the ICP torch and the tip is situated a few mm from the base of the plasma. Theoretically, 100% transport efficiency may be obtained and, as the nebuliser operates at very low liquid flow-rates (30–120 $\mu l\ min^{-1}$), plasma instability is not significant. Detection limits should theoretically be improved thirty fold at a flow-rate of 100 $\mu l\ min^{-1}$ but, as there is no desolvation, local plasma cooling occurs and detection limits are only improved by a factor of 2.5 [33].

The effect of different spray chambers on sample transport efficiency should also be considered when optimising sample transport efficiency. Several different spray chambers have been examined by Rivas et al. [34]. Seven different single pass, double-pass and cyclone-type spray chambers were compared

when connected to a concentric nebuliser and it was found that a transport efficiency of 7.5% could be obtained with the cyclone-type spray chamber.

2.2. Reversed-phase chromatography

2.2.1. Introduction

Reversed-phase (RP) chromatography is one of the most commonly used LC techniques. It achieves the desired separation of analytes using columns where the stationary phase surface is less polar than the mobile phase.

The retention mechanism in RP-HPLC arises due to the relative hydrophobicity of the analyte. The separation selectivity is a result of interactions of the solute with the stationary and mobile phases. Adjustment of the selectivity may be achieved by altering the type and quantity of the organic modifier (solvent) in the mobile phase. Common organic modifiers include methanol and acetonitrile although other organic solvents have been selected to control retention and selectivity. These solvents may be used in binary, tertiary or quaternary combinations with water. Marked differences in separation selectivity may be observed by changing the organic solvent. However, the use of a particular organic modifier is determined according to plasma stability and instrument performance upon solvent aspiration.

The most common stationary phases used in reversed-phase chromatography are prepared from silica-based compositions, usually siloxanes, where the R group of the siloxane may be a C_{18}, C_8 or C_1 hydrophobic hydrocarbon. The C_8 and C_{18} stationary phases are normally used for the separation of relatively low molecular mass analytes and the C_1 phase may be used for the separation of larger molecules.

When performing reversed-phase separations, care should be taken to avoid pH values of greater than 7.5 as hydrolysis of the siloxane stationary phase will occur, resulting in gradual degradation of the packing material. Buffer solutions may be used to control the pH of the mobile phase when coupled to ICP-MS. Phosphate or acetate salts are common, although care must be taken to maintain a minimum level of these compounds in the mobile phase to prevent clogging of the sampler cone.

2.2.2. Environmental and general applications

Reversed-phase chromatography coupled with ICP-MS has been employed extensively for the analysis of environmental and general analytical samples.

Dauchy et al. [35] used RPLC–ICP-MS for the speciation of butyltin compounds which are used extensively in polyvinylchloride production, fungicides, bactericides, insecticides and other general applications. A 0.1% (m/v) tropolone in a methanol–water–acetic acid solution (80:14:6) was used as the mobile phase after optimization studies were performed using different solvent volume combinations. Monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT) compounds were separated using isocratic elution with absolute detection limits of 0.24 ng, 0.24 ng and 0.15 ng (as tin), respectively. Triethyltin was added as an internal standard to improve reproducibility and analysis time, which did not exceed 11 min.

The speciation of inorganic lead (Pb^{2+}), trimethyllead (TML) chloride, triethyllead (TEL) chloride and triphenyllead (TPhL) chloride was achieved using RPLC–ICP-MS by Al-Rashdan et al. [36]. Optimum chromatographic separations were achieved using ICP-AES detection with an acetate buffer and a step gradient of 10–70% methanol. Upon coupling to the ICP-MS, however, an isocratic elution was employed, owing to plasma instability as the organic component of the mobile phase changed. An isocratic separation with a 30% methanol mobile phase was found to give the best compromise between plasma stability and chromatographic resolution (Fig. 1). A C_{18} column was used and pH effects were studied. Detection limits using ICP-MS as a detector were improved by three orders of magnitude when compared to ICP-AES detection. Isocratic RP-HPLC showed superior detection capabilities when compared to ion-pair and cation-exchange chromatography.

In the work of Bushee [37], ICP-MS was used in the reversed-phase separation of mercury compounds. The method was subsequently applied to the determination of methylmercury in an NBS SRM-50 Albacore tuna sample with good agreement between experimentally obtained and certified values. Liquid chromatography and flow injection techniques were found to be favourable in comparison to direct ICP-

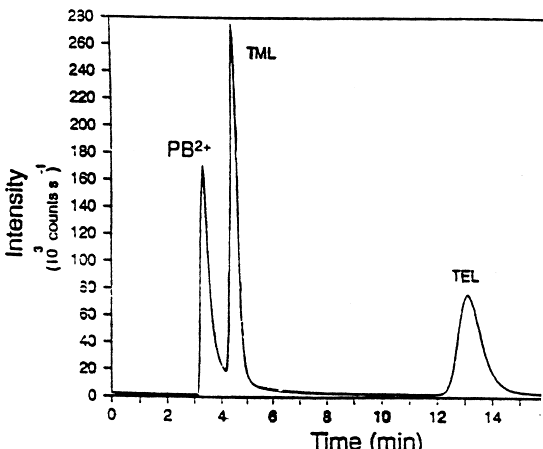

Fig. 1. LC–ICP-MS chromatogram of a standard mixture of organolead and inorganic lead compounds (Pb^{2+}, trimethyllead and triethyllead) using reversed-phase HPLC. Mobile phase, 0.1 *M* ammonium acetate and 0.1 *M* acetic acid at pH 4.6, 30% methanol. Flow-rate, 1 ml min^{-1}. Reprinted from Al-Rashdan et al. [36] by permission of Preston Publications, a division of Preston Industries.

MS because of a 'large persistent, mercury memory problem in conventional ICP-MS'.

Mercury speciation has received a great deal of attention in recent years due to environmental and toxicological effects which are dependent upon the particular form of the metal in the sample. Huang and Jiang [38] used RP-HPLC coupled to ICP-MS with ultrasonic nebulisation for the determination of methylmercury, ethylmercury and inorganic mercury. Absolute detection limits were in the range 70–160 pg of Hg which were ten times better than results achieved using conventional pneumatic nebulisation and were comparable to values obtained using cold vapour generation. The concentration of methyl mercury in NRC Dorm-1 Dogfish muscle reference sample and inorganic mercury in a waste water reference solution were determined and results were comparable to certified values with precisions less than 8% R.S.D. for all determinations.

Ebdon et al. [39] described the analysis of geoporphyrins by RPLC–ICP-MS. Geoporphyrins occur in oils, oil shales and sediments as Ni^{2+} and vanadyl (VO^{2+}) complexes and are used in oil exploration and as oil maturity indicators. Julia Creek and Serpiano nickel porphyrins were analyzed using both LC–ICP-MS and HPLC–UV–Vis. The two methods of detection showed good quantitative agreement of analytical data of the nickel porphyrin fractions. The authors state that "...the system was limited for routine use by the need for high oxygen concentrations in the plasma gas". Limits of detection were not reported therefore no indication is given regarding the best method of detection. However, the authors realised the potential for multielement, multiisotopic detection for future studies.

Tellurium speciation has been described by Klinkenberg et al. [40] using reversed-phase LC–ICP-MS for the analysis of tellurium in samples from a wastewater treatment plant. A method was developed for the separation of TeO_3^{2-} and $HTeO_4^-$ and, although at least 11 different organic Te compounds were detected, no attempt was made to identify them. This is essentially a problem with ICP detection since all structural information is lost by plasma sample decomposition.

2.2.3. Clinical applications

RPLC–ICP-MS has been used for clinical analyses by a number of workers, particularly with regard to speciation studies.

Takatera and Watanabe [41] used this technique for the speciation of iodide ion, I^-, and five iodo amino acids (monoiodotyrosine (MIT), diiodotyrosine (DIT), 3,3,5-triiodothyromine (T_3), 3,3,5′-triiodothyromine (rT_3), and thyroxine (T_4)) which are all found in thyroid hormones. The speciation of these compounds in clinical samples such as blood plasma and urine may assist in the identification of thyroid diseases. The RPLC–ICP-MS system was able to detect all of the I-containing compounds with no interferences. Detection limits were in the range 35–130 pg for the six compounds using a 50% methanol eluent. Detection limits were better for species eluted at a shorter retention time since the peak shapes were sharper. The detection limits calculated were an order of magnitude lower than for methods where UV absorbance detection was used.

Owen et al. [42] used RPLC–ICP-MS to separate Zn-containing species in an in vitro gastrointestinal digest of chicken meat that had been isocratically labeled with Zn both intrinsically and extrinsically. Single ion monitoring was used for two separate isotopes, ^{66}Zn and ^{68}Zn. Aqueous Zn eluted from C_8

and C_{18} columns connected in series, with separate peaks observed for the chicken meat (Zn associated with 3 fractions) and enzyme blanks (zinc associated with a single fraction). Data handling using time-resolved acquisition was found to be cumbersome and it was not possible to evaluate directly the net peak area. The authors recognised the need for further work to standardise the chromatographic runs with the use of a post-column internal standard. The effect of gradient elution on plasma stability was also a further problem to be addressed. Detection limits were not reported in this study.

ICP-MS was used for the detection of biologically significant metalloporphyrins separated by RP-HPLC by Kumar et al. [43]. Cobalt protoporphyrin (CoPP), iron protoporphyrin (hemin) and zinc protoporphyrin (ZnPP) were separated using a C1 column (due to the relatively large molecular mass of the compounds) and a mobile phase optimised with 68% methanol at pH 4.5 (Fig. 2). Detection limits were 0.1 ng, 5.8 μg and 4.6 ng for CoPP, hemin and ZnPP, respectively. R.S.D. values for each porphyrin were within 5%. The technique was then used to quantify zinc protoporphyrin from the blood of a lead-poisoned patient. The authors indicated that the method could be applicable to detection of metalloporphyrins in urine and other body fluid extracts.

The analysis of thimerosal (sodium ethylmercurithiosalicylate), a mercury-containing antimicrobial agent, in biological products has been described by a number of authors [29,37,44]. Bushee [37] described the speciation of several organomercury species and the subsequent identification and quantification of thimerosol using RPLC–ICP-MS. It was found that all the mercury was in the thimerosol form. The detection limits for four organomercury compounds were stated to be in the range 7–20 ng ml^{-1} Hg. A C_{18} column with 3% acetonitrile mobile phase was utilised for the separation at pH 6.8 for the methylmercury and pH 4.3 for the

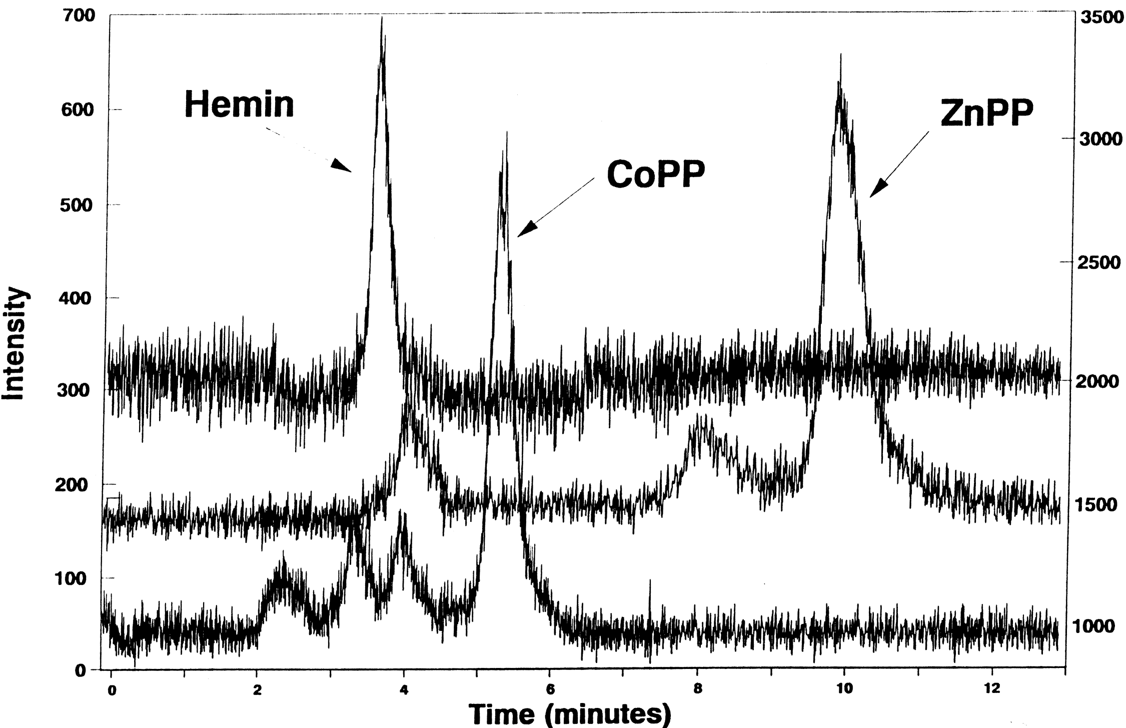

Fig. 2. Multielement chromatogram of metalloporphyrins. The intensity scale on the left corresponds to CoPP and ZnPP; the intensity scale on the right corresponds to hemin. Reprinted from Kumar et al. [43] by permission of Preston Publications, a division of Preston Industries.

thimerosol studies. In a further study, Bushee et al. [44] also determined thimerosol in injectable biological products (influenza virus vaccine and tetanus toxoid) and thimerosol decomposition products [methyl mercury chloride, dimethylmercury and mercury(III) chloride]. The group employed the same C_{18} column and mobile phase as used in the previous study [37] and flow injection was used to measure total mercury levels to confirm that all mercury species were determined by liquid chromatography. Evidence of long-term degradation of thimerosol was demonstrated.

The speciation of platinum compounds in chemotherapy drugs using RPLC–ICP-MS has been demonstrated by Cairns et al. [25]. A novel desolvation interface comprising a membrane drier and Peltier condenser situated between the chromatographic module and the ICP mass spectrometer was described. The desolvation device enabled 100% organic solvents such as methanol and acetonitrile to be used, as well as solvent gradients, with minimal baseline drifts. A new generation drug, JM-216, was shown to completely metabolise in the human body into a number of compounds. The drug was separated from its metabolites using a C_{18} column with a solvent gradient of 95:5 to 30:70 water–acetonitrile in 25 min at a flow-rate of 1 ml min^{-1}. Peak broadening was attributed to the increased dead volume of the desolvation system. Detection limits were 0.6 ng ml^{-1}, representing an actual mass of 120 pg of Pt.

A number of selenide species have been separated by RPLC–ICP-MS using an oscillating capillary nebuliser [29]. Five organoselenium compounds, phenyl-2-aminoethylselenide (PAESe), 4-hydroxyphenyl-2-aminoethylselenide (HO-PAESe), 4-fluorophenyl-2-aminoethylselenide (F-PAESe), phenyl-2-acetamidoethylselenide, *N*-acetylPAESe and (RS)α-methylphenyl-2-aminoethylselenide (RS)-MePAESe were separated using a C_{18} column. Each selenide was shown to have a different response factor. The mobile phase was optimised for organic modifier concentration, pH, ionic buffer concentration and the elution gradient. The absolute limits of detection of the selenide compounds were reported to be in the range 30–400 pg Se and were dependent on the solvent flow-rate.

The various applications of RPLC are listed alphabetically in Table 1 according to sample type. The chromatographic parameters, instruments used for detection and elements analyzed are summarised.

2.3. Ion-pair liquid chromatography

2.3.1. Introduction

A variation of reversed-phase chromatography, known as ion-pair (or paired ion) chromatography (IPC) is one of the most widely employed chromatographic techniques to be interfaced with ICP-MS.

Ion-pair chromatography may be used for the separation and determination of ionic and non-ionic species. The technique may be carried out in either normal-phase or reversed-phase modes, however only the latter mode has been used with ICP-MS detection. The stationary phase in reversed-phase IPC is a standard silanised silica packing such as that used in conventional reversed-phase chromatography, e.g. C_8 or C_{18}. The mobile phase is comprised of an aqueous buffer such as a phosphate or acetate salt, an organic modifier (commonly methanol or acetonitrile) and an ion-pairing reagent. The counter ion of the ion-pairing reagent combines with the analyte in question to form an ion-pair which is then retained by the reversed-phase column. Elution and separation of the analytes is then achieved using the aqueous solution with organic modifier. Commonly used ion-pairing reagents are long chain alkyl anions (such as tetraalkylammonium salts or triethyl C_5–C_8 alkyl ammonium salts) or cations (such as C_5–C_{10} alkylsulphonates). The concentration of ion-pairing reagent used typically varies from 0.001 to 0.005 *M*. In general, increasing the concentration of the counterion in the mobile phase causes an undesirable increase in capacity factor (k') values for reversed-phase IPC. The principle consideration when selecting a counterion for a particular separation is the charge compatibility. The counterion of the ion-pairing reagent should ideally be soluble in the mobile phase, univalent, aprotic and should be non-destructive to the chromatographic system as a whole. In addition, pH changes may affect the hydrophobic interactions governing the separation resulting in significant changes in the chromatogram. It is therefore important to buffer the aqueous phase with respect to both pH and concentration of the counterion to avoid peak tailing or multiple peaks. In

Table 1
Liquid chromatography applications

Sample	Chromatography	Detector	Elements	Limits of detection	Ref.
Blood	Reversed-phase Hypersil SAS C_1 column 68% methanol pH 4.5	VG Plasma Quad PQS 1500 W FP Column connected to cross-flow nebuliser	Co, Fe and Zn	0.1, 5.8 and 4.6 ng, respectively (as porphyrin)	[43]
Blood	TSK G 3000 SEC column with 0.1 *M* Tris HCl buffer	Perkin-Elmer SCIEX ELAN 250 1300 W FP <5 W RP Cross-flow nebuliser, Scott-type spray chamber and Sciex 'long torch'	Pb	0.15 ng ml^{-1}	[111]
Bovine thyroglobulin	Reversed-phase Shiseido C_{18} SG120 10% or 50% methanol–0.1 *M* $(NH_4)_2HPO_4$	1300 W FP <5 W RP for 10% methanol eluent 1700 W FP <5 W RP for 50% methanol eluent Meinhard nebuliser and STDP spray chamber, water-cooled	Iodine (speciation)	35–130 pg as iodine	[41]
Chemotherapy drugs	Reversed-phase In-house PEEK column packed with Hypersil Phenyl 5 μm silica Mobile Phase acetonitrile–water (25:75)	Fisons Plasma Quad 2+ 1500 W FP Membrane drier and Peltier driven condenser used as desolvation device Meinhard Nebuliser and cyclone spray chamber	Pt (speciation)	0.6 ng l^{-1} (120 pg of Pt)	[25]
Chicken tissue	PEP RPC HR reversed-phase column. Mobile phase: 5% methanol:95% 0.01 *M* orthophosphoric acid	VG PlasmaQuad 2 1300 W FP 10 W RP. Concentric glass Meinhard nebuliser. Water-cooled Scott-type spray chamber	As	Limit of quantification 25 ng g^{-1} in solid sample	[78]
Coal fly ash	Wescan Anion/R IC column. 2% propanol eluent and 50 m*M* carbonate buffer used at pH 7.5	VG Plasma Quad 2 with concentric nebuliser and double-pass Scott spray chamber at 5°C 1350 W FP <2W RP	As (speciation), V and Ni	Not reported	[74]
Coastal seawater (CASS-2), lobster hepatopancreas tissue LUTS-1, harbour sediment PACS-1	Whatman cation-exchange column 1400 W FP Thermostatted nebuliser spray chamber used for organic solvent introduction	Perkin-Elmer SCIEX ICP-MS 1200 W FP Glass concentric nebuliser and thermostatted spray chamber	Cr, Ni, Cu, Zn, Mo, Cd, Pb, V, Sr, Hg, Sn	5–12 ng for Sn speciation	[95]
Contact lens solution	Reversed-phase Waters PicoTag C_{18} 0.06 *M* ammonium acetate, 3% acetonitrile and 0.005% (v/v) 2-mercaptoethanol pH 6	VG PlasmaQuad 1.3 kW FP Turbomolecular pumps used instead of original diffusion pumps. Additional ventilation for temperature stability	Hg (speciation)	40 ng ml^{-1} (as thimerosal)	[37]
Cooked cod	Polysphere IC AN-2 column and guard column Mobile phase: 5 m*M* salicylate adjusted to pH 8.5 with TRIS	VG PlasmaQuad 2 Turbo Plus 1350 FP <1 W RP	Se (speciation)	0.008 mg kg^{-1} in dry solid	[81]
Dogfish muscle	C_{18} reversed-phase column with mobile phase 3% (v/v) methanol, 1.5% acetonitrile, 0.1% 2-mercaptoethanol containing 0.06 mol l^{-1} ammonium acetate	Elan 5000 ICP-MS 1200 W FP Ultrasonic nebuliser used with condenser temp. 0°C and desolvation tube temperature 100°C	Hg (speciation)	70–160 pg of Hg	[38]

Table 1. Continued

Sample	Chromatography	Detector	Elements	Limits of detection	Ref.
Dogfish muscle DORM-1	Benson strong anion-exchange column resin. Mobile phase: 1 m*M* K_2SO_4 for 3 min then 50 m*M* K_2SO_4 at pH 10.5	VG Plasma Quad 2 with high solids nebuliser 1500 W FP <10 W RP	As (speciation)	Not reported	[76]
Dogfish muscle DORM-1	Anion-pairing HPLC, anion-exchange HPLC and cation-pairing HPLC techniques all used. Various columns, ion pair reagents, buffers and organic modifiers used	Perkin-Elmer SCIEX ELAN 250 Shorter ICP torch used and an *x*, *y*, *z* translational stage	As (speciation)	50–300 pg of As	[46]
Dogfish muscle DORM-1	Ion-pair chromatography Pierce C_{18} column Mobile phase: 10 m*M* sodium dodecyl sulphate solution, 5% methanol and 2.5% glacial acetic acid	Perkin-Elmer SCIEX ELAN 250 1400 W FP Column directly interfaced with Teflon tubing to nebuliser	As (speciation)	0.3 ng of As	[45]
Drinking water	Dionex AG10 column and 100 m*M* eluent	Fisons PlasmaQuad 2	Br (as bromate)	0.1–0.2 ng ml^{-1}	[90]
Fish and sediment extracts	Hamilton PRP X100 anion-exchange column. Mobile phase: 10 m*M* ammonium dihydrogenphosphate and 10 m*M* diammonium monohydrogenphosphate at pH 6.5–7.5	VG Plasma Quad 2+ 1400 W FP Fassel torch, Meinhard nebuliser and Scott-type spray chamber	As (speciation)	10–30 pg As	[75]
Fly ash (SRM 1633a)	Ion-pair reversed-phase separations. Isocratic separation on Waters PicoTag C_{18} column Mobile phase 0.4 *M* HIBA, 0.02 *M* octanesulphonic acid at pH 3.8	VG Plasma Quad 1300 W FP	Lanthanides	0.4–5.0 ng ml^{-1}	[65]
Fuel (SRM 2715)	Ion-pair chromatography. Nucleosil C_{18} column. Gradient elution from 40 to 90% methanol over 10 min then isocratic elution for 20 min. Sodium pentane sulphonate mobile ion-pair reagent used. pH optimised for separation	VG Plasma Quad 1400 W FP <20 W RP SIM used at *m*/*z* 208	Pb (speciation)	0.14–3.9 ng of Pb	[56]
Harbour water sample	Ion-pair separation Perkin-Elmer C_8 column. Mobile phase 5 m*M* sodium 1-pentanesulphonate, 5% acetic acid and 50% methanol	Perkin-Elmer SCIEX ELAN 5000 1150 FP. Ultrasonic nebuliser used with condenser temp. −10°C and desolvation temp. 80°C	Sn (speciation)	2.8–16 pg Sn	[59]
Human blood	Bio-Sil TSK 250 size-exclusion column with isocratic mobile phase of 25 m*M* Tris buffer at pH 7.7	Perkin-Elmer SCIEX ELAN 250 ICP-MS 1300 W FP <5 W RP Meinhard Nebuliser and Scott-type spray chamber	Au	35 pg Au	[112]
Human serum	Ion pair chromatography Hamilton PRP1 column. Mobile phase: methanol–water (98:2) and 10^{-4} *M* $C_5H_{11}SO_3^-$ pH 4.5	Perkin-Elmer SCIEX ELAN 5000 Cross-flow nebuliser and Ryton double-pass spray chamber 1100 FP. ^{82}Se monitored	Se (organoselenium compounds)	<1 ng ml^{-1} for each species	[54]

