Food authenticity and traceability

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Edited by
Michèle Lees

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Contents

Part II Authenticating and tracing particular foods

Contributor contact details

Chapter 1

Prof. N. Marmiroli, Dr C. Peano and Prof. E. Maestri
Dept of Environmental Science
Division of Genetic and Environmental Biotechnology
Parco Area delle Scienze 11/A
43100 Parma
Italy

Tel: +39 0521 905606
Fax: +39 0521 905665
E-mail: marmirol@unipr.it

Chapter 2

Dr J. A. Lenstra
Faculty of Veterinary Medicine
Utrecht University
PO Box 80154
3508 TD Utrecht
The Netherlands

Tel: +31 30 2534992
Fax: +31 30 2518126
E-mail: j.a.lenstra@vet.uu.nl

Chapter 3

Prof. E. Märtlbauer
University of Munich
Institute of Milk Hygiene
Veterinaerstr. 13
D-80539 Munich
Germany

Tel: + 49 89 2180 3672
Fax: +49 89 2180 2106
E-mail: E.Maertlbauer@mh.vetmed.uni-muenchen.de

Chapter 4

Dr S. Vaidyanathan and Dr R. Goodacre
Department of Chemistry
UMIST
PO Box 88
Sackville Street
Manchester M60 1QD
UK

Tel: +44 (0)161 200 4480
Fax: +44 (0)161 200 4519
E-mail: R.Goodacre@umist.ac.uk

Chapter 5

Dr I. Benson
Sales & Marketing Director
NDC Infrared Engineering
Bates Road
Maldon
Essex CM9 5FA

Tel: +44 1621 852244
Fax: +44 1621 856180
E-mail: ibenson@ndcinfrared.co.uk

Chapter 6

Dr G. Le Gall and Dr I. J. Colquhoun
Institute of Food Research
Norwich Research Park
Colney
Norwich NR4 7UA

Tel: +44 (0)1603 255353
Fax: +44 (0)1603 507723
E-mail: ian.colquhoun@bbsrc.ac.uk

Chapter 7

Dr S. D. Kelly
Institute of Food Research
Norwich Research Park
Colney
Norwich NR4 7UA
UK

Phone: +44 (0)1603 255000
Fax: +44 (0)1603 507723
Email: simon.kelly@bbsrc.ac.uk

Chapter 8

Prof. M. Meurens
Université Catholique de Louvain
Faculte d'ingénierie biologique, agronomique et environnementale
Unité de biochimie de la nutrition
Croix du Sud, 2 (8)
1348 Louvain-la-Neuve
Belgium

Tel: (32) 10 473726
Fax: (32) 10 473728
E-mail: meurens@bnut.ucl.ac.be

Chapter 9

Dr E. Forgács and Dr T. Cserháti
The Institute of Chemistry
Chemical Research Centre
Hungarian Academy of Sciences
PO Box 17
1525 Budapest
Hungary

E-mail: forgacs@chemres.hu

Chapter 10

Dr L. Nollet
Hogeschool Gent
Department of Engineering Sciences
Schoonmeersstraat 52
B9000 Gent
Belgium

Tel: 00 32 92424226
Fax: 00 32 92438777
E-mail: leo.nollet@hogent.be

Chapter 11

Prof. G. Henniger
Lindenstr. 25
D-82065 Baierbrunn

Tel/fax: (+49) 89 7930035
E-mail: guenther.henniger@t-online.de

Chapter 12

Dr P. Patel and Dr C. Beveridge
Leatherhead Food International Ltd
Randalls Road
Leatherhead
Surrey KT22 7RY
UK

Tel: (+44) 01372 822200
Fax: (+44) 01372 386228
E-mail: ppatel@leatherheadfood.com

Chapter 13

Dr R. Leardi
Department of Pharmaceutical and Food Chemistry and Technology
University of Genoa
Via Brigata Salerno (ponte)
16147 Genoa
Italy

Tel: +39 010 3532636
Fax: +39 010 3532684
E-mail:riclea@dictfa.unige.it

Chapter 14

Dr C. Sotelo and Dr R. Pérez-Martín
Instituto de Investigaciones Marinas
Eduardo Cabello 6, 36208
Vigo, Spain

Phone: +34-986-214471
Fax: +34-986-292762

Chapter 15

Dr M. Lees and Dr B. Popping
Eurofins Scientific Analytics
Rue Pierre Adolphe Bobierre
PO Box 42301
44323 Nantes Cedex 3
France

E-mail: michelelees@eurofins.com

Chapter 16

Dr F. Ulberth
European Commission
DG Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg
B-2440 Geel
Belgium

Tel: +32 14 571600
Fax: +32 14 590406
E-mail: franz.ulberth@irmm.jrc.be

Chapter 17

Dr G. Downey
Consumer Foods Department
TEAGASC
The National Food Centre
Ashtown
Dublin

Tel 353 1 805 9500
Fax: 353 1 805 9550
E-mail:
gdowney@NFC.TEAGASC.IE

Chapter 18

Dr R. Singhal and Prof. P. Kulkarni
Food & Fermentation
Technology Department
University of Mumbai
Matunga
Mumbai 400 019
India

Tel: 91 22 414 5616
Fax: 91 22 414 5614
E-mail: rekha@udct.org

Chapter 19

Dr B. Popping
Eurofins Scientific Analytics
Rue Pierre Adolphe Bobierre
PO Box 42301
44323 Nantes Cedex 3
France

Tel: +44 7768 16673
Fax: +44 870 168 8047
E-mail: bertpopping@eurofins.com

Chapter 20

Assistant Professor I. Arvanitoyannis
Department of Agriculture
Crop Production and Rural
Environment
School of Agricultural Sciences
University of Thessaly
Fytoko Str
Nea Ionia 38446
Magnesia
Greece

Tel: +30 421 93104
Fax: +30 421 93144
E-mail: parmenion@uth.gr

Chapter 21

Dr C. Morrison
Youngs Bluecrest Seafoods Ltd, UK

E-mail: cliff_morrison@biscuits.com

Chapter 22

Professor A. Furness and
Professor K. A. Osman
Centre for Automatic Identification
and Intelligent Systems
Technology Innovation Centre
Millennium Point
Birmingham B4 7XG
UK

Tel: +44 121 331 5400
Fax: +44 121 331 5401
E-mail: keith.osman@tic.ac.uk

Chapter 23

Dr M. Dillon and M. M. Thompson
Humber Institute of Food and Fisheries
Westside Road
Alexandra Dock
Grimsby
NE Lincs DN31 3TD
UK

Tel: +44 (0) 1472 500220
Fax: +44 (0) 1472 500221
E-mail: thompsonm@grimsby.ac.uk
E-mail: dillonm@grimsby.ac.uk

Chapter 24

Dr E. Larsen
Danish Institute of Fisheries Research
Technical University of Denmark
Soltofts Plads
DTU
Bygning 221
DK-2800 Kgs. Lyngby
Denmark

Tel: 45 45 25 25 46
Fax: 45 45 88 47 74
E-mail: epl@dfu.min.dk

Chapter 25

Dr S. Notermans and I. H. Beumer
TNO Nutrition and Food Research
Utrechtseweg 48
PO Box 360
3700 AJ Zeist
The Netherlands

Tel: 31 30 694 49 43
Fax: 31 30 695 49 03
E-mail: notermans@voeding.tno.nl

Chapter 26

Dr L. Pillonel and Dr J. Bosset
Federal Dairy Research Station
Chemistry and Physics Unit
Schwarzenburgstr. 161
CH-3003 BERN

Tel: 41 31 323 85 87
Fax: 41 31 323 82 87
E-mail: Jacques-olivier.bosset@fam.admin.ch

Chapter 27

A. Holst-Jensen
Section of Feed and Food Microbiology
National Veterinary Institute
Ullevaalsveien 68
PO Box 8156 Dep
0033 Oslo
Norway

Tel: +47 23 21 60 00
Fax: +47 23 21 60 01
E-mail: arne.holst-jensen@vetinst.no

The Humber Institute of Food & Fisheries

The Humber Institute of Food & Fisheries (HIFF), a Division of Grimsby College, was established because of a very real demand for expert training and consultancy within the food and fishing sectors.

A team of nationally and internationally recognised personnel currently works to provide companies with first class assistance and advice. Through its own resources and those of its partners, the Institute's consultants can advise companies on the most up-to-date practices and legal requirements within the sector and offers consultancy services over a wide range of disciplines:

- Building Cost Effective Food Control
- Improving Operational Efficiency
- Enhancing Refrigeration Performance
- Improving Business Performance

Located in the heart of Grimsby's dockland the Institute offers over 35 different courses including NVQs, Degree programmes and Post-graduate Diplomas and also has the capacity to design and deliver tailor-made programmes based on specifications from companies regionally, nationally and internationally. An advice and drop-in Food Skills Centre was created within the Institute and is available to support local small and medium enterprises who wish to research new technologies, assess potential of new software and investigate new product and process development particularly with relation to food and hygiene.

The Institute co-ordinates a responsive partnership which has recently been nominated for Centre of Vocational Excellence (CoVE) status. The Humber Food CoVE provides first/frontline management with focused techniques, information and education customised to business needs and is specifically designed to improve skills and productivity. Funding provided by the CoVE has also led to the setting up of Food Dynamo, a dedicated training factory, which provides an access point to learn practical factory skills.

In a fast moving, high profile sector, HIFF is able to provide a range of solutions to constructively enhance development of food skills and experience with an industry-focused team.

Part I

Methods for authentication and traceability

1

Advanced PCR techniques in identifying food components

N. Marmiroli, C. Peano and E. Maestri, University of Parma, Italy

1.1 Introduction

The development of fast low-cost DNA synthesis procedures, which produce a new fragment of DNA with a specific nucleotide sequence has greatly accelerated molecular cloning and DNA characterisation. The Polymerase Chain Reaction (PCR) developed by Kary Mullis (U.S. patent 4683202) has also had a major impact. The possibility of generating great quantities of DNA by amplifying fragments of genomic or cloned cDNA has greatly increased the possibility of screening gene-banks, analysing mutation, mapping chromosomes and thousands of other applications (Saiki *et al.*, 1985). The Polymerase Chain Reaction (PCR), the repetitive bi-directional DNA synthesis via primer extension of a region of nucleic acid, is simple in design and can be applied in seemingly endless ways. PCR amplification of a template requires two oligonucleotides primers, the four deoxynucleotides triphosphates (dNTPs), Magnesium ions in molar excess of the dNTPs, and a thermostable DNA polymerase to perform the DNA synthesis (Dieffenbach and Dveksler, 1995).

The PCR reaction has a great efficacy, but this must be measured also by its specificity, efficiency and accuracy that depend on a number of parameters.

In vitro DNA replication has been accomplished from many different sources (Saiki *et al.*, 1985; Mullis and Faloona, 1987; Keohavong *et al.*, 1988; Saiki *et al.*, 1988) The initial PCR procedure described by Saiki *et al.* (1985) used the Klenow fragment of *Escherichia coli* DNA polymerase I. This enzyme was heat labile and fresh enzyme had to be added during each cycle following the denaturation and primer hybridisation steps. Introduction of the thermostable

Taq polymerase, the DNA polymerase obtained from *Thermus aquaticus*, in PCR (Saiki *et al.*, 1988) resolved this problem and made possible the automation of the thermal cycling in the procedure. Virtually all forms of DNA and RNA are suitable substrates for PCR. These include genomic, plasmid, and phage DNA, previously amplified DNA, cDNA, and mRNA. Samples prepared via standard molecular methodologies (Sambrook *et al.*, 1989) are sufficiently pure for PCR, and usually no extra purification steps are required. In general the efficiency of PCR is greater for smaller-size template DNA than for high molecular weight DNA.

For many applications of PCR, primers are designed to be exactly complementary to the template. However, for other applications, such as allele specific PCR, the engineering of mutations or of new restriction endonuclease sites into a specific region of the genome, and cloning of homologous genes where sequence information is lacking, base pair mismatches are introduced either intentionally or unavoidably (Kwok *et al.*, 1995). In either case, an ideal pair of primers should hybridise efficiently to the target sequence with negligible hybridisation to other related sequences that are present in the sample. Studies have shown that different DNA polymerases have distinct characteristics that affect the efficiency of PCR. For example Taq Polymerase does not have the 3′-5′ exonuclease proofreading function, and as a result, it has a relatively high error rate in PCR (Lawyer *et al.*, 1989).

1.1.1 How PCR techniques work

The PCR reaction allows the million-fold amplification of a specific target DNA fragment framed by two primers (synthetic oligonucleotides, complementary to either one of the two strands of the target sequence). The PCR is a multiple-process with consecutive cycles of three different temperatures, where the number of target sequences grows exponentially according to the number of cycles. In each cycle (Fig. 1.1) the three temperatures correspond to three different steps in the reaction (Dieffenbach and Dveksler, 1995).

In the first step the template, the DNA serving as master copy for the DNA polymerase is separated into single strands by heat denaturation at ~95ºC. In the second step the reaction mix is cooled down to a temperature of 50–60ºC (depending on the composition of the primers used) to allow the annealing of the primers to the target sequence. Primer hybridisation is favoured over DNA/DNA hybridisation because of the excess of primers molecules. In the third step, the annealed primers are extended using a *Thermus aquaticus* (Taq) polymerase at the optimum temperature of 72ºC. With the elongation of the primers, a copy of the target sequence is generated. The cycle of these three different temperatures is then repeated from 20 to 50 times, depending on the amount of DNA present and the length of the amplicon (amplified DNA fragment).

In order to use PCR, the analyst must know the exact nucleotide sequence that flank both ends of the target DNA region. Any PCR-based detection strategy will thus depend on the selection of the oligonucleotide primers and the detailed

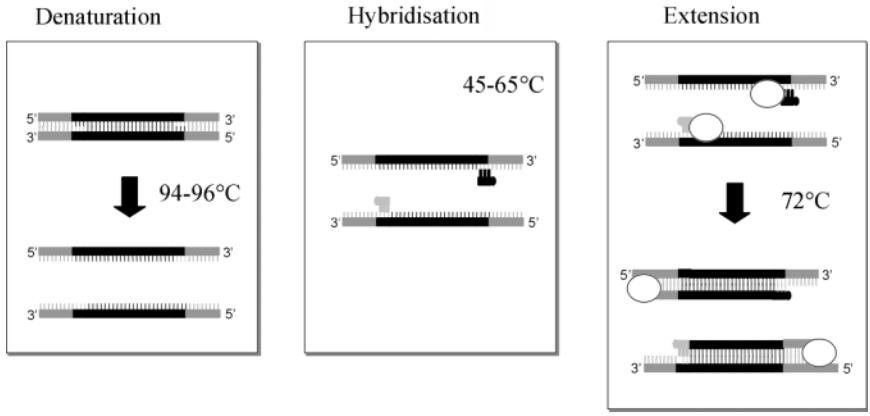

Fig. 1.1 Schematic representation of the three steps in PCR amplification: denaturation of the template DNA, hybridisation of the primers, extension by Taq polymerase. Temperatures in the hybridisation (or annealing) step may vary.

knowledge of the molecular structure and DNA sequences used. Faster cycling with better temperature control using capillaries in air heated thermal cyclers has improved PCR specificity. 'Rapid cycle' PCR requires amplification cycles of 20–60 sec and the whole procedure of amplification, 30 cycles, only 10–30 min. Rapid cycle is based on a 'kinetic' rather than an 'equilibrium' paradigm for PCR. Whereas in the equilibrium mode 3 reactions occur at 3 temperatures for 3 times during each cycle, in the kinetic mode both temperature transition and denaturation and annealing are in a constant state of change (Table 1.1).

Confirmation/verification of the identity of the amplicon is necessary to ensure that the amplified DNA really corresponds to the chosen target sequence and is not a by-product of un-specific binding of the primers. For this purpose several methods are available such as gel electrophoresis to verify if the PCR products have the expected size and purity. However, this is not always enough and the PCR products should additionally be verified for their restriction endonuclease profile. Analysing PCR products during amplification has become known as 'real-time PCR'. Even more reliable is a Southern Blot assay in which the amplicon is separated by gel electrophoresis, transferred onto a membrane

Table 1.1 Suggested temperatures and times parameters for 'rapid cycle' PCR

	Temperature (ºC)	*Time (sec)*
Denaturation	94[1]	0
Annealing	30 + 0.5	0
Extension	74[2]	0.03[3]

1. For products with GC domains consider adding DMSO or formamide and/or increasing the temperature.
2. Need to be lower for products with high AT domains.
3. For products < 100 bp an extension time of 0 sec must be used.

and hybridised to a specific DNA probe. Another possible control is to subject the PCR product to a second round of PCR. This technique is called nested PCR and exploits two different sets of primers, an outer and an inner pair that are used in two consecutive rounds of PCR amplifications. This technique reduces nearly to zero the possibilities of un-specific amplifications (Zimmermann *et al.*, 1994).

Nevertheless the most reliable way to confirm the authenticity of a PCR product is its sequencing. This method depends on DNA quality and purity. DNA quality is determined by fragment length and degree of damage. Damage may be caused by exposure to heat, low pH, nucleases that cause hydrolysis, depurination and enzymatic degradation. DNA isolated from processed foods and certain agricultural matrixes is usually of low quality and available target sequences may be rather short (Ahmed, 2002); thus an appropriate choice of primers to obtain short amplicons should be made. DNA purity can also be severely affected by various contaminants in food matrices (Ahmed, 2002). Contaminants may be substances that originate from the material under examination: polysaccharides, lipids and polyphenols or chemicals used during the DNA extraction procedure (CTAB- cetyltrimethylammonium bromide or hexadecyltrymethyl ammonium bromide). The Taq Polymerase, the key enzyme used in the PCR reaction is inhibited by contaminants such as polysaccharides, EDTA, phenol and SDS. All these compounds can thus affect the amplification reaction.

1.2 Qualitative and quantitative PCR techniques

1.2.1 Qualitative techniques

The polymerase chain reaction (PCR) has been used in many different applications because it has a very great flexibility in the field of molecular biology. Its principal use is to generate a large amount of a desired DNA product starting from a given template, but it can be used also to amplify very long fragments of DNA and in such a way to synthesise whole genes, to amplify and quantify specific RNA species, to produce RNA fingerprinting, or PCR mediated cloning, to screen DNA libraries and to produce DNA sequences.

Long-distance PCR

This method amplifies and detects routinely and specifically PCR products ranging in size from less than 1 Kb to more than 50 Kb (Foord and Rose, 1995), regardless of target template sequence or structure. The ability to amplify fragments up to 20–50 Kb enables the isolation of an entire gene from a cDNA probe, thereby obviating the time consuming task of screening a genomic library for the target gene. Large genomic fragments can be isolated from complex genomes, as well as from hybrid cell lines or from microdissected or flow-sorted chromosomal regions. Long-distance PCR facilitates the amplification of eukaryotic genomic DNA segments containing introns of varying number and lengths, thus permitting the definition of intron/exons boundaries.

In situ PCR

About 10 years ago, direct cellular localisation of a DNA or RNA target was routinely achieved by in situ hybridisation. This method has been dramatically improved in its sensitivity. However, despite these improvements the relatively high detection threshold of in situ hybridisation of about 10 copies per cell limits its usefulness (Nuovo, 1994). The advent of PCR made possible the detection also of the low copy events. With PCR one can routinely detect one copy in a background of 1 μg of total cellular DNA. Recently there has been a dramatic improvement in the technology, combining the high sensitivity of PCR with the cell localising ability of in situ hybridisation. Although in situ PCR is relatively new, many groups have published protocols and data using in situ PCR techniques (Nuovo, 1995).

PCR starting from RNA

The amplification and quantification of specific RNA species can be performed by several methods, including: RNA-PCR/RT-PCR, QC-PCR etc ... These methods detect or measure a defined RNA species, ranging from mRNAs for gene products to the level of viral RNA in plasma (Rashtchian, 1994). RNA-PCR is particularly useful if very low quantities of mRNAs are available. RNA-PCR is a good method for screening cells and tissues for the expression of an mRNA (Fig. 1.2). If quantitative information is required and mRNA amounts are limited, then RNA-PCR could be employed using a synthetic mRNA control amplified with the same set of primers as the mRNA for quantification.

RNA fingerprinting using arbitrarily primed PCR

The extension of arbitrarily primed PCR (AP-PCR) fingerprinting to RNA has resulted in a tool with exciting potential for detecting differential gene expression (Liang and Pardee, 1992). It is now possible to obtain a partially abundance-normalised sample of cDNA produced in a single tube in a few hours. Fragments of differentially expressed genes can be cloned directly from PCR-amplified products isolated from the gel. The method can provide a complex phenotype reflecting changes in the abundance of hundreds of RNAs under various conditions. Comparison of RNA fingerprinting from different treatment groups allows one to draw inferences regarding gene regulation.

PCR-mediated cloning

Under standard PCR conditions, sufficient sequence information from a template is required to design two primers that hybridise to each strand of the DNA. When attempting to clone a previously uncharacterised cDNA or gene fragment, a limited quantity of genetic sequence may be available, and thus only one primer can be designed. Under these circumstances PCR can be used to create the second site for primer annealing, making possible to clone the desired fragment (Dieffenbach and Dveksler, 1995).

A PCR-based method for screening DNA libraries

Screening DNA libraries of high complexity for rare sequences is one of the fundamental techniques of molecular biology. When applied to the screening of

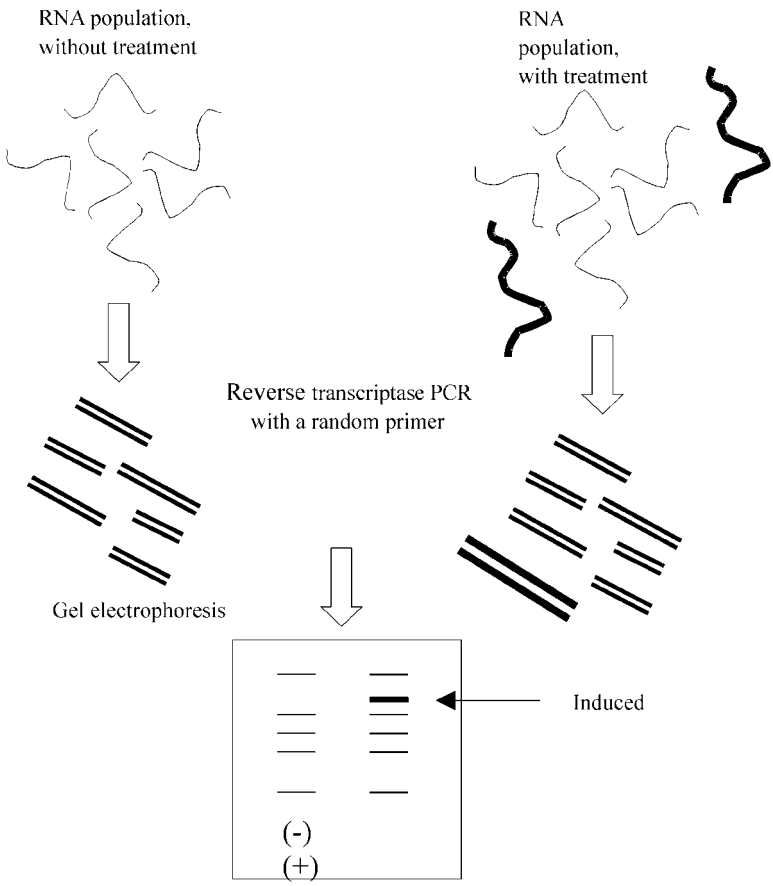

Fig. 1.2 An example of PCR starting from RNA: application of reverse transcriptase PCR to the identification of differentially expressed genes.

highly complex DNA libraries contained within either bacteriophage or plasmid vectors, PCR offers the opportunity to identify rare DNA sequences in complex mixtures of molecular clones by increasing the abundance of a particular sequence (Israel, 1993). A pool that contains the desired clone is subsequently subdivided into smaller pools, each of which is then screened by using the PCR protocol that was applied for the first screen. After several cycles of subdividing and screening, the initially rare clone is greatly enriched and can be easily obtained as a pure clone.

PCR Cycle sequencing

This technique employs a thermostable DNA polymerase in a temperature cycling format to perform multiple rounds of dideoxynucleotide sequencing on the template (Murray, 1989). The result of the temperature cycling is linear amplification of the sequencing product, leading to an increase of the signal

generated during the sequencing protocols. Cycling the sequencing reaction results in several advantages:

- the amount of template necessary for the sequencing reaction is greatly reduced
- screening reactions can be performed on minimally prepared templates
- the high temperature at which the sequencing reactions are run allows the DNA polymerase to synthesise through areas of secondary structures
- the multiple heat denaturation steps allow double stranded templates to be sequenced without a separate denaturation step.

1.2.2 Quantitative techniques

A PCR reaction profile is characterised by three segments: an early background phase, the exponential growth or log phase, and a plateau. During log phase the amplification proceeds according to equation

$$T_n = T_0(E)^n$$

where T_n is the amount of target sequence at the cycle n, T_0 is the amount of target at time zero, and E is the efficacy of amplification. A major drawback of conventional PCR is the lack of accurate quantitative information due to the effect of the amplification efficiency (*E*). If the reaction efficiency for each amplification cycle would remain constant, the concentration of DNA following PCR would be directly proportional to the amount of initial DNA target. Unfortunately, *E* is not a constant parameter, but varies between different reactions, particularly in the later cycles of PCR, when products are formed with an unknown reaction rate and in a non-logarithmic fashion. Conventional PCR relies on end point measurements, when often the reaction has gone beyond the exponential phase because of limiting reagents (Cha and Thilly, 1995). Some PCR-based techniques that address the problem of establishing a relationship between the concentration of target DNA and the amount of PCR product generated by the amplification have been recently developed. The two principal techniques of quantitative PCR in use at the moment are: QC-PCR (quantitative-competitive PCR) and real time PCR.

The QC-PCR is the co-amplification of a target analyte with an internal standard. In particular, it involves the co-amplification of unknown amounts of an internal control template in the same reaction tube by the identical primer pair (Fig. 1.3, Studer *et al.*, 1998). The reaction conditions can be maintained to generate amplification products that should not differ by more than 40 bp. Multiple PCR reactions are needed as each sample is amplified with increasing amounts of competitor, while maintaining constant the sample volume/concentration. Quantification is achieved by comparing the equivalence point at which the amplicon from the competitor gives the same signal intensity of the target DNA on stained agarose gels (Studer *et al.*, 1998; Hardegger *et al.*, 1999).

Real time PCR was originally developed in 1992 by Higuchi *et al.* (1992) and has rapidly gained popularity due to the introduction of several real-time

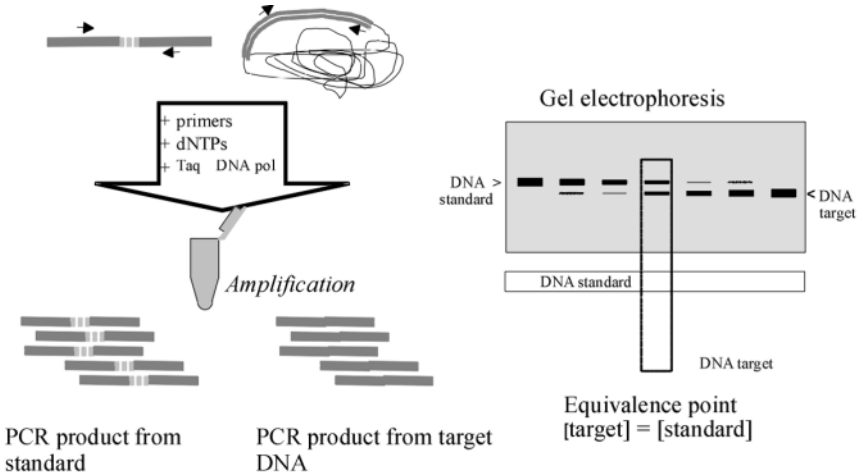

Fig. 1.3 Schematic representation of quantitative-competitive PCR. On the left, a mixture of target DNA and internal standard DNA is subjected to PCR amplification with specific primers. Different mixtures are prepared with different amounts of target DNA. On the right, results on the electrophoresis gel show the equivalence point when concentration of target DNA equals the concentration of standard DNA.

complete instruments and easy-to-use PCR assays. With this technique the amplification of the target DNA sequence can be followed during the whole reaction by the indirect monitoring of the product formation. Real time detection strategies rely on continuous measurements of the increment in fluorescence generated during the PCR reaction. The number of PCR cycles necessary to generate a signal that is significantly and statistically above noise level is taken as a quantitative measure and is called cycle threshold (Ct). As long as the Ct value is measured at the stage of the PCR where the efficiency is still constant, the Ct value is inversely proportional to the log of the initial amount of target molecules. Typical real-time PCR as performed on the LightCycler is shown in Fig. 1.4. It takes 10^{10} copies of PCR products to produce a signal above background. From the above equation it can be predicted that if one has 1000000 copies at the beginning of PCR with an efficiency of 1.9, the first signal can be seen at cycle 14. With 1000 copies, the signal is seen at cycle 25 and with a single copy at the beginning, the signal will be seen after 30 cycles. However, the reaction does not proceed linearly, but plateaus in later cycles. The reasons for this are:

- the reannealing of PCR products competing more efficiently with hybridisation of primers
- the inhibition of reactions by reaction by-products
- the limiting of the polymerising *compounds*.

The easiest and simpler way to follow amplification during PCR is fluorescence. Monitoring fluorescence each cycle is a powerful way to quantify the initial

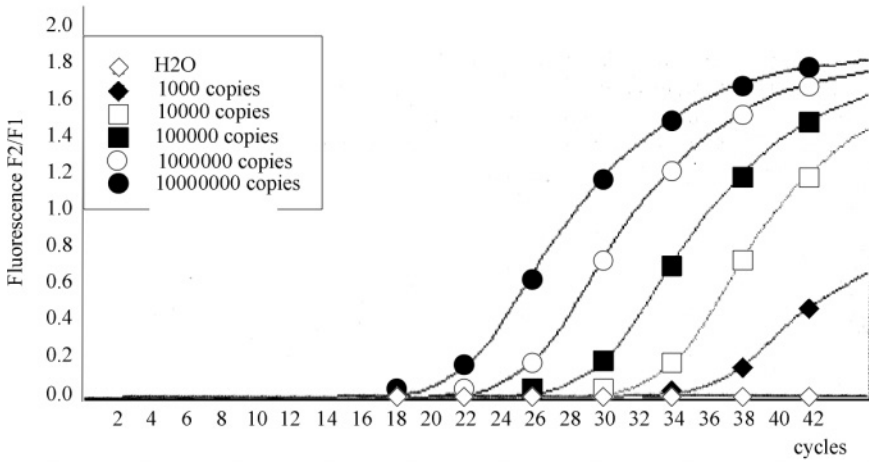

Fig. 1.4 Monitoring in real time PCR. Dilution series of purified PCR products show that the fluorescent signal increases with time according to the quantity of the amplified product.

number of templates. Many applications are based on the double strand specific dye SYBR® Green but certain applications require greatest sequence specificity. For this purpose exonuclease (TaqMam®) probes and hybridisation probes have been developed. One of the most used assays for real time PCR is the TaqMam® or 5′-exonuclease assay, which employs a fluorogenic probe consisting of an oligonucleotide with both a reporter and a quencher dye attached (Figs 1.5(c) and 1.6, Grove, 1999). When the probe is intact, the reporter fluorescence is quenched by the proximity of the quencher dye. Due to its target specific sequence, the probe anneals specifically to the amplification product (target DNA) between the forward and the reverse primers. If hybridisation has occurred, the 5′-3′ exonuclease activity of the Taq polymerase cleaves the internal probe during the extension step of amplification. The cleavage reduces the quenching effect and the fluorescent signal of the reporter dye becomes a measure of the amount of amplification product generated. The specificity of real time PCR detection is considerably higher than that of the conventional PCR since the development of the fluorogenic reporter signal takes place only if both the PCR primers and the TaqMam® probe anneal to the target DNA. The relative quantification of the target gene is made possible by preparing a standard curve from known quantities of an additional endogenous gene and extrapolating from the linear regression line. For relative GMO concentrations in food mixtures, the quantification of a GM marker has to be normalised to a plant-specific reference gene. Relative quantification might be achieved by a combination of two absolute quantification reactions: one for the GMO specific gene and a second for the plant specific reference gene (Hubner *et al.*, 2001).

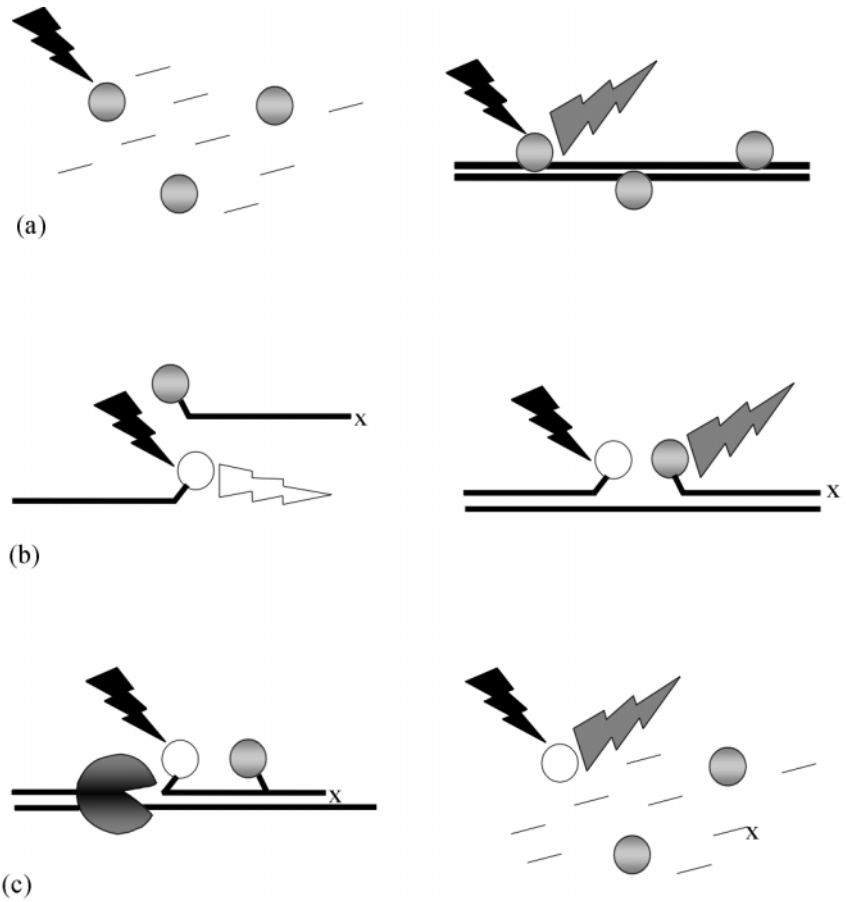

Fig. 1.5 Three probe systems compatible with the LightCycler: (a) SYBR® Green I; (b) hybridisation probes; (c) TaqMam® probes. On the left, conditions of inefficient fluorescence emission, on the right, conditions for efficient fluorescence emission.

In addition to the TaqMam® assay, various other techniques for the indirect monitoring have been recently described.

SYBR® Green

This is a dsDNA binding dye (Figs 1.5(a) and 1.6). The dye has been employed in place of ethidium bromide as a double stranded DNA dye in agarose gels to reduce background and allow better real time monitoring of product formation. It is thought to bind the minor groove of dsDNA and upon binding increases in fluorescence over 100-fold. It is important to note that at very high concentrations it starts to inhibit the PCR reaction. It binds to any type of dsDNA and no probe design is necessary. The disadvantage is that specific product, non-specific products and primer dimmers are detected with SYBR® Green (Wittwer *et al.*, 1997).

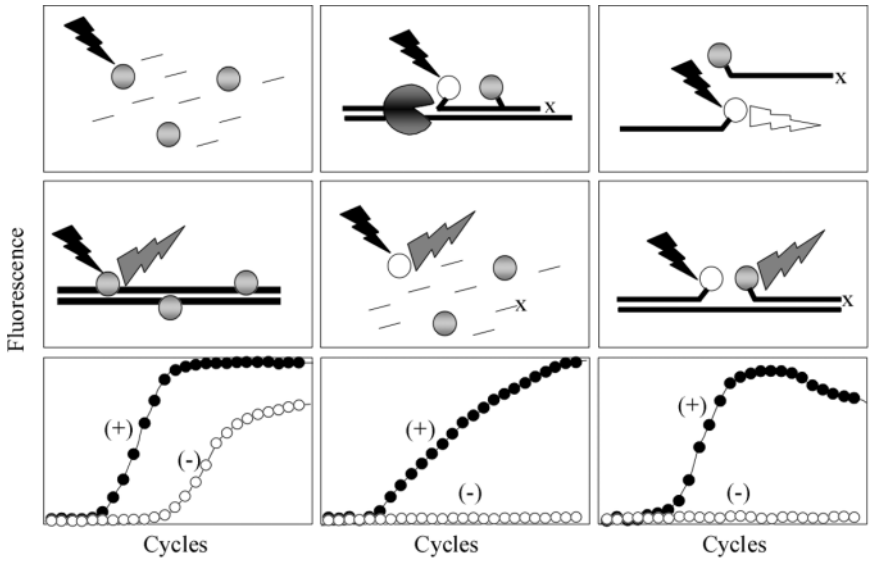

Fig. 1.6 Three fluorescence monitoring schemes for PCR, utilising three different probes: left, dsDNA dye; centre, exonuclease probe; right, hybridisation probe. Upper row, before amplification; middle row, after amplification; bottom row, monitoring each cycle of PCR by fluorescence emission.

Hybridisation probes

If sequence specific recognition is required, hybridisation probes allow detection only of the specific product. In this case two probes are designed that hybridise side by side on the PCR product (Figs 1.5(b)–1.6). The 3′ end of the upstream probe is labelled with fluorescein, which functions as a fluorescence resonance energy transfer (FRET) donor, whereas the 5′ end of the downstream probe is labelled with an acceptor dye. The FRET signal is observed only when the specific hybridisation event occurs (Wittwer *et al.*, 1997).

Rules for probe design

The LightCycler Hybridisation probe method uses two fluorescent labelled oligonucleotide probes that hybridise in a head to tail arrangement to adjacent sequences on the target DNA. Interaction between the labels occurs only when both probes are bound. As a design rule it is optimal to choose neighbouring sequences on the same strand with a gap of one to five bases. In this case the energy transfer process is distance-dependent. It is also necessary to block the 3′ termini of probes against polymerase extension and label the adjacent ends of the probes. Another recommendation is to use balanced sequences, avoid the same base stretches, the simple repetition sequence and the GC-rich sequences, as well as palindromes and complementarity within each probe. It is also necessary to avoid complementarity between probes, between the 3′ termini of the primers and the probes and avoid T_ms much greater than extension

temperature. For quantification probes it may be better to stay away from the primer in the same orientation. The T_m of the probe should be 5–10ºC higher than the T_ms of both primers. The T_m of the probes should be higher than the annealing temperature but not much higher than the extension temperature. In fact, one primer may compete with the binding of the two probes and the probes might block primer extension.

Molecular beacons
These have been successfully employed in real time PCR and for the generation of melting curves, including the multiplex PCR format, and they are widely used for discriminating single base pair differences (SNP). They may be tailored for the detection and quantification of new GM-crops that feature single nucleotide genetic modifications (Seitz, 2000). The growing number of commercially available real-time thermocyclers is an indicator of the success of this technology. Presently, real-time quantification can be considered as the more powerful tool for the detection and quantification of GMOs in a wide variety of agricultural and food products (Hubner *et al.*, 2001).

1.3 Method validation

Analytical methods used by enforcement laboratories, especially where legal proof may become necessary, should be subject to validation procedures for reliable and repeatable results. The objective of validation is to demonstrate that the defined system produces acceptably accurate, precise and reproducible results for a given analyte (Hubner *et al.*, 2001). These studies must be carried out according to harmonised international protocols and cover as a wide range of laboratories as possible, usually considered to be no fewer than eight. The choice of the parameters to be used during the validation process is determined by the intended use, as well as the nature of the method in question. Typically GMO detection will require more parameters to be carefully defined, due to the unknown concentrations of the analyte in the test samples, combined with the increasing demand to detect at lower levels and for comparison of results with labelling thresholds. Specificity, sensitivity, detection limit, matrix effect inhibition, precision and robustness have to be established for identification/screening purposes. At the same time accuracy, quantification limit, linearity, working range, coefficient of variation and measurement of uncertainty have to be evaluated for a quantitative detection system (Anklam *et al.*, 2002).

The validation parameters for a qualitative method are:

- **Specificity:** the probability to obtain a negative result given that the analyte is not present. It can be established by determining the percentage of correct classification as GMO negative of samples that does not contain GMO.
- **Sensitivity**: the probability to obtain a positive result given that the analyte is present. It is established by determining the percentage of correct classifi-

cation as GMO positive of a sample that contains GMO. Two additional controls should be used routinely: a negative control as a test of contamination and a positive control close to the detection limit as a test of sensitivity.

- **Limit of detection:** the minimum level at which the analyte can reliably be detected and by analysis of samples containing known concentrations of the analyte (Kay and Van den Eede, 2001).
- **Precision:** inter-laboratory variation should be determined by repeating the experiments over a short time and over a prolonged time period (repeatability). The inter-laboratory variations need to be determined in a ring trial (reproducibility).
- **Robustness:** the reliability of the method should be demonstrated with respect to deliberate variations in method parameters.
- **Overall Accuracy:** defined as the probability to obtain a correct result.

The definitions for the requested parameters for a quantitative method are:

- **Accuracy:** the percentage recovery by the assay of known added amount of analyte or as the difference between the mean and the accepted value of the reference material.
- **Limit of Quantification (LOQ):** determined by the analysis of known samples and establishes the minimum level at which the analyte can be quantified.
- **Linearity and working range:** to demonstrate the proportionality of the signal to the amount of the reference material it is necessary to calculate a regression line with the adequate statistical method. The linear range thereby obtained may define the working range.
- **Precision:** demonstrated in terms of repeatability, intermediate precision and reproducibility.

1.4 Advanced PCR techniques

1.4.1 Multiplex PCR

Multiplex PCR can be a two-amplicon system or it can amplify 13 or more separate regions of DNA. It may be the end point of the analysis, or it may be preliminary to further analysis such as sequencing and hybridisation. Producing some multiplex PCR systems may be as simple as combining two sets of primers for which reaction conditions have been determined separately. However, other multiplex PCRs must be developed with careful consideration for the regions to be amplified, the relative sizes of fragments, the dynamics of the primers and the optimisation of PCR technique to accommodate multiple fragments (Edwards and Gibbs, 1995). The expense of reagents and the preparation time are less in multiplex PCR than in systems where several tubes of uniplex PCRs are used. Multiplex PCR assays can be tedious and time consuming to establish and lengthy optimisation procedures such as adjusting primers concentration, Mg^{2+} concentration and amount of enzyme are often necessary. Novel developments in the reaction chemistry have

made multiplex PCR more simple and straightforward. A multiplex reaction is ideal for conserving costly polymerase and templates in short supply. Multiplex PCR methods exhibit great flexibility in experimental design and in overcoming limiting primer kinetics and fragment competition.

In the traceability field multiplex PCR could be a powerful instrument to identify at the same time and in the same sample DNA deriving from different origin (like different GMOs, or different cultivars). Typically it is used for genotyping applications where analysis of multiple markers is required such as typing of normal and genetically modified animals and plants, detection of pathogens or genetically modified organisms (GMOs) or for microsatellite analysis or even to amplify different targets of the same transgenic construct to implement the specificity of the analysis (Matsuoka *et al.*, 2001). Multiplex PCR can be a qualitative and a quantitative instrument of analysis. The use of multiplex PCR for quantitative determination is made possible by the utilisation of different reporter dyes, which can be detected separately in one reaction tube thanks to the TaqMam® chemistry. Multiplex reactions are often economical and allow accurate relative quantifications. With a Multiplex reaction a direct correlation between results of a real time PCR and percentage of GMO can be established. This reduces the variation and allows accurate data interpretation by simple statistical evaluation of the quantification results. Due to the above-mentioned advantages, multiplex real-time PCR is increasingly applied in genetic analysis.

1.4.2 PNA/LNA PCR

Peptide nucleic acid (PNA) is a DNA mimic that was originally developed as a reagent for sequence specific recognition of double stranded (ds) DNA by a conventional triple helix type principle (Nielsen *et al.*, 1991). PNA has turned out to be an extremely good mimic of DNA, forming very stable duplex hybrids with Watson-Crick complementary DNA, RNA (or PNA) oligomers. PNA has a pseudo peptide backbone. However, it is much more related to proteins (peptides) than to nucleic acids (Fig. 1.7(a)). Nevertheless PNA is biologically stable and not degraded by normal proteases and peptidases. These properties of PNA have attracted the interest of many researchers inspiring the development of a variety of techniques exploiting these properties of PNA (Nielsen, 2001). PNA clamping (Fig. 1.7(b)) has been introduced as an elegant way of modulating PCR reactions, for example, by efficient and specific inhibition of amplicons only differing by a single base mutation, which is important in genetic diagnostics (Ørum *et al.*, 1993). This technology is now gaining wider acceptance in the study, for example, of tumour mutations, as a mean of suppressing the amplification of the wild type gene in detection of low levels of oncogenes, or just for the suppression of the one allele, and most recently also in clinical diagnostic studies of genetic variations. The technique is so powerful, in fact, that it also can be used to detect single base pair gene variants on a background of up to a 100-fold excess wild type. Not surprisingly, PNAs may also be constructed as detection beacons (Fig. 1.7(c)), for example for real time monitoring of PCR reactions (Seitz, 2000). The

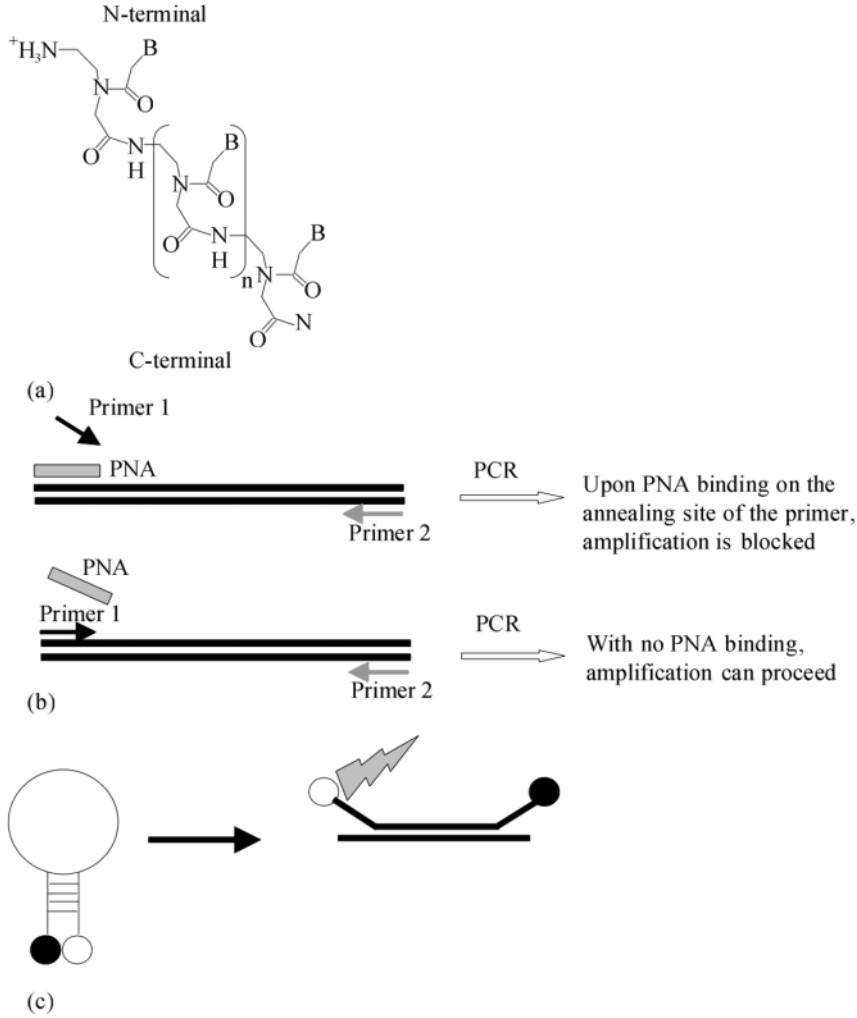

Fig. 1.7 Peptide nucleic acids and PCR. In (a) the structure of a PNA backbone. In (b), a scheme of an experiment of PNA-directed PCR clamping, in which binding of PNA blocks PCR amplification from specific primers. In (c) the concept of a PNA molecular beacon, in which the molecule fluorophore is quenched when the molecule self-anneals; upon binding to the target, the fluorophore can emit intense fluorescence.

PNA based technique such as the PCR Clamping and the PNA beacons could find also a great field of application in GMO traceability.

Locked nucleic acid (LNA™) are DNA analogs with improved hybridisation characteristics and biostability LNA oligos obey Watson-Crick base pairing and Hoogsten triplex formation rules. The LNA modification leads to enhance specificity and duplex stability with complementary nucleic acid targets (Singh *et al.*, 1998). The LNA advantages are: increasing of the thermal stability of

duplex due to its RNA-like structure, in fact LNA:LNA duplex formation constitutes the most stable Watson-Crick base pairing system yet developed. LNA enhances hybridisation performance relative to native DNA and RNA, and PNA probes, it presents a better mismatch discrimination. LNA can be designed to enhance a wide variety of molecular biology applications that rely on the use of oligonucleotides such as different kind of PCR.

1.4.3 Array and PCR

Microscopic array of oligonucleotides or cDNA containing up to several hundred thousand different sequences are starting to influence methodologies and paths to discovery in genomics (Graves, 1999). Microarrays of DNA and oligonucleotides are beginning to have the same impact on the biological sciences that integrated circuits have already produced on the physical sciences, and for similar reasons they can do many things in parallel, with very little material and with a modest investment of labour. They can be used, for example, in expression analysis, polymorphism detection, DNA resequencing and genotyping on a genomic scale (Graves, 1999). These arrays consist of many microscopic spots, each of which contains identical single stranded polymeric molecules of deoxyribonucleotide attached to a solid support such as glass or a polymer. Each spot contains many copies of a particular sequence, which can range in length from ten or twenty bases up to one or two thousands. The utility of these spots arises from the tendency of their component bases to pair up or hybridise with a second strand containing a complementary sequence. It is easy to see how an array of different sequences can be used to identify one or more pieces of DNA or RNA in a solution. These unknown sequences are all tagged by attaching a fluorescent dye to them, and then exposed to an array containing hundreds or thousands of different sequences, each in a known location. When the array is examined, one can tell which molecules are present in the solution by determining which spots fluoresce. PNA arrays could also form powerful tools for hybridisation based DNA screening assays due to some favourable features of the PNA molecules (Weiler *et al.*, 1997).

DNA chips can find a great applicability also in agricultural biotechnology. Microarrays will assist plant biotechnology companies by allowing rapid analysis of transgenic plants (Lemieux *et al.*, 1998). The link existing between microarray and PCR is great, in fact PCR product could be tested directly on DNA chip rendering easy and very fast the phase of screening even for different target sequences in the same reaction; this technique will find a great field of applicability in GMO traceability.

1.5 Applying PCR techniques: identifying genetically modified organisms in food

Genetically modified organisms (GMOs) entered the European food market in 1996. The first product to appear on UK supermarket shelves was a genetically

modified tomato puree. This product was clearly labelled and therefore anticipated the European Commission's Novel Foods Regulation (EC) No. 258/97 (European Commission 1997) established in 1997, under which products containing GMOs must be labelled if they differ substantially from their conventional counterpart (principle of substantial equivalence). Since two other products – Roundup Ready™ soybeans and Bt176 maize – were already authorised for marketing within Europe before the Novel Foods Regulation came into force, a specific labelling regulation was established in 1998 (EC) No. 1139/98 (European Commission 1998). This Regulation requires labelling if transgenic DNA or newly expressed proteins can be found. For this purpose qualitative methods for detection of GMOs are required. It is also important to investigate whether the GMO found is authorised or not; consequently, specific methods for identification of GMO are needed (Hardegger *et al.*, 1999; Ahmed, 2002). The labelling regulation was amended two years later by fixing a threshold of 1% of adventitious contamination of GM-material in a non-GM background (EC) No. 49/2000 and 50/2000 (European Commission 2000). In order to determine whether a food product or food additive contains more than 1% GMOs, quantitative analytical methods are necessary (European Commission 2001/18 (EC)).

The need to monitor and verify the presence and the amount of GMOs in agricultural crops and in food products has generated a demand of analytical methods capable of detecting, identifying and quantifying either the DNA introduced or the proteins expressed in transgenic plants. Several laboratories have developed methods based on DNA detection using the PCR technique, or based on protein detection using enzyme linked immunosorbent assays (ELISA). The development and application of reliable detection and quantitative analytical methods is essential for the implementation of labelling rules (Anklam *et al.*, 2002).

An immunological assay is based on the specific binding between a protein and antibody, and any conformational changes in the tertiary structure of the protein render the test ineffective. Such conformational changes are induced frequently during food processing and processed foods are generally analysed with the PCR method. Irrespectively of a variety of potentially available methods for DNA analysis, so far only PCR in its different formats has found broad application in GMO detection analysis as a generally accepted method for regulatory compliance. As DNA is a rather stable molecule, it is the preferred analyte for almost any kind of sample (raw materials, ingredients, processed foods). PCR, which is the most common DNA detection method, is very sensitive and therefore, very small aliquots of plant material are required for the analysis. In general traceability procedures consist of three different steps:

1. detection
2. identification
3. quantification (European Commission, 2001).

Detection (screening of GMOs)

The objective is to determine if a product contains a GMO or not. For this purpose a screening method can be used such as qualitative PCR. For routine

screening purposes one should focus on target sequences that are characteristic for the group to be screened. Genetic control elements such as the Cauliflower Mosaic Virus 35S Promoter (P-35S) and the *Agrobacterium tumefaciens nos* terminator (NOS3′) are present in many GMOs currently on the market (Hardegger *et al.*, 1999). The result is a positive/negative statement. However, a few approved GMOs are not screenable/detectable with the P-35S or the NOS3′ primers and additional target sequences are needed to guarantee a complete screening procedure, including a second phase of screening whenever necessary.

Identification

The purpose of identification is to reveal how many different GMOs are present and if they are authorised or not. The identification of DNA deriving from different varieties of GMOs can be achieved with qualitative PCR, but specific information and molecular details on the make-up of the transgenic constructs of the different GMOs have to be available. In the identification phase it is very important to know the correct sequences of the transgenic constructs contained in all the varieties of GMO analysed to design specific primers for the amplification of specific DNA sequence characteristics of each GMO. For unequivocal identification, primer selection has to be based on target sequences that are characteristic for the individual transgenic organism. Examples include the cross border regions between integration site and transformed genetic element of a specific GMO, or the cross border regions between different elements constituting the entire transgenic constructs such as promoter and intron or gene and terminator, etc.

Generally one should avoid target sequences that may occur as natural contaminants in the sample as DNA from plant viruses and bacteria, because of the risk of false positives. Only a continuous survey of all data available on GMOs, especially the introduced genetic elements and their integration sites, not only for GM products approved for the market release but also for any other GMO released for field trials worldwide, can guarantee a complete/comprehensive monitoring/detection of GMOs.

Quantification

If a food product has been shown to contain (one or more) authorised GMOs, then it becomes necessary to assess compliance with the 0.9% threshold regulation by the determination of the amount of each of the GMOs present in the individual ingredients from which it has been prepared. For this purpose it is possible to use quantitative-competitive PCR techniques or better to use real-time quantification techniques. At the moment it is possible to reliably quantify GMOs at a limit of quantification (LOQ) as low as 0.1% using real time PCR. Until now it has been possible for the companies seeking approval of their GMOs in Europe to request that the sequence information in the dossiers describing the GMO be kept confidential. Consequently unavailability of sequence data has made it difficult for many laboratories to develop detection methods. Similarly it has been difficult to obtain material of the majority of the GMOs (approved as well as non approved). Such material is absolutely

necessary to validate the detection methods. A collaborative attitude from the GMO companies to competent authorities and control laboratories, the recent revision of EC directive 90/220 (new EC directive 18/2001) and ongoing revisions of other EC directives and regulations, may lead to better access to sequence information and GMO material in the future.

Better methods for isolation of the molecules to be detected, and for qualitative and quantitative analysis of GMO are also needed and under development. Increased specificity may be obtained with:

- PCR methods targeting the junction between integration site and target DNA
- GMO specific fingerprinting methods similar to those used to identify criminals
- diagnostic microchips similar to those used for human diseases diagnosis
- possibility of multiplexing detection reaction and quantification of different GMO targets in a single reaction.

Finally, GMO analysis laboratories should participate in proficiency tests organised by independent bodies, to regularly test and demonstrate that their analyses are reliable and accurate.

Determination of GMO content

This may be defined as the ratio that the mass of GMO bears to the mass of the relevant species in pure product such as seeds. In practice this can be calculated as the percentage stemming from the ratio that the initial total quantity of DNA of the GMO bears to the initial total quantity of DNA of the species in question.

Limit of detection

This may be defined as the lowest analyte concentration that can be detected but not quantified under the test conditions specified in the method. It is often defined in comparison with the pure product or on a pure reference double-stranded DNA.

Quantification limit

This is the lowest analyte concentration or content that can be quantified with an acceptable uncertainty under the test condition specified in the method. The practical detection limit of the PCR technique is at least 1 to 20 target copies of DNA per tube, this often represents also the quantification limit.

Risk of a false negative

Finding a GMO-containing sample to be negative depends on three factors: (i) the number of seeds in the original sample, (ii) the number of particles ground for DNA extraction, and (iii) the number of templates in the PCR reaction. As an example, the probability of including a GMO particle in 10 000 seeds with 0.1% concentration is 0.9999. If 10 000 particles are used for one DNA extraction, the probability of NOT including a GMO particle is 0.001. If two extractions are done, the probability that both are negative is 0, whereas the probability that one

of the two is positive is 1. With 20 000 templates, the probability of including a GMO particle is 1. Therefore, the overall probability of detecting a GMO particle in at least one of the two reactions is

$$0.9999 \times 1 \times 1 = 0.9999, \text{that is} > 99.9\%$$

On 37 000 seeds, the probability of including a GMO particle at 0.01% concentration is 0.975. If 20 000 particles are used for one DNA extraction, the probability of NOT including a GMO particle is 0.135. If two extractions are done, the probability that both are negative is 0.018, whereas the probability that one of the two is positive is 0.982. With 40 000 templates, the probability of including a GMO particle is 0.9816. Therefore, the overall probability of detecting a GMO particle in at least one of the two reactions is

$$0.975 \times 0.982 \times 0.9816 = 0.9398, \text{that is } 94\%$$

1.6 Applying PCR techniques: molecular markers and identification of cultivar or breed

Genetic markers are heritable traits with multiple states for each trait: every diploid individual can possess only two states for each trait or locus. All types of markers give information about differences in the sequence of DNA, with a compromise between precision and convenience. Some markers could also be sensitive to environmental interferences, which can mask genetic diversity. A marker can be defined as genetic when the phenotype identified during analysis can be assigned unambiguously to one genotype. A general distinction can be made among multilocus or single-locus molecular markers. Multilocus markers usually show dominance. Specific parameters are the multiplex ratio, how many markers are generated per reaction, their information content, and how many alleles there are per marker. A high multiplex ratio is an advantage in applications aimed at typing of individuals or measuring genetic diversity, whereas high information content is required for heritability tests and mapping. A second distinction can be made among molecular markers based on hybridisation or on amplification. Advent of PCR amplification and its application to molecular markers detection have brought about several advantages: wider accessibility for specific genomic regions, requirement for lower amounts of DNA, a greater facility in operations.

Application of PCR to molecular markers technology was first initiated around 1989 with application of locus-specific PCR to amplification of microsatellites and minisatellites (Weber and May, 1989). This application was devised to simplify the detection of allelic variants at specific loci previously studied by restriction and hybridisation. Allele-specific amplification requires the design of specific primers, and therefore knowledge of the sequence to be amplified. A major breakthrough came in 1990 with the simultaneous advent of AP-PCR and RAPDs (Welsh and McClelland, 1990; Williams *et al.*,

1990). In this case the primers used in the PCR reaction have an arbitrary and precise sequence (incorrectly called random) which is not based on previous sequence knowledge of the target. There are several targets amplified for each reaction which is usually carried out at low stringency in the annealing phase, and they can be very different from each other in length, sequence, function and genomic localisation. Polymorphism can arise from the loss or gain of annealing sites for the primer, from genetic rearrangements, from insertions and deletions between the two annealing sites. The great advantage conferred by these techniques and successive modifications is the possibility of amplifying perfectly usable markers from genomes for which no previous knowledge was available. Additional advantages are the avoidance of radioactive labelling, and technical simplicity, which makes the technique usable by non-trained personnel. This is manifest in the enormous number of publications that are still produced in which RAPDs and similar markers are applied to all types of organisms for genetic mapping, measuring genetic diversity and targeting specific loci with genetic markers (Tingey and del Tufo, 1993), even if recently some scientific journals have started to reject most papers using RAPDs. The limitations of this approach are usually recognised to be the dominant characteristic of the polymorphic markers and the limited reproducibility of the technique when conditions, reagents and machines differ (Jones *et al.*, 1997). Moreover, RAPDs are almost impossible to transfer between species, even if very similar. In order to bypass these limitations, several improvements and modifications of these techniques have been developed, culminating with the invention of AFLPs (Vos *et al.*, 1995), which combine the variability mechanism at the base of RFLPs, i.e. variation in sequence evidenced by restriction cutting, with an amplification step to visualise the polymorphism. A recent review lists at least 26 different methods of genotyping that require PCR as one of the steps (Drabek, 2001).

1.6.1 PCR based molecular markers

RAPD

Random amplified polymorphic DNA (RAPDs) are in fact just one example of a whole set of PCR-based molecular markers, which have been collectively termed as MAAP, multiple arbitrary amplicon profiling (Caetano-Anollés, 1993). These approaches have in common the use of one, usually, or two primers of random sequence to amplify multibanded fingerprints from a complex genome (Fig. 1.8). The techniques differ in the length and sequence of the primers, number of amplification cycles, temperature of the annealing stage and methods for evidencing polymorphisms (Caetano-Anollés, 1993, 1994). The 'classic' RAPD technique (Williams *et al.*, 1990) uses one single primer, 10-nt long, with a GC percentage between 50 and 70%, to amplify in a low-stringency reaction (annealing at 34–36ºC) sequences encompassed by imperfect inverted repeats of the primer. This technique has been applied to all types of organisms for different purposes. Its main advantages are that it is fast, easy to perform and

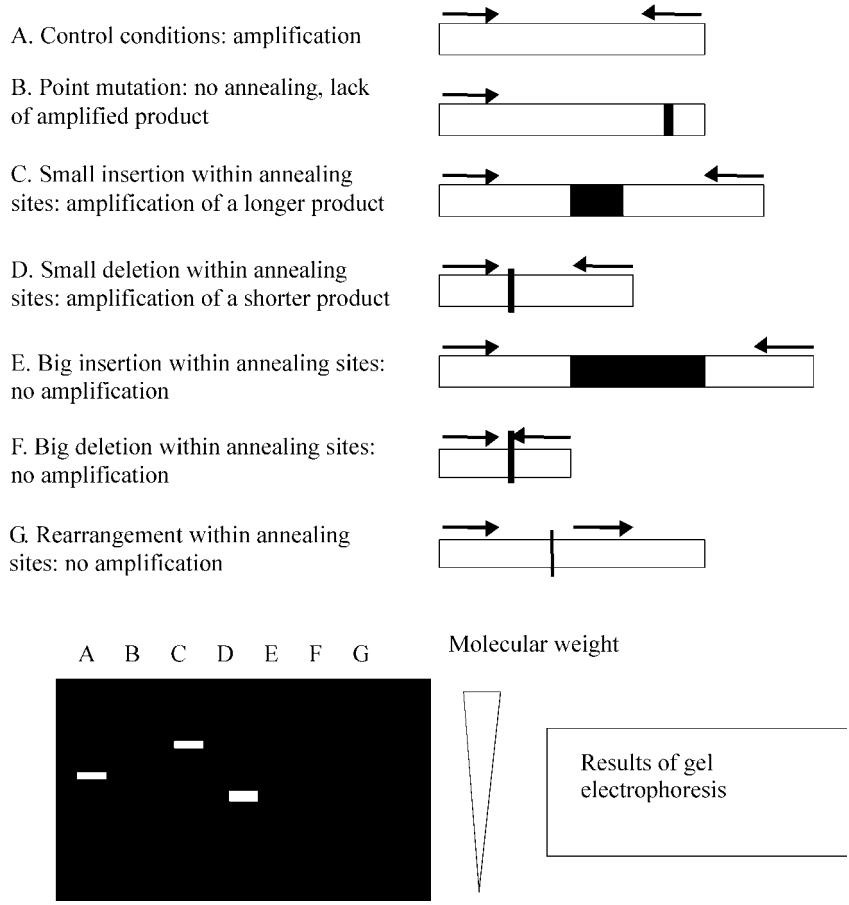

Fig. 1.8 Possible outcomes of PCR amplification from a single randomly chosen oligonucleotide in RAPD or AP-PCR technique. Results of gel electrophoresis evidence appearance or disappearance of bands in the profile according to different types of mutation in the template DNA.

requiring small amounts of DNA. The disadvantages are the dominance of RAPD markers and the poor reproducibility of RAPD patterns in different laboratories. The AP-PCR technique (Welsh and McClelland, 1990) uses a longer primer of arbitrary sequence in a reaction that includes some cycles at a lower annealing temperature. A particular modification could involve the use of two different random primers, thereby increasing the number of bands in the fingerprint (Micheli *et al.*, 1993; Hu *et al.*, 1995; Diaz and Ferrer, 2003).

SCAR

Sequence characterised amplified regions (SCARs) were first described in 1993 (Paran and Michelmore, 1993), shortly after the invention of RAPD markers. A

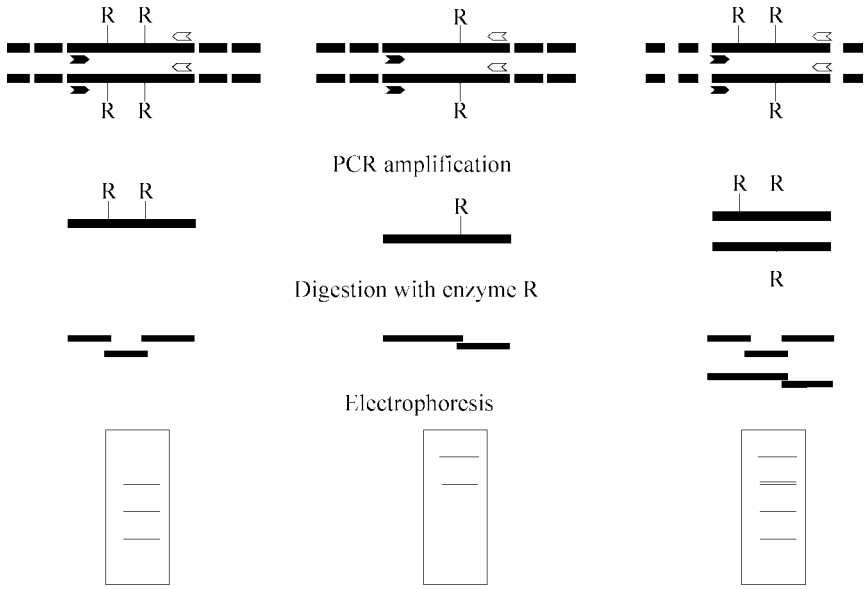

Fig. 1.9 Application of SCAR markers, PCR amplification followed by restriction with endonucleases evidences the homozygotic (left and centre) or heterozygotic (right) state of the individual.

SCAR is identified by two specific primers that amplify a well-defined genetic locus, derived by sequencing a RAPD product. This type of technique (Fig. 1.9) exploits first the advantages of RAPDs in terms of simplicity and rapidity. When the interesting RAPD markers have been singled out, they are sequenced, also partially, and the sequence information used to design long primers, 24–25 nt, to amplify that locus in a specific way. The polymorphism between SCAR bands is determined by variation in length of the sequence between the two primers, or by lack of annealing. In some cases therefore SCARs can be co-dominant. However, SCARs amplified by different individuals can also be of the same length, because the longer primers will anneal even in presence of few mismatches, as opposed to the short primers used in RAPDs. In this case, polymorphisms in the intervening sequence can be evidenced by restriction with enzymes with four-bp recognition sequences (PCR-RFLP). Not all RAPDs markers are amenable to transformation into SCAR markers. RAPD polymorphisms can also arise with different mechanisms, for example rearrangement of secondary structures during amplification rounds (Caetano-Anollés, 1993).

SSR

SSR (simple sequence repeats) are also called microsatellites, and consist of 1–10 bp units repeated in tandem, with a variable number of repetitions (Tautz, 1989). Usually they represent a single locus, which is hypervariable and highly

polymorphic in different individuals. They can be detected by amplification from specific primers annealing to the unique flanking sequences and this can make them amenable to transfer to related species. Their discovery implies the construction of genomic libraries that are then selected with oligonucleotides containing the repetitive motif and enriched in several ways. Sequencing of the positive clones yields information on the length and on the flanking sequences, which have to be unique in the genome. Since microsatellites variability derives from 'slippage' during the replication process, the same problem can also occur during the PCR reaction, producing a profile of 'stuttering' bands, differing in length of 1–5 repetitions. Detection of SSRs is accomplished with a sequencing apparatus, either on gel or in capillaries. SSRs are an example of locus-specific PCR, but they have been also transformed into a 'random' approach by employing a microsatellite sequence as primers, adding 3–4 selective bases (Inter-SSR PCR, Zietkiewicz *et al.*, 1994).

AFLP

AFLPs, amplified fragment length polymorphisms (Vos *et al.*, 1995), are based on the detection by PCR amplification of restriction fragments generated from a genome. The amplification of the restriction fragments is obtained by ligating specific adapters to the fragments' ends and utilising primers complementary to the adapters. To decrease the number of bands amplifiable from a complex genome, the primers include at the 3′ end 1–3 selective bases extending into the restriction fragments past the adapter. Therefore, a subsample of restriction fragments is 'selected' during amplification. By separating the amplification products on long polyacrylamide gels, 50–100 different bands can easily be detected and scored. The origin of polymorphism in AFLP is the same as in RFLP: changes in the restriction site sequence or insertions/deletions between two adjacent restriction sites. Additionally, polymorphisms in the adjacent bases will prevent amplification, according to the selective bases of the primers. AFLPs are also dominant markers, and they are more laborious to produce: DNA has to be of high quality and high molecular weight, the production involves several steps, and detection requires technical ability in casting the gel and staining it.

SNP

SNP, single nucleotide polymorphism, are single base changes in the DNA sequences, the most abundant type of mutation. In general, they represent biallelic systems, with two allelic forms in each locus, but systems with higher numbers of alleles have been described (Wang *et al*, 1998). The informational content is not very high, but it can be increased by the occurrence of several adjacent SNPs in one sequence. They can be identified by sequencing, and then a procedure for identifying them with PCR is designed. For instance, if the 3′-most base of one primer anneals at the SNP site, different oligonucleotides can discriminate between alleles, since only the matching primer will be able to amplify the sequence. The search for SNP can be more effectively carried out by hybridisation on chips in macro- or microarrays, using oligonucleotide probes

(Lemieux *et al.*, 1998). The advantage of SNPs is mostly in their high number and density along the genome, giving the possibility of developing haplotyping systems for genes of interest.

1.6.2 Application of molecular markers to food

The elective method for identification of DNA in food matrices has been PCR, which can be applied if there is a previous knowledge of the partial or complete sequence of some unique genetic element to be utilised as a target (Hubner *et al.*, 2001). The main aim of PCR detection in foods concerns in fact traceability of DNA fragments identifying precisely plant or animal species, cultivars, varieties or breeds. Many important species relevant for food production, both animal and plants, have been characterised for several types of molecular markers during the course of breeding programmes. This type of analysis may be required for detection of fraud or for certification of origin.

A recent application concerns the 'fingerprinting' of barley cultivars in malt. After identification of several specific sequence-tagged markers differentiating two-row and six-row malting or feed barley cultivars, PCR has been applied to bulk DNA isolated from malt allotments, allowing detection of contamination from offtype cultivars, with a resolution limit of 1% (Habernicht and Blake, 1999).

A different example can be derived from the identification of marine mammals (seal, whale) in processed seafood products performed with species-diagnostic molecular markers, including RAPDs (Martinez and Danielsdottir, 2000). Similarly, a polymorphism in a specific gene affecting coat colour allowed detection in cheese of milk coming from a particular breed, not allowed in the production of Registered Designation of Origin cheeses (Maudet and Taberlet, 2002).

A specific problem as already mentioned, is the traceability of GMOs. The main objective in this case is to facilitate withdrawal of products, should an unforeseen risk to human health or the environment be established, targeted monitoring of potential effects on human health or the environment, and control and verification of labelling claims. In order to recognise transgenic DNA in food matrices, a minimal prerequisite is the complete or partial knowledge of their DNA sequences. In reality it is difficult to achieve this knowledge for every single gene involved in transgenic procedures, whereas it is simpler for some of the DNA sequences involved in regulation of expression: CaMV 35S promoter, polyadenylation signals, NOS terminator are just some examples. Other suitable targets may be the sequences of the selection markers which are introduced in the constructs and used for selecting transgenic plants during their development. In these cases, the targets for PCR amplification are fragments which are common to many different transgenic plants, checked with the help of internal controls.

For these and similar applications, PCR has several advantages in comparison with other methods (e.g. analysis of specific proteins) but it also has some drawbacks:

1. It needs the presence in the sample of DNA with some integrity in order to allow the amplification, which is commonly performed using 18–25 nucleotide primers.
2. It is extremely sensitive to environmental conditions and both false positives and negatives can be produced by adventitious contamination and by the presence of inhibitors in the PCR reaction.
3. A limited stability after the formation of the primer-DNA complexes can give false positives and negatives.

The first issue in the search for DNA in food products concerns sampling. Key issues include: the type of material to be sampled (raw materials, ingredients, finished processed products); and the dimensions of the sample (sub-samples from a greater sample or several small independent samples). For each material there are specific heterogeneity problems due to mixing between different sources of material, which have to be taken into consideration during sampling. The amount of sampled material is also subjected to variation according to the purpose of the analysis. Moreover, each food product to be marketed has a particular production and processing chain, divided into phases in which sampling can be performed, especially when separate ingredients are involved.

A necessary prerequisite of every type of PCR amplification is the extraction of high quality DNA from the sample, avoiding additional contamination and the likely causes of DNA degradation. For this reason, every traceability analysis requires amplification assays utilising as targets known genes of the plant species. The success in amplification of fragments from these 'reference' genes guarantees the good quality of the sample DNA. DNA extraction techniques from complex matrices such as those found in food products require extensive improvement. Moreover, it is absolutely necessary that DNA is conserved during the different food chain phases. For some food products, preparation involves drastic extraction procedures, heating and other treatments which could denature or degrade the DNA making an amplification technique unsuccessful, if it is based upon the availability of complete sequences spanning some hundreds base pairs: examples are oils, fats and refined sugars. It is also possible that matrices to be analysed contain substances which inhibit the PCR reaction. These interfering substances could be removed with purification procedures, which however can decrease the final amount of DNA isolated.

Some technological improvement have increased the applicability of PCR to the analysis of DNA in the food chain by molecular markers and by molecular fingerprinting (Hubner *et al.*, 2001; Matsuoka *et al.*, 2001). Real time PCR, and the LightCycler technology have been utilised to assess DNA degradation at different stages of food processing and to identify and quantify DNA in food samples (Al Robaiy *et al.*, 2001). If PCR amplification originates a product of the expected length, based on previous sequence information, several validation steps are required. The amplified product must be subjected to restriction analysis, nested PCR or Southern blot hybridisation in order to confirm its identity, to identify 'false positives'. 'False negatives' must be instead identified

by parallel control amplification performed on specific and diagnostic genes for the species of interest, such as soy lectin or maize zein: an unsuccessful amplification in this case suggests the presence of PCR inhibiting substances.

1.7 Future trends: PCR and identity preservation of foods

Identity preservation (IP) differs from traceability, since it concerns a complete system for production, handling, and marketing structured to maintain the integrity and purity of agricultural commodities. It is a process by which a crop is grown, handled, processed and delivered under controlled conditions, in such a way that the end user of the product is assured that it has maintained its unique identity from farm to end-use. It is particularly important when a crop has unique properties and quality traits, and its marketing has to maintain its added value. A different aspect of IP concerns organic products, to be produced following specific criteria and controls and to be marketed in segregation from other products. Finally, IP has acquired a new meaning with the advent of biotechnologies, since in some countries labelling laws may require that GM crops have to be kept segregated from other crops. IP programmes have therefore to certify the identity and composition of agricultural commodities, with a complex system of standards, records, and auditing throughout the entire process of crop production, including harvesting, handling, and marketing. Seed purity and certification is the first step. However it is generally recognised that no system can guarantee 100% pure seed free of any contamination: tolerance of contamination depends on the purity standard required for the final product, since seed purity must be equal or higher. Planting of seeds requires knowledge about the previous crops cultivated on the same site, as incompatibilities may be generated. During growth, crops must be kept isolated from potential pollen sources, with practices that depend on the pollination biology of the crop itself. All equipment must be controlled and inspected to avoid any possible contamination. Sampling and analysis are required at different stages to ensure the identification and the purity level. It is very important to ensure representative sampling and reliability of testing techniques.

The role of PCR-based techniques in testing for IP processes concerns mainly GM crops, since it is relatively easy to devise suitable targets for detection of the presence or absence of GM materials in commodities. It has been estimated (Bullock *et al.*, 2000) that a quantification of the GM content in corn can be attained by a PCR test allowing assessment of the total percentage of GM corn at $370 per sample. A qualitative test to prove by PCR the absence of all GM events available in the USA but not approved for import in the EU would for instance add $900 to the cost of segregation between GM and non-GM corn (Bullock *et al.*, 2000). DNA testing can be also applied for IP programmes of other commodities, as can be the case of the IP of rice varieties produced in California (Sundstrom *et al.*, 2002): special types of rice are becoming increasingly important and fulfil the needs of different market niches. It may be

necessary to certify that different varieties of rice have not been mixed and all rice varieties will have to be provided with an IP certification system according to rice certification regulations. Attributes that can be certified include verifiable characteristics, including those traits that can be verified with a DNA test, such as herbicide resistance.

1.8 References

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2

DNA methods for identifying plant and animal species in food

J. A. Lenstra, Utrecht University, The Netherlands

2.1 Introduction

The verification of the authenticity of food starts at the species level. Prevention of mislabelling or undeclared admixture, either deliberate or accidental, is relevant for economic reasons as well as for public health and for respecting religious beliefs. In addition, wildlife management often requires the identification of animal remains (Murray *et al.*, 1995; Baker *et al.*, 1996; Hsieh *et al.*, 2001). Furthermore, the same method can be useful for the verification of the origin of cell types (Parodi *et al.*, 2002).