(Continued on p. 94)

Table 1. Continued

Sample	Chromatography	Detector	Elements	Limits of detection	Ref.
Metalloprotein	Asahipak GFA-30F size-exclusion guard and analytical column Mobile phase 0.2 *M* $(NH_4)_2SO_4$, 0.05 *M* Tris-HCl and 1 m*M* EDTA	Seiko ICP-MS	Hg, Zn, Cd	Not reported	[102,108–110]
Metalloproteins in biological samples	Spherogel SW 2000 size-exclusion column and guard column Mobile phase 0.06 *M* Tris-HCl, 0.05% NaN_3	VG PlasmaQuad PQ1 1250 W FP Concentric Meinhard nebuliser and cooled double-pass spray chamber Ames torch	Cd, Zn, Ga, Y and Cu	8.75, 46, 18, 0.21 and 378 pg for Cd, Zn, Ga, Y and Cu, respectively	[104]
Methamphetamine	SAM3-125 anion-exchange column and guard column. Mobile phase: 4.4 m*M* sodium carbonate and 1.2 m*M* sodium bicarbonate	Yokogama Model PMS 100 ICP-MS 1500 W FP	Na, Pd, Ba, I and Br	4–60 ng ml^{-1}	[91]
Metalloporphyrins in coal extracts	Ion-pair chromatography. C_{18} reversed-phase column with 15% 1 m*M* tetrabutylammonium dihydrogenphosphate in methanol mobile phase	VG Plasma Quad 2 1750 FP. 25 W RP Ebdon v-groove nebuliser and cooled double-pass spray chamber. Oxygen with nebuliser gas flow	Ga	64 pg s^{-1}	[64]
Natural waters	Ion pair separation Hamilton PRP1 resin based, reversed-phase column. Mobile phase: 0.5 m*M* tetrabutylammonium phosphate (ion-pair reagent), 4 m*M* $Na_2HPO_4 \cdot 2H_2O$, adjusted to pH 9 with dilute ammonia. Methanol added to increase signal sensitivity	VG Elemental Plasma Quad Turbo 2+ 1350 W FP 1 W RP Meinhard concentric glass nebuliser and water-cooled Scott double-pass spray chamber	As (speciation)	1.0–3.0 ng ml^{-1}	[51]
Nuclear fuels	Various cation-exchange columns used for different elemental separations, along with various mobile phases. Isocratic elution in all cases	Perkin-Elmer Elan 5000 ICP-MS Modified glove box used for handling of hazardous substances	Cs, Ba, lanthanides, actinides	0.002–0.100 ng ml^{-1}	[98]
Pig kidney	Superose-12 size-exclusion column Mobile phase 0.12 *M* Tris–HCl at pH 7.5	VG PlasmaQuad	Cd (speciation)	Not reported	[107]
Proteins in human serum	SynChropak GPC 300 size-exclusion column with mobile phase 0.1 *M* Tris-HCl at pH 6.9	Perkin-Elmer Elan 250 with modified short torch 1400 W FP	Na, Cu, Fe, Zn, Pb, Ba and Cd	0.5–3 pg of metal	[32]
Sea water	Dionex MetPac CC-1 column Various eluent compositions investigated. pH 5.5	Perkin-Elmer ELAN 5000 ICP-MS 1050 W FP	Rare earths, Co, Cu, Mn, Ni, Zn, Pb and U	1–50 pg ml^{-1}	[100]
Sea water and human urine	Dowex 1-X8 resin column. Mobile phase: dilute nitric acid eluent (sea water) and acetate eluent (serum)	VG PlasmaQuad 1350 FP <5 W RP. Meinhard-type nebuliser and cooled double-pass spray chamber	As and Se	Not reported	[79]
Seafood samples	Cation-exchange column Ionosphere-C Mobile phase 20 m*M* pyridinium ion adjusted to pH 2.65 with HCO_2H ION 120 anion-exchange column with 100 m*M* NH_4HCO_3 adjusted to pH 10.3 with NH_4OH	Perkin-Elmer Sciex ELAN 5000 ICP-MS MS with cross-flow nebuliser and double-pass spray chamber. 1300 W FP	As	10–50 ng g^{-1} (dry mass)	[94]

Table 1. Continued

Sample	Chromatography	Detector	Elements	Limits of detection	Ref.
Sediment reference material PACS-1	Ion-pair separation Perkin-Elmer C_8 column. Mobile phase 5 m*M* sodium 1-pentanesulphonate, 5% acetic acid and 50% methanol	Perkin-Elmer SCIEX ELAN 5000 1150 FP. Ultrasonic nebuliser used with condenser temp. −10°C and desolvation temp. 80°C	Sn (speciation)	2.8–16 pg Sn	[59]
Soils, percolate water, sewage and human serum	Dowex 1-X8 anion-exchange resin column Mobile phase 0.0014 mol l^{-1} HNO_3	VG PlasmaQuad 2 1350 W FP <5 W RP Meinhard concentric nebuliser and water-cooled double-pass spray chamber	Cl and S	Not reported	[89]
Thimerosal	C_{18} reversed-phase column with mobile phase 3% (v/v) methanol, 1.5% acetonitrile, 0.1% 2-mercaptoethanol containing 0.06 mol l^{-1} ammonium acetate	Perkin-Elmer Elan 5000 ICP-MS 1200 W FP Ultrasonic nebuliser used with condenser temp. 0°C and desolvation tube temperature 100°C	Hg (speciation)	70–160 pg of Hg	[38]
Tuna	Reversed-phase Waters PicoTag C_{18} 0.06 *M* ammonium acetate, 3% acetonitrile and 0.005% (v/v) 2-mercaptoethanol pH 6.8	VG PlasmaQuad 1.3 kW FP Turbomolecular pumps used instead of original diffusion pumps. Additional ventilation for temperature stability	Hg (speciation)	40 ng ml^{-1} (as thimerosal)	[37]
Uranium materials	IonPac CS10 column and guard column used Linear 18 min gradient from 0.04 to 0.265 *M* HIBA used to separate lanthanides 2 *M* HNO_3, 1 *M* HCl and 0.4 *M* HIBA gradient used for actinide separation	Fisons PlasmaQuad 2+ Glove box used for handling and measuring toxic radioactive samples 1350 W FP	Lanthanides and actinides	Not reported	[99]
Urine	Ion-pair chromatography. Hamilton PRP-1 column 3% methanol, 5 m*M* tetrabutylammonium phosphate mobile phase at pH 7.6	Perkin-Elmer ELAN 5000 ICP-MS Cetac U-5000 ultrasonic nebuliser	Se	22–74 pg Se	[52]
Urine	Ion pair chromatography Hamilton PRP1 column. Mobile phase: methanol–water (98:2) and 10^{-4} *M* $C_5H_{11}SO_3^-$ pH 4.5	Perkin-Elmer SCIEX ELAN 5000 Cross-flow nebuliser and Ryton double-pass spray chamber 1100 W FP. ^{82}Se monitored	Se (organoselenium compounds)	<1 ng ml^{-1} for each species	[54]
Urine	Micellar LC separation Alltech RP metal free column and guard column. 0.05 *M* Cetyltrimethyl-ammonium bromide (CTAB) and 10% propanol mobile phase. pH 10	VG Plasma Quad PQ2+. C-1 type concentric nebuliser and double-pass spray chamber cooled to 5°C	As (speciation)	90–300 pg for various species	[68]
Urine	Anion-exchange on a weak anion-exchange column Adsorbosphere-NH_2 column. Mobile phase: 30% ethanol, 15 m*M* $NH_4H_2PO_4$ and 1.5 m*M* CH_3COONH_4 at a pH of 5.75	VG Elemental Plasma Quad. 1500 W FP and <10 W RP. Concentric nebuliser and Scott double-pass spray chamber with cooling jacket	As (speciation)	36–96 pg	[70]

(Continued on p. 96)

Table 1. Continued

Sample	Chromatography	Detector	Elements	Limits of detection	Ref.
Urine	Anion-exchange. Wescan Anion/R IC column and guard column Mobile phase of 5 m*M* phthalic acid at pH 2.7	VG Elemental Plasma Quad. 1350 W FP <5 W RP Concentric nebuliser and cooled double-pass spray chamber	As (speciation)	3.4–7.0 ng ml^{-1} for various species	[71]
Urine (human)	Ion-pair chromatography Cetac C_{18} reversed-phase packing material Various ion-pairing reagents investigated and converted to ammonium salts to avoid clogging of DIN	Perkin-Elmer SCIEX Elan 250 1400 W FP Modified SCIEX short torch and DIN used	Hg and Pb (speciation)	0.2 pg Pb and 7 pg Hg	[31]
Urine (human)	Anion-exchange: ION 120 column with 0.10 *M* NH_4HCO_3 at pH 10.3 with NH_4OH Cation-exchange: Ionosphere-C column with 0.1 *M* pyridinium ion at pH 2.65 with HCOOH	1350 W FP cross-flow nebuliser with sapphire bits. Spray chamber maintained at 20°C	As (speciation)	3–6 ng ml^{-1} for cations and 7–10 ng ml^{-1} for anions	[73]
Urine, freeze dried	Dionex CS5 mixed mode column and Dionex AS11 anion-exchange column Mobile phase: 6 m*M* 2,6-pyridinedicarboxylic acid and 8.6 m*M* LiOH at pH 6.8	VG PlasmaQuad 2 1350 WFP 1 W RP Concentric nebuliser and double-pass spray chamber	Cr (speciation)	3 pg for each species	[82]
Urine, freeze dried		VG PlasmaQuad	Cr, V, Ni	0.042 and 0.017 for Cr (III) at *m*/*z* 52 and 53, respectively 0.055 and 0.022 ng for Cr (VI) 0.031 ng for Ni	[96]
Urine, wine, club soda	Wescan Anion/R IC column Carbonate buffer mobile phase	VG Plasma Quad ICP-MS. 1350 W FP <5 W RP Type C-1 concentric nebuliser and double-pass spray chamber cooled to 5°C	As (speciation)	3–10 ng ml^{-1} for various species	[72]
Vaccines and toxoids	Reversed-phase Waters PicoTag C_{18} column Mobile phase 0.06 *M* ammonium acetate, 3% acetonitrile and 0.005% (v/v) 2-mercaptoethanol pH 5.3	VG PlasmaQuad Spray chamber cooled to 8°C. 60 cm of FEP tubing used to connect column to nebuliser	Hg	Not reported	[44]
Waste water	C_{18} reversed-phase column with mobile phase 3% (v/v) methanol, 1.5% acetonitrile, 0.1% 2-mercaptoethanol containing 0.06 mol l^{-1} ammonium acetate	Perkin-Elmer Elan 5000 ICP-MS 1200 W FP Ultrasonic nebuliser used with condenser temp. 0°C and desolvation tube temperature 100°C	Hg (speciation)	70–160 pg of Hg	[38]
Waste water treatment stream	Nucleosil 120-5 C_{18} column. 4% propanol in water, 10^{-2} *M* H_3PO_4, 0.1 ppm Rh	Perkin-Elmer Sciex ELAN 500 Meinhard-type nebuliser and DSM organic spray chamber	Te (speciation)	Not reported	[40]
Water	Ion-pair chromatography. Nucleosil C_{18} column. Gradient elution from 40 to 90% methanol over 10 min then isocratic elution for 20 min. Sodium pentane	VG Plasma Quad 1400 W FP <20 W RP SIM used at *m*/*z* 208	Pb (speciation)	0.14–3.9 ng of Pb	[56]

Table 1. Continued

Sample	Chromatography	Detector	Elements	Limits of detection	Ref.
	sulphonate mobile ion-pair reagent used. pH optimised for separation				
Water samples	Vydac 201TP C_{18} column. Mobile phase: 2% methanol, 1 m*M* tetrabutylammonium phosphate, 2 m*M* ammonium acetate at pH 5.99	Perkin-Elmer ELAN 5000 1100 FP Cross-flow pneumatic nebuliser and Scott-type spray chamber with cooling system	As (speciation)	11–51 pg ml^{-1}	[50]
Yeast	Ion pair chromatography Hamilton PRP1 column. Mobile phase: methanol–water (98:2) and 10^{-4} *M* $C_5H_{11}SO_3^-$. pH 4.5	Perkin-Elmer SCIEX ELAN 5000 Cross-flow nebuliser and Ryton double-pass spray chamber 1100 FP. ^{82}Se monitored	Se (organoselenium compounds)	<1 ng ml^{-1} for each species	[54]

reversed-phase IPC, maximum k' values are obtained at intermediate pH. At lower pH values a smaller number of ion pairs are formed in the stationary phase and sample compounds elute more quickly, thus improving peak shapes. Selectivity of a separation may be effectively controlled by varying the pH of the mobile phase.

Solvent strengths may be varied by changing the mobile phase polarity, i.e. by varying the relative concentrations of a binary organic modifier. The selectivities of the solutes for an ion-pair chromatographic technique depend on the mobile phase composition and on the organic solvent selected. Generally, as the amount of water in the mobile phase is decreased, the solvent becomes stronger and k' values decrease.

2.3.2. Environmental, clinical and general applications

IPC coupled to ICP-MS has been used by Beauchemin et al. [45,46] for the identification and quantification of arsenic species in a dogfish muscle reference material, DORM-1. A C_{18} column and a mobile phase of 10 m*M* sodium dodecylsulphate solution ion pair reagent, 5% methanol and 2.5% glacial acetic acid was used at pH 2.5 in both studies. It was found that the aspiration of organic solvents into the plasma required a slightly higher RF power to maintain the sensitivity normally acquired using aqueous arsenic solutions. The species identified were monomethylarsenic (MMA), dimethylarsinic acid (DMA), arsenobetaine (AB), arsenocholine (AC), As (III) and As (V). AB was found to be the principal As species in DORM-1 (84% of total). The detection limit reported for AB in the earlier paper [45] using LC–ICP-MS was 300 pg of As which was found to be 25 times better than that determined using ICP-AES detection.

Early studies investigating the feasibility of employing ICP-MS as a detector for HPLC were performed by Thompson and Houk [47]. IPC was successfully used to separate six As and Se species with detection limits approaching 0.1 ng of the element. The ion-pair reagents used were sodium pentanesulphonate (PIC-B5) and tetrabutylammonium phosphate (PIC-A) and methanol was employed as the organic modifier. A C_{18} type column was used. In addition, the same authors analyzed a 15 element mixture using the same IPC system and multiple ion monitoring with similar (0.1 ng) detection limits.

Shibata and Morita [48] reported a preliminary separation of arsenobetaine and cacodylate arsenic compounds using IPC–ICP-MS. An Internal ODS-2 RP column was used for the separation with tetraalkylammonium ion (TRA) as the ion-pairing reagent and a malonic acid buffer.

Arsenic is a monoisotopic element (molecular mass 75) which is known to suffer from an isobaric interference during ICP-MS analyses owing to the presence of $ArCl^+$ if chlorine is introduced to the plasma as a concomitant species. This is particularly apparent for samples such as sea water, serum and urine and the problem has been addressed by Story et al. [49] when analysing ultra trace As concentrations. These workers used hydride generation to reduce this interference (Fig. 3). The element was acidified and then reacted with sodium borohydride to form a

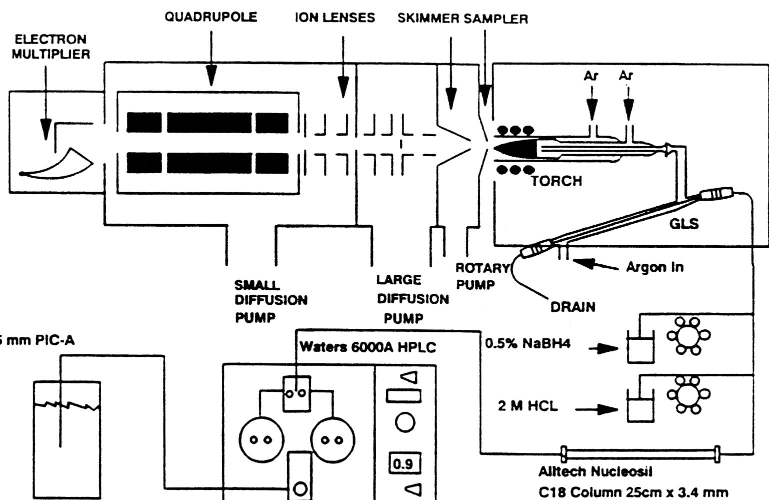

Fig. 3. Schematic diagram of a LC–ICP-MS interface with hydride generation system. Reprinted from Story et al. [49] by permission of Preston Publications, a division of Preston Industries.

volatile hydride which, in turn, was transported to the plasma more efficiently. The authors made use of a polypropylene tube as a gas liquid separator for selective transportation of the hydrides and hydrogen and removal of the argon chloride. PIC-A was used as the ion pairing agent for the reversed-phase chromatographic separation of three As species in an estuarine certified reference sample. Total As concentrations measured using the hydride generation technique fell within the certified precision values whereas values obtained using conventional pneumatic nebulisation did not. The authors concluded that the gas liquid separator eliminated the chloride interference to the point that samples did not require matrix matching.

In a similar study ionic compounds, containing arsenic, in several natural water samples were separated using IPC–HPLC [50]. The compounds separated were As(III), As(VI), dimethylarsonic acid (DMAA) and monomethylarsonic acid (MMAA). The ion-pairing reagent used was PIC-A (1 m*M*) with 2% methanol as the organic modifier and 2 m*M* ammonium acetate at pH 5.99. A post-column hydride generation system was again utilised and optimised using flow-injection analysis. Superior detection limits were obtained when compared to LC–ICP-MS with conventional nebulisation (11–51 $ng\,l^{-1}$). The total amount of arsenic present in the four species agreed with the certified values for the reference water samples analyzed.

Thomas and Sniatecki [51] also performed an analysis of trace amounts of arsenic species in natural waters using hydride generation IPC–ICP-MS. Six arsenic species were determined with detection limits in the range 1.0–3.0 $\mu g\,l^{-1}$ and total arsenic was determined using hydride generation by atomic fluorescence detection. It was found that the predominant species present in bottled mineral water samples was always As(V) with very low levels of As(III). The authors described how the system required "... further work using special chromatographic software ... to improve the quantitative measurement at a natural level."

Three recent papers [52–54] have used IPC–ICP-MS for the speciation of Se in environmental and clinical samples. Yang and Jiang [52] determined selenite, selenate and trimethylselenonium using a 3% methanol, 5 m*M* PIC-A mobile phase at pH 7.6 with ultrasonic nebulisation of the eluent into the ICP. Absolute detection limits for the three species were in the range 22–74 pg Se which corresponds to relative values of 0.11–0.37 $ng\,ml^{-1}$. In the analysis of urine, selenite was found to be the principle selenium species although trimethylselenonium was detected in a number of samples. The formation of several unidentified chromatographic peaks was at-

tributed to the presence of selenoamino acids. Total selenium values calculated agreed well with certified concentrations. Detection limits in the urine samples were slightly higher (0.17, 0.76 and 0.53 ng ml^{-1} for $TMSe^+$, Se(IV) and Se(VI), respectively) and this was attributed to an increase in background noise caused by the injection of the highly ionic urine sample.

The study by Muñoz Olivas et al. [54] has addressed the speciation of organic selenium species, in particular the separation and identification of two selenoamino acids: selenomethionine (SeMet) and selenocystine (SeCys), and the trimethylselenonium ion ($TMeSe^+$) (Fig. 4). The technique used was based on that presented by Jiang and Houk [55] for sulphur amino acid separation. The counter ion used was anionic (PIC-B5 was the ion-pairing reagent) so that cationic species such as $TMSe^+$ were retained on-column, and a methanol–water mixture was used as the mobile phase (pH 4.5). The concentration of mobile phase and ion-pairing reagent, along with the ionic strength and pH were optimised to give the best plasma stability and chromatographic separation. The method was evaluated by measuring the concentration of the various Se species in an enriched yeast sample. The total selenium concentrations (the sum of the concentrations of the various species) agreed well with the total selenium value measured using direct sample introduction into the ICP-MS system. Detection limits were calculated to be 0.20, 0.60 and 0.20 $\mu g\,l^{-1}$ (as Se) for SeCys, SeMet and TMSe, respectively. A certified serum standard and human

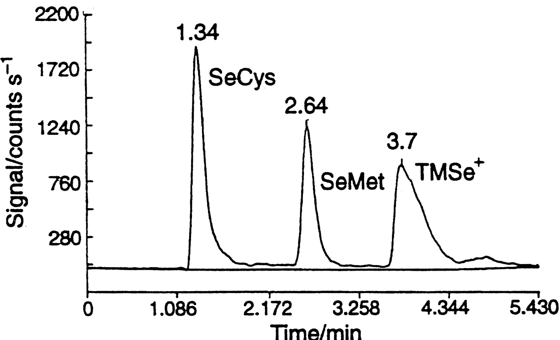

Fig. 4. Chromatogram obtained for separation of SeCys, SeMet, and $TMSe^+$ (100 $\mu g\,l^{-1}$ as Se) under optimum experimental conditions. Reprinted from Munoz Olivas et al. [54] by permission of The Royal Society of Chemistry.

urine sample were also analyzed using the same technique. A single peak was identified for the serum sample and was attributed to be SeCys, however the authors explained that, due to the poor retention of SeCys on column, other species may have been co-eluting and, therefore, further studies were required. As shown by previous workers [52] the chromatograms for the urine samples showed increased noise levels. Again, the main peak was attributed to SeCys. The main limitation of the system was the inability to separate both inorganic and organic species together.

The ability to separate a number of lead compounds by IPC–ICP-MS has received attention in recent years [31,36,56,57]. Al-Rashdan et al. [36] described the separation of inorganic lead (Pb^{2+}) and several trialkyllead species [trimethyllead (TML) chloride, triethyllead (TEL) chloride and triphenyllead (TPL) chloride]. Reversed-phase, ion-pairing and ion-exchange LC modes were compared as well as both ICP-MS and ICP-AES as detection methods. For ion-pairing studies, a C_{18} column with a methanol mobile phase was used. The ion-pairing reagent was sodium pentane sulfonate. In the reversed-phase studies, a C_{18} column was again used with an acetate buffer containing varying percentages of methanol. Cation-exchange LC was used with an acetate buffer containing methanol. For all three modes, organic modifier concentration and pH was optimised. As expected, LC with ICP-MS detection gave detection limits improved by three orders of magnitude when compared to ICP-AES. Isocratic RP-HPLC was the method of choice and was able to yield a separation of Pb^{2+} from TML whereas attempts to perform this separation using IPC were unsuccessful. Strong cation-exchange also did not give an adequate separation. A separation of the two peaks was observed using RP-HPLC but resolution was poor. An obvious answer to improve the resolution would be to use a gradient elution; however, an unstable plasma and high reflected power were obtained so only isocratic elution was feasible.