Conventional methods for species identification are based on immunoassays with antisera raised against food extracts or on the generation of species-specific protein electrophoresis patterns (Rehbein *et al.*, 1995; Cooke, 1995). In more modern methods, the species origin is identified by the detection of specific DNA sequences (Lenstra *et al.*, 2001; Popping, 2002), which has the following advantages:

- The experimental behaviour of DNA is predictable and does not depend on the species, which allows the use of universal assay formats.
- DNA is relatively stable and can be tested in samples that have been heated to up to 120ºC.
- The diversity of DNA allows the differentiation of closely related species or even of subspecies or populations.

The first DNA-based methods for species identification of meat samples were based on DNA hybridisation to species-specific probes. In the future this may become relevant again if implemented in a microarray test format. However, most current methods are based on PCR, which offers the advantages of

versatility and extreme sensitivity. In the following sections we elaborate the various methods used for meat species identification and review similar methods for the analysis of dairy products, feedstuff, fish, plants and cell lines.

2.1.1 DNA isolation procedures

Several methods for lysis of tissue and subsequent purifying of DNA can be found in the standard books on DNA methodology. A common procedure for animal tissues is a lysis by proteinase K and sodium dodecyl sulphate, followed by removal of the proteins by phenol/chloroform organic and precipitation of DNA by alcohol. Adaptations of these methods to isolation of DNA from food for PCR assays have been reported (Dickinson *et al.*, 1995). Lysis of plant material is often accomplished by CTAB (hexadecyltrimethylammonium bromide). However, these methods are too laborious for routine tests. For hybridisation assays DNA can be extracted by a rapid alkaline treatment (Buntjer *et al.*, 1995). This yields single-stranded DNA (6 to 15 μg DNA/g meat), which can be spotted directly on a positively charged nylon membrane for hybridisation. Several kits are now available for fast purification of double-stranded DNA, which exploit the specific binding of DNA to proprietary resins under chaotropic conditions. Wang *et al.* (2000), Lahiff *et al.* (2001) and Prado *et al.* (2002) optimised the extraction of DNA from meat and bone meals. As a positive control on the DNA isolation, Meyer *et al.* (1994a) developed primers for a highly conserved region of the 18S rDNA gene, which generate a 137-bp product.

2.2 Meat species identification

2.2.1 Hybridisation methods

In the first DNA-based meat identification tests, genomic DNA was used as a species-specific hybridisation probe (Bauer *et al.*, 1987; Chikuni *et al.*, 1990; Winterø *et al.*, 1990; Ebbehøj *et al.*, 1991). The subsequent development of probes derived from the species-specific satellite repetitive DNA improved the specificity of the assay and allowed the detection of admixtures of 5% or less. Most species-specific probes are derived from the tandem repeated satellite DNA, which accounts for up to 20% of the total genome and has a species-specific sequence.

Three hybridisation procedures with satellite-specific probes (Buntjer *et al.*, 1995, 1999; Hunt *et al.*, 1997; Janssen *et al.*, 1998; Lenstra and Buntjer, 1999) have been reported. Oligonucleotide probes can be coupled directly to alkaline phosphatase (Buntjer *et al.*, 1995, 1999) or digoxigenin (Hunt *et al.*, 1997) and detected by immunochemical procedures. Longer probes (200–1000 bp) can be prepared and labelled with digoxigenin by PCR amplification of satellite DNA segments (Janssen *et al.*, 1998). These probes are less sensitive to variations in the hybridisation conditions than oligonucleotides and have been validated for the most common meat species.

The sensitivity for admixtures was in the range of 1 to 5%. The chief limitation of the satellite hybridisation procedure is that it does not differentiate closely related species (like sheep and goat, bovine species, deer species) and that probes have to be developed for each species to be detected (Waye and Haigh, 1992).

2.2.2 PCR

As in clinical genetics, most DNA-based assays in food inspection are based on PCR amplification, which is driven by the extension of two primers in opposite orientations. Thus one primer generates the substrate for the other and vice versa, which results in a chain reaction. Since this may be initiated by only a small amount of template DNA, PCR is inherently sensitive, while the requirement of the binding of two primes ensures a high specificity. The main drawback of PCR is that the occurrence of false-positives caused by contamination of the reagents is difficult to control.

For differentiation of animal species, mitochondrial DNA is the obvious choice because it has accumulated about ten times as many mutations per generation as nuclear DNA and is present in thousands of copies per cell. So amplification of a mitochondrial DNA is relatively sensitive and generates a DNA fragment with several species-specific mutations. However, the maternally inherited mitochondrial DNA may give misleading results in case of species hybridisation, which can be detected by analysis of satellite DNA (Nijman *et al.*, 1999; Nijman and Lenstra 2001; Verkaar *et al.*, 2001, see below) or other nuclear markers (Nijman *et al.*, 2003). Further, it should be checked that there is no interference by (co)amplification of copies of mitochondrial DNA integrated in the nuclear genome (Perna and Kocher, 1996; Wolf *et al.*, 1999a; Partis *et al.*, 2000).

2.2.3 PCR-sequencing

The most straightforward method for detection of species-specific mutation is sequencing. Originally this was termed FINS for forensic informative nucleotide sequencing (Bartlett and Davidson, 1992). It has been applied to the identification of whale and dolphin products (Baker *et al.*, 1996), exotic meat species (Forrest and Carnegie, 1994) or of remains of endangered mammals (Hsieh *et al.*, 2001). A BLAST search in the Genbank will identify immediately the species or the most related species for which a homologous sequence is available. However, sequencing is not suitable for analysing samples of mixed species composition.

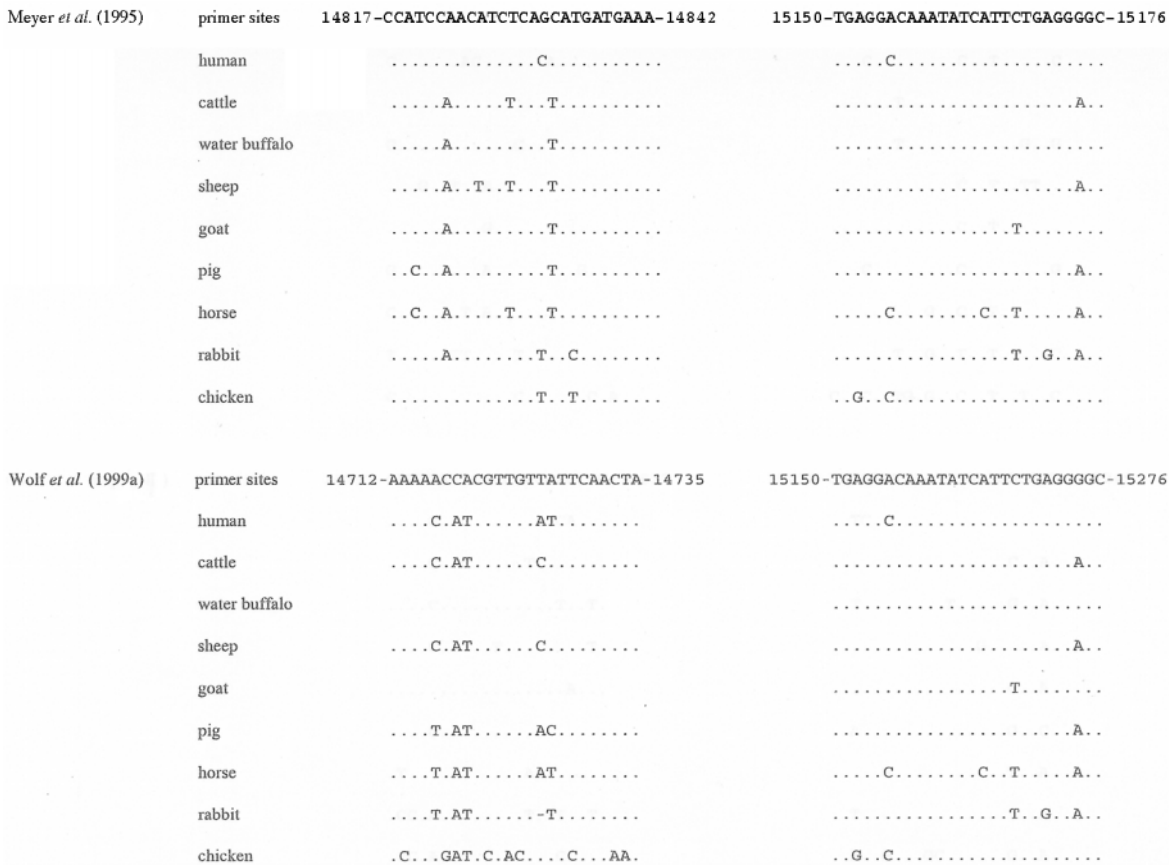
2.2.4 PCR-RFLP on mitochondrial DNA

A convenient strategy for the differentiation of a panel of known sequences is PCR-RFLP (restriction enzyme length polymorphism): PCR, digestion with an

enzyme having a recognition sequence that is either created or abolished by the mutation, and analysis of the cleavage pattern by agarose gel electrophoresis. This method is also suitable for the detection of admixtures with a sensitivity of about 5%. The most common target gene for the PCR-RFLP assays is the mitochondrial cytochrome *b* (Meyer *et al.*, 1995, 1996; Burgener and Hübner, 1998; Verkaar *et al.*, 2001). The primers described originally by Kocher *et al.* (1989) yield a 359-bp fragment in all species relevant for food inspection, which can be differentiated by restriction digestion (Meyer *et al.*, 1995, 1996; Partis *et al.*, 2000; Abdulmawjood and Bülte, 2002). Amplification of a slightly longer fragment reduced interference by nuclear copies of the mitochondrial sequence and allowed PCR-RFLP identification of several game species (Burgener and Hübner, 1998; Wolf *et al.*, 1999a). Verkaar *et al.* (2001) optimised the primers for beef-producing species. Murray *et al.* (1995) differentiated various ungulates by PCR-FLP of the mitochondrial D-loop, while a similar assay for authenticitation of snails has been based on sequence variation in the mitochondrial 12S and 16S rRNA regions (Borgo *et al.*, 1996; Abdulmawjood and Bülte, 2001).

PCR-RFLP provides a universal format for species detection and, better than methods based on species-specific primers (see below), allows discrimination between substitution and admixture. However, there are a number of caveats (Verkaar *et al.*, 2001):

1. The primers do not match completely the mitochondrial DNA of all species to be analysed. Figure 2.1 indicates the relative positions and mismatches of generic primers (Meyer *et al.*, 1995; Wolf *et al.*, 1999a) and of primers designed for bovine species (Verkaar *et al.*, 2001) or for bovid and cervid species (Van Cann, unpublished laboratory results), respectively. In general, mismatches near the 3′-OH end of the primer have more influence than near the 5′-OH end and may cause a preferential amplification of one of the components in a sample of mixed origin (Partis *et al.*, 2000). So for analysis of admixture, the effect of mismatches should be checked and, if necessary, primers should be designed that amplify all components of the mixture equally well.
2. It should also be verified that the diagnostic restriction sites are not variable within the species (Wolf *et al.*, 1999a). For instance, the restriction site reported to discriminate wild boar and domestic pigs (Meyer *et al.*, 1995) reflects intraspecies polymorphism (Kijas *et al.*, 1998). Furthermore, a *Hin*fI site described to be diagnostic for cattle is not present in the cytochrome *b* gene of related bovine species (Verkaar *et al.*, 2001). So at least two diagnostic restriction sites should be used for an unambiguous identification.
3. Failure of restriction digestion for technical reasons may lead to a wrong assignment. So material from the appropriate reference animals should be analysed in the same experiment and again, identification should be based on at least two sites.



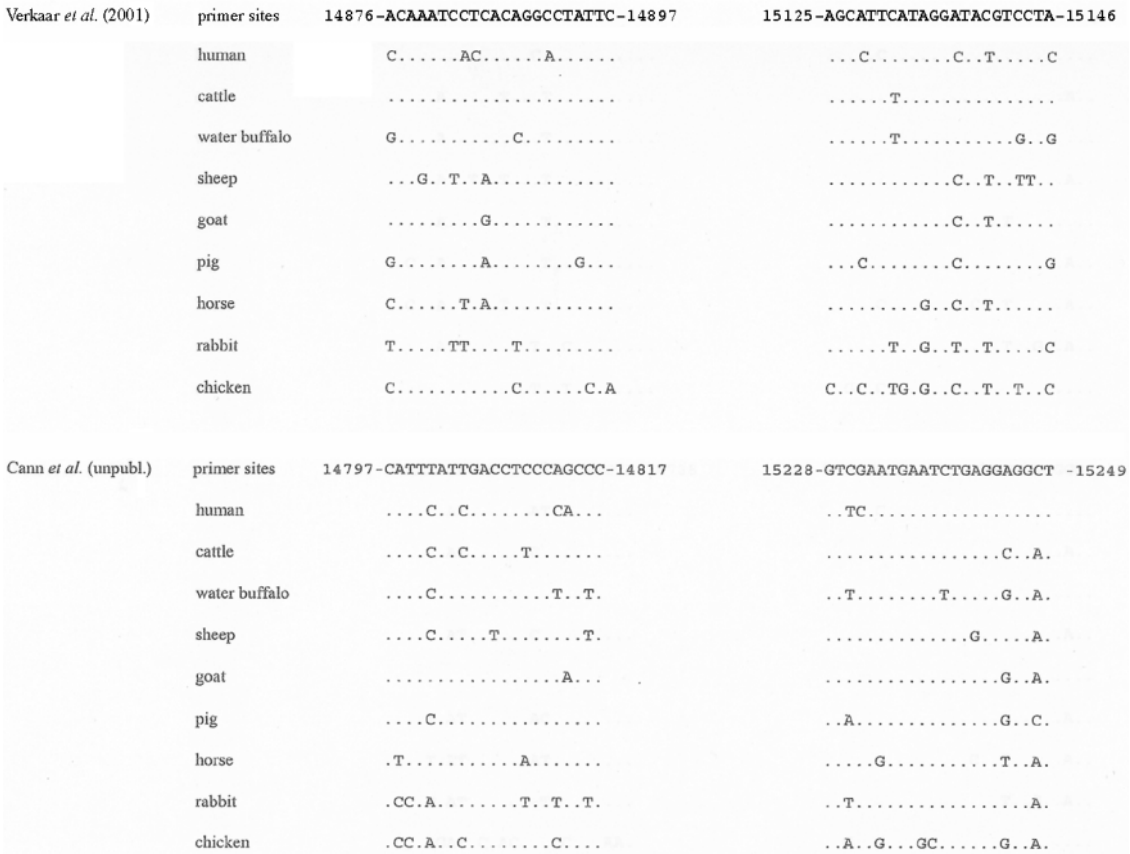


Fig. 2.1 Mismatches of primers for amplification of cytochrome *b* sequences described by Meyer *et al.* (1995), Wolf *et al.* (1999a), Verkaar *et al.* (2001) and Van Cann (unpublished laboratory results) to primer binding sites of the indicated species. For the right primers, the complementary sequences are shown. Dots indicate matches to the human sequence (Genbank). The position numbers refer to the same Genbank entry.

With the appropriate controls PCR-RFLP is, however, a fast and convenient method of species detection and requires substantial investments in equipment only for high-throughput implementations or for quantification of the restriction patterns.

2.2.5 Species-specific PCR on mitochondrial DNA

Special mitochondrial PCRs without subsequent restriction enzyme digestion have been designed for the differentiation of ostrich and emu meat (Colombo *et al.*, 2000; see also Abdulmawjood *et al.*, 2002) or for a sensitive detection of porcine material (Montiel-Sosa *et al.*, 2000). Dedicated methods for detecting bovine mitochondrial DNA have been developed for the inspection of feeding stuff (see below). In general, these approaches require a careful check of the amplification of traces of DNA from the reagents, which in PCR-RFLP assays with generic primers are suppressed by the amplification of the sample DNA. Matsunaga *et al.* (1999) described a competitive multiplex PCR with one generic primer and six primers specific for cattle, sheep, goat, pig, horse and chicken, respectively, which generates amplicons of different lengths for the six respective species.

2.2.6 PCR of repetitive elements and the detection of hybrid species

Because if their abundance in the genome, repetitive elements are also suitable targets for sensitive PCR assays. Generally, interspersed SINE repeats are specific for a mammalian order or suborder, while centromeric satellite tandem repeats are unique for the species. Both categories of repetitive elements typically occupy 5 to 20% of the total genome. Detection of pork has been based on the amplification of the porcine SSPRE SINE element (Calvo *et al.*, 2001, 2002a) and assays for beef has been based on the bovine satellite IV (Guoli *et al.*, 1999; Calvo *et al.*, 2002b). Similar assays have been used for feeding stuff (see below) and are most useful in a quantitative format in order to differentiate between positive samples and insignificant contaminations

Cross-fertile species have similar satellite DNA, but the frequencies of mutations in the repeat unit depend on the species (Chikuni *et al.*, 1994; Nijman and Lenstra, 2001). This can be visualised by restriction digestion and forms the basis of the SFLP (satellite fragment length polymorphism). In SFLP assays, the ratio of repeat units with and without restriction site differentiates related species and their hybrids. This has been implemented for African taurine cattle-zebu hybrids (Nijman *et al.*, 1999), for South-East Asian zebu-banteng hybrids (Nijman *et al.*, 2003) and for sheep-goat hybrids (Nijman *et al.*, 2002). Verkaar *et al.* (2001) described an SFLP-based differentiation of bison and cattle beef as complementary to the mitochondrial PCR-RLP test. In this case, tests based on maternally transmitted mitochondrial DNA are misleading if the bison individual carries the taurine mitochondrial genotype as a consequence of taurine introgression in the American bison population. Since SFLP relies on the

simultaneous amplification of several heterogeneous repeat units, it is less suitable for the analysis of critical DNA samples than mitochondrial PCR assays.

Naito *et al.* (1992) generated species-specific patterns for forensic purposes using primers for the ribosomal RNA gene. Buntjer and Lenstra (1998) used primers specific for the mammalian MIR interspersed repeat element for generating species-specific fingerprints. Species identification then relies on an automatic matching of the pattern with patterns in a database. It was shown that species identification was possible with autoclaved meat samples, but the test was not suitable for the detection of admixtures.

2.2.7 Single-copy nuclear genes

Although mutations in any gene are informative for species identification, assays based on single-copy genes are inherently less sensitive than assays based on mitochondrial DNA or on repetitive elements. However, the copy number of both mitochondrial and repeated DNA is variable and for quantitative assays nuclear assays may be a better measure of the amount of animal tissue. Furthermore, variation in nuclear DNA with effect on gene expression offers the option to relate the test to phenotypic variation, which is relevant for differentiation at the sub-species level (see section 2.4.2).

Meyer *et al.* (1994a) developed primers for the porcine growth hormone gene in order to detect 2% or less pork in beef. Using the TaqMam®, or 5′-nuclease assay, Laube *et al.* (2001) have described a quantitative PCR assay of the bovine phosphodiesterase gene. As positive control, an amplification was carried out with primers specific for conserved regions of the mammalian and avian myostatin genes.

2.3 Identifying species in dairy products, feedstuff and fish

Dairy products produced in Europe originate from either taurine cattle, water buffalo, sheep or goat. Plath *et al.* (1997) used restriction sites in the ovine or caprine β-casein in order to detect cattle or water-buffalo material in ewe or goat cheese. More sensitive is the mitochondrial PCR-RFLP assay (Meyer *et al.*, 1995), which discriminates between all four species (Branciari *et al.*, 2000). Less than 0.1% of cattle milk used during the manufacturing of goat cheese can be detected by PCR with primers specific for the cattle mitochondrial control region (Maudet and Taberlet, 2001). Duplex PCR methods with three (Rea *et al.*, 2001) or four (Bottero *et al.*, 2001) primers have been designed for verifying the buffalo origin of mozzarella cheese.

2.3.1 Analysis of feedstuff

The use of sheep material in feedstuff for cattle has probably caused the recent emergence and spread of BSE (bovine spongiform encephalopathy and has led

to a ban on material from any mammalian species in feedstuff. This has created a need for sensitive tests on feedstuff for detecting the species-specific DNA after extreme heating during the destruction of the animal material to be included in the feedstuff. Although the conditions prescribed for the inactivation of the infective prions (133ºC for 20 min, 3 bar) are likely also to degrade the DNA, in practice heating has not always been carried out according to the regulations.

Bellagamba *et al.* (2001) and Kingombe *et al.* (2001) applied the PCR-RFLP methods with generic primers developed by Meyer *et al.* (1995) and Wolf *et al.* (1999a), respectively. However, this is not likely to achieve an optimal sensitivity because of the mismatches of the primers and the length of the amplification products (359 and 454 bp, respectively). Kingombe *et al.* (2001) also reported positive results with the competitive multipex PCR of Matsunaga *et al.* (1999), generating a 274 amplicon for bovine DNA.

Tartaglia *et al.* (1998) targeted a test of feedstuff towards a 271 bp fragment of the bovine mitochondrial ATPase subunit. This test was combined with a rapid DNA extraction protocol with Chelex-100 (Wang *et al.*, 2000). Validated results showed that 0.125% bovine material could be detected (Myer *et al.*, 2001; Krcmar *et al.*, 2001). Similar tests on the porcine, ovine and chicken mitochondrial ATPase genes have been designed by Lahiff *et al.* (2001).

An alternative method for species detection was based on the amplification of ruminant, porcine and chicken-specific SINE repeats (Tajima *et al.*, 2002) and detected in test samples admixtures of 0.01% or more.

However, scoring the success of the amplification on agarose gel does not differentiate between substantial admixtures and insignificant contaminations. Therefore, Lahiff *et al.* (2002) developed a quantitative real-time TaqMam® PCR assay of the bovine 271-bp ATPase gene fragment, reaching a detection limit of 0.001%. However, Frezza *et al.* (1993) showed that amplification of a shorter DNA fragment of the ATPase subunit gene improved the performance of the PCR with samples subjected to extreme heat treatment.

In order to detect DNA from any ruminant with an optimised sensitivity, Verkaar (unpublished) developed as a complementary assay a TaqMam® assay on an 88-bp fragment from the relatively homogeneous and ruminant-specific Bov-A2 SINE repeat. This assay had the predicted specificity and detected 10 fg DNA in feedstuff samples, but has to be validated in practice.

2.3.2 Fish species

As with meat species, protein-based methods for fish species identification (Rehbein *et al.*, 1995) do not differentiate related species and are unsuitable for products that have been subjected to heat treatment. Bossier (1999) reviewed several DNA based methods. DNA fingerprints generated by AFLP and RAPD are informative for the species and are less likely to be disturbed by admixtures than with meat species. However, both methods are not suitable for fragmented DNA extracted from food products. In addition, AFLP is rather laborious and

RAPD is notoriously irreproducible. Most DNA-based methods for fish species identification are again based on the amplification of a mitochondrial DNA fragment, followed by the detection of species-specific mutations. Since only a single species has to be detected, the methods for fish appear to be rather tolerant with regard to the use of generic PCR primers that match only partially the target sequence.

Mutation detection can be accomplished directly by sequencing (Unseld *et al.*, 1995; Bartlett and Davidson 1991), but this is expensive for screening of many samples. Species-specific PCR primers have been used for differentiation of caviar from sturgeon species (De Salle and Birnstein 1996; Birstein *et al.*, 1998). SSCP (Bossier 1999; Rehbein *et al*, 1997; Céspedes *et al.*, 1999a) has the advantage that mutations that do not change a restriction pattern can also be detected, but require carefully controlled electrophoresis conditions and generated patterns with low information content.

Not surprisingly, PCR-RFLP is also the most widely used method for fish. The use of the generic cytochrome *b* primers of Meyer *et al.* (1995) has been demonstrated for tuna (Bartlett and Davidson, 1991), flatfish (Céspedes *et al.*, 1998a), salmon and trout (Carrera *et al.*, 1998). Partially overlapping primers have been described for differentiation of snappers (Chow *et al.*, 1993) and tuna (Chow and Inoue, 1993). Several publications describe methods based on the cytochrome *b* primers of Burgener and Hübner (1998), which generate a 464 bp product for sturgeon (Wolf *et al.*, 1999b), salmon (Russel *et al.*, 2000; Hold *et al.*, 2001a), flatfish species (Sotelo *et al.*, 2001) and several other fish (Hold *et al.*, 2001b).

However, for canned products better results are obtained with primers that generate shorter fragments of the mitochondrial cytochrome *b* genes from tuna (Ram *et al.*, 1996; Quinteiro *et al.*, 1998), flatfish (Céspedes *et al.*, 1998b) or sardines (Jérôme *et al.*, 2003). A 156-bp fragment from the mitochondrial control region was amplified for the PCR-RFPL analysis of related hake species (Quinteiro *et al.*, 2001). Primers specific for the mitochondrial 12S gene from snails (Borgo *et al.*, 1996) appeared also suitable for flatfish (Céspedes *et al.*, 2000) and shrimps (Bouchon *et al.*, 1994).

As mentioned, differentiation of salmon and trout has been accomplished by PCR-RFLP of cytochrome *b* (Carrera *et al.*, 1998; Russell *et al.*, 2000; Hold *et al.*, 2001a), but also by PCR-RFLP of the mitochondrial 16S and cytochrome oxidase genes (Carrera *et al.*, 1999a, 1999b, 1999c) as well as by PCR-RFLP of the nuclear *p53* gene (Carrera *et al.*, 2000). This may provide additional evidence in cases of criminal prosecution. Likewise, several assays are now available for flatfish: mitochondrial PCR-RFLP assays using cytochrome *b* fragments (Céspedes *et al.*, 1998a, 1998b; Sotelo *et al.*, 2001) or a 12S fragment (Céspedes *et al.*, 1999b, 2000) and PCR of the nuclear 5S rDNA gene (Céspedes *et al.*, 1999c). The last gene has also been used for differentiation of perch, grouper and wreck fish (Asensio *et al.*, 2001).

2.4 Identifying plant species, cell lines and animal breeds

Plant identification is usually at the subspecies (cultivar) level rather than at the species level. Cooke (1995) reviewed the available methods for the identification of plant varieties that are based on protein electrophoresis and predicted a wider use of DNA-based methods. In plant genetics DNA fingerprinting by ALFP and RAPD are used more widely than in animal genetics. Wünsch and Hormaza (2002) review the use of RFLP, RAPD, AFLP and microsatellites for fruit species. Legumes (Weder, 2002) and rice (Verma *et al.*, 1999) have been differentiated by RAPD. Microsatellites have been used for the typing of potatoes (Ashkenazi *et al*., 2001) and rice (Olufowote *et al.*, 1997; Popping, 2002). However, differentiation on the basis of organelle DNA is more robust. The chloroplast *rbcL* has proven to be informative for within-species variation (Dumolin-Lapegue *et al.*, 1997) and fruit species differentiation (Popping, 2002). An alternative target for differentiation of plant species is the 5S ribosomal RNA gene (Ko and Henry, 1996).

Discrimination between durum-wheat, the component of authentic pasta, and the common hexaploid wheat for bread via amplification of a durum-specific repeat sequence has been described by Bryan *et al.* (1998). Shen *et al.* (1998) have developed an allele-specific PCR as well as a PCR-RFLP for identification of beet species based on the variation in the internal transcribed sequence (ITS1) of the nuclear rDNA gene and proposed that this is also suitable for other crops.

DNA-based methods for food testing have also been developed for the detection of allergens of plant species. Detection of wheat, barley and rye in gluten-free food has been accomplished by quantitative competitive PCR with primers specific for the chloroplast *trnL* gene (Dahinden *et al.*, 2001). Meyer *et al.* (1994b) designed a PCR of the lectin *Le1* gene in order to detect 1% or more soya in meat. Holzhauser *et al.* (2000, 2002) developed highly sensitive (10 ppm) methods for the detection of hazelnut residues in food based on the amplification of the gene encoding the major hazelnut allergen.

2.4.1 Cell lines

The same methods that are suitable for food specie detection can be used for the verification of the origin of laboratory cell lines, which occasionally are overgrown after contamination with better growing cells. Parodi *et al.* (2002) developed primer pairs specific for the mitochondrial cytochrome c oxidase subunit I of human, cat, dog, mouse, rat, horse, rabbit, African Green monkey and the Chinese hamster cytochrome *b* gene.

2.4.2 Animal breeds

Testing the breed identity of meat species would be useful for complete traceability of the food chain and for food products for which authenticity depends on the breed or region, like several local European beef and cheese products. However, farm animal breeds are genetically much less homogeneous

than plant cultivars and have typically 70–80% of the total genetic diversity of the species.

Assignment of samples to breeds can be accomplished by typing with a large panel of microsatellites if the allele frequencies in the breeds to be differentiated have been established. It is estimated that up to 98% of the chicken samples from 20 breeds can be assigned on the basis of 27 microsatellite genotypes per animal (Rosenberg *et al.*, 2001). Ciampolini *et al.* (2000) was able to discriminate Holstein-Friesian cattle from Italian beef breeds on the basis of typing with a panel of 20 microsatellites.

An unambiguous discrimination between breeds is possible if genes that encode the breed phenotype have been identified. This has been demonstrated for the melanocortin receptor (MC1R) gene, which determines coat colour and has an allele specific for the wild boar (Kijas *et al.*, 1998). Maudet and Taberlet (2002) used an MCR1 allele associated with the black coat colour of cattle to detect 1% or more Holstein milk in French cheeses for which the Registered Designation of Origin (RDO) does not allow the use of Holstein milk.

Methods for sexing of beef on the basis of Y-chromosomal sequences have been surveyed by Zeleny and Schimmel (2002). Two methods, an amplification of the *AMELX/AMELY* genes and a duplex PCR on bovine satellite 1.715 and the Y-chromosomal repeat OY11.1 have been evaluated in a ring trial, in which only 1 of our 375 samples was misidentified (Zeleny *et al.*, 2002).

2.5 Comparison and validation of methods

The choice between the several available methods depends both on the specific application and on considerations of convenience. Satellite hybridisation is suitable for screening many samples simultaneously, but has a limited flexibility. For PCR, the mitochondrial or chloroplast DNA offers a better sensitivity than nuclear single-copy genes DNA. For many applications generic primers can be used or primers can be adapted to the species to be differentiated. PCR-RFLP is the most simple and flexible method for detection of species-specific mutations. Sequencing of the PCR product is more expensive, but is much less likely to be disturbed by intraspecies variation.

Amplification of species-specific nuclear elements, preferably in combination with online detection, is often suitable for sensitive detection of a given species. Assays based on single-copy nuclear genes can be informative for the phenotype of the individual animal or plant that has been the source of the food sample.

2.5.1 Considerations of validation

The large diversity of biological materials to be analysed almost precludes a rigid standardisation of the protocols and the establishment of validation parameters. However, the standard of test accuracy that is required for criminal

prosecution can be accomplished by using appropriate positive and negative controls according to normal scientific standards, by performing independent assays and by testing methods in ring trials.

For all applications a representative sampling is crucial and tests should have good reproducibility. Further, all qualitative and quantitative DNA-based tests depend only on the inherently variable DNA content of the different components of the samples. For instance, in samples containing equal amounts of muscle and liver the latter component will be over-represented in the DNA.

Many applications of DNA-based species identification require only the identification of the major species component(s). If the state of the sample permits the extraction of amplifiable DNA, the specificity is limited only by the taxonomic range of the mutations that are detected. With regard to sensitivity, PCR-RFLP usually allows a detection level of 5% admixture, which is above the level that is expected for fraudulent species substitution. However, selectivity, i.e., the detection of species in the presence of material from other species, needs to be checked since the use of generic PCR primers may very well lead to a preferential amplification of one of the components of a mixture. This can be prevented by using primers dedicated to the detection of a given set of species and checking their performance in reference samples of mixed composition.

Different considerations of validation apply if the presence of a species has to be excluded, for instance if consumption of this species imposes a health risk or is forbidden on religious grounds. These tests need to give quantitative results and to be validated with regard to sensitivity (detection level), specificity, selectivity and linearity.

2.6 Future trends

Technological advances continually offer new possibilities. Ongoing trends appear to lead to, first, the use of high-through automatic sequencing for species identification; secondly, the development of more quantitative PCR tests that are based on online fluorescent monitoring of the PCR reaction; and thirdly, the development of more tests on the subspecies level, either by typing of anonymous genetic markers (microsatellites, AFLP) or by detection of mutations that are associated with the breed. Still to be initiated for species detection is the use of microarrays of species-specific sequences. An array of sequences derived from the species-specific repetitive element may for many applications allow the direct testing of food extracts without any DNA amplification.

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3

Enzyme immunoassays for identifying animal species in food

E. Märtlbauer, University of Munich, Germany

3.1 Introduction

Besides biomedical research and clinical chemistry, enzyme immunoassays have been used in a broad range of applications in food analysis, including analytes of low molecular weight such as mycotoxins, anabolics, antimicrobial drugs, pesticides and vitamins, as well as macromolecules like bacterial toxins, enzymes, hormones, food proteins, and even living organisms including bacteria and moulds (Fukal and Kas, 1989, Hitchcock, 1988). This chapter is focused on enzyme immunoassays for the detection of proteins occurring in food that allow either the identification of the animal species or give an indication on adulteration of processed food. After a general introduction about antibody production and the principles underlying all immunochemical methods, the most prevalent assay formats are described briefly. The second part of the chapter tries to give an overview to the applicability of enzyme immunoassays with particular emphasis on food of animal origin. Although during the last decade molecular biological methods have been adopted in this area of food analysis, immunoassays still show advantages in terms of speed together with the simplicity of manipulation involved in the procedure (good practicability) and good cost-effectiveness. This situation is reflected by numerous publications about the application of enzyme immunoassays to prove the authenticity of meat and meat products, milk and milk products and to a lesser extent of other food. After discussing the main advantages and disadvantages of enzyme immunoassays, additional information for further reading is given including internet addresses containing information on available commercial products.

3.2 Principles of enzyme immunoassays

Immunochemical methods are based on the ability of antibodies (immunoglobulins) to recognise three-dimensional structures and play a major role in biochemical research. Being primarily a part of the immune system in most classes of vertebrates (Stanworth and Turner, 1979), immunoglobulins have been utilised as the key substances in any immunoassay for more than 40 years now. Molecules capable of inducing the production of immunoglobulins in a certain species are called immunogens or antigens. An immunogen must have a minimum size (molecular weight of about 5000 daltons) and contain structures which are 'foreign' to the challenged animal species. Haptens on the other hand are substances which do not elicit an immunological response but are able to react with antibodies. Antibodies against haptens are induced by using artificial immunogens, e.g., haptens coupled to a carrier protein (macromolecule).

Antibodies represent a group of glycoproteins possessing two distinct types of polypeptide chains linked by both covalent and non-covalent bonds. Both the light chain and the heavy chain show a variable region at their amino terminal end of about 110 amino acid residues, whereas the remaining part of the polypeptide chain is referred to as the constant region. The variable regions of both chains contain a hypervariable part which represents the antigen binding site (antibody combining site) or 'paratope'. After folding and combining of the light and heavy chains, this hypervariable region of the immunoglobulin shows a structure complementary to the corresponding part of the antigen molecule, which is referred to as antigenic determinant or 'epitope'. The antibodies produced in an animal species are polyclonal in nature because usually a number of B-lymphocytes is stimulated by the immunisation process. The antibodies are then synthesised and secreted by plasma cells, derived from these lymphocytes. In 1975, Köhler and Milstein developed a technique in which antibody secreting lymphocytes are fused with myeloma cells, a type of B-cell tumour. The myeloma cells provide the genes for continued cell division, whereas the lymphocytes provide the functional immunoglobulin genes. The fused cells, which possess the properties of both parent cell lines, are called hybrid cells or hybridomas, and produce monoclonal antibodies (Goding, 1986; Peters and Baumgarten, 1992). The main advantages of monoclonal compared with polyclonal antibodies are unrestricted availability once a cell line is established, homogeneity, and often improved specificity. Recent advances permitting manipulation of antibody genes using recombinant DNA techniques may offer additional advantages for specific applications in the future (Lillehoj and Malik, 1993).

The reversible association between antibodies and their corresponding antigens is called the immunological reaction. The binding forces involved are weak molecular interactions like Coulomb- and Van der Waals forces, as well as hydrogen bonds and hydrophobic binding. The antigen-antibody reaction is based on the law of mass action, and the amount of antigen or antibody present in the reaction mixture may be inferred from the extent of the reaction.

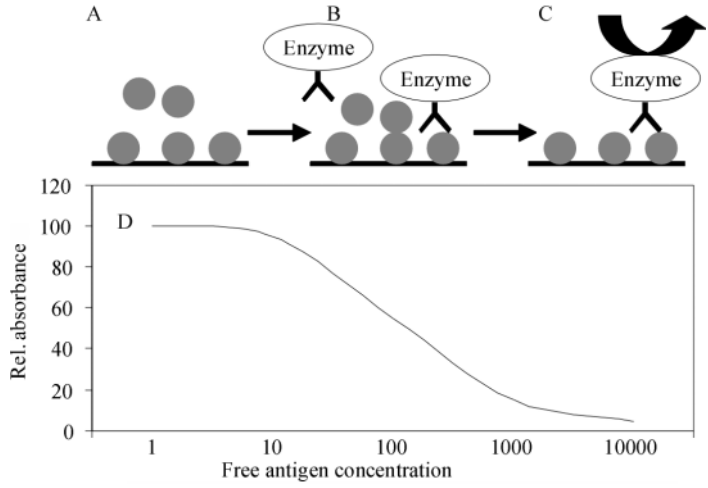

Fig. 3.1 Competitive enzyme immunoassay.
In the first step the microtiter plate is coated with a known amount of target antigen. Then the unknown sample is added (A). After addition of the enzyme labelled antibody, free and solid-phase bound antigen compete for the antibody combining sites (B), unbound reagents are removed by a washing step. In the last step, enzyme substrate and chromogen are added, and the colour formation is estimated visually or by using a photometer (C). The assay response is inversely proportional to the concentration of the free antigen (D).

To permit a sensitive observation of the antigen-antibody reaction, either labelled antigens or labelled antibodies are used, thus improving the sensitivity of immunoassays by several orders of magnitude compared to marker-free methods such as simple agglutination techniques. After introduction of enzymes as labels in the early 1970s the enzyme immunoassay has become the most important immunochemical method in food analysis.

Concerning the principle of the method, a distinction is to be made between competitive methods and non-competitive methods. Competitive methods are based on the competition of free and labelled or solid phase bound antigen for a limited number of antibody combining sites. In most cases the assay response represents the bound labelled antigen, but any other measure of the distribution of the labelled antigen is in principle possible. In a typical competitive enzyme immunoassay (see Fig. 3.1), free and solid phase bound antigen compete for a limited number of antibody combining sites. Using a solid support (e.g. a microtitre plate) provides the possibility to remove all reagents, which are not bound to the solid phase antigen, by a 'washing' step. The assay response represents the solid phase bound antigen, and is therefore inversely proportional to the concentration of the free antigen.

In non-competitive methods a limited number of antigen molecules is bound by the specific (partly) labelled antibodies. In most cases, the assay response is represented by the bound labelled antibody, in order to obtain highest sensitivity. Two typical variants of this type of assay are mainly used in food analysis. The so-called sandwich enzyme immunoassay (see Fig. 3.2) can be used only for the

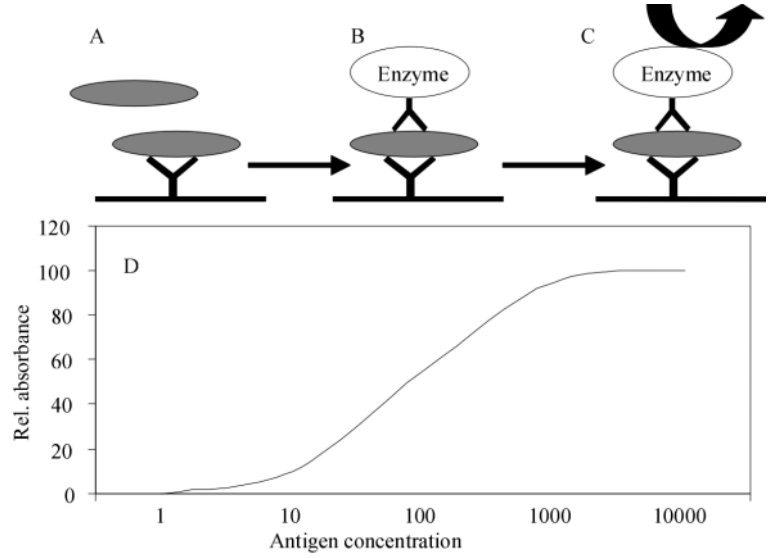

Fig. 3.2 Non-competitive enzyme immunoassay (sandwich assay). In the first step the sample is added to the microtiter plate coated with specific antibodies (A). Then the enzyme labelled antibody is added and allowed to bind to the antigen (B). After a washing step, enzyme substrate and chromogen are added and the colour formation is estimated visually or by using a photometer (C). The assay response is directly proportional to the concentration of the antigen (D).

detection of macromolecules, such as proteins, having at least two antigenic determinants in suitable steric positions, enabling two antibodies to bind to the antigen. In both variants, the target antigen is bound by the specific antibodies. In the first type of assay, a part of the antibodies (capture antibody) is bound to a solid-phase (microtitre plate) as the immunosorbent, so all the reagents that are not bound by the antibody can be easily removed by 'washing' the solid phase. The other part of the specific antibodies (enzyme-labelled) is subsequently added, in order to monitor the extent of the immunological reaction. In the second variant (see Fig. 3.3) the solid phase is coated directly with the antigen and the amount of antigen bound is determined using the specific enzyme-labelled antibodies. In both cases the assay response is directly proportional to the concentration of the target antigen. The antibody used for the detection of the bound antigen may be directly labelled with a suitable enzyme. Alternatively, a second antibody-enzyme-conjugate, enzyme-conjugated protein A or an antibody-biotin conjugate detected with an avidin-enzyme conjugate could be used.

For the detection of macromolecules both the competitive and the non-competitive assay format may be used. For the detection of low molecular compounds that possess only one antibody binding site (epitope) the competitive assay format is mandatory. To provide distinction between unreacted and complexed reactands most assays use either antibody or antigen bound to a solid-phase as the immunosorbent. So all the reagents, which are not specifically bound by the antibody can be easily removed by 'washing' the solid phase.

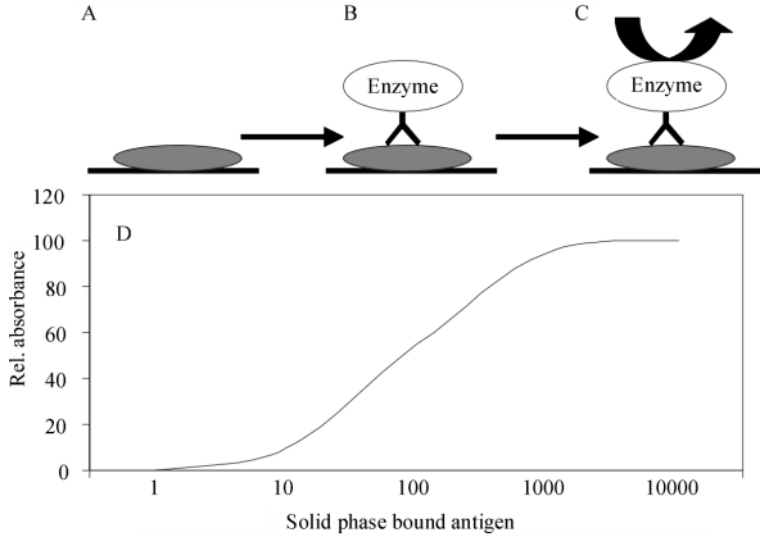

Fig. 3.3 Non-competitive enzyme immunoassay (antigen coating). The microtiter plate is coated directly with the sample extract solution (A). If the sample contains the target antigen, the specific enzyme labelled antibody is bound to the solid phase via the target antigen (B), and – after a washing step – enzyme substrate and chromogen are added and the colour formation is estimated visually or by using a photometer (C). The assay response is directly proportional to the concentration of the solid phase bound antigen (D).

3.2.1 Assay formats

The most prevalent test format of enzyme immunoassays is still the microtitre plate assay, which is usually performed employing (semi-)automated absorbance measurement and calculation of the results. Depending on the individual test specificity these assays are either quantitative or qualitative methods which can easily be performed in routine laboratories. Although a range of rapid on-site immunochemical tests in various test formats has been developed in clinical chemistry, in food analysis only few such tests have been described. Some of the most promising approaches use membrane-based test devices either in a dipstick enzyme immunoassay or in a flow-through 'immunofiltration' system. These tests have been basically designed as visual tests which require only low-cost instrumentation, some are even self-contained.

Dipstick tests use either membranes (nitrocellulose, nylon, etc.) or plastic materials as the solid phase. Depending on the pre-treatment of the solid support, antigens or antibodies are bound covalently or just adsorbed by multiple non-covalent bonds on the surface of the membrane as dots or lines. The tests give qualitative or semi-quantitative results in a relatively short time (<1 h). The working steps involved in this procedure are essentially the same as for the microtitre plate assay, except that usually the antibody coated membrane is incubated in the individual test solutions rather than pipetting the solutions into the wells of a plate.

A further reduction of assay time was achieved by employing immunochemical, membrane-based flow-through systems, in which unbound reagents and sample matrices are removed by absorbance through a cellulose pad. Usually, the tests are performed in a plastic test device, in which antibody-coated nylon membranes are pressed tightly to an absorbent cellulose layer. Sample extract solution, antibody-enzyme-conjugate, and enzyme substrate/chromogen solution are sequentially added onto the membrane. The test can be evaluated visually by comparing the intensity of the resulting coloured dot with that of a negative control. Qualitative results can be obtained within 10 min. These assays find their ideal application as an on-site test to detect the presence of the target molecule at a defined threshold level.

3.2.2 Sensitivity and specificity

The potential sensitivity of any immunoassay is directly related to the affinity of the antibody and may be calculated if the equilibrium constant is known. Since the antigen-antibody reaction may be described using reaction kinetics as well as thermodynamic equations, reaction time and temperature also influence assay sensitivity. Relying on antibody affinity there is a significant difference between competitive and non-competitive methods. In practice, non-competitive methods may show sensitivities which are one or two orders of magnitude greater then comparable competitive assays (Jackson and Ekins, 1986). The dominant factor causing the difference in sensitivity between both assay formats is the use of excess reagents in non-competitive assays. This theoretical advantage of non-competitive over competitive assays, however, can only be utilised if markers with high specific activity, such as horseradish peroxidase or alkaline phosphatase, are used for the labelling procedure.

Next to sensitivity, the specificity of an immunochemical method is important for the performance of the assay. In principle, a 'specific reaction' in immunology may be defined as follows: in the presence of different molecules the specific antibodies must complex only one kind of molecules. The probability of forming a 'wrong' complex determines the specificity of the reaction. Specificity is determined by the steric (three-dimensional) match of antigen and antibody, as well as by the number of molecular interactions taking place between both molecules. Discussion of specificity requires that both the structure of the antigen and the homogeneity or heterogeneity of the antibodies be considered. An antibody preparation is homogeneous if all the antibodies bind only to one and the same epitope, although possibly with different affinity. This definition applies to monoclonal antibodies and to antisera against compounds of low molecular weight (haptens). On the other hand, an antibody preparation is heterogeneous if it contains different antibody populations specific for different epitopes. A typical example for the latter is an immune serum obtained after immunisation with a high molecular weight protein such as an immunoglobulin. But also a mixture of different monoclonal antibodies represents a heterogeneous antibody preparation. From a practical point of view the term 'monospecific' is frequently used in publications dealing with species identification.

In the context of this chapter, a monospecific antiserum is defined as an antiserum reacting with only one (or more) proteins from a single animal species. Rabbits immunised with bovine IgG will produce antisera that contain antibodies against bovine, ovine, and caprine IgG, because the IgG molecules of these phylogenetically closely related species share common epitopes. In order to make such an antiserum monospecific, it may be either absorbed with serum of sheep and goats, or purified by immunoaffinity chromatography. In its widest sense a monospecific antiserum is therefore a heterogeneous antibody preparation containing active antibodies only against epitopes specific for one single animal species. Therefore, even monoclonal antibodies need not necessarily be monospecific, because they may be directed against an epitope which is common to several animal species.

It is important to keep in mind that non-specific influence on the assay caused by, e.g., matrix effects, often cannot be distinguished from the specific influence of cross-reacting substances. In both cases the assay response may be the same and a false positive or a false negative result is obtained. Due to the immunoassay principle, however, false negatives are unlikely in competitive assays but may occur in non-competitive assays. False positive results may be observed in both assay types.

3.3 Applications: identifying animal species in meat, dairy and other foods

One main area for application of antibody techniques is the authenticity of food of animal origin. Most applications in this field focused on identification of meat and milk of numerous animal species, either directly in the raw material or in processed food. Until 15 years ago immunoelectrophoretic methods and immunodiffusion techniques were predominant, immunodiffusion methods still representing an ideal tool for routine species identification of raw meats. In the meantime numerous applications of enzyme immunoassays have been described and some have become commercially available (see 3.5), thus allowing this method to be implemented in routine analysis.

3.3.1 Meat and meat products

The target antigens used in most studies on meat and meat products are blood or serum proteins, such as albumin. Some authors also used muscle proteins, preparations of adrenals, and sarcoplasmatic extracts to produce specific polyclonal antisera in rabbits, goats, and sheep. So far, in only a few studies were mice immunised for the production of monoclonal antibodies. The proteins used for the immunisation procedure cover a wide range of animal species, such as horse, cattle, pig, sheep and exotic species like impala and topi. Usually reliability and sensitivity of these assays decreases with increased heating of the samples, even when relatively stable proteins, such as myoglobulin (Hayden,

Table 3.1 Enzyme immunoassays for species identification in meat and meat products

Antigen (preparation)	Detection limit	Reference
Serum, albumin (pig)	1–10%	Ayob *et al.* (1989)
Skeletal muscle extract (pig, chicken)	250 ppm (pork) 126 ppm (poultry)	Berger *et al.* (1988)
Desmin (chicken)	10%	Billet *et al.* (1996)
Serum (cattle)	< 10%	Griffiths and Billington (1984)
Heated muscle protein (pig)	0.5%	Hsieh *et al.* (1998)
Serum albumin (pig)	1–3%	Jones and Patterson (1985)
Serum albumin (cattle, horse, pig)	ca. 3%	Jones and Patterson (1986)
Serum albumin (cattle, horse, pig)	ca. 3%	Kang'ethe *et al.* (1982)
Albumin (cattle, horse, pig, chicken)	0.6%	Macedo *et al.* (2000)
Sarcoplasmatic extracts (pig)	1%	Martin *et al.* (1998)
Soluble muscle proteins (chicken)	ca. 1%	Martin *et al.* (1998)
Glycoprotein (pig)	0.06%	Martin *et al.* (1998)
Muscle protein (pig)	1%	Morales *et al.* (1994)
IgG (pig, chicken, cattle)	–	Myers *et al.* (1997)
Serum proteins (buffalo, goat, donkey)	0.1–1%	Patterson and Spencer (1985)
Serum proteins (sheep, goat, cattle, buffalo, kangaroo, horse, camel, pig)	< 1%	Patterson *et al.* (1984)
Autoclaved muscle extract (pig)	0.5–1%	Sawaya *et al.* (1990)
Heated adrenals and muscles (buffalo, cattle, sheep, goat, pig)	1%	Sherikar *et al.* (1993)
Heat treated chicken muscle proteins	ca. 1%	Sheu and Hsieh (1998)
Chicken red bone marrow	–	Stevenson *et al.* (1994b)
Turkey muscle LDH	1%	Wang and Smith (1995)
Serum protein horse, cattle, heep, kangaroo, pig, camel)	< 10%	Whittaker *et al.* (1983)

1979), adrenal preparations (Hayden, 1981) or troponin (Schweiger *et al.*, 1983) are used as the target antigens.

Particularly for use in enzyme immunoassays, polyclonal antisera have to be treated by absorption or immunoaffinity procedures to provide sufficient specificity. A water or buffer solution extract of meat or meat products is sufficient for most enzyme immunoassay variants described. Similar to immunodiffusion methods, enzyme immunoassay is best suited to analyse raw meat or mildly heated meat products. A selection out of the variety of enzyme immunoassays described so far is presented in Table 3.1.

The most sensitive assays have been established using the sandwich enzyme immunoassay format. Compared to the non-competitive assay format, utilising direct coating of the antigen to the solid phase, the sandwich enzyme immunoassay avoids variation of the assay response due to variations in coating efficiency if different types of samples are analysed. In addition, binding

of the antigen to the solid phase via a capturing antibody results in better presentation of the antigen for binding by the second (labelled) antibody. A disadvantage is that antibodies against two epitopes in suitable steric positions must be present in the test antiserum in order to enable both capturing and detection of the analyte.

Enzyme immunoassays utilising blood or serum proteins, such as albumin, as the target antigen show limited suitability in testing heat-treated sample materials. A progressive loss in activity is observed with increased heat treatment due to denaturation of the antigen (Goodwin, 1992). Another drawback is that the amount of blood may be different in various types of beef, and that the antigenic properties of blood and serum proteins may change on storage (Griffiths and Billington, 1984). On the other hand, a number of assays have been described based on antibodies against heat stable or heat treated antigens (Berger *et al.*, 1988; Patterson and Jones, 1989; Sawaya *et al.*, 1990; Sherikar *et al.*, 1993). The main drawback of these methods is the need for complex extraction steps. Furthermore, the assays show reduced specificity and provide no quantitative results.

3.3.2 Milk and milk products

The milk proteins used to produce specific antisera are either caseins or whey proteins. Caseins have the advantage that they represent the main part of the protein fraction in milk and that they are more or less heat stable. This means that caseins may successfully be used as target antigens in heat treated (pasteurised or UHT) milk and milk products. The main disadvantage of caseins is their poor immunogenicity (Perez *et al.*, 1992). In addition caseins are susceptible to proteolytic degradation. In contrast, whey proteins are less susceptible to proteolysis and mostly good immunogens, but are less heat-stable than the caseins.

To avoid the cross-reactivity of the antisera against cows' milk proteins with proteins in ewes' or goats' milk, either sheep or goats were immunised (Levieux, 1977; Radford *et al.*, 1981; Garcia *et al.*, 1989), or the antisera were absorbed against bovine casein by adding ovine or caprine casein (Rodriguez *et al.*, 1990) or more complex mixtures (Gombocz *et al.*, 1981). Immunoaffinity chromatography was used to purify polyclonal antisera by Aranda *et al.* (1988), Sargeant *et al.* (1989) and Garcia *et al.* (1991). Antibody preparations obtained after these purification steps could also be used to establish non-competitive (Sandwich) enzyme immunoassays for the detection of about 1% or less of cows' milk in ewes' milk (Table 3.2). A very sensitive assay was established by immunising sheep with bovine IgG (Sauer, 1992). The resulting antiserum was highly specific for bovine IgG and cross-reacted neither with homologous immunoglobulins of closely related species (caprine or ovine IgG) nor with other bovine whey proteins.

Most applications utilised conventional microtitre-plate techniques and instrumental test evaluation for the qualitative and quantitative determination of the respective analyte. Milk samples can be directly applied in most assays or

Table 3.2 Microtitre plate enzyme immunoassays for the detection of cows' and goats' milk

Antigen	Detection limit	Reference
β-Casein	–	Anguita *et al.* (1995)
β-Lactoglobulin	0.5% cows' milk	Beer *et al.* (1995)
κ-Casein fragment (139–152)	0.25% cows' milk	Bitri *et al.* (1993)
Bovine IgG	0.0008% raw cows' milk 0.0015% past. cows' milk	Çan (1996)
Bovine whey proteins	1% cows' milk	Castro *et al.* (1992)
Bovine whey proteins	1% cows' milk	Garcia *et al.* (1990)
Bovine whey proteins	1% cows' milk	Garcia *et al.* (1991)
Caprine whey proteins	0.5% cows' milk	Garcia *et al.* (1993)
Caprine whey proteins	1% goats' milk	Garcia *et al.* (1994)
Goat α-S2-casein	0.5% goatśmilk	Haza *et al.* (1997)
Bovine β-lactoglobulin	0.0001% cows' milk	Levieux & Venien (1994)
γ_3-Casein	0.2% cows' milk	Richter *et al.* (1995)
Bovine caseins	1% cows' milk	Rodriguez *et al.* (1990)
Caprine caseins	1% goats' milk	Rodriguez *et al.* (1991)
Bovine caseins	0.5% cows' milk	Rodriguez *et al.* (1993)
Caprine caseins	1% goats' milk	Rodriguez *et al.* (1994)
Bovine α_{s1}-casein fragment (140–149)	0.125% cows' milk	Rolland *et al.* (1993)
Bovine whey proteins		Sargeant *et al.* (1989)
Bovine IgG	0.001% cows' milk	Sauer (1991)
Bovine plasmin	0.0002% raw cows' milk 0.0004% past. cows' milk 1.3% UHT cows' milk	Schilk (1995)
Caprine IgG	0.002% raw goats' milk	Schilk (1995)
Caprine IgG	1% cows' milk	Spencer and Patterson (1986)

need a simple defatting step, such as centrifugation. During the past few years, several membrane based assays for the detection of cows' and goats' milk have been described (Table 3.3). The detection limit provided by these tests is sufficient under practical aspects, although compared with microtitre-plate assay they show a reduced sensitivity. On the other hand rapid immunochemical methods are excellent tools to check milk delivered to dairy plants before processing, thus preventing financial losses for the cheese producing industry. Some of these assays have become commercially available (see 3.5).

3.3.3 Other applications

As well as the identification of meat and milk of different mammalian species, only a few other applications, such as an enzyme immunoassay for distinguishing between crustacean tail meat and white fish (Taylor and Jones, 1992) have been described. Substitution of canned sardine with other species and adulteration of canned tuna with bonito were two other specific authentication issues, which have been adressed (Taylor *et al.*, 1994). Also a

Table 3.3 Membrane based immunoassays for the detection of cows' and goats' milk

Antigen	Detection limit	Reference
β-Casein	0.5% cows' milk	Addeo *et al.* (1995)
β-Casein	1% cows' milk	Anguita *et al.* (1996)
Bovine caseins	0.1% bovine casein	Aranda *et al.* (1988)
Caprine IgG	0.5% cows' and goats' milk	Aranda *et al.* (1993)
β-Lactogobulin	1% cows' milk	Molina *et al.* (1996)
Bovine caseins	0.1% cows' milk	Perez *et al.* (1992)
γ_3-Casein	0.2% cows' milk	Richter *et al.* (1995)
Bovine IgG	1% cows' milk	Sauer (1992)

relatively low number of applications was focused on the detection of plant proteins, e.g., soy in meat products (Gonzalez *et al.*, 1998) or soy, wheat and pea proteins in milk powder (Haasnoot *et al.*, 2001). An area with increasing importance is the application of immunoassays to test for allergens in food, e.g., for almonds (Roux *et al.*, 2000) or hazelnuts (Blais and Phillippe, 2001) and particularly for gliadins (Andrews and Skerrit, 1994; Stevenson *et al.*, 1994a; Chirdo *et al.*, 1998). It is interesting to note that enzyme immunoassays for the detection of bovine IgG, which were intended to be used for the detection of adulteration of sheep and goat's milk, have been successfully used for classification of bovine milk according to the thermal treatment. The assays served for the easy identification of UHT milk, because the denaturation of IgG is markedly enhanced at temperatures above 73ºC (Rosenthal *et al.*, 1999). In a similar manner immunoassays for specific groups of glutenins and gliadins could be used for the analysis of biopolymer quality in cereals. Quantitative immunoassays allowed the prediction of aspects of dough strength and extensibility, while qualitative assays could be used to screen for products of particular wheat or translocated rye genes associated with specific dough qualities (Skerritt *et al.*, 1994).

3.4 Advantages and disadvantages

The main advantages of enzyme immunoassy over immunodiffusion or immunoelectrophoresis procedures are reduced assay time (usually less than four hours), requirement of only small amounts of antisera, and the possibility of obtaining quantitative results. In addition the enzyme immunoassy microtitre plate assay may be automated, thus allowing a large number of samples to be processed, whereas rapid tests like dipsticks (immunosticks) may be used as field tests to screen suspicious samples.

Compared to physico-chemical methods of analysis, microtitre tests – as well as immunochemical techniques in general – have advantages in aspects of the sample treatment necessary prior to analysis. The sample extract clean-up in particular can be simplified or even totally omitted. This is mainly due to the

fact that immunochemical methods are highly sensitive and specific for their target molecule. Liquid materials such as milk present an ideal sample matrix for immunochemical assays and milk samples may be analysed without any extraction step necessary. For analysing meat and meat products in most cases the preparation of an aqueous extract including a defatting step is sufficient.

A general disadvantage of antibody techniques in this particular area is the limited availability of commercial test kits. The main reason for this limitation is the particular structure of the market for immunochemical methods in food analysis, which is characterised by a broad range of potential products requiring a high degree of innovation but having relatively low sales per product. Within Europe only a few companies are selling enzyme immunoassay systems for species identification, e.g., Cortecs Diagnostics (UK) provided one of the first manufacturer dipstick (F.A.S.T., immunostick) assays for a range of animal species, including beef, horse, pork, sheep/goat, poultry, rabbit, kangaroo, goat, chicken, turkey and buffalo. The F.A.S.T. system can be used for testing raw meat and meat products, milk, animal tissue and serum.

To analyse cooked samples, a microtitre plate assay system (Biokit) is marketed for beef, pork, poultry and sheep by the same manufacturer. The Biokit system is also available for raw sample specimen originating from cattle, horse, pork, sheep, poultry, rabbit and kangaroo. The detection limit for all assays is in a range of 1–3%, quantification is, however, not possible. In a similar way one of the first and probably most often used microtitre plate and immunofiltration assay for the detection of cows milk (RIDASCREEN® CIS and RIDAQUICK® CIS) was made commercially available more than ten years ago by R-Biopharm GmbH (Germany). Although the availability of commercial reagents and test kits is still limited, the number of new developments during the last ten years is promising and could lead to a broad spectrum of reliable and rapid methods suitable for the increasing number of routine analyses, which are required by new regulatory limits, new sampling strategies and consumer demand for safe and authentic food.

3.5 Sources of further information and advice

The development and application of enzyme immunoassays includes principles of immunology, immunochemistry, analytical chemistry and statistics. For further information on immunology and the theory of immunoassays several textbooks are available, e.g., Roitt *et al.* (1985), as well as valuable papers on specific topics, like sensitivity (Halfman and Schneider, 1981; Jackson and Ekins, 1986) and specificity (Berzofsky and Schechter, 1981).

In view of the application of these assays textbooks about enzyme immunoassay techniques, e.g., Walker (1984), Clausen (1988) and Edwards (1996), could be helpful. Additional information on the specific application of antibody techniques for food authentication is provided by Lumley (1996) and Märtlbauer (1998). For the discussion of statistical parameters influencing the

'quality' of immunoassays it should be noted that general statistical methods to define the limit of detection, the limit of quantification, reproducibility, etc., must also be applied to immunoassays. There are, however, some parameters influencing assay precision (repeatability) and accuracy of immunoassay results, which are typical for enzyme immunoassays set up on 96-well microtitre plates (Bunch *et al.*, 1990; Märtlbauer, 1993).

Most users of enzyme immunoassays have to deal with routine analyses and will therefore be forced to use commercial assays. Due to changes in strategy of the manufacturers, introduction of new products, improvement of products, as well as withdrawal of products it is nearly impossible to give an overview about the availability of test kits. There are however several sources of information available on the worldwide web, either on the home pages of the test kit producing companies or on the home page of the AOAC International (www.aoac.org), which provides an up-to-date database containing information on probably most commercial available assays relevant in food analyses.

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4

Proteome and metabolome analyses for food authentication

S. Vaidyanathan and R. Goodacre, UMIST, Manchester, UK

4.1 Introduction

The advent of genomics and genomic technologies has resulted in a paradigm shift in the emphasis of analytical research of biological systems. The traditional approaches of biochemistry and molecular biology, where the cellular processes were investigated individually and often independent of each other, is giving way to a more global approach of analysing the cellular compositions in parallel and in its entirety, in order to obtain a 'holistic' picture. Subsequent to the genetic revolution, which resulted in the systematic sequencing of the genetic make up of several living organisms (http://www.tigr.org/tdb/mdb/mdb.html), including microbes, plants and animals, analysis at the level of the gene products, such as mRNAs, proteins and metabolites is increasingly becoming relevant for characterisation and identification purposes. This is especially true with the realisation that analysis beyond the genetic level is required to elucidate gene function. Developments in analytical techniques are progressing rapidly to enable simultaneous high-throughput measurements of several analytes, at the level of the transcript (transcriptomics), proteins (proteomics), and metabolites (metabolomics).

These developments are being driven primarily by the requirements in the healthcare sector. However, given the ongoing debates on genetically modified (GM) organisms and foodstuffs made thereof, and subsequent concerns with respect to food safety, the so called ''omics' approaches are relevant to food authentication and traceability, and offer potential for food characterisation. Developments in proteome and metabolome analyses will form the subject matter of this chapter, with emphasis on a survey of recent developments in analytical strategies. Techniques that enable differences to be delineated

between different biological systems or even between different states of a system may be useful for characterisation and identification purposes, even when a complete knowledge of the genetic make up of the system is not available. In this regard, fingerprinting approaches that provide protein and metabolic 'snapshots' would offer the potential for rapid assessments of biological systems at the functional level. Some of the techniques that are currently being used and those that show promise for protein and metabolic fingerprinting will also be discussed in this chapter, as will be the relevance of proteome and metabolome analyses to food authentication and traceability.

4.2 The importance of proteomics and metabolomics

DNA sequencing of organisms is progressing at a swift pace. Since the first complete sequencing of a free living organism in 1995, that of *Haemophilus influenzae*, a bacterium of relevance to human health and disease, genome sequences of over 150 living organisms have been completed and many more are in progress (http://wit.integratedgenomics.com/GOLD/). These include over 100 bacterial genomes, the human genome and the plant genomes of *Arabidopsis thaliana* and *Oryza sativa* (rice). The availability of the genetic make up of living organisms has enabled comparative assessments to be made between and within species, for instance to understand pathogenic basis, and to gain insights into evolutionary traits. However, DNA sequence information alone does not tell one how cells work, or explain complications resulting from cellular function or dysfunction. It is now known that the total number of human genes does not differ substantially from the number of genes of the nematode worm *Coenobacter elegans*, and that there are considerable similarities in the genetic make up of diverse species, suggesting that contextual combination of gene products confers complexity and diversity to the functional genome. Therefore, the goal in genomics cannot only be the simple provision of a catalogue of all the genes and information about their function, but also the generation of an understanding of how the components come together to comprise a functional entity such as a cell or organism. Consequently, in the 'post-genomic' era there is greater emphasis on technologies that would elucidate the functional aspects of genes and gene products.

Since mRNAs, proteins and metabolites effect the flow of information from gene to function (Fig. 4.1), ''omic' technologies that enable large-scale assessment at the levels of mRNAs (transcriptomics), proteins (proteomics) and metabolites (metabolomics) have emerged and are continually being developed. Subsequent to the availability of DNA sequence information, annotation of the genes necessitated the development of high-throughput technologies to monitor gene expression. Initial efforts were expended on technologies that would enable large-scale assessment of gene expression via the transcriptome (the spatial and temporal accumulation patterns of mRNAs). Subsequently, array-based methodologies emerged for gene expression monitoring. Nucleic acid arrays

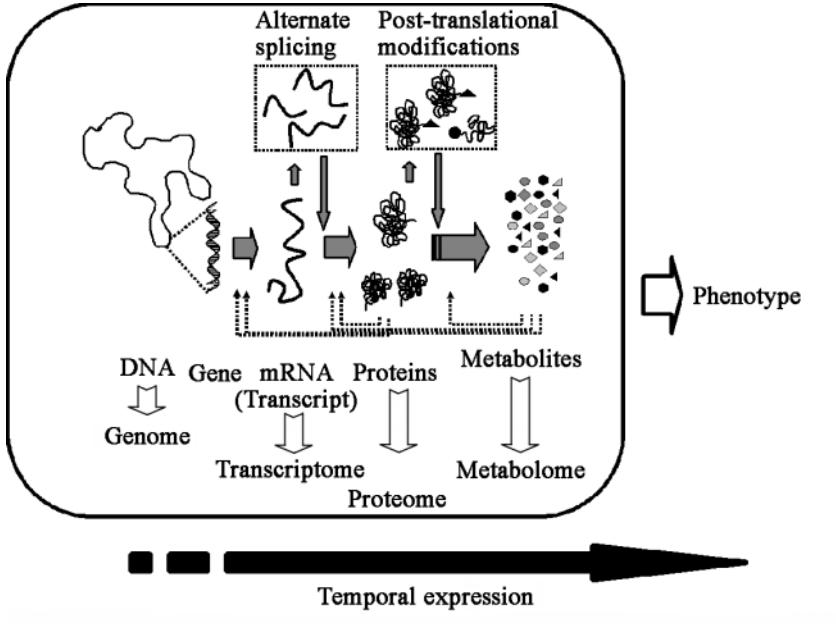

Fig. 4.1 Schematic of ''omic' organisation. Dotted arrows indicate interactions regulating respective ''omic' expression.

produced by the robotic deposition of PCR products, plasmids, or oligonucleotides onto a glass slide or *in situ* synthesis of oligonucleotides using photolithography have been used. A key advantage of using arrays, especially those that contain probes for tens of thousands of genes is that a holistic (rather than targeted and potentially biased) view of cellular response can be obtained, as compared to looking for specific genes, without a priori knowledge of which genes or mechanisms are important (Lockhart and Winzeler, 2000). These and other tools, such as SAGE (sequential analysis of gene expression), can be used for monitoring differences in gene expression that are responsible for morphological and physiological/phenotypical differences, and can be indicative of cellular response to environmental stimuli and perturbations. However, mRNA is only an intermediate in translating the genetic information to cellular response and function (Fig. 4.1). Proteins are at the business end and carry out the activities of the cell at the biochemical level.

Whilst the genome sequence can give an idea of which proteins the cell has a potential to make, and the mRNA profiles can indicate which proteins are being produced, analyses beyond the genomic and transcriptomic levels are required to gain information regarding what the expressed proteins do to carry out cellular function at the molecular level. The existence of an open reading frame (ORF) does not necessarily imply the existence of a functional gene. In addition, it is now recognised that the relationship between genes and gene products (i.e. proteins) is not necessarily linear, and that a given gene can express more than

one protein. Even mRNA levels do not necessarily correlate to protein levels (Gygi *et al.*, 1999b). Cellular regulation can be mediated at the protein (e.g., post-translational modifications) or metabolic (e.g., localised changes in metabolite concentrations) levels. Besides, complex networks of interactions often mediate cellular response, and the nature of the response is dependent on the cell type and state, aspects that cannot be accounted for by investigations at the genomic or transcriptomic levels alone. In order to link genomic information with phenotypic variations biochemical characterisation may be required. These are some considerations that have necessitated characterisation of organisms beyond the genomic and transcriptomic levels. Consequently, technologies for assessing the accumulation patterns for proteins (proteomes) and metabolites (metabolomes) are currently receiving greater attention.

4.3 Proteome analysis

Proteins serve as the structural and functional entities in cells, contributing as catalysts, secondary messengers, transporters, receptors, etc., and are of considerable importance in cellular characterisation. The 'proteome' has been defined as the entire protein complement of a cell, or a tissue type (Wilkins *et al.*, 1996). Proteomics is the large-scale study/analysis of proteins. It involves the assessment of the total cellular protein content, in terms of protein type, nature and quantity, and has been extended to include characterisation of subcellular localisations and protein-protein interactions that help in defining cell function. In a general sense, comprehensive proteomics would thus translate to mapping the cellular proteins in a spatial and temporal manner. Unlike the genome, where the information content is conserved for a given organism, with the proteome the information content is dynamic, depending on the cell type and state. The nature of the proteins can also vary, depending on post-transcriptional (splice variants) and post-translational (phosphorylation, glycosylation, proteolysis, etc.) modifications. In addition, in many cases multi-protein complexes act as functional units in carrying out cellular activites. There are thus different aspects of the proteome that can be analysed.

Three major analytical strategies are available for proteomic analysis (Fig. 4.2): (a) two-dimensional gel electrophoresis (2D-GE) based separation followed by mass spectrometric (MS) identification of separated protein spots; (b) liquid chromatography (LC) or capillary electrophoresis (CE) based separation of proteins/digested peptides, followed by MS, and (c) protein microarray technology.

4.3.1 2D-GE-MS

2D-GE is the most widely used tool for large-scale proteomics, as is evident from the databases available on the web (*http://www.expasy.ch*, for instance). In 2D-GE, cellular extracts of proteins are separated based on charge (p*I*) in the

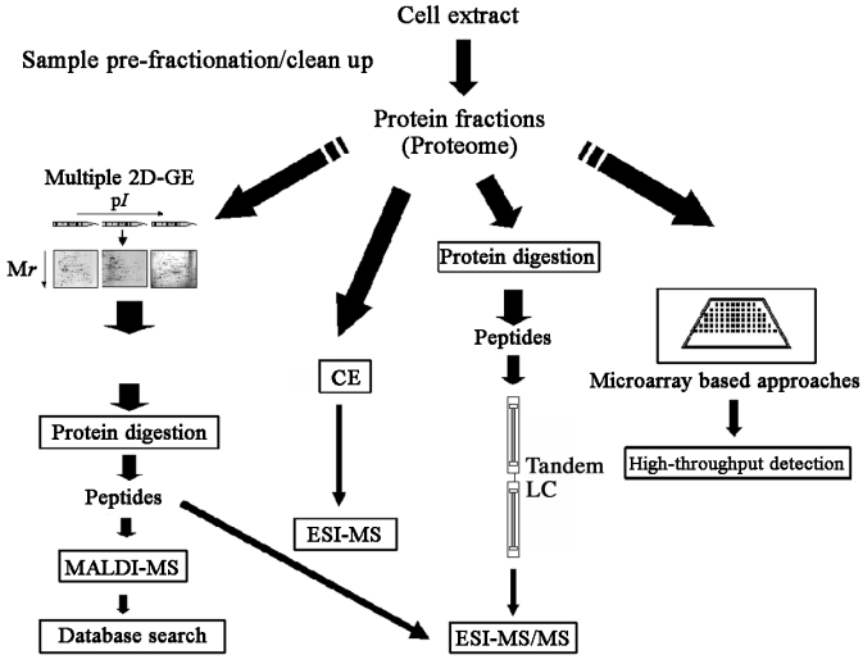

Fig. 4.2 General strategies for proteome analyses (refer to text for abbreviations).

first dimension using isoelectric focusing, and by size (M*r*) in the second dimension using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The gels can be used at an analytical scale to look for prominent signals, or at a micro-preparative scale for identification and further characterisation of separated proteins. The advent of immobilised pH gradient strips (IPG) has improved resolution and reproducibility of analysis when analysing mixtures containing milligram quantities of proteins. In-gel sample loading and the development of sensitive protein stains, such as ammoniacal silver, radioactive stains, and fluorescent dye tags, have contributed to improvements in the technique over recent years. For comprehensive proteome analysis, it is required that the analytical technique be capable of separating and resolving the different protein types, ideally with minimal sample preparation. The difficulties of using 2D-GE in this regard arise from the fact that there can be enormous variations in the amount of different proteins, for instance ranging from as much as 2,000,000 copies/cell to as little as 100 copies/cell, as in *Saccharomyces cereviseae*, leading to variations in protein concentrations of up to 10,000 fold (Futcher *et al.*, 1999).

Unlike genomics, where PCR can be used to amplify DNA, there is no ready-made amplification strategy in proteomics, making it difficult to detect low abundance proteins, although protein enrichment strategies such as using hydroxyapetite chromatography, prior to 2D-GE (Fountoulakis *et al.*, 1999)

could help towards minimising this problem. Besides, differential protein processing (resulting in more than one spot per protein) and co-migrating spots present problems for both quantitative protein expression and database matching (Gygi *et al.*, 2000). The proteins constituting a proteome may also have a heterogeneous distribution in the type of protein with respect to size (M*r*) and charge (p*I*), necessitating analysis over narrower p*I* and M*r* ranges, thereby increasing the number of gels that need to be run, for a given sample. There is also difficulty in relating positions on various gels, for comparative purposes. Despite these shortcomings, 2D-GE is still the most widely practised option, and is useful in qualitatively, and perhaps semi-quantitatively assessing proteomes, at least with respect to the most abundant proteins.

For 2D-GE resolved proteomes, the next stage of analysis is the identification of the protein spots using MS. Matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI) (more increasingly nano-ESI) are the common ionisation methods employed (see section 4.5.3). Two main approaches can be followed to analyse and identify the excised protein spots. In 'peptide mass fingerprinting' (Henzel *et al.*, 1993) the protein spots (bands) are subjected to in-gel digestion by a sequence-specific protease, usually trypsin, after destaining, reduction, alkylation and washing steps. This is followed by analysis of the eluted peptides by MALDI-MS (Shevchenko *et al.*, 1996). The set of masses from the MS analysis is then compared to theoretically expected tryptic peptide masses in a database to identify the protein. The requirement for protein digestion prior to mass spectrometric analysis arise from the relative difficulty in eluting gel-separated proteins and the poor mass accuracy and resolution of high molecular weight proteins, compared to those of peptides. A match of at least five peptide masses and 15% protein coverage would indicate unambiguous identification, with mass accuracies of 10—50 ppm (Mann *et al.*, 2001).

The second approach involves peptide sequencing using tandem mass spectrometry, followed by analysis of fragmented peptides. This approach usually uses tandem ESI-MS/MS (section 4.5.3). The peptides in liquid phase are electrosprayed into the mass spectrometer, a precursor ion corresponding to the ionised peptide mass is chosen using the first mass analyser (usually a quadrupole) and this is fragmented by the application of a collision gas (such as argon) under pressure to give product ions that are separated by a second mass analyser (usually a ToF), thus generating a fragmentation pattern that can be used to sequence the peptide and in turn identify the protein. Peptide chemistry dictates that there is a propensity for certain fragments to occur in preference to others, enabling the deduction of rules for sequence identity.

Larger peptides often fragment efficiently providing long ion series, but due to multiple charge distribution of the precursor ion intensity, may have poor sensitivity (Mann *et al.*, 2001). More than 500 proteins (corresponding to about 30% of the predicted number of ORFs) have been identified in the proteome of *H. influenzae*, using peptide mass fingerprinting (Langen *et al.*, 2000). Protein identification has been reported even in cases where the genomic and proteomic

database is not available as yet, which will be particularly valuable since most of the raw ingredients for foodstuffs are not yet sequenced. Mass spectrometric identification by peptide mass fingerprinting and by tandem mass spectrometry combined with database search has been used to identify proteins from brewing yeast strains that have homology to those of *S. cereviseae* from a protein database (Joubert *et al.*, 2001). Mass spectrometry can also be performed after the first dimension of 2D-GE, by performing surface analysis on the IPG strips using MALDI-MS, to construct a virtual 2D-gel (Loo *et al.*, 2001).

4.3.2 MS of peptides after LC or CE separations

MALDI-MS and ESI-MS are sensitive techniques. However, the nature of the ionisation process, which is still not completely understood, necessitates that the sample analysed is clean (i.e., presence of little or no unwanted chemical species other than the analyte of interest). In samples containing mixtures of analytes, the chances of observing and quantifying the analyte(s) of interest decreases when the number of analytes present increases. This is due to competition for ionisation and ion suppression effects, especially with ESI-MS (Sterner *et al.*, 2000). It is therefore common to employ a sample clean-up or an analyte separation stage prior to MS, when analysing protein mixtures or protein digests. Liquid chromatography (LC) and capillary-electrophoresis (CE) can be employed for these purposes. ESI-MS is especially suited to coupling with LC, and the application of LC-MS or LC-MS/MS (Pflieger *et al.*, 2002) for proteomic studies on peptide mixtures derived from 2D-GE is common. LC-MS (MS/MS) (Li *et al.*, 1999) and CE-MS can also be used directly on intact protein mixtures for proteomic analysis, obviating the need for 2D-GE separation. A combination of capillary isoelectric focussing (CIEF) with MS enabled the measurement of several proteins in cell lysates of *E. coli* (Jensen *et al.*, 2000).

Advanced approaches of combining similar or different separation strategies with mass spectrometry, for e.g., LC-LC-MS(/MS) (Davis *et al.*, 2001, Washburn *et al.*, 2001) and CE-LC-MS can also be potentially applied to analyse protein or peptide mixtures. Reverse-phase, ion-exchange (Davis *et al.*, 2001, Washburn *et al.*, 2001), affinity and hydrophobic interaction columns (Langen *et al.*, 2000) have been applied in isolation or in tandem prior to mass spectral analysis. Yates and co-workers have developed tandem LC separation strategies of global peptide digests followed by database search, which they term multi-dimensional protein identification technology (MudPIT), to analyse complex proteomes, as an alternative to 2D-GE (Link *et al.*, 1999, Washburn *et al.*, 2001). This has shown that protein coverage increased using MudPIT as compared to using 2D-GE alone, when applied to a survey of the rice proteome (Koller *et al.*, 2002).

The use of capillary LC systems provides greater efficiency of separation. Multiple capillary systems using serially connected dual-capillary columns can be more effective as they eliminate time delays for column regeneration. Over 100,000 peptides could be detected in yeast cytosolic tryptic digests using this

strategy combined with MS (Shen *et al.*, 2001). Similarly, microcapillary LC columns have been multiplexed to perform parallel separations and reduce chromatographic time (Lee *et al.*, 2002). Chromatography can also be used as a pre-concentration step to increase the sensitivity of detection for peptides derived from protein digests of 2D-GE spots (Langen *et al.*, 2000, Timperman and Aebersold, 2000). Most of these advanced strategies, however, are still in the proof-of-principle stage, and although they have all been shown to have significant potential, further validation and improvements with respect to practical application will be needed before they come into routine operation.

4.3.3 Protein microarrays

Microarray-based approaches involve miniaturisation of standard assay procedures in multiple arrays to allow simultaneous analysis of multiple determinants/analytes. Such assays are very popular in transcriptomics, but have also been extended to proteome analyses. Microspots of 'bait' molecules are immobilised in rows and columns onto a solid support and exposed to samples containing the corresponding binding molecules. The complex formation within each microspot can be detected using readout systems based on fluorescence, chemiluminescence, electrochemistry, mass spectrometry, or radioactivity. Large-scale assessment of protein profiles can be carried out by the use of immunoassays on microarrays (Schweitzer and Kingsmore, 2002, Blagoev and Pandey, 2001). The analysis involves a scale-up of enzyme-linked immunoassays (ELISA) that have been in use for protein analysis. Antibodies immobilised in an array format onto specifically treated surfaces act as 'baits' to probe the sample of interest to detect proteins that bind to the relevant antibodies, using, say, fluorescence detection.

Protein microarrays can also be developed for assessing protein-protein, enzyme-substrate, and other protein-metabolite interactions (MacBeath and Schreiber, 2000). In one study (Zhu *et al.*, 2000), 119 yeast protein kinases were arrayed in microwells and examined for kinase activity with 17 substrates, demonstrating the value of microarrays in multiplexed protein functional assessments. Carbohydrate-based microarrays have also been devised (Wang *et al.*, 2002). Microarray technology for the examination of proteins on a large scale is still in the developmental stage (Ringeisen *et al.*, 2002, Schweitzer *et al.*, 2002, Ng *et al.*, 2002, Hodneland *et al.*, 2002) and there are several challenges to be considered particularly with respect to reproducibility and quantification, compared to the technology used for nucleic acids (Templin *et al.*, 2002, Talapatra *et al.*, 2002, Mitchell, 2002).

4.3.4 Protein-protein interactions and post-translational modification

The definition of protein function in the post-genomic era has to be considered in the context of its interactions with other proteins in the cell (Eisenberg *et al.*, 2000). In fact, clues to the function of a protein can be obtained by assessing the

proteins it interacts with, the concept of guilt-by-association (Oliver, 2000). In addition to qualitatively and quantitatively analysing the total cellular protein content (or protein expression), information regarding protein-protein interactions, protein localisation and post-translational modifications are required to assess and understand protein (and in turn cell) function, and hence form a significant part of proteomic analysis. It is now known that many cellular activities are carried out by multi-protein complexes, and that protein-protein interactions mediate many aspects of cell behaviour.

Yeast has been the test bed for characterising protein-protein interactions and protein complexes. The yeast two-hybrid (Y2H) system (Fields and Song, 1989) is a simple and powerful genetic method to identify protein-protein interactions, and has been used extensively, for instance in the analysis of *H. pylori* proteome, where over 1,200 interactions were identified, connecting 46% of the proteome (Rain *et al.*, 2001). Alternatively, tagged proteins can be used as baits for high affinity capture of complexes, followed by subsequent identification of the protein components using MS (Gavin *et al.*, 2002, Ho *et al.*, 2002). Such analyses provide the database for further characterisation by integrating datasets (Legrain, 2002, Bader and Hogue, 2002). Other than protein-protein interactions, the subcellular localisation of proteins (e.g., Kumar *et al.*, 2002) will provide a valuable database for defining protein function.

Post-translational modifications (PTMs) are protein processing events in which the nascent (translated) protein is modified covalently in order to confer or abstract functionality, allowing for diversity in the regulation of protein function. These include proteolytic cleavage (e.g., active insulin from proinsulin), phosphorylation (e.g., kinase mediated signalling cascades), glycosylation (e.g., excreted proteins, transcription factors), ubiquitinisation, acetylation, methylation, etc. Although over a few hundred PTMs have been documented, methods for monitoring them on a proteomic scale are still in early days of development. Four basic strategies that can be used with varying degrees of success have been identified (Mann and Jensen, 2003) as being currently available for monitoring PTMs. These are (a) 2D-GE based separations followed by MS identification, (b) affinity-based enrichment of modified proteins followed by MS of protein mixtures, (c) LC-MS/MS of enzymatically digested proteins (peptide mixtures), and (d) selective derivation of peptides followed by affinity purification.

MS forms a vital part in current techniques for identifying PTMs. For instance phosphorylation, which is a common post-translational modification, can be detected in protein and peptide mixtures, based on the fact that phosphopeptides and phosphorylated proteins are heavier than their unphosphorylated counterparts, and the mass spectra can be scanned for neutral losses of 98 (H_3PO_4) or 80 (HPO_3). Depending on the nature of the sample several strategies can be employed to detect and even quantify the phosphorylated proteins (Mann *et al.*, 2002). More than 1,000 phosphopeptides were detected using a MS-strategy applied to the analysis of whole cell lysates of *S. cerevisae* (Ficarro *et al.*, 2002). It has also been possible to identify post-

translational modifications such as loss of initiating methionine, acetylation, methylation, and proteolytic maturation in yeast ribosomal proteins (Lee *et al.*, 2002). Other strategies that utilise molecular recognition to induce measurable events (e.g., ribozyme activation (Vaish *et al.*, 2003)) are also being investigated. Systematic unambiguous identification of post-translational modifications in proteins from their mass spectral profiles may require the application of software tools (Wilkins *et al.*, 1999).

4.3.5 Quantification in proteomics

Quantification of expressed proteins is another important aspect of proteomic analysis. Quantification of 2D-GE spots is error prone and not easy to assess in a reproducible manner, due to the poor resolution of staining intensities on the gels. Alternatively, methods based on the incorporation of stable isotopes open the possibility of quantification using MS. The methods rely on the principle that stable isotope incorporation shifts the mass of the peptides by a predictable amount. The ratio of the analyte between the isotope incorporated and the non-incorporated state can then be determined accurately by the measured peak ratio between the underivatised and the derivatised sample. Isotope labelling can be introduced pre-experiment, at the growth phase (metabolic labelling), where the proteins are labelled as they are synthesised, or post-experiment, using external stable isotope labelling of a particular amino acid residue or of the N- or C-terminus of proteins.

More recently, tandem mass spectrometry of protein digests and the targeted isolation of selected peptides from the complex mixture using isotope coded affinity tags (ICAT) (Gygi *et al.*, 1999a), that differentially labels and enables selective isolation of Cys-containing peptides have been described. Another strategy developed by Cagney and colleagues (Cagney and Emili, 2002) utilises mass-coded abundance tagging (MCAT), where lysine is modified to homoargenine in one of the two peptide-mixtures that are compared by MS. Several other labelling procedures that minimise complexity of peptide digests and enhance mass spectral identification are also emerging. Alternatively, strategies that involve protein identification by correlating tandem mass spectra of peptides with sequences from a database, followed by quantification based on peak area calculations have also been described (Bondarenko *et al.*, 2002). Despite these developments, quantitative proteomics currently lacks the coverage (in a relative sense) of a proteome that array analysis provides of a transcriptome.

4.4 Metabolome analysis

The metabolome is defined as the metabolic complement (low-molecular weight intermediates of metabolism) of a cell, tissue or organ type. Like the proteome and unlike the genome it is contextual and would vary from condition to condition, with the potential to be more dynamic in composition between

different states of the cell. It is a relatively new arrival to the repertoire of ''omic' technologies. Although techniques for the measurement of metabolites have been in existence since the early days of biochemistry, high-throughput technologies for large-scale monitoring of metabolites have lagged behind those of genomics, transcriptomics and proteomics.

The dynamic nature of the metabolic composition of cells, and the subsequent difficulty in precisely defining 'metabolic states' of a cell, is a probable reason why it has been less attractive for characterisation purposes. Besides, its proximity to cell phenotype in a temporal sense (Fig. 4.1) makes it less attractive as an indicator of the potential course of a cellular activity/function (or malfunction), for monitoring purposes. However, there are instances when the cellular responses to environmental stressors may not be reflected in gene expression profiles, and take place post-expression, for example, when regulation is at the level of enzyme inhibitions, when analysis of the metabolome would be more useful. Recent investigations and observations (Ideker *et al.*, 2001, Fell, 2001, ter Kuile and Westerhoff, 2001) raise doubts about whether transcriptomics and proteomics data would suffice in assessing biological function, pointing that metabolomic data may be beneficial and emphasising the need for investigations at the metabolomic level.

Profiling the metabolome has the potential not only to discover novel genes but also to ascribe functions to genes (Trethewey, 2001). Functions to orphan genes may be elucidated using metabolome data in combination with *in silico* pathway analysis data (Forster *et al.*, 2002). It is even argued that for simple organisms like prokaryotes, it may be possible to identify gene function and regulatory networks based on mRNA expression and metabolomics, without the need for proteomics (Phelps *et al.*, 2002). An integrative 'biological systems' approach that incorporates metabolome data with those from genomic, transcriptomic and proteomic platforms is the likely way forward in unravelling gene function (Oliver, 2002, Fell, 2001).

Alterations in cells induced in response to environmental or developmental stimuli, or to a genetic mutation, would result in changes in the quasi-steady-state levels of intermediate metabolites of pathways or in the final accumulation levels of terminal metabolites. Capturing these changes would require monitoring the metabolites and their levels, both spatially and temporally. Although it would be useful to have information on the entire metabolic complement of a cell, there may be instances when it would suffice to monitor only a portion of the metabolome. Accordingly, approaches to metabolome analysis may be classified, as suggested by Fiehn (2001), as *metabolite target analysis* (restricted to analysis of metabolite(s) of say an enzyme system), *metabolite profiling* (analysis of a group of metabolites, of say a specific pathway), *metabolomics* (comprehensive analysis of the entire metabolome, under a given condition), and *metabolic fingerprinting* (classification of samples based on their biological relevance or origin). Although other terminologies, such as *metabolic profiling* and *metabonomics* exist, the above classification highlights the options available for monitoring the metabolome.

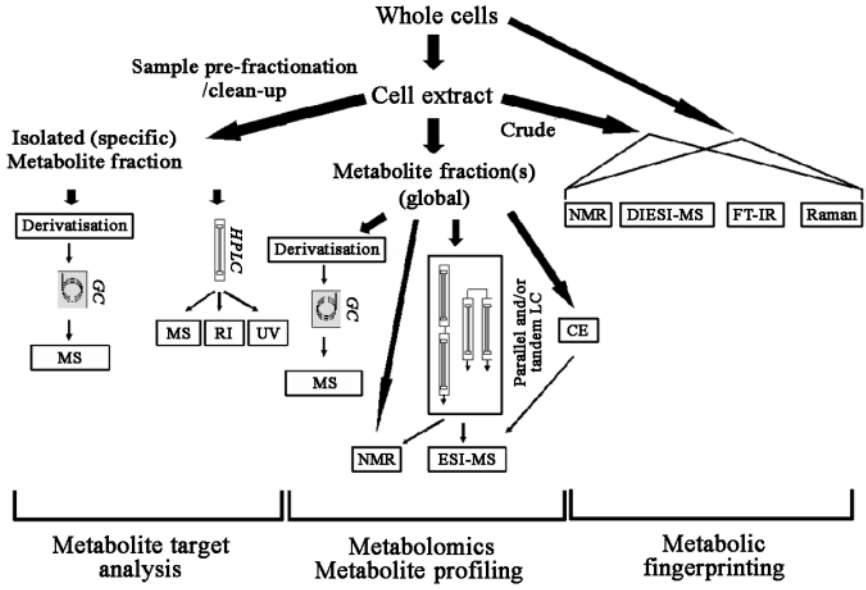

Fig. 4.3 General strategies for metabolome analyses. DIESI: direct-infusion ESI (refer text for other abbreviations).

Although the concept of analysing the 'metabolome' is a recent one (Tweeddale *et al.*, 1998), metabolic profiling of body fluids, such as urine, involving the determination of several metabolites, as a diagnostic method is not new (Matsumoto and Kuhara, 1996, Ning *et al.*, 1996). Metabolite profiling of specific pathways has also been in existence in the realms of metabolic engineering (Cameron and Chaplen, 1997). The potential for metabolic (metabolite) profiling in medical applications is highlighted by the capability of devising methods to follow disease progression (Griffin *et al.*, 2001), identifying metabolic steps that control cell proliferation for assessing anti-cancer drug targets (Boros *et al.*, 2002), and in studying drug toxicity (Nicholson *et al.*, 2002).

In a general sense, strategies for the analysis of the metabolome (Fig. 4.3) currently involve a combination of analyte separation strategies, such as gas or liquid chromatography, and detection techniques such as MS, NMR, UV, IR, etc., followed by chemometric analysis of the multivariate data to extract relevant information (Nicholson *et al.*, 2002, Kell, 2002, Taylor *et al.*, 2002, Fiehn, 2001). While the ultimate goal of metabolomics may be to detect reliably and quantify every metabolite in a cellular extract, it is unlikely to be attained by any single analytical technique available at present. Some metabolite selection is inevitable in all methodologies. Metabolic profiles generated from GC-MS data have been shown to reflect genotypic variations in plant extracts (Fiehn *et al.*, 2000). NMR has also been used for the characterisation of metabolomes, non-invasively and in a quantitative manner using ^{13}C stable isotope labelling (Schmidt *et al.*, 1999, De Graaf *et al.*, 1999, Dauner *et al.*, 2002). Differences

between glucose and fructose fed fermentations in *Zymomonas mobilis* were found to relate to global alterations in intracellular levels of phosphorylated metabolites (De Graaf *et al.*, 1999), using ^{31}P NMR, an approach that can be classified as metabolite profiling. Capillary electrophoretic methods can also be used to profile the metabolome (Terabe *et al.*, 2001).

For comprehensive analysis of the metabolome (metabolomics), it is essential to employ strategies that have a wider coverage in terms of the type and number of metabolites analysed. Sample preparation may have to be elaborate, incorporating sample clean up and pre-fractionation steps in order to capture even minor changes, and compensate for the wide dynamic range of cellular metabolites. Combination of several analytical techniques may have to be employed for such analysis. For example, parallel LC separations could be coupled to MS and/or NMR based detections. Methods for rapid quenching of metabolism and extraction of metabolites will be required due to rapid cellular turnover of some metabolites like ATP. It may also be necessary to retain the subcellular location of metabolites while extracting them, due to their contextual significance in terms of functions such as regulation or inhibition. Most extraction procedures reported in the literature so far are less comprehensive and biased, missing out on some metabolite or the other, resulting in only a portion of the metabolome being measured (in other words, they are metabolite profiles) but even in these investigations the information content obtainable points to the potential of monitoring metabolomes comprehensively.

4.5 Fingerprinting techniques

Fingerprinting approaches are those that provide a means for rapid and high-throughput monitoring and would be ideally suited for rapid characterisations if prominent changes can be captured in a reproducible manner. Short and simple protocols that still provide the required information will be useful for the purposes of monitoring food safety and authenticity. Techniques capable of handling a large number of samples with minimal sample preparation, but still capable of providing relevant chemical information would be ideally suited for generating rapid fingerprints. In such approaches it may not be required to identify and quantify the relevant proteins or metabolites, but to obtain a consistent fingerprint pattern for monitoring a given food, be it packaged or processed. Any deviations from the fingerprint outside the statistically relevant confidence thresholds would be indicative of safety concerns or loss of authenticity. Techniques that can be used to obtain fingerprints are thus those that are capable of providing rapid analytical information.

4.5.1 Vibrational spectroscopies

Vibrational spectroscopic techniques comprise those that are based on molecular bond vibrations to detect chemical species, including near-infra-red (NIR), mid-

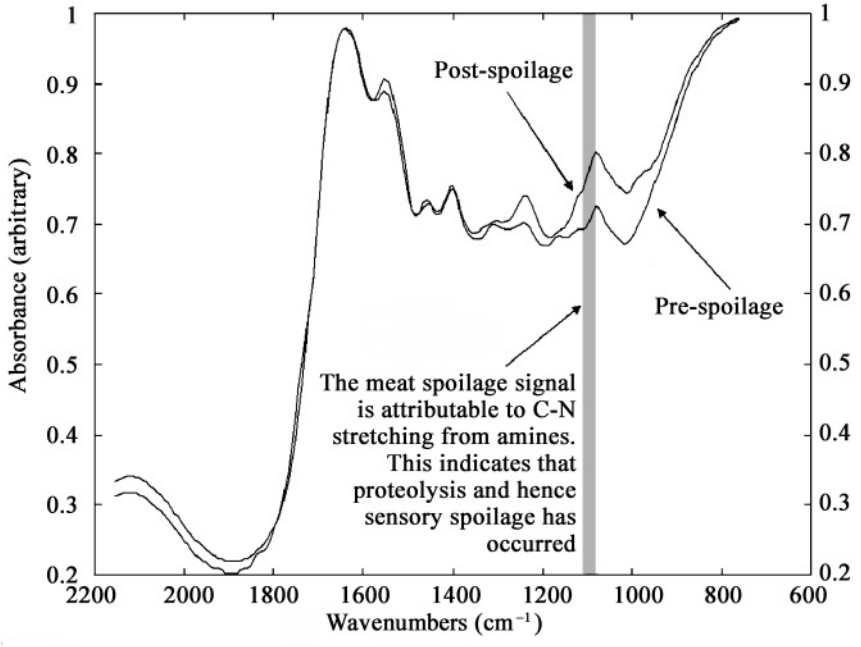

Fig. 4.4 Typical FT-IR absorbance spectra from pre- and post-spoilage chicken. Also shown is the area of the FT-IR spectra that can be used to predict spoilage. Spectra were collected using a ZnSe HATR accessory (Spectroscopy Central Ltd, Warrington, UK) on a Bruker IFS28 infra-red spectrometer (Bruker Electrospin Ltd, Coventry, UK).

infra-red (MIR) and Raman spectroscopic techniques. NIR spectroscopy in combination with chemometrics has been in routine use in the food industry, for instance as a secondary method of monitoring total proteins, moisture content, total fat directly in several foods, such as wheat and milk. But, because the vibrations in the NIR region are combinations and overtones of the fundamental vibrations that occur in the MIR region, they are relatively weak and would lack sufficient signal resolution for protein and metabolic fingerprinting purposes. However, Fourier-transformed (FT) MIR (or FT-IR) spectroscopic signals may be used to capture differences in biological samples that arise due to differences in protein or metabolic profiles if background variations are minimal, and when combined with chemometric methods of data extraction, as has been shown recently for detection of microbial spoilage in meat samples (Fig. 4.4) (Ellis *et al.*, 2002).

Such FT-IR fingerprints can be useful for assessing bacterial contamination of meat (Ellis and Goodacre, 2001, Goodacre, 2002), and for confirming food authenticity in general (Downey, 1998, Goodacre and Anklam, 2001). Metabolite information obtained from FT-IR fingerprints of mutant strains may also be useful in evaluating and assessing gene function (Oliver *et al.*, 1998) or changes in physiology (Goodacre *et al.*, 2000). The major advantage of this technique is its rapidity and ease of spectral acquisition, enabling non-

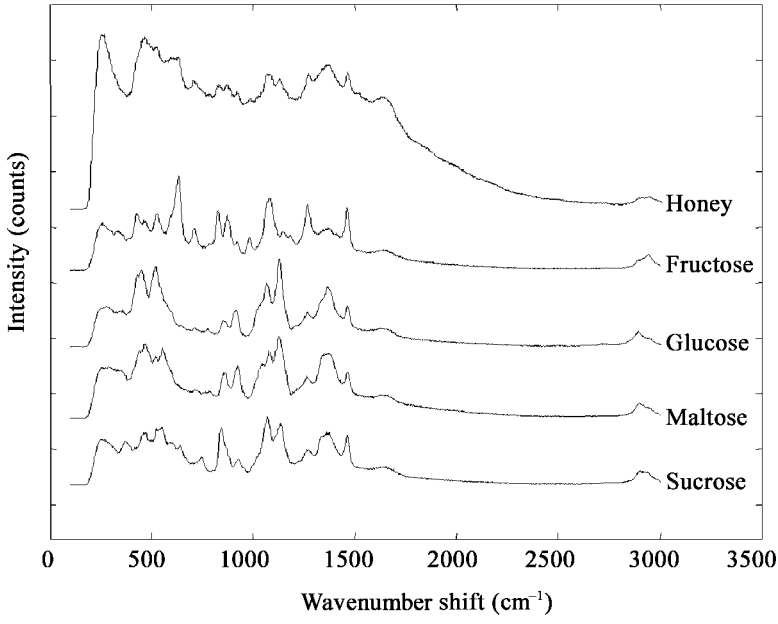

Fig. 4.5 Raman spectra of honey and its sugar components (typical % weight); fructose (38.5%), glucose (31.0%), maltose (7.2%), and sucrose (1.5%). Spectra were collected on a Renishaw System 100 dispersive Raman spectrometer (Renishaw, UK), with a near-infra-red 780-nm diode laser with the power at the sampling point typically at 80 mW.

invasive measurements to be made with little or no sample preparation. However, sufficient signal resolution is to be ascertained for the desired effect to be monitored, in order to use spectral information as protein or metabolic fingerprints.

Another vibrational spectroscopic technique that has the potential to be able to capture metabolic or protein snapshots is Raman spectroscopy. It is a technique that monitors Raman scattered photons (resulting from vibration mediated scattering of incident light on analytes), which are orders of magnitude less intense than the more common Raleigh scattered photons. The resulting signal is therefore weak to generate sufficient discrimination for protein and metabolic fingerprinting. However, signal enhancements can be obtained by using metal colloids as in surface enhanced (resonance) Raman or by using laser of wavelength (in the UV region) at which a co-analyte absorbs and resonates to generate enhanced signals (UV-resonance Raman) (Ferraro and Nakamoto, 1994). The information content even in Raman spectroscopy (Fig. 4.5), which has been demonstrated to have the capability for discriminating honey of different floral and geographical origins (Goodacre *et al.*, 2002a), and the authentication of extra virgin olive oils using FT-Raman spectroscopy (Davies *et al.*, 2000) point to the potential of Raman spectroscopy to the rapid fingerprinting for food authentication (LiChan, 1996).

4.5.2 Nuclear magnetic resonance spectroscopy

NMR is another technique that has been shown to have the potential for generating rapid metabolic fingerprints, non-invasively (Raamsdonk *et al.*, 2001, Nicholson *et al.*, 2002). It is based on the fact that nuclei such as ^{1}H, ^{13}C, and ^{31}P can exist at different energy levels in a strong magnetic field, as they possess nuclear spin. If such nuclei are subject to a magnetic field and pulsed with radio frequency energy, the absorption and re-emission of energy as they change energy levels can be measured as chemical shifts, the NMR spectrum being a series of peaks representing the chemical environments within a molecule. It is thus valuable in getting structural information. ^{1}H, ^{13}C, ^{31}P NMR can also be used to trace metabolites along pathways. However, ^{1}H NMR spectra can be complex due to several contributing analytes, and lengthy 2D NMR is necessary to attempt to assign the chemical shifts to specific metabolites. ^{13}C or ^{31}P NMR are ideally suited to monitor biochemical activities, but ^{13}C NMR would require growing cultures in media containing isotopically labelled substrates in order to enhance sensitivity of detection, and although ^{31}P NMR can be used for *in vivo* studies, it would require high cell densities for sensitive measurements.

A metabolic fingerprinting approach using NMR has been shown to have potential in detecting 'silent genes', genes that do not overtly show a phenotype, such as changes in growth rate (Raamsdonk *et al.*, 2001). The study also highlights the possibility of revealing functions when 'metabolic snapshots' from strains deleted for unstudied genes are compared to those deleted for known genes. Metabolite fingerprinting using proton NMR and LC as a basis for evaluating substantial equivalence of transgenic crops has been suggested (Noteborn *et al.*, 2000).

4.5.3 Mass spectrometric methods

Mass spectrometry involves the detection of gas phase ions, based on its mass and charge. It has been a major technique in chemical analysis over the last century, and has been used to identify and characterise chemical compounds. Simplistically it consists of (a) an ionisation source to generate gas phase ions, (b) a mass analyser to separate the ions based on its mass and charge, and (c) a detector to detect the separated ions. The advent of ESI and MALDI techniques has enabled efficient 'soft' ionisation of large macromolecules with minimal fragmentation, and has widened the scope of biomolecular analysis, the discoveries being acknowledged by Nobel prizes in Chemistry for 2002 to John Fenn and Koichi Tanaka.

Sample desolvation and ionisation in ESI takes place when a liquid sample is allowed to flow through a narrow capillary, the tip of which is connected to a voltage supply, to generate charged aerosols that can be desolvated with the assistance of temperature, and a coaxial flow of gas. In MALDI, samples are presented as dried spots, mixed with a matrix. Pulsed application of a laser at a wavelength at which the matrix absorbs enables desorption of the sample-matrix co-crystals from the surface into the analyser as charged ions. One of four

analyser types is in common use (a) (magnetic) sector (b) quadrupole (Q) filters and ion traps, (c) time-of-flight (ToF), or (d) Fourier-transform ion cyclotron resonance (FT-ICR). ESI is usually coupled to Q or ToF analysers, while MALDI is coupled to a ToF. More recently FT-ICR analysers that provide high mass resolutions have become available.

A unique feature of MS analysis is the capability of combining one or more of these analysers to create tandem mass spectrometers (MS/MS). Each mass spectrometer can be used to scan, select one or all ions, and between two mass analysers the selected ions can be subjected to collision with neutrals, gases, acceleration, deceleration, etc., leading to several possible MS/MS experiments. This is particularly useful in characterising bio-macromolecules. Two common tandem MS geometries are the triple quadrupole and the Q-TOF. Tandem MS experiments are also possible with ion-traps and FTICR analysers. Here, the successive MS experiments are separated in time instead of space, enabling MS^n possibilities. Combinations with sector instruments are also possible, but these are generally costly.

Compared to vibrational and NMR spectroscopies, analysis by MS offers greater sensitivity and selectivity of measurements, although the technique is invasive. Therefore, the information content can be expected to be greater with MS for fingerprinting applications. An example of relevance to food authentication is the demonstration of discriminatory capability of direct-infusion ESI-MS in authenticating vegetable and nut oils (Fig. 4.6 (Goodacre *et al.*, 2002b)). Microbial fingerprinting using MS of crude cell extracts and whole cells offers potential for monitoring microbiological safety in the food and environment. Such methods usually involve few sample preparation steps and offer sufficient information for discrimination or identification purposes.

Early investigations in this direction involved the application of pyrolysis-MS (Goodfellow, 1995, Magee, 1993, Guillou *et al.*, 1999). However, the analytes measurable in the commonest PyMS are small (typically $< \mathrm{m/z}\,200$), and information on the structure and identity of the molecules producing the pyrolysate is lost, due to the *in vacuo* thermal degradation step (Goodacre and Kell, 1996), unless the metabolite is of very low molecular weight (Goodacre *et al.*, 2000). Alternatively, the application of desorption techniques, such as plasma desorption (Cotter, 1988) and fast-atom bombardment (FAB) (Drucker, 1994, Heller *et al.*, 1987) allows the analysis of involatile fractions, and extends the measurable mass range. However, with the advent of MALDI- and ESI-MS, the ease of microbial fingerprinting using these techniques is advancing further, and is a subject of several investigations (Claydon *et al.*, 1996, Demirev *et al.*, 2001a, Krishnamurthy *et al.*, 2000, Vaidyanathan *et al.*, 2001) and reviews (vanBaar, 2000, Fenselau and Demirev, 2001, Lay, 2001). In particular, MALDI-MS of whole cells (Claydon *et al.*, 1996, Fenselau and Demirev, 2001, Krishnamurthy *et al.*, 2000, Lay, 2001) is attractive because of its ability to characterise the proteome, albeit only a fraction of it, directly without 2D-GE separation and match these proteins to those in databases (Demirev *et al.*, 2001a, Ryzhov and Fenselau, 2001).

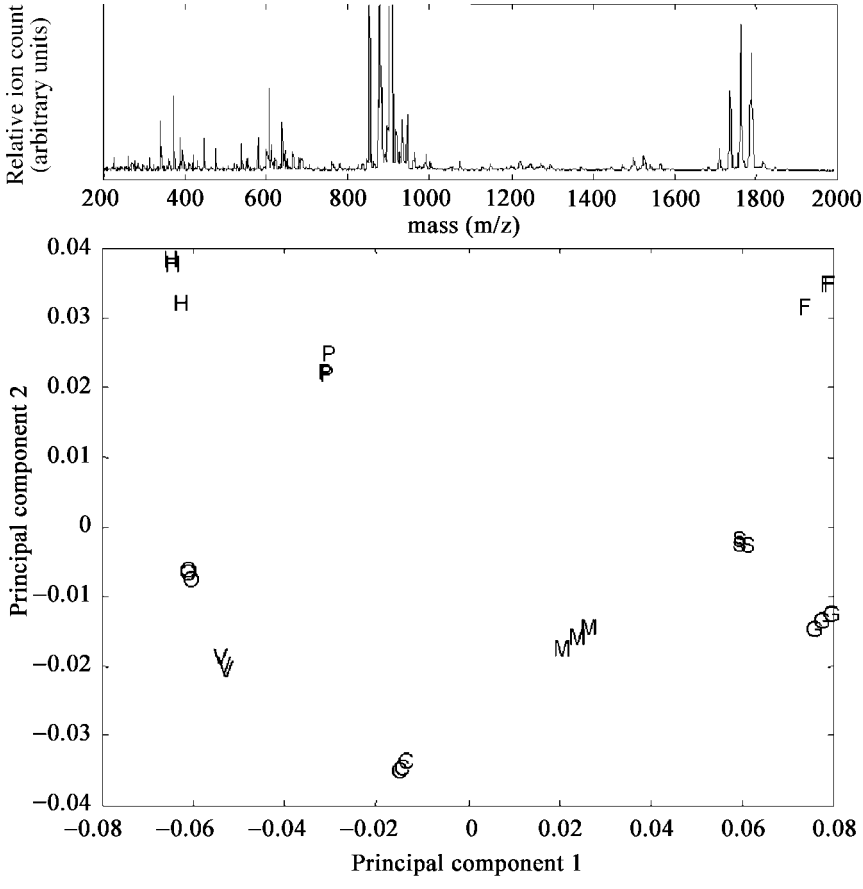

Fig. 4.6 (a) A typical ESI-MS spectrum from olive oil (O). (b) PCA on direct infusion ESI-MS spectra from corn (C), grapestone oil (G), husk oil (H), olive oil (O), extra virgin olive oil (V), peanut oil (P), soya oil (S), sunflower oil (F) and a mixed oil (M) comprising peanut, sunflower and soya oils. The first two PCs are plotted which account for 70.1% and 12.6% of the total explained variance respectively. The oils were analysed by diluting them 1,000-fold in 60% dichloromethane (CH2Cl2): 40% 10mM ammonium acetate (NH4OAc) in methanol (CH3OH). Spectra were collected in the positive ion mode (ES+) on a Micromass QTof^{TM} ESI-MS(-MS) spectrometer (Wythenshawe, Manchester, UK).

The majority of the investigations have concentrated on methods development and on demonstrating the applicability of the technique to relatively well-characterised microorganisms. Proteins contribute predominantly to the mass spectral signals in whole cell MALDI-MS analyses. Low mass proteins (typically less than 30 kDa) are readily observed in whole cell MALDI-MS. However, it is also possible to observe high mass proteins in some cases (Madonna *et al.*, 2000, Vaidyanathan *et al.*, 2002b), albeit with relatively poor mass resolution and accuracy. Although such rapid methods are not

comprehensive in terms of information content, a sizeable number of signals (typically 40–50 peaks for Gram-negative, and 10–20 for Gram-positive microorganisms) are observed for fingerprinting purposes, and have been shown to have discriminatory value.

The mass spectral signals appear to have contributions from cold-shock proteins (Holland *et al.*, 1999), cell-wall or membrane associated proteins (Dai *et al.*, 1999), as well as those from the cytosol (Ryzhov and Fenselau, 2001). ESI-MS of intact viruses (Siuzdak, 1998) and whole bacterial cells (Goodacre *et al.*, 1999) has also been shown to contain information-rich spectra, the latter with strain level discriminatory capabilities (Vaidyanathan *et al.*, 2001), and fully automated via flow-injection (Vaidyanathan *et al.*, 2002a). An advantage of using ESI-MS for fingerprinting purposes is the possibility of using MS/MS for global characterisation (Xiang *et al.*, 2000) and biomarker identification (Demirev *et al.*, 2001b).

A more recent addition to the proteomic MS based tools for fingerprinting applications is the surface-enhanced laser desorption ionisation (SELDI) MS (Merchant and Weinberger, 2000, Chapman, 2002). It involves the capture of proteins by biochemical and intermolecular interactions on chromatographic surfaces and subsequent direct mass spectrometry from the surface, usually by ToF-MS. Reproducible protein profiles can be generated from crude biological samples like cell lysates and body fluids. Such systems are commercially available (Ciphergen) and have been applied mostly in medical analysis (von Eggeling *et al.*, 2001, Issaq *et al.*, 2002).

4.5.4 Bioinformatics and chemometrics

In the post-genomic era, there has been a deluge of information available for processing. Proteomic (and to a lesser extent metabolomic) databases are catching up with genomic databases, but there are considerable challenges in accruing information from these later developing fields, particularly with respect to database infrastructure, data curation and quality checks (and the same is also true of DNA array data), remembering that user requirements must still be clarified. The subject of bioinformatics is dedicated for such efforts and rapid strides are being made for storing and retrieving data, thanks to developments in computing technology. Given the multivariate nature of analysis in '-omic technologies there is also a greater emphasis in research on the application of chemometric techniques for extracting relevant information (Lavine and Workman, 2002).

In addition to the increasing urgency for good databases, there is a concomitant need for improved chemometric methods for the analysis of metabolome and proteome data. This chapter deals with the analytical processes used and in development for the production of these data, and for in-depth discussions on chemometrics we direct you to Martens and Næs (1989), Massart *et al.* (1997), Shaw *et al.* (1999) and Beavis *et al.* (2000) for excellent introductory texts and reviews as well as the three relevant chapters in this book.

4.6 Applications: rapid authentication of food components

The application and utility of the technologies discussed above to food authentication and traceability can be appreciated from the factors that drive analytical efforts in this area of food science. Food, processed or packaged, is generally of plant or animal origin. Therefore, a key issue in food authenticity is the reliable authentication of the species of origin, especially with meat and meat products. In some cases, for example honey, geographical origin is an identification criterion that is often used as a quality measure. Another issue in food authentication is the adulteration of foods for economic gains. Although an age-old problem, it is still persistent and is driven by such factors like demand for higher value 'speciality' foods, global trading, and price fluctuations, providing opportunities for illegal profits to be made from adulteration. A third issue in food authentication, more of a food safety one, is that of microbial contamination resulting in food spoilage and the detection of foodborne pathogens.

More recently, other issues such as authentication of organically produced products and genetically modified produce have also become important within the context of 'substantial equivalence' (*vide infra*). Therefore, it can be stated that the broad objective in food authentication is to identify unique markers or groups of markers to characterise the authenticity of foods or their potential adulterants/contaminants, and use these to resolve authenticity issues. In this regard, DNA based methodologies (Popping, 2002, Luthy, 1999) have been used, but protein and metabolite fingerprinting approaches have the potential to be useful for rapid screening on a routine basis and can be complemented by DNA based methodologies for more elaborate assessments. Some of the examples include:

- the potential for FT-Raman spectroscopy (Davies *et al.*, 2000), NMR spectroscopy (for the detection of minor metabolites) (Mannina and Segre, 2002) and mass spectrometric methods (Goodacre *et al.*, 2002b), all in combination with chemometric techniques, for the authentication of olive oils
- capillary electrophoresis in fingerprinting metabolites (organic acids) for authentication of orange juices (Saavedra *et al.*, 2000)
- Raman spectroscopy combined with chemometrics for the authentication of honey (Goodacre *et al.*, 2002a)
- FT-IR combined with chemometrics for the detection of microbial spoilage of food (Ellis *et al.*, 2002)
- the modern mass spectrometric methods that are under development for bacterial identification (section 4.5.3 above).

Finally, since proteins form a substantial fraction of organic matter in meat, fish, or their products, protein-fingerprinting approaches would be particularly suitable for authentication of these foodstuffs. For processed foods, metabolic and protein fingerprinting approaches can be useful in authenticating quality of produce at different stages of production.

A major issue in food traceability is that of genetically engineered crops. It has been a subject of intense debate ever since field trials were introduced in the 1980s. There has been extensive experience of GM crops in commercial agriculture over the past decade and there are scientific and economic concerns with respect to transgenic crops (Smyth *et al.*, 2002), especially with respect to gene flow resulting in undesirable transfer of traits from crop to wild species, and the importance of environmental and toxicological studies on the raw foodstuff (Snow, 2002, Schubert, 2002, Dale *et al.*, 2002, Chassy, 2002, Smyth *et al.*, 2002). Introduction of the same gene in different systems can have different consequences, indicating that gene transfer is not necessarily a localised effect and that it can have undesirable consequences on a global scale. For, instance, over-expression of a gene involved in pectin synthesis had no effect in tobacco, but caused leaf shedding in apple trees (Atkinson *et al.*, 2002). It is argued that introduction of any gene, whether from a different species or the same cultivar, usually significantly changes the overall gene expression and therefore the phenotype of the recipient cell. This raises obvious concerns that such perturbations could lead to biosynthesis of molecules that are toxic, allergenic, or carcinogenic (Schubert, 2002). Therefore, the food safety assessment of genetically engineered foods requires the assessment of how safe the modified food is with respect to its traditional counterpart.

As a starting point of the safety assessment, the OECD introduced the concept of 'substantial equivalence' as a means of establishing a benchmark for safe foods in 1993. It is said to involve comparison between the modified crop and its non-modified counterpart, with respect to substances relevant from a toxicological, nutritional, or 'wholesomeness' point of view (Custers, 2001). The broadness of its definition makes it an indirect method for assessing differences, and as there are no standardised lists yet of what substances to compare, substantial equivalence is open to subjective interpretation. This has prompted scepticism in the scientific community with regards to its acceptance (Millstone *et al.*, 1999). However, it has been argued that it is only a conceptual tool for food producers and government regulators and is not intended as a scientific concept (Miller, 1999), and that it is a useful tool as a guiding principle and not as a substitute for safety assessment (Kearns and Mayers, 1999). In the absence of an alternative, it is advocated as an aiding tool for subsequent toxicological investigations that may be required for food safety assessments (Kuiper *et al.*, 2001).

It has been proposed that an operational definition of food safety assessment include a minimum list of macro- and micronutrients, anti-nutrients, inherent plant toxins, secondary metabolites, and allergens to be analysed for each GM crop species and of their baseline concentrations in conventional varieties (Schenkelaars, 2002). In this regard, it may be essential to generate metabolomic and proteomic databases and use these in making food safety assessments, especially as metabolites and proteins confer toxicity or allergenicity and are suitable candidates as markers for monitoring purposes. In addition, aspects such as taste and nutrition may be traced at the phenotypic level by monitoring the metabolic and protein profiles of food, and can be used for authenticating the

quality of raw materials that can in turn be useful in assessing the potential quality of the end products.

4.7 Future trends

The genomic revolution has grown in phases, driven partly by the realisation of our paucity of knowledge of the biology of living organisms; for example, when the microbiologist's pet organism *Escherichia coli* was sequenced (Blattner *et al.*, 1997) it was discovered that a staggering 38% of its genes had never been encountered in the laboratory! And the thirst for knowledge has been further accelerated due to the production last year of draft sequences of the human genome. Consequently, analytical efforts have to a large extent mirrored the knowledge base and subsequent hypotheses. Initial efforts were driven by the need to unravel the genetic make-up of living organisms (DNA sequencing). This was followed by the need to define genes and know which genes are expressed/transcribed (transcriptomics). Subsequent knowledge that not all transcribed genes contribute to cell function and that function is mediated by activities beyond transcription have necessitated directing analytical efforts towards capturing the proteome (proteomics), the complexity of which is occupying a significant portion of current analytical efforts (as is evident from section 4.3). This is the phase we are currently in. Technologically, challenges with respect to increasing the number of proteins detected per analysis, the sensitivity of detection to identify proteins of low abundance, the dynamic range of analysis, miniaturising large-scale protein separation strategies to increase sample throughput and proteome completeness, will be addressed in the near future. Concomitant with this will be efforts towards creating and maintaining public repositories of proteomic data and standardisation of protocols to enable ease of comparison of proteomes obtained from different laboratories (http://psidev.sourceforge.net/meetings/2002–10/report.htm). Greater efforts will be expended in developing technologies for comprehensive estimation of protein function as compared to those for protein expression.

Although metabolites in themselves are less complex than proteins and nucleic acids and techniques for their determination have been in existence since biochemical analyses began, analytical efforts for monitoring metabolomes are lagging behind, in part due to insufficient understanding of metabolic networks and their contribution to cell function. The diversity and dynamic nature of metabolomes poses considerable challenges for future analytical efforts in developing rapid, high-throughput technologies; for example, there are estimated to be up to 200,000 different metabolites in the plant kingdom (Fiehn, 2002). Techniques that incorporate miniaturised analytical strategies for high-throughput analysis, such as micro total analytical systems (Greenwood and Greenway, 2002), will play a useful role in expanding metabolomic analyses. Current technologies enable only a portion of the metabolome to be analysed in a single go. Future efforts will have to be directed towards

increasing the size and range of metabolites analysed. For this, technical challenges with respect to reliable and comprehensive metabolite extraction strategies will have to be addressed in the near future. Hyphenated techniques that incorporate tandem and parallel combinations of one or more techniques will receive greater attention for comprehensive analyses. Non-invasive techniques that can provide reliable information in a rapid, high-throughput format will receive greater attention for devising fingerprinting strategies.

Subsequent to the knowledge gained by the use of ''omic' technologies, food authentication and traceability in the post-genomic era will increasingly be driven by database-based assessments. The motivations will certainly be influenced by developments in the medical field, where ''omic' technologies have found wider application, for instance, the growth of crops for health rather than for food or fibre (Raskin *et al.*, 2002), and the development of functional foods (Mollet and Rowland, 2002). To a greater extent, stringent regulations driven by public awareness of genetically modified foods will influence the application of ''omic' technologies, especially those of proteomes and metabolomes, to food authentication and traceability.

4.8 Sources of further information and advice

Comprehensive access to information regarding complete and ongoing genome projects around the world can be had from 'genomes online database' (GOLD) http://wit.integratedgenomics.com/GOLD/. The Institute for Genomic Research (TIGR) provides web-based access to microbial databases (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl), with a listing of published microbial genomes and chromosomes and those in progress (http://www.tigr.org/tdb/mdb/mdb.html).

The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://www.expasy.ch) is dedicated to the analysis of protein sequences and structures as well as 2-D PAGE analysis data (http://us.expasy.org/ch2d/2d-index.html). Protein sequence information is contained in SWISS-PROT/TrEMBL databases that can be accessed from the server (http://ca.expasy.org/sprot/sprot-top.html). Information on biologically significant protein domains, and on patterns and profiles that help to identify the protein family (if any) a new sequence belongs to can be obtained from the PROSITE database (http://ca.expasy.org/prosite/). The Munich Information Centre for Protein Sequences (MIPS), hosted by the Institute for Bioinformatics (IBI) supports and maintains a set of generic protein databases as well as the systematic comparative analysis of microbial, fungal, and plant genomes (http://mips.gsf.de/).

Other proteomic databases include EXProt (database for EXPerimentally verified Protein functions), a non-redundant database containing protein sequences for which the function has been experimentally verified (http://www.cmbi.kun.nl/EXProt/); a proteome analysis database (http://www.ebi.ac.uk/proteome/) for

comprehensive statistical and comparative analyses of predicted proteomes, including InterPro, a database of protein families, domains and functional sites (http://www.ebi.ac.uk/interpro/); databases from Incyte Genomics (http://www.proteome.com/databases/); protein information resource (PIR), a database of functionally annotated protein sequences (http://pir.georgetown.edu/pirwww/).

A compendium of protein fingerprints (groups of conserved motifs used to characterise a protein family) is available from PRINTS (http://www.bioinf.man.ac.uk/dbbrowser/PRINTS/). Current news articles on proteomics are available from http://www.bioexchange.com/news/proteomics.cfm. BioCyc knowledge library (http://biocyc.org/) is another database collection that describes genomes and metabolic pathways. Database search engines like BLAST (of National Centre for Biotechnology Information) and FASTA (of the European Bioinformatics Institute) are useful for assessing sequence similarity and for homology searching against nucleotide and protein databases. Specific search engines and tools for mining sequence databases in conjunction with mass spectrometry data are also available (e.g., ProteinProspector (http://prospector.ucsf.edu/), and Mascot (http://www.matrixscience.com/cgi/index.pl?page=../home.html)).

Several books on proteomics methodology are increasingly being published (e.g., *Proteomics in Practice: A Laboratory Manual of Proteome Analysis*, by R. Westermeir and T. Naven, published by John Wiley & Sons, 2002; and *Proteomics: From Protein Sequence to Function*, edited by S. Pennington and M. J. Dunn, published by Springer Verlag, 2001). Relatively less information is available on metabolome analyses, although some books are beginning to appear (e.g., *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*, edited by G. G. Harrigan and R. Goodacre, published by Kluwer academic publishers, 2003). Whilst functional genomics with respect to phytochemistry has recently been highlighted in Vol. 36 of *Recent Advances in Phytochemistry* (edited by J.T. Romeo and R.A. Dixon, published by Pergamon, 2002). Journals that publish on proteome and metabolome analyses include *Proteomics*, and *Comparative and Functional Genomics*. Current trends and challenges in proteomics are discussed in the March 2003 issues of *Nature* (Vol. 422, Iss. 6928) and *Nature Biotechnology* (Vol. 21, Iss. 3).

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5

Near infra-red absorption technology for analysing food composition

I. B. Benson, NDC Infrared Engineering, UK

5.1 Introduction

Near infra-red (NIR) measurement technology offers an amazingly diverse capability for the analysis of many different constituents or properties of food products. Moisture, fat, protein and sugar content are perhaps the most well known applications in products such as grain, flour, cereals, dairy products, snacks and coffee but NIR has also found application for the measurement of chocolate thickness on refiner rollers, the thickness of sausage casings, the alcohol content of beverages, the maturity of peas and even the quality of fruit juices. The technique can also provide food technologists with information regarding the thickness of critical barrier layers in multi-layer packaging films, protective coatings applied to plastic, paper or metal food packaging materials which all can impact on food storage properties if not controlled.

The emphasis today, and for the future, is towards on-line measurement and control of food manufacturing processes and NIR lends itself extremely well to this. It is fast, continuous, non-destructive and can be applied relatively simply in many industrial processes. However, for it to be of value in an increasingly automated world the control loop needs to be closed and the emphasis nowadays has to be on robust, easy to apply measurement solutions, which can be depended upon. Achieving these goals is the major preoccupation of both instrument suppliers and users. To date the food processing industry has generally been slow, with few exceptions, on the uptake of on-line NIR measurement in terms of closed-loop process control. Many factors have contributed to this such as confidence in the reliability of the technique, obtaining access to the product to allow a reliable measurement (e.g. for products transported in pneumatic or gravity fed ducts), the fact that many

processes are batch operations where the product is batch analysed prior to processing and the willingness or ability to invest in automation. This situation is changing as with the demand for ever more efficient manufacturing of the highest quality, consistent product becomes the norm along with legislative demands for food product constituents to be traceable. This in turn has necessitated instrument manufacturers developing product solutions that can be depended upon and has required serious effort in the re-education of potential users that the technique indeed has a place in modern industry.

NIR measurement is a well-established analytical technique, and many examples of its applications can be found in the literature going back as far as the 1950s and before. Specifically, early laboratory applications of NIR concentrated upon quantitative and qualitative studies of liquids and solvent mixtures, and such applications are cited in a review by Kaye in 1954. From the same era McCallum (1961) and Miller *et al.* (1949) provide extensive general coverage of NIR spectroscopy, while Willis (1979) reviews industrial analytical applications of the technique. The most significant and active area of development over the last twenty years has been the application of NIR for the compositional analysis of solids, particularly cereals and foodstuffs where the product is analysed by a reflectance type measurement. The chemical complexities of most foodstuffs or natural products, however, have necessitated the accompanying development of a variety of statistical and mathematical signal-processing methods. These techniques have enabled useful measurements to be obtained from spectral reflectance data, which characteristically show many overlapping, broad absorption features of seemingly low information content.

The uses of NIR within the food and cereals industries are far too numerous to be comprehensively listed, and therefore a selection of references to pertinent reviews and papers is given (Star *et al.* 1981; Norris and Hart 1965; Osborne and Fearn 1986; Norris and Williams 1987; Hunt *et al.* 1977; Biggs 1979; Osbourne *et al.*, 1993; Burns and Ciurczak, 2001). These generally refer to laboratory-based analysis, although on-line analysis is discussed in other reports (Edgar and Hindle 1971; Bruton 1970; Benson 1989).

NIR measurement has broadly evolved on two fronts, these being laboratory and on-line application of the technique. This division has resulted from the very different demands that the two approaches place upon the instrument design and specification. Laboratory measurement has the benefit of offering very controlled measurement conditions. The product can be appropriately prepared; for example it can be ground to a specific particle size and consistently presented to the instrument, usually in some form of windowed cell. Also an acceptable time for each measurement may be 30 seconds or more, which is obviously faster than the laboratory wet chemical equivalent, but slow in terms of a continuous on-line analysis.

The on-line NIR gauge must operate very much more quickly, making many measurements each second. For an on-line measurement, product presentation can rarely be controlled. The distance of the product from the gauge will almost certainly fluctuate, and the quantity of product passing the point of measurement

may vary. The form of the product is rarely uniform; there may be variations in the appearance or particle size of powders and fibrous materials. The ambient conditions are also rarely controlled with variations in product or ambient temperature, humidity and lighting levels being commonplace. The on-line gauge must therefore be designed to ignore such variations and be sufficiently robust to survive the rigours of the factory environment.

The application requirements for off-line and on-line measurement have also widely differed over the years. The most active areas of on-line analysis have been concerned with measurements where, if the component concentration changes, a corrective action can be taken in the process. The most obvious example is moisture, where dryers or conditioners can be readily controlled. Off-line analysis, on the other hand, has tended to concentrate upon providing a rapid replacement for some of the time consuming, wet chemical analyses such as those for protein, fat or carbohydrates.

The authors' expertise and interest have been strongly biased towards on-line measurement and the following text will therefore concentrate on this area. However, off-line measurement possibilities will be discussed and a novel approach to the concept of NIR laboratory analysis, using many of the key features derived from an on-line philosophy, will be described.

While moisture measurement remains a dominant on-line requirement, food industry needs are developing and the possibility of controlling, in real time, parameters other than moisture is becoming a requirement. The fat content of meat, protein levels in flour or the oil content of snacks are such examples. Additionally, quality traceabilty issues place demands upon manufacturers to show that the other product constituents have been measured and quantified. On-line instrumentation has undergone significant development and improvement and is now capable of offering far more than the early analysers. Many of the conventional laboratory-based NIR analyses can be performed online at least as well as in the laboratory.

Theory and instrumentation are discussed, often illustrated with details for moisture measurement, but equally applicable to other component analysis. Emphasis has been placed upon describing the characteristics and limitations of the NIR technique, and providing the reader with many actual examples of its application in the food industry for both moisture and non-moisture applications.

5.2 Principles of measurement

5.2.1 Theory behind NIR measurement

Infra-red light is part of the broad spectrum of energy known as electromagnetic radiation. Figure 5.1 shows the relative wavelengths and energies in the electromagnetic spectrum that are used in spectroscopy. While X-rays are of extremely high energy, capable of promoting inner electron transitions in high atomic number elements, the infra-red region is of relatively low energy and upon interaction with molecules, causes inter-atomic vibrations. Near infra-red

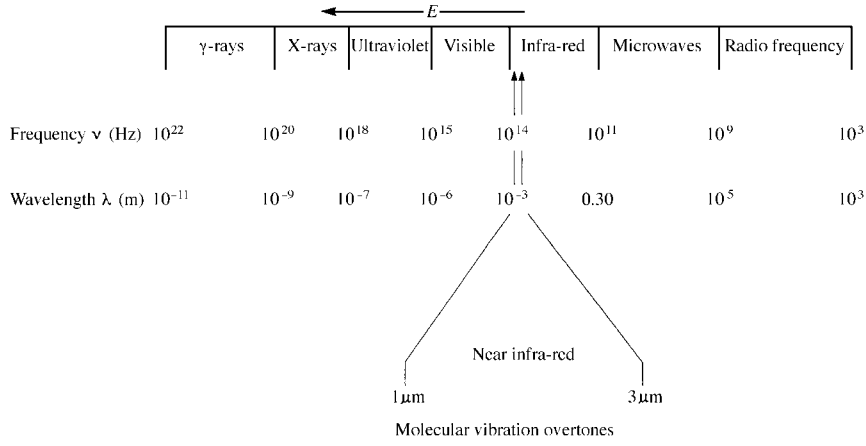

Fig. 5.1 Effect of instrument response time.

spectroscopy is concerned with a specific region of the infra-red, namely the 1–2.5 micrometres (μm) (10,000 to 4,000 cm^{-1}) range, adjacent to the red end of the visible spectrum.

The frequency of this wave oscillation in the infra-red region is about $10^{13}-10^{14}$ Hz, which is of the same order as the natural mechanical vibrational frequencies of many chemical groups. (The wavelength λ (m) of electromagnetic radiation is related to the frequency v (Hz) by $c = v\lambda$, where $c \approx 3 \times 10^8$ m s^{-1} is the velocity of light.) The water molecule is one of the best known examples of an NIR absorber and exhibits several distinct vibrational modes which relate to infra-red absorption at well-defined wavelengths or frequencies. The frequency of oscillation of any mode is dependent upon the atomic masses and bond strengths of the $-$OH group. Provided that the vibration in question results in a change in dipole moment at its extremes of movement, NIR radiation at the appropriate frequency will be absorbed by the group, causing it to vibrate at this same frequency. Energy is thus taken from the radiation field and is dissipated as heat by frictional or collision damping. The mechanism described is known as infra-red absorption and is covered comprehensively in other texts (Banwell 1983; Herzberg 1945).

These fundamental or natural vibrations are the basis of the familiar mid-infra-red spectroscopy commonly used by chemists in qualitative analysis. The mid-infra-red is, however, generally unsuitable for on-line compositional analysis for a number of reasons. In particular, the extremely low reflectivity of most solids above 2.5 μm results in unusable signal-to-noise levels for meaningful interpretation. While Fourier transform infra-red (FTIR) analysis in the mid-IR has been demonstrated to be a useful technique, this method does not lend itself to on-line analysis (Benson *et al.* 1988). Secondly, the absorption bands in the mid-IR are extremely intense and often display considerable fine structure, both being features that do not lend themselves to quantitative

analysis. The NIR spectral region, on the other hand, deals with absorptions, which are a result of vibrations at harmonic or combination frequencies of the fundamental absorptions. The NIR region is therefore often referred to as the overtone region. The types of absorption that dominate the NIR are hydrogenic absorptions such as −OH, −NH and −CH vibrations. These types of absorption are displayed by moisture and virtually all other major constituents of foodstuffs.

Since these different molecular groups are made up of atoms of different masses and bond strengths, each one exhibits a unique set of absorptions at specific vibrational frequencies or wavelengths. This absorption specificity of the technique is one of the most important features in increasing the scope of its application in the food industry, allowing the analysis of various product constituents independently and simultaneously.

The amount of infra-red absorption due to a particular absorber relates in a well-defined way to the quantity of absorber present. The change in absorption with an increase in moisture within a material is illustrated in Fig. 5.2, which shows diffuse reflectance spectra for milk powder at two different moisture levels. Such spectra for solid food products are obtained using a diffuse reflectance NIR scanning spectrophotometer. The spectral information shown in Fig. 5.2 is a plot of percentage reflectance as a function of wavelength. Water shows three principal absorptions in the near infra-red at 1.45, 1.94 and 2.95 μm. The most commonly used water absorption band at 1.94 μm is evident in Fig. 5.2,

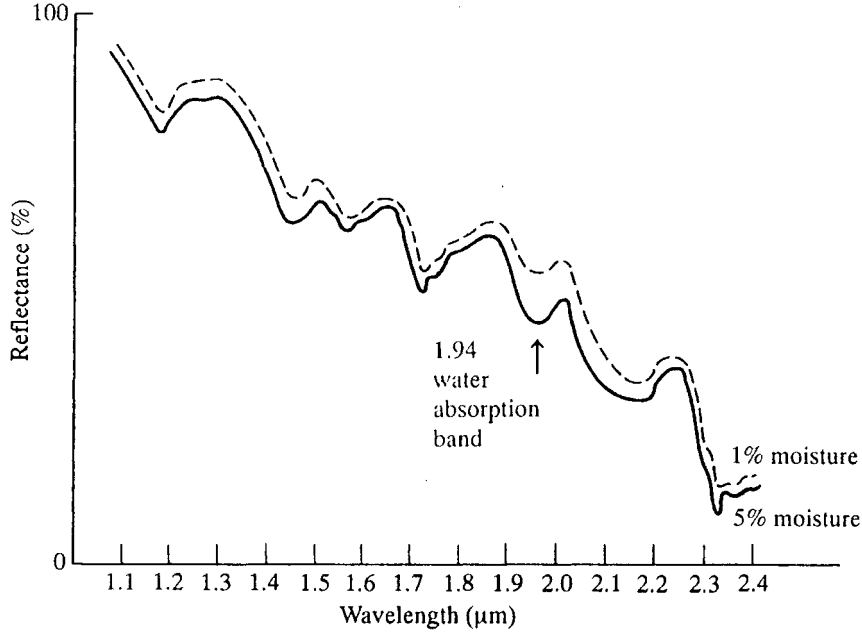

Fig. 5.2 Milk powder NIR reflectance spectra at two different moisture contents.

and as the moisture content increases there is a corresponding deepening of the band, that is a reduction in reflectivity consistent with increased infra-red absorption.

The absorption of infra-red energy by solids approximates to the Beer-Lambert exponential law for transmission, of the form

$$I_t = I_0 \exp(-kx)$$

where x is the path length of the radiation, k is the absorption coefficient, I_0 is the incident infra-red energy and I_t is the transmitted infra-red energy. This can be written as

$$\log(I_o/I_t) = kx \tag{1}$$

Therefore there is a simple linear relationship between the amount of absorber and the logarithm of the ratio of incident and transmitted energies. Making use of this principle requires a design of instrumentation capable of accurately measuring absorption changes at a number of wavelengths in the NIR corresponding to the absorber to be measured. Various techniques have been devised over the years to achieve this in both hardware design and the subsequent mathematical evaluation of the signal as discussed in section 5.3.

For reflectance measurements, while potentially complicated by light-scattering effects, a similar logarithmic relationship can be applied successfully in many instances. With some applications where light scattering plays a significant role, it will be shown that a more sophisticated approach is necessary to compensate for the effects that changes in the scattering characteristics of a product can have upon a measurement.

The reflectance spectra of milk powder in Fig. 5.2, in addition to the absorption bands due to water at 1.45 and 1.94 μm, show the presence of many other overlapping absorption bands. These are due to the various −OH, −NH and −CH stretches associated with the lactose, casein and fats present in typical milk powders. Although these can complicate a measurement of water content, requiring correction for the effects that their variation may have on the moisture measurement, they also provide an opportunity to measure these other constituents in addition to moisture.

5.2.2 Characteristics of NIR technology

The measurement range of the NIR is well suited to the needs of food processing since a wide range of different constituents may need to be measured, such as moisture, fat, sugar, caffeine, oil and protein. In the NIR the most important and prominent absorptions are due to the −OH, −NH and −CH groups. These absorption features are very specific to the constituent in question as discussed above and so the technique readily lends itself to quite detailed discrimination of the constituent parts of a foodstuff. In addition the technique has one further important strength, that of a choice of absorption sensitivities for a given constituent. Each of the absorbing groups characteristically exhibits three main

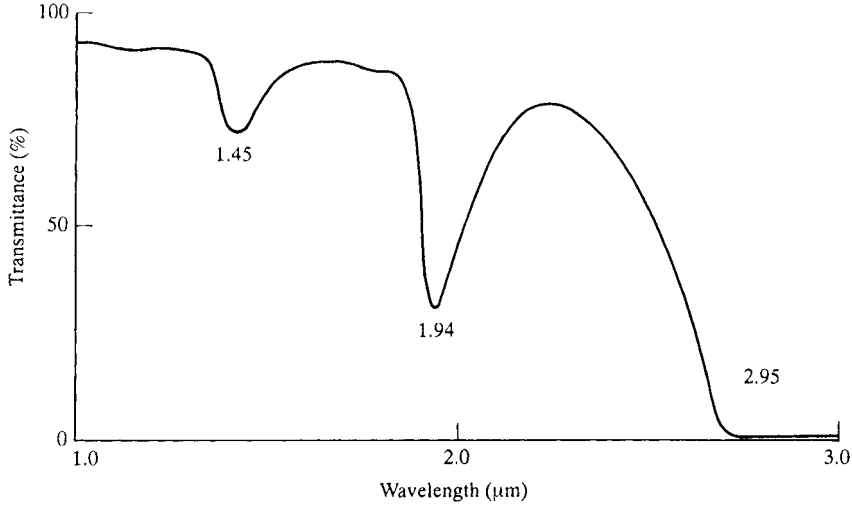

Fig. 5.3 Near infra-red transmission spectrum of water.

absorption bands in the NIR. For water, these absorptions occur at 1.45, 1.94 and 2.95 μm as is shown in Fig. 5.3, which is a transmission spectrum of pure water. The relative intensities for the three bands are 1:3:90 respectively. Fats or oils show corresponding absorptions at 1.7, 2.3 and 3.4 μm with a similar range of relative intensities to water. To generate the spectrum of water in Fig. 5.3 a cell path length of 150 μm was used. Clearly with this quantity of water the absorption at 2.95 μm is of no use in practice because it exhibits total absorption, whereas both absorption bands at the shorter wavelengths would provide usable changes if the cell path length was changed. This means that a wide range of moistures can be measured in the NIR by selecting the appropriate absorption band, which shows the greatest level of change at the product moisture level.

The NIR effectively provides a huge range of absorption sensitivities that can be selected to meet the application requirement. For example, with products at extremely low moistures, perhaps less than 0.5 per cent moisture, use might be made of the highly sensitive 2.95 μm absorption band. On the other hand for a 'wet' material of around 80 per cent moisture, e.g. sugar beet pulp, the weak 1.45 μm absorption would be more appropriate, to provide an adequate absorption change as a function of water content at these high moisture levels. This is a real strength of the technique, enabling an instrument to be tailored for the application. As stated above, the same characteristic holds true for −CH or −NH absorptions of materials such as fats or proteins; they each exhibit at least three regions of absorption in the NIR of different intensities and, importantly, at wavelengths different from the −OH absorptions of water.

The choice of the absorption band to use for a particular application depends upon many considerations. The degree of penetration into the product and the way in which it is scattered will affect the 'path length' of the radiation in the material and hence the amount of absorption. While the classical Beer-Lambert

absorption law ignores scatter characteristics, in fact many food products where measurement is required are solids and the scatter characteristics become equally or more important. The scatter/absorption models proposed by Kubelka and Munk (1931) show that the effective Beer Lambert equivalent absorption coefficient for an absorber in a solid matrix is better described as per the following equation:

$$K_{\text{eff}} = \sqrt{2K_{\text{act}}S}$$

Where: K_{eff} = the effective absorption coefficient, K_{act} = the water absorption coefficient (in the case of moisture) and S = the scatter coefficient for the product.

The choice is not always obvious. For example granulated sugar moisture, which is required to be dried below 0.1% moisture, has to be measured at 1.94μm because there is a tremendous level of absorption in a reflectance measurement due to the high penetration and scatter characteristics of the product. Similarly, breakfast cereals around 2–3% moisture require the use of the weak, 1.45μm band because the moisture absorption characteristic is so intense. For the determination of water contents between the levels discussed above, it is usually a matter of selecting the band that shows the greatest absorption change with a given change in moisture content. The absorption of NIR energy follows an exponential characteristic and therefore the maximum rate of change of absorption occurs at reflectance levels of around 60 per cent. This means that there is a point for a given moisture level above which greater sensitivity would be obtained by using a weaker absorption band. For food applications, the most commonly used water absorption is the 1.94 μm band. Apart from the 'anomalous' examples cited above, this band can generally be used for moisture levels between 1 and 30 per cent. These subtleties require the skills of applications engineering specialists correctly to configure an on-line instrument so that a user gets a suitable solution for the application in question. It is important, as discussed in section 5.6, that such capabilities are looked for when selecting a potential supplier of on-line NIR instrumentation.

5.2.3 Technologies for implementing NIR measurement

There have been a variety of concepts and technologies tried over the years for implementing NIR technology and this probably remains one of the interesting areas for development in instrument hardware. The theme of this chapter is very much on-line measurement and to date the author firmly believes that the fixed filter based technology as discussed in the next section, still leads the field in meeting the requirements for most applications, especially in light of some of the recent advances in instrument design and detector technology. Since fixed filter technology receives detailed coverage in the next section the following provides an overview of interesting and/or promising, alternative technology.

The use of LEDs for generating the necessary infra-red wavelengths is of great interest because this approach would eliminate the issues of source lamp life. For example, a solid state device designed by McFarlane employed infra-

red light-emitting diodes (IREDs) to generate the relevant NIR wavelengths for the measurement. These IREDs, however, emit a broad band of NIR energy and therefore fixed interference filters are still necessary to produce narrow bands of infra-red light. The physical displacement between the devices (one for each wavelength) also makes even illumination of the sample under analysis optically difficult. A more serious drawback for IREDs is that their spectral outputs change with temperature, which clearly, without sophisticated compensation, would have dire effects upon calibration stability. The wavelength range of operation of IREDs is perhaps the most severe limitation at the moment, since they are only economically available up to the 1.45 μm water absorption, thus missing the principal 1.94 μm band. In their favour, IREDs can be rapidly switched, thus providing a high-speed measurement in a modulated form without any moving parts but the very poor signal-to-noise ratio all but eliminates this benefit.

The German Rembe GmbH company once offered an LED-based device for the measurement of minerals and building products. However, it is required to be operated at very long response times with sophisticated signal processing to interpret the signals. For many food applications this speed of response issue and implied lack of precision would be a problem. Solid-state laser diodes offer another potentially interesting means of wavelength selection with a narrower band of emission of NIR energy. However, they are still affected by some of the limitations described above.

Another concept of potentially great interest is the use of acousto-optical tunable filters (AOTFs) to generate the relevant NIR wavelengths. The technique relies upon polarised light being diffracted by optically active materials under the influence of high-frequency acoustic waves. The radiation transmitted through such a material is preferentially polarised at a certain wavelength dependent upon the acoustic frequency. Thus, by using a polariser on the outgoing light beam, it is possible to select any number of wavelengths very quickly and without any moving parts. The technology is currently expensive and the concept is physically complex to implement, especially on line. There are also issues about the lifetime of the crystal used and the effects of vibration. Finally, containment of the high-energy RF emissions used in the generation of the acoustic wave present a challenge in meeting the containment requirements of the CE directives on EMC. However, systems are commercially available particularly for laboratory use. Some of these have been adapted for on-line use where product is essentially presented to an adaption of a laboratory device (e.g. Brimrose). No doubt with time this technology will become more practical and cost-acceptable in its implementation.

Various forms of spectrometer-based products are available which operate by generating full spectrum data. These may be based upon grating systems or Fourier transform technology. The price of such technology has been radically reduced over the years but the concept still has significant shortcomings in terms of signal-to-noise performance and therefore measurement observation times need to be lengthy (many seconds) to achieve useful results. The whole subject

of whether full spectrum analysis offers benefits over fixed filter systems is outside of the scope of this discussion but is covered in detail elsewhere (Hindle and Smith 1996). It is the contention of the authors that many so-called full spectrum analytical techniques actually use various data reduction techniques such as partial least squares (PLS), principal components analysis (PCA) or neural networks resulting in only partial use of the spectral information relevant to the product and constituents in question.

The fixed filter approach as discussed in section 5.3 is highly practical because in most on-line measurement situations the user knows what measurement is required and therefore the application is well defined and the appropriate wavelengths for the measurement are few and easily accommodated by such a device. To date, the fixed filter technology provides the best signal-to-noise ratio solution, important when looking at providing a dynamic measurement, with a need to reveal instantaneously short- and long-term information about the inherent variation in a process.

5.3 Instrumentation

5.3.1 On-line NIR Instrumentation

For an on-line NIR measurement to be acceptable in most industrial processes, severe constraints are imposed on the instrument design. For example, while most off-line analysers require the product to be carefully presented, pressed against a glass cell window, this is not practical in an on-line situation where a non-contacting system is much more appropriate. An on-line gauge must also be able to tolerate variations in operating conditions. Ambient temperature, relative humidity and the level of factory lighting are all factors that will change with time, yet they should not influence a measurement. Additionally, while certain processes may be impeccably clean and dust free, this is not usually the case; therefore instrument design must take into account the need to be able to operate in a mixture of dusty, damp, steamy and oily conditions!

Figure 5.4 shows a schematic drawing of the MM710 back scatter on-line NIR Gauge (NDC Infrared Engineering Ltd), which is designed for use in a diversity of applications in food constituent measurement. A quartz halogen lamp provides a broad spectrum of visible and infra-red light. The source is underrun to prolong its life, and is optically pre-aligned in a lamp holder to ensure maximum energy throughput. The light from the lamp is collected by an off-axis mirror which ensures maximum energy throughput. The focused light passes through a rotating filter wheel, which contains optical interference filters (McCloud 1986). The wheel also contains visible light-transmitting filters to allow the light beam patch of the instrument to be seen. The interference filters are designed to transmit infra-red energy at the wavelengths chosen for the measurement.

Typically, filters with bandwidths of between 1 and 5 per cent of the centre wavelength may be used. The number of wavelengths needed for a measurement

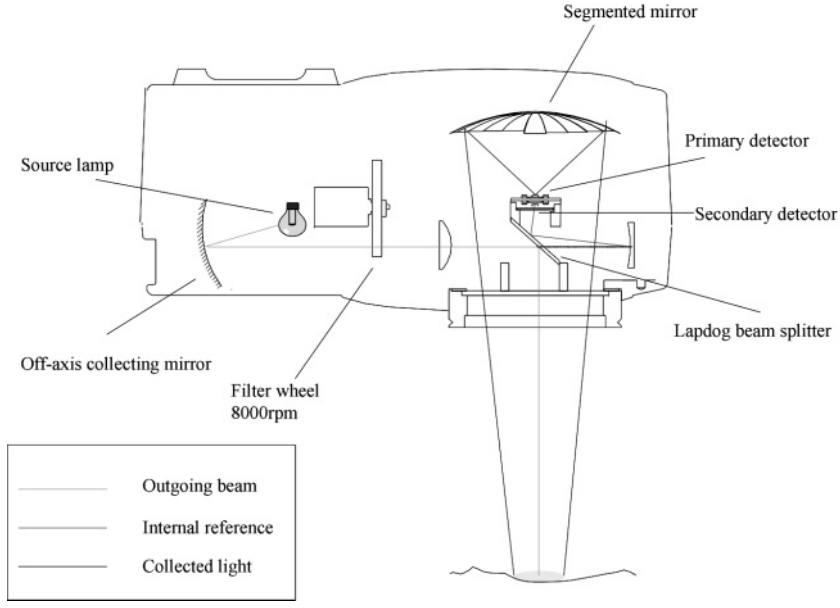

Fig. 5.4 MM710 optical sensing head schematic (NDC Infrared Engineering Ltd).

is dependent upon the application, as will be discussed later. These 'colour' filters take the form of thin glass disks, about 10mm square, upon which multi-layer dielectric coatings have been vacuum deposited. The major benefits of using these devices for wavelength isolation are their compactness and efficiency with respect to energy throughput at the chosen wavelength. They are also robust elements and offer extremely good long-term stability, both mechanically and in terms of their transmission, centre wavelength and bandwidth characteristics, all of which are important for long-term instrument stability.

The rotation rate of the filter wheel depends upon instrument design but in the case of the new generation gauge such as the MM710, is up to 8000 rpm providing for an extremely fast measurement. This high-speed performance is very important for number of reasons. In general a faster measurement is desirable because by high-speed sampling of the product the accuracy achievable is enhanced through making more measurements in a given time. Accuracy will be improved a $\sqrt{n}$ factor so if a product is sampled say 10 times more than another instrument there will be a potential $\sqrt{10}$ fold (i.e., 3 times) improvement in accuracy and reduction therefore in measurement noise. This means that the faster gauge is able to provide far more detailed information about a process and its variation and is not likely to be the limiting factor in the ability to control a process or monitor it using normal SPC procedures. In applications where the product flow is discontinuous, such as biscuits, the high-speed nature of the gauge in conjunction with an optical gating system allows analysis of the individual pieces as they pass under the gauge. These characteristics should be considered when choosing an on-line gauging system.

Immediately prior to the filtered light leaving the gauge, the secondary lead sulphide detector, located behind the beam splitter, samples a small percentage. The secondary detector monitors the optical characteristics of the instrument, in particular those of the lamp and interference filters. The lamp emission decreases with time while interference filters show changes in transmission with temperature variation. Both changes will be detected by the secondary detector, thus providing for subsequent correction. The importance of this concept becomes clearer shortly. The majority of the light passes from the gauge and lands on the product to be analysed. Some of the radiation is absorbed by the product while the remainder is scattered. The multi-faceted, concave collecting mirror in the gauge is arranged so as to collect a portion of the back scattered light and to focus it onto the primary lead sulphide detector. The lead sulphide photoconductive device is ideal for NIR applications since its detectivity peaks at wavelengths of about 2 μm at room temperature, and it is a fast responsive element offering good signal-to-noise performance. Advances in detector technology, patented by the authors' company, allow the device to be run at incredibly high speeds offering the benefits of greater product sampling and the chance to measure discontinuous product more meaningfully as discussed above.

Because the primary and secondary detectors are matched in sensitivity and kept at the same temperature, when the two sets of detector signals are ratioed an instrument output will be obtained that is self-compensated for the effects of temperature on the sensing head optical components and for the ageing (colour temperature change with time) of the lamp. For example, a drop in lamp emission at one of the measuring wavelengths would be detected by both primary and secondary detectors and seen as the same percentage change in signal level, cancelled by ratioing the detector outputs. The ability to design an instrument that is unaffected by temperature variation is vital because in practical situations temperature changes occur with daytime and seasons.

To date the sensor design described above has been the most successful all-round approach for meeting the requirements of an on-line NIR process analyser. However, other techniques have been developed, as discussed in section 5.2, which, if advances in technology permit, are likely to make an impact on basic gauge design.

Consideration of equation (1) suggests that, irrespective of the optical system, to make use of NIR absorption requires a knowledge of the incident and reflected energies at the measuring wavelength. In practice it is not possible to measure how much energy falls on to the measured sample, that is the incident radiation. Therefore for on-line measurement, in the simplest case, this is approximated by making a two-wavelength measurement. Figure 5.5 shows a schematic diagram of the technique and takes moisture as an example. One wavelength would be centred on the 1.94 μm absorption band of moisture. The other would be chosen from a part of the spectrum where there is little or no absorption due to moisture; commonly, a wavelength close to 1.80 μm is chosen as this reference. The ratio of the reflected signals at the two wavelengths provides a measure of the peak height

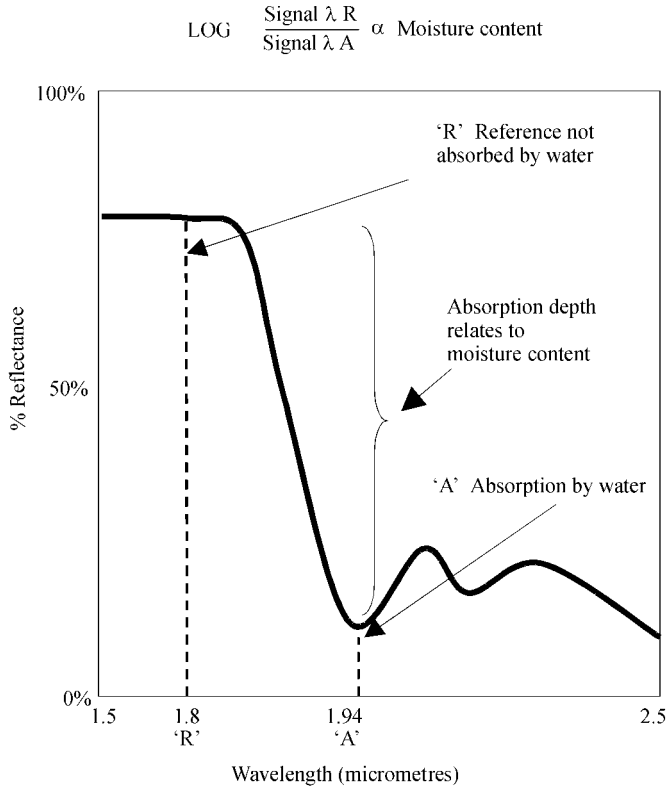

Fig. 5.5 Schematic of a two-length measurement with reference to the product NIR spectrum.

of the absorption. The logarithm of the ratio will relate linearly to moisture content as follows:

$$\log[\text{sig } \lambda_{\text{ref}}/\lambda_{\text{abs}}] \propto [H_2O] \tag{2}$$

where sig (λ_{ref}) is the signal at the reference wavelength, sig (λ_{abs}) is the signal at the absorption wavelength and [H_2O] is the moisture content. Clearly, as moisture content increases the absorption increases, there is a corresponding reduction in sig (λ_{abs}), and thus the overall term increases.