In a further study [56] the same group used IPC with a C_{18} column, methanol–water mobile phase and PIC-B5 for the separation of inorganic lead (Pb^{2+}), TEL, TPLL and tetraethyllead (TTEL). Detection limits were 0.37, 0.14, 0.17 and 3.9 ng of Pb for the four compounds, respectively. Method

evaluation was performed by calculating the TEL concentration in Standard Reference Material Lead in Fuel and inorganic lead in a water Quality Control sample from the US Environmental Protection Agency. The results obtained experimentally compared well with reference values. It was found that a post-column derivatisation method could probably be used to further increase sensitivity of the method for studies on the environmental fate of alkyllead species in the environment.

Brown et al. [57] described the development of a coupled LC isotope dilution ICP-MS method for lead speciation. Pb^{2+}, TML and TEL were separated using IPC on a C_{18} column using a mobile phase gradient of 10:90 to 30:70 methanol–buffer eluent with 0.1 mol^{-1} sodium acetate, 0.1 mol^{-1} acetic acid and 4 $mmol^{-1}$ PIC-B5 as the ion-pairing reagent. Following on from the work of Al-Rashdan [36] the principal aim of this work was to optimise the separation of Pb^{2+} from TML. The gradient elution employed affected the chromatographic baseline but, with respect to the TML and Pb^{2+} separation, the fluctuations were deemed irrelevant. An artificial rainwater sample was analyzed to assess the method accuracy with encouraging results. A lack of suitable 'customised' software for LC–isotope dilution-ICP-MS was identified as an unresolved problem, however the authors manipulated their data using a graphics package off-line.

Shum et al. [30] investigated lead and mercury speciation using ion-pair microbore column LC–ICP-MS with direct injection nebulisation. Inorganic lead, two trialkyl lead species, inorganic mercury and three organomercury species were separated using an acetonitrile–water (20:80, v/v) mobile phase with 5 m*M* ammonium pentanesulphonate ion-pairing reagent at pH 3.4. Detection limits for all the lead compounds were 0.2 pg of Pb. The detection limits were 7, 18, and 16 pg of Hg for inorganic mercury, $MeHg^{+}$ and $EtHg^{+}$, respectively. Peak areas were used in these calculations. The separation method was evaluated by measuring Pb and Hg species in human urine (NIST SRM 2670 freeze dried urine). Inorganic Pb^{2+} was retained permanently on the column (as the column was not completely end-capped) and was removed by flushing the column with EDTA. Only inorganic lead was found in the urine sample at a concentration of 10.3 $\mu g\,l^{-1}$ Pb which compared well to the certified value (10 $\mu g\,l^{-1}$). Spiked urine samples were used to evaluate the feasibility of the remaining lead compounds using this method. Similarly, no organomercury species were identified in the urine sample and the experimentally measured inorganic mercury concentration (28 $\mu g\,l^{-1}$) compared well with the certified concentration (28 $\mu g\,l^{-1}$). Again a spiked urine sample was analyzed to determine method performance for the organomercury samples. In this instance, the sensitivities for $MeHg^{+}$ and $EtHg^{+}$ were reduced by a factor of two in the urine matrix due to an easily ionisable element interference from Na which is present at a concentration of 1000 $mg\,l^{-1}$. Sensitivity for Hg^{2+} was not affected as the Na^{+} was retained by the anionic pairing reagent and so eluted after Hg^{2+}. Conversely, the tail of the Na^{+} chromatographic peak interfered with the inorganic species.

Suyani et al. [58] compared ICP-MS and ICP-AES as detection methods for organotin speciation and, in addition, evaluated the separation using both cation-exchange and ion-pair HPLC. Detection limits obtained using ICP-MS were, not surprisingly, three orders of magnitude lower than those measured using ICP-AES for trimethyltin chloride (TMT-Cl), tributyltin chloride (TBT-Cl) and triphenyltin acetate (TPhT-Ac). The linear dynamic ranges were three orders of magnitude for cation-exchange and two orders of magnitude for ion-pair HPLC, with the exception being TPhT-Ac due to poor resolution. When the two chromatographic modes were compared it was observed that ion-exchange chromatography provided better resolution but the separation time was longer. Conversely, ion-pair chromatography gave a shorter analysis time but poorer resolution.

Yang et al. [59] also studied the speciation of tin compounds using IPC–ICP-MS. Inorganic tin, trimethyltin (TMT), triethyltin (TET), tripropyltin (TPT), tributyltin (TBT) and triphenyltin (TPhT) were separated in less than 6 min using a C_8 column and a 50% methanol mobile phase with 5% acetic acid and 5 m*M* PIC-B5 added. Calculated detection limits were in the range 2.8–16 pg Sn for the various species. Various tin species in harbour sediment reference sample PACS-1 (prepared using a tropolone–benzene extraction) and in a harbour water sample were analyzed. TBT was the principal

species in the sediment sample with Sn(IV) and an unidentified species also present. The concentration of Sn as TBT obtained experimentally agreed with the certified value. TPhT and TBT were identified in the harbour water sample and recoveries approached 100% for TPT, TPhT and TBT.

Kumar et al. [60] described the effect of inorganic tin chloride on the separation of trimethyl-, tributyl- and triphenyltin chlorides using IPC–ICP-MS. Two columns, a PRP-1 and a silica-based column were investigated. For the latter, inorganic tin was held on-column and did not affect the separation unless the concentration exceeded 1 $\mu g\, g^{-1}$ inorganic tin chloride. There was less retention of inorganic tin on the PRP-1 column. A good separation between inorganic and organotin compounds was achieved at pH 6 using the PRP-1 column. Detection limits were 1.6 pg, 1.5 pg and 2.3 pg as Sn for TMT, TBT and TPhT, respectively.

The same group also used supercritical fluid extraction (SFE) to extract TBT and TPhT from biological samples in about 15 min [61]. IPC was subsequently used to separate the compounds in the extracts. Extraction temperature, pressure and modifier were optimised. The amount of sample used was reduced to 0.14 g using SFE as compared to 2.5–5.0 g. Low recoveries, however, were obtained, indicating the need for procedural modifications. Further work by Vela et al. [62] described the optimisation of cartridge size, modifier type and restrictor temperature for SFE before analysis of the same organotin compounds by IPC–ICP-MS. This analysis indicated that the extracted species varied with the type of modifier employed. Lower extraction efficiencies were obtained with 'real samples' indicating that modifications to the extraction procedure were required for further work.

Limited work has been performed in the area of chromium speciation using IPC–ICP-MS which is surprising as the element is widely distributed in the environment owing to its use in industrial applications. Jakubowski et al. [26] used IPC–ICP-MS with hydraulic high pressure nebulisation for the separation of Cr(III) and Cr(VI). A 5 μm Eurospher 100-C_{18} column was used with a mobile phase of 25% methanol, ammonium acetate and tetrabutylammonium acetate as the ion-pairing reagent. Carbon interferences, caused by the use of organic solvents, were reduced upon oxygen addition to the nebuliser gas flow and by the use of desolvation. Detection limits were 0.6 and 1.8 $ng\, ml^{-1}$ Cr for the two species, respectively, which were better than those obtained using ICP-AES detection and when nebulisers such as the DIN and USN were used.

Jiang and Houk [55] investigated the separation of anionic compounds of phosphorus and sulphur using ion-pair chromatography with ICP-MS detection. For the separation of inorganic phosphates a PRP-1 divinylbenzene co-polymer column was used with 0.005 *M* triethylammonium nitrate as the ion-pairing reagent. MeOH (2%) was used as the organic modifier at pH 6. Orthophosphate $(PO_4)^{3-}$, pyrophosphate $(P_2O_7)^{4-}$ and tripolyphosphate $(P_3O_{10})^{5-}$ were successfully separated with detection limits of 0.4, 0.6 and 1 ng P, respectively. These detection limits were superior by a factor of 200–2000 compared to the best LC–ICP-AES results reported in the literature for the same compounds. For the analysis of adenosine phosphates, the same column was used but with 0.01 *M* triethylammonium bromide as the ion-pairing reagent. The nucleotides AMP, ADP, ATP and cyclic 2,3-AMP were separated successfully although resolution was not ideal for the separation of ADP and ATP. Detection limits ranged from 0.8–4.0 ng P which, again are superior to detection limits reported using ICP-AES. This chromatographic system was also employed for the separation of sulphur containing amino acids, this time with 1% acetonitrile as an organic modifier at pH 7.5. Cysteine and methionine were successfully separated with estimated detection limits of 150 $ng\, ml^{-1}$ sulphur. Finally, inorganic sulphates were separated using a silica-based C_{18} column with 0.005 *M* PIC-A and 5% methanol at pH 7.1. It was found that sulphate and sulphite could not be separated under any conditions and the authors suggested that this could be due to sulphite converting to sulphate during the chromatographic process.

Heumann et al. [63] used isotope dilution mass spectrometry as an element specific detector for the determination of iodide and iodate in mineral water using an IPC system and a species specific spiking method. A known quantity of a ^{129}I enriched iodide and iodate spike was mixed with the sample before injection into the chromatograph. Detection limits were stated to be in the $pg\, ml^{-1}$ range although

detection limits for the specific analytes were not reported.

IPC–ICP-MS has been used by Pretorius et al. [64] for the determination of gallium porphyrins in coal extracts. As certified reference materials with accurate metalloporphyrin concentrations are not available, the technique was compared to HPLC with UV–Vis detection. Compounds were separated on a Novapak C18 column using 1 m*M* PIC-A as an ion-pair reagent and 15% methanol. The technique was applied successfully to the analysis of gallium porphyrin distributions in three coal extracts. The estimated detection limit was 64 pg s^{-1}, although peaks were rather broad.

A study carried out by Braverman [65] involved the use of IPC–ICP-MS for the separation of 14 rare earth elements. An isocratic separation on a C_{18} column was performed with a mobile phase of 0.14 *M* 2-hydroxy-2-methylpropanoic acid (HIBA) and 0.02 *M* octanesulphonic acid at pH 3.8. Detection limits were in the range 0.4–5.0 ng ml^{-1} for the elements. The technique was used for the analysis of NIST Fly Ash. All elements except for Er, Tm, Yb and Lu exhibited good recovery. The author suggested the use of isotope dilution or an improved clean up separation stage in the analysis to improve upon this.

Zhao et al. [66] used IPC–ICP-MS with platinum specific detection to determine cisplatin (an anti-tumour drug) and its possible metabolites in the human body. The reaction products of cisplatin with various proteins and cisplatin hydrolysis products were separated using a ODS C_{18} column and 1-heptanesulphonate negatively charged ion-pairing agent. In order to retain the thiol containing Pt complexes, the pH was adjusted to 2.6. The authors concluded that, compared to conventional detection techniques, the use of ICP-MS as a detector is more efficient in terms of selectivity and sensitivity with respect to Pt species. Using heptanesulphonate, all Pt complexes were resolved and the method was applied to the determination of cisplatin and its metabolites in urine and blood samples.

The various applications of IPC listed alphabetically in Table 1 according to sample type. The chromatographic parameters, instruments used for detection and elements analyzed are summarised.

2.4. Micellar liquid chromatography

2.4.1. Introduction

Another variation on reversed-phase and ion-pair chromatography is micellar liquid chromatography (MLC) where the counter ion is a relatively high concentration of a surfactant (detergent). The counter ion in MLC possesses a long-chain hydrocarbon ‘tail’. Formation of micelles occurs in aqueous solutions when the concentration of these counter ions exceeds what is known as the ‘critical micelle concentration’. Around 40–100 ions aggregate, forming spherical particles with the hydrophobic tail directed towards the centre and the hydrophillic head directed towards the outside so they are in contact with water molecules. In this way a ‘second phase’ is created and uncharged species may be solubilised as micelles. If a sample containing compounds of varying polarity is introduced into such a system, the compounds will partition between the aqueous and hydrophobic phases and, thus, a separation may be achieved. Using such a system, both ionic and non-ionic compounds may be separated.

2.4.2. Applications

Suyani et al. [67] used micellar liquid chromatography for the speciation of alkyltin compounds [TMT-Cl, triethyltin-chloride (TET-Cl) and tripropyltin-chloride (TPT-Cl)] using a micellar mobile phase of 0.1 *M* sodium dodecylsulphate (SDS) and a C_{18} Spherisorb column. The detection limits obtained were 27, 51 and 111 pg (Sn), respectively. For the separation of monoethyltin chloride (MET-Cl), dimethyltin chloride (DMT-Cl) and TMT-Cl the concentration of the mobile phase was increased to 0.02 *M* SDS. In this instance the detection limits were 46, 26 and 126 pg (Sn), respectively. SDS is a negatively charged surfactant. The use of a positively charged or nonionic micelle mobile phase results in the elution of compounds in the void volume or irreversible absorption onto the stationary phase due to low electrostatic interactions. The authors suggested that the concentration of the surfactant should not exceed 0.1 *M* in order to avoid clogging of the torch and sampling cone orifice in the ICP-MS.

Ding et al. [68] employed micellar LC for arsenic speciation using ICP-MS detection. Dimethylarsenic

acid (DMA), monomethylarsonic acid (MMA), As(III) and As(V) were separated using a mobile phase of 0.05 *M* cetrimide (cetyltrimethylammonium bromide) as the micelle forming agent, 10% propanol and 0.02 *M* borate buffer (Fig. 5). Cetrimide was used as it exhibited favourable electrostatic interactions between the solutes and micelles. Again, the k' values decreased with increased micelle forming agent, which favoured the chromatography, but care was taken not to introduce excessive amounts of salt that would result in sample cone clogging. The detection limits for the four compounds ranged from 90–300 pg (As) and R.S.Ds. were all below 5%. The dynamic range was linear to three orders of magnitude for each of the species. Urine samples (so-called 'dirty samples') were easily analyzed with little back pressure increase and the total arsenic concentration (0.52±0.02 ppm) agreed favorably with the certified value (0.48±0.10 ppm).

The various applications of micellar liquid chromatography are listed alphabetically in Table 1 according to sample type. The chromatographic parameters, instruments used for detection and elements analyzed are summarised.

2.5. Ion-exchange chromatography

2.5.1. Introduction

Processes involving ion-exchange chromatography (IEC) are based upon the utilisation of exchange equilibria between charged solute ions and oppositely charged ions on the surface of a stationary phase. Solute ions and ions of equivalent charge in the mobile phase compete for the counter ion on the stationary phase and the extent of this competition determines the relative retention of the ions. A commonly used strong cation-exchange resin contains the sulphonic acid group $-SO_3H$ and a typically weak cation-exchange resin contains the carboxylic acid group $-COOH$. Anion-exchangers commonly use quaternary or primary amine groups.

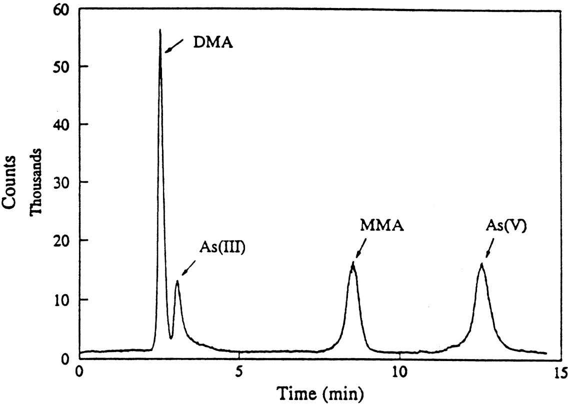

Fig. 5. Chromatogram of mixture of four arsenic standards using micellar liquid chromatography. Reprinted from Ding et al. [68] by permission of Elsevier Science.

Ion-exchange packing materials are traditionally formed from the emulsion copolymerisation of styrene and divinylbenzene, the latter polymer is used to provide cross linking and thus increase the rigidity of the beads. Ionic functional groups are chemically bonded to this backbone. Pellicular silica-based packing materials may also be used which are then coated with a synthetic ion-exchange resin but these tend to have comparatively less sample capacity.

Ion-exchange chromatography may be used to separate ionic species at a particular pH. Ion-exchange packings may also be used to separate charged species from uncharged species. Factors, other than simple coulombic interactions, may influence retention in IEC. For instance, when organic ions are injected into an IEC, hydrophobic interactions between the nonionic carbon backbone of the stationary phase and the sample cause organic ions to be retained in a manner characteristic of reversed-phase chromatography. The sample may then diffuse to the charged region of the support where an ionic interaction may occur.

The retention of a sample by a column and the resultant column efficiency is principally dependent on the rate of diffusion of the analyte through the column. The diffusion rate is dependent upon both the size and porosity of the resin beads and the viscosity of the eluent. The mean free path of an analyte through a column is increased when the resin particle diameter is reduced and also if the stationary phase resin is more porous. The combination of these diffusion mechanisms is the rate determining step in IEC.

The resolution of an ion-exchange separation may

be adjusted by optimising the ionic strength, pH, buffer concentration, organic modifier concentration, temperature and liquid flow-rate. Resolution may be improved by increasing the counter ion concentration (and thus the ionic strength) of the mobile phase so that there is more competition between the sample and counterions for the exchangeable ionic centres. The selectivity of the separation may be greatly influenced by small variations in the pH of the mobile phase. A pH change will affect the ionisation of the sample and the equilibria between the analyte buffer and stationary phase. Both ionic strength and pH gradients may be used to optimise a separation.

An increase in the column temperature may result in increased column efficiency and large changes in separation selectivity due to improved solute diffusion and mass transfer. Column efficiency may also be achieved using low flow-rates. The pH of the mobile phase for ion-exchange chromatography is most effectively controlled by the use of a buffer. The type and concentration of the buffer are of importance since the ionic strength, which determines the relative retention, affects the competition between the analyte and mobile phase ions. An organic modifier at a concentration of less than 10% may also influence the selectivity of the separation by affecting the mechanism controlling the hydrophobic interaction of the solutes with the matrix. Owing to the number of papers published in the area of IEC, anion-exchange and cation-exchange chromatography will be discussed separately. The various applications of ion-exchange liquid chromatography are listed alphabetically in Table 1 according to sample type. The chromatographic parameters, instruments used for detection and elements analyzed are summarised.

2.5.2. Applications of anion-exchange chromatography

Anion-exchange chromatography has been used extensively for arsenic speciation studies. Arsenic exists in many forms which vary in toxicity according to the following order arsenite (most toxic)>arsenate>monomethylarsonic acid (MMA>dimethylarsinic acid (DMA)>arsenobetaine (least toxic). Branch et al. [69] described preliminary results for the speciation of these five arsenic species using a column packed with Benson 7–10 μm anion-exchange resin with a 50 m*M* K_2SO_4 mobile phase at pH 10.5. The sulphate concentration was not detrimental to the cones and the plasma was found to be stable for up to 4 h.

A number of workers have investigated the speciation of As in urine using this technique [70–73]. Heitkemper et al. [70] separated As(III), As(V), DMA and MMA using a weak custom made anion-exchange column with Adsorbosphere-NH_2 packing. A guard column dry packed with a pellicular amino packing was used along with a presaturation column. Initial studies using ICP-AES as a detector used a buffered mobile phase of 30% methanol, 50 m*M* ammonium dihydrogen phosphate and 5 m*M* ammonium acetate was used with good results. However, when ICP-MS was used as the detector, clogging of the sampling cone and rapid erosion occurred due to the high buffer concentration. A mobile phase of 30% methanol, 15 m*M* ammonium dihydrogen phosphate and 2 m*M* ammonium carbonate at pH 5.75 was finally used. In order to achieve a satisfactory separation of MMA and As(V), a flow-rate change was programmed. Absolute detection limits ranged from 20 to 91 pg As in aqueous media. The method was subsequently applied for the analysis of urine. Standard additions were used to determine the four species in two freeze dried urine standards. Detection limits in the real samples were found to be slightly higher (36–96 pg). The R.S.Ds. for each species was found to be less than 10%. Chloride interference ($^{40}Ar^{35}Cl$) resulted in an interfering peak which complicated the determination of As (III).

In a subsequent paper [71], the same research group discussed overcoming the ArCl interference which was detrimental in the former study. A Wescan anion-exchange column was used with a mobile phase of aqueous phthalic acid. The authors explained that the concentration of sodium chloride in urine is about 0.15 *M* and that normal levels of As are in the region of 100 ppb. Thus, chloride concentrations are five orders of magnitude higher than any As species and the potential $AsCl^+$ interference may be substantial. The anion-exchange column was used to chromatographically separate Cl^- from arsenic. A high dilution of the urine samples was used so that column overloading did not occur. Drawbacks to this procedure include an increase in

detection limits (3.4–7 ppb) due to the dilution factor and an incomplete separation between DMA and MMA.

The next study by this group [72] extended the chloride removal technique for the separation of four As species (As(III), As(V), DMA and MMA) (Fig. 6). A Wescan Anion IC column was again employed with a 2% propanol, 50 m*M* carbonate mobile phase at pH 7.5. Urine samples and spiked beverage samples were analyzed. The authors investigated the potential of using a He–Ar mixed gas plasma to reduce detection limits. This alternative ionisation source improved detection limits but also intensified the $ArCl^+$ interfering signal. Chromatographic methods were again used to eliminate the argon chloride interference. It was found that arsenite oxidised to arsenate in urine samples at high concentrations, but this problem was overcome by diluting one part urine with four parts water. The ClO^+ ion was monitored and it was found that the chloride eluted 100 s after the last analyte peak. Cation-exchange chromatography was also used in this study to separate four arsenic cationic species: arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AsC) and tetramethylarsonium ion (TMAs). An ionosphere column was used in this instance with a pyridinium ion (0.1 *M*) mobile phase. Detection

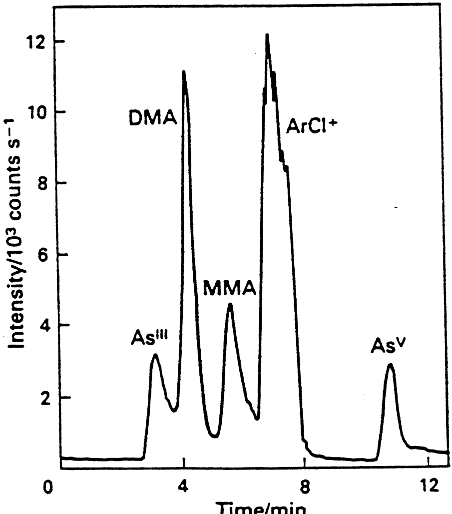

Fig. 6. Separation of four As species and chloride by ion chromatography with ICP-MS detection. Reprinted from Sheppard et al. [72].

limits for the anions in the urine sample were 7–10 ng ml^{-1} and 3–6 ng ml^{-1} for the cations with the recovery of all arsenic species in urine approaching 100%.

Wang et al. [74] reported the conversion of As(III) to As (V), especially at low pH, during the speciation of arsenic in coal fly ash. The authors suggested that oxidation may be attributed to harsh sample preparation techniques or by co-existing high oxidation state elements in the solution extraction procedures. Oxidation was not attributed to the presence of atmospheric oxygen from air entrainment.

Demesmay et al. [75] used anion-exchange chromatography, again for speciation of six arsenic species. Three anionic species (DMA, MMA and As (V)), nonionic As (III), a cationic species (AC) and a zwitterion (AB) were separated using a 2% acetonitrile mobile phase and a Hamilton PRP X100 anion-exchange column at pH 6.5. Detection limits ranged from 10 to 30 pg As and both R.S.Ds. and linearity were good. The method was extended for the analysis of As species in a fish extract. No significant interference effects were noted for the presence of chloride in the fish sample and this was attributed to the organic solvent having a masking effect.