The infra-red detector, which is a photoconductive device, generates signals that are proportional to the reflected intensities at each wavelength in an alternating current (a.c.) form. In the MM710 gauge, which is an intelligent sensor, these signals are processed and a calibrated output is provided direct from the gauge itself. The MM710 is pre-calibrated for the application under consideration thus greatly reducing the effort required by users to get the device commissioned and operational. The final gauge output of a simple two-wavelength measurement would be of the form

$$\text{Output} = a + b \log [\text{sig } \lambda_{\text{ref}}/\lambda_{\text{abs}}]$$

where a is the offset or zero adjustment and b is the gain or span adjustment.

The measurement ratio calculation fulfils another fundamental requirement for on-line measurement. Many factors such as product-to-sensor distance (pass height variation) or dust build-up on external optical surfaces cannot be controlled. Changes in such factors would affect the size of the detected signals. However, both reference and absorption signals will be affected equally, and therefore the integrity of the ratio is maintained and the measurement is unaffected.

The description above concentrates on a simple two-wavelength measurement. This approach was used in the early days of NIR gauging but it quickly became apparent that to achieve greater calibration robustness or to measure more than one component, more infra-red wavelengths were necessary and even the format of the algorithm needed to be more sophisticated. This will be discussed in the next section with application examples. Earlier on-line instrumentation was based upon analogue electronic design with limited mathematical processing facilities. However, modern instruments such as the MM710, based upon microprocessor electronics and with greater sophistication in optical design allow far greater capability and the opportunity to develop calibrations based upon more sophisticated algorithm models.

The information generated by the on-line infra-red gauge nowadays needs to be output in a format that can be used by modern factory control systems. In addition to the traditional analogue outputs (e.g. 4–20 mA current loop proportional to the parameter being measured) connectivity to a process computer is now provided by any of the commonly used databus protocols such as ProfiBus, DeviceNet, ModBus or Ethernet TCPIP. This connectivity allows the user to use the gauge to perform some form of automatic feedback control to a dryer or a moisture conditioning process via the factory computer DCS system.

The main thrust in instrument design by the author's company is towards simplifying the implementation of a measurement system and ensuring that it is easy to use. Modern manufacturing facilities do not have the time or resources to apply to developing measurements and calibrating a gauge. For this reason the MM710 gauge is provided pre-calibrated for the application. In this way the gauge can be used virtually straight from the box without lengthy laboratory testing and configuration – a major time saver. Generally for proven applications, all that will be required is a simple offset correction to the laboratory reference method favoured by the user. This aspect is discussed in greater detail in section 5.6.

The instrument described above is designed for the measurement of powders, flaky or fibrous solids yet NIR measurement technology can be applied to liquids measurement. In this case a transmission gauge is more suitable. Figure 5.6 shows a fibre optic based analyser known as Liquidata which has been used in applications in the beverage industry for the measurement of alcohol and original gravity in beers, lagers and ciders. In this instrument the generation of

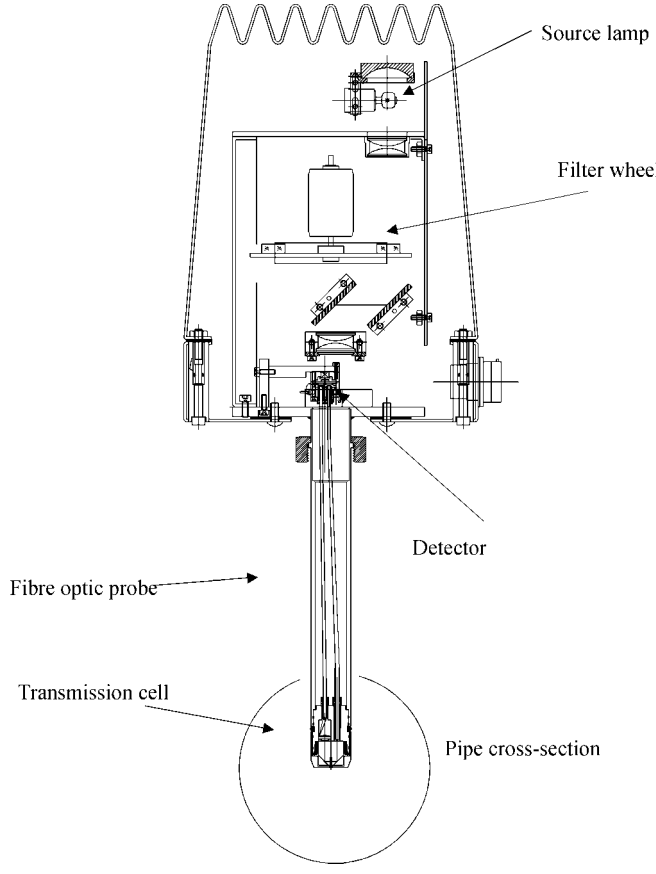

Fig. 5.6 Schematic of a liquid analyser (Liquidata NDC Infrared Engineering Ltd).

NIR light and the appropriate measurement wavelengths still makes use of the interference filter concept described above but the light is passed through fibre optics to a transmission cell which forms part a probe which is inserted into a pipeline. There are severe design requirements for this type of device in the food industry, namely the need for its tolerance to the aggressive clean in place (CIP) regimes that the industry uses (e.g. caustic soda cleaning at 80ºC). Such instruments have proven capability and provide highly accurate measurements in a liquid stream.

5.3.2 Laboratory NIR instrumentation

The principles and functions of a laboratory analyser will clearly be similar to those of on-line devices, albeit with less emphasis being placed upon the tolerance to varied sample presentation and operating conditions, as discussed in the introduction. However, the techniques that have been and are being used for

wavelength selection, detection and subsequent signal handling can differ. Benson *et al.* (1988) discuss the various techniques that have been employed for both off-line and on-line analysers, describing the relative merits of each approach.

For laboratory-based analysers, optical filter technology is also used; however, clearly this restricts the choice of wavelengths, especially for a device that may be used for research and development. Full spectrum analysers are commonly used; these are based upon scanning monochromators or Michelson based interferometers. In many cases for solids measurement the optical configuration is based on reflectance. However, devices do exist which operate in transmission mode at wavelengths short of 1.5 μm; these have been principally used for analysing whole cereal grains, meat and liquids or slurries. They incorporate silicon detectors for greater sensitivity at the extremely short wavelengths (0.8–1.0 μm) necessary to achieve penetration (or forward scatter) through the sample. The schematic diagram of a typical laboratory reflectance analyser in Fig. 5.7 shows the key elements of the device and the difference in the collection of reflected radiation.

The signal processing methodology after data collection has been the area where the greatest energies have been devoted over the years. While on-line analysers tend to be based upon log (reflectance) mathematics or variants approximating more closely to Kubelka-Munk scattering equations (Kubelka and Munk 1931; Kubelka 1948), the off-line analysers have also been configured to perform first- and second-derivative spectral analysis; more

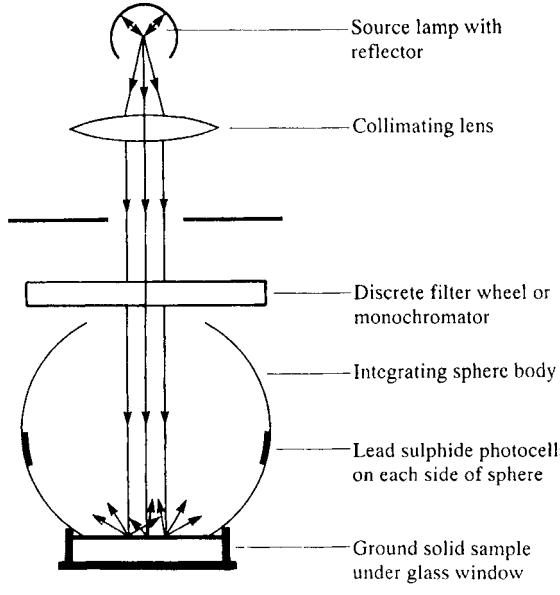

Fig. 5.7 Typical laboratory NIR analyser optical configuration.

recently, Fourier transformation has been proposed as a useful way of analysing the spectral data collected by a scanning instrument (Giesbrecht *et al.* 1981; McClure *et al.* 1984). The latest areas of interest for data analysis include principal component analysis (Cowe and McNicol 1985), Mahalonobis distances (Mark 1986) and partial least squares analysis (Martens and Martens 1986). The concepts behind these ideas are discussed in a short review by Davies (1987).

With all of the above instrumentation, the biggest criticism is the need, in most cases, for careful sample preparation and presentation, especially for materials, which are not in a powdered form. This inevitably leads to the result potentially showing a high degree of operator dependence, since it is unlikely that different individuals will carry out the test in exactly the same way. For certain materials, especially those at higher moistures such as cheese, this would prevent a reliable measurement because of the impracticalities of grinding damp materials. Also the size of the sample analysed is commonly only a few grams, and this must raise the question of how representative this would be when a process producing many tonnes of product per hour is monitored for quality control purposes. This problem is of course not restricted to off-line NIR analysis but to any laboratory analytical technique which is being used to track a manufacturing process.

A novel approach to overcome many of these shortcomings has been introduced in the form of a laboratory instrument which incorporates many of the lessons learnt from an on-line analytical background. One such device is the Infralab (NDC Infrared Engineering), which is a filter based instrument built exactly according to the technology described in the previous section. Importantly, the instrument uses a large sample tray (140 mm $\times$ 140 mm square) containing 50 grams or more of sample material, which the instrument then scans automatically, taking many readings and providing an average value for the variable being measured. As a consequence, sample presentation is no longer critical; the height of the product in the tray need not be controlled, and the sample does not require any specific preparation. The speed of measurement of the on-line sensor technology used in this instrument means that multiple measurement can be carried out rapidly, providing results, even for a large sample area, within 15 seconds. Since the instrument scans a large sample area, it effectively provides statistical information (mean and standard deviation of the multiple measurements) about that sample. It thus helps in judging its quality and reliability as a representative sample from the product flow, and minimises the need for replicate analyses.

Digital multi-wavelength instrumentation, as for on-line analyses, simplifies the operation of off-line instrumentation. However, principally because of the better control of off-line measurement conditions (temperature, humidity) and the length of time available for a measurement, complex multi-component analysers have been in use for many years.

Finally, and very important conceptually, an approach in which the sample is automatically scanned provides a measurement which is no longer operator dependent.

5.4 Multi-component analysis of food products

While on-line moisture measurement using NIR technology remains the principal need within the food industry, other measurements are becoming increasingly important for a number of reasons. Legislation and product in-pack specification issues are becoming ever more important. The continuing trend to produce low fat/oil products means measurement of this parameter is more important. While moisture control is practical through more or less drying in a process and therefore the benefit of measurement is obvious the ability continually to adjust other parameters is possible in some cases and this demands ability to measure them on-line.

Traditionally, many of these other parameters would have been measured off line possibly using NIR on the basis of the belief that their variation was limited enough to make batch testing representative enough. In reality there is variation in most process streams and so on-line measurement is the only way to be certain that the product is adequately quantified. In processes where a constituent other than moisture can be adjusted, on-line measurement offers tremendous potential to reduce production costs through not over-specifying and optimising product quality. In most cases such measurements will be required simultaneously with moisture since this allows dry weight calculations to be performed in instances where another component may be added to a process stream. For example, in chocolate manufacture cocoa butter which is very expensive may be added to the bulk.

The requirement in these instances is for a multi-constituent NIR analyser which can provide measurement of more than one component. The MM710 from NDC Infrared Engineering, described in section 5.3 is able to provide a measurement of up to four constituents simultaneously. This capability now allows food technologists and processors to implement on-line NIR measurements that in the past would have been restricted to laboratory instruments. While non-moisture measurements are generally more demanding because their infra-red absorptions are weaker and less well defined, current on-line instrumentation, as described, is more than capable of achieving high accuracy on a wide range of different components.

The only word of warning on multi-component measurements is to constrain oneself to the realms of measurements based upon sound chemical/physical rationale. Despite the explosion of interest in NIR applications for almost any constituent in food products using laboratory analysers there is, in the authors' view, need for caution in deciding which applications may be successfully tackled on-line. The need of a production facility is to have a measurement which is robust with time and which does not require constant attention or adjustment especially if the measurement output is to be used in closed-loop control. In a laboratory environment it may be acceptable with skilled personnel to accept that frequent calibration correction is part of a procedure. In practice this means that measurement calibrations with correlation coefficients of less than 0.95 just will not stand up to the test of time in a production environment.

The subtleties of measurement development are outside the domain of this discussion and are handled in other articles (Honigs 1985), but a broad understanding of the topic is important to those considering the use of NIR multi-component instrumentation in a new application. The quality and robustness of a calibration, that is, the long-term dependability of the measurement, will be strongly related to the soundness of this initial approach. The authors and their colleagues strongly favour an approach where the measurement is based upon sound chemical/physical principles. There are instances described in the literature where NIR measurement of a component, not existent in the material, is apparently being made, probably because of a correlation with some material in the natural product within the sample set collected for the analysis. The measurement of the ash content of wheat, the tar content of cigarette tobacco, the bakeability of bread (Hagburg factor) and the sensory qualities of peas are such examples. These measurements would be unlikely to succeed as on-line applications in the long term, and frequent recalibration of an instrument would be symptomatic of this lack of robustness.

Examples of the on-line multi-component applications that are successful are plentiful. Those that have been selected for discussion highlight the breadth of the technique and should provide a theme for consideration of others, which may not have been developed to date. Fat/oil and moisture measurements in products probably is the most commonly applied multi-component measurement with snack foods such as potato chips, tortilla chips, extruded snacks and pretzels being the most significant examples. These are demanding measurements principally due to the environment where the ambient temperature is extremely high and the atmosphere laden with oil mist and humidity. Nonetheless, on-line instruments can be designed to handle such hostile locations in a process.

Fat and moisture in chocolate processing is a very successful and important application at most stages of the process from cocoa nib though to finished chocolate. An interesting application arising from this capability has been the use of on-line NIR to measure the thickness of chocolate on the refiner rollers to ensure correct product texture. The fat measurement provides the basis for measuring layer thickness, which relates to the roller gap and therefore the creaminess of the chocolate.

Milk powder can also be measured comprehensively with on-line NIR gauging, moisture fat and protein and perhaps lactose being four measurements that virtually characterise a formulation. Protein content in flour can be an important on-line measurement in cases where a mill adjusts protein level through gluten addition. Low levels of constituents can be measured such as the caffeine content in coffee. While de-caffeinated product would be difficult to quantify to an adequate accuracy, the 1–2% levels in beans is practical with an on-line gauge.

Oil extraction applications provide another example of where on-line measurement is of great potential benefit to a user. In the production of extra virgin olive oil the olive pulp is pressed in a centrifuge and extraction efficiency has a great economic impact on the process. Extraction efficiency depends upon

Table 5.1 A selection of on-line multi-component measurements in the food industry

Product	Components
Animal meals: fish, meat, soya	Moisture, fat, protein
Biscuits	Moisture, oil
Chocolate products	Moisture, fat
Cocoa powders	Moisture, fat
Cheese	Moisture, fat, protein
Flour	Moisture, protein
Grain products	Moisture, protein
Maize products: meal, flakes	Moisture, protein
Milk powders	Moisture, fat protein
Non-dairy creamers	Moisture, fat
Olive pulps and other pressed oil seeds	Moisture, oil
Meat minced (beef, pork, turkey, chicken, lamb)	Moisture, fat, protein
Snack foods, all types	Moisture, oil

the decanter performance and this is affected by pulp moisture content as well. On-line NIR is being successfully applied to measure residual oil content at levels around 1–4% and simultaneously moisture at about 60%. The technique may be applicable to other oil extraction processes such as palm, soya, rape and sunflower oils.

The fat content in ground meats is another area of considerable interest again due to the potential cost savings and quality improvements through better control of the process. On-line NIR measurement has been successfully deployed in measuring ground meats such as beef, turkey pork and chicken. Typically the process involves the blending of lean and fatty cuts with the aim of achieving a specified fat level. The on-line measurement allows control of this blending process to a high degree of accuracy.

The cheese measurement mentioned earlier is principally concerned with moisture measurement but fat and protein may also be measured simultaneously. Its application to date has been 'at-line' mainly but the purpose has been to control the production process and its speed has eliminated the need for the expensive, time consuming and somewhat hazardous wet chemistry techniques.

A list of on-line multi-component applications is provided in Table 5.1 to illustrate the current status in the market. However, there are certain to be other requirements and hopefully the examples mentioned above highlight the possibilities and scope of the technique.

5.5 Advantages and disadvantages

When considering the possibility of using the NIR technique to solve a particular measurement problem, it is necessary to appreciate both its scope and its limitations. The non-contact characteristic is considerably attractive, since the measurement will not normally interfere with product flow. Also, for food

processing, non-contact measurements are favoured by hygiene considerations. The non-destructive nature of the technique minimises product wastage associated with conventional laboratory methods and for some high cost products, this feature alone can justify the use of an instrument if it can replace the majority of quality assurance testing.

On-line NIR gauges provide a continuous or near-continuous output that varies in real time according to the variations in the constituent being measured. The speed of the rotating filter wheel, see section 5.3.1, and hence the resulting measurement time is very fast when compared to the speed of products conveyed by the more usual methods (ranging from belt conveyors to pneumatically conveyed powders. This is ideal for control purposes because all the product viewed by the gauge is sampled and the average moisture (for example) derived can be extremely representative of the product stream, even at very fast response times (second to second).

NIR measurement is unaffected by changes in the electrical properties of foodstuffs, for example electrical conductivity or dielectric behaviour; such parameters can easily change if the salt or other ionic material content varies. This provides a distinct advantage over alternative methods of on-line moisture measurement based upon monitoring the electrical properties of the product, such as capacitance or conductance which can be related to moisture content. For carefully constructed algorithms, (see section 5.3), product density does not generally influence the measurement but will certainly affect techniques based upon monitoring electrical capacitance properties. Similarly, product temperature will not directly influence NIR measurement whereas electrical techniques are temperature sensitive. Although there will be an increase in infra-red emission from the product with temperature, this will be a relatively small change at the operational wavelengths and a continuous emission of DC form. It will therefore be ignored by a properly designed AC coupled detection system in an NIR instrument. However, if temperature variations in the process cause compositional changes in the product it is possible that the NIR gauge would register this as a change in the calibration of the gauge.

It seems ironic that an NIR gauge can measure moisture precisely but when compared to a technique that essentially measures volatiles it will appear to have product type/variation sensitivity. The effect that is being observed is that the non-moisture volatile materials present in the product are also removed to a lesser or greater extent during oven testing. These losses contribute to the apparent moisture content. This can be avoided to a great degree by comparing NIR measurements to water specific tests such as Karl Fischer.

In the application of NIR to moisture determination, the response to free or associated moisture and bound water (water of crystallisation) should be appreciated. In most materials, the difference in wavelength between the absorption bands for these two forms of moisture is very small and therefore they cannot usually be treated separately. Whether this is an advantage or a drawback depends on the requirements of the individual application. For example, with whey powders the moisture content will be distributed between

free and bound moisture, the latter being present as water of crystallisation in the alpha-lactose. However, the storage stability of whey is only influenced by the free moisture and therefore an NIR measurement is of limited value.

Whether an infra-red gauge is sensitive to changes in ambient lighting levels is critically dependent on the instrument design. The form of the blocking filter, which shields a detector from visible and irrelevant parts of the infra-red spectrum, plays an important role in reducing the influence of ambient lighting. Signal processing techniques are able to eliminate ambient lighting completely, but if ambient lighting changes are found to affect the measurement it is always possible to shield the measuring area from those changes. This shield is an additional expense and invariably causes access problem to the measuring unit.

There are huge variations in atmospheric moisture or relative humidity (RH) in any food-processing environment. The effect of variations in RH should not cause any measurement problems for a carefully constructed algorithm. Condensing steam or water vapour is different and any moisture gauge will require installation away from sources of condensing water. This does not usually present a problem due to the flexibility of mounting orientations and the use of effective air purge windows. Inspection of Fig. 5.8, which shows the transmission spectra of liquid water relative to water vapour, shows that although the bands overlap, they do absorb at different wavelengths. Any overlap can be compensated for by careful wavelength selection and appropriate cross correction within the algorithm.

The major limitation of the NIR measurement is the limited penetration of the infra-red radiation into the product. Although this is dependent upon the water

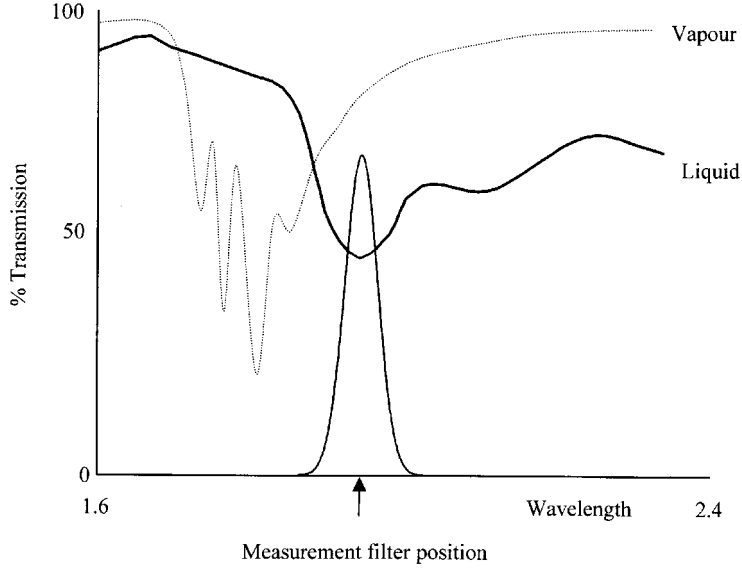

Fig. 5.8 Water spectra in the liquid and vapour phase.

absorption band chosen (shorter wavelength radiation is more penetrating), it usually amounts to no more than a few tenths of a millimetre into the material. The scattering characteristics of the product primarily determine the level of light penetration in a given wavelength region. Fortunately in many practical applications this does not present a problem since the bulk of the foodstuff, especially if made up of fine particles (powders, granulates, etc.), is well represented by its surface layer. For example, in milk powder with particle sizes ranging from below 50 μm to over 200 μm, the individual particles are adequately penetrated by the radiation to give a meaningful moisture measurement. However, it is important that the surface of the product bed viewed by the gauge is representative of the bulk of the material which will not be seen. If the surface material tends to dry off relative to the bulk, because of long periods of exposure to the air or from having emerged hot from a dryer, precautions must be taken. The particulate product must be turned over prior to measurement, by installing the gauge at a point where a change in conveyors occurs or even by ploughing the surface, this latter method being particularly suitable for fine powders.

The concern for materials with large particle size, especially those that have recently been dried or steam conditioned, is whether the surface moisture represents the internal condition. Indeed, it is frequently the case in drying processes that the surface and internal moisture levels of bulky materials differ. In many situations, this potential difficulty is not a problem because there is some form of relationship between the surface and total moisture content which can be exploited to provide a measurement. It is often the relatively constant conditions employed to dry a foodstuff that ensure this assumption is valid! The classic example of this is the on-line measurement of biscuit moisture. After oven drying, the surface moisture content of biscuits is very low, and will even vary across the surface, while the internal moisture is at a higher level. However, the oven drying characteristics are very consistent and therefore the on-line measurement of surface moisture provides the biscuit producer with useful information for a process control system. It is sensible to question whether very large particle size materials are suitable because of the almost certain difference in surface characteristics from the interior condition. Block or slab materials such as finished chocolate or even freshly baked bread provide extremely doubtful examples.

Microwave measurement can be a useful alternative technique when the limited penetration of an NIR measurement is a problem. However, apart from the difficulties of engineering a microwave emitter/receiver on-line, often in intimate contact with the product, the technique is still susceptible to product temperature and density variations unless appropriate signal correction is applied. Sophisticated instruments are available which take these difficulties into account. The range of moistures over which a microwave system operates can be very limited owing to excessive attenuation of the microwave radiation by water. However, new techniques utilising stripline or coaxial gauges can in part overcome these shortcomings.

Of course, there are instances where NIR measurement may not be successful. Backscatter gauges work on the absorption characteristics and if they are subject to specular energy from shiny surfaces the measurements can become noisy and ultimately worthless. The light directly reflected has no absorption information and products such as caramels, syrups and fondants fall into this category. In these instances it is possible to consider a special optical arrangement for the gauge to ignore specular light, or even to use a transmission configuration.

5.6 On-line applications

The ideal optical configuration for most on-line NIR measurements will be based upon a back scatter (reflectance) configuration of the gauge, this being the most suitable approach to the analysis of powdered, granular or flaky materials. Backscatter measurement is non-contact, the gauge being mounted some 250 mm away from the product surface. Careful optical design and the characteristic of the ratio measurement can eliminate the non-uniformity of NIR detectors to the point where quite large variations in the product pass height can be tolerated. Variations in height of ±100 mm will not cause problems and in fact larger ranges can be acceptable, especially if they are short term and random rather than systematic over longer periods of time. Installation is therefore simple and requires only a continuous flow of product so that the gauge does not view the conveyor belt. In instances where this is not possible, such as in biscuit production, a special version of the on-line gauge with electronic gating is available.

For powdered, granular or flaky materials the alternatives for maintaining a continuous product stream include product ploughs to create a build-up or sampling devices to capture a representative sample, presenting it to the gauge and returning it to the product flow. Since the diffuse reflectance technique depends upon the gauge measuring light that has interacted with the product and has been partially absorbed, the measurement of shiny materials needs special consideration, especially when the amount of reflected light reaching the gauge is changing appreciably. In these instances the gauge must be arranged to avoid seeing the large surface reflected light component, which of course contains no absorption information. Locations meeting these relatively undemanding requirements can be found on most plants. Open band conveyors are ideal especially after the transition from one conveyor to the next, where a fresh representative surface minimises errors in providing a representative reading.

On occasions it is necessary to monitor the product through a window in a duct. The use of toughened glass or sapphire allows this and, provided it is kept clean and a moving flow of product is maintained against the window, the window will not affect the measurement. Maize grits and meals, soya meals, coffee powders and milk powder are examples of products which have been successfully measured through viewing windows.

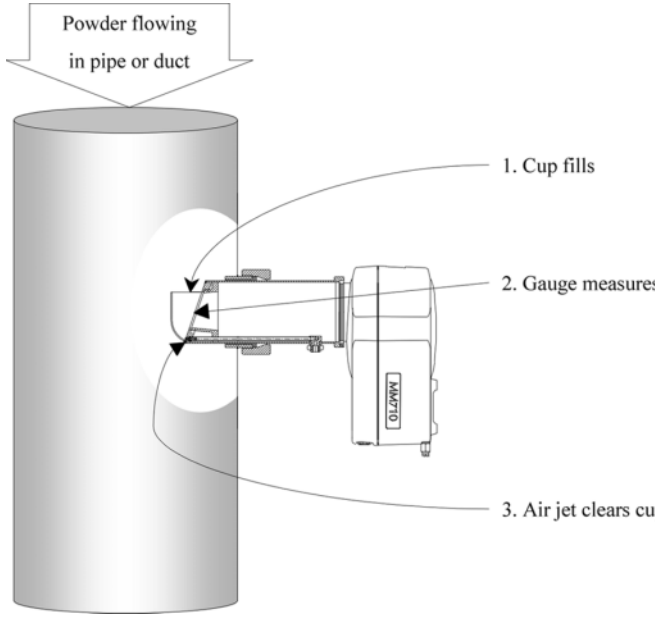

Fig. 5.9 NDC Infrared Engineering PowderVision system.

One of the aims in processing is to eliminate dust from the open plant environment, making access to the product somewhat challenging. Powders are often conveyed within a pneumatic system or enclosed ducting. A simple viewing window would be sufficient if it was always completely covered by fresh product. Usually the product flow will be intermittent and consistent presentation of the product to a viewing window cannot be guaranteed. Devices exist that perform a sample and hold routine on the product, within the enclosed conveying system. Figure 5.9 shows NDC Infrared Engineering's PowderVision system, which was designed to meet these challenges.

Products in a screw conveyor may be measured without interference from the screw blade by removing a small section of the blade and mounting the gauge away from the axis screw shaft. This usually gives a product presentation with minimal height variation and no interference from the metal parts of the screw.

It is worth restating the need for the gauge to be viewing a surface that is truly representative of the bulk moisture content of the product. This consideration should also be extended to include the need for careful positioning of a static gauge on a wide band of food product. The chosen point for such an application must be where the gauge provides an output that correlates well with the average moisture content irrespective of any profile that may exist across the width of product. Alternatively the gauge may be scanned automatically. Whatever location is chosen, the point must always enable access for sample collection close by and immediately downstream from the gauge for the inevitable cross-

checking of the instrument with the laboratory test. This will be particularly important in the early commissioning stages after installation.

In many cases, the food industry demands certain hygienic standards to be met; given the often dusty and high humidity environments or even the aggressive nature of some food products, the gauges must be designed accordingly. Since the optical gauge is a self-contained device connected only by a single cable to the outside world, this is relatively simple to achieve. Gauges are available in stainless steel with flush fitting joints to avoid harbouring dirt. For high temperature operations methods of cooling using air or water can be applied to allow the unit to operate within its specified working temperature. As far as protection against dust or moisture ingress is concerned, an on-line gauge is usually built to standards such as the recognised IP65 or NEMA4 ratings. In steamy or dusty environments it is necessary to keep the optical windows clean, and this can be achieved by using an air purge system. The air purge device is attached to the gauge window. The purge is made up of two concentric tubes, the inner one being porous. Compressed air is passed through an aperture in the outer tube and this creates a steady flow of air through the inner tube, keeping the optical window clean. If it is necessary to install the gauge where there is an explosion hazard, a purge and pressurisation system can be used to ensure explosive vapour or dust is kept away from the electronics. If the pressure within the purge system falls below a set point, the equipment is shutdown safely.

One of the attractions of the on-line NIR gauge is the variety of seemingly adverse environments it can tolerate. This is largely attributable to the ruggedness of the design and the fact that the optics can be built from materials such as glass or quartz rather than the more fragile components usually associated with the longer wavelength mid-infra-red region. It is only necessary to ensure that the instrument is not subject to excessive vibration, as with any optical system. The response time of the gauge is usually an exponential time constant, effectively providing a running average of the moisture content of the product stream. By definition, this type of response time is the interval during which the gauge will have made a 63 per cent change towards the new value following a step change in moisture. The longer the chosen value, the more slowly the gauge will respond to a sudden change in moisture.

The chosen response time therefore depends on how variable is the product moisture, and is usually derived empirically by adjustment until a sufficiently stable output is obtained for practical use. For the control of a dryer, where the time needed to provide a change in drying conditions may be of the order of minutes, a gauge response time of perhaps 10 or 20 seconds would be appropriate. However, on a potato crisp production-line, where it can be important to quickly identify ‘wet’ batches of product so that they can be immediately diverted, a shorter response time of 2 to 5 seconds might be used to maximise the ability to detect the out-of-specification product. The ideas expressed above are schematically shown in Fig. 5.10. Using the longer response time, the amplitude of the process variations is reduced and the peak values are

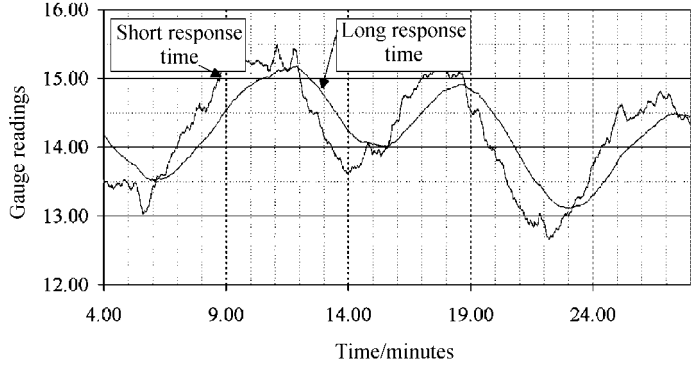

Fig. 5.1 Effect of instrument response time.

shifted in time. Long response times can reduce the ability to detect out-of-specification product.

5.7 Future trends

NIR measurement has been shown by examples in the foregoing text to be a very powerful technique for food analysis of moisture and other constituents in a hugely diverse range of applications. Emphasis has been placed upon the way on-line NIR technology has evolved over the years in terms of how the technology is evolving to make its application simpler and the measurements more robust. It is shown how the technology in some instances has not met user expectations and how this has led to a lack of belief in the technique. The reasons for this are candidly discussed and hopefully readers will now be able to make an informed judgement and carefully select the right supplier and to remain open to the technology and what it can do for them.

The main effort nowadays from leading NIR instrument manufacturers is to concentrate on making the supplied solution ever simpler to implement and to try to minimise the need for users to become involved in calibration work, a major weakness of the technique in the past. It is clear that users nowadays do not have time to devote to the niceties of checking on-line instruments and furthermore need as much assistance as possible about comparative checking methods and informative methods for decision-making about whether a calibration indeed needs correction or should be just left alone. This chapter has highlighted the sort of hardware and software products available to achieve such goals and in particular offers solutions to overcome the nightmare of 'calibration hunting'.

The benefits of on-line measurement have been highlighted and in particular the information that can be gained about a process demonstrated with typical examples. It will be clear now that on-line measurement should be implemented wherever possible in favour of batch/historic off-line analysis because of the

need for continuous real-time information about a process and the far more representative nature of the information about a batch of product that is gained.

While moisture measurement has been the historically important NIR measurement, on-line examples in the text show how this is evolving and that other parameters can be successfully controlled and product quality improved by their measurement.

The future for the application of on-line NIR technology looks very good with increasing opportunity for its application as the food industry becomes ever more concerned with accurate process control. Moisture is likely to remain the principal application for an on-line gauge because it has such obvious possibilities for control and has far-reaching impact upon on product quality, keeping characteristics, yield optimisation and process energy usage. However, other parameters such as oil or fat where their control can be affected are and will continue to become more important. In the days of increasing specification of packaged foods composition it is clear that to have traceability through on-line measurement is going to be ever more important. In the words of process personnel in one key international food manufacturer: 'We are just waiting for our board to direct that product will be monitored routinely for its key constituents.'

For instrument manufacturers the need is to continue to strive to simplify the implementation of on-line measurement. While some significant steps towards this have been achieved by the author's company with instrument precalibration, there is still scope for even simpler implementation. The holy grail would have to be a moisture gauge which was able just to measure moisture without recalibration or configuration for whatever product was being measured! With advances in scattering theory and the continual improvements in microprocessors this may be attainable one day!

Instrument technology continues to develop even in areas where it was previously thought that significant improvements could not be made. Alternative technologies for the wavelength generation and detection are continuously under investigation. Techniques such as AOTF and even the Michelson Interferometer, which can generate NIR full-spectrum information with good signal-to-noise ratios may become commercially viable and open up even greater capability on-line. Having said this it is contended by the authors that for a given constituent and the multi-wavelength nature of the modern filter gauge this is a difficult approach to beat due to its high speed and excellent signal-to-noise performance. We shall see!

New measurements will continue to evolve and it is hoped that this chapter has provided stimulus to its readers to consider NIR technology as at least a potential approach for a newly arising application. Finally, all things that were confined to laboratory NIR analysis should be viewed as contenders for on-line NIR measurement but preferably with the cautions described in the text being borne in mind. Remember the on-line gauge has to perform day in day out and without calibration drift for whatever reason so that it can be depended upon for control of a process. Provided this basic philosophy of measurement is

remembered the technology has a great future and a strong contribution to make to the developing needs of food technologists and manufacturers worldwide.

5.8 References

(Sources for cheese production figures: ZMP, National Statistics; USDA; FAO; EUROSTAT. European Commission.)

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6

NMR spectroscopy in food authentication

G. Le Gall and I. J. Colquhoun, Institute of Food Research, UK

6.1 Introduction

NMR spectroscopy is nowadays being used more and more to analyse foods. Advantages such as the simplicity of the sample preparation and measurement procedures, the instrumental stability and the ease with which spectra can be interpreted have contributed to the growing popularity of the technique. Standard ^{1}H, ^{13}C and now high resolution magic angle spinning (HR/MAS) NMR spectra can give a wealth of chemical information on liquid foodstuffs and even semi-solid foods. Almost any hydrogen-containing molecules are observable provided that the compound concentration is above the detection limit of the technique.

NMR spectra of food products can act as 'fingerprints' that can be used to compare, discriminate or classify samples. Selected variables (NMR peak heights or integrals) that characterise the samples in some specific way are also used instead of the whole spectra. Chemometric techniques are often employed to analyse the data as the information contained in the spectra is of a high degree of complexity. These statistical techniques serve several purposes in comparing and classifying samples (authentic or not?) or in quantifying adulterants using calibration sets.

Several reviews have already been published on the subject of NMR and food[1–3] and on NMR and chemometrics.[4] The main purpose of this chapter is to report on the latest developments of NMR in food authentication. It covers practical considerations, the potential limitations and how to overcome them. It also comprises a review of recent NMR applications on foods such as vegetable oils, fish and meat, milk, cheese, wheat, fruit juices, coffee, green tea, wine and beer. The diversity of these applications highlights the versatility of NMR as an

analytical and structure elucidation tool. A majority of these studies report on the use of high resolution liquid state ^{1}H and ^{13}C NMR but a few make use of ^{31}P NMR, ^{1}H HR/MAS NMR or even liquid chromatography nuclear magnetic resonance (LC/NMR). The conclusion includes a short commentary on future trends.

6.2 Using NMR spectroscopy: sample preparation

The principles of NMR spectroscopy will not be addressed in this chapter. The books by Gunther[5] and Claridge[6] and the article by Mannina and Segre[7] offer descriptions of NMR theory and practice. In this section we emphasise some of the practical steps that must be taken regarding both sample preparation and instrument operation in order to use NMR to make true quantitative comparisons across a series of different (but related) samples.

There are several ways of preparing food samples for NMR, depending on the nature of the sample itself (solid, liquid) and on what type of analysis is to be carried out. Some targeted analyses tend to include an extraction or fractionation step (e.g., extraction of triacylglycerols from milk[8]) while other samples are used as they are for non-targeted analyses. For high resolution ^{1}H, ^{13}C or ^{31}P NMR of aqueous liquids (fruit juices, degassed beer, wine, skimmed milk) the samples are often prepared simply by adding 5–10% of D_2O to the liquid. Deuterated solvents provide a signal for magnetic field stabilisation (field-frequency lock) and allow optimisation of the resolution of the NMR peaks.

Solid samples (e.g., fruits, vegetables, green tea) are freeze-dried and/or ground and then extracted into a deuterated solvent. High protein samples (fish muscle, meat, cheese) may be homogenised in hydrochloric acid. In both cases the samples are centrifuged, then the supernatant is collected for analysis. Other samples, such as oils or instant coffees are simply dissolved at the desired concentration in a suitable deuterated solvent. Standard procedures should be followed to ensure repeatability and comparability when preparing a series of samples.

Sometimes, the same type of food has been prepared differently for different studies. Rapp *et al.*[9] concentrated 100 ml of wine to 10 ml while Kosir and Kidric[10–11] either freeze-dried the material or simply added D_2O. The latter procedure is the most convenient while freeze-drying minimises the residual NMR signal for water. Concentrating the sample is the best way to optimise the NMR signals of minor compounds but this gain in 'NMR time' is at the expense of sample preparation time, which could be important if many samples have to be prepared. D_2O is the most common solvent used. For freeze-dried and other solid samples an extraction mixture of d_4-MeOD/D_2O allows the solubilisation of many metabolites such as sugars, organic, amino and fatty acids and phenolic compounds.[12] Oils and oil extracts are dissolved in $CDCl_3$ or DMSO-d_6.

A measured amount of a reference compound such as tetramethylsilane (TMS) for organic solvents and sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4

(TSP) or sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) for aqueous solutions is often added to the sample. This serves both as chemical shift and intensity reference. The isolated reference peak at 0 ppm is also useful as a check on the resolution and lineshape of the NMR signals obtained. Other internal standards, e.g., pyrazine in oil samples in $CDCl_3$ have been used for quantitative analysis.[13] In aqueous food samples, the chemical shifts of many compounds (such as the organic acids) can be affected by the pH. Several ways to overcome this problem are discussed in section 6.5.

The amount of sample to prepare varies according to the type of spectrometer available. The same measured volume should be placed in the NMR tube for all samples in a given series. Usually, volumes required are 0.5 to 0.8 ml for ^{1}H NMR in conventional 5 mm o.d. NMR tubes. Larger volumes might be used (e.g., 3 ml in 10 mm o.d. tubes) for ^{13}C or ^{31}P NMR to compensate for the lower sensitivity of these nuclei. Some modern systems allow NMR tubes to be dispensed with altogether.

Flow injection systems and LC/NMR share a similar probe design: the sample is pumped via capillary tubing into a static cell (volume 250 μl or less), held there while the measurement is made, and then pumped to waste or collected. Samples are transferred to the probe from plates or vials held in a liquid handler (flow injection NMR) or are transferred automatically after chromatographic analysis (LC/NMR).[14] These systems have not yet been widely used for authenticity applications but flow injection is ideal for screening many samples when the data acquisition time is short since it is intrinsically highly repeatable and the changeover time between samples can be made very brief. Because of its separation ability and high sensitivity LC/NMR may help with the identification of novel marker compounds even when these are available only in very small amounts (LC/NMR detection limits are now in the 10–100 ng range).

6.3 Data recording and processing

Data sets for NMR/authenticity studies are commonly acquired under automation using a sample changer to acquire spectra for 20–60 samples in a batch (this assumes a conventional rather than a flow injection system). Prior to any data acquisition, it is recommended to perform the tuning of the NMR spectrometer on the first sample of a series (as long as it is representative this will be good for the whole series), to check the 90º pulse length of the instrument and to optimise the field homogeneity. Auto-shimming (at least Z1 and Z2) is carried out for each sample in order to obtain consistent quality of the NMR signals in terms of lineshape. At the end of the run it is advisable to check that all the spectra have acceptable linewidth and lineshape (as mentioned above the reference peak is suitable for this check). The pulse sequence presented in Fig. 6.1 is the classical sequence used to record a one-dimensional NMR spectrum (^{1}H as shown; ^{13}C etc., usually with ^{1}H decoupling).

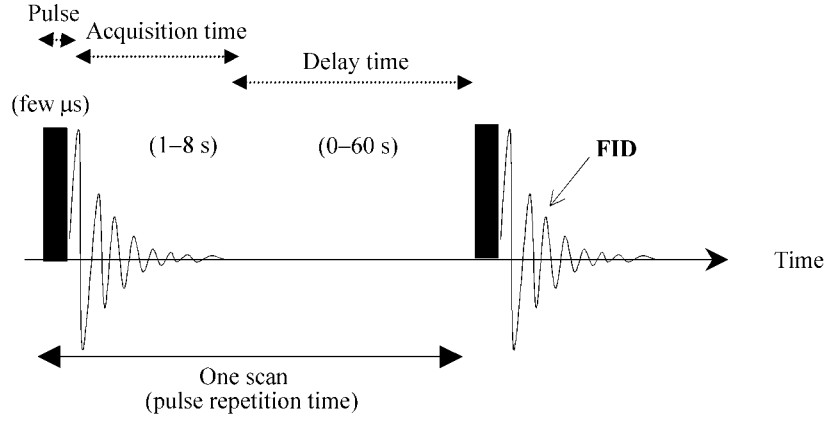

Fig. 6.1 Typical 1D NMR sequence: the range of acquisition and delay times shown is taken from articles reviewed here and covers both ^{1}H and other nuclei.

Choice of pulse angle and pulse repetition time are important in determining the signal-to-noise ratio (S/N) that can be achieved in an NMR experiment in a given fixed time. In practice, a 90º pulse angle is a common choice in ^{1}H NMR but 30 to 50º pulse angles are sometimes used in combination with a reduced pulse repetition time, especially for NMR of other nuclei. In experiments where multiple scans are acquired to improve the S/N, the pulse angle (Ernst angle) that gives maximum signal intensity is $\cos^{-1}[\exp(-t_r/T_1)]$ where t_r is the pulse repetition time and T_1 the longitudinal relaxation time of the nucleus in question. It is also worth noting that if 90º pulses are used the maximum signal intensity for a fixed total acquisition time is obtained by setting the repetition time to $1.3T_1$.[6] Of course in any given sample the nuclei of interest will cover a range of T_1 values so some prior knowledge or trial experiments must be used to choose the acquisition parameters.

Following the pulse irradiation, the acquisition time is the part of the sequence where the free induction decay (FID) signal is recorded. The acquisition time is dependent on the digital resolution required. The smaller the resolution, the higher the number of data points, e.g., for a spectral width of 5000 Hz, typical for a ^{1}H spectrum at 400 MHz, 32,768 data points are required for a digital resolution of 0.15 Hz. The corresponding acquisition time would be 6.6 s (1/0.15). Better digital resolution gives better peak definition and more precise integration but it also means longer acquisition times and larger files. In practice however the acquisition time in ^{1}H NMR is often set to be about $3T_2^*$, where T_2^* is the effective transverse relaxation time, determined by the magnet shimming.[6] At this stage the FID has decayed to 95% of its original value and acquisition of further data only reduces the eventual S/N. Thus acquisition times of 2–5 s are commonly used for ^{1}H spectra.

The delay time allows additional time for the nuclei to come back partly or completely to the equilibrium position before applying another pulse. In the case

of quaternary carbons, the T_1 value can be very long but most of the protons in the liquids have T_1 of 3–4 s or less. For quantitative analysis by NMR however the repetition time needs to be $5T_1$, where the T_1 is that of the nucleus with the longest relaxation time. This could mean a repetition time of 20 s for ^{1}H NMR, potentially much longer for ^{13}C. Such long repetition times are rarely used, at least for ^{1}H, when acquiring series of spectra for chemometric analysis. Repetition times of 4–5 s are more common, reflecting the need to optimise S/N in the time available. Whilst observance of the $5T_1$ condition ensures that correct relative signal intensities are obtained within a single spectrum, the condition may be relaxed if the aim is only to maintain true relative intensities for corresponding signals across different spectra. Care must still be taken however to ensure that experiments are repeatable, potentially across large numbers of samples and across time for different batches of samples.

The S/N increases as $\sqrt{n}$ where n is the number of FIDs acquired and added. The choice of the number of scans, which will determine the duration of the overall sample recording depends on the type of information needed. In theory it depends on the concentrations of the molecules present in the smallest amounts that one would wish to detect. For quantitative purposes, it is said that a S/N of at least 35 is desirable[15] but this figure is achieved for only a few compounds in a typical mixture analysis of a food. A S/N value of 10 has been given as a limit for quantification.[16] For non-targeted ^{1}H NMR analyses of mixtures of 'unknown' composition, 300 scans has been found suitable for detecting many compounds with an overall experiment duration of 30 min.[17]

In aqueous samples, water is by far the strongest peak of the ^{1}H spectrum, almost obscuring the rest of the signals. The water signal is most easily suppressed by presaturation. A low power rf irradiation at the water frequency, applied during the delay time, allows the suppression of the peak. Several other suppression sequences exist which can give improved results (the NOESY presaturation sequence for single suppression and the WET sequence for multi-suppressions where water and other peaks have to be removed).[10]

6.3.1 Data processing and file conversion

Once the experiment is finished the total FID (the NMR signal as a function of time) is transformed into a frequency domain spectrum by Fourier transformation (FT). Multiplication of the FID prior to the transformation (exponential, gaussian and trapezoidal functions are most commonly used) can be used either to reduce the linewidth of the peaks or to increase the S/N but the improvement of one parameter is done to the detriment of the other. Since improvement of S/N is the most important consideration in NMR/authenticity applications, ^{1}H FIDs are usually multiplied by a decaying exponential (equivalent to say 1 Hz additional line broadening) prior to the FT. After the FT, the baseline of the spectra is usually corrected with one of several baseline correction functions from the spectrometer software. Finally, in some cases, the spectra are converted to ASCII or JCAMP file formats (most NMR processing

packages now allow this) and transferred to a PC for further analysis. For the statistical analysis of a series of spectra, it may be important to align spectra by horizontal shifts of a few data points, lining up on the reference peak. With D_2O, because the lock signal is rather broad, the different spectra may not be lined up perfectly, hence this additional step is needed. The task is not required for samples in a mixture of deuterated methanol/water as the lock is on the very sharp methanol signal.

6.3.2 Data preparation for chemometrics

There are several independent approaches for the conversion of the raw NMR spectra into data suitable for chemometric analysis. One procedure is to transfer the transformed spectra to a PC and use them as they are (after having optionally removed the regions of the spectra that do not contain signals and any regions that contain unwanted signals).[17–19] Another option is to divide the spectra into segments (called 'buckets') and to sum the intensities of the data points in each segment.[4, 20–21] Some authors exclude buckets where the integrated signal never exceeds five times the noise level (cf. comments on limit for quantification).[16] There is also the popular alternative of selecting a series of signals from the NMR spectra using the spectrometer's own 'peak-picking' routine which gives peak positions and heights.[22–27] In this case the investigator has to decide which signals in the different spectra have the same origin. Whether this is feasible depends on the nature of the spectra and how well resolved they are: for example it will tend to be more practical with ^{13}C than with ^{1}H spectra. Sometimes, when the whole spectra are used as inputs, individual NMR peaks or multiplets need to be aligned prior to chemometric analysis. This aspect of the pre-processing is developed in section 6.5.

There are other types of data pre-processing (mathematical manipulations commonly used in chemometrics, whatever the origin of the data), all aimed at enhancing the relevant information contained in the data matrix. First pre-processing can remove arbitrary or unwanted differences in magnitudes (normalising). One normalisation procedure is to sum the intensities for each sample and scale to a constant total, usually unity. It may also help to remove any intensity variations caused by differences in equipment performance. Normalising the data is optional and one may leave the data unchanged if absolute values are meaningful (e.g., the spectral intensity should directly reflect the amount of a given compound). It is also possible to normalise the spectra to the reference if a fixed amount of reference is present in every sample. This preserves the relationship between intensity and amount of compound whilst correcting for any instrumental variability (see section 6.5). Pre-processing can also involve mean centring and scaling the variables. The mean-centred matrix is obtained by subtracting the mean spectrum (mean intensity for each of the variables) from each spectrum. Mean-centring is always used when applying a data reduction technique to a data matrix.[28] Additionally, it may sometimes be desirable to scale the variables by dividing the entries in the mean-centred

matrix by the respective column standard deviations (auto scaling). This allows the smaller spectral features to influence the result as much as the larger ones.

6.4 Signal assignment and chemometrics

6.4.1 Signal assignment

A chemical shift reference chart may be the first tool used to assign signals but it is really suitable only for giving a pointer to the types of molecule that may be present. To deal with complex mixtures, there is a need for more detailed and precise information. Some literature compilations giving the chemical shifts and signal multiplicity of common metabolites have proven to be useful.[29] The next option is to acquire spectra of compounds that are expected to be present in the sample as reference standards and then to compare their chemical shifts with those of the sample spectrum. It is necessary to run both standard and real samples under similar conditions because some experimental parameters, such as the pH, cause chemical shift changes. Some peaks in the food samples will inevitably be from unexpected and unidentified compounds. Further information about those unknowns can be obtained through the analysis of 2-dimensional (2D) spectra. They show the links (via $^{1}H-^{1}H$ and $^{1}H-^{13}C$ couplings) between signals belonging to the same molecule. The basic techniques to obtain 2D spectra are the COSY (Correlation SpectroscopY) and the HOHAHA (HOmonuclear HArtmann-HAhn, also called TOCSY, TOtal Correlation SpectroscopY) sequences for, respectively, the short and long range $^{1}H-^{1}H$ correlations, plus HMQC (Heteronuclear Multiple Quantum Coherence spectroscopy) and HMBC (Heteronuclear Multiple Bond Correlation spectroscopy) sequences for the short and long range $^{1}H-^{13}C$ correlations.[6]

6.4.2 Chemometrics

In chemistry, it is possible to record many pieces of information such as spectroscopic or chromatographic intensities for each sample. Therefore, most chemical experiments are multivariate. The way to deal with such a wealth of information is to use multivariate analysis, which includes much of applied statistics. One definition of chemometrics is as follows: 'The chemical discipline that uses mathematical and statistical methods for handling, analysing, interpreting and predicting chemical data'.[30] Chemometrics represents a wide range of statistical methods aimed at tackling three different main objectives: simplifying complex and massive data sets (data reduction), classifying objects (by supervised or unsupervised methods) or predicting analytical parameters (calibration methods).

Data reduction methods include Principal Component Analysis (PCA) and Partial Least Squares (PLS).[31] There are several types of supervised classification technique such as Soft Independent Modelling of Class Analogy (SIMCA), Linear Discriminant Analysis (LDA) and Canonical Variates Analysis (CVA) and unsupervised techniques (cluster analyses). Principal

Component Regression (PCR) and PLS used as a regression tool[32] are the main techniques used to create calibration models. For more information about chemometrics, see the books by Sharaf *et al.*,[33] Krzanowski,[34] Massart *et al.*,[35] Brereton[36-37] Martens and Naes,[32] Beebe *et al.*[28] or Kemsley.[38]

From the applications review presented in section 6.6, it was found that several types of software have been used to carry out chemometrics on NMR data for authentication purposes. Analysts have used SCANWIN (Minitab Inc., State College, 1995) to perform PCA,[39] S-plus statistical system[22] to perform PCA and cluster analysis, and SPSS for Windows (v.9.0.1; Chicago, IL)[27] to perform Stepwise Discriminant Analysis (SDA). Statistica for Windows ('97 Ed. by StatSoft, Inc., Tulsa, OK) is also widely used[24–26, 40–43] to execute cluster analysis and LDA. Matlab (The Math Works Inc., Natick, Massachusetts, USA) and Win-DAS (software and accompanying book[38]) packages have been regularly applied to analyse spectroscopic data at the Institute of Food Research.[17] The main advantage of using Matlab is its flexibility: PCA, LDA, PLS for calibration or discrimination, ANOVA and cluster analyses can be performed using appropriate macros (some available in the Statistics or third-party Toolboxes). The disadvantage is that macros for some purposes have to be written in-house so a good understanding of chemometrics and the software itself is necessary. WinDas is more readily usable for beginners interested primarily in discriminant analysis (it includes PCA, PLS as reduction methods, SIMCA and CVA).

One last point of importance can be stressed, namely, the assessment and validation of chemometric models. It is important to test the reliability of a classification or a calibration model with either an independent test set (samples not used to build the model) or by using a cross-validation method (also called 'leave-one-out' or 'jack-knife'). The latter consists in removing a sample from the data set, then using it to test the model produced by the $n-1$ remaining samples; this procedure being repeated for all of the n samples in turn. For more reading, see articles by Kemsley,[38, 44] Defernez and Kemsley[45] or Martens and Dardenne.[46]

6.5 Advantages and disadvantages of the NMR technique

The first limitation in using NMR for food authentication (and the most prohibitive one) is the cost of the equipment. A new 500 MHz NMR spectrometer might cost 7–8 times as much as a new HPLC/UV-DAD system. The second limiting reason is the relatively low sensitivity of NMR compared to other techniques such as HPLC or GC. However, the versatility of the technique means that the initial high cost may well be overridden by a number of advantages that other techniques may not provide. The first of them is obviously the power of structural elucidation of the technique. Food authentication research is constantly looking for techniques that can identify marker compounds to permit the detection of adulteration or testify to the quality of a

high price food product. The second advantage is that NMR is probably the best non-targeted technique to use for the screening of food extracts: all the main metabolites (fatty, amino and organic acids, sugars, aromatic compounds) can be detected in a single spectrum with minimal and non-destructive sample preparation (this is the third advantage). The recording can also be completed in less than an hour. This allows the possibility of analysing a large number of samples. With the detection of several tens of compounds and the structural elucidation power of NMR, a new marker compound is more likely to be identified. The sensitivity of NMR is good enough to observe the main primary and secondary metabolites in a simple whole food extract.

For the detection of minor components, larger amounts of the starting material can be extracted initially and then fractionation steps can be added to the sample preparation in order to concentrate certain types of compound.[27, 47] Also, high field NMR instruments allow more compounds to be detected since they provide improved sensitivity and signal dispersion, and the introduction of cryo-probes will give significantly increased sensitivity on existing instruments. Another advantage of using NMR is the fact that it is quantitative. A number of analyses have been carried out on oils and wine using this attribute.[7, 23, 48–49]

The measurements are easily repeatable and reproducible over the long term. The instrumental parameters that need careful attention from the analyst and that may vary from one data recording session to another are the tuning and the resolution. A difference (even small) in tuning will affect the intensity of all peaks in the spectra. In order to compare quantitatively sets of spectra recorded at different times, all the spectra can be normalised to the reference peak provided that a fixed amount of internal standard is added to all the samples. The problems of repeatability and reproducibility of chromatographic techniques that are caused by the ageing of columns (affecting separation performances and changing retention times), temperature fluctuations, etc., are not applicable to NMR. The disturbances that can occur are the small shifts of the NMR peaks caused by differences in pH in the NMR spectra of food extracts or the broadening of certain signals due to chemical exchange of protonated and non-protonated species and metabolite-metal interactions (complexation with Ca^{++} or Mg^{++} ions). To overcome the variable pH problem, it is possible to manually adjust the pH to the same value for all samples by adding drops of NaOH or HCl to the mixture with a micropipette while monitoring the pH with a microelectrode. Phosphate buffers in D_2O can also be used as solvent[12, 47] but they may not be strong enough to control the pH of some samples when used in a methanol/water or similar mixed solvent. EDTA is effective in reducing line broadening of citric acid signals when this is due to metal complexation.

The other solution to the problem of chemical shift displacements is to treat the NMR signals after recording. Vogels *et al.*[50] proposed a program called Partial Linear Fit (PLF) that lines up peaks affected by small displacements. As a matter of fact, it has been possible to create macros in Matlab software (partly derived from the PLF algorithm) that enable the lining up of NMR peaks (Defernez[51]) but these procedures currently require considerable operator input

and it has not yet proved possible to automate them. The consequences for chemometric analysis of ignoring changes of lineshape and chemical shift displacements between spectra have been explored (Defernez and Colquhoun[52]).

6.6 Applications: authenticating oils, beverages, animal and other foods

The authenticity of a food product is essentially defined by legally recognised descriptions that concern its characteristics (quality, origin, process, etc.) An authentic food product has to be properly labelled according to the appropriate (inter)national regulations when presented for sale in the marketplace. Mislabelling may arise, for example, when the legal definition of a given product traded on the global market differs from one country to another. The list of mislabelling problems is potentially endless. It can concern the truthfulness of the claimed geographical origin, e.g., for wine and olive oil; the species of plant or animal, e.g., beef or pork in sausage; the processing used, e.g., whether food has been irradiated or not; the quality claimed, e.g., farmed versus wild, natural versus artificial, organic versus conventional, etc. Along with mislabelling, there are other food authenticity issues such as the fraudulent addition of substances or the illicit replacement of high priced material with cheaper substitutes, the freshness of a food product, etc.

Food adulteration is definitely a growing concern for the food industry, regulatory authorities and the consumer. Recently, the growing awareness of the public has also influenced the EU and national scientific research programmes. The need for advanced detection techniques is more than ever essential. In response, the investigation of NMR methods for authenticating food has blossomed during recent years. The following sections present a variety of pilot applications some of which could potentially lead to an official adoption of the technique. The area where this seems most likely at present is in the authentication of oils.

6.6.1 Vegetable oils

Virgin and extra virgin olive oils are high price commodities that have triggered a considerable amount of authenticity research in the last ten years. Olive oil has been extensively studied because it can easily be adulterated by cheaper oils and analytical tools are needed to control the quality and genuineness of those oils. Moreover, there is also the need for authenticating the geographical origin of extra virgin olive oils as some of these are entitled to Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) labels under EC Regulation 2081/92.[53] Such oils are distinguished by certain characteristic features (cultivar, geographical origin, agricultural practice, production technology and organoleptic properties).

High resolution ^{13}C NMR has been proposed to discriminate virgin olive oils originating from several countries from other high oleic oils (refined olive,

'lampante' olive, refined pomace olive, high oleic sunflower, hazelnut and rapeseed oils) and high linoleic oils (sunflower, corn, soybean, grapeseed and peanut oils).[54] Mixtures of 5–50% hazelnut oil in virgin olive oil were also successfully separated from the pure virgin olive oils using the same procedure. The authors however, stressed that although the procedure gave satisfactory results for this particular sample data set, additional data (such as information on minor components) may be needed to tackle more complicated mixtures. Mavromoustakos *et al.*[13] have used the olefinic signals obtained by ^{13}C NMR to differentiate virgin olive oils from oils adulterated with soybean, cottonseed, corn and sunflower seed oils. Samples with an adulteration level of 40% or more were discriminated. Vlahov[49] used the distortionless enhancement by polarisation transfer (DEPT) pulse sequence[6] to detect olive oil adulteration with soybean oil. The author claimed that the technique helps to overcome the sensitivity limitations of conventional ^{13}C spectroscopy and reduces the experiment time. Under the conditions used limits of detection for soybean oil in olive oil were less than 10% by integration of olefinic signals in oleyl and linoleyl chains but more than 10% using linolenyl olefinic signals, making this chain less suitable for the detection of soybean oil adulteration. High resolution proton NMR has been employed too.

Fauhl *et al.*[41] discriminated virgin olive oils from hazelnut oils, sunflower oils and more importantly from olive oil samples adulterated by the two other oils (10–55% adulteration). Results suggested that the olive oil adulteration could be detected down to a level of 10% (sunflower) or 25% (hazelnut). ^{1}H NMR (600 MHz) has been combined with gas chromatography (GC) in a procedure that detects hazelnut adulteration in olive oil.[40] The method is based on the evaluation of the linolenic acid amount via the ratio between the NMR signals of one of the ^{13}C satellites of the main methyl resonance and the signals of the terminal methyl of the linolenic chains. If the linolenic acid value is anomalously low, there is suspicion that hazelnut oil has been added (the linolenic acid content of hazelnut oil is much lower than that of olive oil), in which case GC is used to characterise the fatty acid composition of the oil. Then chemometrics is applied to the GC data to establish the level of adulteration (a semi-quantitative model has been built giving a 10% limit of detection).

Recently another procedure has been proposed to detect hazelnut oil in olive oil. Ruiz del Castillo *et al.*[55] proposed a suitable off-line LC/NMR method to detect filbertone, a marker compound present in hazelnut oil but not in olive oil. Fractions from an HPLC separation (five 100 μl injections) of a hazelnut oil sample were combined and concentrated on an SPE cartridge, then the concentrate was analysed by ^{1}H NMR. Virgin olive oils have been differentiated by high resolution ^{1}H, ^{13}C and ^{31}P NMR on the basis of both the olive variety and the geographical origin.

Sacco *et al.*[24] discriminated oils from five cultivars originating from the Italian region Apulia on the basis of the fatty acid composition (GC data) and a selection of aldehydic, vinylic and aromatic proton peaks measured on phenolic extracts of the oils by ^{1}H NMR. The fatty acid data separated oils only according

to their variety while the NMR of the phenolic extracts separated the oils according to both the variety and the location origin (limited sample numbers). Sacchi *et al.*[22] and Mannina *et al.*[25] showed that virgin olive oils from different regions in Italy and oils from the same region (Tuscany) could be differentiated on the basis of ^{1}H signals arising from minor compounds (squalene, β-sitosterol, terpenes, aldehydes and other volatile compounds). Measurements were made directly on the oils diluted in $CDCl_3$ plus a little d_6-DMSO at high field (600 MHz). However, due to the structure of their database, the influential factor (varietal or geographical) could not truly be determined.

In another article, Mannina *et al.*[26] suggested that the geographical origin might be the major influential factor but Vlahov *et al.*,[23] who compared virgin olive oils from PDO areas using a ^{13}C DEPT pulse sequence, concluded that the cultivar more than the geographical origin appeared to be the major discriminant factor. In fact the two methods are based on quite different information, one on minor components (^{1}H) and the other on fatty acid composition (^{13}C).

Zamora *et al.*[27] have recently proposed a new ^{13}C NMR method with an extraction step that allowed concentration of polar lipid components and the detection of twice as many signals (135) as in complete oils. These signals arose from newly assigned minor compounds (polymeric and oxidised triacylglycerols, di- and mono-glycerols and free fatty acids). Such developments may help in authenticating the geographical origin of PDO labelled virgin olive oils.

The quality of Greek virgin olive oils was also assessed by ^{31}P NMR.[56] The method is based on the phosphitylation of the free hydroxyls of the diglycerides with a phospholane derivative. The quality of olive oils was determined on the basis of the content of 1,2-, 1,3- and total diglycerides (DGs). The results suggested that some commercial oils were of relatively poor quality (adulterated?). Statistical analysis revealed that DGs alone were not sufficient to discriminate oils according to their geographical origin.

6.6.2 Fish oils

In recent years, the beneficial effect of omega-3 polyunsaturated fatty acids (PUFAs) has led to the introduction on the international market of new fish products such as fish oil capsules which are used to enrich foodstuffs. As this market expands, there is a need to control the quality and content as declared on the label of those products.[57] Igarashi *et al.*[58] showed that high resolution ^{1}H NMR was an appropriate alternative to GC for quantifying docosahexaenoic acid (DHA) and the total PUFA content in fish oils. Aursand *et al.*[59] had already reported the possibility of using both ^{13}C and ^{1}H for evaluation of the distribution and content of PUFAs from fish muscle (the techniques give complementary information) and Sacchi *et al.*[60] proposed a rapid way for determining the proportion of PUFAs expressed as a percentage of total fatty acids.

6.6.3 Fruit juices

Belton *et al.* highlighted in 1996[61] the potential of high resolution ^{1}H NMR to authenticate fruit juices. The sample preparation and acquisition were straightforward and the clear differences in the chemical composition of juices like grape, apple, pineapple, orange and grapefruit suggested that authenticity problems could be tackled. Another preliminary study followed[62] where juices from different apple varieties were discriminated on the basis of their ^{1}H NMR spectra. Vogels *et al.*[63] have demonstrated the potential of NMR to discriminate pure orange juice from samples adulterated by pulp wash or sugars. Their work constituted a pilot study, as the number of samples did not exceed 20.

In later work,[17] orange juices have been discriminated from pulp wash (over 300 samples overall) by applying chemometrics to the ^{1}H NMR spectra of the juices. Pulp wash is a secondary product produced in the manufacturing of orange juice (it is used to make squash drinks for instance). It is cheaper than orange juice and its addition to orange juices labelled 'pure juice' is illegal in Europe. The statistics showed that, among others, the compound *N*, *N*-dimethylproline, was mainly responsible for the discrimination of the two orange products. The large number of samples permitted a statistical evaluation of the relative amounts of the compound in orange juice and pulp wash. It was found in significantly higher concentrations in pulp wash but the range of natural variation prevented it from being used as a marker compound on its own.

Fourier Transform Infra Red (FTIR) has been used to detect the adulteration of raspberry and strawberry purées with cheaper fruits and sugar solutions.[64–65] Similarly, the potential of ^{1}H NMR in discriminating adulterated from pure raspberry juices has been investigated.[66] Five types of adulterants were studied: apple, plum, pear and two sugar syrups commonly used in the food industry. Samples were either supplied by industrial companies or prepared from whole fruits in the laboratory. A preliminary analysis showed that pure raspberry and potential adulterant juices differed considerably in their contents of sugars, sorbitol, malic, quinic and citric acids. The ^{1}H NMR spectrum of a typical raspberry juice is dominated by signals arising from glucose, fructose and citric acid, while the other juices contain less citric acid, more malic acid, more fructose, sucrose and sorbitol (the two latter being rarely present in raspberry juice) .

It can be seen in Fig. 6.2 that the chemical composition of each juice is different; however no previously unrecognised marker compound was detected. The success of this study was limited. Raspberry juice samples were deliberately adulterated by juices of apple, plum and pear as well as two syrup solutions at adulteration levels of 5, 10 and 20% v.v. From the chemometric analyses performed on the spectra, it was established that plum, pear and apple could be detected at a minimum level situated between 10 and 20%, while the detection limit of the sugar adulteration was above 20%. ANOVA was performed on a selected number of compound peak heights and the only significant differences observed between pure and adulterated samples involved the known compounds sorbitol, malic and quinic acids. No noticeable differences were to be observed

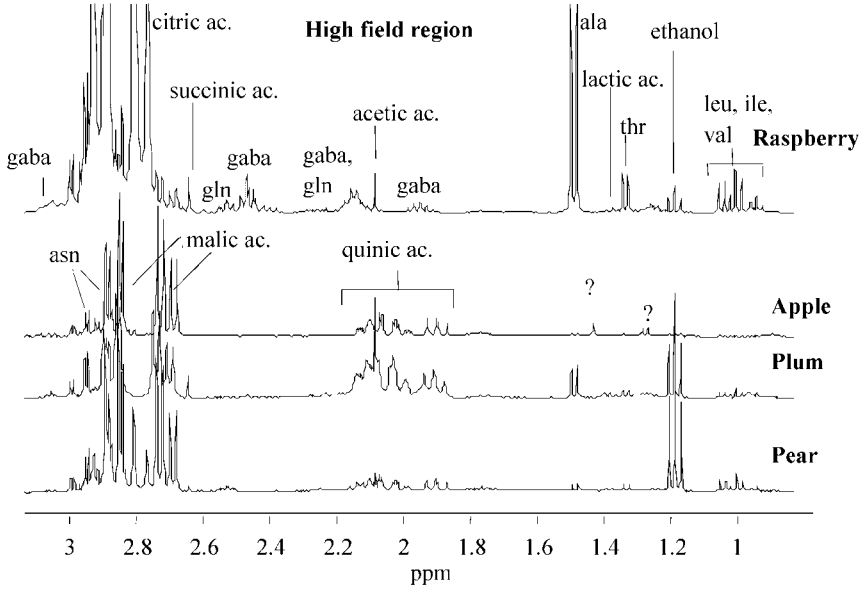

Fig. 6.2 400 MHz ^{1}H NMR spectra of typical raspberry, apple, pear and plum juices: high field region. Key to assignments: leu, leucine; ile, isoleucine; val, valine; thr, threonine; ac., acid; ala, alanine; gaba, γ-amino-butyric acid; gln, glutamine; asn, asparagine.

in the levels of fructose and glucose apart from the samples adulterated by pear, which significantly differed from the pure raspberry ones in fructose level. Although a 10% detection limit may be seen as a worthwhile result, HPLC analyses that target the sorbitol, malic and quinic acid content are likely to reach lower detection limits.

This study showed that the natural variability in chemical composition of raspberry is great. It seemed that the adulteration had an impact on the results but the natural variability in the composition of raspberry juices made it difficult to detect adulteration by a profiling method. Note that the chemometric models were validated using independent test sets, a procedure which limited the number of factors used in the models. There were only a limited number of samples in each subgroup and some of these were removed for the test set. It would be possible instead to use an appropriate discrimination technique and test it by cross-validation in order to make use of all the samples for building the models.

6.6.4 Wine/beer

Both ^{13}C and ^{1}H NMR have been applied to freeze-dried wine extracts dissolved in D2O to successfully classify white wines from three German regions[67] and red wines from three areas of the Apulia region in Italy.[42] The same procedure

has also been employed to analyse wines from different parts of Europe and their amino acid content was evaluated by ^{13}C NMR.[48] More recently Kosir and Kidric[11] applied hierarchical cluster analysis to a selection of proton signals arising from amino acids in order to classify wines from different regions of Slovenia. Like amino acids, anthocyanins are known to play a role in determining the flavour of wine. The authors assigned the NMR spectra of standards of anthocyanins in order to analyse in future studies the composition of liquid chromatographic fractions of wine extracts.

Beer has also been analysed by ^{1}H NMR. Duarte *et al.*[18] have identified around thirty compounds in degassed beer samples and identified as many unassigned spin systems. Ale and lager beers were compared and although the high and mid-field regions (amino, fatty and organic acids plus fermented sugars and dextrins) allow some separation, it was the low field region (aromatic compounds) that gave the best discrimination. This preliminary work suggests that the technique could be used to address authentication issues (geographical origin) as well as quality issues (processing, reproducibility within different brewing sites).

6.6.5 Coffee

Bosco *et al.*[68] have investigated the potential of ^{1}H NMR to detect as many components as possible from a coffee extract. Their work showed that the quality of the spectra of water extracts was improved by adding small amounts of HCl and DMSO-d_6. The amount of information in the spectrum was quite high but deuterated chloroform extracts yielded even more signals. From the water extract, organic acids, caffeine and aromatic heterocyclic compounds such as trigonelline were identified while in $CDCl_3$, certain low field signals were tentatively assigned to several aldehyde compounds originating from the roasting of the coffee beans. This study showed that ^{1}H NMR could be quite promising for tackling authenticity and quality problems in coffee. The work by Charlton *et al.*[19] further demonstrated it. The authors used high resolution ^{1}H NMR to discriminate spray-dried coffees obtained from three different producers. Two products from the same manufacturer were also separated by applying chemometrics to their ^{1}H NMR spectra. The main factor that separated the two products was the level of the compound 5-(hydroxymethyl)-2-furaldehyde.

6.6.6 Green tea

The ^{1}H NMR spectra of green teas (ground tea leaves extracted in a mixture of methanol/buffered D_2O) contained the signals of an appreciable number of metabolites including those of fatty, amino and organic acids, sugars, flavanols (more than ten compounds were identified in 1D and 2D spectra), flavonols, caffeine, and phenolic compounds such as p-coumaroyl quinic acid and theogallin (3-galloyl quinic acid).[66] Figure 6.3 shows the ^{1}H NMR spectrum of a

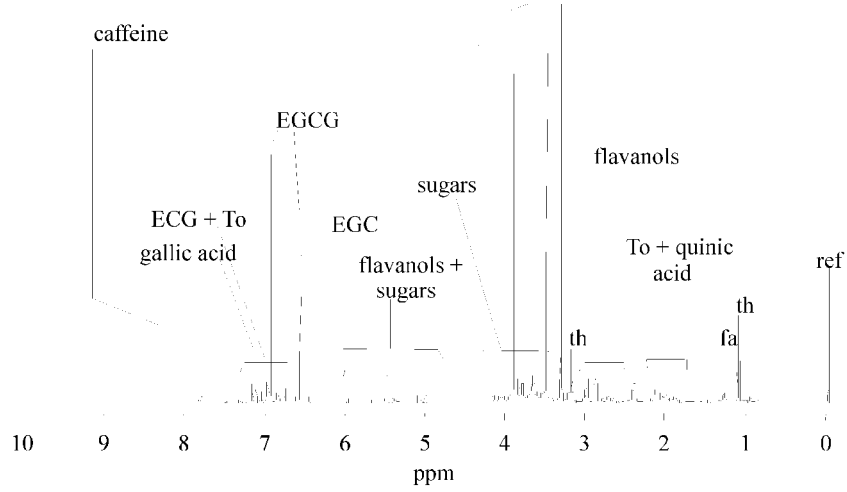

Fig. 6.3 400 MHz ^{1}H NMR spectrum of a 'Longjing' (or 'Dragon Well') Chinese green tea. Key to assignments: ref, reference; th, theanine; fa, fatty acids; To, theogallin; EGC, (−)-epigallo-catechin; ECG, (−)-epicatechin-3-gallate; EGCG, (−)-epigallo-catechin-3-gallate.

'Longjing' (or 'Dragon Well') Chinese green tea. The caffeine and some flavonol peaks shifted significantly from one spectrum to another,[52] therefore a program was used to align them before attempting chemometric analysis (see section 6.5). PCA was applied to a data set comprising NMR spectra of 38 Longjing 'high quality' teas and 61 roasted lesser quality teas (data not shown).

The quality of tea is highly (but not only) related to the age of the tea leaves when picked: the younger the leaves are, the better the quality is. The cheapest Longjing tea (4th grade) cost £4/50g while the price of other teas ranged from £2.5/50g for superfine to £0.80/50g for 4th grade. Teas originated from different places in China but a high proportion came from the Zhejiang county near Shanghai (region with the biggest national production). PCA showed that a distinctive separation occurred along PC1 (data not shown). The corresponding loading suggested that more than fifteen compounds could be involved in the quality of green tea.

Analysis of specific signals by ANOVA showed that Longjing teas have higher levels of theanine, theogallin, gallic acid, caffeine, epicatechin gallate, 2-*O*-(*β*-L-arabinopyranosyl)-myo-inositol and seven minor sugar signals, and lower levels of fatty acids, quinic acid, epigallocatechin and sucrose compared to the roasted teas. Although some of the compounds listed above may not directly contribute to the taste, they remain good indicators of the maturity of the tea shoots used to make a given bulk lot of tea. They could prove to be useful in authenticating tea bulk lots made from broken leaves.

6.6.7 Dairy products

The application of NMR spectroscopy to milk and dairy products has been recently reviewed.[69] The use of ^{13}C NMR to distinguish milks from different animal species has been reported.[8,70] The technique could provide a test on milk authenticity, an issue particularly important in the region of Naples where 'Mozarella di bufala' a PDO cheese exclusively made of buffaloes' milk is produced.[70] Belloque *et al.*[71] used ^{31}P NMR to simultaneously detect and quantify in milk all phosphorus-containing compounds in a single spectrum. The technique seems to be a good alternative to the current colorimetric technique and has the advantage of detecting polyphosphates, added compounds used as preservatives.

Cheese has also been studied by ^{1}H NMR.[72] The technique was used to assess the change in composition of amino acids in Grana Padano cheese, a popular Italian cheese. Preliminary work conducted at the Institute of Food Research showed that it was possible to differentiate mature from mild cheddar cheeses on the basis of their ^{1}H NMR spectra (Wang and Colquhoun, unpublished) (Fig. 6.4). Extra mature cheddars contain more free amino acids than the mild ones. PC2 also separates supermarket branded matured cheese (positive scores) from farm produced PDO cheddars (negative scores).