Six different fish samples were analyzed for As species by Branch et al. [76]. Five species were separated [AB, DMA, As(III), MMA and As(V)] using a Benson strong anion-exchange resin. The predominant arsenic compound was found to be AB which is non-toxic. Arsenic levels were in the range 1.0 mg kg^{-1} dry mass in mackerel to 187 mg kg^{-1} dry mass in plaice. Nitrogen addition was used to remove $ArCl^+$ interference at m/z 75.

Chen et al. [77] studied metabolites of dimethylarsinic acid in the urine of rats exposed to DMA in drinking water. Anion-exchange chromatography was used to monitor the increased concentrations of arsenite, DMA, trimethylarsine oxide and an unidentified compound in the urine. Results showed that DMA was demethylated to inorganic As which is achieved by the action of intestinal bacteria.

Analysis of the growth promoter 4-hydroxy-3-nitrophenylarsonic acid (roxarsone) has been investigated by Dean et al. [78]. Tissue, sampled from chickens with a roxarsone supplemented diet was digested using a trypsin enzymolysis technique. Anion-exchange chromatography was used to per-

form bulk matrix separation and three anion-exchange columns were used in series. The anionic roxarsone was retained whereas, at the particular pH, non-ionic and cationic species had no affinity for the columns. The roxarsone concentration was then determined by reversed-phase HPLC with a 5% methanol mobile phase and 0.01 *M* orthophosphoric acid. The limit of quantification was 25 ng of roxarsone per gram of muscle tissue. The authors found that no roxarsone was detected in muscle tissue from chickens fed with the growth promoter following a seven day withdrawal period.

Goossens et al. [79] described an anion-exchange separation method for the determination of As and Se with ICP-MS detection. A Dowex-IX8 anion-exchange column was used to separate As and Se in nitrate form from Cl^-. The As and Se species were eluted with 0.03 mol l^{-1} HNO_3 whereas Cl^- was retained. In this way the $ArCl^+$ interference was eliminated. The method was applied to the determination of As and Se in sea-water, human serum and human urine samples. For the latter sample, dilute acetic acid was used instead of nitric acid as the eluent. As and Se results compared well with certified values whereas, for the human serum sample, experimental concentrations did not fall within the certified range.

Shum and Houk [32] described the use of a direct injection nebuliser (DIN) for use with packed microcolumns for anion-exchange chromatography coupled to ICP-MS. A 5 μm anion-exchange resin with a mobile phase of 5 m*M* NH_4HCO_3 and 5 m*M* $(NH_4)_2CO_3$ was used for the separation of SeO_3^{2-} [Se(IV)] and SeO_4^{2-} [Se(VI)]. Calculated detection limits were 14 and 15 pg of Se for Se(IV) and Se(VI), respectively. The isotopes ^{74}Se and ^{78}Se were monitored during the chromatographic separation. The detection limits were an order of magnitude better than those obtained using a traditional pneumatic nebuliser. Isotope ratios were calculated using the area under the chromatographic peak for each isotope. The R.S.Ds. obtained improved when the amount of sample injected was increased.

The application of anion-exchange chromatography for the speciation of Se has been well documented in the literature. Cai et al. [80] performed on-line preconcentration of Se(IV) and Se(VI) in aqueous matrices followed by IPC or anion-exchange chromatography and ICP-MS detection. It was found that detection limits and background levels were superior for the anion-exchange chromatography compared to IPC. A preconcentration column was coupled on-line with the anion-exchange system and used in the analysis of spiked water matrices. Detection limits obtained using the preconcentration method were in the range 0.16–0.42 and 0.08–0.19 ng ml^{-1} of Se for selenite and selenate, respectively. These detection limits were improved by two orders of magnitude when compared to those obtained without the preconcentration step.

Crews et al. [81] investigated the speciation of Se in in vitro gastrointestinal extracts of cooked cod using anion-exchange HPLC–ICP-MS. Four Se standards were measured, selenomethionine, selenocystine, sodium selenite and sodium selenate, along with a gastrointestinal extract. A Polysphere IC AN-2 column was used with a 5 m*M* salicylate mobile phase at pH 8.5. The ^{82}Se isotope was used throughout the study. 12% of total Se measured in the cooked cod was found to be in the form of selenite. The remaining Se did not correspond to any of the Se standards measured but was believed to be an organoselenium compound.

Chromium speciation has been the subject of several recent chromatographic studies where ICP-MS was used as a detector. Chromium(III) is an essential metal that must be incorporated into the diet to aid in the metabolism of insulin and other biological systems. Conversely, chromium(VI) is highly toxic in the human body and has the ability to traverse biological membranes. Exposure to Cr(VI) may result in dermal reaction. The speciation of chromium, is, therefore, essential to gauge if toxic or nontoxic forms of the metal are prevalent in a sample.

Zoorob et al. [82] used a direct injection nebuliser and anion-exchange LC–ICP-MS for chromium speciation (Fig. 7). A Dionex ASII microbore column was used with a mobile phase of 6 m*M* 2,6-pyridine dicarboxylic acid (PDCA) and 8.6 m*M* lithium hydroxide at pH 6.8. Chromium(III) was pre-complexed with PDCA by warming for 2 h at 65°C until the colour changed from pink to purple indicating formation of $Cr(PDCA)_2$. Chromium(VI) did not require complexing. Detection limits were calculated to be 3 pg Cr. The technique was success-

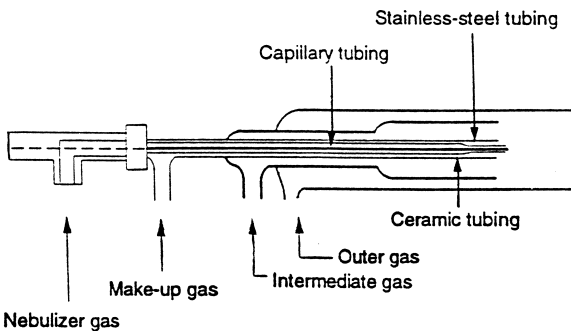

Fig. 7. Schematic diagram of a direct injection nebulizer. Reprinted from Zoorob et al. [82] with permission of the Royal Society of Chemistry.

fully used for the analysis of chromium species in a freeze dried urine certified reference material.

In another study, Byrdy et al. [83] also performed chromium speciation by anion-exchange HPLC using ICP-AES detection for preliminary studies and ICP-MS for final work. Again Cr(III) and Cr(VI) were separated, this time using an EDTA chelation procedure to stabilise Cr(III), an ammonium sulphate-ammonium hydroxide mobile phase and an IonPac AS7 mixed mode column and guard column. A high SO^+ polyatomic interference was observed, therefore the more abundant m/z 53 isotope was monitored instead of the m/z 52 isotope. This served to reduce the mobile phase background, however it should be noted that m/z 52 may be monitored to avoid possible chloride interference at m/z 53. The absolute detection limits were 40 pg for Cr(III) and 100 pg for Cr(VI) in aqueous standards, based on peak height calculations. The linear dynamic range extended to approximately three orders of magnitude for both species. A certified water sample reference material was analyzed for the presence of Cr(III) and the species was detected at the 20 $\mu g\ l^{-1}$ level. The use of gradient elution to reduce the duration of chromatographic runs was identified as a region for future studies.

Inoue et al. [84] also used EDTA chelation to stabilize Cr(III) prior to separation by anion-exchange chromatography and detection by ICP-MS at m/z 52 and 53. No $ArCl^+$ and ClO^+ interferences were observed and detection limits were $8.1 \cdot 10^{-5}$ and $8.8 \cdot 10^{-5}$ $\mu g\ ml^{-1}$ of Cr for Cr(III) and Cr(VI), respectively, with a linear dynamic range of four orders of magnitude.

Pantsar-Kallio and Manninen [85] optimised a unique coupled cation- and anion-exchange chromatographic system for Cr speciation. The need for Cr(III) species conversion by oxidation was eliminated by the use of dilute nitric acid eluents. Detection limits using the system were 0.3 $\mu g\ l^{-1}$ for Cr(III) and 0.8 $\mu g\ l^{-1}$ for Cr(VI) in lake water samples.

Roehl and Alforque [86] developed a method for the determination of hexavalent chromium in aqueous samples by isocratic anion-exchange-HPLC. A Dionex AS4A anion-exchange column was used with a 6 mM $(NH_4)_2SO_4$ mobile phase at pH 9. Detection limits and linear dynamic ranges compared well to an existing method of detection comprising a post-column reactor and colorimetric diphenylcarbohydrazide complex. Arar et al. [87] also determined the isotopic composition of hexavalent chromium in wastewater sludge incinerator emission.

Ding et al. [88] used anion-exchange chromatography–ICP-MS to determine different forms of chromium in chromium picolinate products which are used as dietary supplements and appear to assist in weight loss. A Dionex AS7 anion-exchange column was used to separate Cr(III)–EDTA chelate and Cr(VI) in the supplements. Only 1% total chromium recoveries were obtained and this was attributed to retention of the chromium species on-column. The use of RP-HPLC proved to be more effective and complete chromium recoveries were obtained, based on the amounts stated on the manufacturer's product labels.

Tomlinson and Caruso [28] also performed the speciation of Cr(III) and (VI) using a Dionex AS-11 anion-exchange microbore column and 6 mM 2,6-PDCA–8.6 mM lithium hydroxide mobile phase. A thermospray source was used as the interface between LC and ICP-MS. Absolute limits of detection were at the pg level for both species using this instrument assembly.

Goossens and Dams [89] developed a method to separate chlorine and sulphur interferents from V, Cr, Cu, Zn, As and Se in various samples using a Dionex-1 anion-exchange column and dilute nitric acid eluent. The analytes were collected in the eluent whereas Cl^-, ClO_4^-, SO_4^{2-} and SO_3^{2-} interferents

were retained on the column in the NO_3^- form. This procedure illustrates a method for the simultaneous separation of cations and anions from S and Cl.

The determination of bromate in drinking water by anion-exchange LC–ICP-MS was described by Creed et al. [90]. Bromate is a carcinogenic agent and is found in drinking water owing to the oxidation of bromide by ozone. A Dionex AG10 column and 100 m*M* NaOH mobile phase was found to be suitable for the determination of bromate in a 1000 ppm chloride matrix. In addition, sulphate and nitrate, which are the principle anions found in drinking water, did not interfere with ICP-MS detection. Excellent recoveries were obtained in the presence of high concentrations of chloride, nitrate and sulphate with R.S.Ds. of less than 6%. Preconcentration of samples was attempted and yielded detection limits in the 0.1–0.2 $\mu g\, l^{-1}$ range. With an ultrasonic nebuliser the detection limit was 50 $ng\, l^{-1}$ for bromate but the precision of samples was degraded due to an adjacent peak interference.

A species specific spiking method has been demonstrated by Heumann et al. [63] for the determination of iodide and iodate in mineral water using anion-exchange ion chromatography. An exact known quantity of an ^{129}I enriched iodide and iodate spike was mixed with the sample before separation. Iodate concentrations were determined in the range of 0.5–20 $ng\, ml^{-1}$ and for iodide in the range 0.1–5 $ng\, ml^{-1}$. For the analysis of two mineral water samples, the sum of the two inorganic iodine species was comparable to the total iodine concentrations. In a third sample, a third peak was identified and attributed to an organo-iodine species.

A number of inorganic impurities was identified in the analysis of methamphetamine by anion-exchange–ICP-MS [91]. A SAM3-125 anion-exchange column was used with a 4.4 m*M* sodium carbonate and 1.2 m*M* sodium bicarbonate mobile phase. The 21 elements (including metals and non-metals) were identified and separated. The authors identified the potential of this technique for the discrimination of various methamphetamines by inorganic elements in forensic studies.

Jiang et al. [92] separated metal oxide ions of titanium, molybdenum and uranium from singly charged metal species. Diatomic oxide ions, MO^+ cause overlap interferences in ICP-MS at m/z 16–18 units above M^+ and are particularly problematic for elements such as tungsten and the rare earth elements which have a high tendency to form these species in plasmas. The authors used an anion-exchange column to retain interfering molybdenum, titanium and uranium complexes while copper, zinc and cadmium were eluted rapidly. The complexes were readily washed from the column. This technique may potentially be used to separate various other interfering elements such as Sn, W, Hf or Zr.

Anion-exchange chromatography with ICP-MS detection has been used for the determination of gold drug metabolites and related metals in human blood [93]. Gold-based drugs are often used in the treatment of rheumatoid arthritis but metabolism of the drugs in the human body and their action upon the disease is not completely understood. A weak anion-exchange column was used with a mobile phase linear gradient starting with 20 m*M* aqueous Tris buffer at pH 6.5. Human blood serum was analyzed for Au, Zn and Cu. Three Zn species, two Au species and at least four Cu containing species were identified. The chromatographic retention times were not consistent from patient to patient, and, therefore identification of the species was difficult.

2.5.3. Applications of cation-exchange chromatography

Arsenic speciation was perfomed in a rigorous study by Larsen et al. [94] for the analysis of several seafood sample extracts. Several arsenicals were detected with an Ionosphere cation-exchange column using the trimethylselenonium ion as an internal chromatographic standard and a mobile phase of 20 m*M* pyridinium ion, adjusted to pH 2.65 with acetic acid. The cationic compounds AsC, TMA, trimethylarsine oxide, two unknown cationic substances and inorganic As were detected and quantified at low levels. The concentration of each species (as arsenic) relative to total arsenic was 19–98% (arsenobetaine), 0–0.6% (arsenocholine and trimethylarsonium ion) in the seafood samples. The first unknown cationic species was present at 3.1–18% in shell fish and lobster and the second was present at 0.2–6.4% in all the samples. The concentration of arsenite and arsenate was 0–1.4%, dimethylarsinate constituted 8.2–29% whereas 0.3% monomethylarsonate was detected in oyster tissue. It

was concluded that the concentration of toxic arsenic species in the samples was low, and, therefore, tolerable in the human diet according to levels set by the World Health Organisation.

Suyani et al. [58] employed ICP-MS as a detector for the speciation of organotin compounds by both cation-exchange and IPC. TMT-Cl, TBT-Cl and TPhT-Cl were separated using a Spherisorb ODS-2 C_{18} column for IPC and an Adsorbosphere SCX column for cation-exchange LC studies. It was found that ion-exchange chromatography gave a linear dynamic range of over three orders of magnitude while IPC was two orders of magnitude due to poor resolution.

In the work of McLaren et al. [95] butyltin species in the certified reference harbour sediment, PACS-1 were separated by cation-exchange. TBT and DBT species were speciated with a Whatman SCX column and a step gradient elution of 0.3 *M* ammonium acetate in 60:40 methanol:water in which the pH was altered from 6 to 3 after 1 min elution. The limits of detection reported for TBT and DBT in sediment samples were 5 ng g^{-1} and 12 ng g^{-1}, respectively.

Tomlinson, Wang and Caruso [96] used an Alltech Adsorbosphere cation-exchange column for the speciation of vanadium (IV) and (V) with ICP-MS detection. Single ion monitoring at *m/z* 51 was used and the mobile phase consisted of 7 m*M* 2,6-pyridine carboxylic acid and 9.6 m*M* lithium hydroxide at pH 4.0. Absolute detection limits were 0.024 ng and 0.114 ng for vanadium(V) and (IV), respectively. Other metals, nickel(II), chromium(III) and chromium(VI) were separated using the same column but with a slight modification to the mobile phase. Detection limits for chromium(III) at *m/z* 52 and 53, respectively, were 0.042 and 0.017 ng whereas for chromium(VI) the detection limits were 0.055 and 0.022 ng for the same masses. The limit of detection for nickel was 0.031 ng.

Trace amounts of rare earth elements that exist as impurities in other materials have also been analyzed using cation-exchange HPLC [97]. Ion chromatography was used to separate 14 rare earth elements and to eliminate interferences from polyatomic ions upon direct introduction of the eluent into the HPLC system (Fig. 8). The detection limits of the elements were in the range 1–5 pg ml^{-1} in solution and ng g^{-1} in the solid. The linear range extended to six orders of magnitude from 10 pg ml^{-1} to 10 μg ml^{-1}. R.S.Ds. were also favourable (±1% for Lu).

Fission products and actinides in spent nuclear fuels have also been analyzed using cation-exchange LC [98]. Chromatography was essential in order to separate fission Cs from Ba for the analysis of the lanthanides and to eliminate isobaric interferences in the separation of the actinides. Separation of fission

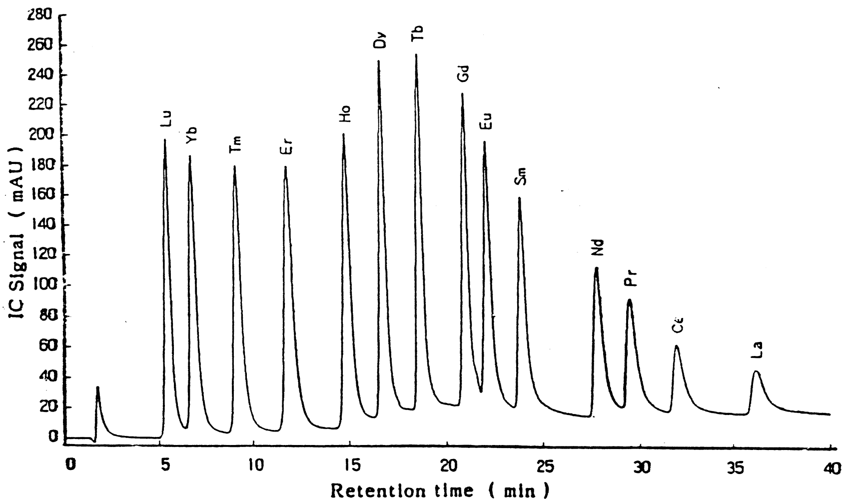

Fig. 8. Chromatograms of rare earth elements using ion chromatography, each 5 μg ml^{-1}. Reprinted from Kawabata et al. [97] by permission of the American Chemical Society.

Cs and Ba was achieved using a Dionex CS3 column with 1 *M* nitric acid as an eluent in about 10 min. The sample, which contained fission Cs of a different isotopic composition to natural Cs, was an acidified leachate from spent nuclear fuel. The separation of the lanthanides was achieved using 0.1 *M* oxalic acid in 0.19 *M* LiOH with a Dionex CS5 mixed bed column. The lanthanides were found to elute in order of increasing atomic number. Röllin et al. [99] recently published a study for the determination of fission product isotopes in irradiated nuclear fuels using the same technique. High U and Pu concentrations in such samples are known to suppress the signals of trace elements and this study offers a method for the measurement of Nd, when high concentrations of U and Pu are present and, similarly, a method of eliminating isobaric overlaps by separating U, Am and Pu. An IonPac CS10 analytical column was used for these separations with 1 *M* HCl as an eluent. Separation of the lanthanides was achieved using an 18 min gradient from 0.04 to 0.26 *M* HIBA. The method proved to yield a very reliable and efficient separation, comparable to standard techniques for the calculation of the burn-up of a nuclear fuel.

Cation-exchange chromatography has been used to determine 20 elements including the rare earths and Co, Cu, Mn, Ni, Zn, Pb and U with ICP-MS at ultra-trace levels [100]. Multielement standards were used for calibration of the analytical system in order to increase sample throughput. The detection limits for the elements were in the range 1–50 pg ml^{-1}.

2.6. Size-exclusion chromatography

2.6.1. Introduction

Size-exclusion chromatography (SEC) is a separation method where the retention of a solute depends on molecular size. In addition, the retention of a particular compound may be controlled by molecular interactions between the solute and the mobile and stationary phases. If the solute has an equivalent affinity for both phases, then the selectivity and retention of the system will only be dependent upon the physical characteristics of the stationary phase, such as pore size distribution. If the chromatographic system is suitably calibrated it also may be possible to identify the approximate molecular masses of the particular components being separated.

In SEC, smaller molecules are able to sample a larger effective pore volume compared to bigger solute molecules. Larger particles of higher molecular mass therefore spend relatively less time in the stationary phase pores and so are eluted before the smaller particles. Solvent molecules are normally the smallest molecules in the eluent system and are thus eluted last at a retention time known as the 'dead-time', t_0. This is in contrast to the liquid chromatographic methods described previously where the components are eluted after the t_0 peak.

Size-exclusion chromatography has a number of advantages over other LC methods. First, samples of unknown molecular mass are known to elute before t_o and so the end of a chromatographic run may be predicted. Second, the retention time of an unknown compound is predictable in a calibrated chromatographic system. Third, SEC is a 'gentle' method of chromatographic separation and does not normally result in sample analyte loss or on-column reactions.

Conversely, SEC has a number of disadvantages associated with the technique. The separation is determined upon molecular size; therefore, the whole separation must be eluted within the excluded volume and the dead volume. To obtain an adequate separation, the peaks must be sufficiently narrow as the column exhibits limited peak capacity. For a complex multicomponent system, complete resolution of the peaks is normally not achieved. A further disadvantage is that the method is only applicable to certain samples that are not of similar size and do not absorb onto the column packing materials.

SEC has been used frequently to separate and identify biological macromolecules using hydrophillic column packings and aqueous mobile phases. In addition, hydrophillic packings and organic mobile phases have been used to separate small organic molecules and to obtain the molecular mass distribution of some polymers.

2.6.2. Applications of size-exclusion chromatography

A number of studies investigating the metal content of metalloproteins have been performed using SEC–ICP-MS [32,101–108]. Dean et al. [103] used a Superose-12 'prep grade' SEC column and

Tris HCl mobile phase to separate metallothionein and ferritin in horse kidney in an attempt to determine the cadmium content in the two metalloproteins. In a following paper, the same group used SEC for the speciation of Cd in retail pig kidney samples [107]. Three discrete peaks were observed in uncooked kidney, and their molecular masses calculated. Samples of cooked kidney and a simulated gastric digestion of the cooked kidney were also analyzed, and it was found that the majority of soluble cadmium in retail pig kidney is associated with a metallothionein-like protein which survives cooking and simulated in vitro gastrointestinal digestion.

Mason et al. [104] used SEC for the analysis of metallothionein protein standards of known elemental composition for Cd, Zn and Cu. Zn was displaced from the protein molecule during chromatography and substituted with Cu. Full recovery of Cd was obtained. The technique was used for the analysis of the metal content of cytosolic metal binding proteins from the polychaete worm *Neanthes arenaceodentata*. Cu, Zn and Cd were determined, using EDTA complexation and a Tris-HCl mobile phase, at levels of 42.178 μg Cd, 4.523 μg Zn and 2.368 μg Cu per mg of protein. The same group [106] also used directly coupled SEC–ICP-MS for the quantitative analysis of environmentally induced perturbations in cytoplasmic distributions of metals in *Neanthes arenaceodentata*. Specific binding patterns in the marine organisms were identified and it was concluded that ligands in metallothioneins preferentially bind Cu>Cd>Zn.

Owen et al. [101] described a preliminary study of metals in proteins using SEC–ICP-MS where multielement and multi-isotope determinations were made using time resolved software. A mixture of known proteins was separated on a Superose 12 column and the distribution of associated elements was measured. Isotope retention times were found to be reproducible.

Three papers by Takatera and Watanabe [102,109,110] have described studies for the analysis of Zn(II), Cd(II), Cu(II) and Hg(II), in induced metallothionein compounds of the metals, found in cyanobacterium. Temperature and light conditions were optimised for metallothionein biosynthesis. Valuable information regarding the preferred incorporation of the metals and interactions during metallothionein synthesis was obtained using the SEC–ICP-MS system. The same authors [108] used the technique to determine sulphhydryl groups (SHs) in chicken ovalbumin (OVA) after the conversion into mercaptides using organomercury compounds. SEC–ICP-MS may be used to separate excess organic mercurial reagents that are not consumed in the reaction. Five commercially available organic mercurial reagents were compared regarding their reactivity towards SHs in OVA.