6.6.8 Fish and meat

Sitter *et al.*[73] have used ^{1}H NMR to evaluate the freshness of halibut on the basis of the level of metabolites such as adenosine triphosphate (ATP), adenosine

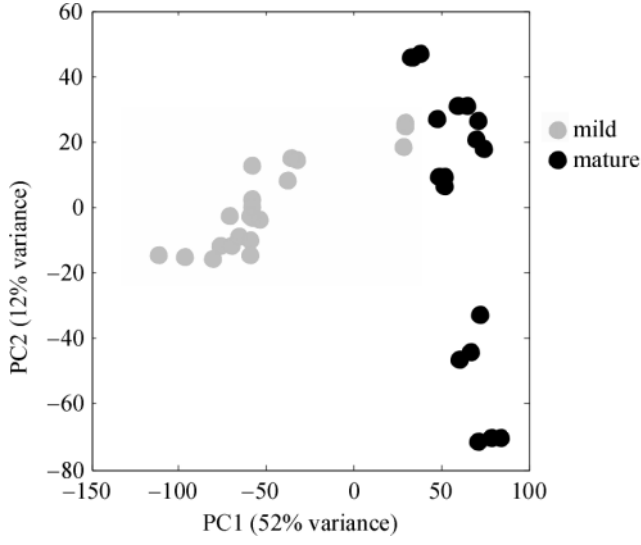

Fig. 6.4 PCA results for 21 mild and 18 extra matured cheddar cheeses. Variables have been mean centred and autoscaled. Cheese samples were homogenised in a 1M HC1 solution and ^{1}H NMR spectra of the supernatants (with added D_2O) were recorded at 400 MHz.

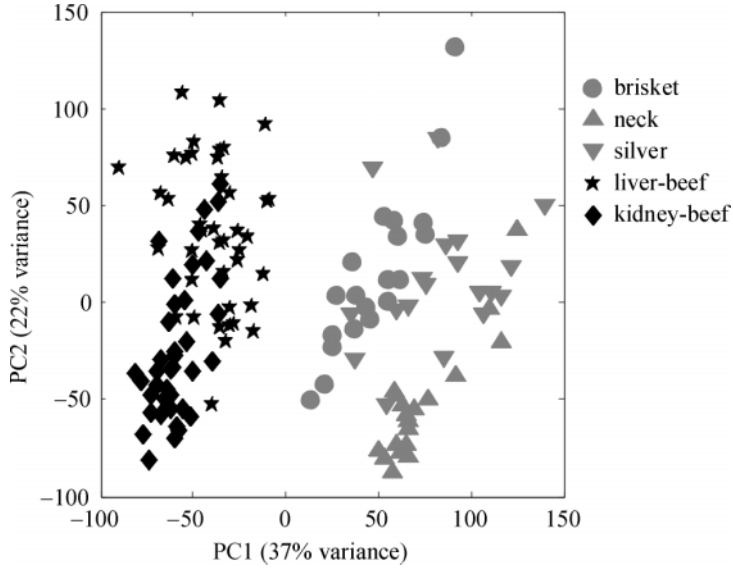

Fig. 6.5 PCA results for three cuts of beef muscle (19 'brisket', 19 'neck', 19 'silverside') and two groups of 41 adulterated samples (adulterants are kidney and liver with adulteration levels of 10–90%). Variables have been mean centred and autoscaled. Samples were homogenised in a 1M HC1 solution and ^{1}H NMR spectra of the supernatants (with added D_2O) were recorded at 500 MHz.

diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine and hypoxanthine. Amounts of ATP, ADP and AMP decreased with time while IMP, inosine, and hypoxanthine increased. Trimethylamine oxide was not detected and phosphocreatine disappeared after Day 1 as expected. In another preliminary study, wild and farmed salmon, Scottish and Norwegian salmon, mackerel and halibut were discriminated on the basis of their ^{13}C NMR lipid profiles.[74]

Al-Jowder *et al.*[75] showed that it was possible to discriminate pure beef muscle from samples adulterated by liver or kidney using ^{1}H NMR (Fig. 6.5). The technique even allowed the partial separation of the different types of 'pure' muscle sample. Many metabolites were identified. PLS models were built to quantify amounts of added liver or kidney and correlation coefficients of 0.99 were obtained. Adulteration of muscle tissue could be detected down to a level of 10%.

6.6.9 Miscellaneous studies

Wheat flour has been studied by ^{1}H HR/MAS NMR.[39,43] The authors assigned spectra of durum wheat flour and discriminated flours from different areas from the Apulia region in Italy. Limiroli *et al.*[76] studied the vegetation water of three Italian cultivars of olive fruits by ^{1}H NMR. Results showed some compositional differences between the cultivars. Gil *et al.*[77] evaluated the compositional

changes occurring during the ripening of mango using high resolution ^{1}H HR/MAS of fruit pulp and compared it with conventional liquid state NMR of the juice. More than 40 metabolites were identified.

6.7 Future trends

This chapter has shown that there are several important advantages in using NMR: it has great structural elucidation power (spectra are interpretable), it is quantitative, the preparation of the sample is rapid and simple, it is a non-targeted technique, it yields much information in a single spectrum and the measurements are reproducible over the long term. Because of the flexibility of the technique, a wide range of foods can be analysed and (potentially) authenticated, not just the liquid foods (oils, juices) that formed the basis of the earliest studies. An extract of almost any whole or freeze-dried food can be prepared to give high quality NMR spectra and is likely to yield useful information on the main metabolites present in the extract, sometimes in combinations that are not readily analysed by a single chromatographic technique. Preliminary studies such as the ones on cheese, meat, wine, beer or coffee presented in section 6.6 have highlighted this. From the papers published in the last few years and reviewed here the following conclusions and trends may be noted.

- Most work on 'conventional' NMR and authenticity is still at the level of pilot studies. No one method has yet been taken up as 'official' or is in regular use by commercial companies (contrast the case of SNIF NMR).
- There has been increasing use of high field spectrometers (500 and 600 MHz) for authenticity work. This gives access to more minor components (without recourse to lengthy fractionation procedures), which are proving useful, e.g., in the characterisation of PDO olive oils.
- Chemometrics is now used almost routinely to analyse NMR data, either the whole spectrum or combinations of selected signals. New chemometric methods will allow systematic selection of signals giving better discrimination performance and leading to the discovery of new marker compounds.
- NMR is unlikely ever to be used by local enforcement bodies or for screening raw materials at the factory gate. Current developments (higher fields, higher throughput, but not lower cost of the hardware) indicate more a role for central laboratories undertaking statistically based analysis/profiling studies of very large sample collections to establish reference databases or conduct surveillance exercises.

6.8 Sources of further information and advice

Colquhoun and Lees reviewed earlier work on NMR and food authenticity[3] in a chapter that also included a discussion of SNIF NMR. Another review[7] has dealt

with the applications of NMR to various aspects of olive oil quality. Several general texts on NMR[5, 6, 15] are recommended as sources of practical advice. Although dealing mainly with biomedical applications the article by Lindon *et al.*[4] provides much useful information on the use of chemometrics in high resolution NMR. The book by Kemsley,[38] with accompanying Win-DAS software, is one of the few chemometrics (as opposed to general multivariate analysis) texts to concentrate on discriminant analysis. Its examples are mostly drawn from the area of food authenticity and FTIR spectroscopy but the general principles are equally applicable to NMR. Other chemometrics books that have been found useful are referenced.[28, 30, 32–37]

The web sites of the main instrument manufacturers (Bruker, JEOL, Varian) are useful sources for information on new developments in analytical NMR (hardware, automation, etc.) Of the many web sites devoted to chemometrics the one from Umea http://www.acc.umu.se/~tnkjtg/chemometrics/index.html has links to most of the software companies offering the packages mentioned in the text, tutorial and editorial articles (editorials for September and October 2002 cover chemometrics and NMR), and Matlab macros to download.

Food science journals include an increasing number of articles that feature NMR and a significant number of these deal with authenticity. Probably the most prominent journal in this area is the *Journal of Agricultural and Food Chemistry* (http://pubs.acs.org/journals/jafcau/). The biennial conferences on 'Applications of Magnetic Resonance in Food Science' usually include several papers on authenticity and proceedings are published in the year following the conference by the Royal Society of Chemistry. Articles from several of these volumes are referenced here.[16, 73, 75]

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7

Using stable isotope ratio mass spectrometry (IRMS) in food authentication and traceability

S. D. Kelly, Institute of Food Research, UK

7.1 Introduction: stable isotopes

Isotope ratio mass spectrometry (IRMS) has been used to detect economic fraud in food production since the early 1970s. Yet in many conferences dedicated to gas isotope mass spectrometry, presentations reporting the use of IRMS in food authentication are often relegated to the 'miscellaneous sessions'. For the majority of users IRMS techniques are still the domain of geologists, clinicians and environmental scientists. More recently however, the technique is gaining wider acceptance in food control laboratories as the reliability and array of 'on-line' techniques is culminating in the use of IRMS analysers as 'another detection system'. Ultimately IRMS can offer unequivocal evidence of food adulteration such that it may be used as a practical everyday tool by control laboratories and enforcement agencies in the prosecution of dishonest traders. This chapter will review the historical development of IRMS in food authenticity control and two of the major emerging techniques in IRMS. However, the first part of the chapter will describe some of the definitions and principles fundamental to the understanding and application of the IRMS technique.

7.1.1 Definition of stable isotopes

The word *isotope* originates from the Greek language (*isos* equal, *topos* place)[1] and means *equal places*. This denotes that isotopes occupy the same position in the periodic table of the elements. Thus, isotopes may be defined as atoms possessing the same number of protons but different numbers of neutrons in their respective nuclei. The conventional atomic annotation may therefore be

Table 7.1 Mean terrestrial abundance of the stable isotopes – principal elements of interest in food authenticity studies (adapted from reference 2)

Element	Isotope	Abundance (atom %)
Hydrogen	^{1}H	99.985
	^{2}H	0.015
Carbon	^{12}C	98.89
	^{13}C	1.11
Nitrogen	^{14}N	99.63
	^{15}N	0.37
Oxygen	^{16}O	99.759
	^{17}O	0.037
	^{18}O	0.204
Sulfur	^{32}S	95.00
	^{33}S	0.76
	^{34}S	4.22

used to describe any given isotope. For example $^{12}_{6}C$ represents the neutral isotope of carbon with a mass number of 12 and an atomic number of 6. Isotopes exist in both stable and unstable (radioactive forms). Table 7.1 lists the mean natural abundance of the principal light bio-elements used in food authenticity studies.

7.1.2 Isotope terminology and delta scales

Fractionation (isotope effects)

The electronic structure of an element primarily governs its chemical reactivity. Although, as a first approximation, the nucleus is responsible for its physical properties.[2] All isotopes of a given element possess the same electronic configuration and consequently there are marked similarities in their behaviour. However, this similarity is not limitless. Natural variation in isotope abundance or *fractionation* is a physical phenomenon and is dependent upon thermodynamic equilibria and kinetic processes affecting a given isotope. In both cases, fractionation is a function of variation in the physico-chemical properties of the isotopes and is proportional to differences in their masses respectively.[2] As such, these *isotope effects* are most evident in the light elements, e.g., the melting point of water, at 1 atmosphere pressure, is by definition 0ºC. Whereas, the melting point of deuterium oxide (D_2O) under the same conditions is 3.82ºC.

Equilibrium isotope effect (EIE)

Equilibrium isotope effects (EIE) cause the heavy isotope to build up in a given component of a system at equilibrium. In such a system the heavy isotope

accumulates preferentially in the chemical compound in which the element is bound most strongly. The size of an EIE may be characterised by an equilibrium constant or *fractionation factor*. In the majority of cases, fractionation factors relate to equilibrium constants of exchange reactions in which a single atom is exchanged between two species.[3] In this scenario the ratio of the isotope ratios for the exchanged positions in the reaction, is numerically equivalent to the fractionation factor. For example, when CO_2 is equilibrated with water and oxygen atoms are exchanged, via bicarbonate intermediates, ^{18}O is concentrated in the CO_2.[4] The CO_2/H_2O equilibrium is shown below in equation 7.1:

$$C^{16}O^{16}O_{(g)} + HC^{18}O^{16}O^{16}O^-_{(aq)} \rightleftharpoons C^{16}O^{18}O_{(g)} + HC^{16}O^{16}O^{16}O^-_{(aq)}.. \qquad 7.1$$

For this exchange reaction, the fractionation factor (α) is given in equation 7.2 and is numerically equal to the equilibrium constant

$$\alpha = \frac{(^{18}O/^{16}O)_{HCO_3^-}}{(^{18}O/^{16}O)_{CO_2}} \qquad 7.2$$

Kinetic isotope effect (KIE)

Qualitatively, many observed deviations from the simple equilibrium processes can be interpreted as consequences of the various isotope components having different rates of reaction. Isotope fractionation measurements taken during irreversible chemical reactions always show a preferential enrichment of the lighter isotope in the products of the reaction. If the sensitivity to isotopic substitution exists at the position where chemical bonds are broken and formed during the reaction, the KIE is defined as *primary*. Furthermore, a *normal* KIE is one in which the species (or isotopomer) containing the lighter isotope reacts more rapidly. All *primary* KIEs involving elements heavier than hydrogen are *normal*.[5] The nature of this fractionation stems from the lower ground state vibration frequency of the heavy isotope. Hence, more energy is required to destroy a molecule bearing the heavy isotope. For an explanation of this phenomenon it is necessary to discuss the quantum mechanical effects underlying bond fission. The energy of a molecule is described in terms of the electronic energy plus the translational, rotational and vibrational energy of the molecule. The electronic translation and rotation energies are more or less equal for isotopes of the same element. Therefore, molecular vibrations are the ultimate source of isotope effects. Figure 7.1 shows schematically the effect of replacing protium (^1H) with deuterium(^2H) on a reaction in which the breaking of the C-H bond is the rate-determining step. The x-axis or 'reaction co-ordinate' equates to the stretching of the C-H bond. When deuterium is substituted for protium, the principal change is the reduction in the zero-point energy (zp) of the bond.

This is because the bond is restricted to certain discrete energy levels and the zero-point is equivalent to $\frac{1}{2}hv$, where h is Plank's constant and v is the frequency with which the C and H atoms vibrate with respect to one another.[3] As the vibrational frequency of the bond is inversely proportional to the masses

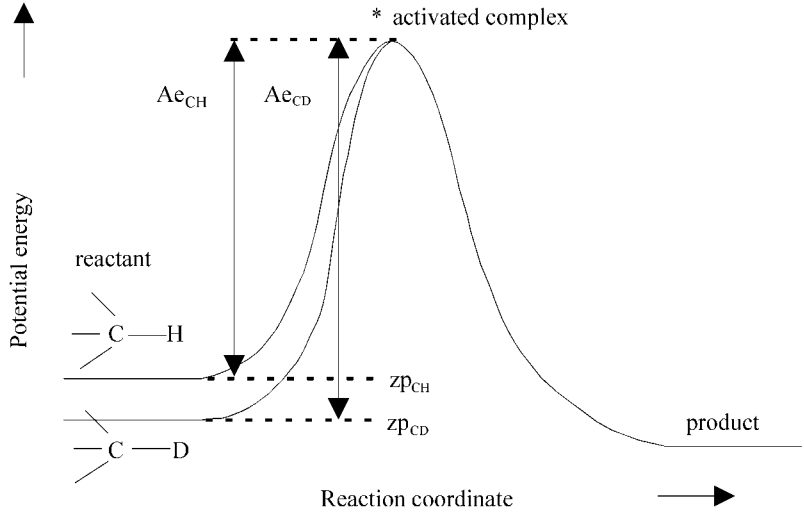

Fig. 7.1 Changes in the reaction potential energy profile when a bond undergoing cleavage is deuterated. The only significant change is to the zero-point (zp) energy of the reactants, which is lower for C-D than for C-H. As a result the activation energy (Ae) is greater for C-D than for C-H (adapted from reference 6).

of the C, H and D atoms, the C-D bond will have a lower zp energy than the equivalent C-H bond. This means that the activation energy required to break bonds with light isotopes is less than bonds involving heavy isotopes. It should be noted that the whole reaction profile is not lowered. This is because the corresponding vibration in the activated complex (*) has a very low force constant. Essentially the zp energy has little or no effect in either the protium or deuterium forms of the complex.[6]

The size of a kinetic isotope effect can be represented in terms of the ratio of the rate constants. The relationship between the magnitude of an isotope effect and the isotopic fractionation is rarely straightforward. However, there are some general observations that can be described. These are that even the largest isotope effect possible will not cause any fractionation if the substrate in a reaction is quantitatively converted to the product. Examples of this are the fermentation of sugars to ethanol by resting yeast cells, nitrogen assimilation and the assimilatory sulfate reduction.[7] In such cases the isotope effects in the reaction are not *expressed*. An isotope fractionation will, however, always be observed when a reaction has an isotope effect and the formation of the product is not quantitative or there is metabolic branching. In this latter case the products must show depletion and enrichments that are inversely proportional to their yields as demanded by an isotopic mass balance. For example, the depletion of ^{13}C in glycerol during fermentation is a compensation for small enrichments in the corresponding positions of hexoses formed during the metabolic fluxes of dihydroxyacetone phosphate. This results in the $\delta^{13}C‰$ of glycerol being 4 to 5‰ more negative than carbohydrates from the same botanical source.[8]

Other processes that can lead to isotope fractionation

Many other natural phenomena, classed as physico-chemical effects, can lead to isotope fractionation. For example, evaporation and condensation, crystallisation and melting, absorption and desorption, diffusion and thermodiffusion. Isotope fractionation (or discrimination) is usually expressed as the difference between the source and product of a reaction, as shown for carbon in equation 7.3 below:

$$\text{Fractionation } (\Delta\delta) = \frac{\delta^{13}\text{C‰}_{\text{source}} - \delta^{13}\text{C‰}_{\text{product}}}{1 + (\delta^{13}C‰\ \text{source}\ .10^{-3})} \qquad 7.3$$

This fractionation has units of ‰, but to avoid confusion with δ^{13}C‰ values is denoted as $\Delta\delta$. The fractionation has a positive sign when ^{12}C is transformed into the product more rapidly than ^{13}C, which is the situation for most physico-chemical processes. Of special interest in food authenticity studies are the evaporation-condensation and evapotranspiration processes and the information this provides with respect to the geographical and botanical origin of foods respectively.

7.1.3 'Delta' notation and isotope reference materials

Differences in isotope effects mentioned in the previous section are usually of the order of a few percent. Consequently, changes in the isotopic ratio at natural abundance levels often occur around the third or fourth significant figure. Isotopic analysis therefore requires very precise measurement and this is achieved by measuring the ratio of the heavy and light stable isotopes in the test material and comparing it to a reference compound of nominal isotope ratio. This is done principally because measurements of absolute isotope ratios are not sufficiently stable over longer periods.[9] Since the instrumental requirements are so strict for IRMS, the stability of measurements is affected by, for example, subtle changes in the geometry of the flight tube induced by temperature fluctuations. These changes can however be negated over the long-term if the sample is compared to a reference, as the observed difference between the sample and reference will remain constant as they are equally affected by the instrumental changes.

The approach of using differential comparison between the sample and a suitable reference has been a fundamental part of IRMS since its introduction and formal definition over 50 years ago.[10] Thus, isotopic abundance of a sample relative to a reference is normally expressed by the differential equation 7.4:

$$\delta\, ref = \left(\frac{Rsamp - Rref}{Rref}\right).1000 \qquad 7.4$$

Which can be simplified to equation 7.5

$$\delta\, ref = \left(\frac{Rsamp}{Rref} - 1\right).1000 \qquad 7.5$$

Where δ *ref* is the isotope ratio of the sample expressed in delta units relative to the reference material. *Rsamp* and *Rref* are the absolute isotope ratios of the sample and reference material respectively. Multiplying by 1,000 converts the value to *parts per thousand* (‰), or the more commonly used expression *per mil* (derived from the Latin *mille* meaning one thousand and used in an analogous way to *per centum* or *per cent*). In essence the use of equation 7.4 or 7.5 facilitates the comparison of isotope ratios especially at the natural abundance level when the differences being examined are small. For example, the natural flavour chemical vanillin may typically have a $^{13}C/^{12}C$ isotope ratio of 0.010989786 whereas synthetically derived vanillin from petrochemical precursors may be 0.01089989. The difference in the fourth decimal place is masked to some extent by the similarity of the preceding numbers (0.010). However, when these ratios are expressed using the delta notation they become −22.0‰ and −30.0‰ respectively relative to the Pee Dee Belemnite reference material, that has a $^{13}C/^{12}C$ ratio of 0.011237. The difference in ^{13}C abundance between the two sources of vanillin is thus much more apparent.

There are four primary isotopic reference materials for the *light* bio-elements. These are listed in Table 7.2.[11] Neither SMOW nor PDB are available to calibrate isotopic measurements as the original materials have been used up. Consequently, the International Atomic Energy Agency (IAEA, Vienna) has carefully calibrated alternative reference materials that compare closely to the primary standards or have defined δ-values relative to the original primary standards. For example the IAEA prepared VSMOW which has an isotopic composition very close to that of the original primary reference SMOW. Examples of these reference materials are given in Table 7.3.[12]

In practice, routinely used *working* or *laboratory standards* are calibrated against the appropriate reference materials listed in Table 7.3 in order to preserve the limited stocks of these materials. A reference material is then

Table 7.2 Isotopic compositions of the *primary* reference materials (adapted from reference 11)

Primary reference material	Isotope ratio	Accepted value ($\times 10^6$, ppm) (with 95% Confidence interval)
Standard Mean Ocean Water (SMOW)	$^{2}H/^{1}H$	155.76 ± 0.10
	$^{18}O/^{16}O$	2005.20 ± 0.43
	$^{17}O/^{16}O$	373 ± 15
Pee Dee Belemnite (PDB)	$^{13}C/^{12}C$	11237 ± 9.0
	$^{18}O/^{16}O$	2067.1 ± 2.1
	$^{17}O/^{16}O$	379 ± 15
Air	$^{15}N/^{14}N$	3676.5 ± 8.1
Cañon Diabolo Troilite (CDT)	$^{34}S/^{32}S$	45004.5
	$^{33}S/^{32}S$	8100.0

Table 7.3 List of reference materials available for analysis of stable isotope ratios of light elements at environmental levels (extracted from reference 13)

Name	Material	Status	δ-value (‰)	δ-value (‰)	Remarks
VSMOW	water	CM	$\delta^2H = 0$	$\delta^{18}O = 0$	by definition
SLAP	water	CM	$\delta^2H = -428.0$	$\delta^{18}O = -55.5$	by convention
GISP	water	RM	$\delta^2H = -189.5 \pm 1.0$	$\delta^{18}O = -24.8 \pm 0.05$	
NBS19	limestone	CM	$\delta^{13}C = +1.95$	$\delta^{18}O = -2.200$	by definition
IAEA-CH-6	sucrose	RM	$\delta^{13}C = -10.4 \pm 0.2$		old name Sucr. Anu
IAEA-CH-6	polyethylene	RM	$\delta^{13}C = -31.8 \pm 0.2$	$\delta^2H = -100.3 \pm 2.0$	old name PEF-1
NBS22	oil	RM	$\delta^{13}C = -29.7 \pm 0.2$	$\delta^2H = -118.5 \pm 2.8$	
IAEA-S-1	Ag_2S	RM	$\delta^{34}S = -0.30$		by definition
NBS127	$BaSO_4$	CM	$\delta^{34}S = +20.3 \pm 0.4$	$\delta^{18}O = +9.3 \pm 0.4$	
IAEA-N-1	$(NH_4)_2SO_4$	RM	$\delta^{15}N = +0.4 \pm 0.2$		
USGS32	KNO_3	RM	$\delta^{15}N = +180 \pm 1$		

Notes: All δ-values for isotope abundance in the list are given in deviation from a zero reference point defined by a calibration material (CM), i.e., versus VSMOW for hydrogen and oxygen in water samples; versus VPDB for carbon and oxygen in solid materials; versus VCDT for sulfur and air-N_2 for nitrogen. CM = calibration material and RM = reference material.

analysed at intervals to control the quality of isotopic measurements. The δ-value measured relative to the laboratory standard may be converted to the appropriate international scale using equation 7.6[13]:

$$\delta_i^s = \delta_w^s + \delta_i^w + \left(\delta \frac{{}^s_w \cdot \delta_i^w}{1000}\right) \qquad 7.6$$

Where:

δ_i^s is the δ-value of the sample (s) on the international scale (i)
δ_w^s is the δ-value of the sample (s) relative to the working standard (w)
δ_i^w is the δ-value of the working standard (w) on the international scale (i).

Again, in practice, the manufacturer's proprietary software supplied with most modern IRMS instruments requires only δ_i^w to be entered into the appropriate *standard table*, for δ_i^s to be automatically calculated.

7.2 Principles of operation of IRMS

Charged atoms and molecules are separated in a mass spectrometer on the basis of their mass-to-charge ratio ($^m/_z$). $^m/_z$ is the common notation used to define a dimensionless quantity derived by dividing the mass number (m) of an ion by its total charge (z). The mass number is the sum of the total number of protons and neutrons in an atom, molecule or ion. The relative abundance of the ions is then determined by measuring the currents produced by the spatially separated ion beams. Most of the mass spectrometers in use for isotope ratio measurements are based on the design of Nier,[14] whose mass spectrometer design achieved a level

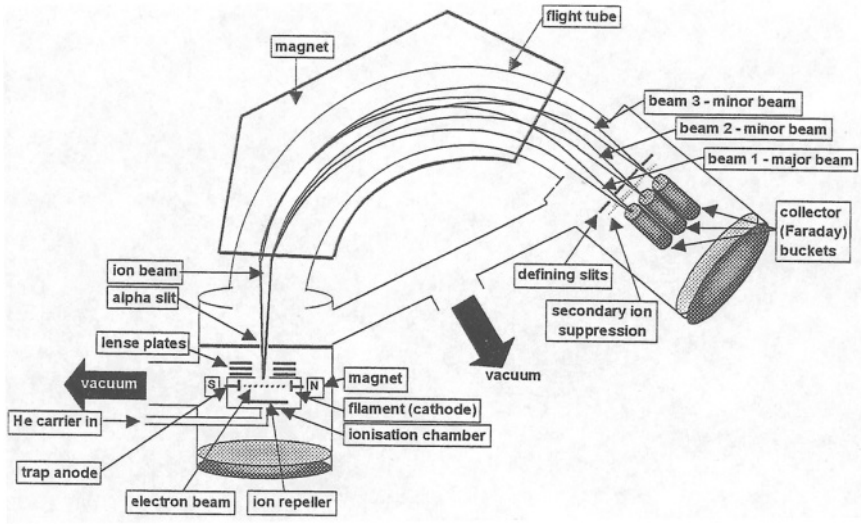

Fig. 7.2 Components of an isotope ratio mass spectrometer.

of precision and reliability of operation that set the bench-mark for isotope mass spectrometry. Although subsequent improvements by McKinney *et al.* may be considered to have fully facilitated the precise determination of $^{2}H/^{1}H$, $^{13}C/^{12}C$ $^{18}O/^{16}O$ and $^{34}S/^{32}S$ isotope ratios in geological and biological samples.[15] The modern Nier-type mass spectrometer has three basic components – an ion source, a mass analyser and an ion detector (collector) system. The basic parts of the IRMS are represented schematically in Fig. 7.2 (modified from reference 16):

Gas samples, typically H_2, N_2, CO, CO_2 and SO_2 are introduced into the source through a narrow capillary. The gas molecules then undergo ionisation, as a result of impact with a stream of electrons generated by a thoria coated tungsten filament $\sim$70 eV. Interaction of the gas molecule with an energetic electron generally removes an electron from the valence orbital of the molecule according to equation 7.7:

$$M \xrightarrow{e^-} M^+ + 2e^- \qquad 7.7$$

Excess energy transferred to the newly formed positively charged ion can cause bond dissociation (fragmentation or cracking). For example, during the ionisation of CO_2, significant quantities of CO are formed, which has the same mass as N_2. It is therefore imperative that during the isotope analysis of N_2 the gas is free from all traces of CO_2, otherwise the measurement will be inaccurate.

After ionisation of the sample gas, the positively charged ions are accelerated and collimated (ions moving in a parallel path) by a series of electrode 'lenses' to which variable potentials may be applied. At this stage the ion beam is

unresolved and enters the magnetic sector of the flight tube, which is designed to provide low-mass resolution of 1 atomic mass unit (amu) with an average resolution between any two peaks of 10% relative to the peak height. The Field (B) generated by the permanent or electromagnet deflects the ions into circular trajectories with radii proportional to the mass of the respective isotope, i.e., the heavier gas isotopes (isotopomers) are deflected less than the lighter isotopes. Using carbon dioxide as an example, the major ion $^{12}C\ ^{16}O\ ^{16}O$ (m/z 44) with the relatively lowest mass follows the trajectory with the shortest radius and the heavier isotope m/z 45 ($^{13}C\ ^{16}O\ ^{16}O$) follows a 'central' trajectory and the heaviest isotope $^{12}C\ ^{16}O\ ^{18}O$ (m/z 46) follows the trajectory with the largest radius. The resolved beams then continue along the flight tube to the collector assembly.

The ion beam collides with the inside walls of a metal box (Faraday cup) and all secondary ion emission is suppressed. An accurate measure of the charge deposited by the ion beam is obtained via an electron current that flows through resistors of 10^9–$10^{12}\ \Omega$ depending on the relative intensity of the ion beams. The subsequent voltage generated across the resistors is amplified and converted into a digital output to a personal computer. The ion beam defining slits are wider than the ion beams, resulting in the characteristic flat top peaks that are insensitive to drift, which may result from temperature variation affecting the geometry of the flight tube and electronic components used in mass selection.[16] The overall result is stable, well-defined beams that can be utilised for precise measurement of the relative abundance of the isotopomers.

7.2.1 Ion separation – equations of motion

Ions carrying the same charge (z) are accelerated in the ion source by a potential difference (V). The kinetic energy ($K.E.$) acquired by an ion of mass (m) is defined by:

$$K.E. = zV = \frac{1}{2}\, mv^2 \tag{7.8}$$

Where v is the ion velocity. As a consequence of this relationship, it can be seen that ions of different masses will have different velocities. Rearranging equation 7.8 gives:

$$v = \sqrt{\frac{2zV}{m}} \tag{7.9}$$

In a magnetic field of strength B, the ions experience a force zvB perpendicular to the direction of motion. This results in a circular trajectory (with radius) within the magnetic field, such that the centrifugal force balances the deflecting force:

$$zvB = \frac{mv^2}{r} \tag{7.10}$$

By eliminating v^2 from equations 7.8 and 7.10 we have:

$$\frac{2zV}{m} = \frac{z^2B^2r^2}{m^2} \tag{7.11}$$

and

$$\frac{m}{z} = \frac{B^2r^2}{2V} \tag{7.12}$$

The equation shows that if B and V are constants the radius (r) of the trajectory of the ion is proportional to the square root of the mass (m). Furthermore, solving equation 7.6 for V demonstrates that the accelerating voltage or the magnetic field can be altered to cause an ion of mass m and charge z to describe a trajectory of radius r.

7.2.2 Dual inlet – IRMS (DI-IRMS)

The most significant difference between an IRMS and a high resolution mass spectrometer is the use of a dual-inlet system in the former. The dual-inlet comprises two variable volume gas reservoirs, constructed of concertinaed stainless-steel bellows. One volume is used to store a calibrated gas of known isotopic composition, whilst the other volume holds the sample of test gas. These two gases are allowed to leak into the source of the analyser system, through 'balanced' capillary tubes under viscous flow conditions. The capillaries ensure viscous rather than molecular flow. In viscous flow, gas molecules behave in a predictable and 'fluid-like' manner at pressures greater than 1×10^{-2} millibar. Many mass spectrometer techniques utilise molecular flow at lower pressures, however this condition means that molecules are so far apart that they no longer behave as a fluid and their motion is random. This situation is not suitable for isotopic measurements as it results in isotopic fractionation of the gas under test. The capillaries from the two variable volumes flow through a change-over valve into the ion source. The change-over valve switches the flow between the reference and sample gas. The two measurements are then used to determine the difference in isotopic abundance between the two gases. The relative difference is expressed in delta notation and then converted to the relevant international standard using the equations 7.4 to 7.6 above.

7.2.3 Preparation of samples for DI-IRMS

Although the reproducibility and accuracy of IRMS measurements of any given pure gas are constrained by the quality of the sample presented to it, the major requirement is that the conversion of an inorganic or organic substrate into a gaseous product must be as complete as possible to prevent isotopic fractionation.

Hydrogen

The off-line preparation of hydrogen gas for dual inlet measurement has been achieved using a wide range of mainly metallic reducing agents including

carbon, chromium, magnesium, manganese, tungsten, uranium and zinc. These methods have been extensively reviewed by Wong and Klein.[17] The equilibration technique requires the use of a platinised hydrophobic polymer which catalyses the exchange of hydrogen in the gas phase and the hydrogen present in water.[18,19] Two automated hydrogen preparation devices have been described which were directly interfaced with dual inlet mass spectrometers (DI-MS) allowing increased sample throughput compared to the classical off-line techniques whilst retaining the high-precision of DI measurements. These systems used chromium[20] and manganese[21] to convert water and organic material to hydrogen gas.

Nitrogen

Classical preparation of N gas for ^{15}N DI-IRMS analysis generally employed Kjeldahl digestion on the compound of interest. The resulting ammonium salt is then oxidised by the hyper-bromite reaction to generate dinitrogen gas (equation 7.13):

$$2NH_4^+ + 3BrO^- + 2OH^- \longrightarrow N_2 + 5H_2O + 3Br^- \qquad 7.13$$

The determination of ^{15}N in inorganic nitrite and nitrate can be facilitated by their conversion to N_2O via reduction with a cadmium/copper foil. The oxidised nitrogen species are reduced in potassium chloride solution to nitrite and hydroxylamine which undergo further reduction to form nitrous oxide (equation 7.14):

$$NO_3 \longrightarrow NO_2 + NH_2OH \longrightarrow N_2O \qquad 7.14$$

By measuring the 45/44 ratio of the N_2O it is possible to determine the $^{15}NO_3/^{14}NO_3$ ratio of the original substrate.

Carbon

Carbon dioxide is generated from organic molecules by evacuated sealed tube combustion using a range of oxidants, including copper oxide. The CO_2 is purified by cyogenic trapping in liquid nitrogen and transfer of the purified gas to a suitable container for DI-IRMS. CO_2 is prepared from inorganic carbonate by the action of 85–100% phosphoric acid under vacuum. This process has been subjected to complete automation in the majority of cases such that 3–4 analyses can be completed per hour.

Oxygen

The oxygen ^{18}O content of water is still measured predominantly by using the CO_2/water equilibrium technique.[22] This technique involves evacuating small bottles containing the aqueous sample and refilling the headspace with carbon dioxide. An equilibrium is established between the ^{18}O isotope in water, carbon dioxide and carbonic acid (H_2CO_3). The headspace is transferred via capillary to the duct inlet. Since the $\delta^{18}O$ of the CO_2 added to the headspace is accurately known, the isotope ratio of the water test sample can be calculated by measuring

the change in the $\delta^{18}O\%$ of the headspace CO_2 after equilibrium has been reached, usually 6–8 hours. This process is usually automated to facilitate batch analysis of a large number of aqueous samples equilibrated simultaneously. The $\delta^{18}O$ of carbonates is determined by preparing CO_2 , as described above, for ^{13}C analyses by the action of phosphoric acid.

Preparation of organic materials for $\delta^{18}O$ analysis is most often performed by pyrolising the test material under vacuum with mercury (II) chloride followed by cryogenic purification. The mercury II chloride technique was developed by Rittenberg and Ponticorvo and has undergone various subsequent modifications.[23]

7.2.4 Continuous flow – IRMS (CF-IRMS)

The combination of on-line or continuous flow preparation techniques with IRMS, and its origins and developments, has recently been extensively reviewed by Midwood and McGraw.[24] It was the work of Preston and Owens[25] in coupling an elemental analyser system with an IRMS that first permitted the dual-inlet to be bypassed. Continuous flow IRMS led to a significant evolution in on-line sample preparation techniques based around two principal instruments, the elemental analyser and the gas chromatograph-combustion interface. Between the two principal preparation modes it is possible to determine the isotopic content of a wide variety of volatile and non-volatile solids and liquids in which the analyte concentration may vary widely. Just in the same way that DI-IRMS requires a pure gas sample, so does CF-IRMS. Under these 'continuous flow' conditions the gas produced from the sample is a transient signal and the helium carrier gas fulfils the requirement for viscous flow conditions.

7.2.5 Recent instrumental developments in CF-IRMS

Over the past five years there have been significant developments in the design of isotope ratio mass spectrometers to permit the determination of stable hydrogen isotope ratios in continuous flow mode, that is, in the presence of a large excess of helium carrier gas. Under these conditions $^4He^+$ is approximately 10^5 times more abundant than $^2H^1H^+$. In conventional isotope ratio mass spectrometers the very large helium ion beam at m/z 4, produces a significant overspill into the m/z 3 collector bucket, making reproducible $^2H/^1H$ measurement difficult. Advances in design of isotope ratio mass spectrometers have been necessary to permit precise $^2H/^1H$ analysis by continuous-flow. This has evolved through the alteration of the geometry of the mass spectrometer's flight tube, to achieve greater dispersion between the $^4He^+$ and $^2H^1H^+$ ion beams.[26] In addition, an Electro-Static Filter (ESF)[27] or a retardation lens[28] have been mounted in the entrance of the m/z 3 ($^2H^1H$) collector to remove any residual $^4He^+$ overspill.

These advances in instrument design have been accompanied by an equally rapid development in peripheral devices for the on-line production of hydrogen from water and organic samples. Since 1997 pyrolysis, or high temperature

conversion, has been shown to provide the most reliable means of quantitatively producing hydrogen in both bulk and compound-specific analysis. Commercially available pyrolysis interfaces for GC-IRMS are descended from the configuration described by Tobias and Brenna,[29] in which an empty alumina tube, maintained at a temperature of 1150ºC, was used for conversion of light hydrocarbons to hydrogen gas. An improved system for conversion of GC separated low molecular weight alkanes and alcohols to hydrogen, in a pyrolysis reactor consisting of a graphitised alumina tube heated to 1430ºC was demonstrated by Burgoyne and Hayes.[30] The use of this system in ^{2}H analysis by GC-IRMS has subsequently been reported by Hilkert *et al.*[31] and Sessions *et al.*[32] Scrimgeour *et al.*[33] used a GC system fitted with a wider-bore open tubular reactor operating at 1200ºC for preliminary analysis of $^{2}H/^{1}H$ in deuterium labelled fatty acids. D/H ratios of a range of chromatographically separated compounds were measured with an external precision of <2‰.

A desire to determine the $\delta^{2}H$ in bulk materials by continuous flow-isotope ratio mass spectrometry has resulted in the developments of pyrolysis interfaces based upon the elemental analyser. These Py/CF-IRMS systems have used an inert packing material, such as glassy carbon, for thermal decomposition of sample to H_2 at temperatures in excess of 1000ºC.[31,34,35]

In 1996 Werner *et al.* reported on the use of a modified elemental analyser in continuous-flow mode to determine the $\delta^{18}O$‰ value of carbohydrates and aromatic compounds.[36] The reactor tube was packed with highly inert glassy carbon and operated at 1080ºC. These conditions achieved a rapid pyrolysis of the organic samples producing carbon monoxide as the measuring gas in the helium carrier stream. The results obtained gave satisfactory agreement with $\delta^{18}O$‰ values obtained by the Rittenberg-Ponticorvo method and with known $\delta^{18}O$‰ values of standards. The internal precision of the method was typically 0.8‰ or better. The possibilities of using this technique to obtain position-specific $\delta^{13}C$ values from organic compounds with a carbon:oxygen ratio greater than 1, were also alluded to.

One of the problems that the previous on-line system presented was the calibration of $\delta^{18}O$‰ for organic samples. The inertness of the reactor tube packing meant that it was not possible to quantitatively convert the accepted water standards, VSMOW, GISP and SLAP to CO for calibration. Furthermore, carbonate standards could not be used because the pyrolysis temperature was insufficiently high to quantitatively convert these materials to CO. Koziet developed a higher temperature system based around a Leco elemental analyser operated at 1300ºC.[37] Carbon black powder was supported on a bed of carbon felt in a glassy carbon tube. The method was validated with GISP and the carbonate standard NBS 19. The values obtained were −24.8‰ and 27.3‰ which were in good agreement with IAEA reference values of −24.8‰ and 28.7‰ respectively. The potential of the method was demonstrated by measuring the $\delta^{18}O$‰ value of beet and cane sucrose and vanillin.

Farquhar *et al.* achieved similar results to Koziet with a lower temperature elemental analyser reactor (1080ºC) containing nickelised carbon as a catalyst.[38]

This configuration permitted the use of water standards as calibrants, however, the system suffered from memory effects between samples differing by more than 20‰. The internal precision of the method was typically 0.2‰.

The use of a specially constructed high temperature elemental analyser coupled to a CF-IRMS was reported by Kornexl *et al.*[39] This system achieved quantitative conversion of water, carbonate, ammonium sulfate, barium sulfate potassium nitrate, potassium hydrogen phosphate, silver phosphate, lithium carbonate and organic samples to the measuring gas carbon monoxide at 1400ºC over a small quantity of nickelised carbon. The excellent accuracy of the system was demonstrated by analysing a wide range of IAEA reference materials and the internal precision was usually better than 0.5‰. Furthermore the system could be used to accurately determine the δ^{15}N‰ and δ^{18}O‰ value of inorganic nitrogenous compounds in the same sample analysis (e.g., nitrates).

One of the potential drawbacks of the above systems is the use of carbon monoxide as a measuring gas. CO is toxic and thus continuous-flow systems that employ a CO reference gas injection for internal calibration require specially ventilated interfaces. Loader and Buhay elegantly overcame this difficulty by utilising the first reactor in an elemental analyser to pyrolyse cellulose and silver nitrate samples over glassy carbon at 1040ºC.[40] A second reactor containing nickel powder facilitated a non-contributive partial catalytic oxidation of CO to CO_2 at a temperature of between 550 and 600ºC. The internal precision was better than 0.2‰ and showed excellent agreement with off-line methodology for both cellulose and silver nitrate samples.

7.3 Current applications: adulteration of fruit juice, honey and wine

Photosynthetic CO_2 assimilation via the C_3, C_4 and CAM pathways is of primary importance in the use of carbon stable isotope ratio analysis in food authenticity control. The detection of commercial C_4 cane and corn derived sugar syrups in C_3 agricultural products (e.g., fruit juice, honey, maple syrup) are thus facilitated by characteristic differences in δ^{13}C‰ values. The technique is necessarily comparative, as it must take into account the natural variation of δ^{13}C‰ values in authentic products due to environmental factors, such as water availability and light intensity. Nitrogen isotope ratios have had limited application in authenticity studies. However, δ^{15}N‰ values provide an insight into the use of synthetic fertilisers. Consequently, nitrate-assimilating vegetables that have been exposed to artificial fertilisers may possess low δ^{15}N‰ values relative to untreated or 'organic' vegetables. In addition, ^{15}N signatures of *agricultural practices* may provide supplementary information in geographical origin assignment and diet reconstruction. In a similar way, assimilatory sulfate reduction does not produce significant fractionation, such that organic sulfur is clearly related to its source. Therefore, the soil or sulphate fertiliser from which it is derived can provide useful geographical origin

information (e.g., dairy and meat products with protected designation of origin).

Both ^{18}O and ^{2}H are valuable isotopic probes for both geographical and botanical origin discrimination. Fractionation associated with the global hydrological cycle, local climatic conditions, evaporation, condensation, transpiration and metabolism ultimately give rise to useful characteristic isotopic signatures for empirical origin determinations. In particular the $\delta^{2}H‰$ value of the non-exchangeable hydrogen atoms in carbohydrate are used to detect the addition of C_3 beet sugar syrups to other C_3 products, such as fruit juice. This section looks directly at how these principles have been applied in practice historically to the authentication of specific food commodities.

Measurements of the natural variation in stable isotope abundance were first applied to food quality control by Bricout and Merlivat in 1971.[41] They determined the deuterium content of orange juice water to distinguish between fresh and ground water reconstituted concentrate. Two of the most comprehensive reviews of the early research into the application of SIRA to food authenticity are those of Krueger and Reesman[42] and Winkler.[12] This section also details the chronological development of stable isotope methods for specific bio-elements, applied to a selection of premium commodities.

7.3.1 Apple juice

The sugars fructose, glucose and sucrose account for over 95% of the soluble solids in apple and the following ranges have been determined for each sugar: Fructose, 5.7 to 7.5%w/w; glucose, 2.2 to 3.1%w/w and sucrose 1.1 to 3.2%w/w.[43] These natural compositional ranges render conventional analyses, (e.g., HPLC) of little use as an indicator of adulteration of apple juice with moderate amounts of high fructose corn syrup (HFCS) – a cheap commercial sugar syrup containing about 50% glucose and 40% fructose. Since apple trees, like most commercial crops, utilise the C_3 pathway to fix CO_2 and the corn plant adopts the C_4 pathway, the possibility of using stable carbon isotope ratio analysis (SCIRA) in the detection of added HFCS was thus exploited.

White and Doner established that the mean $\delta^{13}C‰$ of HFCS was $-9.7‰$ with a standard deviation (sd) equal to 0.14‰.[44] Subsequently Doner *et al.* investigated whether the $\delta^{13}C‰$ values of authentic apple juice were sufficiently homogeneous that SCIRA could be used to detect adulteration with HFCS.[45] They observed that the mean $\delta^{13}C‰$ value of juices from different varieties of apple and cultivated in different geographical locations was $-25.4‰$ (sd, 1.2‰). No significant correlation either between the variety of apple, or geographical origin, and ^{13}C content was established. It was concluded that SCIRA could be used in principle to detect adulteration with HFCS since the two products had been shown to have measurably different and reasonably consistent $\delta^{13}C‰$ values. Doner and Phillips then went on to demonstrate the linear relationship between $\delta^{13}C‰$ and apple juice content in controlled mixtures of apple juice and HFCS.[99] On the basis of previously established mean $\delta^{13}C‰$ values of apple juice and HFCS above, test

samples with δ^{13}C‰ values less negative than −20.2‰ could be classified as adulterated with a high degree of certainty.

Lee and Wrolstad investigated whether determining the δ^{13}C‰ value of apple juice components could increase the sensitivity with which adulteration could be detected.[46] They found that the mean δ^{13}C‰ value of whole apple juice was −24.2‰ (sd, 0.6‰) and that apples from Argentina, Mexico and New Zealand did not differ significantly from those grown in the USA. The δ^{13}C‰ value of the fruit pulp was found to be the same as those for the corresponding whole juice. Sugars had slightly more positive δ^{13}C‰ values (mean, −23.3‰; sd, 1.0‰) than the whole juice. Whereas the non-volatile acids and phenolic fraction had more negative mean values of −27.0 and −28.8‰ respectively, reflecting their relative positions in the plants metabolic chain. The ratios of $\delta^{13}C_{ACIDS}$:δ^{13}C‰$_{JUICE}$ and δ^{13}C‰$_{PHENOLICS}$:δ^{13}C‰$_{JUICE}$ demonstrated that this was consistent for all samples investigated.

The use of a chemical compound, other than that suspected of adulteration, as an *internal isotopic reference (IIR)* point has important implications in food authentication. The majority of authentication techniques require a database of authentic isotopic values to which test samples can be compared. Compiling such a database is time consuming and expensive. Furthermore, if the isotopic parameters being investigated exhibit significant seasonal variations, as is the case with hydrogen and oxygen, the database must necessarily be updated annually. The careful choice of a suitable IIR that is highly correlated with the product of interest is extremely valuable and obviates the need for comparison with an authentic database. Subverting this kind of technique is also more difficult.

Jamin *et al.* exploited this concept in order to improve the detection of corn and cane syrup addition in apple juice.[47] The major non-volatile acid in apple juice (l-malic) was defined as a suitable internal standard for the determination of the ^{13}C content. Pure malic acid was isolated by preparative reversed-phase liquid chromatography (LC). A correlation between the carbon isotope ratios of sugars and malic acid was observed, and cut-off points for the difference between the δ^{13}C‰ of the apple juice and malic acid were defined. The method was applied to apple juices from different origins and years of production. They demonstrated that the detection limit, of cane or corn derived syrups, could be as low as 5%, whereas the conventional method of determining the δ^{13}C‰ of the whole juice was typically 10% or greater.

7.3.2 Honey

Honey is a relatively expensive commodity. Its dry weight consists mainly of the carbohydrates glucose and fructose in approximately equal amounts. It is therefore susceptible to extension with cheaper sources of sugar, in particular HFCS, which can be purchased at a fraction of the cost and closely resembles the carbohydrate composition of honey. Since the nectar collected by bees to make honey is mostly derived from C_3 flora, the possibility of detecting HFCS derived from the C_4 corn plant has been extensively investigated.

White and Doner measured the $\delta^{13}C‰$ values of US honeys and found that the mean value was −25.4‰ (sd, 0.98‰).[48] They also measured a large number of HFCS samples that had a mean value of −9.7‰ (sd, 0.14‰). Furthermore, they established the linear relationship between $\delta^{13}C‰$ values and the HFCS content of mixtures of corn syrup and honey. However, they observed that the wide range of $^{13}C/^{12}C$ ratios of authentic honey resulted in a considerable degree of uncertainty within which no positive judgement could be made about purity without other chemical tests. White suggested that samples of honey having a $\delta^{13}C‰$ value between −23.4‰ and −21.5‰ could not be classified as adulterated without a positive thin layer chromatography (TLC) test.[49] However, the time-consuming test required subjective assessment and did not detect the more highly purified HFCSs with low oligosaccharide content.

Burroughs and Otlet measured the $\delta^{13}C‰$ values of authentic UK honeys to establish their relationship with US honeys.[50] The average $\delta^{13}C‰$ value of UK honeys was found to be −25.5‰ (sd, 0.82), that is, very similar to that of US honeys. No significant correlation was observed, either between location and $\delta^{13}C‰$ or between the plant species, from which the nectar was derived, and $\delta^{13}C‰$ value. It was suggested that adulteration with HFCS could be confirmed if the $\delta^{13}C‰$ value of a test honey was more positive than −23‰, although this could correspond to the addition of 15% HFCS to a honey with a naturally depleted ^{13}C content. It was also recognised by the author that the adulteration of honey with a hydrolysed beet sugar would go undetected, since this carbohydrate was synthesised via the Calvin cycle.

To improve the sensitivity with which an adulterated honey could be detected by SCIRA, White and Winters developed an internal standardisation technique based on the determination of the $\delta^{13}C‰$ value of the pollen, or protein fraction, in the honey.[51] The mean difference between the whole honey and protein fraction was 0.13‰ (sd, 0.22‰). White and Winters proposed that a difference of 1.0 (equivalent to approximately 7% cane or corn syrup addition) should be adopted as the limit for deciding that a test sample was adulterated.

Roßmann *et al.* confirmed the correlation between the $\delta^{13}C‰$ honey protein and the $\delta^{13}C‰$ of the whole honey sample.[52] They observed that a mean difference of +0.1‰ (range +1.1‰ to −0.1‰) with more negative differences indicating the addition of C_4 plant sugars. They also noted that the addition of C_3 plant sugars could not be proven by this method. However; they noted that for certain types of honey, authenticity may be confirmed by stable hydrogen isotope ratio analysis. Furthermore, they suggested the possibility of measuring other isotopic abundances in honey such as ^{18}O and ^{15}N to obtain a more secure classification of authenticity.

7.3.3 Orange juice

Beet medium invert syrup (BMIS) is commercially produced from the partial hydrolysis of beet sucrose and contains sucrose, glucose and fructose in the ratio 2:1:1, the same as that of natural orange juice. The extension of orange juice

with BMIS is therefore difficult to detect using classical techniques. Furthermore, SCIRA is ineffectual in its detection because the beet and orange plant share the same C_3 photosynthetic pathway. However, Doner *et al.* found that the mean δ^2H‰ value of carbon bound hydrogen in sucrose isolated from beet sugars was −143‰ (sd, 19‰) whereas the mean δ^2H‰ value of orange juice sucrose was −27‰ (sd, 9.0‰).[53] Since sucrose from beet and orange juice had exhibited markedly different δ^2H‰ values, it was concluded that SHIRA would identify orange juice adulterated with beet sugar. Doner developed a discriminatory formula describing a 99.99% confidence ellipse about authentic orange juice sucrose δ^2H‰ and δ^{18}O‰ values. Bricout and Koziet conducted similar experiments and observed that the δ^2H‰ value of the total sugar nitrate-esters of authentic orange juices were −22.1 (sd, 10‰).[54] Commercial beet sugars were found to have δ^2H in the range −109 to −163‰. In addition they analysed a number of commercial cane and corn sugars which possessed δ^2H‰ values, much closer to orange juice sugars, of −31 to −63‰. It was concluded that this latter observation was a result of the similar tropical climates in which oranges and cane sugar were cultivated.

Roßmann *et al.* extended the use of nitrate-esters of sugars to examine the ^{2}H/^{1}H ratio of the carbon-bound hydrogen atoms in a range of fruit juices.[55] The mean δ^2H‰ values of the citrus juices examined (lemon, grapefruit and orange) ranged between −30.5 and −55.0‰. Whereas the apple juices possessed a mean δ^2H‰ value of −69.0‰, the commercial sugar syrups derived from potato, beet and corn had mean δ^2H‰ values of −141.0‰, −118.4‰ and 43.0‰ respectively. This research demonstrated that the addition of beet syrups to fruit juices could be readily detected using SHIRA. However, detection in crops, such as apples, cultivated in temperate climates was more difficult due to the smaller difference in the δ^2H‰ values. The isolation of the sucrose by preparative HPLC and conversion to the corresponding octa-nitrate ester prior to combustion and reduction meant that this technique was cumbersome and time consuming. Furthermore, the sugar nitro-ester derivatives were unstable and so the procedure was not widely adopted as a routine screening method.

7.3.4 Wine

The adulteration of wine by the addition of inexpensive commercial sugars must be accompanied by the addition of l-malic acid, to maintain the sugar acid ratio, and dilution with water in order to maintain the appropriate soluble solids concentration. Since the water used to dilute the wine is likely to be ground water, this results in a decrease in the overall ^{18}O/^{16}O ratio of the water present in the wine. As a result the determination of the δ^{18}O‰ value of wine water is a routine test in wine control laboratories and is conducted as one of the European Wine Databank procedures.[56]

Dunbar *et al.* conducted a preliminary study on factors that affected the ^{18}O enrichment in wine water.[57] Several observations were made which proved beneficial in interpretation of δ^{18}O‰ values with respect to enforcement

exercises. Especially environmental effects prior to or during harvesting which may have been used to 'explain' uncharacteristically low δ^{18}O‰ values. The main findings were that

- the δ^{18}O‰ value of the juice from the grapes does not change significantly during the course of 24 hours;
- the δ^{18}O‰ value of the juice from the grapes does not change significantly as the grapes ripen and up to 14 days before harvest there is no detectable change;
- rain will affect the grape juice δ^{18}O‰ values only when the fruit is ripe. Uptake of water in ripe grapes has a diluting effect, lowering the δ^{18}O‰ value of the juice;
- the δ^{18}O‰ value of several grape juice concentrates prepared by rotary evaporation in the laboratory were found to be very similar to those of control wines;
- as harvested grapes lose water by evaporation through the skin, the remaining water in the juice becomes more enriched in ^{18}O;
- fermenting grape juice (must) into wine does not appreciably alter the δ^{18}O‰ of the water.

Holbach *et al.* reported a range of δ^{18}O‰ values for ground water from 15 locations around the world. Whilst these data varied from −5.21 (Corfu) to −17.4‰ (Argentina) the majority of countries had ground water δ^{18}O values between −5.5 and −9‰.[58] In the case of wine, evaporation by vine leaves and grapes arising from their transpiration is a fractionating step which enriches the ^{18}O isotope in plant water. However, the extent of this fractionation depends on such factors as relative humidity and temperature. Although year to year climatic changes will also affect the ^{18}O content of water within a grape, the grape must from any geographical location will normally show an elevated ^{18}O concentration when compared to the local ground water. Furthermore this effect is maintained during the fermentation process. Therefore, if mains or ground water are added to a grape must or wine the δ^{18}O‰ value of the adulterated product will be more negative than that of an authentic product.

7.4 New applications: determining the geographical origin of foods

Consumers are increasingly interested in the provenance of the foods they consume. The reasons for this vary from (a) patriotism; (b) specific culinary or organoleptic qualities associated with regional products; (c) decreased confidence in the quality and safety of products produced outside their local region, country or the EU or (d) concern about animal welfare and 'environmentally friendly' production methods more often adopted by smaller regional producers. Recent food scares such as BSE, Foot and Mouth and the malpractices of some international food producers have increased public

awareness regarding the validity of claims about the origin of their food. As an example, the UK Food Standards Agency has consulted the public on a number of key issues relating to food labelling. Their findings have clearly demonstrated that 'country of origin labelling' is 'high on the consumers' list of demands for change'.[59] An increasing number of research papers have been published detailing the use of natural stable isotope abundance as a geographic probe to determine the provenance of food and these investigations are mainly based around the systematic variations of stable hydrogen and oxygen isotope ratios in combination with elemental concentraions, heavy isotope variations (e.g., strontium-87 [60, 61]) and other chemical geographic indicators.

Oxygen-18 (^{18}O) and deuterium (^{2}H) are especially interesting for geographical identification of a range of different food products. Hydrogen has two stable isotopes with atomic weights 1.0079 (^{1}H) and 2.01 (^{2}H). The heavier isotope, deuterium, has a relatively low natural abundance of 150 ppm, when compared to the heavy isotopomers of other bio-elements. The relatively large difference in the mass of the isotopes of hydrogen gives rise to markedly different physical properties including rates of reaction. This means that biological processes, involving enzyme catalysed reaction and physical processes, such as evaporation, results in a larger range of variation in the natural abundance of deuterium. Oxygen is composed of three stable isotopes, ^{16}O (99.8%), ^{17}O (0.04%) and ^{18}O (0.2%). For the purposes of IRMS studies it is the abundance of the heaviest isotope (^{18}O) that is routinely determined. The relatively smaller difference in the mass of the oxygen isotopes means that the effects of fractionating processes mentioned above do not bring about such a pronounced variation in ^{18}O natural abundance.

The principal source of all organic hydrogen and oxygen is the hydrosphere. This water has passed through the meteorological cycle of evaporation, cloud condensation and precipitation, ultimately making up ground water and exhibiting a consistent geographical isotope variation. Decreasing temperatures cause a progressive heavy-isotope depletion of the precipitation when the water vapour from oceans in equatorial regions moves to higher latitudes and altitudes. Evaporation of water from the oceans is a fractionating process that decreases the concentration of the heavy isotopomers of water ($^{1}H^{2}H^{16}O$, $^{1}H^{1}H^{18}O$) in the clouds compared to the sea. As the clouds move inland and gain altitude further evaporation, condensation and precipitation events occur decreasing the concentration of deuterium and oxygen-18. Consequently, the ground water reflects this isotopic gradient from the coast to inland areas.

For land plants, a further pre-assimilation affects the isotopic composition of the water substrate. The hydrogen and oxygen present in plant material originates from the water taken up by the roots. The water is transported through the plants xylem system. The isotopic composition of the xylem water is the same as that of water taken in by the roots, and the water is taken into the leaves without a change in isotopic composition. Evapotranspiration of water through the leaf stomata enriches the remaining water in the heavier isotopomers. Therefore, it is expected that growing regions with relatively low humidity,

where the rate of evaporation from the leaf is higher, result in plant materials with relatively enriched δ^2H‰ and δ^{18}O values. Although oxygen from carbon dioxide is initially fixed into carbohydrate during photosynthesis, this oxygen exchanges completely with leaf water prior to assimilation and therefore reflects the isotopic composition of the leaf water.

Another very important factor which affects the extent of enrichment for plants growing in similar temperate climates is the surface area of leaves. This affects the rate of evapotranspiration such that the botanical origin of agricultural products can be differentiated from plants grown in similar geographical locations. The differences that exist in the hydrogen isotope composition of plant material have previously been exploited in IRMS studies to determine the botanical origin of sugars as mentioned above.

There has been an increased awareness by consumers across Europe regarding the origin of the vegetable oils they buy. There are many branded goods declaring provincial high-quality characteristics and especially for virgin olive oil. In order to safeguard these products European Protected Denomination of Origin (PDO) legislation has been introduced as a guarantee of quality (Council Regulation (EEC) No 2081/92). However, a wide number of chemical and sensory parameters, such as fatty acids, triglycerides, unsuponifiable matter, iodine value and so on, have been combined with multivariate statistics, but they do not provide a secure classification of geographical origin to verify PDO labelling.[62] This is where geographical classifications based on the stable isotope ratios of oxygen and hydrogen, when combined with other multi-element data, offer the best analytical stratergy for accurately verifying the geographical origin of cultivation.

Initial research into the use of ^{18}O-pyrolysis continuous-flow IRMS, to obtain information about the geographical origin of olive oil samples, has been carried out by Angerosa *et al* .[63] They determined the δ^{13}C‰ and δ^{18}O‰ values of whole olive oil, sterols and aliphatic alcohol fractions from fruits of Olea europaea L. produced in Greece, Italy, Morocco, Spain, Tunisia, and Turkey. The results permitted provincial classification of the oils. However, there was some misclassification observed for oil samples coming from neighbouring countries with similar climates. There is growing evidence that if these provisional assessments of the geographical origin of olive oil are supplemented with *heavy* isotope ratios (e.g., ^{87}Sr/^{86}Sr) and multi-element data, gathered by inductively coupled plasma-mass spectrometry, that a secure geographical classification of this product may be achieved. Thus ensuring that the consumer is not defrauded, and that the honest trader is not disadvantaged by having their PDO oils misrepresented by inferior products.

A further issue of concern regarding geographical origin is subsidy fraud. Regional origin assignment of highly valuable milk products such as butter, is of considerable importance for legal, fiscal and trade controls within the European Union (EU) It is also of value for ensuring fair competition and as a means of protecting consumers against fraud due to mislabelling. Rossmann *et al.* conducted stable isotope ratio determinations of the light elements (C, N, O, S; bioelements) and the heavy element, strontium as a means of classifying the

origin of butter and milk.[64] They discussed these elements in natural cycles, their variations due to climate and geology, the abiotic and biological fractionation of isotopes and the reasons for regional differences in multi-element stable isotope ratios of butter. They reported data from several European countries and from outside the EU. The results indicate that stable isotope ratios and subsequent discriminant analysis based on data for samples of certified origin can enable the reliable detection of the regional provenance of butter. Furthermore, this methodology has recently proved sufficiently robust to be admissible in criminal prosecutions in the German court of law.

Preliminary results on the analysis of Pecorino Sardo cheese by Manca *et al.* have demonstrated the usefulness of stable isotope analysis in the absence of ^{18}O and ^{2}H data.[65] The $\delta^{13}C‰$ and $\delta^{15}N‰$ of casein and a number of free amino acid ratios (His/Pro, Ile/Pro, Met/Pro, and Thr/Pro) determined by high performance liquid chromatography in samples of ewes' milk cheese from Sardinia, Sicily, and Apulia were reported as parameters independent of maturation time. Multivariate statistical analysis performed by applying both unsupervised (principal component analysis and cluster analysis) and supervised linear discriminant analysis (LDA) demonstrated reliable discrimination for the Pecorino Sardo cheese according to geographical production area. Certain variables were reported as having greater discriminatory power than others, in particular the variables Ile/Pro, Thr/Pro, $\delta^{13}C‰$ and $\delta^{15}N‰$ which resulted in 100% discrimination and classification of the samples by LDA.

Kelly *et al.* have reported the use of Isotope Ratio Mass Spectrometry and Inductively Coupled Plasma Mass Spectrometry to determine the origin of rice samples cultivated in the USA, Europe and Basmati regions in India and Pakistan.[66] Nine key variables (carbon-13, oxygen-18, boron, holmium, gadolinium, magnesium, rubidium, selenium and tungsten) were identified by canonical discriminant analysis as providing the 100% discrimination between rice samples from these regions. High levels of boron (> 2500 ppb) were associated with rice samples from America and notably high levels of holmium were found in rice samples from the state of Arkansas. European rice samples generally contained relatively high levels of magnesium and Indian/Pakistani samples were characterised by relatively low ^{18}O abundance characteristic of the high altitude at which these latter rice crops were cultivated.

The authenticity and geographical origin of wines produced in Slovenia were investigated by Ogrinc *et al.* using a combination of IRMS and SNIF-NMR methods.[67] Grapes and wines produced in the three different wine-growing regions of Slovenia in 1996, 1997, and 1998 were analysed. The stable isotope data were evaluated using principal component analysis (PCA) and linear discriminant analysis (LDA). Discrimination between coastal and continental regions was achieved with the deuterium/hydrogen isotopic ratio of the methylene site in the ethanol molecule (D/H)(II), which relates mainly to the fermentation water from the grapes and delta C-13 values. Furthermore, inclusion of $\delta^{18}O‰$ in the principal component analysis and linear discriminat Analysis resolved the two continental regions Drava and Sava.

7.5 Future trends: position-specific isotope analysis (PSIA)

The measurement of *site-specific* or *position-specific* isotope ratios has long been the domain of quantitative nuclear magnetic resonance (NMR) spectroscopy.[68, 69] Whilst this approach is acknowledged as supplying valuable information on intra-molecular isotopic distributions and a practical tool for authenticity studies, its limitations may be recognised in terms of measurement acquisition times and the quantities of analyte required. However, it is possible to conduct position-specific isotope analysis (PSIA) by IRMS. This approach has been used previously in food authentication[70, 71] but has not gained wide acceptance because the technique requires time-consuming off-line chemical cleavage and recovery of molecular fragments without introducing isotopic fractionation. Nevertheless, IRMS does offer advantages in terms of measurement precision, the small quantities of sample required and the speed of instrumental analysis. Consequently, it has been used to provide valuable information on the isotopic distribution of ^{13}C within glycerol that can be used for plant metabolism studies, botanical source determinations and the detection of fraud.

Weber *et al.*[8] reported that the average $\delta^{13}C‰$ value of glycerol from a botanical origin was 4 to 5‰ more negative than that of carbohydrates from the same source. This depletion was related exclusively to a large depletion at the C_1 position of the glycerol molecule. This PSIA was performed after degrading the glycerol with periodic acid. The stoichiometry of the reaction is such that one mole of periodic acid is required to cleave each pair of adjacent hydroxyl groups on the glycerol molecule. As a result, one mole of glycerol yields two moles of formaldehyde and one mole of formic acid.[72] The formaldehyde retains the carbon isotopic signature of the C_1 and C_3 atoms of the parent glycerol, whereas the formic acid retains the C_2 information. Weber *et al.* determined the $\delta^{13}C‰$ value of the volatile products directly by GC-Combustion-IRMS.

In addition, Weber *et al.* demonstrated that the depletion of glycerol was a compensation for small ^{13}C enrichments in the corresponding positions of hexoses formed during the metabolic fluxes of dihydroxyacetone phosphate. The numerical difference between the average $\delta^{13}C‰$ and the C_1 position of glycerol was reliable indicator of plant, animal and synthetic origin. More recently, Zhang *et al.*[73] conducted an investigation into the equivalence of ^{13}C SNIF-NMR and PSIA-IRMS measurements of glycerol derived from plant and animal sources. They compared direct measurement of ^{13}C abundance of $C_{1,3}$ and C_2 of glycerol by NMR (using a complex least squares curve-fitting algorithm) with position-specific isotope ratios measured by IRMS (after periodate degradation). The repeatability and reproducibility of both analytical techniques were estimated. A good agreement was found between the two isotopic data sets that confirmed the dependability of the two analytical approaches.

As mentioned above, off-line preparation of degradation products for PSIA by IRMS has hindered the use of the technique for investigating intra-molecular isotopic distributions. Another confounding factor may be the availability of a

relatively straightforward synthetic transformation that yields the position specificity. However, as with the development of on-line gas preparation devices for IRMS in the early 1980s, there has been an analagous development in the late 1990s of on-line pyrolytic techniques to provide molecular fragments for position specific analysis by IRMS. Two pyrolysis techniques were investigated by Dennis *et al.*,[74] for providing position-specific isotopic information for vanillin. A Curie Point pyrolyser was used to fragment vanillin samples of known carbon isotopic composition. Hydroxy benzaldehyde, phenol and catechol were produced and identified by GC-MS. Their carbon isotopic ratios were measured by GC-C-IRMS. The extreme conditions required to produce this fragmentation and the presence of the cobalt Curie Point wire led to isotopic scrambling such that, under the conditions employed, the fragmentation products did not retain isotopic information which could be used for authentication purposes.

A second pyrolytic technique generating carbon monoxide under continuous flow conditions for isotope ratio mass spectrometry (Py-CF-IRMS) was also used to study vanillin samples. This system generated carbon monoxide (CO) from samples in a modified elemental analyser. The $^{m}/_{z}$ 29 : $^{m}/_{z}$ 28 ratio of CO provided an average δ^{13}C‰ from the three specific carbon atoms bonded to oxygen at the methoxy, carbonyl and hydroxyl moieties, in the parent vanillin molecule. Comparison of carbon isotope data measured using this procedure agreed well with the known values of standard vanillin materials. In addition the The $^{m}/_{z}$ 30 : $^{m}/_{z}$ 28 ratio of CO provided an average ^{18}O abundance from the oxygen isotopes present in the vanillin. The oxygen isotopes provided useful information on the source discrimination of vanillin samples from fossil fuel and plant sources. Py-CF-IRMS therefore provides a rapid and convenient technique for obtaining PSIA δ^{13}C‰ data for carbon directly attached to oxygen in organic molecules.

The feasibility of a generally applicable technique for on-line automated preparation of molecular fragments for PSIA by IRMS, was first reported by Thomas Brenna's group at Cornell University.[75] Free radical fragmentation of methyl palmitate (Me 16:0) was achieved by pyrolysis in an open tube furnace maintained at 550ºC after separation from the hexane solvent in one gas chromatograph. They observed two series of peaks corresponding to an olefinic and methyl ester fragment breaking from each end of the parent-molecule. These were analysed by GC-C-IRMS after cryofocusing and separation in a second gas chromatograph. The repeatability of δ^{13}C‰ analysis of the fragments was reported as less than 0.4‰. In addition, Brenna and co-workers conducted a series of Isotopic labelling experiments to demonstrate the absence of rearrangement during activation and fragmentation. These data clearly demonstrated the feasibility of automated δ^{13}C‰ PSIA for molecules contained in complex mixtures.

In a subsequent publication Brenna *et al.* explored the possibility of combining PSIA with a novel derivative of palmitic acid to establish intramolecular isotope variations. They reported the reduction of methyl

palmitate to 1-hexadecanol with lithium aluminium hydride. This facilitated high-quality chromatographic separation, without adding exogenous carbon in the form of methyl derivatives.[76] This is particularly useful in GC-C-IRMS as carbon added from derivatising groups cannot be differentiated from carbon in the parent compound, by chromatography-based CSIA or PSIA. After the initial GC-pyrolysis step, 1-hexadecanol fragmented into a series of monounsaturated alcohols and alpha-olefins analogous to the fragmentation observed for methyl palmitate, in the same system, above. In addition a pyrolytic dehydration product, 1-hexadecene was formed. The repeatability of the δ^{13}C‰ GC-C-IRMS analysis was again less than 0.4‰. Furthermore, their investigations showed no evidence of isotopic scrambling in the molecular fragments of 1-hexadecanol.

The most recent publication by Brenna's team further increased the scope of PSIA to toluene and n-alkanes (C_5 to C_{10}).[77] Toluene was found to reproducibly fragment to benzene and methane and the n-alkanes yielded methane and α-olefins. Furthermore, the precision of the analysis was improved such that pyrolysis product isotope ratios were determined with a precision of < 0.2 and were not significantly correlated with pyrolysis temperature. Their data also demonstrated that PSIA of hydrocarbons were indicative of the source of the material.

7.6 Conclusion

Despite the proliferation of regulations regarding the quality and labelling of food, cases of economic fraud have been widespread throughout the last three decades. Ultimately, the driving force behind this situation has been the economics of food production. Isotope ratio mass spectrometry has developed into a powerful analytical tool for the food authentication scientist over the same period. It can supply unequivocal evidence of food adulteration that cannot be obtained using other analytical techniques. Consequently it has contributed to the detection of millions of pounds worth of food fraud in Europe and the United States. The underlying sensitivity of the technique means that it is not only difficult to subvert, but often uneconomic to do so if the adulteration requires the use of isotopically enriched ingredients. Its limitations are derived from the natural variation in isotopic composition of premium products themselves. However, authentication strategies involving the use of multi-isotopic parameters (^{2}H, ^{13}C, ^{15}N and ^{18}O) facilitated by increasingly rapid measurement procedures, means that authentic variation is more readily constrained.

The most powerful methods are those that combine as many of the isotopic signatures as possible, into a multi-dimensional authenticity matrix that is difficult to subvert. This is particularly the case for some of the future challenges facing analytical chemists who wish to verify the geographical origin of food. In this scenario a deeper understanding is required of the way in which our food is affected by meteorological and geological factors during cultivation. From an instrumental perspective automated systems based on multidimensional gas

chromatography offer great potential for accurately and reliably determining the intramolecular distribution of isotopes in specific compounds present in complex mixtures. This is currently not offered by any other technique or system in an automated on-line configuration. Furthermore, on-line low-temperature pyrolysis coupled with high-precision IRMS may provide detailed isotope information and thus a greater understanding of biosynthetic processes and is therefore likely to become a powerful weapon in the fight against economic food fraud.

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8

Spectrophotometric techniques

M. Meurens, Université Catholique de Louvain, Belgium

8.1 Introduction

The origin and history characteristics are important food quality criteria. A continuing demand exists for rapid, inexpensive and effective techniques for measurement of these criteria of food quality. Twenty years ago, spectrophotometric techniques were considered to be promising tools for rapid sample screening. However, the difficult chemical interpretation of spectra and the need for prior calibration, among other reasons, limited their application in industrial processes. The generation of high performing photonic instruments and the improvement in data processing by chemometric methods has led to a boom in these techniques in the last few years.

Spectrophotometric techniques cover the ultraviolet (UV), visible (VIS) and infra-red (IR) spectra. In contrast to UV and IR spectroscopy which are based on phenomena of light absorption, fluorescence and Raman spectroscopy are based on the phenomena of light emission. The principles, instruments and applications of these four spectrophotometric techniques in the authenticity and traceability control of food products, such as milk, meat, fish, cereals, fruits, honey, fats and oils, are described in this chapter in order to give a general overview of their potential in this field.

8.1.1 Principles of spectrophotometric techniques

Spectrophotometric techniques of analysis are based upon the production or interaction of electromagnetic radiations with matter. Emission techniques measure light radiations produced when the analyte is excited by thermal, electrical, or radiant energy. Absorption techniques are based upon the

attenuation of a beam of light radiations as a consequence of its interaction with and partial absorption by the analyte.

Absorption by polyatomic molecules, particularly in the condensed state, is a complex process because the number of possible energy states is large. Here, the total energy of a molecule is given by the equation: $E = E_{electronic} + E_{vibrational} + E_{rotational}$ where $E_{electronic}$ describes the energy associated with the various orbitals of the outer electrons of molecule, while $E_{vibrational}$ refers to energy of the molecule as a whole due to interatomic vibrations, and $E_{rotational}$ accounts for the energy associated with the rotation of the molecule around its centre of gravity. Ultraviolet (UV) and visible (VIS) spectroscopy concerns the molecular absorption of light increasing $E_{electronic}$. Infra-red (IR) spectroscopy concerns the molecular absorption of light increasing $E_{vibrational}$ and $E_{rotational}$.

Fluorescence refers to the light emitted by some electrons of the illuminated molecules during the period in which they are excited by photons. This emitted light is from the electrons excited in singlet states and returning to the ground state. Because some energy is lost in the brief period before emission can occur, the emitted fluorescence radiations are of longer wavelength than the absorbed radiations.

When a transparent and homogeneous medium is traversed by a beam of light, laterally diffused radiations may be observed. This phenomenon is termed scattering of light by the medium. When the scattered light has the same wavelength as the exciting light, it is referred to as Rayleigh scattering. In 1928 Raman discovered, in the scattered light, weak radiations of discrete frequencies differing from those of the Rayleigh scattering. This frequency difference is due to an addition or a subtraction of vibrational energy to the excitation photons. It results from inelastic shocks between these photons and the atoms. The Raman scattering is much weaker than the Rayleigh scattering and the fluorescence but become significant when high-intensity light sources such as laser are used.

8.2 Ultraviolet spectroscopy: detecting fruit and vegetable oil adulteration

The wavelength at which a molecule absorbs depends upon how tightly its various electrons are bound. Thus, the shared electrons in single bonds such as C-C or C-H are so firmly held that their excitations require energies corresponding to wavelengths in the vacuum ultraviolet region at wavelengths less than 180 nanometres (nm). This region is not readily accessible because components of the atmosphere also absorb; as a result, absorption by single bonds of these types has not been important for analytical purposes. The unshared electrons in sulphur, bromine and iodine are less strongly held than shared electrons of a saturated bond between carbon atoms. Organic molecules containing these elements frequently have absorption peaks in the UV region (180–350 nm) as a consequence.

Electrons of double and triple bonds in organic molecules are relatively easily excited by radiation; thus, species containing unsaturated bonds generally exhibit useful absorption peaks. Unsaturated organic functional groups that absorb in the ultraviolet and visible regions are termed chromophores. When two chromophores are conjugated, shifts in peak maxima to longer wavelengths usually occur. Finally, peaks in the ultraviolet region are ordinarily broad because of vibrational and rotational transitions occurring in the same time. UV spectra, which usually consist of broad absorption bands, have less value for identification but great utility for quantitative analysis.

8.2.1 UV spectrophotometer

The components of a spectrophotometer depend on the region of the light spectrum. Historically there are specific components, sources and detectors for each spectral region. This is especially true of the UV region in which transmission optics must be made of very pure silica, reflection optics must have a special coating, the source is generally a deuterium lamp and the detector must be UV-enhanced. In fact the cross-over from the UV to the VIS region occurs at about 350 nm, where the visible tungsten-halogen lamp stops emitting and the deuterium or xenon lamp is required. At very short wavelengths, ultra-pure silica is needed for adequate transmission.

Monochromators are devices that separate the polychromatic radiations coming from the light source into monochromatic radiations. In nearly all modern dispersive instruments, this step is achieved via diffraction gratings. A grating is a reflective surface that is ruled, or scratched, with parallel lines that are spaced by a distance about the same as the wavelength of light to be analysed. There are two different types of grating UV spectrophotometers; those with moving grating and mono-channel (phototube or photomultiplier) detector and those with stationary grating and multi-channel (diode array or charge transfer CCD) detector.

8.2.2 UV control of food

Normally for authenticity and traceability of food products, UV spectroscopy is not used alone but in combination with the HPLC technique. However, some publications on UV spectroscopy, as the sole analytical technique, can be found. For example, this spectrophotometric technique is used to detect adulterations of fruit products and vegetable oils.