SEC may yield significant information regarding protein bound metal distributions in blood plasma and serum samples. Shum and Houk [32] described the separation of proteins in human serum, without the need for sample pretreatment, using a direct injection nebuliser. Pb, Cd, Cu, Fe and Zn metalloproteins were separated using a SynChropak SEC column and 0.1 *M* Tris–HCl mobile phase at pH 6.9. Six metal binding molecular mass fragments were observed of viscosity 15 000–650 000. Possible proteins responsible for these molecular mass fractions were postulated, e.g. ceruloplasmin at 130 kDa, however some are still unidentified. Detection limits for the metals in the metalloproteins ranged from 0.5 to 3 pg of metal and are superior by two orders of magnitude to previous values obtained by ICP-MS.

Lyon and Fell [105] used ^{63}Cu:^{65}Cu isotope ratio measurements to measure blood plasma and serum by ICP-MS. Polyatomic species such as $ArNa^{+}$ and PO_2^{+} were found to 'swamp' the Cu measurements. As copper is principally bound to proteins in blood, SEC was used to overcome the interferences.

Gercken and Barnes [111] also used the technique for the determination of lead and other trace element species in human blood. Lead was found in at least 3 molecular mass fractions, the major fraction being coincident with the copper signal at M_r 140 000. This fraction was attributed to ceruloplasmin. Analysis of rat serum samples was also performed and both iron and zinc concentrations were quantified. The detection limit for lead in the protein fractions was 0.15 $\mu g\,l^{-1}$ with a precision of ±10%.

A study attempting to separate Au, Zn and Cu bound immunoglobulins in blood plasma was investigated by Matz et al. [112]. No conditions could be found using SEC–ICP-MS where the metals were released from the column in a single run. Resolution

was poor and low MW fragments were retained on the column. Anion-exchange studies were more effective.

Rottmann and Heumann [113] used SEC–isotope dilution-ICP-MS to determine the interactions of different molecular mass fractions of dissolved organic matter with Cu and Mo in a water sample. Three Mo species, which interacted with the humic substances, were identified and were of high molecular mass. Two species of Cu were detected and were found to interact with the low-molecular mass fraction. Total elemental levels were found to agree well with the sum of the concentrations of the speciated fractions, emphasising the reliability of the method.

The various applications of size-exclusion chromatography are listed alphabetically in Table 1 according to sample type. The chromatographic parameters, instruments used for detection and elements analyzed are summarised.

3. Gas chromatography

3.1. Introduction

The technique of gas chromatography (GC) involves vaporisation of a sample, injected onto a GC column which is then eluted by a gaseous mobile phase. This mobile phase does not interact with the sample analyte—its only function is to transport the analyte through the column. There are two common types of gas chromatography: gas-liquid chromatography and gas-solid chromatography, depending on the physical state of the mobile phase. Gas-solid chromatography is seldom used and the studies reported in this review all employ the technique of gas-liquid chromatography, where the analyte is partitioned between the mobile phase (gas) and a liquid phase which is retained onto a finely divided inert solid support such as a diatomaceous earth. The liquid phase should ideally possess a low volatility (so that it does not volatilise with the analyte), be thermally stable, be chemically inert and have solvent characteristics such that k' is favorable. The mobile phase gas must be chemically inert so that no interaction occurs with the analyte. Common gases included, helium, argon, nitrogen and carbon dioxide and the choice of gas is principally determined by the detection system used. For an argon ICP, argon would be used as the mobile phase.

The sample is injected via a microsyringe through a rubber septum into a flash vaporiser port situated at the top of the GC column. This vaporiser port is maintained at a temperature high enough to quickly vaporise the sample, normally 50°C higher than the boiling point of the least volatile analyte in the sample to be separated.

There are two basic types of GC column commonly used in coupled techniques: the packed and capillary column (otherwise known as an open tubular column). The latter type is becoming more popular and several studies will be described where such columns have been utilised. Columns are usually between 2 and 50 m in length and are coiled for practical insertion into the GC oven where the temperature is controlled. Temperatures commonly used should be just above the boiling point of the sample so that elution of the analytes occurs in a reasonable time whereas in LC separations may be optimised by using gradient elution. In GC, temperature gradients may be programmed to improve chromatographic resolution.

Earlier reviews concerning GC–ICP-MS [2,4] reported the limited use of GC–ICP-MS as an analytical tool due to the limited sample applications associated with the technique. The number of studies published recently, however, indicates that the technique is gaining popularity, probably due to the fact that speciation information is now often required when analysing samples.

There are a number of advantages associated with the use of GC–ICP-MS for the separation of volatile species and these are summarised in an excellent paper by Peters and Beauchemin [114]. Due to the gaseous state of the sample being introduced into the plasma, there is approximately 100% sample transport efficiency from the GC to the plasma. This results in low detection limits and excellent analytical recoveries. The analyte is also more efficiently ionised in the plasma as it is already in the vapour form thus requiring no desolvation and vaporization upon aspiration into the ICP. Due to the absence of an aqueous mobile phase, GC suffers less isobaric interferences than LC. Water and organic solvents in LC also increase the load on the plasma and as solvents are physically separated before the analyte

reaches the plasma. Buffers containing a high salt content are also not required in GC; therefore, erosion of the sampler and skimmer cones is not as prevalent.

3.2. Interface designs and GC–ICP-MS applications

One of the first reported couplings of GC–ICP-MS was by Van Loon et al. [115], who used a coupled system for the speciation of organotin compounds. A Perkin-Elmer Sciex Elan quadrupole mass filter instrument was used as the detector with 1250 or 1500 W forward power. The GC system comprised a Chromasorb column with 8 ml min^{-1} Ar/2 ml min^{-1} O_2 carrier gas flow with an oven temperature of 250°C. The interface comprised a stainless-steel transfer line (0.8 m long) which connected from the GC column to the base of the ICP torch. The transfer line was heated to 250°C. Oxygen gas was injected at the midpoint of the transfer line to prevent carbon deposits in the ICP torch and on the sampler cone. Carbon deposits were found to contain tin and thus proved detrimental to analytical recoveries. Detection limits were in the range 6–16 ng Sn compared to 0.1 ng obtained by ETAAS, but the authors identified areas for future improvements in detection limits and scope of the coupled system.

The next reported use of GC coupling with ICP-MS was by Chong and Houk [116]. A special interface oven was used which involved connecting the end of a packed GC column to the innermost tube of the ICP torch with glass lined stainless-steel tubing. The thermal conductivity and inert surface of this tubing prevented adsorption of analytes and condensation. The authors found that a stable plasma was obtained if the tip of the stainless-steel tubing was placed 2 cm below the tip of the injector tube (positioning of the tube higher into the load coil caused arcing). Volatile organic compounds were analyzed and elemental ratios of C, N, Cl, Br, S, B and Si were calculated. Detection limits for the various elements were in the range 0.001–400 ng s^{-1} with the best detection limits reported for elements with relatively low ionisation energy.

Two papers by Kim et al. [117,118] described the use of a capillary column for the speciation of organometallic compounds. The first paper [117] described the construction of a capillary GC–ICP-MS heated transfer line. The central cone of the transfer line was made of an aluminium rod with a longitudinal slot for column insertion. The transfer line was grounded and the temperature monitored using four thermocouples. The length was kept to a minimum to limit dead volume. Helium was used as the carrier gas for capillary GC and argon was added as a make-up gas for efficient sample transport into the plasma. Again, the distance of the capillary column from the tip of the injector was important, along with alignment of the torch and transfer line. The quantitative analysis of five alkyllead compounds in a complex naptha hydrocarbon mixture was described (Fig. 9). The detection limit was 0.7 pg s^{-1} (measured at 50 pg) and the authors stated that the method was applicable for the analysis of more involatile organometallic compounds.

In the second paper [118] tin, iron and nickel organometallic compounds were separated using aluminium-clad high temperature columns coated with 0.1 μm films of HT-5. Various column lengths and temperature gradients were used to separate the species. Helium was used as the carrier gas. The same GC–ICP-MS set up was used as in the previous paper with slight modification of the transfer line for the analysis of nickel diethyldithiocar-

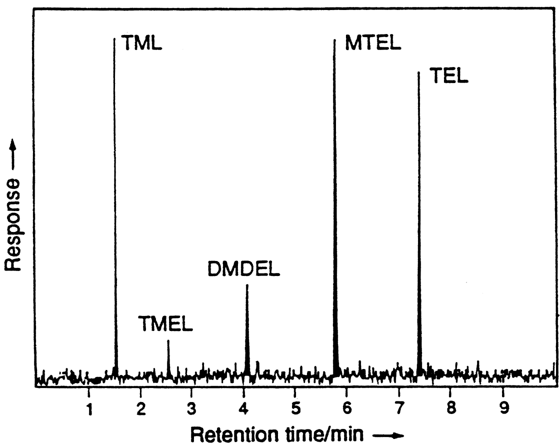

Fig. 9. GC–ICP-MS chromatogram (^{208}Pb) of naptha sample containing five alkyllead components: TML (tetramethyllead), TMEL (trimethylethyllead), DMDEL (dimethyldiethyllead), MTEL (methyltriethyllead) and TEL (tetraethyllead). Reprinted from Kim et al. [117] by permission of The Royal Society of Chemistry.

bamate ($Nidt_2$). Separation of six organotin chlorides was achieved with detection limits in the range 3–6.5 pg s^{-1} and retention times of 4.4–12.4 min. The method was applied to the analysis of a harbour sediment sample. Ferrocene gave a detection limit of 3.0 pg s^{-1} in hexane (mean retention time 7.75 min) and $Nidt_2$ a detection limit of 6.5 pg s^{-1} (mean retention time 10.45 min).

Peters and Beauchemin [114] described the use of a novel design interface for GC detection which allowed the analysis of nebulised solutions using a 'zero dead-volume' switching T-valve. The valve could be rotated to permit standard aerosol introduction into the plasma. The transfer line was constructed from glass-lined stainless-steel tubing which was heated with heating tape. A 'sheathing' (make-up) gas was again used and introduced tangentially into the device. Ion lenses were optimised using solution nebulisation. This interface was used to separate dichloromethane, 1,1,1-trichloroethane and trichloroethylene while detecting the $^{35}Cl^+$ ion. Detection limits of 2.6, 2.2 and 2.6 ng were obtained for the three compounds, respectively. The performance of the interface for the direct aspiration of aqueous solutions was also assessed and degradation of detection limits for most elements was observed.

In a further paper [119], the authors stated that the interface constricted aerosol flow from the spray chamber to the plasma torch resulting in condensation in the sheathing device, reduced aerosol flow to the torch and ultimately poor sensitivity. A new sheathing device was constructed with an enlarged inner diameter which reduced the aerosol constriction and improved sensitivity. The central channel was also extended to overcome 'pooling' of water and sputtering. Detection limits for 1,1,1-trichloroethane and trichloroethylene were much improved compared to the previous paper.

Pretorius et al. [39,120] described the development of a high temperature GC–ICP-MS interface for the analysis of geoporphyrins. The first paper described a modification of the system used by Kim et al. [117,118]. The retention index (I) of the established system for most retained analytes was approximately 3400 (due to cooling effects of the argon nebuliser gas flow) whereas metalloporphyrins have typical I values in the region of 5000. This established the need for a modified interface. The main requirement for improved Is demands heating of the argon which is difficult owing to its low heat capacity. An argon heater was constructed from nichrome wire inside a silica tube which was heated to 'red-heat' with the argon gas passing through the heater. Dead volume was kept to a minimum where possible. This GC interface with ICP-MS proved to be a selective and sensitive method for profiling metalloporphyrins in geological samples. In the next paper by the group [39] a second interface was described which gave similar chromatographs and detection limits but eliminated potential 'cold spots' by uniformly heating the entire interface.

The use of GC–ICP-MS with a 'purge-and-trap' method for the analysis of methylmercury species in sediment samples has been described by Hintelmann et al. [121]. The ICP-MS detector was used to measure specific isotopes in methylmercury compounds after GC separation. Detection limits were 1 pg (as Hg) or 0.02 ng g^{-1} Hg in dry sediment with an R.S.D. of 4%. Accuracy of the method was verified by analysis of a certified reference material harbour sediment.

In a recent paper by Prange and Jantzen [122], the development of a GC–ICP-MS interface was described for the determination of organometallic species. A quartz transfer line, heated to 240°C was used to direct a capillary column to a short distance in front of the argon plasma. Helium was used as the carrier gas in the gas chromatograph, and a temperature gradient was used for the separation of organotin, lead and mercury compounds. Limits of detection for tetraethyllead, diethylmercury and tetrabutyltin were 100, 120 and 50 fg, respectively, which were at least 3 orders of magnitude superior to previously reported detection limits. This highlights the excellent applicability of GC–ICP-MS for the analysis of volatile organometallic compounds. Van Loorn et al. [115] used a short stainless-steel tube interface in a similar study.

De Smaele et al. [123] described the coupling of GC and ICP-MS via a commercial transfer line and a custom-made transfer line (Fig. 10). The former consisted of a stainless-steel tube, a teflon tube and a fused-silica capillary tube — the teflon served to prevent capillary breakage. A variable voltage supply was employed to heat the stainless-steel tube and the

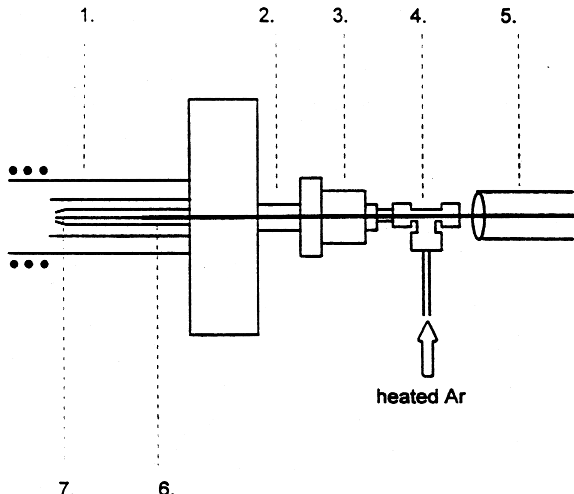

Fig. 10. Scheme of coupling of the GC with the ICP-MS instrument: 1, torch; 2, injector supply; 3, PTFE piece+PTFE Swagelok adapter; 4, Swagelok T-joint; 5, commercial transfer line; 6, stainless-steel transfer tube; 7, transfer capillary. Reprinted from De Smaele et al. [123] by permission of Elsevier Science.

temperature monitored using a built-in thermocouple. The end of the transfer line was situated 5 cm from the torch injector tip to prevent arcing. Again, a heated Ar tube was used to heat the Ar make-up gas which was introduced via a T-joint at the base of the torch. The use of this T-joint caused peak broadening due to solvent condensation from insufficient heating, therefore a custom made transfer line was constructed using a miniaturised T-joint design where heating was more isothermal. In addition, the initial transfer line was of ‘stiff’ construction and the capillary was prone to breakage. The transfer line was bent over 360° to obtain flexibility for easy coupling of the GC and ICP-MS. In a further paper by the same workers [124], the ‘flexible’ interface was used for the analysis of alkyltin compounds. This time Xe was used as a make-up gas. A 30 m capillary column was used for the separation of several organotin compounds. Results were not reported for a certified reference material therefore no indication of the accuracy of the method could be ascertained. Detection limits were very low and in the 15–35 fg range.

A recent paper by Pritzl et al. [125] described a convenient interface for capillary GC–ICP-MS which could be set up in under a minute. The transfer line was, again, a stainless-steel capillary tube, thermostated between 20–350°C and connected to the torch injector. To enable easy coupling, the GC oven was placed on a guide way sledge. The performance of the system was evaluated using various phosphorus, arsenic and tin species along with a certified marine sediment sample. Speciation of three organotin species gave detection limits in the range 0.1–1.0 pg.

The various applications of GC–ICP-MS are shown in Table 2. Samples are listed alphabetically according to sample type and it can be seen that the technique may be utilised for the analysis of a range of environmental and geological samples.

4. Supercritical fluid chromatography

4.1. Introduction

A supercritical fluid exists when a substance is heated above its critical temperature and pressure and is unable to be condensed to a liquid by pressure alone. A typical supercritical fluid is carbon dioxide, which, at temperatures above 31°C and pressures above 73 atm, exists in a supercritical fluid state where individual molecules of the compound are held by less restrictive intermolecular forces and molecular movement resembles that of a gas (1 atm=101 325 Pa).

Supercritical fluid chromatography (SFC) has gained increasing popularity in recent years as an alternative to liquid chromatography and gas chromatography. SFC is faster than LC due to the lower viscosity of the mobile phase and high diffusion coefficients of the analytes. It also yields chromatographic peaks which have less band broadening when compared to GC. Many LC techniques require the use of organic solvents and have relatively long on-column residence times which are improved by the use of SFC. In addition, compounds which are traditionally difficult to separate by GC such as thermally labile, non-volatile and high-molecular-mass compounds may be separated with relative ease.

SFC has been performed using instruments similar in design to those used with HPLC with provision for pressure control, the primary variable. Gradient

Table 2
Gas chromatography applications

Sample	Chromatography	Detector	Elements	Limits of detection	Reference
Fuel	Aluminum clad high-temp. column with siloxane carborane stationary phase (25 m×0.32 mm I.D.) Temperature program: 40°C–180°C Helium carrier gas, 6 ml min^{-1} at 150°C	VG Plasma Quad 2. 1500 W FP <5 W RP In-house transfer line constructed	Pb (as alkyllead compounds)	0.7 pg s^{-1} for various species	[118]
Geo-porphyrins	High-temperature gas chromatography. HT-5 aluminum clad fused-silica column (12 m×0.32 mm I.D.) or DB-1 HT polyimide-coated fused-silica (15 m×0.32 mm I.D)	VG Plasma Quad 2 1500 W FP <5 W RP Stainless-steel transfer line	Co, Cr, Fe, Ni, Ti, V, Zn (metallo-porphyrins)	Not reported	[39]
Harbour sediment	25 m×0.32 mm I.D. aluminium-clad high-temperature column Helium carrier gas, 2 ml at 200°C Temperature gradient (40–320°C)	VG Plasma Quad 2 1500 W FP <5 W RP Stainless-steel transfer line at 190–337°C (depending on compound)	Sn (organotin compounds) Fe (ferrocene) Ni ($Nidt_2$)	3.0–6.0 pg s^{-1} (retention time 4.4–12.4 min) for organotin compounds 3.0 pg s^{-1} (ret. time 7.75 min) for ferrocene 6.5 pg s^{-1} (ret. time 10.4 min) for $Nidt_2$	[87]
Harbour water	RSL–150 capillary column (30 m×0.25 mm I.D.) Gradient temperature program used H_2 carrier gas at 30 p.s.i. inlet pressure[a]	Perkin-Elmer Elan 5000 ICP-MS 1300 W FP Heated transfer line at 250°C	Sn (as organotin compounds)	15–35 fg	[124]
Metallo-porphyrins	Aluminium clad high-temp. column (10 m×0.32 mm) Helium carrier gas, 3 cm min^{-1} Temperature gradient program used	VG Plasma Quad 2 1500 W FP <5 W RP Stainless-steel transfer line at 190–337°C (depending on compound)	V, Mn, Fe, Ni, Cu, Zn (as metalloporphyrins)	0.10–0.55 ng on-column for various species	[109]
Sediment (PACS–1)	DB1701 quartz capillary column (30 m×0.32 mm I.D.). 80–280°C, 30°C min^{-1} He carrier gas used	Perkin-Elmer Elan 5000 ICP-MS 1000 W FP Heated transfer line at 240°C	Sn (as organotin compounds)	50–120 fg absolute for various species	[122]
Sediments	J&W 22 m DB5 0.25 mm×0.25 μm column used	PE Sciex Elan 5000 1200 W FP	P, As, Sn, Sb, Sn (as organo compounds)	0.1–1.0 pg for organotin compounds	[125]
Sediments	OV-3 on carbowax column (40×0.4 cm) at 105°C. Ramp heating to 200°C	PTFE transfer line Perkin-Elmer Elan 5000 ICP-MS 1200 W FP	Hg (organomercury speciation)	1 pg (as Hg) absolute	[121]

[a] 1 p.s.i.=6894.76 Pa.

elution may be achieved by adjusting the pressure in SFC as well as using mobile phase gradients and temperature gradients to achieve an optimum separation with good k' values and resolution. Both packed and open tubular columns may be used, although the latter generally lead to better column efficiency. Columns are longer than those used with LC (10–20 m length, 50–100 μm diameter) and are commonly made from fused-silica with chemically bonded polysiloxane coatings. The most commonly used mobile phase for SFC is carbon dioxide, although ethane, pentane, dichlorodifluoromethane, diethylether and tetrahydrofuran have also been employed.

Most preliminary SFC-plasma coupled techniques employed microwave-induced plasmas (MIPs), however the use of ICP-MS is now increasing in popularity. Carey and Caruso [126] have discussed in detail the use of plasma spectrometric detection for SFC. Flame ionization detection has been traditionally used with SFC, however ICP-MS detection offers improved sensitivity and is element selective,

so speciation information may be easily obtained. Detection is performed after the decompression zone of the system. At this point the supercritical fluid changes state to a gas. A restrictor, a length of fused-silica tubing (30–120 cm long) with a porous frit end, is connected to the end of the SFC column to maintain a linear mobile phase velocity so that significant band broadening does not occur [20].

Carey and Caruso [126] also summarised the two main approaches to interfacing the SFC restrictor with the ICP torch. The first method, used with packed SFC columns, introduces the restrictor into a heated cross-flow nebuliser and the nebulised sample is subsequently swept into the torch by the nebuliser gas flow. Where capillary SFC systems are used, a second interface design is commonly employed where the restrictor is directly introduced into the central channel of the torch. This interface is more widely used with SFC–ICP-MS coupling [20]. The restrictor is passed through a heated transfer line which connects the SFC oven with the ICP torch. The restrictor is positioned so that it is flush with the inner tube of the ICP torch. This position may, however, be optimised to yield improved resolution. The connection between the transfer line and the torch connection must be heated to prevent freezing of the mobile phase eluent after decompression when exiting the restrictor. A make-up gas flow is introduced to transport the analyte to the plasma. This flow-rate is normally similar to traditional nebuliser gas flow-rates. The make-up gas flow-rate and temperature must be optimised for improved analytical sensitivity and resolution.

The introduction of the SFC mobile phase into the plasma has the effect of reducing sensitivity due to quenching of the plasma, rather like the effect of introducing organic solvents with LC. Polar modifiers, however, do not have a serious deleterious effect on the plasma which enables the polarity of the mobile phase to be changed with no significant loss of sensitivity or resolution. This enables the analysis of compounds which are too polar for adequate separation with pure CO_2 as the mobile phase [126]. It should be noted that the use of CO_2 may cause background interference from $^{12}C^+$, $^{12}C^{16}O_2^+$ and $^{40}Ar^{12}C^+$ [20].

4.2. Applications

The first instance of SFC coupled to ICP-MS was reported by Shen et al. [127] for the speciation of tetraalkyltin compounds. Liquid CO_2 was used as the mobile phase and the SFC column was completely inserted through the transfer line and connected to a frit restrictor (Fig. 11). The restrictor was heated to approximately 200°C by a copper tube inserted into the ICP torch. Tetramethyltin (TMT), tetrabutyltin (TBT), tetraphenyltin (TPT), tributyltin acetate

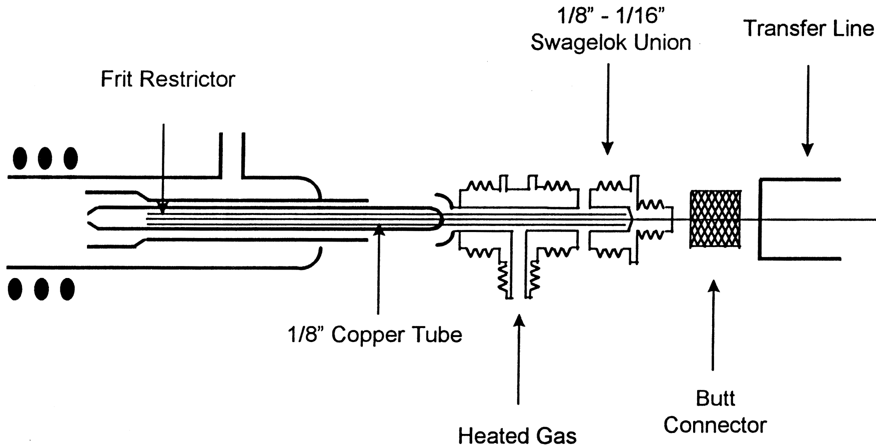

Fig. 11. SFC–ICP-MS interface. Reprinted from Shen et al. [127] by permission of the American Chemical Society.

(TBTA) and dibutyltin diacetate (DBTDA) were separated. CO_2 was introduced as the auxiliary flow to the ICP torch. The Sn signal was found to decrease with increasing CO_2 flow and increase with increasing forward power and so was optimised to give an optimum signal with minimal carbon deposition on the sampler cone. Only TBT and TPT could be separated, despite pressure gradients being applied, due to insufficient interaction with the stationary phase (Fig. 12). Detection limits were 0.034 pg TBT and 0.047 pg TPT. The addition of an organic modifier to increase solvent polarity, increasing the solvent strength, use of an alternative stationary phase and a longer column were postulated as potential areas for future studies.

A further study by Vela and Caruso [128] evaluated the effects of interface temperature, oven temperature, CO_2 pressure, mobile phase composition and column length in order to optimise the separation of several tetra and tri organotin compounds. The same interface, described by Shen [127], was used. It was found that the introduction of CO_2 did not require nebuliser flow-rate and RF power optimisation if the ion lenses were tuned sufficiently. The addition of a polar solvent to the non-polar mobile phase did not yield any improvement in resolution. Longer columns were found to yield broader chromatographic peaks. Absolute detection limits for TBT, tributyltin chloride, triphenyltin chloride and TPT were in the range 0.20–0.80 pg Sn.

The same workers [129] compared flame ionisation and ICP-MS as methods of detection for organometallic compounds separated by capillary SFC. TBT, tributyltin chloride, triphenyltin chloride and TPT were separated using the same SFC–ICP-MS interface, used for the previous studies [127,128]. Resolution, detection limits, linear dynamic range and reproducibility were all compared for the two detection systems. Fluctuations in transfer-line temperature resulted in degradation of resolution for ICP-MS. Detection limits were improved by an order of magnitude using ICP-MS and were in the range 0.20–0.80 pg Sn. Reproducibility was also improved using SFC–ICP-MS compared to SFC–flame ionization detection (FID) (1.3–3.4% compared to 3.2–6.4%). Temperature control of the SFC–ICP-MS interface was found to be critical which was not the case with SFC–FID.

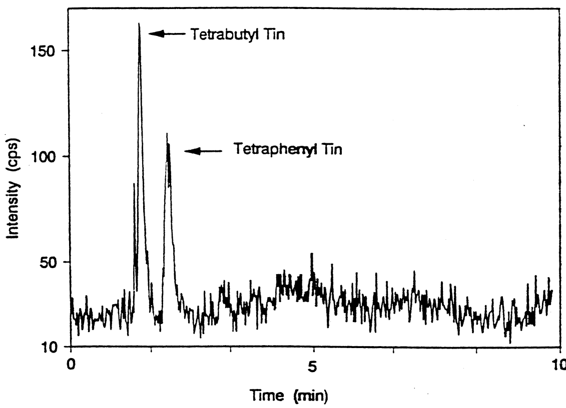

Fig. 12. SFC-ICP-MS chromatogram for a 1 pg injection of tetrabutyltin and tetraphenyltin. Reprinted from Shen et al. [127] by permission of the American Chemical Society.

Kumar et al. [130] used SFC–ICP-MS for the speciation of organometal compounds of arsenic, antimony and mercury. The study was performed using the interface developed by Shen et al. [127]. Trimethylarsine (TMA), triphenylarsine (TPA), triphenylarsenic oxide (TPAO), triphenylantimony (TPSb) and diphenylmercury (DPHg) were all separated in a single chromatographic injection. A mixed gas He/Ar plasma was used to improve detection limits of As compounds by reducing the $^{40}Ar^{35}Cl^{+}$ interference and methanol was used as the solvent instead of methylene chloride. SFC parameters such as pressure and temperature were optimised for ICP-MS detection. The element selective detection of ICP-MS enabled the TMA peak to be distinguished from the solvent peak, a feat not possible using FID. Detection limits were in the subpg–pg range.

Carey et al. [131] also investigated the feasibility of multielement detection for organomercury and organolead compounds. Simultaneous multielement detection of chromatographic peaks was compared to results obtained by single ion monitoring using the Shen interface [127]. Detection limits using the two modes of data acquisition were always better when single ion monitoring was used. This difference was attributed to the large mass differences between the elements which results in increased scan time of the quadrupole and lower duty cycle for each element during multielement analysis.

The same authors used SFC–ICP-MS for the separation of a pair of β-ketonate chromium com-

pounds and a thermally labile organochromium dimer [132]. Flame ionisation detection and ICP-MS detection were again compared. In this instance the thermally labile complex was only detectable using FID. ICP-MS gave superior detection limits for the separation of the β-ketonate complexes. The labile dimer may have thermally decomposed in the restrictor and could have been irreversibly bound to the capillary tube walls. The use of CO_2 as a mobile phase resulted in the formation of $^{40}Ar^{12}C^{+}$ which isobarically interfered with the major isotope of chromium at m/z 52. Nitrous oxide, was, therefore, chosen as an alternative mobile phase and yielded a simpler background spectrum.

Two papers by the Raynor group [133,134] described a capillary SFC–ICP-MS system for organometallic analysis. In the former paper [133], an interface was described in detail which was easy to assemble and caused minimal disruption to the SFC or ICP-MS instruments. The SFC oven was placed as close to the ICP as possible, in order to keep chromatographic efficiency at a maximum, and the restrictor temperature was optimised. Tetrabutyl and tributyl tin were separated using a CO_2 mobile phase with a pressure gradient. The mobile phase change did not interfere with the analysis. Detection limits were 0.025 pg and 0.035 pg for tributyltin and tetrabutyltin, respectively. These results compared well with those reported by Shen et al. [127]. The addition of small amounts of a modifier such as formic acid may improve the peak tailing exhibited in the chromatograms. In the second paper [134], the same interface was used to analyze a series of organotin, organoarsenic and organoiron compounds. The effect of analyte concentration and, again, restrictor temperature on peak intensity was investigated along with the effect of the CO_2 mobile phase.

5. Capillary electrophoresis

5.1. Introduction

The coupling of capillary electrophoresis (CE) to ICP-MS is a technique that has started to receive attention in recent years; the first papers describing the technique were written in 1995 [135–137] by the Olesik, Barnes and Lopez-Avila groups. CE is sometimes known as high-performance capillary electrophoresis (HPCE) since it has far greater efficiency of separation in comparison with conventional slab-gel electrophoresis. The most widely used mode in CE is capillary zone electrophoresis (CZE) since it offers ease of operation and can be used for a wide range of analytes. Other modes of CE include capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), capillary isoelectric focusing (cIEF) and capillary isotachophoresis. The separation of analytes depends upon the solutes' mobilities in an electric field as opposed to distributions between a mobile and stationary phase. The technique offers the analyst a valuable separation tool suitable for the separation of ionic and neutral compounds with many potential applications.

The general mechanism for CE separations is based upon differences in the mobilities of solutes which are transported along a capillary tube by a high DC voltage. Both ends of a fused-silica capillary, filled with a suitable buffer, are immersed in two reservoirs of the same buffer. The capillary is normally 50–100 cm long with an I.D. of 25–100 μm. A high potential difference of 20–30 kV is applied across two platinum electrodes in the reservoirs. The sample (several nl) is injected into the capillary and the components of the sample migrate toward the negative electrode. Owing to the small cross-section of the capillary, a high surface to volume ratio is achieved which dissipates heat, generated by joule heating, to the surroundings. Convective mixing within the capillary is, therefore, not significant and band broadening is minimised resulting in efficiencies of several hundred thousand plates.

Electroosmotic flow (EOF) of the solvent occurs in the capillary in the direction of the positive to negative electrode. It arises due to the electric double layer that is formed at the silica capillary surface/solution interface. The capillary surface has a net negative charge owing to the dissociation of the functional groups on the capillary surface. Positive ions are attracted from the buffer solution and a double layer scenario results. Mobile positive ions, present at the capillary surface, are attracted to the negative electrode and solvent molecules are transported concurrently. Capillary electrophoresis flow profiles are flat rather than parabolic owing to this

phenomenon and peak broadening contributions from the EOF are not problematical.

Separations in CE arise from differences in electrophoretic mobilities of the analytes which are principally determined by the mass-to-charge ratios of the analyte, physical dimensions of the analyte, viscosity of the medium and the interaction of the analyte with the buffer [21]. Positive species are attracted to the negative electrode and therefore migrate at a velocity greater than the EOF. Conversely, negative species are repelled by the cathode and migrate more slowly than the EOF. Neutral species move with the EOF and are generally unseparated unless some modification to the surface charge of the analyte is made, for instance by the addition of a surfactant.

Traditional detectors used in CE are genuinely the same as those employed with HPLC. However, for high resolution, the volume of sample injected onto the capillary must be small relative to the capillary volume and usually is in the 5–50 nl range. Detection of these very low levels of separated analytes requires a method that is both sensitive and specific. ICP-MS is potentially suitable, however two major challenges for coupling of the technique need to be addressed and have been described by Tomlinson et al. [21]. The flow-rate (typically of the order of $\mu l\ min^{-1}$) and low sample volume of a typical CE separation is the first potential problem, as most nebulisers for ICP-MS are designed to operate at flow-rates in the $ml\ min^{-1}$ range. Glass frit, direct injection, oscillating capillary and ultrasonic nebulisers are suitable for such low flows. Another factor to consider is that the end of the capillary will no longer be immersed in a buffer reservoir upon coupling to ICP-MS and a method of 'grounding' the electrode must be achieved. The construction of such an interface is an important consideration so that a high transport efficiency is achieved.

5.2. CE–ICP-MS interfaces and applications

Olesik et al. [135] reported the first instance of a CE–ICP-MS coupled system for rapid elemental speciation. The paper is a very comprehensive study which may be used as a guide for any analyst attempting CE–ICP-MS for the first time. The aim of the study was to develop a technique for 'quantitative elemental speciation in less than 1 min with detection limits in the low $ng\ ml^{-1}$–sub-$ng\ ml^{-1}$ range for a range of sample types'. In addition, the authors sought to determine the concentrations of free ions with different charge states, metal-ligand complexes and organometallic species.

It was noted that there is no need to electrophoretically separate species that contain different elements from each other as the ICP-MS detector serves to individually identify the metals present. Speciation studies are of primary interest for CE–ICP-MS as only 'ions, complexes or molecules containing the same element need to be separated'. Therefore, for different element ions of similar mobilities, electrophoretic resolution will be poor but the ions will be adequately identified by the ICP-MS element specific detector. It was identified that for CE–ICP-MS there is a need for an interface with low dead volume to reduce peak broadening. The actual interface used was simple in construction and the capillary was ground by coating the last 5 cm with silver paint to complete the electrical connection. The thickness of the paint layer was controlled. Contamination of the capillary effluent may occur using this method of grounding. The EOF was ~0.05 $\mu l\ min^{-1}$ and, due to the formation of a slight vacuum from the gas exiting the nebuliser, a natural aspiration rate of 2 $ml\ min^{-1}$ was achieved and no make-up flow was required. This increased laminar flow resulted in peak broadening due to the formation of a parabolic shaped velocity profile but could have been minimised using a capillary with smaller internal diameter. A smaller capillary may, however, degrade detection limits. The effect of conductivity of the electrolyte upon resolution was discussed along with the choice of electrolyte, the effect of nebuliser gas flow-rate and the effect of injection volume on peak width and peak area. Detection limits for ions were in the range 0.06–2 $ng\ ml^{-1}$ for all elements investigated. These were a factor of 20 worse than those obtained using continuous sample introduction and may have been principally caused by the use of a spray chamber which had significant dead volume and low sample transport efficiency.

Liu et al. [136] used a direct injection nebuliser (DIN) in a CE–ICP-MS interface where the CE capillary was placed concentrically inside the DIN

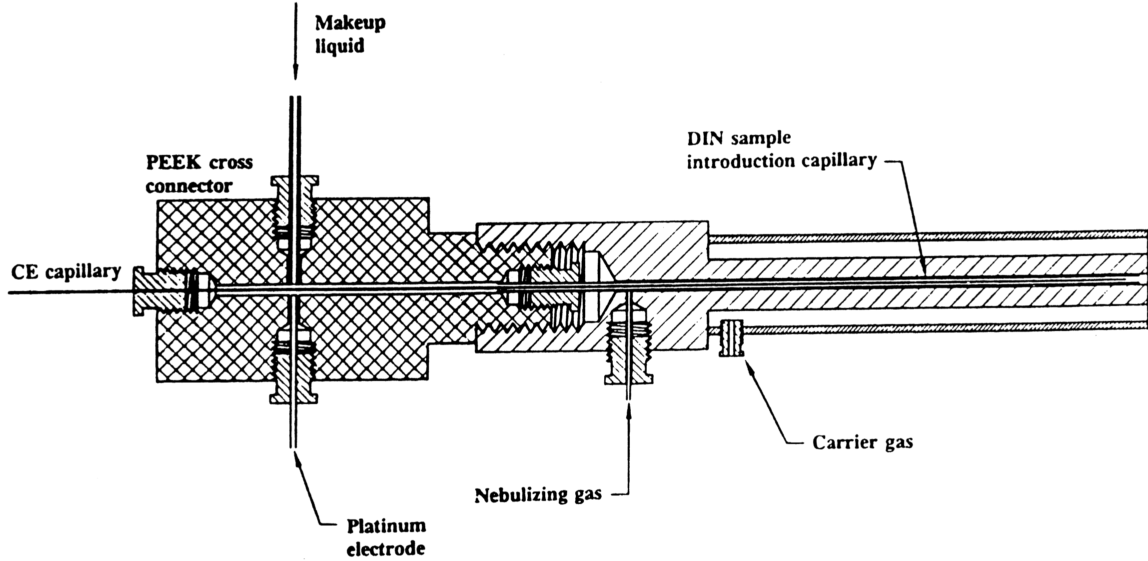

Fig. 13. Block diagram of the CE–ICP-MS interface. Reprinted from Liu et al. [136] with permission of the American Chemical Society.

sample introduction capillary so that the liquid sample was directly nebulised into the central channel of the ICP torch (Fig. 13). In this way 100% sample transport efficiency may be achieved and low sample flow-rates (10–100 μl) may be accommodated. In addition, memory effects are minimal due to fast sample washout and a small internal volume. A three-port PEEK cross-connector was specially made to accommodate the DIN sample introduction capillary (through which the CE capillary was inserted), a platinum grounding electrode and a make-up liquid which flows outside the CE capillary to establish the electrical contact. The make-up liquid was directly nebulised, along with the EOF, into the ICP torch and ensured that operation of the DIN was independent of the EOF.

The EOF and make-up flow were combined at the exit of the capillary before nebulisation. In this way, no suction was observed and band broadening was not significant. Alkali, alkaline-earth and heavy metal ions were analyzed at concentrations of 2–100 ng ml^{-1}. Detection limits were worse for CE–DIN-ICP-MS compared to continuous DIN-ICP-MS although the authors did not explain why this should occur. The feasibility of using the system for speciation studies of As and Se was demonstrated with excellent resolution of peaks and sensitivity.

Lu et al. [137] used a concentric glass nebuliser interface, similar to that used by the Olesik group, for their CE–ICP-MS studies along with a conical spray chamber. A conductive co-axial liquid sheath of electrolyte solution in the nebuliser was used to provide ground contact to the capillary. It was found that the CE capillary position inside the glass nebuliser had a significant influence on signal intensity, resolution and migration time. Peak widths narrowed and migration times shortened as the CE was inserted into the nebuliser. The technique was used to separate metal-binding proteins and to determine bound metal concentrations. Metallothionein isoforms and ferritin concentrations were determined. Detection limits were in the subpg range.

A further study by the Olesik group [138] used an interface with a laminar flow in the direction of the detector. The interface was a stainless-steel tee with the capillary threaded through the colinear ends of the tee. A sheath electrolyte was delivered through the lower arm of the tee with a peristaltic pump. Both a high efficiency nebuliser (HEN) and a concentric glass nebuliser were used in the study; the former was used with a conical spray chamber and the latter with a Scott double-pass spray chamber. Increasing the sheath electrolyte flow-rate enabled the laminar flow to be eliminated, therefore improv-

ing electrophoretic resolution. With higher sheath flow-rates, the laminar flow direction was reversed (away from the detector), effectively retarding migration of charged species. This may be useful where high injection concentrations are required to obtain low detection limits as band broadening may decrease, resulting in resolution enhancement, by reversing the laminar flow. The sheath flow-rate should therefore be optimised to obtain ideal resolution with satisfactory analysis times. The HEN had a lower sheath flow-rate than the concentric nebuliser for elimination of the laminar flow. Improved aerosol transport efficiencies were obtained with a lower sheath flow-rate so this nebuliser was used preferentially.

An ultrasonic nebuliser has been described by Lu and Barnes [139] for a CE–ICP-MS interface. As in their previous paper [137] the CE ground path was provided using a sheathing electrolyte flow around the capillary. Modification of the USN was required to accommodate the CE assembly of the two capillaries. Also described previously, the position of the CE capillary was variable inside the outer capillary. The arrangement gave improved separation resolution and sensitivity compared to a concentric nebuliser. Detection limits were not improved significantly, however, as the signal background with the USN was noisy. The authors postulated that the interface may be altered in order to transport a more uniform aerosol, yielding lower noise levels.

Preliminary studies by Michalke and Schramel [140] employed a Meinhard nebuliser interface for CE–ICP-MS. The interface was not described in significant detail. A co-axial sheath flow provided the electrical connection and no detectable 'suction' flow was identified. The method was used for the separation of Se compounds and adequate electropherograms were obtained.

A recently published paper by Magnuson et al. [141] described the use of a CE hydrodynamically modified electroosmotic flow with hydride generation ICP-MS for arsenic speciation. This modified EOF was similar in operation to the 'sheath electrolyte flow' described by Kinzer et al. [138]. The electroosmotic flow was modified by applying hydrodynamic pressure in the opposite direction to the EOF. This enabled the injection of large quantities of analyte, which normally cause peak broadening, by offsetting the EOF thus yielding improved peak resolution. Four hydride species were separated using this technique with post capillary hydride generation to convert the species to their hydrides before analysis by ICP-MS. Detection limits were in the range 6–58 ppt for four arsenic species. Two drinking water samples were analyzed using this technique.

6. Other plasma mass spectrometric systems used as chromatographic detectors

Although ICP-MS is the most commonly used plasma mass spectrometric technique in the analytical laboratory, other plasma sources have been used as mass spectrometric detectors and will be briefly discussed.

6.1. Use of the helium microwave induced plasma as a chromatographic detector

A number of papers by the Caruso group [142–147] have highlighted the attributes of a He-MIP as an alternative ion source for chromatographic detection by mass spectrometry. A review by Olson and Caruso [142] discussed instrumentation, chromatographic techniques and the advantages associated with He-MIP plasmas. The main advantages of using MIPs as alternative plasma sources are principally the reduced gas flows and power consumption needed to sustain the plasma. Alternative plasma gases may also be used which form plasmas of more efficient excitation and ionisation energies, thus improving sensitivity for elements with higher ionisation potentials. For non-metals this is significant as Ar-ICPs only ionise approximately 50% of the sample in the plasma. Isobaric interferences from polyatomic species are also problematical in Ar ICPs and may prevent the detection of lower mass elements due to argon, oxygen, nitrogen and hydrogen combinations [142]. These interferences are essentially eliminated when helium is used as the plasma gas.

The most common method of sample transport into the He-MIP is by gaseous introduction. The use of MIP-MS as a gas chromatographic detector has been well described by the work of Caruso et al.

[142,143,145–147]. Both non-metals and metals have been analyzed by GC–MIP-MS. The GC–MIP-MS interface used in these studies was made from a 1/6 in. stainless-steel tube, wrapped with heating cord and insulated with fibre glass tape (1 in.=2.54 cm). The transfer line was maintained at 300°C.

Helium MIP-MS has also been used as an element selective detector for supercritical fluid chromatography of halogenated compounds [144]. Compared to the Ar-ICP, the He-MIP offers spectral simplicity and, for halogens, more efficient ionisation. The design of the interface is important [144] as the temperature of the frit restrictor must be maintained above 100°C to avoid condensation of the analytes. Again, a heated stainless-steel transfer line should be used. Typical plasma power settings for such a coupled system are in the region of 100 W with a low (5 $l\,min^{-1}$) plasma gas flow-rate. To date, liquid chromatography has not been coupled with He-MIP-MS as the high liquid flow-rates involved tend to quench the plasma. The use of microbore LC coupled to MIP-MS may be a more promising technique [148].

6.2. Use of low-pressure helium ICP-MS as a chromatographic detector

Another alternative ion source for mass spectrometry is the helium low-pressure ICP [149–151]. Again, this helium plasma gas has a higher ionisation energy (24.6 eV) compared to the argon ICP (15.8 eV) and isobaric interferences are minimised. The use of a low-pressure plasma eliminates air entrainment and consumes less gas than an atmospheric pressure ICP (<500 $ml\,min^{-1}$) and radio frequency (RF) powers (100 W) may be used.

Evans and Caruso [152] developed a low-pressure argon ICP with a water-cooled low-pressure torch interface for gaseous sample introduction. Further studies by the same workers [149–151] realised the potential of He as the plasma gas. These plasmas were coupled to GC systems for the analysis of organotin and organohalide compounds [149–153]. Recent papers have realised that it is possible to sustain the plasma using the carrier gas from the gas chromatograph alone [150,151]. In addition, mass spectra may be obtained that yield more molecular information as the degree of fragmentation of the organic species may be controlled by altering the plasma gas flow and forward power [150,151].

Castillano et al. [154] investigated the feasibility of using solution nebulisation with a low-pressure He-ICP with mass spectrometric detection. The importance of reduced solvent loading to avoid quenching of the plasma was realised. Multielement solutions containing As, Se, In, Cs and Pb were aspirated and detection limits were in the low ppb region. Obviously this technique has great potential for direct coupling of microbore liquid chromatography to the low-pressure plasma for speciation studies.

6.3. Use of ion spray mass spectrometry as a chromatographic detector

Corr and Anacieto [155] recently described the successful separation of a mixture of ions by capillary electrophoresis and ion-exchange chromatography with mass spectrometric detection using an ion-spray atmospheric pressure ionisation source. Ion-spray is similar to electrospray except that the ionisation of the analyte is pneumatically assisted. Such an atmospheric pressure ionisation technique is able to produce analyte ions in the gaseous phase directly from solution [155]. Using an ion-spray source, ion-adduct declustering and molecular fragmentation may be controlled selectively, thus yielding both elemental and molecular information depending on the instrumental settings.