Fruit products

UV-VIS absorption spectra of alcoholic solution of frozen orange concentrates and single strength orange juices can give qualitative detection and quantitative approximation of orange pulp wash in orange juice. With fluorescence spectra they have been adopted as official first measurements for detecting adulteration of Florida orange juice with pulp wash (Petrus and Attaway, 1985). Fruit juices

and wines contain polyphenols whose identity and concentration can be determined from the UV spectrum around 280 nm.

Vegetable oils

The authentication of virgin olive oil can be achieved by UV measurement at the wavelength 268 nm corresponding to the maximum absorption of conjugated trienes produced during refining. However, the storage of virgin olive oil leads to the formation of chemical compounds that absorb in the same spectral region. The measurement at 315 nm is another approach because there is an absorption peak characteristic of the conjugated tetraenes. About 5% adulteration of virgin olive oil by refined oils can be detected by absorption measurement at 315 nm (Kapoulas and Andrikopoulos,1987) or better by the ratio of the spectrum slope on the two sides of the absorption peak (Passalaglou-Emmanouilidou, 1990). The spectral region 310–320 nm is also used to detect marine oils in various vegetable oils such as linseed, palm kernel, cottonseed, olive, peanut, poppy seed, sesame, rapeseed, pumpkin and sunflower oils (Franzke, 1964).

8.3 Infra-red spectroscopy for food authentication

Vibrational absorption occurs in the infra-red region, where the light energy is insufficient for electronic transitions. The relative positions of atoms in a molecule are not fixed; instead, they fluctuate continuously as a consequence of a multitude of different types of vibrations. These vibrations are quantified in the sense that their frequencies can assume only certain values. A vibrational energy transition corresponding to an IR absorption requires that the radiation frequency exactly matches the vibration frequency of a bond. Thus, IR absorption typically consists of narrow peaks, each one of which corresponds to a vibrational frequency of a bond in the molecule. A requirement for IR absorption is that the vibrational motion about a bond must cause a change in dipole moment. Only then can the alternating field of the radiation interact with the bond and cause a change in amplitude of the vibration. Most organic and inorganic molecules contain bonds between atoms of differing charge density. Therefore, most molecules, both inorganic and organic, exhibit IR absorption peaks.

8.3.1 IR spectrophotometer

There are two main categories of instruments used in IR spectroscopy; dispersive and interferometric Fourier Transform (FT) spectrophotometers. In the dispersive instrument, only one spectral element is sampled by the detector at a time. In contrast, in the interferometric FT instrument, all the radiations arriving at the detector are examined simultaneously. The main class of commercial instruments are FT systems based on the principle of the Michelson interferometer. The original FTIR spectrometers, available in the 1960s, were produced for far infra-red measurements. However, in general, today it is

possible to extend the technology to include all measurements from the UV region, right through to the far infra-red. The infra-red light sources used are globar, Nernst glower, silicon carbide filaments, and mercury arc lamps.

As for the UV spectrophotometers, the greatest recent improvements in the IR spectrophotometers have been in the area of detectors They are transducers of radiation. They change radiation into electrical power which can be amplified by the accompanying electronics. The methods of transduction can be separated into two groups; thermal detectors and photon detectors. The responsive element of thermal detectors is sensitive to changes in temperature brought about by changes in incident radiation. The response element of a photon detector is sensitive to changes in the number or mobility of free charge-carriers, electrons and/or holes, that are brought about by changes in the number of incident IR photons. The different thermal detectors are the bolometric, pyroelectric, thermopneumatic and thermovoltaic detectors. As with the UV spectrophotometers, the photon detectors are photoconductive and photovoltaic.

The near IR spectra are constituted by first, second and third overtones located at multiple frequencies of the mid-IR fundamental bands. The combination bands of the near IR spectra correspond to frequency summations or differences of the same fundamental bands. These overtone and combination bands are at least one to two orders of magnitude weaker than the fundamental absorption bands. In consequence the sample thickness crossed by light is generally measured in micrometres, or tenths of millimetres in mid-IR, whereas millimetres and centimetres are appropriate in near-IR to measure comparable levels of light absorption. This simplifies sampling requirements for all condensed-phase materials, and in particular for powdered or granular solids. Also, common silica materials such as quartz and glass are transparent throughout the near IR (NIR), in contrast to the mid IR. This eliminates the necessity for hygroscopic or exotic materials, for sampling and instrument optics, that are commonplace in the mid IR region.

8.3.2 IR control of food

IR spectroscopy is nowadays the main spectrophotometric technique used in control of food authenticity and traceability. A number of publications describe the application of this technique to control the origin and history of different food products such as milk, meat, cereals, fruit products, vegetable oils, honey, and coffee.

Milk

Near and mid IR spectroscopy are referenced as utilisable spectrophotometric techniques to detect vegetable and animal fats in milk fat by Collomb and Spahni (1991). Giangiacomo *et al.* (1998) have shown interesting results for near and mid IR spectroscopy and chemometric data treatment by discrimination between naturally produced thickening agents and thickening additives in fermented milks.

Meat and fish

Discrimination between fresh and frozen beef by NIR reflectance spectroscopy was reported in 1997 by Thyholt and Isaksson and by Downey and Beauchêne (1997). Hildrum *et al.* (1994) correlated the sensory quality of beef by NIR spectroscopy. Similar studies have been conducted on turkey meat by Swatland and Barbut (1995). Rannou and Downey (1997) described the discrimination of raw pork, chicken and turkey meat by spectroscopy in the visible, near and mid-infra-red ranges.

Cereals

Details of the authentication of Basmati rice using NIR spectroscopy have been published by Osborne *et al.* (1993) and Krzanowski (1995).

Fruit products

FT spectroscopy operating in the mid infra-red distinguishes the fruit types in fruit purées (Kemsley *et al.*, 1996). It also allows detection of the variety, the ripeness and the freshness of fruits used for purée making (Defernez *et al.*, 1995). The FTIR reflectance spectra of jam solids washed on filter papers are characteristic fingerprints of fruits (Wilson *et al.*, 1993). A rapid screening of fruit juice authenticity by NIR spectroscopy of dry extract on fibreglass discs according to the DESIR method of Meurens (Thyholt and Isakson, 1997) has been proposed by Scotter and Legrand (1994). Interesting results of fruit juice screening with this method have been published by Twomey *et al.*, (1995) and Li *et al.*, (1996). Classification of red wines analysed by mid infra-red spectroscopy of dry extract according to their geographical origin is reported by Picque *et al.* (2001).

Vegetable oils

In the intervals 3,000–3,600 cm^{-1} and 700–1200 cm^{-1}, FTIR spectra show profile differences between oils of peanut, sesame, sunflower, first pressed olive oil and refined solvent extracted olive oil and synthetic oil. These differences are of value in detecting oil admixtures and adulterations (Bottini and Sapetti, 1958, Bartlett and Mahon, 1958). Metal derivatives, particularly lead salts of fatty acids have been suggested for detecting frauds such as adulteration of olive oil with groundnut oil (Gelli and Pallotta, 1959). The tendency of urea to form a cristalline complex with fatty acids on the basis of chain length and unsaturation has been used to detect adulteration of mustard oil with groundnut oil and linseed oil, and of coconut and sesame oils with groundnut oil (Mehta and Gokhale, 1965). Methodologies based on FTIR or NIR spectroscopy and chemometrics for authentication of olive oil have been published by Safar *et al.* (1994), Sato (1994), Bewig *et al.* (1994), Lai *et al.* (1995), Wesley *et al.* (1995, 1996), Marigheto *et al.* (1998) Hourant *et al.* (1999), and Tay *et al.* (2002).

Honey

Near and mid IR spectroscopy are described as advantageous techniques to determine the parameters of honey authenticity and traceability like moisture,

sugar profile (Robertson *et al.*, 1989; Piekut *et al.*, 2000; Irudayaraj and Sivakesava, 2001; Sivakesava and Irudayaraj, 2001a,b,c; Davies *et al.*, 2002), and specific markers (Dvash *et al.*, 2002).

Coffee

The main coffee authenticity issues concern the botanical and geographical origin of the green and roasted beans, the proportions of Arabica and Robusta in the blend, and the non-declaration of the presence of coffee substitutes. One potential approach to the problem of authenticity confirmation and adulteration detection involves the use of spectrophotometric methods such as near and mid infra-red techniques and several feasability studies have appeared (Cesar *et al.*, 1984; Kemsley *et al.*, 1995; Dupuy *et al.*, 1995; Suchanek *et al.*, 1996; Briandet *et al.*, 1996; Downey *et al.*, 1997).

8.4 Fluorescence spectroscopy for food authentication

As explained in the interaction of light with matter, fluorescence is an emission process in which atoms or molecules are excited by absorption of a beam of electromagnetic radiation; radiant emission then occurs as the excited electron species return to the ground state. The resulting emission spectrum serves as the basis for analysis. Molecular fluorescence spectroscopy is particularly useful for quantitative work because of its high sensitivity and good selectivity.

8.4.1 Spectrofluorimeter

Generally, fluorescence is observed at a 90-degree angle to the excitation beam. This must be monochromatic and produced either by xenon lamp and monochromator or by violet-blue light-emitting diode (LED) and laser source. Original spectra of fluorescence induced by argon laser at wavelength of 350 nm and measured with a stationary grating and a coupled charge device (CCD) detector are presented in Fig. 8.1.

8.4.2 Fluorimetric control of food

Fluorescence spectroscopy has been used for a long time in the control of some food products like milk, meat and fish, but until now, because of the high cost of spectrofluorimeters and the difficulty of interpreting fluorescence spectra, it remains a marginal technique of food quality control. However, recent results with laser induced fluorescence spectroscopy on vegetable fats lead us to believe that spectrofluorimetry could be an important technique for food authentication within a few years.

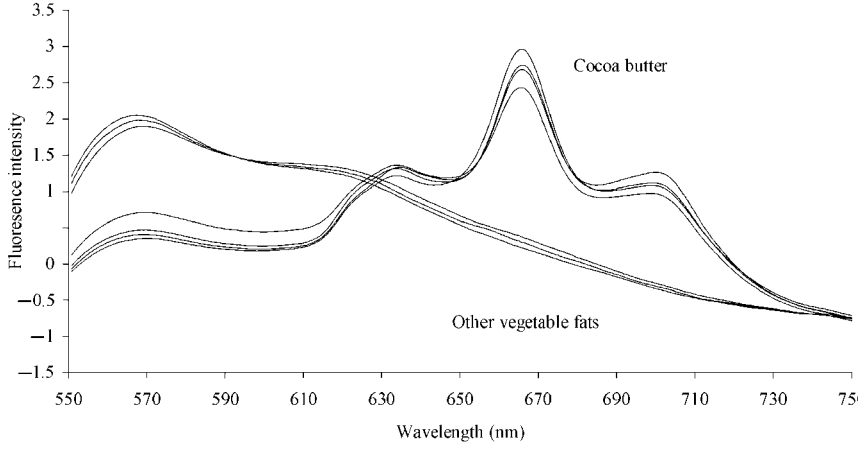

Fig. 8.1 Difference of profile between laser induced fluorescence spectra of cocoa butter and other vegetable fats used in chocolate making.

Milk

Examination of the milk fluorescence spectrum is a suitable preliminary test for mastitis udder disease (Schonberg, 1943).

Meat

Poultry skin used as a low cost filler in processed poultry products can be detected by measuring the fluorescence of collagen (Swatland and Barbut, 1991).

Fish

Studies on fresh minced meat of different fish species have shown that fish shelf life can be predicted from fluorescence spectra (Miyazawa *et al.*, 1991).

Vegetable oils and fats

Recently, Papadopoulos *et al.* (2002) have reported investigations of the adulteration of extra virgin olive oils with seed oils using their fluorescence spectra. Our experiments with laser induced fluorescence spectroscopy on different fats used in chocolate making and on lipidic extracts of green and roasted coffee beans has shown that this technique is able to distinguish the botanical origin of vegetable fats because of the differences in polyphenol, sterol and chlorophyll composition. Spectra of cocoa butter and other vegetable fats used as substitutes for cocoa butter in chocolate are presented as an illustration of this observation in Fig. 8.1.

8.5 Raman spectroscopy for food authentication

The Raman effect is observed as rays at both lower and higher frequencies than the excitation frequency. These rays, known as Stokes and anti-Stokes rays

respectively, are very weak. Their intensity is several orders of magnitude less than the intensity of the excitation ray. Similarly, the intensity of the anti-Stokes rays are weaker than the Stokes rays, in this case, by up to a factor of ten, dependent on the temperature of the sample.

The Raman effect is the result of the molecule undergoing vibrational transitions, usually from the ground state to the first vibrational energy level, giving rise to the Stokes rays, observed as spectral lines occurring at a lower frequency (longer wavelength) than the incident beam. Because the effect involves a vibrational transition, with a net gain in energy, it is comparable to the absorption of photon energy experienced in the generation of the infra-red vibrational spectrum. Therefore both techniques, infra-red and Raman spectroscopy, are derived from similar energy transitions, and the information content of the spectra will have some commonality. However, the spectra are not identical because not all the energy transitions are allowed by quantum theory. These are usually discussed in terms of infra-red active and Raman active vibrations, and are defined as follows: for a molecular vibration to be infra-red active, there must be a net change in dipole moment during the vibration; for a molecular vibration to be Raman active there must be a net change in the bond polarisability during the vibration.

The result of these different rules is that the two sets of spectral data are complementary. In general, molecules that have polar functional groups and low symmetry tend to exhibit strong infra-red spectra and weak Raman spectra, and molecules with polarisable functional groups with high symmetry provide strong Raman spectra. In practice, for most molecular compounds reality is somewhere between these two extremes. As a consequence, most compounds produce unique infra-red and Raman spectra that feature a mixture of strong, medium and weak spectral bands. Typically, a strong feature in one spectrum will either not show, or may be weaker in the other spectrum, and vice versa.

Like the infra-red spectrum, the Raman spectrum is also presented in wavenumbers, cm^{-1}, as a displacement from the main excitation, or Rayleigh line. In concept, the spectral range starts from zero, and extends to the full range of the fundamental molecular vibrations, out to a nominal $4000\,cm^{-1}$. In practice, the extreme intensity of the main excitation line limits how close one may get to zero wavenumbers, and dependent on the properties of the instrument optics used, this may be anywhere from $15\,cm^{-1}$ to $200\,cm^{-1}$. Likewise, the upper end of the spectrum may or may not extend out to $4000\,cm^{-1}$, and is often limited by detector response. In reality, there are no significant Raman-active vibrations above $3500\,cm^{-1}$, and so this is seldom an issue. Unlike the traditional infra-red measurement, the Raman effect is an emission phenomenon, and is not constrained by the laws of absorption. The intensity of a recorded spectral feature is a linear function of the contribution of the Raman scattering centre, and the intensity of the incident light source.

8.5.1 Raman spectrophotometer

Like the infra-red spectrophotometers the Raman spectrophotometers fall into two main categories: the dispersive ones and the interferometric FT ones. The light source in Raman spectroscopy is usually a NIR laser in order to minimise the fluorescence which masks the Raman spectrum. The Raman scattering is collected and focused into a wavelength sorting device, which can be a monochromator in a dispersive instrument or an interferometer in Fourier transform methods. The main characteristics of the Raman spectrophotometers are the powerful laser source of several hundred mW, the high resolution and sensitivity of the spectrophotometric detection system. The advent of very efficient laser line rejection or notch filters has helped to solve the major problem of interference between excitation and the Raman scattered lights.

8.5.2 Raman control of food

The application of Raman spectroscopy in authenticity control of food is relatively recent.

Milk

The first results suggesting the possibility of food authentication by Raman spectroscopy have been published on fermented milk (Meurens, 1995).

Cereals

Discrimination between glutinous and non-glutinous rice by Raman spectroscopy has been presented by Ootake *et al.* (1998).

Vegetable oils

Studies of the use of FT Raman spectroscopy for the authentication of extra virgin olive oil have been published by Baeten *et al.* (1996), Aparicio and Baeten (1998), Marigheto *et al.* (1998), and Davies *et al.* (2000). According to these results and the recent experiments in researching reliable methods to detect, for example, the adulteration of olive oil by hazelnut oil, fluorescence spectroscopy has to be applied on an unsaponifiable fraction rather than on a complete oil sample. Laser induced fluorescence spectra of an unsaponifiable fraction of olive oil and hazelnut oil are shown in Fig. 8.2 in order to show the significant differences of spectral profile between the unsaponifiable fractions of the two vegetable oils.

Honey

In 2002 De Oliveira *et al.*, Goodacre *et al.* and Paradkar and Irudayaraj presented experiments on honey authentication by FT Raman spectroscopy.

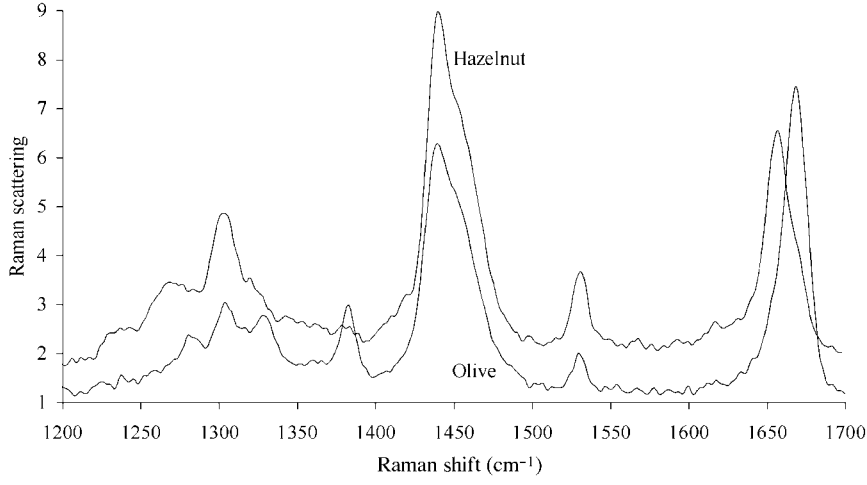

Fig. 8.2 Profile difference between Raman spectra of an unsaponifiable fraction of olive and hazelnut oils.

8.6 Conclusion

Following this overview of spectrophotometric applications in food authenticity and traceability control, we can conclude that these techniques are recognised as interesting and sometimes really useful in the control of food products. Less known than infra-red absorption spectroscopy, fluorescent and Raman emission spectroscopy offer advantages of sensitivity and specificity that could be better exploited if more research on their potential can be initiated and finalised, notably in food authenticity and traceability control.

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9

Gas chromatography

E. Forgács and T. Cserháti, Hungarian Academy of Sciences

9.1 Introduction

Chromatographic techniques have been developed for the rapid and reliable separation of molecules with extremely similar chemical characteristics even from complex matrices. Because of the high separation capacity, chromatographic procedures have found increasing acceptance and employment in food science and technology for the quantitative analysis of numerous molecules such as normal constituents of foods, legal or illegal additives, pollutants, etc. Chromatography is a common name for techniques based on the adsorption and/or partition of the molecules to be separated between a mobile and a stationary phase. Separation is the result of different strengths of adsorption of the different partition of molecules between the two phases.

Stationary phases are generally solids (inorganic or organic), mobile phases are liquids or gases. Chromatographic techniques are classified according to the character of both the stationary and the mobile phases, the form of the stationary phase and according to the driving forces of separation. The methods most frequently used in food analysis are gas chromatography (GC) (solid stationary phase and gas mobile phase), thin-layer chromatography (TLC) (planar solid surface, liquid mobile phase moving by capillary forces or by a pump system), high-performance liquid chromatography (HPLC) (stationary phase filled in column, liquid mobile phase moved by pump), and various electrically driven procedures (capillary zone electrophoresis, micellar electrokinetic chromatography, etc.

The objectives of this chapter are the compilation and concise survey of the most important results in the field of the application of various GC methodologies for the assessment of food authenticity and traceability, the

brief elucidation of the theoretical principles and the critical discussion of the practical results. The inclusion of the basic knowledge of the principles of GC was motivated by the assumption that it may help food chemists and technologists in the effective solution of any analytical problems employing GC as a tool.

9.2 Principles and technologies

The term gas chromatography (GC) indicates chromatographic techniques applying a gas as the mobile phase and solid or liquid stationary phases (gas-solid chromatography = GSC or gas-liquid chromatography = GLC). However, there are limitations concerning the type of molecules appropriate for GC analysis: compounds need to have an appreciable vapour pressure at temperatures below 350–400ºC, and they have to be easily vaporised without decomposing or reacting with the components of stationary and mobile phases or with other components present in the sample to be analysed.

The distribution of solute molecules between the stationary and mobile phases (carrier gas) is defined by the distribution constants (K_D) which is the ratio of the concentration of the solute molecules in the stationary phase to that in the mobile phase:

$$K_D = \text{compound concentration of stationary phase/compound concentration of mobile phase} \quad 9.1$$

The general expression that describes distribution in terms of column temperature and thermodynamical parameters:

$$\ln K_D = -G^0/RT \quad 9.2$$

where G^0 is the change in Gibbs free energy for the evaporation of a compound from the stationary phase, T is the column temperature and R is the ideal gas constant. Equation 9.2 shows that the differences in the Gibbs free energy for the evaporation of the solutes from the stationary phase result in different distribution of the solutes.

The amount of time that the compound spends in the stationary phase is called the retention time (t_R). Dead time (t_0) is the time needed for a nonretained analyte to travel through the column. The partition ratio, also known as the capacity factor (k') is the time a compound spends in the stationary phase relative to the mobile phase.

$$k' = (t_R - t_0)/t_0 \quad 9.3$$

The separation factor (α) can be defined:

$$\alpha = k_2/k_1 \quad 9.4$$

where k_1 is the partition ratio of the earlier eluting compounds and k_2 is partition ratio of the later eluting one.

Resolution number (R) describes the efficacy of the separation of two neighbouring peaks. Resolution numbers are calculated using either of two equations:

$$R = 1.18(t_{R2} - t_{R1})/(w_{h1} + w_{h2}) \quad 9.5$$

$$R = 2(t_{R2} - t_{R1})/(w_{b1} + w_{b2}) \quad 9.6$$

where t_{R1} and t_{R2} are the retention times of peaks 1 and 2, respectively, w_{h1} and w_{h2} are the peak widths at half height of peaks 1 and 2 respectively, w_{b1} and w_{b2} are the peak widths at the base of peaks 1 and 2, respectively.

Theoretical plate number (N) characterises the separation capacity of chromatographic columns. There are no plates in a GC column; however, this concept, borrowed from the distillation theory, is used to characterise column performance (Kudryashov *et al.*, 2002). The number of theoretical plates is calculated by the following equation:

$$N = 5.545(t_R/W_h)^2 \quad 9.7$$

There are a considerable number of books that are useful references concerning gas chromatography; these can be consulted for further information and details (Rotzse 1991, Grob 1995, Jennings 1987, Hill and McMinn 1995).

9.2.1 The practice of gas chromatography

Common gas chromatographic equipment consists of a carrier gas system, injector, gas chromatographic column, detector and data processing unit. The carrier gas is generally a permanent gas with low or negligible adsorption capacity, i.e., hydrogen, helium or nitrogen. The nature of the carrier gas may influence the separation characteristics of the GC system and can modify the sensitivity of the detection. As the stability and reproducibility of the carrier gas flow-rate is a prerequisite of a successful gas chromatographic analysis, they considerably influence both the efficacy of separation and the quantification of results. Injectors deliver the sample to the head of the GC column. Injectors can be classified into two major groups: vaporisation and on-column injectors. Vaporisation injectors utilise high temperatures (100−300ºC) to vaporise a liquid sample rapidly. Usually a syringe is used to introduce the sample into the thermostated injector. In this case the sample rapidly vaporises, mixes with the carrier gas, and is transported into the column. On-column injectors deposit the sample directly into the column without relying upon vaporisation of the sample and its subsequent transport into the column. Separation of volatile compounds of the injected sample is performed in the GC column.

Columns for gas chromatography can be divided into two distinct groups; packed and capillary columns of various dimensions (Spangler, 2001). A packed column is a rigid metal or glass column filled with small particles which are often coated with a thin layer of a high molecular weight polymer. The most common solid supports are diatomaceous earths, fluorocarbons, graphitised carbon black and glass beads. About 90% of all supports are various types of

diatomaceous earth. The stationary liquid phase of GC columns has to comply with the following requirements: low vapour pressure, high chemical stability and relatively low viscosity at the temperature of analysis; selectivity for the sample components under investigation; good wetting capacity both for the surface of the inert support or for the possibly inert wall of the column. The length of a packed column is limited to about 3 m because of the high pressures that are required to maintain the carrier gas flow rates at velocities necessary for optimal performance. Packed columns have several advantages over capillary columns. Packed columns have 10 to 1,000 times greater sample capacity than capillary columns. This makes packed columns superior for analytes where large amounts of sample need to be analysed. However, packed columns have 25–50% fewer theoretical plates per metre than capillary columns. Coupled with the shorter lengths of packed columns (1–3 m versus 10–60 m for capillary columns) the total number of theoretical plates are substantially lower than that of capillary columns.

A capillary (also called open tubular) column is a glass or fused-silica tube of very small internal diameter (generally between 0.20–0.53 mm). The inner surface of a capillary column is coated with a thin layer of stationary phase so it is still possible for the solute molecules to come in contact with the inner walls of the tubing. Most capillary column stationary phases are cross-linked and covalently bonded to the fused-silica surface. The amount of stationary phase in a capillary column is denoted by its film thickness, which is typically $0.1-5\ \mu$m. Compound retention is proportional to film thickness in capillary columns, retention increases as the film thickness increases, and it decreases as the film thickness decreases. The advantage of capillary columns is their very high separation capacity. This allows the resolution of peaks in complex samples that are not adequately separated by packed columns. Because of better separation performance capillary columns have been more often used in gas chromatography than packed columns. The efficacy of GC analyses can be markedly enhanced by using a column switching technique (Samuel and Davis, 2002).

In order to achieve effective and reliable separation the gas chromatographic column has to be thermostated at a constant temperature (isothermal separation mode) or it can be modified according to a predetermined temperature programme (temperature gradient). The application of a temperature gradient greatly increases the efficacy of the separation (Davis *et al.*, 2000). As the column temperature is one of the most decisive parameters in GC analysis its exact regulation is of paramount importance. Detectors interact with the solute molecules as they exit the column. This interaction is converted into an electrical signal that is sent to a recording or data storage device. A chromatogram is then created which is a plot of the intensity of the signal versus elapsed time. The primary characteristics of detectors are the lowest amount of a compound that is detectable (sensitivity) and which compound at the same amount produces the strongest detector response (selectivity).

Many different detectors (flame-ionisation = FID, nitrogen-phosphorus = NPD, flame photometric = FPD, electron capture = ECD, thermal conductivity

= TCD, atomic emission = AED, electrolytic conductivity = ELCD, chemiluminescence, etc.) have been developed for the sensitive and selective detection and quantification of sample components. FID uses a hydrogen flow mixed with the carrier gas. The mixture is ignited, the analytes are burned and the ions formed during the burning process are collected in a cylindrical electrode at a high voltage applied between the jet of the flame and the electrode. The resulting current is amplified and detected. NPD is similar to FID in its design. It contains rubidium or cesium beads inside a heater coil near to the hydrogen jet. The partially combusted nitrogen and phosphorous molecules adsorb on the surface of the bead reducing the emission of electrons which increases the current. FPD specially detects sulphur and phosphorus compounds. Analytes are burned in the flame. Due to the excitation in the flame, light is emitted at 392 (sulphur) and 526 (phosphor) nm. A filter selects the wavelengths reaching a photomultiplier tube.

ECD employs a low-energy β-ray source for the production of electrons and ions. Electron capturing molecules (halogenated compounds) entering the detector decrease the electron current which can be amplified and registered. TCD responds to changes in thermal conductivity and specific heat using a filament under current placed in the carrier gas flow. Changes in the thermal conductivity and/or specific heat of the current gas caused by the analytes modifies the potential across the filament. AED is suitable for the detection of selected atoms or groups of atoms, ELCD can be specially used for the detection of Cl, N, or S containing analytes. A chemiluminescence detector is mainly employed for the detection of sulphur compounds. In the past decades GC methods combined with various mass spectrometric (MS) detection systems have found increasing application in GC analyses.

MS detection is based on the phenomenon that ions or molecules can be ionised in a high vacuum producing additional charged species. These species can be separated and their relative abundance (their mass spectrum) is characteristic of the original analyte. A mass spectrometer has to generate ionic species then separate and detect them. Ion generation can be achieved by electron impact (EI) and chemical ionisation (CI) techniques. In EI method the fragmentation and charge of analytes is performed by producing collisions between them and the electrons generated from a hot filament.

The CI technique employs a reagent gas such as ammonia or methane ionised by an electron beam. The ionised gas reacts with the analytes forming relatively stable ion-molecule complexes. As the most frequently occurring complexes are simple adducts such as $[M+H]^+$ or $[M+NH_4]^+$ the molecular mass of analytes can be easily calculated. Other portable hyphenated GC instruments have also been developed for field applications (Arnold *et al.*, 2000). The current trends in GC instrumentation and methodologies have been recently reviewed by Yashin and Yashin, (2001).

9.3 Sample preparation

Sample preparation is often the most time-consuming step in chromatographic analysis. In many applications, analytes must be determined in complicated matrices in very low concentration. Liquid-liquid extraction (LLE) is the traditional method of treating samples. The LLE procedure generally requires several steps, making the cleanup process difficult to automate (i.e., Soxhlet extraction). The highly purified solvents that are required are expensive and many of these solvents are suspected of endangering the health of laboratory workers. Several alternative methods that reduce or eliminate the use of solvents are now being used to prepare samples for chromatographic analysis. These include static and dynamic headspace analysis for volatile compounds, solid-phase extraction (SPE) and supercritical fluid extraction (SFE) for semivolatiles or non-volatile pollutants. A new variation of SPE has been developed recently, the so-called solid-phase microextraction (SPME). This technique is normally used prior to GC analysis.

In conventional solid-phase extraction (SPE), a liquid sample is passed over a solid or 'sorbent' that is generally packed in a medical-grade polypropylene cartridge or embedded in a disk. As a result of strong attractive forces between the analytes and the sorbent, the analytes are retained on the sorbent. Later, the sorbent is washed with a small volume of a solvent that has the ability to disrupt the bonds between the analytes and the sorbent. The final result is that the analytes are concentrated in a relatively small volume of clean solvent and are therefore ready to be analysed without any additional sample workup.

However, for the successful application of the SPE method a number of factors must be considered. The analytes can be bonded to the sorbent by nonpolar, polar and ionic (electrostatic) interactive forces. In the majority of cases nonpolar or slightly polar analytes are dissolved in water, a highly polar solvent. For the preconcentration of such compounds nonpolar sorbents can be employed. On the other hand analytes containing polar (acidic or basic) functional groups will be readily retained on sorbents of opposite polarity which means that anionic sorbents should be selected to retain molecules with cationic characteristics and cationic sorbents to retain anionis type compounds. SPE cartridges are commercially available in a wide range of sizes with volumes ranging from less than 1 ml to over 50 ml.

When selecting the optimum cartridge size for particular applications, the ability of sorbent to retain all of the analytes present in the sample, volume of the original sample and final volume of purified sample have to be equally taken into consideration. In general the mass of the analytes and interfering compounds retained by the sorbent should be less than 5% of the mass of the sorbent.

In solid-phase microextraction (SPME) as in conventional SPE, analytes are concentrated by adsorption into a solid phase; however, in practice the two techniques are quite different. This technique utilises a short thin solid rod of fused silica (typically 1 cm long and 0.1 μm outer diameter), coated with an

adsorbent polymer. The coated fused silica (SPME fibre) is attached to a metal rod, the entire assembly (fibre holder) may be described as a modified syringe. In the standby position, the fibre is withdrawn into a protective sheath. For sampling, a liquid or solid sample is placed in a vial, and the vial is closed with a cap that contains a septum. The sheath is pushed through the septum and the plunger is lowered, forcing the fibre into the vial, where it is immersed directly into the liquid sample or the headspace. Analytes in the sample are adsorbed on the fibre. After a predetermined time, the fibre is withdrawn into the protective sheath and the sheath is pulled out of the sampling vial. Immediately after, the sheath is inserted into the septum of a GC injector, the plunger is pushed down, and the fibre is forced into the injector where the analytes are thermally desorbed and separated on the GC column. The desorption step is usually 1–2 minutes afterwards, the fibre is withdrawn into the protective sheath and the sheath is removed from the GC injector. Several adsorbent polymers (polydimethylsiloxane, polyacrilate and carbowax/divinyl benzene) with different adsorption characteristics are available on SPME fibres. The fibres can usually be used for 100 or more samplings.

Supercritical fluid extraction (SFE) uses mobile phases with physicochemical properties between those of liquids and gases. The main advantages of the supercritical fluids are that the mass transfer is very rapid in supercritical state and the dynamic viscosities are near to those found in normal gaseous phases, the diffusion coefficient being more than ten times higher than that of a liquid. The viscosity and diffusivity of the supercritical fluid approach those of a liquid as pressure is increased at a fixed temperature. Diffusivity increases with an increase in temperature at a fixed pressure, and viscosity decreases with increasing temperature. Changes in viscosity and diffusivity are more pronounced in the region of the critical point. Even at high pressures diffusivity are 1–2 orders of magnitude greater than those of liquids. Therefore the properties of gas-like viscosity and diffusivity, liquid-like density, combined with pressure dependent solvating power, provided the impetus for applying supercritical fluid technology in both sample preparation and chromatographic separation process.

Due to its favourable physicochemical parameters, CO_2 constitutes the principal mobile phase for SFE. Supercritical fluids can be used for the more or less selective extraction of analytes of interest from complicated organic and inorganic matrices. The main advantages of supercritical fluid extraction is that it is inexpensive and uses environmentally friendly solvent mixtures. Supercritical fluids may have solvating power similar to that of organic solvents generally employed for liquid-liquid extraction. The solvating power can be adjusted by changing the pressure or temperature, or by adding modifiers to the supercritical fluid.

The extraction power (polarity) of supercritical CO_2 can be enhanced by the addition of various organic modifiers such as methanol, acetonitrile, etc. SFE is a promising sample preparation method, being a solvent-free alternative to conventional extraction methods. SFE techniques provide many advantages over traditional liquid extraction: considerable reduction of the volume of organic

solvents, marked decrease of extraction times and the ability for on-line introduction of the extracted material to chromatographic instruments mainly to a gas chromatograph.

The choice of chromatographic system depends on the nature of the sample under investigation but, generally, the analytes extracted using supercritical carbon dioxide are nonpolar or only moderately polar with sufficient volatility to be analysed by GC. Thus, the most popular supercritical fluid on-line technique is SFE-GC. On-line SFE-GC is an attractive approach to coupling the extraction and analysis procedures of real-world samples and offers the analyst a rapid, sensitive, quantitative, and yet selective analytical method. A quantitative SFE-GC analysis including the loading and assembly of the extraction cell and performing the extraction and GC analysis can be routinely undertaken in less than one hour. SFE-GC unquestionably offers an excellent alternative to conventional sample preparation techniques and has the potential to become a routine problem-solving tool for the analytical chemist.

9.4 Applications: identifying flavour compounds

The chromatographic techniques employed for the analysis of macro- and microcomponents in foods and food products have been recently compiled and critically evaluated (Cserháti and Forgács, 1999). These cover the differentiation of cassia, cinnamon, ginger, caraway fruits, turmeric powder, bell peppers, onion, garlic, wine, beer, hops, coffee, tea, dairy products, meat, fish, oils, juices, corn-based snacks and peanuts.

An analysis of volatile compounds in different edible oils is often required as they are responsible for the characteristic flavour properties of the oils and, as is widely known, the aroma plays an important role not only in the overall quality of the product, but also in the acceptance of it by the consumer. Although many methods have been developed for isolating volatile compounds from foods, several difficulties may be observed when analysing volatile compounds from oils, because some of these compounds are fat-soluble and, hence, the efficiency of the isolation procedure can be significantly reduced. The use of purge and trap techniques and headspace sampling (Morales and Aparicio, 1993), thermal desorption-gas chromatographic analysis (Oveton and Manura, 1995) as well as simultaneous purging and solvent extraction have been proposed, but the development of new methods is necessary to overcome the difficulties associated with the lipophilic nature of most volatile compounds. In this respect, simultaneous distillation-solvent extraction (SDE) has received much attention in the past few years as successive modifications of the original design resulted in a micro version which allows operation with small amounts of extraction solvents without requiring a subsequent concentration of the extract, and hence, losses of volatile compounds can be reduced.

Identification of volatile compounds in different oils (i.e., olive oil, almond oil, hazelnut oil, peanut oil and walnut oil) was performed using simultaneous

distillation-solvent extraction followed by gas chromatographic-mass spectrometric analysis (Table 9.1). An alternative approach allows the direct injection of the oil sample and involves the use of on-line coupled reversed-phase liquid chromatography with gas-chromatography (RPLC-GC) for a more reliable and rapid determination of specific compounds (Caja del Mar *et al.*, 2000). The advantages of on-line RPLC-GC are the flexibility of transferred volume section as well as the ease of elimination of problems associated both with risk of degradation of thermolabile compounds and the lipophilic nature of some volatile components. Thus, on-line RPLC-GC enhances reliability and saves time in the analysis of volatile compounds in oils.

The volatile components present in the Citrus peel essential oils largely belong to terpene compounds. Mono and sesquiterpene hydrocarbons together with several oxygenated derivatives, comprising alcohols, aldehydes, ketones, esters and epoxides, are the main compounds. Depending on the particular species and cultivar a more or less significant amount of isoprenoid compounds can be present, the main components being alcohols, esters and aldehydes of aliphatic origin (Table 9.2.). Shaw (1979), Caccioni *et al.* (1998) and Dugo *et al.* (1999) list the components detected by various gas-chromatographic methods in the essential oils of some of the most important Citrus species, namely blond and blood sweet orange (Citrus sinensis L. Osbeck, bitter orange (Citrus aurantium L.) lemon (C. limon L. Burman), mandarin (C. reticulata Blanco), clementine (C. clementina Hort ex Tan.) grapefruit (C. paradisi MacFaden), bergamot (C. bergamia Risso) lime (C. aurantifolia Christm. Swing) and the fruit of the hybrid citrange Carizzo (C. sinensis/Poncirus trifoliata).

Flavour is an important factor in the acceptability of any food product. Vegetables can be eaten raw, cooked or processed and depending on the method of their preparation the volatilies content can vary greatly. The effects of four culinary treatments (steaming and boiling in a covered pot, pressure cooker or a microwave oven) on the volatile component profile of green beans were evaluated. Volatile compounds in raw and cooked beans were analysed by means of dynamic headspace sampling onto an adsorbent, followed by microwave desorption into a gas chromatograph equipped with MS detector. Twenty-seven compounds were identified, including alcohols, aldehydes, ketones, esters, terpenes, sulphur compounds and alkenes (Table 9.3.) All of the thermal treatments caused important changes in the volatile compound profile, in particular an increase in carbonyl compounds and a decrease in alcohol compounds. It was concluded that the change in aroma during the cooking of green beans depends on compounds from lipid oxidation (Rodriguez-Bernaldo De Quirós *et al.*, 2000).

Advances in flavour research and future trends in this field have recently been discussed (Steinhart *et al.*, 2000). Aroma compounds of sparkling wines Cava (Certified Brand of Origin of Spain) were determined by head-space solid phase microextraction and GC-MS method. Five ethyl esters and two isoamyl esters, vitispirane and 2-dihydro-1, 1,1,6-trimethyl-naphthalene were quantified.

Table 9.1 Volatile compounds identified in oil extracts from simultaneous distillation solvent extraction (with permission)

	Compound[a]	Relative peak areas[b]				
		Olive oil	Almond oil	Haselnut oil	Peanut oil	Walnut oil
1.	Hexanal	1.19	0.93	1.45	14.97	3.57
2.	Furfural	–	–	3.16	–	–
3.	1-Acetyl-cyclohexene	1.66	–	–	–	2.17
4.	Saturated hydrocarbon	4.91	–	–	–	–
5.	Hydrocarbon	–	–	–	3.03	
6.	α-Pinene	–	–	–	1.53	–
7.	α-Pinene	–	–	–	3.45	–
8.	(E)-5-Methyl-hept-2-en-4-one	–	–	2.48	–	–
9.	(E)-5-Methyl-hept-2-en-4-one	–	–	3.53	–	–
10.	Pentylfuran	–	–	1.17	3.57	–
11.	Benzaldehyde	–	108.39	–	–	–
12.	Saturated hydrocarbon	0.25	–	–	–	–
13.	6-Methyl-hept-5-en-2-one	0.98	–	–	–	–
14.	2-Octanone	–	–	–	3.36	–
15.	Pyrazine	–	–	3.92	–	–
16.	1.1-Dimethyl cyclopentane	–	–	–	13.19	–
17.	(E)-3-hexen-1-ol	1.49	–	–	–	–
18.	3-Hexen-1-ol-acetate	1.52	–	–	–	–
19.	Octanal	–	–	–	11.37	–
20.	Furancarboxaldehyde	–	–	–	–	2.62
21.	(E.E)-2.4-Heptadienal	1.39	–	–	–	–
22.	2.4-Heptadienal	–	–	–	2.50	3.23
23.	2.4-Heptadienal	0.75	–	–	–	–
24.	2.4 Heptadienal	–	–	–	–	1.22
25.	Furfuryl alcohol	–	–	1.27	–	0.77
26.	Unsaturated aldehyde	–	–	–	8.26	–
27.	Nonanal	1.46	0.62	3.12	27.85	9.24
28.	Phenylacetaldehyde	–	–	2.41	–	–
29.	Saturated hydrocarbon	–	–	–	7.39	–
30.	1-Decene	3.33	–	–	–	–
31.	Sabinene	–	–	0.60	–	–
32.	Octanol	–	–	1045	–	–
33.	2-Nonenal	–	–	–	26.15	–
34.	Saturated alcohol	–	–	–	12.20	–
35.	Decanal	–	–	0.55	–	–
36.	Sbinene	–	–	0.79	–	–
37.	Hydrocarbon	–	–	–	28.76	–
38.	2-Acetylpyrrole	–	–	0.63	–	–
39.	Terpineol	–	–	0.54	–	–
40.	(E)-2-Decenal	0.63	–	–	–	–
41.	(Z)-2-Decenal	–	–	0.76	–	–
42.	Hydrocarbon	–	–	–	43.14	–
43.	Methanol	–	–	–	33.89	–
44.	2.4-Decadienal	0.70	–	0.71	14.48	0.33
45.	Decadienal	–	0.73	–	–	–

Table 9.1 Continued.

Compound[a]	Relative peak areas[b]				
	Olive oil	Almond oil	Haselnut oil	Peanut oil	Walnut oil
46. Hydrocarbon	–	–	–	30.97	–
47. 2.4-Decadienal	–	–	–	73.73	–
48. (E.E)-2.4-decadienal	1.00	1.00	1.00	1.00	1.00
49. 2-Undecenal	0.25	–	0.42	–	–
50. Hydrocarbon	–	–	–	43.14	–
51. (E.E)-a-Farnesene	1.14	–	–	–	–
52. Hydrocarbon	–	–	–	34.61	–
53. Unsaturated hydrocarbon	–	–	–	7.85	–
54. Hydrocarbon	–	–	–	9.52	–
55. Hydrocarbon	–	–	–	20.89	–
56. Hydrocarbon	–	–	–	11.09	–
57. Hydrocarbon	–	–	–	7.10	–
58. Podocarpene	–	–	–	7.63	–

[a] Tentatively identified (on the basis of MS data alone).
[b] Expressed as the ratio of the absolute peak area of each compound to that obtained for (E.E)-2.4-decadienal in the corresponding oil extract.

These flavour components could be used to characterise sparkling wines with different periods of ageing with yeast. This method could be used to identify wines with required nine-month ageing (minimum legal ageing for Cava sparkling wine) and to determine the approximate age of sparkling wines (Francioli *et al.*, 2001).

Investigation of the aroma compound of the essential oils of black pepper (Piper nigrum) and black and white Ashanti pepper (Piper guineense) from Cameroon by means of solid phase microextraction was carried out for the first time to identify the odorous target components responsible for the characteristic odour of these valuable spices and food flavouring products (Jirovetz *et al.*, 2000). By means of GC-flame ionisation detection (FID) and GC-MS the main compounds (concentration of $> 3.0\%$, as calculated as % peak area of GC-FID analysis using a non-polar fused-silica open tubular RLS-200 column) of the SPME headspace samples of P. nigrum (black) and P. guineense (black and white) were found to be: P. nigrum (black)-germacrene D (11.01%), limonene (10.26%), β-pinene (10.02%), α-phellandrene (8.56%), β-caryophyllene (7.29%), α-pinene (6.40%) and cis-β-ocimene (3.19%); P. guieense (black)-β-caryophyllene (57.59%), β-elemene (5.10%), bicyclogermacrene (5.05%) and α-humulene (4.86%) and P. guieense (white)-β-caryophyllene (51.75%), cis-β-ocimene (6.61%), limonene (5.88%), β-pinene (4.56%), linalool (3.97%) and α-humulene (3.29%).

Similarly, headspace solid-phase microextraction (HSSPME) coupled with gas chromatography-mass spectrometry has been used to isolate volatile

Table 9.2 Chemical components of Citrus peel essential oils. Compounds are listed according to the elution order on a polar capillary column (5% phenyl-methylpolysiloxane). All samples are cold-pressed oils, except for Carrizo Citrange oil which was obtained by peel hydrodistillation (with permission).

Compound	Blond sweet orange	Blood sweet orange	Bitter orange	Lemon	Mandarin	Clementine	Grape-fruit	Bergamot	Lime	Carr. Citrange
Hexanol			*							
Tricyclene				*				*	*	
α-Thujene	*	*	*	*	*	*	*	*	*	*
α-Pinene	*	*	*	*	*	*	*	*	*	*
Camphene	*	*	*	*	*	*	*	*	*	*
Thuja-2,4(10)-diene									*	
Heptanol					*					
Sabinene	*	*	*	*	*	*	*	*	*	*
β-Pinene	*	*	*	*	*	*	*	*	*	*
6-Methyl-5-heptene-2-one				*	*			*	*	
Myrcene	*	*	*	*	*	*	*	*	*	*
α-Phellandrene	*	*	*	*	*	*	*	*	*	*
Octanal	*	*	*	*	*	*	*	*	*	*
Decane									*	
Hexyl acetate								*		
δ-3-Carene	*	*	*	*	*	*		*	*	*
α-Terpinene	*	*	*	*	*	*	*	*	*	*
p-Cymene	*	*	*	*	*	*	*	*	*	*
Limonene	*	*	*	*	*	*	*	*	*	*
β-Phellandrene	*	*	*	*	*	*	*	*	*	*
1,8-Cineole				*	*			*	*	
(Z)-β-Ocimene	*	*	*	*	*	*	*	*	*	*
(E)-β-Ocimene	*	*	*	*	*	*	*	*	*	*
γ-Terpinene	*	*	*	*	*	*	*	*	*	*
Cis-Sabinene hydrate	*	*	*	*	*	*	*	*	*	*
Cis-Linalool oxide				*				*		
Octanol	*	*	*	*	*	*	*	*		*
Terpinoolene	*	*	*	*	*	*	*	*	*	*
Trans-Linanool oxide			*				*	*		
Trans-Sabinene hydrate		*	*	*	*	*		*		
Linalool	*	*	*	*	*	*	*	*	*	*
Nonanal	*	*	*	*	*	*	*	*	*	
1,3,8-p-Menthatriene					*					
Endo-Fenchol									*	
Cis-p-Menth-2-en-1-ol									*	
Heptyl acetate								*		
Cis-Limonene oxide	*	*	*	*	*	*	*	*	*	
Allo-Ocimene[a]				*				*	*	
Trans-Pinocarveol									*	
Trans-Limonene oxide	*	*	*	*	*	*	*	*	*	
Camphor				*	*			*	*	
Trans-p-Menth-2-en-1-ol									*	
Isopulegol							*	*		
(E)-Miroxide			*							
Citronellal	*	*	*	*	*	*	*	*	*	*
Borneol			*	*					*	

Table 9.2 Continued.

Compound	Blond sweet orange	Blood sweet orange	Bitter orange	Lemon	Mandarin	Clementine	Grape-fruit	Bergamot	Lime	Carr. Citrange
Cis-Pinocamphone									*	
Terpinen-4-ol	*	*	*	*	*	*	*	*	*	*
Nonanol			*				*			
p-Cymen-8-ol					*					
α-Terpineol	*	*	*	*	*	*	*	*	*	*
Cis-Piperitol									*	
Decanal	*	*	*	*	*	*	*	*	*	*
Dodecane								*	*	
Trans-Carveol	*					*	*			
Nerol	*	*	*	*	*		*	*	*	*
Citronellol			*	*	*					
Cytronellyl formate	*	*								
Carvone	*	*			*	*	*	*		
Neral	*	*	*	*	*	*	*	*	*	*
Piperitone			*	*	*				*	
Geraniol	*	*	*	*	*		*	*	*	*
Linalyl acetate			*		*	*	*	*		
(E)-2-Decenal	*	*			*	*		*		
Perillaldehide	*	*	*	*	*	*	*	*	*	
Geranial	*	*	*	*	*	*	*	*	*	
Decanol			*				*			
Bornyl acetate				*				*	*	
Perillalcohol					*	*				
Trans-Pinocarvyl acetate									*	
Thymol					*	*				
Carvacrol									*	
Tridecane									*	
Undecanal	*	*	*	*	*	*	*	*	*	
(E,E)-2,4-Decadienal	*	*	*		*	*				
Nonyl acetate	*	*	*	*	*			*		
Methyl geranoate				*				*		
δ-Elemene			*					*	*	
Linalyl propanoate								*		
α-Terpenyl acetate	*	*	*			*	*	*	*	*
Trans-Carvyl acetate							*			
Citronellyl acetate	*	*	*	*	*		*	*	*	
Neryl acetate	*	*	*	*	*	*	*	*	*	
α-Copaene	*	*			*	*	*			*
Geranyl acetate	*	*	*	*	*	*	*	*	*	
β-Elemene	*	*			*	*	*			*
β-Cubebene	*	*	*			*				*
Methyl-N-methylanthranilate					*	*				*
Dodecanal	*	*	*		*	*	*	*	*	
β-Caryophyllene	*	*	*	*	*	*	*	*	*	*
Cis-α-Bergamotene				*				*	*	
Decyl acetate	*	*	*	*	*	*	*	*	*	
α-Santalene				*				*	*	

Table 9.2 Continued.

Compound	Blond sweet orange	Blood sweet orange	Bitter orange	Lemon	Mandarin	Clementine	Grape-fruit	Bergamot	Lime	Carr. Citrange
α-Cadinene	*	*				*				
α-Elemene									*	*
Trans-α-Bergamotene			*	*	*		*	*	*	*
α-Humulene	*	*	*	*	*	*	*	*	*	*
β-Santalene				*				*	*	
(Z)-β-Farnesene	*	*	*	*		*	*	*	*	*
(E)-2-Dodecenal			*		*	*	*			
Dodecanol								*		
Germacrene-D	*	*	*		*	*	*	*		
γ-Muurolene		*								
(E)-β-Farnesene				*					*	*
α-Selinene					*				*	
Valencene	*	*		*	*	*				*
Bicyclogermacrene	*	*	*	*	*	*	*	*		
γ-Cadinene		*								
7-epi-α-Selinene	*									
δ-Guaiene		*								
(Z)-α-Bisabolene				*				*	*	
β-Bisabolene			*	*	*		*	*	*	
Aristolene									*	
(E,E)-α-Farnesene	*	*			*	*	*	*	*	*
(z)-γ-Bisabolene				*				*	*	
Tridecanal	*	*								
Undecyl acetate								*		
δ-Cadinene	*	*	*		*	*	*			
(E)-γ-Bisabolene									*	
(E)-α-Bisabolene				*					*	
Elemol	*	*				*	*			
Germacrene-B								*	*	
(E)-Nerolidol	*	*	*		*	*	*	*		*
Germacrene-D-4-ol	*	*		*			*			
Caryophyllene oxide	*	*					*		*	
cis-Sesquisabinene hydrate									*	
Tetradecanal	*	*	*		*	*	*	*	*	
Selin-11-en-4-ol	*	*		*					*	
Campherenol				*				*	*	
β-Bisabolol				*				*	*	
α-Bisabolol				*				*	*	
Cadinol[a]										*
α-Asarone										*
β-Sinensal	*	*				*	*			*
Herniarin									*	
Pentadecanal									*	
(E,E)-Farnesol							*			
(E,Z)-Farnesol							*		*	
α-Sinensal	*	*	*		*	*				
Nootkatone	*	*	*	*			*	*		
Hexadecanal	*	*			*		*		*	

Table 9.3 Identified volatile compounds and their average percentage area (n = 2) of raw and cooked green beans (with permission).

No.	Compound name	Retent. time (min.)	Raw	Covered pot	Steamed	Pressure cooked	Microwave
1	2-Butane	3.318		7.03			
2	3-Metyl-3-butane-2-one	3.685		2.21			
3	Cyclopentanol	3.810		1.09			
4	1-Penten-3-one	3.894		2.55			
5	3-Methylbutanal	4.094		8.92	7.91	13.7	7.34
6	4-Octen-3-one	4.844	1.16				
7	Dimethyldisulphyde	5.311	1.36	5.38	2.54	2.58	2041
8	3-Methylbutanol	5.653		1.93	1.02	7.19	
9	Hexanal	6.629	2.71	15.9	0.215	9.22	17.4
10	Isobutyl acetate	7.036			7.73		
11	4-Hydroxy-4-methylpentan-2-one	7.946	9.15	8.82	24.9	1.54	17.6
12	6-Hepten-1-ol	8.355	1.45				
13	3-Hexeh-1-ol	8.446	1.27				
14	2-Butoxyethanol	10.273	17.3	15.6	46.6	2.79	41.8
15	Benzaldehyde	12.582	0.085	1.04	0.600	3.07	0.915
16	1-Octen-3-ol	13.094	47.1	12.5	3.10	40.8	4.39
17	6-Methyl-5-hepten-2-one	13.391		4.25	2.10		3.05
18	3-Octanone	13.401	6.77				
19	Octanol	13.743	6.60				
20	3-Ethyl-1.4-hexadiene	13.833		3.93			0.820
21	Octanal	14.067		1.85	0.585		0.965
22	3-Hexenyl acetate	14.160	3.81				
23	Hexenyl acetate	14.385	0.545				
24	2-Ethylhexa-1-ol	15.077		0.430		6.85	
25	DL-Limonene	15.446	0.275	0.655	0.475	3.30	0.595
26	Nonanal	18.211	0.225	2.85	1.07	4.76	1041
27	Decanal	22.257	0.210	2.94	1.05	4.12	1025

compounds from three different spices (basil, oregano, bay leaves). SPME conditions were optimised before the experimental extractions, to achieve optimum recovery of the volatile compounds. Typical components of the essential oils of spices and other plant materials, e.g., terpenes, hydrocarbons, and oxygenated terpenes were identified (Diaz-Maroto *et al.*, 2002).

Manual headspace-solid phase microextraction (HS-SPME) gas chromatography-mass spectrometry (GC-MS) was employed for the identification and quantitative analysis of the main volatile constituent in Mango juice. Nineteen compounds were quantified though standard addition. The main volatile compounds of Mango juice were terpenes, including mono- or sesquiterpene hydrocarbons, such as 1R-α-pinene, 3-carene, α-terpinene, limonene, E-α-ocimene, terpinolene, eremo-philadiene, α-caryophylene and guaiadiene (Shang *et al.*, 2002). Recent developments in the determination of aroma components in foods have also been reviewed (Sides *et al.*, 2000).

GC has also been employed for the separation of tocopherols and the results were compared with those of other liquid chromatographic techniques (TLC and HPLC). It was concluded that each method can be used for the effective separation of this class of analytes (Pyka and Sliwiok, 2001). The oxidation of fish oil-enriched mayonnaise has also been followed by a GC technique (Jacobsen *et al.*, 2000). The on-line supercritical fluid extraction-supercritical fluid chromatography-gas chromatography method was applied to determination of volatile compounds of raw and baked Baltic herring (Clupea harengus membrans) (Aro *et al.*, 2002).

After extraction, the volatiles and coeluted lipids were separate on-line using supercritical fluid chromatography and the volatile fractions was introduced directly into a gas chromatograph. In all, 30 compounds were identified from fish samples with mass spectrometry. The most abundant compounds in fresh Baltic herrings were heptadecane and 1-heptadecene (Fig. 9.1). During storage, the proportions of short chain volatile acids increased while the propotions of n-heptadecane and 1-heptadecene decreased significantly (Table 9.4.). After 7–8 days of storage, the most abundant compound was 3-methyl-butanoic acid comprising 36% of all volatiles in the raw herring and 40% in the baked herring. The other main compounds were acetic, propanoic, and 2-methylpropanoic acids. Volatile acids are important components of sauce made from fish and salt by fermentation. Some authors supposed (Peralta *et al.* 1996, Shimoda *et al.* 1996) that these volatile acids were formed from amino acids through bacterial fermentation, but lipid oxidation may also be responsible for some of the acids. Secondary oxydation of aldehydes can also form short chain acids (C_4–C_8) in fish. The volatile acids were probably produced via both mechanisms, oxidation and fermentation. In all probability, 2-methylpropanoic acids and 3-methylbutanoic acids were formed through fermentation because the content of branched fatty acids in Baltic herring is low.

9.5 Advantages and disadvantages of gas chromatography

In the past decades GC has proved to be an excellent analytical tool for the separation and quantitative determination of a wide variety of compounds in various research, development and quality control areas such as environmental protection (Lucia and Luo, 2002), clinical chemistry Hayashi *et al.*, 2002), chemical plant protection (Balakrishnan *et al.*, 2001), theoretical physico-chemistry (Tsibranska and Assenor, 2000), petroleum (Cox *et al.*, 2001) and chemical industry (Becerra *et al.*, 2002). Because of the high separation capacity, velocity and repeatability, GC techniques have found application in food science and technology too. The high number of stationary phases, detectors with different specific sensitivity and high-temperature high resolution techniques make possible the reliable analysis of many macro- and microcomponents, even enantiomers (Schurig, 2002) present in foods and food products facilitating the assessment of the origin and authenticity of the product.

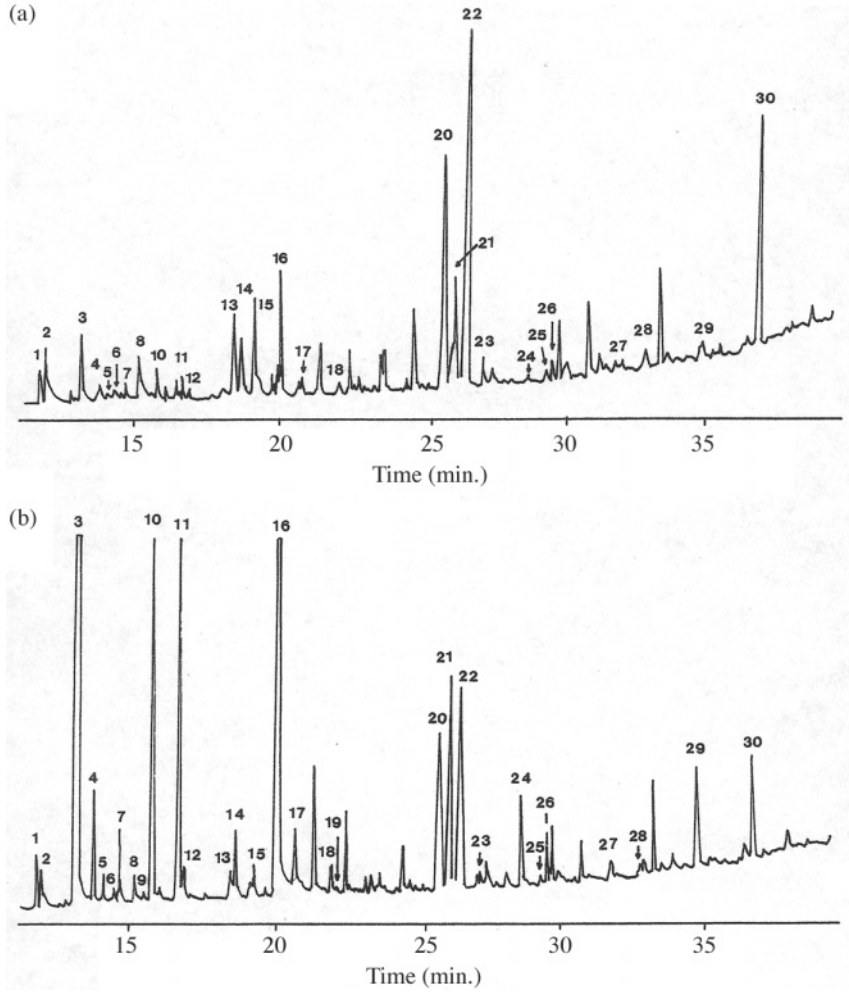

Fig. 9.1 GC chromatograms of the volatiles from raw Baltic herring after 1 day (a) and 6 days (b) of storage (with permission).

The further advantages of the application of GC in food analysis are the low quantity of sample needed for the measurements and the possibility to analyse sample components present in very low concentrations. However, the majority of molecules are not volatile or thermolabile, and cannot be analysed by GC. Much effort has been devoted to the development of derivatisation methods increasing the volatility of analytes and considerable results have been achieved. Although these methods can be successfully used in many cases, derivatisation is sometimes time consuming, increases cost per analysis and represent a possible source of error.

Table 9.4 Proportions of the volatile compounds identified in fresh and stored Baltic herring[a] (with permission)

Peak	Compound	Days after catching		
		1 day (area %)[b]	7–8 days (area %)	Identification[d]
1	2.butoxyyethanol	0,55 ± 0,49	0,29 ± 0,16	MS
2	Nonanal	3,30 ± 2,68	0,99 ± 1,26[C]	MS+RT
3	Acetic acid	2,60 ± 1,18	15,64 ± 5,50[C]	MS
4	2,4-heptadienal	0,49 ± 0,42	0,55 ± 0,28	MS
5	1,5-octadien-3-ol	0,19 ± 0,24	0,18 ± 0,09	MS
6	2-ethylhexanol	0,24 ± 0,23	0,15 ± 0,18	MS
7	2,4-heptadienal	0,19 ± 0,30	0,30 ± 0,13	MS
8	Decanal	3,58 ± 3,39	0,72 ± 0,82[C]	MS
9	3,5-octadien-2-one	0,20 ± 0,24	0,10 ± 0,09	MS
10	Propanoic acid	0,78 ± 0,54	13,23 ± 7,33[C]	MS+RT
11	2-methylpropanoic acid	0,50 ± 0,37	6,37 ± 3,18[C]	MS+RT
12	2,2-dimethylpropanoic acid	0,24 ± 0,17	0,11 ± 0,08	MS
13	Butanoic acid	2,82 ± 1,46	0,52 ± 0,49[C]	MS+RT
14	Pentadecane	3,25 ± 2,85	1,37 ± 0,74[C]	MS+RT
15	1-pentadecene	4,94 ± 3,73	0,75 ± 0,64[C]	MS
16	3-methylbutanoic acid	2,25 ± 1,72	36,33 ± 9,31[C]	MS+RT
17	2,5-octadien-1-ol	0,46 ± 0,32	0,42 ± 0,18	MS
18	Pentanoic acid	0,61 ± 0,67	0,38 ± 0,25	MS
19	Hexadecane	0,19 ± 0,24	0,03 ± 0,06[C]	MS+RT
20	Heptadecane	13,81 ± 3,09	2,27 ± 1,32[C]	MS+RT
21	Heptadecene	3,17 ± 1,30	1,92 ± 0,91	MS
22	1-heptadecene	22,48 ± 20,39	4,51 ± 5,15[C]	MS
23	Benzenemethanol	1,88 ± 1,27	0,33 ± 0,31[C]	MS
24	Benzeneethanol	0,23 ± 0,24	1,72 ± 1,88	MS
25	2-ethylhexanoic acid	2,03 ± 2,77	0,24 ± 0,30 [C]	MS
26	Heptanoic acid	0,75 ± 0,18	0,40 ± 0,14 [C]	MS
27	Phenol	0,84 ± 0,92	1,57 ± 1,61	MS
28	Octanoic acid	0,33 ± 0,37	0,15 ± 0,09	MS
29	Isopropyl myristate	0,77 ± 0,98	0,15 ± 0,13	MS
30	Nonanoic acid	5,00 ± 2,80	1,30 ± 0,55[C]	MS

[a] The fish were analysed as raw.
[b] Mean ± standard deviation for three batches.
[c] Significant difference occurred during storage ($p < 0.05$).
[d] MS+RT are mass spectra and retention time, which were consistent with those of reference compounds. MS is tentatively identified on the basis of mass spectra.

9.5.1 Frequent problems in GC analysis

Instable baseline (drift = baseline movement in one direction during the analysis, wander = baseline movement in different directions, noise = fast fluctuations of baseline) reduces considerably the reliability and repeatability of any measurements. Baseline problems are generally caused by contamination originating from previously injected analytes. Sample components of relatively

low volatility can be collected at the column front and can be slowly released resulting in an unstable baseline. Column bleed also modifies baseline, however, this rising of baseline is caused by the thermal decomposition of the stationary phase at higher temperatures. As the decomposition rate is steady under isothermal conditions, column bleed does not cause baseline drift during isocratic GC measurements. Inadequate conditioning of column and/or detector specially ECD, TCD, NPD, FTIR and MS detector systems, dirty detector and gas may also cause baseline problems.

Slight modifications of retention time (about ± 0.05 min.) are acceptable in routine GC work. The change of flow rate or linear velocity of the carrier gas, leakage of the injector or septum may result in retention time shift. Generally, the concentration of analytes in the sample and the characteristics of the solvent exert a measurable but negligible influence on the retention time. Peak broadening decreases markedly the efficacy of the GC system. It can be caused by incomplete connection between column and injector, inadequate split ratio, too high or too low gas velocity, use of old or damaged column, etc. The loss of separation occurs when the difference between the retention times of peaks decreases while the peak width remains the same. In the case of the loss of resolution both the loss of separation and the peak broadening play a considerable role.

The most common causes of these problems are the deterioration of the column and its contamination with the sample components. The split of peaks is generally caused by an improper injector or injection mode. Tailing of peaks is also related to injector problems or to the binding of polar analytes to the polar active centres of the column not entirely coated with the liquid phase. Many instrumental or technical problems can result in the change of peak size. When each peak behaves uniformly, the changing sample concentration, injection volume or injection method can account for the change. However, in the majority of cases not all peaks behave similarly. This anomaly can be caused by the special adsorption characteristics of the injector wall or the column.

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10

High pressure liquid chromatography (HPLC) in food authentication

L. M. L. Nollet, Hogeschool Gent, Belgium

10.1 Introduction: principles and technologies

The HPLC technique is highly sensitive and very fast in response. The efficiencies of separation are very high. A wide range of compounds may be separated by HPLC because the technique has a wide range of selectivity through the availability of many solvent combinations and packings. New substances to be used as stationary phases are continually developed. No restriction has to be made to sample volatility and derivatization. In most cases sample preparation is small in time and size. Detectors can work continuously and can detect very small amounts. The combination of HPLC and mass detectors opens new and wide horizons. A further trend is the miniaturization of apparatus resulting in less solvent use. In this chapter HPLC techniques on traceability and authenticity of food compounds are discussed. For the detection of phenolic compounds, anthocyanins, and organic acids HPLC is the ideal technique. For other compounds it is 'a' technique not 'the' technique.

10.1.1 Principles and technologies

Chromatography is a separation method of components between two phases. One phase is a stationary bed (the stationary phase) and the other phase is a fluid moving through the stationary phase (the mobile phase). In high performance liquid chromatography or high pressure liquid chromatography (HPLC) the mobile phase is pumped through the column. The parts of a LC instrument are:

- solvent reservoirs
- a solvent delivery system
- an injection device

- the column
- a detector
- a data-acquisition system.

The heart is the column. The majority of the columns have an internal diameter (ID) of 4.6 mm. The stainless steel columns have mostly a length of 10 cm. Guard columns can be coupled with the analytical column to increase lifetime. The packings are 3 μm or 5 μm spherical or irregularly shaped particles.

The trend is to reduce the size of the columns. Microcolumns or microbore columns have a diameter between 0.5 to 1.0 mm; the ID of capillary columns is 100 to 500 μm and the ID of nanoscale LC 10 to 100 μm. The sample is injected via an injection valve with a sample loop of usually 10 μl in the mobile phase. Flow rates are in the range of 0.1 to 10.0 ml/min. Important characteristics are flow rate stability, flow precision and accuracy, and reproducibility.

In adsorption or normal phase liquid chromatography (NPLC or NP-HPLC) we have a polar stationary phase. Polar-bonded phases are widely replacing silica gel or alumina. Diol phases, amino phases, and cyano phases are examples of polar-bonded phases. The best fitted eluent is dependent on many factors such as stationary phase and adsorption. One solvent or a binary mixture of solvents (apolar diluent and polar modifier) are frequently used.

Polar and moderated polar compounds are well separated by NPLC techniques. In reversed-phase liquid chromatography (RPLC or RP-HPLC) the stationary phase is hydrophobic where the mobile phase is highly polar. A wide choice of reversed-phase columns is available. The majority are C_8 or C_{18} silica bonded phases. Alternatives are polystyrene divinylbenzene (PS-DVB) and porous glassy carbon (PGC) phases. Mobile phases consist of water plus organic modifiers such as methanol, acetonitrile, and tetrahydrofuran (THF).

To separate strongly retained molecules gradient elution is performed. A wide range of compounds can be determined by RP-HPLC.

In ion chromatography (IC) three different modes exist:

1. Ion exchange – based on their charges samples are separated by cation or anion exchangers. These exchangers are either strong (SCX, strong cation exchanger, and SAX, strong anion exchange) or weak (WCX, weak cation exchanger, and WAX, weak anion exchanger).
2. Ion exclusion – an ion-exchange packing separates nonionic compounds through differences in acid strength, size, and hydrophobicity.
3. Ion pairing – the separation is a result of the combination RP-phase, mobile phase, and an ion-pairing agent.

In size-exclusion chromatography (SEC or SE-HPLC) the size of the molecules is the separating criterion.

Important factors to be considered when using a detector are, among others, noise, flow sensitivity, detection limit, and quantification limit. The most frequently used detector in combination with LC is the UV absorbance detector. The changes in the absorbance of light are measured in the range of 190 to

700 nm. A filter is used to select a specific wavelength for measurement. For capillary LC a portion of the capillary is adjusted in the light beam of the UV absorbance detector.

In the photodiode array detector (PDA), another photometric detector, the total light is passed through the flow cell and is dispersed by diffraction. An array of photosensitive diodes measures the light. A microprocessor translates the measured values to results. Fluorescence is a process in three stages: excitation, excited state, and emission. Fluorescence emission results in increased selectivity and sensitivity. For fluorescence detection a pre- or postcolumn derivatization step can be necessary. Chemiluminescence, a fourth type of photometric detector, is based on the production of light in a chemical reaction.

In electrochemical detection a distinction is made between conductivity measurements and amperometric measurements. This type of detection is a concentration-sensitive technique. The refractive index (RI) detector is another possible detector. A difference in refractive index between a sample and a reference is monitored. The light-scattering detector can also be used. Three steps are involved in the process: nebulization of the eluent, evaporation of the solvent molecules and measurement of the scattered light.

The mass spectrometry (MS) detector has currently many applications with LC. Different techniques to interface or hyphenate liquid chromatography and mass spectrometry are available. A widely used interface is atmospheric pressure ionization (API). The column effluent is nebulized into an atmospheric ion region. Alternatives are atmospheric pressure chemical ionization (APCI) or ion spray.

Electrospray MS (ESI-MS) has three steps: nebulization of the solution into electrically charged droplets, liberation of ions from the droplets, and transportation of ions into the vacuum of the analyzer. Evidently the cited MS techniques are not exhaustive.

Sample preparation and clean-up are very important steps of analyses. The components of interest have to be isolated from the sample matrix and interfering substances have to be removed. Accuracy, detectability, and selectivity are highly improved. Frequently employed procedures are: lyophilization, ultrafiltration, and liquid-liquid extraction (LLE). Organic compounds are removed from an aqueous solution into a water-immiscible solvent in LLE. The most widely used extraction method is solid-phase extraction (SPE). Analytes are held by a sorbent in a plastic cartridge. A selective solvent washes out the analytes of interest. A great choice of sorbents is available. Offline and online SPE methods are discussed in the literature.

In solid-phase microextraction (SPME) a silica fibre coated with a stationary phase is exposed to the sample. The analytes are attracted by the coating. Next the fibre is transferred to an instrument for desorption. Supercritical fluid extraction (SFE), a powerful extraction technique, has some advantages such as low solvent use, less time consuming, and more selective. In stir bar sorptive extraction (SBSE), a recently developed extraction technique, analytes are

sorbed to a magnetic bar coated with PDMS (polydimethylsiloxane). The compounds are afterwards desorbed by heat in a thermal desorption unit. For further reading on HPLC the reader is directed to refs 1–4.

10.2 Authenticating fruit products

In fruit and fruit products different compounds can be monitored for authenticity purposes: phenolic compounds, organic acids, carotenoids, amino acids, anthocyanins, and sugars. Most of the studies concern the differentiation of citrus fruits and pome fruits.

10.2.1 Organic acids

Hea-Jeung Whang *et al.*[5] discuss a reversed-phase method (YMC ODS-AQ column) on the organic acids contents of apple juice. A laboratory-made apple juice is compared with commercial apple juices. Authors found concentrations of 62–402 mg/L DL-malic acid, 48–360 mg/l L-malic, 1.81–15.74 mg/L citric acid, and not detectable-0.5 mg/L fumaric acid. From the results quality and authenticity of apple juices can be monitored. Adulterated apple juice contains D-malic acid, not present in natural apple juices. Higher amounts of fumaric and citric acids are indicators for adulteration. Cranberry juice contains normally quinic acid, malic acid, and citric acid. D-malic acid is an indication of adulteration; this isomer does not occur naturally.[6]

10.2.2 Phenolic compounds, polyphenols, and dihydrochalcones

Phenolic compounds, a diverse class of compounds containing a hydroxyl group on a benzene ring, include, among others, flavones, flavonols, flavonoids, polyphenols, and chalcones. When quince is scarce the jam can be adulterated by apple and/or pear puree. Silva B.M. *et al.*[7] analyzed by reversed-phase HPLC the phenolic compounds and procyanidin polymers. The detection of arbutin, the characteristic hydroxyquinone of pear, in several samples suggests adulteration with pear purée.

For the detection of dihydrochalcone glycoside (phloretin glycoside and phloretin xyloglycoside) in apple products HPLC-PDA is applied.[8] The detection limit is 1.6 mg dihydrochalcones/l. Dihydrochalcone glycoside is suggested as a quality marker for the identification of apple juice. In the studies of Versara A. *et al.*[8] and Spanos J.A. *et al.*[9] phloretin glycoside or phloridin is regarded as an authenticity factor of apple products. Every plant or fruit has a characteristic flavonoid pattern so differentiation between similar species is possible. Those flavonoid patterns can be used for adulteration studies. Tomas-Lorente F. *et al.*[10] identified the characteristic phenolic and flavonoid compounds in apricot, peach, plum, apples, pear, strawberry, and sour orange jams. To overcome the problems of extraction of those compounds in the

Table 10.1 Phenolic compounds characteristic for quince, apple, and pear

Quince phenols	Procyanidin
	3-O-caffeoylquinic acids
	4-O-caffeoylquinic acids
	5-O-caffeoylquinic acids
	Rutin 3-O-galactoside
	Quercetin 3-O-galactoside
	Quercetin 3-O-xyloside
	Quercetin 3-O-rhamnoside
Apple phenols	Phloretin 2′-xylosylglycoside
	Phloretin 2′-glycoside
Pear phenols	Arbutin

presence of large amounts of sugar and pectin they filter the extracts through Amberlite XAD-2 resins. The same extraction method was used in an authenticity study of quince jelly[11] using HPLC. Similar profiles of more then eight phenols are found (Table 10.1). No characteristic compounds of apple or pear are found. In this study the quince products are not adulturated.

The paper of Ritter G. *et al.*[12] describes the difference in the phenol pattern of apples and sorb apples (*Sorbus domesticus*). Sorb apples are rich in procyanidines and contain phenol carboxylic acid derivatives not occurring in apples. To authenticate apple and pear products, polyphenol patterns, identifying quercetin glycosides, phenolic acids, and dihydrochalcone derivatives, are established.[13] The characteristic quercetin and isorhamnetin glycosides and dihydrochalcones are used to control the authenticity of apple and pear juice.[14] Detection methods are diode array and MS. In this study arbutin appears not to be a specific marker of pear products. Schnull H.[15] discusses HPLC flavonoid fingerprint methods for fruit juice authenticity purposes.

Dihydrochalcone glycosides, characteristic substances of apples, may be useful as authenticity markers. Tomas-Barberan *et al.*[16] identified the substances by a RP-HPLC method. Flavonoid profiles may be used for authenticity purposes of fresh citrus fruits and jams.[17] From RP-HPLC (LiChrochart RP-18) and diode array detection six flavonoids can be identified (Table 10.2).

Table 10.2 Citrus flavonoids

Eriocitrin
Neoeriocitrin
Narirutin
Naringin
Hesperidin
Neohesperidin

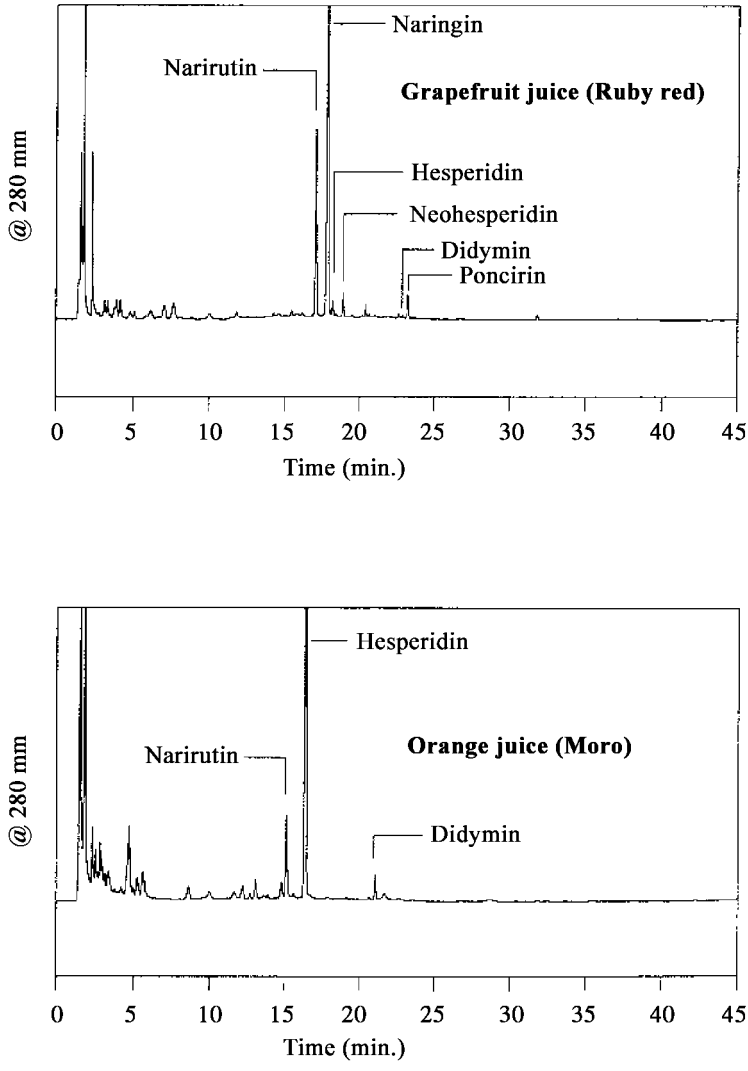


Fig. 10.1 Separation by HPLC of flavanone glycosides in grapefruit juice and orange juice (source: ref 19).

Orange juice contains no naringin or neohesperidin; grapefruit contains both. To detect adulteration of orange juice with grapefruit juice Rouseff R.L.[18] developed a HPLC method based on naringin/neohesperidin ratios. In Fig. 10.1 typical chromatograms of flavanone glycosides in grapefruit juice and orange juice are shown.[19]

Ooghe W. and Detavernier C.[20] developed a method to differentiate sweet orange juices from other citrus juices using flavone glycosides (FG) and polymethoxyflavone (PMF) patterns. The method is a combination of a RP18

column and photodiode array detection. The same group of Ooghe[21,22] used the patterns of flavanone glycosides and polymethoxylated flavones to detect orange juice falsifications. A 15 cm Waters Novapak RP 18 column, gradient elution (aqueous phosphate buffer and acetonitrile) and PDA detection are used.

Grandi R. *et al.*[23] studied the flavonoid composition of lemon juice by HPLC and photodiode detection (270 nm). Hesperidin and especially eriocitrin are characteristic flavonoids of lemon juice. Orange pulpwash can be used as a substitute for orange juice. Phlorin (phloroglucinol monoglycoside) was used as an indicator of pulpwash. Phlorin is present in much greater quantities in pulpwash. Johnson R.L. *et al.*[24] developed a HPLC method (3 μm C_{18} column, UV detection at 214 nm, isocratic 50 mM KH_2PO_4 elution). Myricetin is a marker compound of peach; two coumarins are specific for apricot. In the study of Fernandez de Simon B. *et al.*[25] a binary gradient HPLC method was developed for the separation of phenolics, 3-flavanols, flavonol aglycones, and flavonol glycosides in several juices and nectars of, among others, peach and apricot.

Flavanol glycosides may indicate adulteration of blackcurrant products with red currants[26] and with blackberries.[27] Quercetin-3-O-glycoside is a good marker of adulteration of black currant products with blackberries.[27] Wen L. and Wrolstad R.E.[28] investigated the phenolic composition of pineapple juice by HPLC. On the chromatogram nine peaks are identified: aromatic amino acids, serotonin, 2,5-dimethyl-4-hydroxy-3(2H)furanone (DMHF) and its glycoside and sinapyl derivatives. Three of these sinapyl derivatives are unique for pineapple juice and may be useful markers for authenticity.

10.2.3 Carotenoids

By analysis of the carotenoid content in orange juices[29] non-authentic samples can be detected. The quantity of total carotenoids in hand-squeezed samples is 0.42 mg/l and 0.62 mg/l for frozen concentrated orange juice. The adulterated sample contains only 0.06 mg/l carotenoids. A further indication of adulteration is the presence of sorbic acid in the sample.

10.2.4 Amino acids

In ref. 30 a chromatographic profile of amino acids in fruit juices as (1-Fluoro-2,4-dintrophenyl)-5-L-alanine amide derivatives is established, useful to identify added racemic amino acids. See also refs 31 and 32.

10.2.5 Anthocyanins

The determination of anthocyanin patterns of red fruits to determine the authenticity of natural colourings was standardized in an ISO-gradient technique. Retention times are expressed using the Reversed Retention Index. Garcia-Viguerra C. *et al.*[33] analyzed the anthocyanin content of different berries

(SPE, RP-HPLC, DAD) for authenticity purposes. Anthocyanin profiles have also been used by Lee H.S. and Hong J. for the detection of adulterations in fruit juice products.[34]

10.2.6 Sugars

Cellobiose, maltose, and maltotriose are useful markers of adulteration of fruit juices.[35] Cellobiose is an indicator of illegal use of cellulase during processing; maltose and maltotriose may indicate added sweeteners.

10.2.7 Miscellaneous

Detection of adulteration of cranberry juice products can be performed by HPLC analysis of organic acids (quinic, malic, citric, and fumaric acid), the sugar profile and the anthocyanins.[36] HPLC analysis of glucose, fructose, and sucrose, malic, citric, quinic, shikimic, fumaric, and chlorogenic acids and hydroxymethylfurfural are useful for the verification of authenticity of apple juice.[37] Hofsommer H.-J. discusses different techniques, among others, HPLC and ion-exchange chromatography of different substances (flavonoids, organic acids, sugars, anthocyanins), for authenticity of fruit juices purposes.[38]

10.3 Authenticating oils

The most studied oil is olive oil. A wide variety of methods have been developed to detect the authenticity of olive oils.

10.3.1 Olive oils

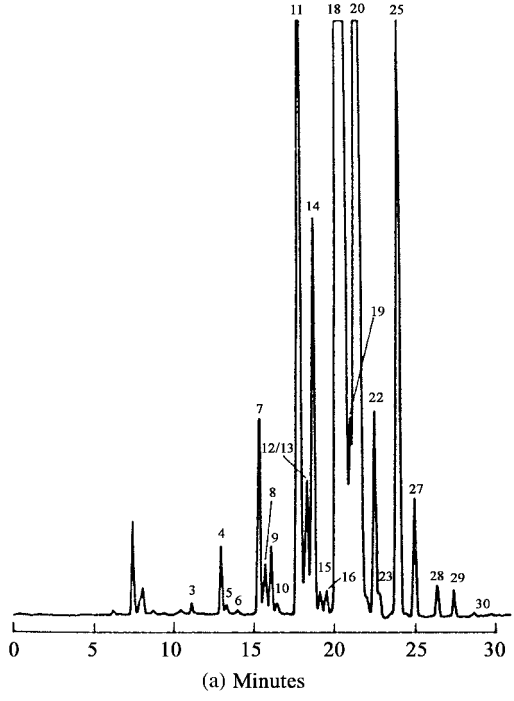
The triglyceride profiles for soybean oil and olive oil are significantly different so the addition of low levels of soybean in olive oil can be detected (see chromatograms in Fig. 10.2).[39] El-fizga[40] developed a method for the detection of oils with high linoleic acid content in olive oil. With the help of RP-HPLC (2 Supelcosil-LC8 columns – aceton-acetonitrile (70:30 v/v) – RI) and an authenticity factor the extent of adulteration can be calculated. The presence of high linoleic acid content vegetable oils in olive oil is measured as an authenticity factor (Au).

$$\mathrm{Au} = \frac{100 - \mathrm{ECN} \times 42(\%)}{\mathrm{ECN} \times 42(\%)}$$

ECN = equivalent carbon number.

$$\mathrm{ECN} = \mathrm{CN} - \mathrm{X.n}$$

CN = total number of carbon atoms; X = number of double bonds; n = factor for double bond contribution.



Peak number	*Triacylglycerol*
1	LnLnLn
2	LLnLn
3	LLnL
4	LLL
5	LLnO
6	LLnP
7	LLO
8	OLnO
9	LLP
10	OLnP
11	OLO
12	OPaO
13	LLS
14	OLP
15	OPaP
16	PLP
17	SLnP
18	OOO
19	OLS
20	OOP
21	PLS
22	POP
23	PPaS
24	PPP
25	OOS
26	SLS
27	POS
28	PPS
29	SOS
30	SPS
31	SOA
32	SSS
33	SSA

P = palmitic acid, Pa = palmitoleic acid, S = stearic acid, O = oleic acid, L = linoleic acid, A = arachidic acid.

Fig. 10.2 (a) Triacylglycerol profile of 10% soybean oil in olive oil (source: ref 39); (b) Triacylglycerol profile of 5% soybean oil in olive oil (source: ref 39).

The ECN 42 triacyl group shows the greatest difference in triacylglycerol content between olive oil and high linoleic acid oils. Virgin olive oil has $Au = 98.2 \pm 3.86$. Authenticity factors for corn, sunflower, and soybean oils are 3.2 ± 0.02, 3.5 ± 0.06, and 3.2 ± 0.19 respectively. The extent of olive oil adulterants can be expressed by following equation:

$$\text{Added oil}(\%) = \frac{\text{ECN42}(\%) - \text{b}}{\text{a}}$$

where a and b are constants according to the added oil.

The determination of the identity of imported and exported olive oil samples is necessary in the EU for legal purposes. Gambacorta G. *et al.*[41] developed a method to determine the similarity between two or more olive oils. The fatty acid fraction is analyzed by high-resolution gas chromatography (screening test) and the triglyceride fraction by HPLC (confirmatory test). Eighty samples (66 exported/14 imported) are examined and 12% of exported samples are partially or totally substituted with other oils.

Pressed hazelnut oil was detected in admixtures with virgin olive oil by HPLC analysis of polar components. Polar fractions from six commercial pressed hazelnut oil and 12 virgin or blended olive oil samples were analyzed by Gordon M.H. *et al.*[42] A component in the hazelnut oil appears in a region that is clear in the chromatogram of virgin olive oil, suggesting that HPLC can be used to detect the adulteration of virgin olive oil with pressed hazelnut oil. This method can also be applied to blends of oils at a level above 2.5%. The presence of stigmastadienes, of which stigmasta-3,5-diene is the most important, squalene isomers, or wax esters in virgin olive oils is an indication that the oils my have been adulterated with refined oils.[43]

Authors developed a HPLC method enabling the separation of all compounds in one single run. Collected samples are then analyzed quantitatively by HR-GC. Results compare favourably with the EC official method but is less time consuming. For accurate determinations the official method is still preferred. The carotene content and lutein contents of olive oil, determined by HPLC, are found to be direct indications of oil quality, virgin or husk.[44] Spectroscopic measurements correlate with the HPLC data. Both methods are equally suitable for quality or authenticity assessments.

10.3.2 Almond kernel oils

Triglyceride analysis has potential for characterization and classification of almond cultivars. This is of interest for genetic improvement of the species and for adulteration detection. Martin-Carratala M.L. *et al.*[45] reported the use of HPLC for the analysis of triglyceride composition of 19 cultivars. Nine triglycerides are quantified and multivariate techniques and principal component analysis are applied to the data. The American cultivar Texas can be distinguished from others by cluster analysis. The largest group includes most of the Spanish cultivars and the Italian cultivars are grouped together.

10.3.3 Corn oils

Minor components such as free and acylated sterols and steryl glycosides, tocopherols, and isoprenoid alcohols, and triglycerides in corn oil were analyzed by HPLC to determine the components usable for the authentication of corn oils.[46] Pure rapeseed, sunflower, and corn oils and corn oil in the other oils are assessed. Isolation of free and acylated sterols and steryl glycosides and isoprenoid alcohols is followed by derivatization into their 9- and 1-anthroylnitriles. Tocopherols and triglycerides are determined by HPLC, while sterols are isolated from the unsaponifiable matter and determined by capillary GLC as trimethyl silane derivatives. Isoprenoid alcohols are the most decisive factor; tocopherols and triglycerides are also useful.

10.4 Authenticating other foods

10.4.1 Ginseng

The dietary supplements Asian ginseng (*Panax ginseng)* and North American ginseng (*Panax quinquefolius*) are widely used. The latter ginseng is more expensive. A HPLC-evaporative light scattering detection (ELSD) method was used for the identification of both ginsengs. Ginsenoside RF of Asian ginseng and 24 (R)-pseudoginsenoside F11 of North American ginseng can be identified at levels of 32–50 ng.[47] HPLC-ELSD is a sensitive method for the identification of the origin of ginsengs. The same markers were used in the study of Chan T.W.D. *et al.* for the differentiation and authentification of *Panax ginseng* and *Pananx quinquefolius*.[48] Ginsenosides are separated using HPLC with electrospray mass spectrometry detection. Identification is based on positive-ion ionization of the characteristic molecular ions and thermal degradation products. The method is reliable and detection limit is in the nanogram range.

10.4.2 Vanilla and saffron

Solinas M. and Cichelli A. used a HPLC method to assess the authenticity of powdered and stranded saffron.[49, 50] Characterization is based on the flavour and colour components of saffron such as picrocrocin, crocin, safranal and isomers.

Scharrer A. and Mosandl A. analyzed 15 vanilla samples of different locations and harvest years on the content of vanillin, vanillic acid, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid. HPLC and GC methods are compared and give the same results. The ratios of those components are used for the authenticity of vanilla.[51] In the same study the same authors compared extraction efficiency of diethyl ether and ethanol/water. Extraction efficiency is better for ethanol/water. Vanillin concentrations and vanillin/vanillic acid and vanillin/4-hydroxybenzaldehyde ratios are similar for the different origins and production years. Comparison of results for authentic samples with reference values show that the reference values specify too narrow a range. Authors suggest a revision of the reference values to reflect actual concentrations and ratios of vanilla constituents.

The very expensive vanilla extract is often replaced by natural vanillin or synthetic vanillin. A HPLC method has been developed by Herrmann A. and Stoeckli M.[52] The method is based on the separation and identification of the following constituents of vanilla: 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, vanillyl alcohol, vanillin, isovanillin, ethyl vanillin, and coumarin. HPLC conditions are: RP-18 (10 μm) column, gradient elution of 0.2M acetic acid in water and in water-methanol (2:8), UV detection. The HPLC method is well suited for authenticity control of commercial vanilla extracts. A HPLC method for the quantification of coumarins as adulterants in different vanilla flavourings was described by Thompson R.D. and Hoffmann T.J.[53]

10.4.3 Honey

Hydroxymethylfurfural (HMF) is normally present in low quantities in honey; large quantities indicate a heat treatment. To distinguish citrus honey from non-citrus honey the content of methyl anthranilate can be measured. Nozal M.J. *et al.*[54] developed a HPLC method for the determination of HMF, furfural, 2- and 3-furoic acids, 3-furaldehyde and methyl anthranilate in honeys of different origin and honeydew. Conditions of HPLC are: RP, gradient elution of aqueous acetic acid:methanol or acetonitrile:water, and UV detection at 250 nm. In all samples HMF is found in concentrations varying from 1 to 8 ppm. Honey methyl anthranilate is found only in orange blossom.

To establish authenticity of the botanical origin of honeys the phenolic composition could be an interesting factor. In the HPLC method developed by Andrade P. *et al.*[55] heather honeys are characterized by ellagic, p-hydroxybenzoic, syringic, and o-coumaric acids where lavender honeys are characterized by gallic acid. Swallow K. W. and Low N. H.[56] use the minor oligosaccharides as fingerprints for the authenticity of honeys. The developed method proves to be difficult.

10.4.4 Soy products

The combination of isoflavones and tartaric acid results in shoyuflavones. Those products have been isolated by Kinoshita *et al.*[57] and have been used for identification of sauces by different manufacturers by means of a chemometric analysis of HPLC profiles. Authors studied the enzymatic formation of ether linkage producing shoyuflavones from genistein and (+−)-trans-epoxysuccinic acid.

The same authors[58] studied the differentiation of soy sauce produced from whole soybeans and defatted soybeans by pattern recognition analysis of HPLC profiles. Prices of M-soya sauce (whole soya beans) are higher then those of K-soya sauce (defatted soya beans). Ferulic acid is identified as one of the key components for the differentiation. Components associated with types of soya bean are determined by a fractional factorial experimental design combined with multiple linear regression analysis.

10.4.5 Milk

The use of soybean proteins in bovine milk is forbidden in many countries and other countries have regulations on maximum allowance levels. Soy proteins in unheated milk were analyzed by Ashoor S.H. and Stiles P.G.[59] Frutos *et al.*[60–61] used RP-HPLC to separate whey proteins from bovine, ovine, and caprine species. Using a gradient method the detection of cows' milk in ewes' and goats' milk was possible as was the detection of ewes' milk in milk mixtures. Similar studies were carried out by Romero *et al.*,[62] Pellegrino *et al.*,[63] De Noni *et al.*,[64] and Torre *et al.*[65]

Haasnoot *et al.* used casein fractions to detect the presence of ovine milk in bovine milk or vice versa.[66] Adulterations of goats' milk and ewes' milk can be detected quantitatively with bovine α_{S1}-caseine as marker; the marker for the presence of cow casein in sheep and goat milk was the degradation product α_{S1}-I-caseine. Mayer *et al.*[67] used para-κ-casein to determine the percentages of cows', ewes', and goats' milk in cheeses.

EU regulations stipulate that skim milk powder may not contain solids from whey or buttermilk. Olieman and van den Bedem[68] presented a SE-HPLC method of detection of adulteration of skim milk powder. Addition of more than 0.8% of rennet whey total solids to skim milk powder and sweet buttermilk powder was accurately detected. The same authors[69] presented a detection method for glycomacropeptide (GMP). The addition of 0.2% of rennet whey solids can be detected.

10.4.6 Meat

The presence of soy protein, caseinate, and whey protein in unheated beef, pork, chicken, and turkey was possible as was that of added nonmeat protein. Quantitative determinations prove to be difficult. In Cattaneo T.M.P. *et al.*[70] their detection by SE-HPLC in dairy products was described. Added soy protein could also be detected at levels of 6 g/kg in cheese samples. The presence of animal whey proteins in vegetable milks or of soybean in animal milk was possible by a RP-HPLC method.[71]

10.4.7 Essential oils

Quality and authenticity may be established by oxygen heterocyclic compounds such as coumarins, psoralens, and polymethoxylated flavones (PMFs) in citrus essential oils. Dugo P. *et al.*[72] developed a HPLC method in combination with positive-ion atmospheric pressure chemical ionization-mass spectrometry (PI-APCI-MS). Two specific PMFs in bergamot oil, one coumarin in lemon oil, and a different coumarin in bitter orange oil are identified. This method can be useful for authenticity and adulteration purposes. Mondello L. *et al.*[73] linked HPLC and high-resolution gas chromatography (HRGC) for the analysis of the volatile fraction, in casu linalol terpinen-4-ol, of citrus essential oils.

Philipp O. and Isengard H.-D.[74] used a HPLC method for the determination of coumarins and psoralens naturally present in unadulterated lemon oil samples. Analyses are carried out using a Spherisorb ODS column with a gradient of water/acetonitrile (flow rate 1.5 ml/min), methanol/water (flow rate 1.0 ml/min) or water/methanol/acetonitrile (flow rate 2.0 ml/min), and photometric detection. By this simple, rapid, and precise method adulterants such as ethyl p-dimetylaminobenzoate or methyl anthranilate can be detected. The dilution of cold-pressed lemon oil with distilled oil was studied by Marko-Varga G. and Barcelo D.[75] Coumarins exhibit strongly at 313 nm.

10.4.8 Sherry wine vinegar

Sherry wine vinegar is a vinegar produced by traditional methods involving slow ageing in wooden barrels. Garcia Parilla M.C. *et al.*[76] investigated changes in phenolic compounds during ageing of sherry vinegars. The samples are from the two classes *Vinagre de Jerez*, aged for less than two years and *Vinagre de Jerez Reserva*, aged for more then two years. Phenolic compounds are determined by HPLC and photodiode array detection. Statistical analysis by multiple analysis of variance (ANOVA) shows significant differences between the groups. Three compounds show an increasing trend in most of the ageing systems. These are gallic acid, 5-hydroxymethylfuraldehyde, and coumaroyltartaric acid.

10.4.9 Wine

GC-SIRMS (stable isotope ratio mass spectroscopy) analysis of sorbitol concentrations was used for evaluating the authenticity of wine. The SIRMS follows a semi-preparative HPLC purification. The isotope ratios show that in some samples sorbitol is not of grape origin. Concentrations for one country out of twelve are greater then 0.2 g/l even up to 6.0 g/l.[77] The HPLC technique is a useful technique for authenticity and adulteration purposes in wine.[78] In tables and chromatograms the use of organic acids, sugars, and glycols as markers are discussed.

10.4.10 Whiskey

US legislation stipulates that straight whiskey should be aged in freshly charred oak barrels for at least two years, and that caramel should not be added as a colouring agent. However, caramel may be used in blended whiskeys to compensate for loss of colour during the blending process. Therefore, authentication of straight whiskey cannot be performed by measuring colour intensity. Jaganathan J. and Dugar S. M.[79] reported that authentic straight whiskeys have a ratio of furfural to 5-hydroxymethyl-2-furaldehyde (HMF) of 2:1 or higher after the ageing period. They developed a HPLC technique for determining furfural and HMF at low parts per million levels. The method may

be used for investigation of misbranded products and for routine monitoring of whiskey for compliance with legislation.

10.4.11 Coffee

Determination of 5-HMF, furfural, 5-O-caffeoylquinic acid and caffeine in coffee beans is helpful for the authenticity study, characterization of origin, and quality of the beans. A RP-HPLC method with diode-array detection and UV detection at 280 nm was developed by Chambel P.[80] Bohacenko I. and Vesely Z.[81] used as indicators of adulteration of pure instant coffee carbohydrates, free mannitol, free fructose, total glucose, and total xylose. The HPLC method is combined with refractometric detection. Nineteen samples have been analyzed; imported samples in original packaging, bulk-imported samples packed locally, and locally manufactured samples. Of the last two groups only one sample of each is authentic.

10.4.12 Wheat pasta

A HPLC method, involving the separation of gamma-gliadins, was worked out to investigate the authenticity of dried durum wheat.[82] Pasta is traditionally made from *Triticum durum* wheat. The use of *T. aestivum* results in a pasta with inferior texture and quality. The common wheat authenticity limit in this study is 8%. In 249 samples one sample contains more than 8% common wheat, four samples between 3 and 5%, and one sample between 5 and 8%. Polyacrylamide-gel electrophoresis (PAGE) is also used; both methods give same results. McCarthy *et al.*[83] developed also a separation method based on the gliadin fraction. Another RP-HPLC method was proposed by de Noni I. *et al.*[84] on the basis of water-soluble proteins and albumins.

10.4.13 Cheese

Pecorino Sardo is a traditional ewes' milk cheese made in Sardinia, Italy, and is protected under EU rules by a Denomination of Protected Origin. An analytical technique for determining the geographical origin, and hence authenticity, of Pecorino Sardo samples was reported by Manca G. *et al.*[85] Stable isotopes ratios (.sup.1.sup.3C/.sup.1.sup.2C and .sup.1.sup.5N/.sup.1.sup.4N) of casein are measured by isotope ratio MS (IRMS) and free amino acid ratios (His/Pro, Ile/Pro, Met/Pro and Thr/Pro) are determined by HPLC in samples of ewes' milk cheese from Sardinia, Sicily, and Apulia. Values are independent of ripening time. Multivariate data treatments are performed using both supervised (principal component analysis and cluster analysis) and supervised (linear discriminant analysis LDA) methods, and reveal good discrimination possibilities for the cheese according the place of origin. In particular, LD analysis of the .sup.1.sup.5N/.sup.1.sup.4N ratios can be used to achieve 100% discrimination and classification of the cheese samples.

10.4.14 Quillaja

The saponin-rich foaming agents quillaja extracts (QE) of *Quillaja saponaria Molina* are widely used. Low-cost saponin sources or dilution with carriers are possibilities of adulteration. San Martin R. and Briones R.[86] used a RP-HPLC method to measure quillaja saponins. Samples are a number of commercial QEs, the bark of *Quillaja* and whole *Quillaja* wood. Saponin concentrations of the non-refined extracts are in the range of 190–200 g saponins/kg and the semi-refined in the range of 750–800 g saponins/kg.

10.4.15 Cocoa butter

The triglyceride (TG) composition is a parameter for the authentication of cocoa butter. Buchgraber M. *et al.*[87] compared HPLC and GC techniques. For HPLC two columns are coupled in series and the detection mode is ELSD. Both methods result in comparable precision. Capillary GLC has the advantages of higher sample throughput and negligible consumption of chemicals.

The reader is directed for further reading to refs 88 to 91.

10.5 Future trends

Downscaling or miniaturization of columns and sample treatment are the challenges of the future. Miniaturization involves less sample volume, fewer solvents, and less time. Regulations and government commitments require more separation efficiency; the hyphenation of HPLC and MS, allowing the development of new methodologies, is a move in the right direction. Automation of the separation and detection and lower costs of the apparatus will be helpful. A lot of work has to be carried out to find reliable marker compounds to confirm the authenticity of and provide traceability for foodstuffs. Fraud is and remains a temptation often yielded to.

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11

Enzymatic techniques for authenticating food components

G. Henniger, University of Applied Sciences – Lemgo, Germany

11.1 Introduction

Enzymatic methods for the determination of a series of foodstuff ingredients are useful tools in routine food analysis. They allow the specific, reliable, precise, and quick determination of some sugars, acids and salts, alcohols and other compounds, which occur in nature and are contained in foodstuffs. These compounds are either typical for the respective food, or they are often the materials which determine its monetary value; their determination is a part of basic analysis. The inexpensive equipment needed for enzymatic analysis is contained in the laboratories, the reagents are commercially available and they are safe and not dangerous. Skilled workers can do the determinations. Many enzymatic methods are published in laws and regulations, as well as being standardised and recommended by national and international analytical organisations/committees.

This chapter is not a replacement for textbooks in biochemistry/enzymology and for instructions published, e.g., in the pack inserts of test kits. It should contain useful details for analysts already using enzymatic methods as well as information for analysts interested in this methodology.

11.1.1 Enzymatic analysis

Enzymatic analysis is today an important methodology in food analysis. It allows the quick and reliable, i.e., the specific and precise, determination of some food ingredients. The equipment for measurement is contained in the laboratories, the necessary reagents are commercially available. The handling of equipment and reagents is easy and can therefore be done also by semi-

skilled workers. Enzymatic measurement techniques are used in the food industry and in official/governmental laboratories (food inspection) for routine analysis.

Enzymatic analysis as an analytical technique is, first, analysis using enzymes as reagents to measure all substances capable of being transformed by enzymes, the analytes are the metabolites in living organisms and they are the substrates of the enzymes. Secondly, the determination of enzyme activities, e.g., to detect and to control heating processes (e.g., honey, milk, vegetables), to notice animal diseases (e.g., mastitis) and to differentiate between fresh and frozen meat. The activity of an enzyme is measured by the rate of consumption of the reactants (substrates) or the formation of products. (This part of enzymatic analysis will not be dealt with in this chapter.)

Note that in the opinion of the author, enzymatic analysis is not analysis where enzymes are used as auxiliary reagents in chemical procedures and with chromatographic techniques, e.g., during sample preparation (e.g., in the analysis of 'fibre') or in ('post-column derivatisation') detection systems in chromatography. The determination of substances with enzyme-labelled reagents by combination of enzymatic methods with immunological procedures (enzyme-immunoassays) may be said to be a sub-chapter of enzymatic analysis.

The analytes in Table 11.1 are discussed in detail in this chapter. Other analytes capable of enzymatic analysis are listed in Table 11.2.

Table 11.1 Analytes discussed in this chapter

Sugars	Acids/salts	Alcohols	Others
D-fructose	acetate	cholesterol	acetaldehyde
D-galactose	L-ascorbate	ethanol	ammonia
D-glucose	citrate	glycerol	creatine/creatinine
lactose	formate	D-sorbitol	urea
lactulose	D-gluconate	xylitol	
maltose	L-glutamate		
starch	D-3-hydroxybutyrate		
sucrose	D-isocitrate		
	D- and L-lactate		
	D- and L-malate		
	nitrate		
	oxalate		
	succinate		
	sulphite		

Table 11.2 Other analytes capable of enzymatic analysis

Sugars	Acids/salts	Alcohols	Others
inulin D-mannose raffinose	L-alanine L-aspartate phosphate pyrophosphate pyruvate	dihydroxyacetone	L-carnitine choline free fatty acids guanosine-5′- phosphate hydrogen peroxide L-α-lecithin triglycerides

11.2 Analysing enzymes in sugars, acids, salts, alcohols and other compounds

Enzymes are the catalysts of living organisms. They accelerate the speed at which the equilibrium of a chemical reaction is reached without having an influence on the balance of this reaction. The other advantage of using enzymatic methods in analysis is their high specificity. Enzymes often react highly specifically, on the other hand not all enzymes can be expected to be 'absolutely' specific. The use of an enzyme with a known non-specificity need not necessarily be a disadvantage. Often a method needs several coupled reactions. In this case only one of the enzymes in the reaction chain has to be specific to make the overall reaction specific. Nevertheless, the specificity of enzymes should always be known when they are used for analytical purposes.

Ideally, enzymes for analysis are absolutely specific and convert only the analyte. A typical example for this group of enzymes in sugar analysis is glucose-6-phosphate dehydrogenase (G6P-DH) which reacts with glucose-6-phosphate (G-6-P) only.

Specificity of a method and accuracy of the results are also given when the less specific reaction of an enzyme is combined with a highly specific enzyme/reaction as shown with the less specific hexokinase (HK) together with the absolutely specific glucose-6-phosphate dehydrogenase (G6P-DH) in the determination of D-glucose: HK reacts with D-glucose, D-fructose and D-mannose in the presence of the coenzyme adenosin-5′-triphosphate (ATP) and the formation of the respective sugar-6-phosphates. From these only G-6-P reacts in the G6P-DH catalysed reaction, which also serves as the indicator reaction in the photometric measurement.

A further type of specificity of enzymes is group-specificity, found primarily in oligo- and poly-saccharide converting enzymes. The enzymes recognise a common group in various different molecules and convert this group. For example, β-fructosidase cleaves β-fructosidic bonds (e.g., in sucrose), α-glucosidase cleaves α-glucosidic bonds (e.g., in sucrose and maltose), β-galactosidase cleaves lactose and lactulose (with greatly reduced speed), and α-galactosidase raffinose and galactinol with the uptake of water and the liberation

of the monomeric sugar(s). (In 1894 these 'glycosidase' enzymes gave Emil Fischer the idea of 'key and lock' when he described the specificity of enzymes: the substrate has to fit into the enzyme like a key into a lock, otherwise no reaction will occur (see *Ber.Dtsch.Chem.Ges*. 27, 2985–2993).

An important type of specificity is stereo-specificity, e.g., in the determination of D- and L-lactate as well as of D- and L-malate. This specificity is based on the use of enzymes of different origins: lactate dehydrogenase (LDH) isolated, e.g., from rabbit or pig muscle reacts with L-lactate only, on the other hand LDH, e.g., from Lactobacillus leichmannii reacts with D-lactate only. This is important, e.g., in the analysis of dairy products like yoghurt. In the case of malate, the L-form that occurs in nature reacts with L-malate dehydrogenase (L-MDH) from pig heart, the D-form that is 'only' a part of the racemic malate reacts with D-MDH isolated from micro-organisms.

Furthermore, specificity is also guaranteed if the substrate of a non-specific reaction is not contained in the sample. An example of this is the enzymatic determination of ethanol. The enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AlDH) also convert n-propanol and n-butanol, but these alcohols are usually not present in foodstuffs or, if present, in only negligible (very low) concentrations. In this case one speaks about a 'relative specificity'.

In some cases (e.g., D-glucose, L-lactate, oxalate), there are two different principles/types of enzymes available for the determination of an analyte: dehydrogenases (substrate DH) and oxidases (substrate OD), in particular, e.g., for the determination of D-glucose and also for di-, oligo-, and poly-glucosides after hydrolysis and liberation of D-glucose: the dehydrogenase G6P-DH and the oxidase glucose oxidase (GOD).

The G6P-DH system is highly specific and reliable because no interference from foodstuff ingredients is known. Together with phosphoglucose isomerase (PGI), the determination of D-fructose is possible after the determination of D-glucose. Foodstuffs, especially of natural origin, mostly contain both D-glucose and D-fructose, which are products of sucrose hydrolysis. The ratio of these mono-saccharides D-glucose and D-fructose may be 1:1, or is often < 1 because D-glucose may be metabolised faster than D-fructose.

The calculation of results is done on the basis of the well-known extinction coefficient of the reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The HK/G6P-DH procedure fulfils all the requirements of a reference method. (It is often used to check, e.g., HPLC systems for specificity. Highly purified enzymes are commercially available, there are no problems with contamination and side effects, and there are no reagent dependent side/creep reactions known.)

When GOD is used for the determination of D-glucose, this enzyme shows a good specificity, but there is a problem with reliability if the sample contains reducing substances like L-ascorbate which interfere with the intermediately formed hydrogen peroxide. Results will be too low. (Many foodstuffs contain vitamin C or are enriched with vitamin C.) GOD methods are often used in

clinical chemistry for the analysis of blood and urine. Impurities/contaminating activities in GOD like 'maltase' (= α-glucosidase), 'lactase' (= β-galactosidase), 'sucrase' (= β-fructosidase), and amylase do not interfere here because the substrates of these enzymes are not present in blood or urine, in contrast to foodstuffs which often contain maltose, lactose, sucrose and starch/partially hydrolysed starch together with D-glucose. False high results have to be expected.

Furthermore, the determination of D-fructose is not possible in combination with GOD. In analysis, when using oxidases the calculation of results has to be done on the basis of standard measurements which introduce further possibilities of errors (e.g., purity and stability of standard materials/solutions) and increase random errors because of a higher number of photometric measurements. The reason for the use of standards is that the light absorbance depends on the actual assay conditions and an extinction coefficient cannot be used for the calculation of results.

This example of the determination of D-glucose with two different enzymatic systems demonstrates that the specificity of an enzyme only is not sufficient for accurate results. The enzyme preparation used in analysis has to be purified to the extent that other ingredients of samples do not react with contaminating activities (i.e., impurities) of the enzyme preparation, and that the measuring/indicator system is reliable and has not interfered with the 'matrix' of the sample.

In the development of enzymatic methods for food analysis, a specific enzyme capable of converting the substance to be determined is located in animal organs, plants or micro-organisms, isolated and subsequently purified. Testing of the specificity is carried out with compounds that either typically occur in foods or are added to them. If then no substance other than the analyte reacts in the determination, and if, in a recovery test, the analyte is determined as being 100% within the range of the error of the method, the method for analysis is said to be specific (selective) and interference-free (reliable).

Enzymes catalyse chemical reactions in a highly specific way under 'physiological conditions' without influencing the balance of the reaction. Thus, in order to achieve a quantitative conversion of the analyte, the appropriate assay conditions have to be chosen.

11.3 Sample materials and equipment

11.3.1 The analytes

All compounds that occur in nature can principally be analysed enzymatically. These are metabolites like sugars, acids or their salts, alcohols and other substances. Living cells contain enzymes capable of synthesizing (anabolism) or decomposing (catabolism) these substances. Thus, if such an enzyme specific to the substrate, which is the analyte in analysis, and a suitable measuring system, e.g., dependent on the concentration of the analyte in the sample can be made

available, the compound can be determined enzymatically. From the economic point of view the analyte should be determined enzymatically so often that capital and man-power investment for the (industrial) isolation and purification of the needed enzyme, as well as for the development of the assay make sense. This is one of the most relevant limitations for enzymatic analytical methods, maybe followed by the availability of an inexpensive measuring system.

11.3.2 Reagents for enzymatic food analysis procedures

Chemicals such as buffer substances and salts used for enzymatic determinations should be of 'A. R.' grade or of similar quality. Enzymes should be purified to the extent that they can be used for the analysis of the highly complex sample materials in food analysis. Enzymes for analytical purposes should not contain contaminating enzymes (impurities, which can be removed during purification) as well as side activities to ingredients of the sample material and ideally should be designed for food analysis by the producer. Note that enzymes of different origin show different specificity characteristics. They may or may not contain 'side activities' which are a sort of a slow non-specificity of the enzyme when compared with the fast main specificity to the substrate which is the analyte in analysis. Therefore, the 'best' raw material has to be chosen in order to isolate and purify the enzyme with the highest selectivity.

Coenzymes like nicotinamide-adenine dinucleotide (NAD(H)) and nicotinamide-adenine dinucleotide phosphate (NADP(H)) in the oxidised and in the reduced forms, adenosin-5′-triphosphate (ATP) and phosphoenolpyruvate (PEP) should also be of suitable purity and also of good stability, especially in aqueous solutions.

In order to obtain solutions of the specified (especially 'microbial') stability, it is recommended that freshly redistilled water from a quartz distillation apparatus or water of comparable quality (e.g., demineralised water, filtered through a membrane filter, and after treatment with activated charcoal) should be used for dissolving the reagents. Demineralised water is, however, adequate for preparing the samples and for the assay mixture.

Test kits for food analysis based on enzymatic methods are available in the market. They contain selected and multi-tested reagents in appropriate quantities. The advantage of using reagents which are ready to use is not only saving of time in the laboratory but also analytical safety involved. This is due to the fact that the ingredients of the test kits have been examined individually as well as together in a comprehensive function test with typical foodstuff samples. Reagents and test kits marked 'store refrigerated' should be kept in the refrigerator at +2 ºC to +8 ºC.

Buffers and aqueous coenzyme solutions can generally be frozen in order to prolong their shelf lives. Suspensions of enzymes, e.g., in ammonium sulphate solution should not be frozen as they lose enzyme activity. Enzyme solutions in glycerol (50% v/v) do not freeze. The stability of the solutions is given in the individual specifications. The expiry date given on the pack label should not be

exceeded. The same applies to auxiliary reagents with a limited stability. Reagent function (e.g., if stored beyond the recommended period) can be checked by carrying out appropriate function tests, e.g., with assay control/standard solutions.

11.3.3 Photometer

The photometer which is used for the absorbance readings should have a measuring range of at least 325–800 nm, a spectral band width of < 10 nm, a linearity (absorbance proportional to concentration) up to 2 absorbance units and a reading accuracy of $+/-0.001$ absorbance unit. The cuvette holder should be able to accept 1.00 cm cuvettes; a multi-cuvette holder is advantageous when carrying out a series of analyses.

11.3.4 Cuvettes

Measurements are carried out in cuvettes with a path-length of 1.00 cm and of a volume of approx. 4 ml. (The use of round-cuvettes cannot be recommended.) Generally, cuvettes made of special optical glass should be used; disposable plastic cuvettes (polystyrene, polymethacrylate) of suitable quality (low light absorption in the near UV range; light path 1.00 cm, deviation $< +/-1\%$) may also be used. They are available on the market in large quantities at a reasonable price. They permit the easy performance of a series of analyses. The light path of plastic cuvettes has to be checked in comparison, e.g., with 'high precision'/ quartz cuvettes. Within one batch of plastic cuvettes, the absorbance of light at the measuring wavelength should be identical within reasonable limits.

The 'soft' plastic material of the cuvettes should not be scratched during handling (mixing the contents with plastic paddles, cleaning the optical glasses with soft paper wipers), care has to be taken with formation of air bubbles in the (especially freshly prepared) solutions; they have to be 'carefully' removed from the optical glasses before the photometric readings. The cuvettes have to be filled to the level that allows the light beam to go through the solution being pholometrically measured and does not 'touch' the surface/meniscus. Always place cuvettes in the same position into the cuvette holder of the photometer.

11.3.5 Pipettes, dispensers

Special enzyme test pipettes (made of glass and graduated to approx. 5 cm from the tip) or, better, piston type pipettes (the 'Eppendorfs') with plastic tips (e.g., in routine analysis with the fixed volumes of 10 μl, 20 μl, 50 μl, 100 μl, 200 μl, and 1000 μl) and dispensers (e.g., from 0.400 ml to 2.000 ml in steps of 0.050 ml) should be used for dispensing reagents, redist. water and sample solutions. They should be used as specified by the manufacturers. A regular check by weighing pipetted/dispensed water is highly recommended. Pipettes and tips should also be flushed several times prior to being used for dispensing the solutions, especially in the case of the sample volume.

11.3.6 The sample material

Routine enzyme analysis originated in biochemical and clinical analysis. The sample materials used – tissue, and especially blood, plasma, serum and urine – are so similar in properties that it was possible to develop methods for analysing a whole series of samples within a very short period of time. In food analysis, however, many different types of samples – clear and turbid liquids, colourless and coloured, neutral or acid solutions, pastes and solids – have to be analysed. An obvious next step, therefore, was to try to adapt proven enzymatic analysis methods as used in clinical chemistry to food analysis and to develop special methods for their use in food analysis, sample preparation included.

Food is very often a complex type of sample and is hence difficult to analyse. The samples involved consist of animal, plant, microbial, inorganic and organic materials. For a number of reasons, preservatives, anti-oxidants, colouring substances, vitamins and a number of other substances are added and the processes involved in the production, e.g., heating, can alter the substances to form new compounds. Samples of this nature are just not found in biochemistry, chemistry or pharmacy. Therefore, highly specific methods are needed to analyse food.

The enzymatic methods used in food analysis are also used for analysis in the non-food area as well as in biochemistry and clinical chemistry for the analysis of body fluids, of animal and plant tissues, of fermentation broths and cell culture media. Whilst interference rarely occurs in food analysis, it may occur when chemicals, cosmetics and pharmaceuticals are analysed. Fortunately, such disturbances are easy to recognise and can often be eliminated by applying suitable sample preparation techniques.

11.3.7 Standardisation and recommendation of methods

Most of the described enzymatic methods have been published as part of national food laws in a number of countries and also in European regulations. In addition, they have been standardised by national and international standards institutes as well as by analysis committees and the American Association of the Official Analytical Chemists (AOAC) (Anonymous, 1997).

11.4 Sample preparation

The working instructions should contain technical details for preparing the sample generally or in the form of application examples. Sample preparation for enzymatic determination is simple and normally based on the measuring principle/equipment mostly used in enzymatic food analysis: for photometry, e.g., almost clear and colourless solutions are required (only ‘almost’ because absorbance differences, i. e., absorbances before and after the addition of the starter enzyme have to be measured, and there is no sample-dependent error in the analysis of opaque and slightly coloured solutions). A separation of interfering substances or even quantitative isolation of the analyte involved (as is

normally the case in classical chemical analysis) are often not necessary due to the high degree of specificity of the enzymes used.

Steps normally involved in sample preparation are: dilution, filtration, neutralisation, grinding and homogenisation, extraction, de-fatting, de-proteinisation with acids or Carrez reagents, de-gassing, and, occasionally, de-colouring.

11.4.1 The sample is liquid, clear, colourless or slightly coloured, neutral

Sample preparation techniques

- use sample directly for the assay
- if necessary dilute (partly fill an appropriate volumetric flask (e.g., 100 ml) with the diluent (often redist. water is used) and pipette the sample (e.g., with a piston type pipette) under the surface of the diluent; fill the flask up to the mark with redist. water and mix.

Reasons for sample preparation

- there must a be a coenzyme excess in the assay to convert the analyte quantitatively
- measurements have to be done in the linear part of calibration curve
- the Lambert-Beer law must be valid
- the measured absorbance difference must be high enough (e.g., > 0.100) to minimise the error of analysis (i.e., precision).

11.4.2 The sample is liquid, coloured

Sample preparation techniques

- decolourise (in a batch technique e.g., by the addition of adsorbents like polyamide, polyvinypolypyrrolidone, gelatine, bentonite, activated charcoal to the coloured solution, mix, incubate for a certain time and filter; alternatively in a column procedure, e.g., with cyclohexyl-silica-adsorbens in a 5 ml-column)
- if necessary dilute (decolourisation is often not necessary after dilution).

Reasons for sample preparation

- the dyestuffs absorb light thus exceeding the optimal range of measurement of absorbances
- changes of the pH in the (e.g., acid) coloured sample solution after the addition to the (e.g., alkaline) assay system may be responsible for creep reactions before the start enzyme is added.

11.4.3 The sample is liquid, turbid

Sample preparation techniques

- filter or centrifuge

- use fluted filter paper or membrane filter dependent on the analytical problem, discard the first volume of the filtrate
- use a 'laboratory centrifuge', decant the supernatant or take the solution with a pipette from the top
- treat with Carrez reagents
- if necessary dilute.

Reasons for sample preparation
- turbidity interferes in the photometric measurement because of refraction, scattering and absorption of light.

11.4.4 The sample is liquid, acidic or alkaline

Sample preparation techniques
- neutralise or adjust to the pH of the assay system (by the addition of KOH/NaOH or acids; incubate, e.g., for 15 min. in the case of coloured samples)
- if necessary dilute (often neutralisation is not necessary after dilution).

Reasons for sample preparation
- enzymatic reactions occur in buffered solutions with pH values optimal for the enzymes and/or the equilibrium of the reaction, the buffer capacity of the assay system must be sufficient to maintain the optimum pH necessary for the enzymatic reaction
- a creep reaction before the start enzyme is added may be caused when a coloured acid sample is introduced into an alkaline assay system.

11.4.5 The sample is liquid, emulsion

Sample preparation techniques
- treat with acid or Carrez reagents, filter or centrifuge
- dilute if necessary.

Reasons for sample preparation

The emulsion of the sample may be responsible for turbidity in the assay system (see 'liquid, turbid samples').

11.4.6 The sample is pasty or solid

Sample preparation techniques
- homogenise or blend, extract, filter or centrifuge
- extract the homogenised sample, corn size < 0.2 mm in the case of solid samples containing plant materials, e.g., with redist. water in a volumetric flask at a temperature, e.g., higher than the melting point of fat contained in the sample
- if necessary dilute.

Reasons for sample preparation

- solid samples cannot be analysed photometrically, therefore the analyte is extracted
- grind samples to obtain representative results
- in the analysis of plant materials, plant cells are broken down when the particle size is smaller than 0.2 mm and can be extracted quantitatively.

11.4.7 The sample is pasty or solid, containing fat

Sample preparation techniques

- homogenise or blend, extract, remove fat in the cold, filter or centrifuge
- extract, e.g., with redist. water in a volumetric flask at a temperature above the melting point of the fat involved, cool to room temperature, fill the volumetric flask up to the mark, place the flask in an ice-bath, e.g., for 15 min., and filter
- use Carrez-clarification
- if necessary dilute.

Reasons for sample preparation

Fat in the sample may be responsible for turbidity in the assay system (see 'liquid, turbid samples').

11.4.8 The sample is pasty or solid, containing protein (enzymes)

Sample preparation techniques

- homogenise or blend, extract, treat with acid or Carrez reagents (see the protocols in the respective instructions for analysis)
- freeze fat, filter or centrifuge
- if necessary dilute.

Reasons for sample preparation

- protein in the sample may be responsible for turbidity in the assay system (see above)
- enzymes in 'living' sample materials have to be denatured to avoid interferences such as creep reactions or consumption of coenzyme(s), e.g., of NAD(P)H by flavin-containing NAD(P)H oxidases.

11.4.9 The sample contains CO_2

Sample preparation techniques

- degas or alkalinise
- filter the sample through fluted filter paper
- stir
- pour from beaker to beaker
- use an ultrasonic bath

- an alternative is the addition of KOH/NaOH up to approx. pH 8 in order to bind CO_2 in the form of bicarbonate;
- if necessary dilute.

Reasons for sample preparation
Solutions containing carbon dioxide cannot be accurately pipetted.

11.4.10 General issues

The Carrez clarification (see Carrez M C, (1909) 'Défécation du lait pour le dosage du lactose par les liqueurs cuprique', *Annales de chimie analytique* 14, 187–8) is a good tool in enzymatic analysis of sugars, alcohols and some acids/salts because the resulting filtrate is clear and the analytes do not get lost. (For details of application see the instructions to the individual methods.) It is applied to sample solutions in order to remove proteins, turbidity and some dyestuffs, as well as to break emulsions like milk. The mechanism can be explained as to the adsorption of materials to small particles, i.e., precipitates *in statu nascendi*.

Protocol: Pipette the liquid sample into a 100 ml volumetric flask which contains approx. 60 ml redist. water, or weigh a sufficient quantity of the sample into a 100 ml volumetric flask (see dilution tables), add approx. 60 ml redist. water, mix. Subsequently, add 5 ml Carrez-I-solution (potassium hexa-cyano-ferrate(II) = ferro cyanide, 85 mmol/l = 3.60 g $K_4[Fe(CN)_6]x3H_2O$/100 ml) and 5 ml Carrez-II-solution (zinc sulphate, 250 mmol/l = 7.20 g $ZnSO_4x7H_2O$/100 ml). Mix by swirling after each addition. A colourless precipitate of $Zn_2[Fe(CN)_6]$ is formed. Adjust to pH 7.5 to 8.5 by the addition of, e.g., 10 ml (in the analysis of almost neutral samples) sodium hydroxide, NaOH (0.1 mol/l), mix. Excess Zn^{++} ions are removed by the formation of insoluble, colourless $Zn(OH)_2$. Fill the volumetric flask to the mark with redist. water, mix and filter.

11.5 Performing an assay

Pipette the reagents, the sample solution and redist. water into the cuvettes. (For the details see the individual instructions of the methods or the pack inserts of the test kits.) Mixing can be done either with a small plastic spatula (mixing paddle) or by closing off the cuvette, e.g., with Parafilm® (registered trademark of American Can Company, Greenwich, CT., USA) and then gently swirling the contents.

11.5.1 Sample volume

The sample volume can be varied to a certain extent if special analytical problems make this necessary (e.g., in order to check for interferences and gross errors, or in the analysis of low analyte concentrations). In these cases, the assay volume must always be balanced with water. The new sample volume is taken into account in the calculation.

Sample volumes (and also the 'main volumes' of the total volume) have to be pipetted with the 'highest' accuracy in order to get a 'minimum' error of analysis. (A regular check, e.g., of the piston type pipette by weighing water pipetted onto the analytical balance can be recommended. In the case of pipetting 0.100 ml, a deviation up to 0.3 μl = 0.3% may be tolerated.)

11.5.2 Reagent blank

For every series of measurements, it is generally necessary to run a reagent blank. Redistilled water should be used here instead of the sample solution. The colour of the starter enzyme is measured in the reagent blank. If the reagents should be contaminated with analyte, too high values for the absorbance difference of reagent blank and sample assay are measured. Correct results are obtained by subtracting the absorbance difference of the reagent blank from the absorbance difference of the sample assay.

11.5.3 Sample blank

Normally, a sample blank is not necessary. A sample blank has to be used in starch analysis (free D-glucose and D-glucose liberated from sucrose and lactose during the sample preparation with DMSO and HCl have to be measured and 'subtracted' from the sample assay). Other procedures containing a sample blank are the determination of L-ascorbate and of oxalate (in the case that 'free' formate in the sample is not of interest). If a sample blank is required, a separate blank has to be made up for each sample.

11.5.4 Reaction temperature

Enzyme-catalysed reactions take place rapidly at room temperature (stated in the instructions as 20 to 25 ºC). For a few methods only (L-ascorbate, succinate), or in the case of automation, a reaction temperature of 37 ºC is recommended in order to increase the reaction rate or to reduce the amount of enzyme needed. (But care should be taken that the enzyme is not denatured before all of the analyte in the assay is converted.) The measurement temperature of these reactions can be 37 ºC or room temperature.

The incubation can be done in an aluminium block put into a water bath. The cuvettes are transferred to the holder of the photometer for measurements. The quality of temperature adjustment is of minor importance in the case of 'end-point procedures' in manual techniques in contrast to kinetic or 'semi-kinetic' measurements, e.g., with automatons, where the temperature has to be adjusted to e.g., +/−0.05 ºC.

11.5.5 Photometric measurement

The enzymatic reaction begins with the addition of the starter enzyme and is followed photometrically.

11.5.6 UV method

All the enzymatic methods of determination referred to as UV methods are based on the measurement of the increase or the decrease in absorbance of the coenzymes NADH (nicotinamide-adenine dinucleotide, reduced form) or NADPH (nicotinamide-adenine dinucleotide phosphate, reduced form), which absorb light in the near UV range (with the maximum at 340 nm). The measurement of analytes with dehydrogenases or with reductases via the formation or the consumption of NAD(P)H is advantageous because the extinction coefficients which are used for the calculation of results are well known and the reactions are to a very large extent free from interferences. The methods are reliable.

Measurement of the formation or the consumption of NAD(P)H has to be made at the absorption maximum of 340 nm when spectrophotometers or filter photometers are used, and at 365 or 334 nm (written as 'Hg 365 nm', 'Hg 334 nm') when spectral-line filter photometers equipped with a Hg-lamp are used (system Eppendorf). The measurements in the flank of the absorption band is allowed only because of the 'absolute monochromassie' of the respective Hg line.

11.5.7 Colorimetric method

The methods referred to as colorimetric methods are also enzymatic methods of determination. The measurement of the concentration is based on the formation of a light-absorbing dye in the visible spectrum. In the determination of D-sorbitol/xylitol, D-3-hydroxybutyrate and L-glutamate the NADH-forming reaction is coupled to a reaction catalysed by diaphorase which transfers the hydrogen to an iodo-nitro-tetrazolium salt (INT). A formazan dye is formed which is measured in the visible range. This system shifts the unfavourable equilibrium of the primary reaction rapidly and quantitatively towards the reaction products.

Other colorimetric methods are used for the determination of L-ascorbate and of cholesterol. In the case of colorimetric methods reaction conditions have to be chosen so that the extinction coefficient is constant from batch to batch of reagents. Then the calculation of results can be done on the basis of the extinction coefficient and not on the basis of the measurement of standards.

11.5.8 Calculation

The calculation of the results is carried out with the general equation for photometric measurements according to the Beer-Lambert law. The constant parameters (extinction coefficient, molecular weight of analyte, light-path, sample volume and total volume) may be combined to form a factor (which should not be confused with the dilution factor F) in order to simplify the calculation.

$$c = \frac{V[ml] \times MW[g/mol] \times F}{\varepsilon[l \times mmol^{-1} \times cm^{-1}] \times d[cm] \times v[ml] \times 1000} \times \triangle A[g/l]$$

V = final/assay volume in ml
MW = molecular weight of analyte in g/mol
F = dilution factor (dilution during sample preparation = volume after dilution divided by the sample/start volume)
A = absorbance
ΔA = $(A_2-A_1)_{sample} - (A_2-A_1)_{blank}$ (when a light absorbing substance is formed during the reaction, such as NADPH in sugar analysis), respectively
ΔA = $(A_1-A_2)_{sample} - (A_1-A_2)_{blank}$ (when a light absorbing substance is consumed during the reaction, such as NADH in the determination of glycerol)
ε = extinction coefficient in $l \times mmol^{-1} \times cm^{-1}$
d = light path in cm
v = sample volume in ml

In the analysis of liquid samples, the result has to be multiplied by the dilution factor (F) if the sample has been diluted prior to analysis.

In the analysis of pasty or solid samples (sometimes also in the analysis of liquid samples like milk) which are weighed out for analysis, the content is calculated and the result is expressed in g/100 g:

$$\text{concentration}_{\text{analyte}} = \frac{\text{concentration}_{\text{analyte}} \text{ in g/l sample solution}}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100\,[\text{g}/100\text{g}]$$

11.5.9 Control of analysis: testing the reagents

The activity or purity of enzymes and coenzymes can be checked by an enzymatic test using a standard. A recovery of 98–100 % is proof that the reagents are in good condition. The standard solutions serve for assay control purposes only and they are not required for the calculation of results. Due to the presence of impurities or moisture in the standard materials, it should not be expected that 100% recovery of the weighed-out quantities of laboratory chemicals will be found.

11.5.10 Testing the methodology (recognising interference during the assay procedure)

Interferences are very seldom observed in enzymatic analysis of foodstuffs. A very simple or simplified explanation for this is that food which is drunk and eaten without interference of the human body will not interfere with the enzymatic reaction in the assay because the nature of enzymes in human metabolism is the same as the nature of the enzymes used in the assay.

Note that analysts are accustomed to interferences in traditional wet chemistry analytical procedures, and those are often referred to 'matrix effects'. This 'scientific' term is only used, to a certain degree, to excuse and circumvent the 'poor specificity' of chemical reactions.

Interferences in enzymatic analysis, e.g., caused by poor or unsuitable sample preparation, may result in a slowed-down reaction, or in no reaction at all, or in the addition of a side-reaction. Interferences in enzymatic analysis can be easily detected. If the conversion of the analyte has been completed within the time given in the working procedures, it can be concluded in general that no interference has occurred. On completion of the reaction, the determination can be started again by adding the analyte (qualitatively or quantitatively). If the absorbance is altered subsequent to the addition of the standard material (e.g., > 0.100 absorbance units), this is also an indication that no interference by ingredients of the sample has occurred and that the assay system contained enough coenzyme.

The reaction cannot be re-started with the analyte in the analysis of maltose, lactose, sucrose and starch, as these analytes no longer react or are no longer cleaved after altering the reaction conditions (change of pH because of the different pH optima of the enzymes). The re-start has to be done with the hydrolysis product which is measured in the indicator reaction: D-glucose, respectively D-galactose in the determination of lactose via D-galactose. Note that this re-start is especially recommended in the case of a 'very high' absorbance difference with the sample assay to see whether or not enough coenzyme is available for quantitative reaction of the analyte.

Operator error or interference of the determination through the presence of substances contained in the sample can be recognised by carrying out a double determination using two different sample volumes; the measured differences in absorbance should be proportional to the sample volumes used. (The reason for this advice is that the increase in the analyte content results in a longer reaction time, and, it is logical, an increase of a possible 'inhibitor' content in the sample solution, while all the other assay conditions are constant, slows down the reaction rate. The incubation time is sufficient when the absorbance differences are proportional to the sample volumes.) Note that this use of different sample volumes in multiple determinations should be the 'standard procedure' when samples have to be analysed, e.g., in duplicate.

Possible interference of the assay caused by substances contained in the sample can be recognised by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with a sample and standard/assay control solution in the same assay (total volume balanced with redist. water). The recovery can then be calculated from the absorbance differences measured (and not from the calculated concentrations or contents of the analyte):

$$\text{recovery} = [(\Delta A_{\text{sample+standard}} - \Delta A_{\text{sample}})/\Delta A_{\text{standard}}] \times 100\,[\%]$$

Note that in the analysis of liquid samples, with or without dilution prior to analysis, the internal standard measurement may be done instead of a double determination with different sample volumes (see above) in order to see possible interferences by the sample matrix or to recognise gross errors in assay performance.

Possible losses of the analyte during the determination, especially during sample preparation, can be recognised by carrying out recovery tests. The sample should be prepared and analysed with and without added standard material. The additive should be recovered quantitatively within the error range of the method. Note that these recovery experiments should be done in the development of either a new analytical procedure or a new sample preparation technique such as deproteinisation or decolourisation. These recovery experiments may not be necessary in routine analysis. In the case of liquid samples the analysis of an internal standard (see above) is sufficient.

11.5.11 Automation

Enzymatic determinations can be carried out by means of automatons as many examples in clinical chemistry demonstrate. Details cannot be dealt with in this chapter, only some general information can be given because of the many machines on the market and the frequent changes in models. The enzymatic determination includes steps that can or cannot be automated with acceptable expenses and costs. The dilution step can be done automatically with diluters in the case of liquid, clear, and carbon dioxide-free samples. All the other techniques for sample preparation have to be applied to the samples before the actual determination.

In continuous flow (segmented flow) analysis a dialysis step is used for dilution and for the removal of compounds with a molecular weight, e.g., of > 1000 Daltons, as well as of 'small' particles responsible for emulsions or opaque solutions like milk or some (filtered) fruit juices. The easy, simple, and automatic sample preparation step 'dilution' is the reason why automatons are mainly or often used in the analysis of liquid and clear (maximum opaque) solutions in enzymatic food analysis.

For the measurement of the enzymatic reaction there are available

- Continuous flow (segmented flow) techniques (systems Technicon, Skalar). Because of the different dyestuffs contained in the different samples, a reagent blank (sample included; reagents without the start enzyme) may be necessary which depends on reaction principle and concentration of analyte.
- Discrete analysers that imitate the manual technique including pipetting, dispensing, mixing, incubation, and photometric reading.
  The determinations are carried out in cuvettes. The automatons 'imitate' the manual technique. In this case the volumes from the manual technique can be applied to automatic analysis, or the volumes may be reduced so that the ratio between the volumes of the solutions will not be changed, or a mix of

reagents (with limitations of stability) is prepared in order to reduce the number of dispensing steps.

- Discrete analysers working with a flow-through cell.
  The incubation is done in tubes, the photometric measurement is done in a flow-through cell. Here one assay has to be carried out for each absorbance reading. Care has to taken that the final volume in all assays is identical. (A simplification may be the measurement of A_2 of the sample assay against A_2 of the blank assay. But in this case the analysis of coloured samples is not possible because results would be too high. This, on the other hand, may be compensated for by the analysis of a sample blank that does not contain the starter enzyme. And this is the above-mentioned situation of 'one assay per absorbance reading'.)

Note that the first step in handling a series of enzymatic determinations should be rationalisation, i.e., the use of piston-type pipettes (with fixed volumes, e.g., 10 μl, 20 μl, 50 μl, 100 μl, 200 μl, 1000 μl), a dispenser with adjustable volumes (0.500 to 2.000 ml in steps of 0.050 ml), disposable cuvettes ($d = 1.00$ cm), and a photometer equipped with a printer. The next step may be off-line data reporting and handling (e.g., calculation), or better on-line if equipment (e.g., multi-cuvette holder) and software is available for the photometer in use.

Decision factors for automation are the number of samples and analytes, availability of samples and the time when the results are needed, costs of man-power, disposables and reagents, as well as costs and depreciation of equipment.

11.5.12 Interpretation of data

The interpretation of analytical results has to take into account the biological fluctuation often demonstrated by natural products, the procedures in production and legal aspects. The error in analysis has also to be considered. (It is or may be often smaller than the biological fluctuation, e.g., of natural raw materials for food production.)

Note that the main error in enzymatic determinations results from the absorbance readings (photometric reading error) followed by the volume error when dispensing the 'small' sample volume into a 'large' buffer and water volume. (A reduction of volumes, e.g., in semi-micro or micro techniques results in an increase of inaccuracy/coefficient of variation, CV value.)

11.6 Routine enzymatic methods for food analysis and authentication

11.6.1 Acetaldehyde

The UV method contained in Swiss law and recommended by MEBAK:

$$\text{Acetaldehyde} + NAD^+ + H_2O - \text{AlDH} \rightarrow \text{acetic acid} + NADH + H^+$$

Acetaldehyde is a product of metabolic processes, e.g., an intermediate in alcoholic fermentation. (Its content indicates the fermentative production of ethanol.) Acetaldehyde is a flavour compound. High concentrations, e.g., in beer indicate the presence of off-flavour compounds. In wine production acetaldehyde is bound to sulphite, e.g., in order to improve the taste. On the other hand, acetaldehyde is the most common aldehyde found in dairy products (yoghurt, cheese, etc.) and is responsible for desirable specific flavour, and flavour defects.

11.6.2 Acetic acid

The UV method contained in Dutch and German laws, standardised by Deutsche Norm DIN, European Standard EN and recommended by IFU and MEBAK:

$$\text{Acetate} + \text{ATP} + \text{CoA} - \text{ACS} \rightarrow \text{acetyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

$$\text{Acetyl-CoA} + \text{oxaloacetate} + \text{H}_2\text{O} - \text{CS} \rightarrow \text{citrate} + \text{CoA}$$

$$\text{L} - \text{Malate} + \text{NAD}^+ \leftarrow \text{L-MDH} \rightarrow \text{oxaloacetate} + \text{NADH} + \text{H}^+$$

Acetic acid is the final product of fermentation processes and the oxidation product of acetaldehyde and ethanol. Acetic acid is the main component of the 'volatile acids' in wine. A high concentration of acetic acid in wine results in spoilage of the product. Acetic acid is used in food production as a preservative and a taste-improver and is the compound determining the monetary value of vinegar.

11.6.3 Ammonia

The UV method contained in German law and recommended by MEBAK:

$$\text{2-Oxoglutarate} + \text{NADH} + \text{NH}_4^+ - \text{GlDH} \rightarrow \text{L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O}$$

High concentrations of ammonia can indicate the (microbial) decomposition of substances like milk, meat and seafood, it is a major component of the off-flavours and odours associated, e.g., with tainted meat and spoiled shrimps. Ammonia also indicates the presence of faeces, urine and micro-organisms in water.

11.6.4 L-Ascorbic acid ('vitamin C')

The colorimetric method recommended by MEBAK:

Sample assay:

$$\text{L-Ascorbate(x-H}_2\text{)} + \text{MTT}^+ - \text{PMS} \rightarrow$$

$$\text{dehydroascorbate(x)} + \text{MTT-formazan} + \text{H}^+$$

Sample blank assay:

$$\text{L-Ascorbate} + 1/2\ \text{O}_2 - \text{AAO} \rightarrow \text{dehydroascorbate} + \text{H}_2\text{O}$$

L-Ascorbic acid is present in varying amounts in all animal and plant cells. Humans cannot synthesise L-ascorbic acid by themselves and are hence dependent on external sources (vitamin C) the main source of which may be fruits and vegetables, e.g., potatoes. For technological reasons, L-ascorbic acid is used as an anti-oxidant in the manufacture of foodstuffs.

L-Ascorbic acid is a relatively 'sensitive' substance; its determination is thus suitable for use in the assessment of the quality of processed food from fruit and vegetable raw materials.

11.6.5 Cholesterol

The colorimetric method contained in German and Swiss laws and recommended by IUPAC:

$$\text{Cholesterol} + O_2 - \text{cholesterol oxidase} \rightarrow \Delta^4\text{-cholestenone} + H_2O_2$$

$$\text{Methanol} + H_2O_2 - \text{catalase} \rightarrow \text{formaldehyde} + 2H_2O$$

$$\text{Formaldehyde} + NH_4^+ + 2 \text{ acetyl acetone} \rightarrow \text{lutidine dye} + 3H_2O$$

Cholesterol is the most important of the animal sterols. It is a component of the cell membranes of higher species of organisms and a precursor to a whole series of steroid hormones. Cholesterol is found in all animal fats. It is a component of egg yolk. Due to the relative constancy of its content, it is frequently used in the determination of the egg content of foodstuffs like bakery goods, noodles and liqueur with egg yolks. Cholesterol is important nutritionally as it is partly ingested in the form of food.

11.6.6 Citric acid

The UV method contained in German and Swiss laws and European regulation, standardised by Deutsche Norm DIN, European Standard EN, Norme Française Homologuée NF; Nederlandse Norm NEN, International Standard ISO, Russian Standard GOST, approved by AOAC and recommended by IDF/FIL, IFU, A.I.J.N., MEBAK, OIV und VDLUFA:

$$\text{Citrate} - \text{CL} \rightarrow \text{oxaloacetate} + \text{acetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ - \text{L-MDH} \rightarrow \text{L-malate} + NAD^+$$

Citric acid is an important metabolite in animals, plants and micro-organisms. It is the best known acid of the fruit acids and is contained in large amounts in, e.g., citrus fruits (approx. 10 g citric acid/l orange juice).

Citric acid is manufactured biotechnologically on a large scale. It is used, e.g., as an acidifier, as an additive in foods, as a complex forming agent for heavy metals (the addition to wine, which may be regulated, prevents turbidity caused by tannin-iron complexes), as an emulsifier (e.g., in the production of processed cheese) and as a flavouring agent in the production of soft drinks and candies.

11.6.7 Creatine/creatinine

The UV method contained in the Swiss law for which no commercial test kit is available:

$$\text{Creatinine} + H_2O - \text{creatininase} \rightarrow \text{creatine}$$

$$\text{Creatine} + \text{ATP} - \text{CK} \rightarrow \text{creatine-P} + \text{ADP}$$

$$\text{ADP} + \text{PEP} - \text{PK} \rightarrow \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ - \text{L-LDH} \rightarrow \text{L-lactate} + NAD^+$$

Creatine/creatinine may be measured for the evaluation of the meat content, e.g., of soups.

11.6.8 Ethanol

The UV method contained in Austrian, Belgian, German and Swiss laws, standardised by Norme Française NF and recommended by ALVA, MEBAK, IFU, A.I.J.N., EBC and ASBC:

$$\text{Ethanol} + NAD^+ - \text{ADH} \rightarrow \text{acetaldehyde} + \text{NADH} + H^+$$

$$\text{Acetaldehyde} + NAD^+ + H_2O - \text{AlDH} \rightarrow \text{acetic acid} + \text{NADH} + H^+$$

Ethanol occurs in nature in practically all organisms, even if in very small quantities. It is the final product of alcoholic fermentation and a 'desired' ingredient of alcoholic beverages (the monetary value-determining component) and the 'undesirable' component in non-alcoholic and low-alcoholic beverages. The presence of ethanol in fruit products like fruit juices indicates that the components used for production may have decomposed. The presence of ethanol is also an indirect indicator for the presence of yeasts.

11.6.9 Formic acid

The UV method contained in German and Swiss laws and recommended by MEBAK:

$$\text{Formate} + NAD^+ + H_2O - \text{FDH} \rightarrow \text{bicarbonate} + \text{NADH} + H^+$$

Formic acid is a metabolite but its concentration is very low. It is the oxidation product of methanol and formaldehyde (formalin). Formic acid is a by-product in acetic acid fermentation in vinegar production; it is not contained in synthetic acetic acid. Formic acid is a part of the 'volatile acids' in wine. As formic acid in low concentrations has both a bactericidal and fungicidal effect, it can be used as a food preservative. Moulds tend to produce formic acid as a metabolite, hence determination of formic acid can give an indication as to the properties, e.g., the degree of decomposition, of samples.

11.6.10 D-Fructose ('fruit sugar')

The UV method contained in Austrian, German, Italian, Swiss laws and European regulation, standardised by Deutsche Norm DIN, European standard EN, Nederlandse Norm NEN, Russian Standard GOST, approved by AOAC, recommended by ALVA, MEBAK, IFU, A.I.J.N. and OIV (see also D-glucose and sucrose):

$$\text{D-Glucose(D-fructose)} + \text{ATP} - \text{HK} \rightarrow \text{G-6-P(F-6-P)} + \text{ADP}$$

$$\text{F-6-P} - \text{PGI} \rightarrow \text{G-6-P}$$

$$\text{G-6-P} + \text{NADP}^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + \text{H}^+$$

Free D-fructose is mostly found in plants, where it is a very important sugar component, e.g., of fruit. D-Fructose is an important ingredient of honey; it is a sugar substitute in food for diabetics. D-Fructose is sweeter than sucrose and causes less formation of dental plaque than does sucrose. D-Fructose is a component of di-, tri- and oligo-saccharides (sucrose, lactulose; raffinose; oligo-β-fructosanes) and is also a component of the poly-saccharide inulin. In food analysis not only the sum of D-glucose and D-fructose ('reducing sugars') is of interest but often the ratio D-glucose to D-fructose (e.g., for the detection of adulterations by the prohibited addition of 'sugar').

11.6.11 D-Gluconic acid/D-glucono-δ-lactone

The UV method contained in German and Swiss laws, standardised by Deutsche Norm DIN, International Standard ISO, Russian Standard GOST and recommended by ALVA:

$$\text{D-Glucono-}\delta\text{ -lactone} + \text{H}_2\text{O} - \text{pH10} \rightarrow \text{D-gluconate}$$

$$\text{D-Gluconate} + \text{ATP} - \text{gluconate kinase} \rightarrow \text{D-gluconate-6-P} + \text{ADP}$$

$$\text{D-Gluconate-6-P} + \text{NADP}^+ - \text{6-PGDH} \rightarrow$$

$$\text{ribulose-5-P} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

D-Glucono-δ-lactone is used in the manufacture of foodstuffs (e.g., sausages and cheese). The reason for this is that the slow hydrolysis of D-glucono-δ-lactone results in a slow pH reduction in production processes. D-Gluconic acid is not (or only in traces) contained in 'healthy' grapes and therefore is also not contained in grape juice. Wines produced from grapes infected with Botrytis cinerea (in German 'Edelfäule') contain reasonable amounts of D-gluconic acid. The ratio glycerol to D-gluconic acid may give an indication of the prohibited addition of glycerol.

11.6.12 D-Glucose ('grape sugar')

The UV method contained in German, Italian and Swiss laws and recommended by ALVA (see also D-fructose and sucrose):

$$\text{D-Glucose} + \text{ATP} - \text{HK} \rightarrow \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + \text{H}^+$$

D-Glucose and D-fructose are main components of the 'reducing sugars' in fruit and vegetable products, whereby the ratio D-glucose to D-fructose may be below 1. The analysis of both mono-saccharides is of great interest in order to detect the prohibited addition of 'sugar' like glucose syrup). D-Glucose is an ingredient of 'starch sugar'/'glucose syrup'/'liquid sugar'/inverted sugar.

In fermentation (techniques) D-glucose is often metabolised faster than D-fructose due to a higher activity or a smaller Michaelis constant of the enzymes. This fact may be of interest in the production of 'modern' wines because the higher sweetness of D-fructose than of D-glucose results in a better taste of the wine. This can be achieved by slow fermentation.

11.6.13 L-Glutamic acid

The colorimetric method contained in Belgian, German and Swiss laws, standardised by International Standard ISO, Russian Standard GOST and recommended by NMKL:

$$\text{L-Glutamate} + \text{NAD}^+ + \text{H}_2\text{O} - \text{GlDH} \rightarrow \text{2-oxoglutarate} + \text{NADH} + \text{NH}_4^+$$

$$\text{NADH} + \text{H}^+ + \text{INT} - \text{diaphorase} \rightarrow \text{NAD}^+ + \text{formazan}$$

The sodium salt of L-glutamic acid (mono-sodium glutamate, MSG) is used as a taste improver and taste intensifier, it is an additive in the production of sausages, canned fish and meat products, frozen and dry products. The addition of MSG gives a salty-sweet taste that may be described as 'mouth satisfaction'. MSG causes the 'China restaurant syndrome'.

11.6.14 Glycerol

The UV method contained in German and Swiss laws and recommended by ALVA, MEBAK, and OIV:

$$\text{Glycerol} + \text{ATP} - \text{GK} \rightarrow \text{L-glycerol-3-P} + \text{ADP}$$

$$\text{ADP} + \text{PEP} - \text{PK} \rightarrow \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ - \text{L-LDH} \rightarrow \text{L-lactate} + \text{NAD}^+$$

Glycerol is a component of high-quality wines. Glycerol is formed as a by-product of fermentation with Saccharomyces cerevisiae; it is an important product of the metabolism of Botrytis cinerea ('Edelfäule'). The sweet taste of glycerol gives the wine 'body'. If the concentration of glycerol is higher, e.g., than 6 to 10% of the ethanol concentration, the wine may be adulterated ('improved') by the prohibited addition of glycerol.

Glycerol is not (or only in traces or small amounts) contained in grape juice from healthy grapes. A higher content of glycerol in grape juice may indicate the

use of spoiled material in production. Glycerol gives vinegar ('Balsamico') a soft and velvety flavour and is used as a moisturiser in the production, e.g., of marzipan.

11.6.15 D-3-Hydroxybutyric acid

The colorimetric method contained in German Law and European regulation:

$$\text{D-3-Hydroxybutyrate} + \text{NAD}^+ - \text{3-HBDH} \rightarrow \text{acetoacetate} + \text{NADH} + \text{H}^+$$

$$\text{NADH} + \text{H}^+ + \text{INT} - \text{diaphorase} \rightarrow \text{NAD}^+ + \text{formazan}$$

An increase in the content of D-3-hydroxybutyric acid in eggs can be detected six days after chickens have been fertilised. This increase continues even after the embryo has died. D-3-Hydroxybutyric acid is thus a typical indicator for fertilised and incubated eggs.

11.6.16 D-Isocitric acid

The UV method contained in German, Swiss and Spanish laws, standardised by Deutsche Norm DIN, European standard EN, Norme Française Homologuée NF, Nederlandse Norm NEN, Russian Standard GOST and recommended by IFU and A.I.J.N.:

$$\text{D-Isocitrate} + \text{NADP}^+ - \text{ICDH} \rightarrow \text{2-oxoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^+$$

The determination of D-isocitric acid has become of importance in the analysis of fruit juices (especially of orange juice) for the detection of illegal additives (adulteration), e.g., of citric acid. Not only the (minimum) content of D-isocitric acid in juices is of interest, but often also the ratio citric acid to D-isocitric acid: a ratio which is too high indicates the addition of citric acid which may not be allowed in the production of fruit juices.

The content of D-isocitric acid in products, e.g., containing orange juice may be used to calculate its content, e.g., in soft drinks with the declaration on the bottle label 'contains orange juice'.

11.6.17 D- and L-Lactic acid

The UV method contained in Dutch, German, Italian, Swiss laws and European regulation, standardised by International Standard ISO, Deutsche Norm DIN, European Standard EN, Russian Standard GOST, recommended by MEBAK, IFU, A.I.J.N., IDF/FIL, OIV and VDLUFA:

$$\text{D-Lactate} + \text{NAD}^+ - \text{D-LDH} \rightarrow \text{pyruvate} + \text{NADH} + \text{H}^+$$

$$\text{L-Lactate} + \text{NAD}^+ - \text{L-LDH} \rightarrow \text{pyruvate} + \text{NADH} + \text{H}^+$$

$$\text{Pyruvate} + \text{L-glutamate} - \text{GPT} \rightarrow \text{L-alanine} + \text{2-oxoglutarate}$$

D-Lactic acid is formed by some micro-organisms only, e.g., from Lactobacillus lactis, Lb. bulgaricus and Leuconostoc cremoris. D-Lactic acid is not formed or

only in traces by 'higher organisms', e.g., by animals. Therefore the presence of D-lactate may serve as an indicator for microbial contamination or spoilage, assuming that fermentation techniques have not been used in the production of the foodstuff. The stereo-specific measurement of the lactate forms is of interest, e.g., in the manufacturing of sour milk products in order to assess the activity of micro-organisms.

L-Lactic acid is often the final product (a 'dead end' or a 'blind alley') in the metabolism of most living organisms. It is formed in muscles by the anaerobic degradation of glycogen (approx. 120 g daily by a human body), not only in the muscle of living animals but also in meat after slaughtering. (L-Lactate is oxidised to pyruvate under aerobic conditions.)

The content of L-lactate in beer indicates the presence of Lactobacilli in production. The content of L-lactate in liquid whole egg or in egg powder gives good information about the hygienic quality of the products. L-Lactate in milk powder indicates the use of neutralised sour milk for the production of milk powder. L-Lactate in wine is also formed during the 'second fermentation' (malo-lactic fermentation, biological de-acidification).

11.6.18 Lactose ('milk sugar')

Via galactose

The UV method, via D-galactose, contained in Austrian, Dutch, German and Swiss laws, approved by AOAC, standardised by Norme Belge-Belgische Norm NBN, Deutsche Norm DIN, Russian Standard GOST and recommended by IDF/FIL and VDLUFA:

$$\text{Lactose} + H_2O - \beta\text{-galactosidase} \rightarrow \text{D-glucose} + \text{D-galactose}$$

$$\text{D-Galactose} + NAD^+ - \text{galactose dehydrogenase} \rightarrow$$

$$\text{D-galactonic acid} + NADH + H^+$$

Lactose is an important carbohydrate component of mammalian milk. Therefore it is of nutritional importance. The lactose concentration of milk from healthy cows is approx. 4.6 to 5 g/100 g. Milk from cows suffering from mastitis shows lower lactose levels. Lactose or products containing lactose, e.g., milk powder, are used, e.g., to improve fermentation, browning, taste and mouth-feeling, texture and keeping freshness.