7. Conclusions and future directions

From the volume of papers published in recent years it is apparent that users of ICP-MS are increasingly employing the instrument for chromatographic detection. Several modes of liquid chromatography, gas chromatography, supercritical fluid chromatography and capillary electrophoresis have all been hyphenated with ICP-MS for improved detection limits compared to other traditional methods of detection such as UV–Vis spectroscopy.

There is a significant demand for speciation information for many elements and the separation ability of chromatography coupled to ICP-MS offers the analyst a versatile tool for such studies. Both

metals and non-metals may be speciated, therefore compounds containing elements such as the halogens may be separated. It is now evident that certified reference materials for speciation studies of various elements must be developed and made available so that speciation techniques may be evaluated for environmental and toxicological studies.

Sample preparation is also an area for future research. Common digestion and extraction procedures are time consuming and may result in the loss of volatile elements. Extraction procedures such as microwave-assisted extraction and supercritical fluid extraction may be employed and even coupled on-line so that samples for chromatographic analysis may be prepared.

Capillary electrophoresis is a technique that offers the analyst potential benefits for speciation analysis. Although capillary electrophoresis is now widely used to perform routine separation of compounds, the coupling of CE with ICP-MS is relatively new and interface designs are still being developed and evaluated. The use of direct injection nebulization and other 'low flow' nebulizers are, without a doubt, the way forward if electropherographic resolution is to rival that obtained with UV detection.

Microbore liquid chromatography is a technique where low flows and small diameter columns may be used to obtain chromatograms of resolution comparable to traditional analytical performance LC columns. These columns may be suitable for performing separations with ICP-MS detection as lower organic solvent concentrations and buffer concentrations would be transferred to the ICP-MS, via a transfer line. This may enable the analyst to use gradient elution of organic solvents for reversed-phase and other modes of LC, without significant loss of plasma stability, an area of chromatography which has proved problematical in the past.

Modern ICP-MS instruments are invariably computer-controlled and chromatograms are usually obtained with on-board time resolved analysis software. This software is often cumbersome and is the limiting factor with regard to resolution and ease of chromatographic data acquisition. Owing to the increasing use of ICP-MS as a chromatographic detector, instrument manufacturers must now meet the demands of the chromatographer and find improved ways for data information acquisition that employ software programs compatible with everyday word processing and presentation packages.

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On-line coupling of ion chromatography with ICP–AES and ICP–MS

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Ion chromatography (IC) and atomic spectrometry are sometimes rivalling and sometimes ideally cooperating techniques. The cooperating applications of the on-line coupling of IC and inductively coupled plasma–atomic emission spectroscopy or –mass spectrometry span from ultra trace analysis utilizing ion exchange as preconcentration technique via speciation applications taking advantage of the unique element specific detection offered by atomic spectroscopy until classical IC applications with atomic spectrometry as a sensitive and selective detector. Characteristics of this type of hyphenated technique are the simple physical coupling, the unique sensitivity for most elements and the superior selectivity obtainable for specific applications. **©2001 Elsevier Science B.V. All rights reserved.**

Keywords: Ion exchange; Ion chromatography; Atomic spectrometry; Inductively coupled plasma–atomic emission spectroscopy; Inductively coupled plasma–mass spectrometry; On-line coupling; Ion chromatography–inductively coupled plasma–(atomic emission spectroscopy,mass spectrometry)

1. Introduction

Generally, the use of a coupling technique is recommended in any case where the analytical information delivered by a single analytical method is not sufficient. A coupling technique can be defined as the combination of two ore more originally independent analytical techniques to a new analytical tool. In case of chromatography as one of the coupling partners, the term coupling or hyphenated technique is limited to more dimensional detectors such as mass spectrometry (MS), nuclear magnetic resonance (NMR), IR and others. This distinguishes it from standard chromatographic detectors such as photometry (UV) or conductivity (CD).

Hyphenated techniques are currently en vogue because of many analytical challenges, which are not resolvable using a single analytical method. For example those challenges are the unquestionable identification of a chromatographic peak as a specific compound. The 'hyphenated' answers to these questions are gas chromatography (GC)–MS, liquid chromatography (LC)–MS, LC–NMR or in case of ionic compounds ion chromatography (IC)–ESI–MS or IC–inductively coupled plasma (ICP)–(atomic emission spectroscopy (AES),MS) [1].

The direct coupling with MS is in case of small ions and IC sometimes the second best choice. ESI–MS for example suffers from calibration problems, the type of eluent is strictly limited when analysing in the negative ion mode and the handling is somewhat difficult [2]. Far better is the combination with an ICP as photon or ion source because of the absence of calibration problems and the perfect element specificity.

Despite this, there are still several limitations remaining in atomic spectrometry:

1. the decision between different species containing the same element or
2. the resolution of spectral or isobaric interferences when using ICP–AES or low-resolution mass filter ICP–MS or
3. by the determination of very low concentrations of trace elements in complex samples.

IC suffers mainly from detection sensitivity and from inadequate resolution of interfering compounds [3]. The selectivity of anion chromatography for example is almost fixed and only minor

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0165-9936/01/$ – see front matter
PII: S0165-9936(01)00082-6
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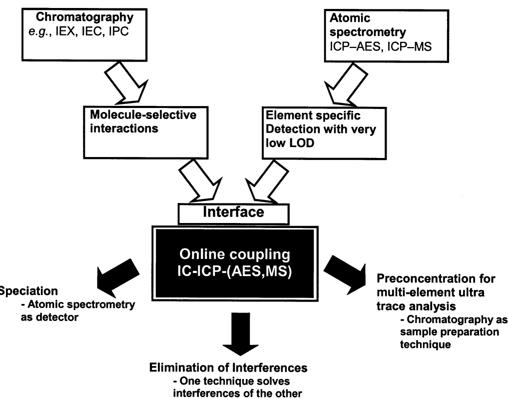

Fig. 1. Basic idea behind the on-line coupling of IC and atomic spectrometry.

changes are realized until now [4]. The main reason for this is the exclusive use of quaternary ammonia groups as exchange site. One requirement for multicomponent analysis with IC is normally non-specific detection such as conductivity. In case of nearby eluting anions such as bromate and chloride we have a non-resolvable selectivity problem and the need for a more selective detection system.

The conclusion of these analytical shortcomings is the basic idea behind IC–ICP–(AES,MS) as outlined in Fig. 1. This paper discusses the usefulness of IC–ICP–(AES,MS) categorized by the three points of shortcomings of atomic spectrometry, whereas point (2) as limited selectivity of IC and point (3) as limited sensitivity of IC are the link to the shortcomings of IC.

The applications based on point (1) are the so-called speciation analysis dealing with organometallic compounds, proteins, elements in different oxidation states and similar analytical problems [5,6].

The point (2) limitations are usually described as spectroscopic and non-spectroscopic interference or more general as matrix effects [7–10]. They are difficult to overcome, but in many cases a time resolution between interfering elements is an acceptable workaround.

The limitations related to point (3) are caused by the increasing demand for ultra pure chemicals for microelectronics industry, for a clean environment without hazardous compounds or by other high tech applications [11–13] and can be generally attributed to the limited sensitivity of all analytical methods.

2. The ICP as source for AES (ICP–AES) and MS (ICP–MS)

Atomic spectrometry methods based on the ICP allow the determination of almost every element. Important restrictions are given by the plasma gas and its impurities (Ar, other noble gases, N, O and to some instance halogens), by the typical solvent used for the liquid solutions (H, O) and by some physical restrictions such as insufficient ionization (F) or emission lines below or above the observable wavelengths (F, Cl, Br, for some instruments the alkali metals) [8].

2.1. The ICP

The ICP is today the most important source for atomic spectrometry [14]. The argon-based plasma is compatible to aqueous aerosols, offers a high amount of energy for drying, dissociation, atomization and ionization of analytes. The temperatures offered by an argon ICP are varying from 4500 to 10 000 K, depending on the type of temperature you mean (kinetic temperature, electron temperature, atomization temperature, ionization temperature and more) and the local position inside the plasma. An ICP is therefore called a non-thermal equilibrium plasma. The temperature in the analytically used inner channel of an ICP is about 5500–6500 K, high enough to destroy all molecular bonds and even high enough to ionize almost every element completely.

The basic set-up and compounds of an ICP–AES and ICP–MS are shown in Fig. 2. The ICP part is almost identical for AES and MS as detection principle. The ICP torch consists of three concentric quartz tubes, from which the outer channel is flushed with the plasma argon at a typical flow rate of 14 l min^{-1}. This gas flow is both the plasma and the cool gas. The middle channel transports the auxiliary argon gas flow, which is used for the shape and the axial position of the plasma. The inner channel encloses the nebulizer gas stream coming form the nebulizer/spray chamber combination. This gas stream transports the analytes into the plasma. Both the auxiliary and the nebulizer gas flow are typically around 1 l min^{-1}. The plasma energy is coupled inductively into the argon gas flow via two or three loops of a water-cooled copper coil. A radio frequency of 27.12 or 40.68 MHz at 1–1.5 kW is used as power source. The plasma is

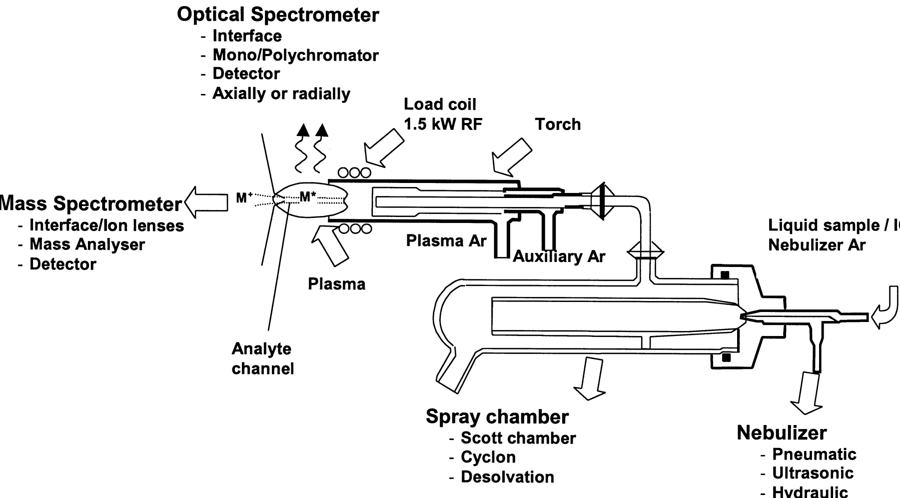

Fig. 2. Sketch of the set-up of ICP–AES and ICP–MS instruments with the typical choices of modern instruments.

formed immediately after an ignition spark was applied to seed electrons and argon cations.

The sample introduction system is usually designed for liquid samples, but solid or gaseous sample are also applicable. The standard configuration of an ICP includes a pneumatic nebulizer for the formation of an aerosol and a spray chamber for the separation of the droplets by size. Only small droplets should be able to enter plasma, otherwise the plasma becomes instable or extinguishes. The ultra fine sample aerosol entering the plasma undergoes rapid desolvation, vaporization, atomization and ionization. Most elements exist as single charged ion in the analytical important part of the plasma. Therefore almost every element is detectable by ICP–MS. Problems in form of isobaric interferences are caused by doubly charged ions and by molecular ion formation in the cooler part of the plasma interface.

In case of photon detection for AES we have to realize that the emission spectra of both the atom and the ion are present leading to numerous emission lines.

A short list of rough estimates of the limits of detection (LODs) for 49 elements in ICP–AES and ICP–MS is given in Table 1.

2.2. *Characteristics of ICP–AES*

AES based on the ICP as photon source is a well established and well understood routine method [8,9]. The photons may be observed radially as the standard set-up or axially as more sensitive alternative. Some commercial instruments offer both variants.

The ICP produces a wavelength dependent optical background due to recombination radiation and other processes inside the plasma. This continuum is overlaid with the emission lines of Ar, of several molecules and radicals such as OH, N_2 etc. The basic problem of ICP–AES is the proper detection of analyte emission lines on this background, which varies with plasma conditions. The optical part of the current spectrometer generation is almost exclusively based on polychromators for the simultaneous measurement of several elements. Their job is the separation of the selected emission line from interfering lines of the other elements or ions. The required resolution spans from less than 3 pm (which is not realizable due to Doppler broadening of the emission lines) to at least 20–30 pm (routine conditions). Typical resolution power of current instruments is between 4 and 20 pm. The optical system is based either on the Paschen–Runge arrangement (a concave grating with the detection of the emission line on the Rowland circle of the grating) or on an Echelle optic with a high order grating and a spectral order separator [8,15]. The detectors are usually photo multipliers, photodiodes, diode arrays or two-dimensional detectors such as CCD or CID chips well known from digital cameras.

Important characteristics of the optical part are the wavelength range, the number of simultaneously observable emission lines and the possibility to measure their backgrounds at the same time, the

Table 1
Rough estimates of the LODs for 49 elements using the most sensitive line in ICP–AES (including sequential and simultaneous types) and the most abundant isotope in ICP–MS (quadrupole ICP–MS, built 1990–1998)

Element	$LOD_{ICP-AES}$ /μg/l	LOD_{ICP-MS} /μg/l
Al	5	0.05
Sb	40	0.005
As	30	0.01
Ba	0.5	0.001
Be	0.1	0.001
Bi	10	0.001
B	3	0.07
Br	800/250	0.05
Cd	2	0.005
Ca	0.1	0.5
Ce	100	0.001
Cr	5	0.005
Cl	250/80	160
Co	3	0.001
Cu	0.3	0.005
Ge	100	0.05
Au	25	0.005
I	100/10	0.005
In	100	0.001
Fe	1	0.1
La	6	0.005
Pb	7	0.001
Li	3	0.005
Mg	0.5	0.05
Mn	0.4	0.005
Hg	20	0.001
Mo	1	0.005
Ni	7	0.005
Pd	70	0.005
Pt	30	0.005
K	80	0.5
P	20	6
Se	50	0.05
Si	5	8
Ag	5	0.005
Na	6	0.05
Sr	0.2	0.001
S	50	50
Te	20	0.005
Tl	50	0.001
Sn	7	0.005
Ti	3	0.05
W	20	0.005
U	400	0.001
V	2	0.005
Zn	1	0.005
Zr	5	0.005

The data are compiled from manufacturers' data and corrected using overlapping elements.

overall noise level and not at least the transmittance. Today's high end instruments allow the observation starting form 120 nm [16] to the near infra red region, offer an almost unlimited number of simultaneously observable emission lines and operate in both the axially and the radially viewing mode.

2.3. Characteristics of ICP–MS

The ICP forms almost exclusively single charged ions of the elements [10]. The extraction of ions from the plasma into the MS part is organized in a multiple stage differentially pumped interface. First part of this interface is a cooled nickel or platinum sampling cone (sampler) with an orifice below 1 mm. A large rotary pump evacuates the expansion chamber to ensure a pressure below 3 mbar in this region behind the sampler. The pressure difference creates a supersonic jet into the mass spectrometer. For preservation of the required high vacuum a second cone with small orifice called the skimmer is placed inside the supersonic jet. Next part of all ICP–MS is the ion optic build of an extraction lense for the acceleration of positively charged ions, a photon stop or alternatively an off-axis mass analyzer and several electrical lenses for focusing of the ion beam [14].

The standard mass analyzer of ICP–MS is still the quadrupole. He allows the resolution of nominal mass units down to 0.2–0.5 mass units and is therefore a low-resolution device. The performance of all ICP–MS instruments is limited by the transmission of the interface and mass analyzer unit, the background count rate due to photons and the remaining gas pressure and the background count rate caused by molecular ions or doubly charged ions. Typical quadrupole instruments offer instrumental background count rates of 10 cps, newer instruments with an off-axis quadrupole show less than 1 cps like high-resolution instruments.

A new generation of quadrupole-based ICP–MS instruments offer an ion focusing and molecular ion destruction device named hexapole, collision cell or dynamic reaction cell depending on the manufacturer [17]. This device increases the transmission and destroys molecular ions with varying efficiency. An example of molecular ions and their impact on the analysis of bromine is shown in Fig. 3.

An intermediate resolution mass analyzer is the time of flight mass filter (TOF) [18,19]. Applied to an ICP it allows resolutions below 1000 $m/\Delta m$. The

main advantage of TOF–ICP–MS is the fast mass spectra recording, which is important for fast transient signals such as obtained from on-line coupling GC, CE, electro thermal vaporization or laser ablation to ICP–MS. For LC the speed of quadrupole instruments is sufficient. The second main advantage is the equal start point for all masses tantamount to an accurate isotope ratio determination.

The high-resolution ICP–MS instruments are all based on a reverse Nier–Johnson geometry with an electrostatic and a magnetic analyzer [14,20]. The routinely observable resolution is between 3000 and 10 000 $m/\Delta m$. This resolution is sufficient for the resolution of a number of molecular ion interferences such as ArO^+ on ^{56}Fe and $ArCl^+$ on ^{75}As as prominent examples. Other molecular ion interferences are still not resolvable.

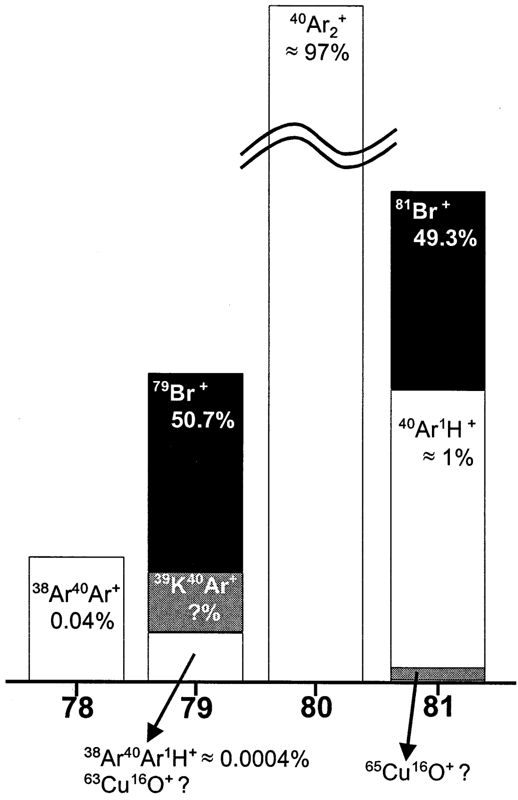

Fig. 3. Example of the constitution of isobaric interferences in ICP–MS. Shown is the situation with the low-resolution ICP–MS instrument for the detection of bromine.

3. Principle of IC as coupling partner

IC is nowadays used as both the short term for ion exchange chromatography (IEX) and as generic term for the three methods ion exchange, ion exclusion and ion pairing chromatography (IPC) all dealing with the separation of charged or potentially charged compounds.

3.1. IEX

Ion exchange is a well known and good understood separation mechanism [21].

Small anions or cations are separated on an ion exchanger of opposite polarity by means of a driving ion of the same polarity. For anion chromatography a quaternary ammonia functionality with hydroxide or carbonate as eluent is used [3]. For cations an additional complexing ligand may be applied to reduce the effective charge and to generate differences in the retention behavior. A typical elution system for cation chromatography consists of H^+ or Na^+ as driving cation and strong complex formers such as dipicolinic acid or tartaric acid [3].

Simple ion exchange processes based on strong acidic or strong basic exchangers are easy to control and show usually no kinetic problems. More difficult is the exclusion of secondary retention mechanisms such as adsorption. Packing materials used for conductivity detection as standard detector for IEX applications are of the low capacity type. Depending on the type of substrate and the constitution of the exchanger is adsorption (polystyrene resins), ion exchange of opposite polarity (agglomerated resins), oxophilic interactions (silica gel) or chelation (methacrylate resins) observable.

The more selective kind of ion exchange, the chelating ion exchange [22], suffers often from kinetic limitations, which limit the application range to cationic compounds with fast ligand exchange kinetic for the inner coordination sphere. Ion exchange is well suitable for preconcentration as well as for separation of chemically similar compounds.

3.2. IPC

The application area of IPC and IC is overlapping, whereas IPC fills the gap between IC and reversed phase chromatography [3]. As one borderline case IPC can be described as IC with dynamically coated exchangers. The ion pairing reagent is simply

adsorbed onto the RP surface and the result is an ion exchanger.

The main advantage of IPC is the ability for the separation of anionic, cationic and non-ionic species in a single separation system. Another favorable feature is the absence of irreversible adsorption of substances on the packing material as it is often observed in IC. Simply moving the ion pair by the usage of strong organic eluents can elute extremely strong bonded ions. Due to these inherent features IPC has become an important separation mechanism for speciation analysis using atomic spectrometry.

A disadvantage of IPC is the long equilibration time caused by the generation of stable, dynamic adsorption equilibria and its sensitivity against highly concentrated samples or too large sample volumes. Directly caused from long equilibrium times the retention time stability of IPC application is not as good as those of IC and RP.

3.3. Ion exclusion chromatography (ICE)

ICE separates the analytes by their pK_a value [3]. The anions of strong acids are excluded from the pore volume of strong acidic cation exchangers due to the repulsion caused by the Donnan membrane. The retention volume increases with increasing pK_a value. This type of ion separation as only separation mechanism has not been used in on-line coupling application until now. A reason for this is the structure of analytes commonly investigated by ICE. The carbon detection capabilities of ICP–AES and –MS are strongly depressed by the CO_2 contents of the plasma argon. Other detection principles such as photometry or conductivity are more favorable.

4. Interfacing IC and ICP techniques

The behavior of ICP spectrometers as detectors in IC is dominated by eluent flow rate and composition. The flow rate of a chromatography system depends strongly on the column geometry. Varying the column diameter from 8 mm inner diameter (i.d.) – wide bore chromatography – via the commonly used 4 and 4.6 mm i.d. – standard bore – down to narrow and microbore columns with 2 or 1 mm i.d. changes the flow rate from 10 to 0.1 ml/min using the same packing material. Advantage of larger diameters is a higher sample amount, which can be analyzed during one run. Small diameters are preferred for small particles and are more difficult to handle because of a higher sensibility to dead volumes, the required low flow pumping system and not at least the more difficult column packing procedure [23].

Most critical units of an ICP for coupling with IC are the nebulizer and the spray chamber. The general requirement for a nebulizer is compatibility with flow rate and eluent composition. Water-based eluents are often deleterious by their salt contents, eluents containing organic modifiers or fully organic eluents tend to affect plasma stability because of the increased solvent vapour pressure. The workaround for water as well as for solvents in ICP–MS is a cooling of the spray chamber for reduction of the vapour pressure. This increases the acceptable amount of organic modifiers and reduces oxide-based molecular ions. Compared to ICP–AES, the ICP–MS needs in general more dilute buffers or lower concentrations of organic solvents.

Pneumatic nebulization as standard equipment of ICP instruments renders the ICP to a mass flow dependent IC detector. The characteristics of common nebulizer with a constant nebulization efficiency at low flow rates and a strongly decreasing efficiency at higher flow rates result in a linear relationship between mass flow of analyte into the nebulizer and observed count rate for the analyte at low flow rates and a constant or slightly decreasing count rate at higher flow rates.

Pneumatic nebulizers are typically operated at conditions where efficiency in terms of the resulting count rate is nearly independent of the flow rate. On-line applications must take notice of this fact when using external calibration. The practical consequence is a strong dependence between the reproducibility and stability of the flow rate and the accuracy of the calibration function for the coupling method [24].

Some easily adsorbed analytes cause problems at very low concentrations when using conventional nebulization. Such difficult elements are the usually as polarizable species existing mercury, iodine, thallium and silver, whose interaction with polymer parts of the sample introduction system is difficult to control or as another example boron, which interacts strongly with the glassy surface of common spray chambers.

A further selection criterion is the increase of peak width caused by the dead volume of the nebulization unit. The typical peak width obtained in chromatographic applications depends on column

Table 2
Nebulizers and spray chambers used or applicable for on-line coupling IC–ICP–(AES,MS)

Type of nebulizer	Mass flow dependent	Sensitivity	Desolvation needed	Applicable for
Conventional pneumatic (Meinhard, cross flow, V-groove)	Yes	Low	No	Water-based eluents, with limitations for organics
High efficiency nebulizer (HEN)	Yes	High	Yes	Water-based eluents, with limitations for organics
USN	Yes	High	Yes	Best for clean water samples, efficiency depends strongly on viscosity and on dissolved solids
High pressure nebulizer (HHPN)	Yes	High	Yes	Best for high viscosity samples, high efficiency for a wide range of samples
DIN	No	Low/high (relative/absolute)	No	All kinds of eluents, useful for Hg, B, I and other elements deleterious for conventional nebulization units
Scott spray chamber	–	–	No	Standard equipment of most ICP
Cyclone chamber	–	–	No	Lower dead volume, faster wash out time
Desolvation	–	–	–	Large dead volume, large surface area, subject to physical and chemical interferences

geometry, flow rate and on efficiency of the separation system. Microbore applications show signal widths below 1 s, semi preparative wide bore applications generate peak widths of about one to several minutes. Standard chromatography generates usually peak widths of 5–30 s at full width half maximum. Those considerations are important for data acquisition and data handling.

Desolvation systems as used for high efficiency nebulizers such as HEN, ultrasonic nebulizer (USN) or hydraulic high pressure nebulizer (HHPN) increase the system dead volume [14]. Coupling interfaces with an extremely low dead volume such as the direct injection nebulizer (DIN) [25] limit the eluent flow rate to 100 μl/min and cause therefore pumping and column problems. Another drawback of the DIN is the somewhat fragile operation characteristic. The flow rate and the salt contents buffer have to be chosen with respect to avoiding clogging and large droplet formation. Table 2 shows the most widely used nebulization units together with their typical features and application areas.

A general advantage of coupling of IC with atomic spectrometry is the constant eluent composition. The ICP–(AES,MS) is operating with the same solution over the whole measurement period. The optimization of ICP–(AES,MS) with respect to forward power, gas composition and flow, instrument specific settings etc. can be done for well defined operating conditions. The outcome of those uniform operating conditions is an increase in the stability of the whole system.

5. Data handling and software issues

Single element detection is an inherent feature of all ICP–AES or ICP–MS instruments. The optical part or the type of mass analyzer influences the multielement detection capabilities of ICP–AES and ICP–MS spectrometers. ICP–AES as coupling partner in multielement applications requires simultaneously operating spectrometers with polychromators or Echelle optics. In case of Echelle spectrometers the design of the array detector and its readout speed are critical selection criteria. The software support by the manufacturers is sufficient for new instruments, for older types a home-made workaround is often required.

The mass analyzers of common ICP–MS instruments are only sequential detectors with the exception of the TOF–MS. The sequential mass analyzers allow for some instances the operation in a quasi-simultaneous mode. From his nature best suited for quasi-simultaneous analysis is the quadrupole mass filter. It is a sequential detection unit with rapid scanning speed over a wide mass range. The elemental mass range of m/z 5–240 is detectable in usually less than 100 ms. Faster scanning is limited by the time needed for the arrangement of a constant electrical field between the quadrupole rods.

The quasi-simultaneous detection behavior of ICP–MS influences the accuracy of peak height and isotope ratio determinations. The relative error in peak height determinations becomes significant if the peak width and the scan time per time

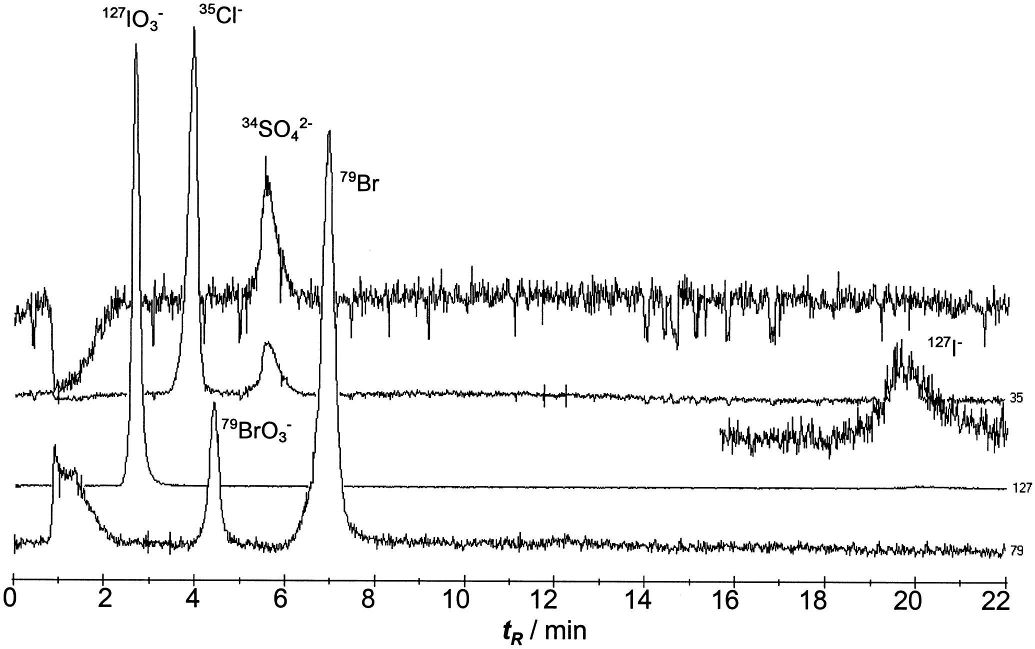

Fig. 4. Example of the simple replacement of conductivity detection by an ICP–MS. The sample was 585 μl of an ozonized tap water. The self-made column P150497 DMEA was operated with 75 mmol/l NH_4NO_3, pH 6 as eluent at a flow rate of 1 ml/min. The detection device was an ICP–MS PQ ExCell in the collision cell mode.

slice are in the same order of magnitude. In typical IC applications this source of error is not significant.

The precision of isotope ratio measurement in on-line applications is rather low as mainly caused by the high noise level of sequential ICP–MS instruments and a time dependent isotope ratio shift caused by the time depending elution signal of IC and the different times at which the masses for isotope ratio determinations are measured [24].

The strategies to measure isotope ratios include peak area and peak height ratioing and on a time slice method with calculation of an isotope ratio for each data point separately as shown in Fig. 4. Peak height is not so suitable because of the high short-term noise of ICP–MS. Best results are obtained for peak area and time slice ratio measurements.

Some practical aspects must be taken into consideration when an appropriate integration time should be chosen. A maximum of sensitivity is given for longer integration times directly on peak, but this time is limited by the number of elements to be determined and the required maximum length of a single time slice. The precision of isotope ratios or of internal standardization should increase with faster scan speed. Disadvantage of faster scanning is a decreasing integration time per peak by an increasing number of dead times resulting from the jump back of the quadrupole and of course an increase of the noise level.

6. Applications of on-line coupling IC–ICP–(AES,MS)

Every coupling application favors one part of the coupling system. A dominating chromatography part leads to the speciation analysis [5,6,26,27]. The elemental specific detection facilities of atomic spectrometry are strongly favored over the multi-element capabilities. An inversion of this construction leads to multielement trace analysis in complex matrices with the use of chromatographic equipment as powerful preconcentration and matrix elimination tool [13]. The ability of chromatography for a further time resolution between the separated traces is not really required because of the excellent elemental specific detection capabilities of atomic spectrometry.

A third group of coupling applications uses the features of coupling of IC and atomic spectrometry in a more balanced way. The separation of the rare earth elements (REE) with help of IC as an example

uses both the excellent separation power of high performance LC (HPLC) and the elemental specific detection [28]. The deleterious influence of barium oxides and oxides of the lighter REE on the heavier ones is eliminated by a (retention) time resolution between them.

6.1. *On-line preconcentration techniques using chromatographic equipment*

Preconcentration or matrix separation is a well known technique in analytical chemistry. Every instrumental method needs or will need such a chemical treatment when the latter does not meet the required detection limit. The literature about older instrumental techniques such as photometry, X-ray fluorescence spectrometry, atomic absorption spectrometry and not at least ICP–AES or the literature about ion exchange and related techniques includes a lot of those chemical treatments [21,29], whereas most of them can be transferred to on-line coupling IC–ICP–(AES,MS). Table 3 summarizes activities using on-line coupled IC–ICP–(AES,MS) for preconcentration and subsequent ultra trace analysis. The main driving power for the development of analytical methods for ultra trace determinations is the environmental research and the microelectronic industry with their rapidly increasing demand for even lower detection limits.

Preconcentration as an extrema of chromatographic separation is the only application of on-line coupling, which offers a double sensitivity enhancement. At first, every chromatographic treatment without included preconcentration step causes a dilution of the analytes of interest. With preconcentration only an on-line coupled detection system offers the ability for the detection of the analytes without lowering the highest possible concentration of the analyte by mixing the different increments of an elution signal. Exactly this happens when off-line separations with fraction collection are to be applied.

Secondly, when matrix elimination is included in the preconcentration procedure, the deleterious interferences and suppression caused by the matrix are absent. Furthermore the implementation of sample pretreatment into a chromatographic system offers some unique advantages:

- The closed system of modern LC reduces the risk of airborne contamination
- Especially for trace analyses modern IC equipment offers a complete metal free design
- Modern IC equipment allows the use of high efficiency packing materials with the main advantage of faster mass transfer during the separation and the elution process
- Highly reproducible flow rates of IC allow the use of nebulization ICP despite its mass flow dependent detection characteristics as quantitative detection system

An extremely powerful application of preconcentration combined with on-line coupling is the analysis of a group of chemicals used for microchip production. The simplest application is the trace

Table 3
Examples of applications of chromatographic preconcentration techniques for on-line coupling IC–ICP–(AES,MS) ordered by the type of matrix

Sample / matrix	Separation mode	Analytes separated	Preconcentration column	Detection limits	Refs.
As, Mo, W, P, Re	Cation, anion	Li, Be, Na, Mg, Al, Ca, Sc, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Sr, Y, Ag, Cd, Ba, REE, Tl, Pb, Bi, Th, U	SCX (AG 50W), SAX (AG1X-8)	0.1–80 ng / g referring to the solid sample	[13,24]
MoSix, WSix	Cation	See As application	After volatilization of SiF_4 SCX (AG 50W)	0.2–200 ng / g solid sample	[38]
Seawater, waste water	Chelation	Cd, Co, Cu, Fe, Mn, Ni, Pb, Ti, V	Iminodiacetic acid	not reported	[39]
Alkali and alkaline earth metals, anions, seawater	Chelation	V, Cr, Ni, Co, Cu, Mo, Pt, Hg, Bi	Bis(carboxymethyl)-dithiocarbamate adsorbed on XAD-4	9–80 ng / l (0.5 ml sample loop, no sample dilution)	[40]
Seawater, riverine water	Chelation	Mn, Co, Cu, Cd, Pb, U, Fe, Mn, Co, Ni, Cu, Zn, Cd, Pb	Iminodiacetic acid, silica immobilized 8-hydroxyquinoline	6–200 ng / l, 0.8 ng / l (with IDMS)	[31,41,42]

enrichment from de-ionized water or hydrogen peroxide as working and etching media. The only problem of hydrogen peroxide analysis is the limited chemical stability of common PS/DVB ion exchangers. Better properties show exchangers based on highly cross-linked PS/DVB combined with a different exchange site for better thermal and oxidation stability [30].

Traces in pure elements represent the next level of complexity. The level of problems is governed by the differences in the chemical behavior of traces to be analyzed and the matrix species and the difficulties of the dissolution procedure including the amount and the purity of the reagents needed for this step [13].

The analysis of complex samples such as seawater requires the use of chelating exchangers [31]. The increased selectivity allows the sensitive analysis of selected groups of analytes. The multielement capabilities are usually decreased when the separation mechanism is changed from ion exchange to chelation.

6.2. Speciation analysis

The field of speciation analysis has been covered by several reviews [6,26,27,32,33] and special issues. The basic methodology is referred to in a number of excellent reviews and monographs [5,6,26]. Most of the work before 1990 uses AAS or ICP–AES as detection unit, whereas newer applications almost exclusively use ICP–MS. Table 4 shows a summary of elements analyzed using ion chromatographic methods as speciation tool. The methodology for the selection of a separation system is based on the chemistry of the species to be separated. Permanent ionic species are most widely analyzed using ion chromatographic techniques, whereas arsenic is the most versatile trace element because of a large number of stable anionic, nonionic and cationic species. Other elements such as lead or mercury are often present as non-ionic species and therefore most likely analyzed by reversed phase and/or ion pair chromatography.

Speciation analysis is beside the elimination of interferences the most important field of coupling applications of IC–ICP–(AES,MS) because of the extended use of IC features. There are practically no alternatives to the speciation applications. The disadvantage of IC for speciation is the limited chromatographic performance of LC with a maximum of 10 compounds that can be separated in a single elution system.

6.3. Elimination of interferences

At an intermediate stage between speciation analysis, which is impossible without chromatographic separation, and preconcentration techniques, which are impossible without a powerful multielement detection system, a third group of coupling applications dealing with the elimination of interferences in atomic spectrometry and IC can be found. In this field on-line coupling IC–ICP–(AES,MS) is only one possible solution. Spectroscopic interferences are a well known problem in

Table 4
Examples for the use of on-line coupling IC–ICP–(AES,MS) for elimination of interferences at the atomic spectrometry or the IC side

Sample/matrix	Separation mode	Eliminated interference ICP	Interference IC	Comments	Refs.
As species in urine	Anion	$^{40}Ar^{35}Cl$ on ^{75}As	–	Prototype of this kind of application	[43,44]
Mo	Anion	MoO_2 on Te	Other anions	Ultra trace determination	[24]
REE, REE oxides	Cation	Oxides and hydrides of lighter REE or heavier ones	Peak overlap due to limited selectivity	HIBA, and lactic acid gradient	[28]
Transition metals in S and Cl matrices	Anion	S- and Cl-based interferences on V, Cr, Cu, Zn, As and Se	Not applicable to IC alone	Off-line application with attempts for on-line coupling	[45]
Se in human urine and serum	Anion	ArCl on ^{75}As and ^{77}Se	Other anions	Both on-line and off-line	[46]
BrO_3^- in water	Anion	Ar_2H on ^{81}Br	Cl^-, NO_2^-	High impact on drinking water	[36]
IO_3^- in water	Anion	–	Early eluting anions	Species-related	[47]
Fission products	Anion/cation	$^{90}Sr/^{90}Zr$, $^{137}Cs/^{137}Ba$, REE	Other anions/cations	Resolution of uncommon isobaric overlaps	[48]

ICP–AES and ICP–MS and several workarounds without a chromatographic technique are being developed. First attempts are simple correction equations using the natural abundances of the isotopes or reference elements for the correction of oxide and double charged ions. More sophisticated is the use of special instrumental features such as cool plasma, collision cells, other detection wavelength or a specific sample introduction system such as hydride generation [8,14].

Cases where such corrections are not successful or impossible increase the application area of on-line coupling. Main attraction for on-line coupling for the prevention from interferences is the analysis of REE (see Table 5). This is a relatively small group of analytes with a very similar chemical behavior among each other and a lot of good usable differences in chemical behavior to common concomitants. The separation of the REE is mostly done by a hydroxyisobutyric acid (HIBA) gradient using a strong acid cation exchanger. The separation of the REE is almost complete and allows the resolution of isobaric ions, of isobaric oxides and as a speciality of isobaric hydrides.

A second type of application deals with the $ArCl^+$ interferences at arsenic, which nature as single isotopic element makes mathematical corrections extremely difficult. Further application areas can be seen in the field of transition metals, whose analysis at trace levels is sometimes extremely difficult or impossible, or in the analysis of anionic traces in anionic matrices such as the refractory metals.

Newer applications deal with the determination of oxyhalides in water samples using both the superior sensitivity of ICP–MS and its element specific detection to solve selectivity and detection problems of IC (Fig. 5). The most prominent example is the determination of bromate, which may guide as a reference for the evolution cycle of on-line applications.

First IC–ICP–MS applications simply adapted an IC application by replacing the conductivity detector by ICP–MS [34]. Optimization on both parts of the coupling system increased speed, accuracy and LOD significantly. On the IC side the commonly used low capacity anion exchangers were replaced by high capacity ones with similar performance [2,35]. This allowed an increase in sample volume and lowered the LOD. The next step is the replacement of the commonly used aqueous NaOH or Na_2CO_3 eluents in concentrations up to 180 mmol/l. These eluents require chemical suppression in order to avoid large amounts of sodium reaching the ICP resulting in decreased plasma stability. Another disadvantage of these strongly basic eluents is the precipitation of alkaline earth metals as hydroxides. The elution system can be optimized with respect to the separation problem because there are almost no limitations in choosing the eluent when ICP–MS detection is applied to IC. An almost ideal eluent for ICP–MS is NH_4NO_3, which disappears completely in the plasma. From the view of IC is nitrate a good choice as eluent anion for moderately retained anions such as chloride and bromate.

The optimization on the ICP–MS side includes specific set-up for bromine determination, the selection of high efficiency nebulizer, the use of collision cell technology for the elimination of interfering molecular ions (Fig. 3) and not at least the use of isotope dilution as calibration technique [36]. The final result is an IC–ICP–MS application,

Table 5
Examples of the use of on-line coupling IC–ICP–(AES,MS) as a tool for the speciation of elements

Element	Separation modes	Separated species	Comments	Refs.
Al	Anion/cation	Al(III) and Al complex species	Chromatographic separation of fragile complexes	[49]
As	Anion/cation	As(III), As(V), anionic or cationic organic As compounds	Most popular application	[50]
Cr	Anion/cation/ion pair	Cr(III), Cr(VI)	Slow kinetics of Cr(III) cause problems	[51]
Hg	Ion pair	Cationic Hg compounds	Critical element in ICP	[52]
Pt	Anion	Anionic Pt complexes	Strong adsorbed anions, catalyst abrasive	[53]
S	Anion	S^{2-}, $SO_3{}^{2-}$, $S_2O_3{}^{2-}$, SCN^-	Detection as $^{32}S^{16}O^+$	[54]
Sb	Anion	Sb(III), Sb(V), anionic organic Sb compounds	Unidentified signals	[55]
Se	Anion	Se(IV), Se(VI), anionic organic Se compounds	Hydride generation possible	[51]
Sn	Cation	Organotin compounds	Mostly sediments	[56]

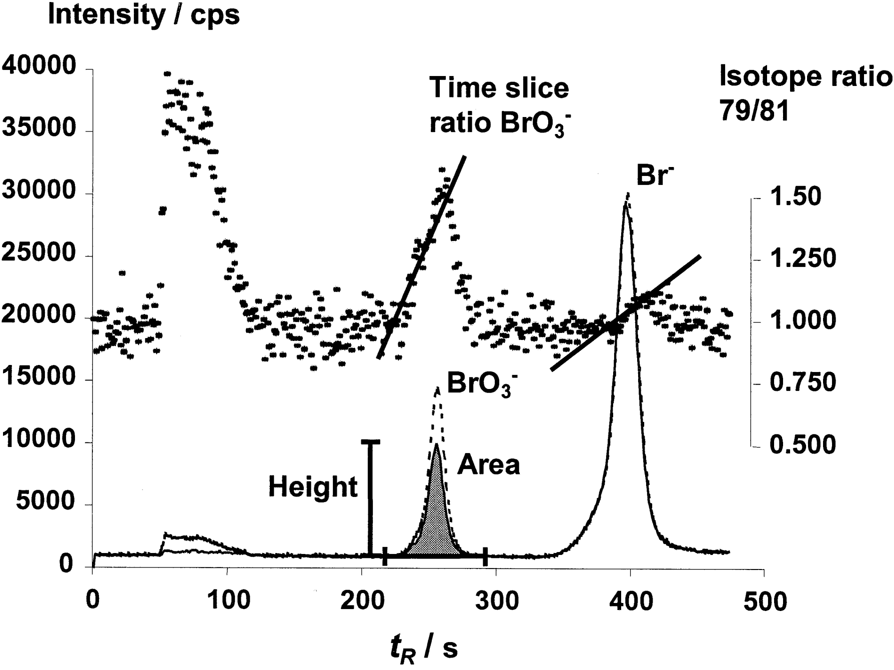

Fig. 5. Strategies for isotope dilution analysis utilizing on-line coupling IC–ICP–MS. Shown is the separation of bromate and bromide by anion IC. The sample was 585 µl of a bottled water, spiked with bromate and a bromate isotope standard. The column, eluent and detection device are as described in Fig. 4. Shown are the mass traces for *m/z* 79 and 81 (dotted line) and the total time slice isotope ratio for *m/z* 79/81.

which allows the highly accurate determination of bromate in almost every water sample within 10 min without any calibration protocol at the sub µg/l level.

6.4. IDMS applications

A specific disadvantage of coupling techniques in contrast to standard ICP–MS applications is the time consumption raising higher costs and even more deleterious a decreased accuracy and reproducibility from long-term drifts. Classical methods for the compensation of long-term drifts such as time dependent calibration functions, internal standards and post column internal standards are not really able to solve drift problems. The ideal way out to eliminate all drift problems is the use of isotope dilution measurements [36,37] as exemplary shown for the determination of bromate in water samples. Using an isotope spike of pure bromate with an enrichment of ^{81}Br generates the chromatogram shown in Fig. 5. The isotope ratio of the background as well as that of the bromide peak equals the natural ratio of approximately 1. The bromate peak shows a strong shift of the ratio, which can be used for quantitation. Detection limits in IDMS are controlled by chemical blanks, isobaric overlaps, purity of the spike isotope and the amount of analyte, which is necessary for accurate determination of the isotope ratios [37].

7. Conclusions and outlook

Today's reality of on-line coupling LC and ICP–MS can be described by a list of advantages and limitations. It should be stressed that a coupling technique is more sophisticated, more complex, more expensive and sometimes difficult to handle. Compared to a single technique it is a more powerful tool and allows trace and speciation analysis at a so far unreached level of sensitivity and selectivity.

The list of advantages includes the following points:

- simple, fast and powerful sample pretreatment technique
- very low detection limits and quite good reproducibility

- large dynamic range of ICP–AES and ICP–MS (up to five orders of magnitude)
- the ICP device is operated with a constant secondary matrix

Like every analytical method on-line coupling IC–ICP–(AES,MS) suffers from several disadvantages and limitations:

- not all elements and all species are detectable (limitations by the chemistry of the chromatography part and by the features of the spectrometer)
- time consuming technique (ranging from 2 to 20 min per sample)
- a large data volume must be handled (ca. 100–1000 data points per measurement)
- high surface area of the separation system, e.g. memory effects must be taken into consideration

Most important features of atomic spectrometry are the element specific detection and the superior sensitivity. Features such as the large dynamic range, the relative freedom from matrix effects even when atomic spectrometry is coupled to chromatography can be used more extensively to save time and to earn more accurate data using coupling techniques.

The calibration of atomic spectrometers can be handled much easier than that of conventional IC detectors using the large dynamic range of ICP techniques. Those simple off-line calibrations had been used for ICP–AES and ICP–MS in on-line preconcentration applications. With its ability to decide between isotopes the ICP–MS is well suited for isotope dilution analysis (IDMS), a calibration tool which increases the accuracy, the results and saves time due to reduced calibration work. The use of IDMS in combination with on-line coupling methods allows a significant speed up of the usually to IDMS applied time consuming separation processes.

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