Lactose is determined in food samples with the declaration 'contains milk', e.g., in order to determine and to calculate the milk content. Food for people suffering with lactose intolerance should not contain lactose.

Via glucose

The UV method, via D-glucose, contained in Swiss food law, standardised by Nederlandse Norm NEN and recommended by IDF/FIL:

$$\text{Lactose} + H_2O - \beta\text{-galactosidase} \rightarrow \text{D-glucose} + \text{D-galactose}$$

$$\text{D-Glucose} + \text{ATP} - \text{HK} \rightarrow \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + NADP^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + H^+$$

11.6.19 Lactulose

The UV method contained in German and Swiss food laws, British Pharmacopoeia, United States Pharmacopoeia BP, United States Pharmacopoeia USP-NF, standardised by Deutsche Norm DIN and recommended by VDLUFA:

$$\text{Lactulose} + H_2O - \beta\text{-galactosidase} \rightarrow \text{D-fructose} + \text{D-galactose}$$

$$\text{D-Fructose} + \text{ATP} - \text{HK} \rightarrow \text{F-6-P} + \text{ADP}$$

$$\text{F-6-P} - \text{PGI} \rightarrow \text{G-6-P}$$

$$\text{G-6-P} + NADP^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + H^+$$

Lactulose is a chemical indicator of the heat treatment intensity to which milk is submitted in industry. The formation of lactulose due to the isomerisation of lactose depends on time and temperature of heating. UHT milk and sterilised milk contain lactulose in different quantities. Lactulose is used in pharmacy because of its laxative effect. It is used, e.g., in the therapy of obstipation and of liver diseases such as cirrhosis, insufficiency and coma hepaticum.

11.6.20 D-Malic acid

The UV method contained in European regulation, standardised by Deutsche Norm DIN, European Standard EN and recommended by IFU, A.I.J.N. and OIV:

$$\text{D-Malate} + NAD^+ - \text{D} + \text{MDH, decarb.} \rightarrow \text{pyruvate} + CO_2 + \text{NADH} + H^+$$

D-Malic acid is a component of the (racemic) D-/L-malic acid as prepared chemically. As natural products are practically free from D-malic acid, the detection of D-malic acid indicates that D-/L-malic acid has been added, e.g., to wine or to fruit juice, which may be allowed or prohibited.

11.6.21 L-Malic acid

The UV method contained in German, Italian, Swiss food laws and European regulation, approved by AOAC and standardised by Deutsche Norm DIN, European standard EN, Norme Française Homologuée NF, Nederlandse Norm NEN, Russian Standard GOST. Recommended by IFU, A.I.J.N., MEBAK and OIV:

$$\text{L-Malate} + NAD^+ - \text{L-MDH} \rightarrow \text{oxaloacetate} + \text{NADH} + H^+$$

$$\text{Oxaloacetate} + \text{L-glutamate} - \text{GOT} \rightarrow \text{L-aspartate} + \text{2-oxoglutarate}$$

L-Malic acid as a component of the citric acid cycle is one of the most important fruit acids and it is the acid with the highest concentration in wine. L-Malic acid

may be used in food production because it is a stronger acid than citric acid. Microbial decomposition of L-malic acid leads to the formation of L-lactate. This can, e.g., be a desirable reaction in the production of wine (malo-lactic fermentation, biological de-acidification), or an undesirable reaction in the case of beer (second fermentation).

11.6.22 Maltose

The UV method contained in German law and recommended by MEBAK:

$$\text{Maltose} + H_2O - \alpha\text{-glucosidase} \rightarrow 2\text{D-glucose}$$

$$(\text{Sucrose} + H_2O - \alpha\text{-glucosidase} \rightarrow \text{D-glucose} + \text{D-fructose})$$

$$(\text{Sucrose} + H_2O - \beta\text{-fructosidase} \rightarrow \text{D-glucose} + \text{D-fructose}$$

$$\text{D-Glucose} + \text{ATP} - \text{HK} \rightarrow \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + NADP^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + H^+$$

Maltose is formed from starch in seed and malt by enzymatic hydrolysis (amylases). Glucose-syrup ('starch-sugar') is produced from starch by means of amyloglucosidase and contains maltose.

11.6.23 Nitrate

The UV method contained in German and Swiss laws, standardised by Deutsche Norm DIN, European Standard EN and recommended by MEBAK:

$$\text{Nitrate} + \text{NADPH} + H^+ - \text{nitrate reductase} \rightarrow \text{nitrite} + NADP^+ + H_2O$$

Some plants, e.g., cabbage, red beets, radish, spinach, and salad have the ability to store nitrate in their tissue ('nitrophil'). The content of nitrate in potatoes is relatively low. High amounts of nitrate may be due to the intensive use of fertilisers. When cooking vegetables a lot of nitrate will be lost with the boiling water.

Nitrate in foodstuffs is of nutritional importance due to its reduction to nitrite and the formation of compounds that attach to haemoglobin. It also forms nitrosamines that are known to be carcinogenic. There are or may be limits for the concentration of nitrate in drinking/tap water (e.g., 50 mg/l in Europe). 'Natural' water contains approx. 1 mg nitrate/l. A higher concentration of nitrate in wine than expected may indicate the addition of tap water.

11.6.24 Oxalic acid

The UV method recommended by MEBAK:

$$\text{Oxalate} - \text{oxalate decarboxylase} \rightarrow \text{formate} + CO_2$$

$$\text{Formate} + NAD^+ + H_2O - \text{formate dehydrogenase} \rightarrow$$

$$\text{bicarbonate} + \text{NADH} + H^+$$

Oxalate in food interferes with resorption and hence availability of calcium and is thus of considerable nutritional importance. Furthermore, oxalate in food influences the level of oxalate in urine. Spinach, rhubarb, peanuts, chocolates, parsley and tea contain high levels of oxalate, whereas beer, coca-cola, cereals and meat are either low or free from oxalate. Foodstuffs containing high amounts of oxalate should not be included in the diet of growing children (spinach!), pregnant women, patients with calcium deficiency and patients who have passed a calcium oxalate stone.

11.6.25 D-Sorbitol/Xylitol

The colorimetric method contained in German law and recommended by IFU and A.I.J.N.:

$$\text{D-Sorbitol} + \text{NAD}^+ - \text{SDH} \rightarrow \text{D-fructose} + \text{NADH} + \text{H}^+$$

$$\text{Xylitol} + \text{NAD}^+ - \text{SDH} \rightarrow \text{D-xylulose} + \text{NADH} + \text{H}^+$$

$$\text{NADH} + \text{INT} + \text{H}^+ - \text{diaphorase} \rightarrow \text{NAD}^+ + \text{formazan}$$

D-Sorbitol, a sugar alcohol, the reduction product of D-fructose, occurs extensively in fruits, e.g., in apples, cherries, pears, plums, but is not contained (or only in traces) in grapes, grape juice and wine. (A remarkable content of D-sorbitol in grape wine, grape juice and vinegar from wine indicates the addition of the prohibited use of apples or apples products in production.) The concentration of D-sorbitol may be used for the calculation of the apple juice content of beverages declared as 'contains apple juice'.

11.6.26 D-Sorbitol

The UV method with NAD and SDH, contained in German law and recommended by OICCC. A commercial kit is not available.

$$\text{D-Sorbitol} + \text{NAD}^+ - \text{SDH} \rightarrow \text{D-fructose} + \text{NADH} + \text{H}^+$$

This is the oldest procedure for the measurement of D-sorbitol. It needs 'quite a lot' of SDH, has quite a long reaction time and a small range of linearity. The measurement of D-sorbitol via D-fructose (section 11.6.27) or with INT/diaphorase (section 11.6.25) may be recommended.

11.6.27 D-Sorbitol

The UV method via D-fructose with SDH, HK, ATP, PGI, NADP and G6P-DH is contained in Italian and Swiss laws. A commercial kit is not available.

An excess of D-glucose and of D-fructose reduces the recovery of D-sorbitol. In this case it is recommended to measure D-sorbitol via INT/diaphorase (section 11.6.25). An alternative is the removal of D-glucose and D-fructose by means of Fehling reagents, and the removal of Cu^{++} by means of Carrez-I solution (see Meier P *et al.* (1985) Mitt.Geb.Lebensm.Hyg. 76, 478–485).

$$\text{D-Sorbitol} + \text{NAD}^+ - \text{SDH} \rightarrow \text{D-fructose} + \text{NADH} + \text{H}^+$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ - \text{L-LDH} \rightarrow \text{L-lactate} + \text{NAD}^+$$

$$\text{D-Glucose (D-fructose)} + \text{ATP} - \text{HK} \rightarrow \text{G-6-P (F-6-P)} + \text{ADP}$$

$$\text{F-6-P} - \text{PGI} \rightarrow \text{G-6-P}$$

$$\text{G-6-P} + \text{NADP}^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + \text{H}^+$$

11.6.28 Starch

The UV method contained in Dutch, German and Swiss laws, standardised by Nederlandse Norm NEN and recommended by MEBAK and A.I.J.N.:

$$\text{Starch} + (\text{n} - 1)\text{H}_2\text{O} - \text{amyloglucosidase} \rightarrow \text{n D-glucose}$$

$$\text{D-Glucose} + \text{ATP} - \text{HK} \rightarrow \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + \text{H}^+$$

Starch is used as a reserve carbohydrate in plants (amylose, amylopectin) and animals (glycogen). Structurally, starch is an irregular polymer consisting of D-glucose units connected by α-1,4- and α-1,6-bonds. Starch is an important ingredient of food. It serves as a binder, e.g., in sauces, filler and thickener, film former, and as a texturiser in the production of foodstuffs.

11.6.29 Succinic acid

The UV method contained in German, Swiss laws and European regulations:

$$\text{Succinate} + \text{ITP} + \text{CoA} - \text{SCS} \rightarrow \text{IDP} + \text{succinyl-CoA} + \text{P}_\text{i}$$

$$\text{IDP} + \text{PEP} - \text{PK} \rightarrow \text{ITP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ - \text{L-LDH} \rightarrow \text{L-lactate} + \text{NAD}^+$$

Succinic acid is a specific indicator of microbial decomposition in eggs and egg products.

11.6.30 Sucrose ('sugar', cane sugar, beet sugar)

The UV method contained in Austrian, Dutch, German and Swiss laws, standardised by Deutsche Norm DIN, Nederlandse Norm NEN, European Standard EN, Russian Standard GOST, Norme Française Homologuée NF and recommended by IFU, A.I.J.N., MEBAK, OICCC and VDLUFA (see also D-fructose and D-glucose):

$$\text{Sucrose} + \text{H}_2\text{O} - \beta\text{-fructosidase} \rightarrow \text{D-glucose} + \text{D-fructose}$$

$$\text{D-Glucose} + \text{ATP} - \text{HK} \rightarrow \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ - \text{G6P-DH} \rightarrow \text{D-gluconate-6-P} + \text{NADPH} + \text{H}^+$$

Sucrose is an important ingredient of foodstuffs. It is not only of monetary value, but also an important sweetener.

11.6.31 Sulphite

The UV method contained in German law, standardised by Deutsche Norm DIN, European Standard EN and recommended by MEBAK and NMKL:

$$\text{Sulphite} + O_2 + H_2O - \text{sulphite oxidase} \rightarrow \text{sulphate} + H_2O_2$$

$$H_2O_2 + \text{NADH} + H^+ - \text{NADH peroxidase} \rightarrow 2\,H_2O + NAD^+$$

Sulphur dioxide, sulphurous acid and its salts (sulphites) occur in very low concentrations in nature. However, they have been used for a very long time in the industrial production of foodstuffs ('sulphurating'). Sulphur dioxide is used as a preservative in the food industry in order to prevent microbial spoilage.

The use of sulphuric acid in the production in wine is one of the more important techniques to improve stability and taste of wine. Among other things, sulphite binds carbonyl compounds, especially acetaldehyde, in the form of compounds which are 'neutral in taste'. Sulphite is regarded as being poisonous for cells; in metabolism, it is rapidly oxidised and excreted.

11.6.32 Urea

The UV method contained in German law, standardised by Nederlandse Norm NEN and recommended by MEBAK:

$$\text{Urea} + H_2O - \text{urease} \rightarrow 2\,NH_3 + CO_2$$

$$\text{2-Oxoglutarate} + \text{NADH} + NH_4^+ - \text{GlDH} \rightarrow \text{L-glutamate} + NAD^+ + H_2O$$

Urea is sometimes added (illegally) to meat products in order to indicate a higher content of muscle protein than is actually present when the protein is measured via 'Kjeldahl nitrogen' without removal of the non-protein-nitrogen compounds fraction.

11.7 Advantages and disadvantages

11.7.1 High specificity

The greatest advantage of using enzymatic methods is their high specificity. This is guaranteed by the enzyme used in analysis. Such a high specificity can only be found within naturally occurring macro-molecules like enzymes and their substrates, antigens and antibodies, DNA and RNA. Enzymes are very specific biological catalysts. From a variety of substances available to them, enzymes convert the substances to which they are specific. This means for practical work that the separation, isolation and identification of the substance to be determined, e.g., the analyte, are not necessary.

A determination is specific, if

- the assay system contains an enzyme which reacts only with the analyte
- a less specific enzyme is combined with a highly specific enzyme
- the specific reaction is very much faster than the unspecific reaction

- the specific reaction is 'only' faster than the side reaction of the enzyme used or the activity of a contaminating enzyme and these cause a slow creep reaction, which can be compensated for.

Specificity is also guaranteed if the substrate of the non-specific reaction is not contained in the sample (one speaks of 'relative specificity' in this case).

11.7.2 Simple sample preparation

Sample preparation refines the sample, so that a reliable analysis can be carried out. Simple sample preparation techniques with inexpensive materials give accurate results and save time and money. Only 'almost clear' and nearly colourless solutions or extracts are necessary for the measuring principle photometry.

11.7.3 Ease of measurement

Running an enzymatic determination is simple. It consists of pipetting buffer and coenzyme solutions, enzyme suspension (or solution), redist. water and the sample solution into a cuvette, following the reaction by photometric measurements and calculating the result acc. to the Beer-Lambert law. Enzymatic reactions proceed quantitatively with known reaction mechanisms and without unknown side reactions. The substrate is quantitatively converted and produces no by-products. Many reactions can be combined with the NAD(P)-NAD(P)H-system. The amount of NAD(P) or other light absorbing substances formed or decomposed is proportional to the amount of analyte. Thus the reaction proceeds stoichiometrically.

The light absorption of the coenzymes NAD and NADP (measured in the near UV range) is different for the reduced and oxidised forms. The reduced forms have an additional absorption peak with a maximum at 340 nm. The absorption at 340 nm is characteristic of these co-enzymes, the extinction coefficient used in the calculations has been determined with great accuracy. Photometric measurements are based on the difference between the light absorbance of the substrates and of the products of enzymatic reactions. The final calculation is based on the Beer-Lambert law.

11.7.4 High precision

The precision of results from enzymatic determinations is good, or in most cases good enough. CV values of 0.5 to 3% are quoted in literature. An analyst achieves a CV value of approx. 1% in the analysis of substances, e.g., determining the monetary value. CV values are higher in the case of trace level compound analysis due to the imprecision of photometric readings.

11.7.5 High sensitivity

Successful enzymatic determinations in trace level compound analysis can be done, when

- a high concentration of the sample in the sample solution is chosen (e.g., D-glucose and D-fructose in sugar)
- the maximum sample volume is used for the assay (e.g., nitrate in tap water)
- photometric measurements are done in the absorption maximum of the dyestuff to be measured (e.g., NAD(P)H at 340 nm)
- NAD(P)H forming reactions are coupled with highly sensitive colour reactions (e.g., L-glutamate, D-3-hydroxybutyrate and D-sorbitol with INT)
- a light path greater than 1.00 cm is used which is often not possible because of the construction of the cuvette holder in the photometer
- the measured signal is amplified in automatons (this also reduces the reaction time)
- the analyte is precipitated from a large volume and dissolved in a small volume (e.g., D-isocitrate in juice and soft drinks containing juice)
- the analyte is extracted from the sample and the solution is concentrated, something often done in modern instrumental analysis.

11.7.6 Rare interferences

Interferences in enzymatic analysis are seldom observed because of the specificity of enzymatic reactions. Interferences in enzymatic analysis are, e.g.,

- a slowed-down main reaction
- no reaction at all
- addition of a side reaction ('creep-reaction')
- a reason for gross errors in the performance of the determination.

11.7.7 Saving of time

Sample preparation is usually very quick for enzymatic analysis because only 'practically clear' and 'almost colourless' sample solutions or extracts are required. The actual time needed for the actual enzymatic analysis includes the time needed for pipetting and for photometric reading. The use of appropriate equipment considerably reduces analysis time. The use of suitable laboratory equipment speeds up the process without influencing the quality of the results. Significant time is saved by using piston type pipettes and dispensers instead of glass pipettes, disposable cuvettes and digital display photometers with printers.

Waiting time for enzymatic determinations is determined by the *incubation time* in the enzymatic reaction. Each reaction requires a specific minimum time, which can only be slightly affected/reduced by reaction conditions.

11.7.8 Low costs

Costs for enzymatic analysis are thought to be high because of the costs of the enzymes, but they should always be considered with other characteristics of the methods, e.g., the known specificity and the ease of performance of sample preparation and assay. The main costs in analysis, the costs for personnel, are favourable in enzymatic analysis. Enzymatic analysis may be regarded as a cost effective reference method.

11.7.9 Rationalisation, mechanisation, automation – flexibility of methods

From manual procedures in enzymatic determinations to completely automatic processing of large numbers of samples are intermediate stages which can be designated as rationalisation, mechanisation or partial mechanisation. Enzymatic methods are flexible with respect to running many samples in series as well as running a list of methods on one sample because the principles are always the same. Only different reagents are necessary for the different methods.

11.7.10 Safe reagents

Most of the reagents used in enzymatic methods for food analysis are not hazardous materials in the sense of the hazardous substances regulations or chemicals laws. However, the general safety measures that apply to all chemical substances and to the work in chemical laboratories should always be adhered to. After use, the reagents can be disposed of with laboratory waste, local regulations must be observed.

11.7.11 'Disadvantages'

There is a limited range of analytes that can be measured enzymatically. The reasons for this are the (commercial) availability of enzymes and of the (inexpensive) measuring equipment. There seems to be no new 'big' test parameter to be developed. The (industrial) development of a new enzymatic method may be in the range of a million Euro, for the isolation of the (specific, selective) enzyme from a suitable raw material, the development of the assay and the check for specificity and interferences by food ingredients, additives, etc., as well as for sample preparation of typical sample materials.

Enzymatic analysis means the discrete determination of individual analytes (in contrast to chromatography). Each analyte has to be measured separately in a reaction vessel, e.g., in a cuvette. Enzymatic analysis in food chemistry seems to have an image of being 'old-fashioned' (because advantages and handling are unknown). Often enzymatic methods are not known or are confused with enzyme immuno assays.

11.8 Future trends

The manual determination carried out as **'end-point' procedure** is the 'standard' in enzymatic food analysis and all other procedures have to be compared with it. The reaction is performed in cuvettes and measurements are made photometrically. Interferences of the analysis can easily be detected by the use of different sample volumes in multiple analysis; very low concentrations of the analyte in trace level compound analysis can be measured by increasing the sample volume. Results are calculated with well-known extinction coefficients.

Enzymatic methods are often in use, e.g., in some countries in Europe and Japan. There is a high potential for manual techniques, when the number of samples to be analysed and methods is quite small, e.g., in America and in Asia. In 'under-developed countries' enzymatic analysis may be the 'methodology of choice' because of the inexpensive equipment and ease of handling (in contrast, e.g., to chromatographic techniques), not forgetting that enzymatic methods often have the characteristic of reference methods.

A large series of determinations, especially in the analysis of liquid samples like fruit juice, milk and wine which need no special sample preparation, can be carried out with **automatons**. These are often developed for clinical chemistry and they should be checked for/adopted to food analysis. There are available continuous flow/segmented flow automatons, as well as discrete analysers that either imitate manual techniques or work with incubation vessels and flow through cells. Results are mostly calculated on the basis of standard/calibration solutions. Automatons for enzymatic analysis have a further good chance in the analysis of fruit juices and of wine (especially in the recognised wine-producing countries), and enzymatic methods may compete successfully with HPLC applications, e.g., in the analysis of wine.

Kinetic measurements which are based on the relationship between substrate/analyte concentration in the assay system and the initial speed/reaction rate of the enzymatic reaction cannot be recommended for food analysis because of the very large differences in the sample matrices of foodstuffs and their possible influences on the enzymatic reaction. As an example of a typical influence on the speed of the enzymatic reaction, phenolic compounds contained in the sample material may be mentioned. These may be compensated for by means of internal standard measurements. Kinetic measurements are much quicker than end-point methods. The equipment is expensive. On the other hand, the Michaelis constant of the available enzymes may be too low for use in kinetic measurements. There may be no need, and also no potential, for this type of measurement in food analysis.

The use of **immobilized enzymes** makes no sense in manual techniques as well as in discrete automation. In continuous flow automatic systems the enzymes are fixed to membranes or to the inside of tubes. Immobilised enzymes may be used in reactors. Systems may use oxidases (e.g., glucose oxidase in sugar analysis) with the already mentioned 'matrix effects'. These systems cannot be recommended because the results of analysis may not be reliable.

Test strips, **enzyme electrodes** and **biosensors** developed, e.g., for clinical chemistry play no special role in food chemistry especially because of their limited availability with respect to the analytes (D-glucose – diabetes; ethanol – forensic medicine; L-lactate – sports medicine). There is no obvious potential for these techniques in the 'typical' analysis of foodstuffs.

11.9 Abbreviations

A.I.J.N.	Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community
ALVA	Arbeitsgemeinschaft der Landwirtschaftlichen Versuchs-Anstalten; Austria
AOAC	Association of the Official Analytical Chemists
ASBC	American Society of Brewing Chemists
BP	British Pharmacopoeia
DIN	Deutsches Institut für Normung (German standard)
EBC	European Brewery Convention
EN	European Norm (European standard)
GOST	Gosstandart Rossii (Standard of the Russian Federation)
ICUMSA	International Commission for Uniform Methods of Sugar Analysis
IDF/FIL	International Dairy Federation
IFU	International Federation of Fruit Juice Producers
ISO	International Standards Organization
IUPAC	International Union of Pure and Applied Chemistry
MEBAK	Mitteleuropäische Brautechnische Analysen-Kommission (Central European Commission for Brewing Technology)
NBN	Norme Belge-Belgische Norm (Belgian standard)
NEN	Nederlandse Norm (Dutch standard)
NF	Norme Française (French standard)
NMKL	Nordisk Metodikkommittée
OICCC	Office International du Cacao, du Chocolat et de la Confiserie
OIV	Office International de la Vigne et du Vin (International Wine Office)
VDLUFA	Verband Deutscher landwirtschaftlicher Untersuchungs- und Forschungs-Anstalten; Germany

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12

In-line sensors for food analysis

P. D. Patel and C. Beveridge, Leatherhead Food International Ltd, UK

12.1 Introduction

Traditionally, within the food manufacturing industries, process performance assessment, fault detection and achievement of consistently high quality safe product have focused on off-line monitoring of quality and safety parameters such as sensory attributes, colour analysis, rheological measurements, and chemical and microbiological analysis. There is now an increasing need for integration of real-time sensors in industrial process monitoring and control that is attributed to a number of factors including legislative drive, consumer pressure for safe and wholesome food and a company's policy for enhancing internal QA programmes. It is necessary to define some terminologies when referring to sensors and process monitoring.

- The term sensor has been defined as a device or system – including control and processing electronics, software and interconnection networks – that responds to a physical or chemical quantity to produce an output that is a measure of that quantity (e.g., pH and ionic strength measurement). A biosensor comprises two distinct elements: a biological recognition element (e.g., antibodies, cell, receptor or nucleic acids) and, in close contact, a signal transduction element (e.g., optical, amperometric, acoustic or electrochemical) connected to a data acquisition and processing system. Thus, the signal resulting from the interaction of the biological element with the corresponding analyte (e.g., antibody-antigen interaction) is converted to a quantifiable signal (e.g., electrical). The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes. Because of the ability to be repeatedly calibrated, a biosensor is distinguished from a

bioanalytical system, which requires additional processing steps such as reagent addition. For further details concerning biosensors in relation to food safety, the reader is referred to the review by Patel (2002).

- The terms on-line, in-line, at-line and near-line measurement in relation to process line application are defined as follows. (i) On-line; a measurement device that is truly part of the main process operation. This could be exemplified by a probe within the line or by an observation made through a transparent window in the line; (ii) in-line: a sampling branch to the process line takes a portion of material for analysis, using the techniques above; (iii) at-line: a portion of material from the sampling branch is isolated to allow sample conditioning to take place (e.g., filtration, pH adjustment, dilution) prior to measurement. Clearly, such an operation mode allows for reagent addition as part of the analysis (e.g., titration); and (iv) near-line: the above sample is transported (manually or automatically) to a work station not connected to the process line, where a wide range of analytical operations can take place.

The overall aim of this chapter is to provide an insight into availability of recent technologies that have the potential to be integrated to provide process line sensors (and biosensors) for real-time measurement of analytes or groups of analytes of direct importance in food quality and safety. The chapter provides a brief overview of the principles and criteria for in-line sensors, points to the main drivers in the area, considers examples of recent commercial analytical technologies that can potentially meet the industrial criteria for process line sensors, explores examples of sample conditioning systems of potential value in line applications and, finally, considers combination technologies (sample conditioning, sensor-based detection) that could provide a basis for the development of process line sensors.

12.2 Requirements for in-line sensors

Figure 12.1 shows a schematic diagram of how the individual elements of the system go together to produce a feedback loop for plant and equipment control. The system would be valid for both continuous and batch processing. Multiple sensing heads with different functions can be accessed and correlated to provide a specific output. The product conditioning is a vital part of the overall sensor system. Without this, the heterogeneous nature of the flowing particulate food stream is likely to give a high noise-to-signal interference in the subsequent sensor-based analysis. This may be attributed to high background fluorescence and signal quenching due to the turbidity and components of the matrix and the general presence of food particulates. A proper sample conditioning system will help reduce or eliminate the interference, thus allowing higher signal-to-noise detection and analysis of the desired signal in the subsequent sensing element. Following the information processing stage, which resolves and collates the

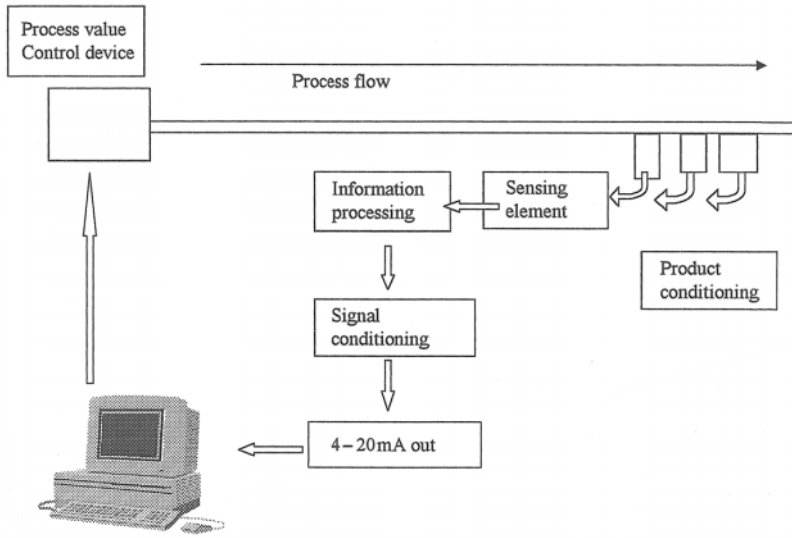

Fig. 12.1 The feedback loop.

desired information from the background interference, the final data is converted to the form that is recognised by the subsequent plant components, e.g., a process valve control device to control the level of the desired raw material in the process line or a computer to alert the process controller of the presence of undesired contaminants.

12.2.1 Operational characteristics

Instrument must be capable of withstanding the rigours of the production line such as resistance to water of at least IP66 or IP67. Operation and commissioning should be simple and where possible self-calibrating. It should have easy communications to display and operating computers, this generally involves signal conditioning to provide a simple 4–20 mA signal or 0–10 V signal. To have maximum utility the unit should be capable of retrofitting to existing plant and plant control systems with minimal installation and commissioning.

12.2.2 Industrial considerations

The majority of the food industry consists of the Small, Medium-size Enterprise (SME) scale of companies and as such has limited financial resources. Instrumentation needs are basic and only that which is absolutely required. Highly sophisticated instruments will generally be rejected on cost and functionality. That being said, an advanced instrumentation system that is cost effective and very simple to operate can be very sophisticated, but its complexity

will be hidden (inside a 'black box') from the operator/user. At present, the acceptable cost of a sensor-based instrumentation will vary significantly depending on the type of information required, but in general this should average at about £1,000 per installed unit. More realistically, however, the final anticipated cost is likely to vary between £5,000–£10,000 per installed unit.

The function of these instruments will normally be applied to matters of food safety and or quality, identification, qualification and quantification. The processes to be measured, for example, can be the cleaning quality, natural or inadvertent contamination of food products (e.g., by allergens, food-spoilage/poisoning bacteria and metabolites, and toxins), chemical analysis, recipe formulation, and ingredient and moisture analysis.

12.2.3 Drivers for process line sensors

There is no doubt that industrial-specification-based sensors will increasingly play a vital role in enhancing the efficiency of a process in order to deliver safe products that are of a consistent quality. Some of the drivers in this regard are summarised briefly.

- *Legislation.* This includes the safety and quality of food, and labelling legislation.
- *Consumer.* Today's consumers are increasingly aware of the potential risks and benefits associated with consumption of varied food types. The demand for wholesome safe food will continue.
- *Global competition.* Food is no longer confined to national boundaries and manufactures are striving to produce high quality products with minimum costs that can be distributed widely. One of the major common limitations is the retrospective nature of the laboratory-based analytical results.
- *Environment.* The environmental issue is another significant driver and acceptance is more likely through waste minimisation and reduction in energy consumption during food production.
- *Enhanced QA procedures.* The manufacturers' constant desire to produce high quality consistent product right-first-time in order to prevent time-consuming and additional costs related to product re-work, give-away or discard all together.

12.3 Current commercial sensor systems

This section considers three categories of commercially available sensor systems, namely those currently used in industrial process applications, examples of some new instrumental systems developed for in-line process monitoring and, finally, examples of low cost analytical instrumentation of potential value for in-line applications.

12.3.1 Commercial in-line instrumentation

A number of relatively expensive spectroscopic and imaging techniques, including NIR, NMR and MRI, have been covered in detail elsewhere in this book. In this section, a brief overview of some of the commercial techniques used industrially for in-line measurement of food constituents has been considered.

The most widely used in-line techniques are based on spectroscopy. These use the absorbency, emission/reflectance and fluorescent characteristics of the components being examined to defined parts of the electromagnetic spectrum, including UV, visible and infra-red and microwave. These systems are largely used for the identification and quantification of groups of chemical species (e.g., proteins, fats and ions) and measurement of particle size and molecular interaction of components.

Reliable monitoring of temperature and humidity is also important to food safety and quality. The control of humidity conditions is important to prevent refrigerated products such as fresh products from losing moisture. Dual-sensor data loggers are used to monitor the temperature and humidity in many applications (e.g., smoking processes for gourmet sausages and environmental conditions in retail stores). The data can be used to optimise energy use and provide a means of traceability. Table 12.1 summarises examples of the technologies available and their industrial applications.

12.3.2 Recent new instruments for process monitoring

Visual process analyser (ViPA) – image analysis

The Jorin ViPA (*www.jorin.co.uk*) is a particle size analysis system that is designed to operate continuously on-line (Fig. 12.2). It uses a video microscope to capture images of the particles in a process flow. The technology has largely been used for water monitoring (e.g., quality, filter efficiency, oil and reservoir), whilst other applications are continually being reported (e.g., chemical dosing monitoring, and polymer manufacturing). The image analysis equipment and

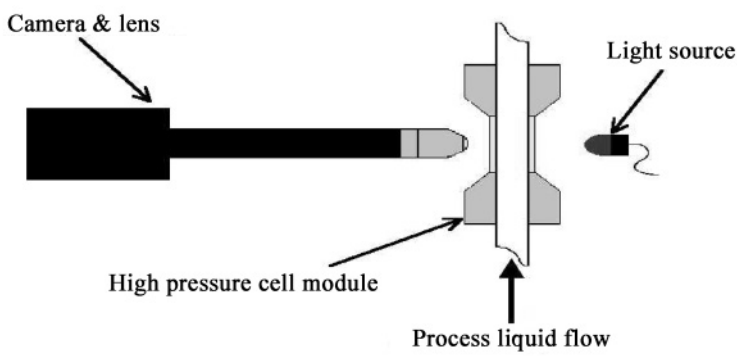

Fig. 12.2 Schematic showing ViPA components (from www.jorin.co.uk).

Table 12.1 Examples of some commercial in-line sensor instrumentation and their applications.

System	Measurement	Application
FTIR/NIR	Protein, fat, fibre, moisture, starch, sugar, salt and solids	Dairy process control for low moisture products: Whey protein concentrate (WPC), cream cheese and skim milk
NIR	Oil and moisture	Snack foods
MRI/NMR	Same	Same
Dual sensor	Temperature and humidity	Smoking process for gourmet sausage
Glass-free pH sensor	Hydrogen ion concentration	Control acidification in yoghurt fermentation
Forward scatter turbidity sensor	Low level particulates	Waste water and chemical processes
FSC402 optical density analyser	Concentration of dissolved matter	Filtered beer, juices and cream
Guided microwave spectrometry	Total ionic chemical species (dielectric constant/ conductivity)	Analysis of moisture and salt in cheese, moisture in cereals/snack-food, sugar and acid level in orange juice and percentage of fat in milk
High resolution ultrasonic spectroscopy	Particle size, monitoring gelation and coagulation processes	Particle size analysis of milk, coagulation point in calcium-fortified milk and droplet concentration of salad dressings
UV analyser	Fluorescent compounds	Flavoured mineral water

software has the potential to examine and characterise particles during food production. Its value in this important area, which includes microbial particles, has yet to be determined.

Two typical images, saved directly from the ViPA analyser are shown in Fig. 12.3. The image on the left shows oil droplets in water and the image on the right shows solids particles in water. From these images it is clear that the solid particles have a very different shape from the oil droplets. The ViPA can use this difference to distinguish between the particle types and catagorise them separately. In this way, and using any or all of the seventeen parameters, the ViPA can differentiate between up to eight particle types in a single liquid flow. Information on the size distribution, concentration, etc., of each of these particle types is then reported on screen and on the optional 4–20 mA ouput.

Impedance analyser ('intelligent pipe')

This technology, developed by Kaiku (*www.kaiku.co.uk*), is based on resonance frequency interrogation of flowing streams allowing for real-time, in-line, non-invasive analysis of the fluid components (Fig. 12.4). The food applications

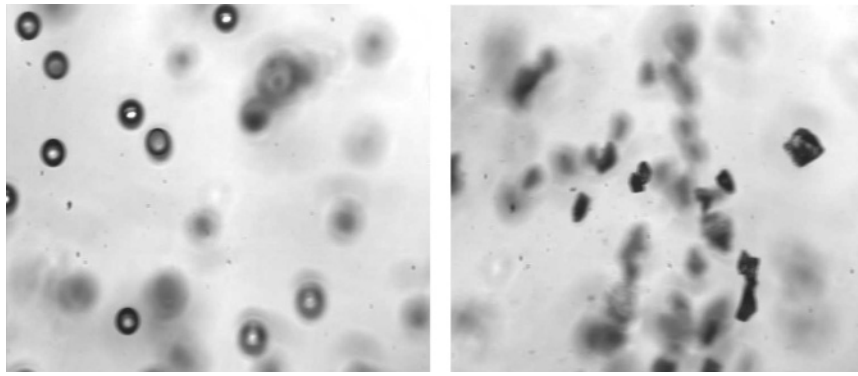

Fig. 12.3 Images of particles in flowing streams (from www.jorin.co.uk).

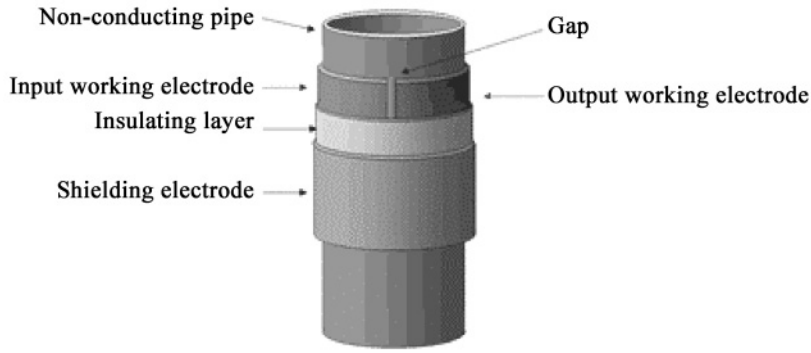

Fig. 12.4 The Kaiku 'intelligent' pipe (from www.kaiku.co.uk).

demonstrated by the manufacturer include determination of water addition to orange juice, starch addition to tomato base, determination of CIP, addition of salt to beers, authenticity testing of orange juices and colas (Fig. 12.5).

12.3.3 Portable analytical instrumentation in process monitoring

Spectroscopic instrumentation

Table 12.2 shows examples of some relatively low cost fibre optic instrumentation available for spectroscopic measurement (e.g., Raman, NIR, UV-Vis and fluorescence) of samples. It also includes additional information on the potential applications of the systems and the company addresses. Most of the instruments are computer linked and hence amenable to process line applications. The value (or otherwise!) of the application of these types of systems has yet to be determined, particularly in relation to set industrial (e.g., ruggedness, line-compatibility, data acquisition and analysis) and system

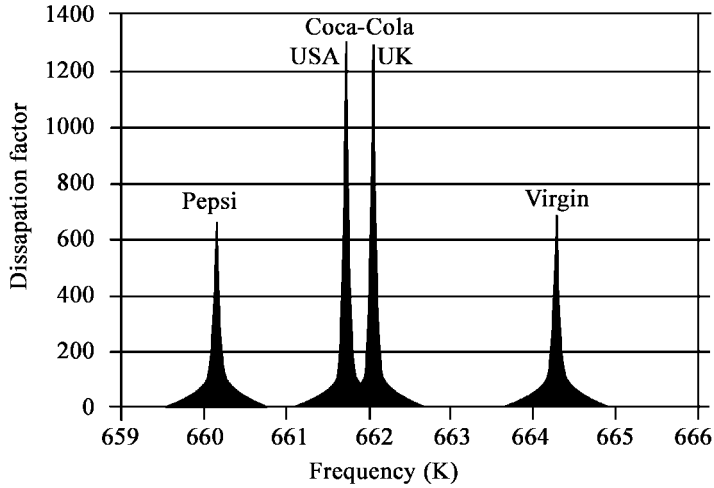

Fig. 12.5 Authenticity of colas determined using the Kaiku 'intelligent' technology (from www.kaiku.co.uk).

performance (e.g., sensitivity, selectivity, specificity and data variability) specifications for a given test analyte.

Electronic nose

An electronic nose can be regarded as a modular system comprising a set of active materials which detect the odour. Associated sensors transduce the chemical quantity into electrical signals, followed by appropriate signal conditioning and processing to classify known odours or identify unknown odours. A range of instruments is commercially available (e.g., 4440B; Agilent technologies and Prometheus; Alpha Mos), including the portable A320 (Cyrano Ssciences) and Kore MS-200 mass spectrometer used as an e-nose.

Research has been carried out into the use of thin and thick film semiconducting (inorganic and organic) materials for odour sensing, e.g., the use of metal oxide thick films for sensing alcohols and ketones, and metal oxide thin films for ammonia and hydrogen. Research effort is now centred upon the use of arrays of metal oxide and conducting polymer as odour sensors. The latter are particularly interesting because their molecular structure can be engineered for a particular odour-sensing application.

There are various applications in which an electronic nose may be used. For example, to monitor the characteristic odour generated by a manufactured product (e.g., drink, food, tobacco, soaps). The electronic nose research group has considerable experience in the analysis of coffee odours (e.g., roasting level and bean type), lager beer odours (lager type and malodours) as well as having analysed tobaccos, spirits, wines, transformer oils, plastics and drinking water. More recent work is on the use of e-noses for medical diagnostics and biotechnology.

Table 12.2 Some commercial portable systems that have potential for in-line applications

Basis	Type	(Potential) application	Company
Fibre optic (FO) spectroscopic systems: (UV/Vis/NIR/RAMAN/ FLUORESCENCE)	1. Avantes FO spectrometer 2. Antaris FT-NI FO spectrometer 3. NetworkIR FO spectrometer 4. S2000 miniature FO spectrometer NIR512 miniature FO spectrometer SF2000 FO fluorescence spectrometer SF 2000 FO fluorescence spectrometer Raman Systems R-2001 FO spectrometer	Remote spectral measurement of components in industries (e.g., chemical, food-processing, biomedical)	Avantes Inc., USA E-mail: info@avantes.com, www.avantes.com Thermo Nicolet, USA E-mail: nicinfo@thermonicolet.com, www.nicoletindustrial.com Clairet Scientific Ltd, UK E-mail: clairet@compuserve.com www.clairet.co.uk Ocean Optics, Inc USA E-mail: info@oceanOptics.com
Fibre optic immersion probes for remote coupling to instrumentation (e.g., UV/Vis and NIR spectrophotometers, fluorimeter, diode array)	Hellma ruggedised immersion probes	Remote monitoring of analytes/chemical composition of process streams under harsh environments (e.g., temperature <200ºC, Pressure < 25 bar)	Hellma (England) Ltd; www.hellma.demon.co.uk
Planar Waveguide Attenuated Total reflectance (ATR) NIR or Vis spectrometer – Computer linked	On-line rugged fibre optic probe At-line PS-1 Portable Spectrometer	Non-invasive chemical spectral measurement (e.g., hydroxyl, isocyanate, rhodamine) in cloudy, viscous liquids	Optical Solutions, Inc., USA E-mail: optsol@ix.netcom.com www.oriel.com
Fluorescence lifetime sensor (FLS) detector – Computer linked	LifeSense LFS for fibre optic biosensors, and on-line, real-time process analysis	High sensitivity detection of popular fluorophores (e.g., FITC, rhodamine, Cy-5) and novel NIR dyes (e.g., Cy-7, IRD-25) which are particularly suited to high background interference in liquids/slurry.	As above
Liquid waveguide capillary cells (LWCC) coupled to fibre optic cable & /remote instrumentation (e.g., UV/Vis/NIR spectrometers, etc.)	The WPI Liquid waveguide capillary cell	LWCC offers increased optical pathlength (50 cm) compared to standard curve (1 cm) and a small sample volume for sensitive measurement of analytes/chemical composition of liquid streams	World Precision Instruments (WPI), Inc, USA www.wpiinc.com
Multiwavelength ellipsometer for real-time process monitoring	Submonolayer-Ellipsometer EL X-1 and M-44/M-88 ellipsometer	For fast (10 min.) detection of immunological reactions. Also, *in-situ* multiwavelength spectroscopic measurement for electrochemistry application	L.O.T.-Oriel, UK www.lotoriel.co.uk

The two main limitations of the e-noses are:

1. *Sensitivity*: Most e-noses have limits of detection in the low ppm range. For practical use, however, limits of detection in the low ppb range or lower are required.
2. *Matrix suppression*: Variable matrices can cause problems. Isolating a known pattern from a highly variable background will require advanced combination of pattern classification (e.g., PCA analysis, see section 12.4) and hardware approaches.

12.3.4 Scope of biosensors for process line applications

Tables 12.3 and 12.4 show examples of commercial biosensor-based analytical instrumentation, together with a summary of their description and applications. For details of the different types and formats of biosensors and their applications in food contaminant analysis, the reader is referred to Patel (2002). The basis of many of these techniques is amenable to development of dedicated instrumentation for process line applications. It would not be possible or, indeed, cost-effective for analytical instrumentation to be simply 'bolted' onto process lines for monitoring specific components or contaminants in flowing food streams. The logical way forward is to develop fit-for-purpose instruments that can be integrated into process lines. This type of developments will inevitably involve bringing together multidisciplinary skills and expertise from different industrial sectors (e.g., defence, medical, microelectronics and agrofood).

One possible approach for developing (bio)sensor-based process line monitoring instrumentation is to use a fibre optic (FO) system linked to optical detection (Fig. 12.6). In the FO (bio)sensor a receptor (e.g., antibodies, nucleic acid, natural binding proteins and plastic molecular imprinted polymers (MIPs) (Patel, 2001) is immobilised to the distal end of an optical fibre. Light is then introduced at the proximal end, which travels to the distal tip by total internal reflection. The emission resulting either directly from the analyte bound to the corresponding receptor or subsequent binding of a fluorescent-labelled reactant (e.g., competitive immunoassay format) is measured by the detector and correlated to the concentration of the test analyte.

12.4 In-line sampling

One of the main factors to consider when developing an in-line sensor for process application is the composition of a sample matrix, in particular potential interference (e.g., due to background components, general turbidity, homogeneity, etc.) that can seriously affect the sensor response and reduce both sensitivity and specificity of the measurement. Other major factors that affect sensor response include the effects of process variables (e.g., temperature, pH, Aw, ionic strength, flow rate and pressure). A schematic of a typical meat homogenate is shown in Fig. 12.7. In-line measurement of trace levels of

Table 12.3 Examples of commercially available SPR-based biosensor instruments

Biosensor type	Biological element	Analyte	Company
BIAcore Q based on immobilisation on a sensor chip	Antibody (also other binding agents can be immobilised (e.g. enzyme, DNA, lectin and receptor))	Folate, biotin, vitamin B_{12} (possible kits for mycotoxins and antibiotics)	Biacore AB, Rapsgatan 7, S-754 50 Uppsala, Sweden *www.biacore.com*
Biacore 1000, 2000, 3000, X	As above	Generic sensor for studying binding interactions (e.g. protein-protein, DNA-protein, lectin-carbohydrate) in real-time	As above
BIOS-1 biosensor based on immobilisation on a waveguide sensor chip	As above	As above	Artificial Sensing Instruments, AG, PO Box 120, Schaffhauserstr. 550, 8052 Zurich, Switzerland
IBIS biosensor based on immobilisation in a cuvette	As above	As above	Intersens Instruments, BV, Scheltussingel 156, 3814 BH Amersfoort, The Netherlands *www.windsor-ltd.co.uk*
Plasmoon biosensor based on immobilisation in a cuvette	As above	As above	BioTul AG, Gollierstrasse 70B, D-80339 Munich, Germany *www.biotul.com*
Spreeta miniature biosensor based on immobilisation in a flow-through cell	As above	As above	Texas Instruments Inc., 12500 TI Boulevard, Dallas, TX 75243-4136, USA *www.ti.com*

NA: Not available.

Table 12.4 Examples of other commercially available biosensors

Biosensor type	Biological element	Analyte	Company
Membrane bound enzyme	Enzyme	Glucose, sucrose, lactate, lactose, ethanol, methanol, glutamate	YSI Inc., Yellow Springs, Ohio, USA *www.ysi.com*
Evanescent-wave fluoroimmunoassay using tapered fibre optic waveguide (Analyte 2000)	Antibody	Staphylococcal enterotoxin B, *E. coli* O157:H7, viruses, spores	Research International, 18706 142nd Ave, N.E., Woodinville, WA 98072, USA *www.resrchintl.com*
As above (Raptor)	Antibody	Biological and warfare agents: Ricin, *B. anthracis, Y. pestis*	As above
Electrochemical biosensor based on electroconductive polymer transducers, which respond to specific analytes (EPSIS)	Antibody, enzyme, receptors and DNA hybridisation	Generic research and development instrumentation for chemical and biological analytes	Abtech Scientific, Inc., P.O. Box 376, Yardley, PA 19067-8376, USA *www.abtechsci.com*
Interdigitated microsensor electrode array device (IME), similar basis to above	As above	IME inert, array microelectrodes for electrochemical and optical chemical and biological sensor development	As above
Hand-held instrument (NanoStat) consisting of a potentiostat for electrochemical detection on a disposable sensor chip	NS	Ascorbic acid (AscoSens) in food applications, e.g. quality control of juices, fruit and vegetables; also, uric acid (UroSens) in clinical samples	Inventus BioTec GmbH & Co, KG, Nottulner Landweg 90, D-48161 Munster, Germany *www.inventus-biotec.com*
In vivo and *in vitro* microdialysis enzyme biosensor with electrochemical detection	Enzyme	Small molecules, e.g. glucose x2 amino acids, glycerol and ascorbate, in clinical matrices	Sycopel International, Rolling Mill Road, Viking Industrial Park, Jarrow Tyne & Wear NE 32 3 DT, UK *www.biotechproducts.com*

(From: Patel, 2000a)

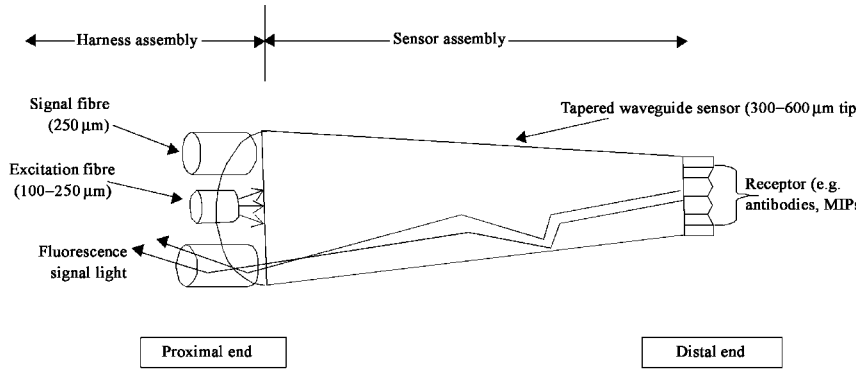

Fig. 12.6 Tapered FO immunosensor (from Patel, 2000a).

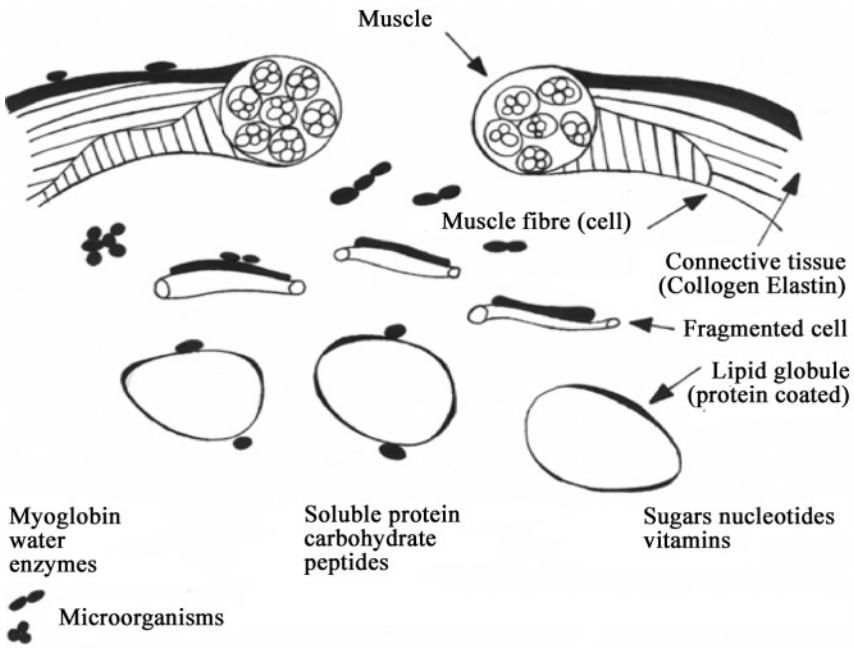

Fig. 12.7 Schematic diagram of a typical meat homogenate.

specific components (e.g., functional protein, micronutrients) or contaminants (e.g., allergen, mycotoxin, or bacterial toxin) present in such a complex matrix would almost certainly require the use of physicochemical techniques for the separation, concentration and resolution of the target analyte prior to measurement.

The following arbitrary definitions are used:

- *Separation:* gross isolation of the target analyte from complex matrices;
- *Concentration*: collection of the target analyte from large volume into smaller volumes; and
- *Resolution*: a specific analyte from a group of analytes and cross-reacting non-target compounds.

The issue of food matrix interference in relation to food microbiological analysis, and implementation of physicochemical and immunological procedures that can reduce interference and enhance the signal-to-noise are covered in detail elsewhere (Patel, 2000b). Examples of some physicochemical techniques and devices that have the potential for use as in-line sample conditioning systems are described below.

12.4.1 Ultrafiltration and dialysis probes

Macromolecules (e.g., proteins and polysaccharides) in solution can be separated from low M.Wt. solutes (e.g., salts, amino acids) by dialysis which utilises a semipermeable membrane to retain macromolecules and allow small solute molecules to pass through (Fig. 12.8). An alternative way of separating macromolecules from low M.Wt. components is by ultrafiltration, in which pressure, vacuum or centrifugal force is used to filter the aqueous medium and low M.Wt. solutes through a semipermeable membrane, which retains the macromolecules (Fig. 12.9). Both of these techniques have been widely exploited in academic settings and by the food industry (e.g., preparation of low lactose milk and hypoallergenic foods).

A biosensor probe integrating dialysis and enzyme-based potentiometric detection (Fig. 12.10), and an ultrafiltration probe (UF, Fig. 12.11) have been used in the medical research field for continuous *in vivo* isolation and detection

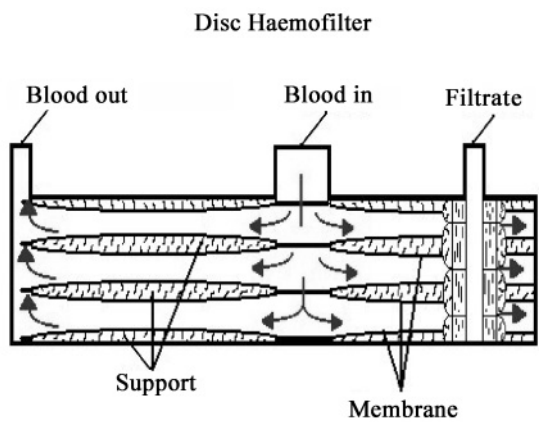

Fig. 12.8 Example of a dialysis cell (disc haemofilter).

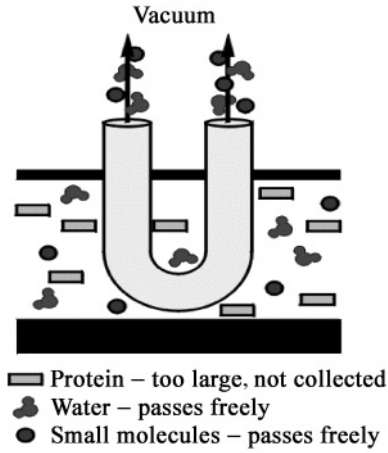

Fig. 12.9 Principle of ultrafiltration.

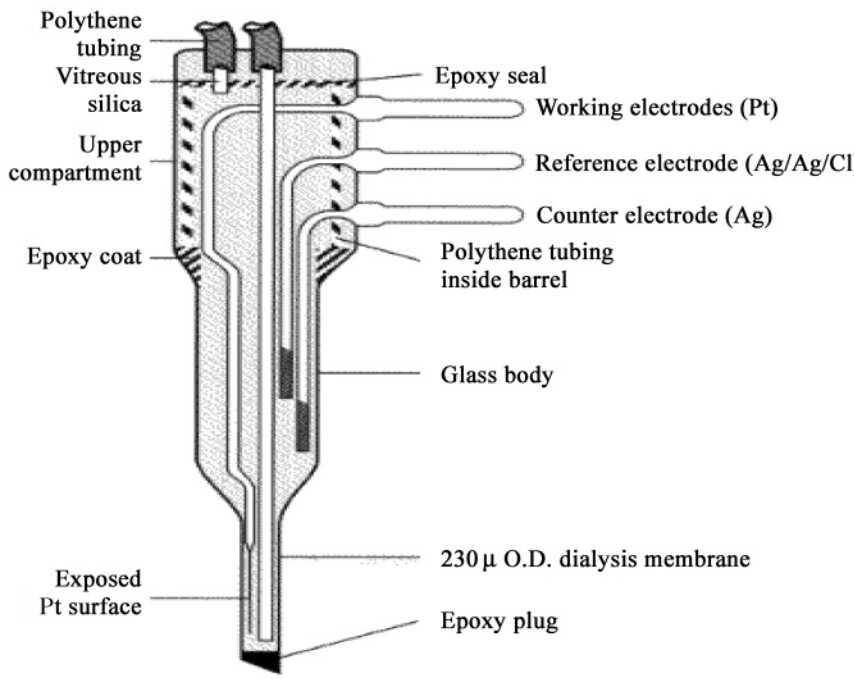

Fig. 12.10 Sycopel microdialysis biosensor.

of low M.Wt. components from flowing blood steams (e.g., glucose, glutamate and acetylcholine). These probes use membranes of 30,000 M.Wt. cut-off value. These types of separation and detection devices with appropriate modifications, including addition of a chemometrics system, can be exploited as novel sample conditioning systems in the development of in-line sensor systems.

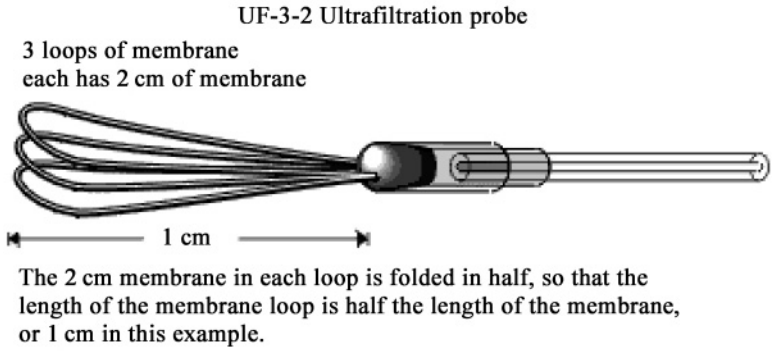

Fig. 12.11 The BAS *in vivo* probe.

12.4.2 Continuous centrifuge

Separation and concentration methods based on batch centrifugation have been widely used both in academia and in industrial plants. A variation of the batch method is the use of continuous centrifugation to separate particles from flowing streams of aqueous media. A recent introduction is the continuous flow centrifugation (Centrifuge^{TM} Stratos, Kendro Laboratory Products Ltd), that has been used for the separation of suspended matter from river water. In this case, the table-top centrifuge connected through a pipe directly linked to a river was shown to achieve a rotor speed of 17,000 rpm with a degree of separation of just over 90%. The system could be valuable in food processing applications, e.g., separation of sugar from molasses. A custom made centrifuge that addresses industrial criteria (e.g., cost, ruggedness, efficiency, calibration) for an integrated in-line sensor could be invaluable as a novel sample conditioning system in the development of a process line sensor.

12.4.3 Ultrasonic standing waves

When particles in suspension are placed in a stationary acoustic field (e.g., 1–3 MHz), they move towards and concentrate at half-wavelength intervals where there is minimum acoustic potential energy (Coakley, 1997). A number of different configurations have been used in order to separate particles and measure their efficiencies. These include the static banding cell, ultrasonic flow cell and ultrasonic. The reported efficiencies for the yeast *Rhodotorula glutinans* in the flow through design were 49–65% retention rate at a concentration of 1×10^7 cfu/ml in aqueous suspensions to 86–96% at 1×10^3 cfu/ml (Zamani *et al*., 1993). However, this rate was shown to be reduced (>60%) with smaller size bacterial cells at a concentration of (10^9–10^{10} cfu/ml; Coakley, 1997). Thus, the higher the biomass the greater the efficiency of retention with the implication that the ability to form clumps at high biomass is an important aspect of harvesting prokaryotes.

The BioSep (AppliSens) is an acoustic device that utilises MHz range ultrasonic waves to separate suspended cells from cell culture medium (Fig.

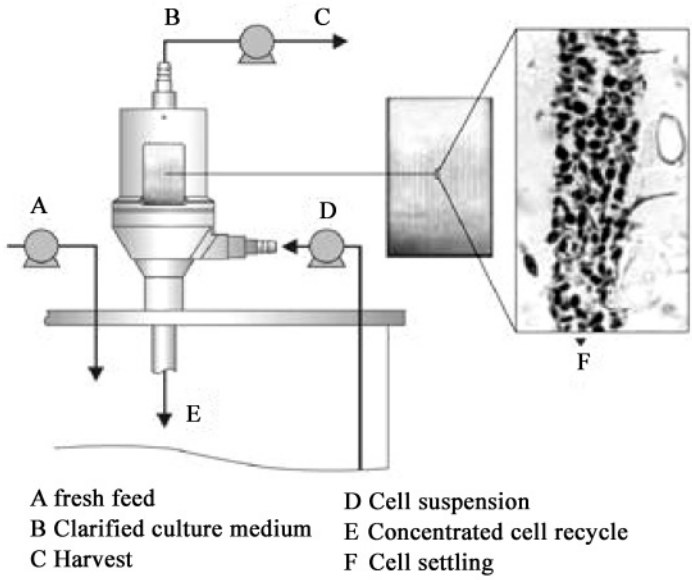

Fig. 12.12 Typical configuration of the AppliSens acoustic cell retention system.

12.12). Cell separation takes place within a defined volume of the BioSep – the resonator. Basically, the resonator is composed of two opposing parallel glass surfaces, one of which is piezoelectrically activated and acts as an ultrasonic source. The resulting standing field captures the cells within the antinodes of the field. The trapped cells typically form loose aggregates settling out of the acoustic field. The cells can immediately be disaggregated if required as in the acoustic perfusion process described briefly below.

The use of BioSep in an acoustic perfusion process involves continuous addition of fresh medium to the bioreactor, while cells are filtered from the harvest stream by the resonator chamber and returned to the bioreactor. The BioSep can be directly mounted onto the bioreactor head plate. A standard mode of operation employs a harvest pump at the exit port of the resonator chamber, and a recirculation pump for the return of separated cells that settled from the acoustic energy field within the resonator chamber. Alternatively the BioSep system can also be set up to allow for semicontinuous operation. In contrast to other cell separation techniques, the BioSep comprises non-contact, non-fouling and non-moving filtration means of separation and concentration allowing for up to thousands of hours of continuous operation.

The Electronic Systems Design Group at the University of Southampton (http://eprints.esc.soton.ac.uk) recently designed and demonstrated a silicon microfluidic ultrasonic separator which could separate and concentrate particles from flowing streams. It is suggested that this offered a functional equivalent of a centrifugal separator for microfluidic systems. The devices are highly

compatible with established microfabrication techniques, allowing low cost mass production. Overall, the combination of ultrasonic device as sample conditioning system and (bio) sensor detection techniques described previously could allow the development of an integrated in-line sensor system for monitoring food components and contaminants.

12.4.4 Free flow electrophoresis (FFE)

FFE is different from the conventional widely used analytical gel-based electrophoresis technique (Patel and Weber, 2003). Thus, in FFE:

- electrophoresis is carried out in free solution in the absence of any stationary phase, there is no transport of analytes inside and/or across a solid matrix such as gel (i.e., no screen segmentation)
- the separation medium and the analytes are transported between electrodes, and the direction of electrical field is perpendicular to the direction of the flow of the separation medium
- the separation of the analytes occurs during a single transit through the electrical field (no recycling process).

The modes of operation in FFE include zone electrophoresis, isoelectric focusing, isotachophoresis, field step electrophoresis and interval mode electrophoresis. Accordingly, FFE can be used to resolve components with net overall charge (e.g., micro-organisms, biopolymers and low M.Wt. ionic species) on a continuous basis.

Some of the major technological developments in the micro-engineering field leading to miniaturisation of analytical systems (also referred to as Lab-on-chip technology) include the following.

- Microfluidics research has resulted in the production of delivery systems (e.g., micro pumps) and associated software to transport samples and reagents accurately in micron-sized channels, where process dynamics are very different from the conventional analytical instrumentation.
- Microfabrication processes based on photolithography and hot embossing techniques have allowed cost-effective mass production of often very complex microstructures on a chip that are comparable to the integrated circuits used in the computer industry.

It must be emphasised that, by miniaturisation, we are not referring to minor size reduction of the various components of laboratory analytical instrumentation. In effect, developments in the above sector are allowing integration of all the functions necessary for carrying out an analytical process on a micro-scale. In the context of process line sensors, such FFE devices in combination with (bio) sensors could provide yet another means of sample conditioning prior to detection of the desired analyte.

12.4.5 Dielectrophoresis (DEP)

DEP has been defined as the motion of a neutral or charged particle (e.g., mammalian or microbial cell) that has undergone polarisation as a result of being placed in a non-uniform electrical field. The non-uniformity of the electrical field results in a non-uniform force distribution on the now polarised particle (known as dipole), causing the particle to move towards the region of highest field intensity. Unlike classical electrophoresis, where movement is largely determined by the overall charge on the particle (or molecule), dielectrophoretic movement is a function of the dielectric properties (conductivity and permittivity) of the particle and the suspending medium. Thus, for DEP to be effective, these parameters must be properly controlled.

DEP uses microstructures for determining the dielectrophoretic properties of cells and the process has been applied widely in biotechnology. This includes dielectrophoretic manipulation of cells, such as plant protoplasts, bacterial cells (e.g., *E. coli*, *Lactobacillus brevis* and *Bacillus subtilis*) and yeast cells (e.g., *Saccharomyces cerevisiae*) from aqueous suspensions. DEP has also been applied to the separation and concentration of micro-organisms from relatively complex food matrices to give a clear microbial suspension suitable for analysis by modern techniques (e.g., ATP bioluminescence, impedimetry and flow cytometry) (Pimbley *et al.*, 1998). In this case, a 3-D flow-through dielectrophoretic chamber was used for rapid (30 min.) separation of total microbial flora from suspensions of various foods (beef, chicken and skimmed milk powder). The overall technique involved rapid (15 min.) desalting of food homogenates (reduction of conductivity from $>2000\ \mu S\ cm^{-1}$ to between 41 and $59\ S\ cm^{-1}$) followed by DEP (15 min.). Further work included DEP application to the separation of spoilage micro-organisms (*Kluyveromyces lactis* and *Pseudomonas aeruginosa*) from lager beer and spores (*Geotrichum candidum*, *Mucor plumbeus* and *Penicillium* spp) from pasteurised whole milk.

DEP has been used in combination with field flow fractionation (DEP-FFF) to separate human breast cancer cells from normal T-lymphocytes. Unlike DEP alone, DEP-FFF has been demonstrated to exhibit a high and electrically controllable discrimination of cell separation. DEP forces produced by microelectrodes at high frequency are used to levitate cells in a thin chamber to equilibrium heights where sedimentation forces balance the vertical DEP forces. A carrier fluid moves through the chamber and establishes a hydrodynamic velocity profile causing cells of different dielectric and density properties to be transported through the chamber at different velocities and thereby separated.

Like FFE, DEP-based microstructures have the potential to be used as sample conditioning systems for process line applications. However, since positive DEP technique is highly dependent on the conductivity of the suspending medium and the particle to be resolved and concentrated, additional procedures involving reduction of sample conductivity must be included prior to DEP and detection of the target particle.

12.4.6 Field flow fractionation (FFF)

The FFF technique is a family of chromatographic-like elution techniques, but without the use of solid phases, in which an external field or gradient (e.g., gravitational, centrifugal, electrical, flow and thermal) causes differential retention of biomolecules (e.g., proteins, peptides and polysaccharides) and particles (latex, microbial and parasites) ranging typically from 1 nm to 300μm (Giddings, 1995).

In practice, FFF takes place in a thin ribbon-like channel. A field applied perpendicular to the channel axis drives components towards one wall (the accumulation wall) of the channel where each forms a steady-state distribution. In most cases, the particles are driven to within 1–10 μm of the accumulation wall. The flow of the sample is laminar and parabolic because of the channel dimensions, ranging from 75–250 μm. The particles are driven to the exit port where they can be detected and characterised using an array of detection systems, including hyphenated detectors (e.g., electron microscope, scan cytometer, photon correlation spectroscopy, multiangle light scattering, FT-IR, ICP-MS).

Four fields have been widely studied.

1. Flow FFF (FFFF) is one of the most universal separation techniques. In FFFF, a cross flowstream of carrier liquid, in which the force orginates from the friction of the cross flow stream across the components results in the separation of the components. The FFFF is rapid (1 min. run time) and can be automated, and has been applied to the fine separation of biomolecules and particles (e.g., single and double stranded DNA, protein dimers) based on differences in the molecule's diffusion coefficient.
2. Sedimentation FFF (SFFF) is when the force is generated usually by gravity or centrifugation. The sedimentation force acts perpendicular to the flow separation axis and is a prominent method for separation and characterisation of colloidal particles.
3. Temperature gradient or thermal FFF (TFFF) is when the perpendicular force is a result of thermal diffusion. The technique has been primarily used for fractionating polymers of high molecular weight, although recently it has also been applied to particles in both aqueous and non-aqueous media.
4. Electrical FFF (EFFF) is when the force is due to the electrical field and the separation depends on the polymer or particle charge and mass. Unlike capillary electrophoresis, in the EFFF the field is perpendicular to the flow of carrier liquid, the potential difference required to produce selectivity is far less stringent since it is applied across the thin gap in the FFF channel and EFFF can process larger sample volumes and larger particle sizes. Unfortunately, the low currents required to keep the electrode polarisation at a minimum, and the fields maintained at reasonable levels, limit the choice of carrier to solutions of low ionic strengths (e.g., 150 μM NaCl).

12.4.7 Improvement of signal-to-noise ratio

Food systems by their very nature tend to be 'noisy' environments. Two main options exist that can extract the information required, the instrumentation can either data mine the information mathematically, or the food process can be sampled in such a way that it isolates only those items of interest. The latter aspect has been covered in detail in the previous section.

Data mining is the colloquial term for statistical techniques that can be used to analyse noisy information and obtain real data on submerged information, particularly in techniques generating complex spectral information (e.g., FT-NIR and Raman). It uses multivariate analytical techniques, which are a major part of chemometrics. These techniques are covered in detail elsewhere in the book and so will be mentioned only briefly. To draw full potential from the observed spectra, techniques such as PCA are used (Hasegawa, 2001). The technique provides solutions to three major issues:

1. quantitative spectral calibration;
2. chemical discrimination analysis with the use of the spectra; and
3. spectral separation into pure chemical component spectra.

The latter is extremely useful as it provides information regarding specific constituents of the mix under analysis. It therefore avoids the need for chromatography and can be considered as chromatography of numerical information. PCA has been found to be very useful at drawing out data relating to trace amounts of chemical species in a mixture.

Spectra obtained by Fourier Transform methods are particularly suited to analysis by PCA as the information from the spectroscope is already digitised. The operation of PCA on the spectrogram (or other multivariate data samples) represents a large part of the functional intelligence of the instrumentation package. PCA is also used in pattern recognition programs and so could be used alongside imaging techniques of analysis.

12.5 Future trends

To date, to the authors' knowledge, there is no commercial technology yet available that can be used for specific industrial in-line process monitoring of trace level components and contaminants of interest or concern to the food industry. A range of technologies, in particular the spectroscopic group, is commercially available for certain compositional analysis and these have been covered in detail elsewhere in the book and briefly in this chapter. Examples of some very recent technologies that have become available for in-line process monitoring have also been included.

The authors' view is that in-line sensors are needed by the food processing industries for a variety of applications, ranging from trace level analysis of low and high M.Wt. analytes (e.g., mycotoxins, bacterial toxins and allergens) to particulate contaminants (e.g., micro-organisms). However, for sensor

technologies to be acceptable by the industry, they must comply with certain strict criteria such as overall cost of the sensors, time to result, continual or continuous measurement, low to unskilled operator requirement, self-calibration and applications to both batch or flowing product streams. It is also the authors' view that 'bolting' an analytical system onto a process line is probably not the way forward. The development is likely to comprise integration of several different technologies and multidisciplinary skills to develop a fit-for-purpose application-specific instrument.

The proposed combination approaches based on sample conditioning systems, many of which are amenable to low-cost mass manufacturing technologies, are discussed in the chapter and are potentially amenable to process line applications. Thus, the proposed future trends are expected to include:

1. simple sample conditioning techniques that resolve or isolate the component or contaminants of interest from complex food matrices;
2. presentation of the relatively 'clean' matrix containing target component to a (fibre-optic) detection system (e.g., spectral detector or biosensor with electrochemical, amperometric, impedemetric or optical detection); and
3. developing the system hardware and software tools for integration into process lines.

Finally, there are new fields of expertise continually being researched and developed in other industries that may well feed into future development in food process development, control and monitoring. For example, microreaction technology opens up new possibilities through the development of small, inexpensive and versatile devices that ensure maximum selectivity, minimum waste and investment, and a better control of the process to create a more efficient process (Ehrfeld, 2000). Some of the basic techniques (e.g., microfabrication, etching and embossing) used for the development of microreactors (e.g., mixers, heat exchangers and reactors) are also common to the development of microanalytical systems. Is it possible that in the quest for process line sensors, microseparation and analytical modules can be developed for microreactors of particular value to the food processing industries?

12.6 Sources of further information and advice

In addition to the references given in the text, the following general references and conference proceedings will be of use to the reader if further information is sought.

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SMYTH C, O'DRISCOLL, B, KURYASHOV E and BUCKIN V (2002), 'High-resolution ultrasonic spectroscopy for dairy analysis', *Eur Fd Scientist*, 25–26.
PROCEEDINGS OF THE 'SECOND WORLD CONGRESS ON INDUSTRIAL PROCESS TOMOGRAPHY', 29–31 August, Hannover, Germany.

Internet sites of value

www.cpact.com: the centre for process analytics and control technology (CPACT) is a multidisciplinary centre formed thorough the UK Foresight Challenge Initiative. CPACT brings together chemical and process engineers, analytical chemists, control systems engineers, chemometricians, signal processing engineers and statisticians, from academia and industry, to research solutions to generic problems in process monitoring and control.

www.flair-flow.com: flair-flow is a network that disseminates food research results in 24 European countries. The Flair-flow 4 synthesis report is entitled 'Food quality sensors' and comprises results from about 20 EU-supported scientific projects.

www.vcipt.org.uk: process tomography.

www.itoms.com: industrial process tomography products and applications.

www.kcl.ac.uk/neuronet: network of excellence in Neural Networks, including international organisations and societies in the field.

www.physics.dcu.ie/PhysicsHome/optronics/fos-en.html: website of the fibre optic sensors European network (FOS_EN).

www.frost.com: market analysis reports on sensors and smart sensors.

www.lab-on-a-chip.com: list of major centres of expertise on microarrays, microfabrication technologies, laboratory automation and micrototal analytical systems. Also includes sites showing lab-on-a-chip consortia, and microarray newsgroups and organisations.

www.microchemicalsystems.co.uk: a company manufacturing microreaction products and equipment. It also has links to major conferences in the micrototal analytical systems area.

www.woice.de/index: site showing microreaction technology 'know how', 'know who' and 'know where'. The microreactors offer a revolutionary alternative to the large-scale production facilities in industrial plants.

12.7 References

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13

Chemometrics in data analysis

R. Leardi, University of Genoa, Italy

13.1 Introduction

In this chapter the fundamentals of chemometrics will be presented by means of a quick overview of the most relevant techniques for data display, classification, modelling and calibration. The goal of the chapter is to make people aware of the great superiority of multivariate analysis over the commonly used univariate approach. Mathematical and algorithmical details will not be presented, since the chapter is mainly focused on the general problems to which chemometrics can be successfully applied in the field of food chemistry.

I am well aware that many of the readers of this book are not familiar with chemometrics, and that a significant percentage among them have never even heard about this 'new' science (it is quite strange that it is still considered a 'new' science, when the Chemometrics Society was founded 30 years ago and the most basic algorithms date back to the beginning of the twentieth century). I also know very well that some among the readers are quite put off by anything involving mathematical computations higher than a square root or statistical tests more complex than a t test.

Therefore, the goal I set for myself in writing this chapter is simply that of being read and understood by the majority of the readers of this book; I will be completely satisfied if some of them, after having read it, could say: 'Chemometrics is easy and powerful indeed, and from now on I will always think in a multivariate way'. Of course, to accomplish this goal in the reduced space of a chapter I must try to highlight the attractive sides of chemometrics. Therefore, I will always try to show the intuitive aspects of each technique, without giving too much relevance to the algorithms.

First of all, what is chemometrics? According to the definition of the Chemometrics Society, it is 'the chemical discipline that uses mathematical and statistical methods to design or select optimal procedures and experiments, and to provide maximum chemical information by analysing chemical data'. One of the major mistakes people make about chemometrics is thinking that to use it one has to be a very good mathematician and to know the mathematical details of the algorithms being used. From the definition itself, it is clear instead that a chemometrician is a *chemist* who can *use* mathematical and statistical methods. If we want to draw a parallel with everyday life, how many among us really know in detail how a TV set, a telephone, a car or a washing machine work? Anyway, everybody watches TV programmes, makes phone calls, drives a car and starts a washing machine. Of course, what is important is that people know what each instrument is made for and that nobody tries to watch inside a telephone, or to drive a TV set, or to speak inside a washing machine or to do the laundry in a car.

Though chemometrics makes available a very wide range of techniques, some of them being very difficult to fully understand and use correctly, the great majority of the real problems can be solved by applying one of the basic techniques, whose understanding, at least from an intuitive point of view, is relatively easy and does not require high-level mathematical skills.

13.2 Data collection and display

Chemometrics works on data matrices. This means that on each sample a certain number of variables have been measured (in the 'chemometrical jargon' we say that each object is described by p variables). Although some techniques can work with a limited number of missing values, a chemometrical data set must be thought of as a spreadsheet in which all the cells are full.

Sometimes, instead, if data are gathered without having any specific project, it happens that the result is a 'sparse' matrix, in which not all the cells contain a value. In that case, if the percentage of missing data is quite high, the whole data set is not suitable for a multivariate analysis; as a consequence, the variables and/or the objects with the lowest number of data must be removed, and therefore a huge amount of experimental effort can be lost.

All the chemometrical software allows the import of data from ASCII files or from spreadsheets. It is therefore suggested to organise the data from the beginning in matrix form as shown in Fig. 13.1, in such a way that the import can be performed in a single step. If, on the contrary, the data are spread in several files or sheets (e.g., one file for each sample or for each variable), then the import procedure would be much longer and more cumbersome.

13.2.1 Data display

The human mind can digest much more information when looking at plots rather than numbers. This is easily demonstrated by looking first at the sequence of

	var. 1	var. 2	var. 3	var. 4	var. 5	var. 6	var. 7	…	var. p
obj. 1									
obj. 2									
obj. 3									
obj. 4									
obj. 5									
obj. 6									
.....									
obj. n									

Fig. 13.1 The structure of a chemometrical data set.

Table 13.1 Ten samples described by one variable

Sample	1	2	3	4	5	6	7	8	9	10
Value	25.3	22.1	25.5	25.6	19.4	25.7	20.2	21.3	25.9	21.8

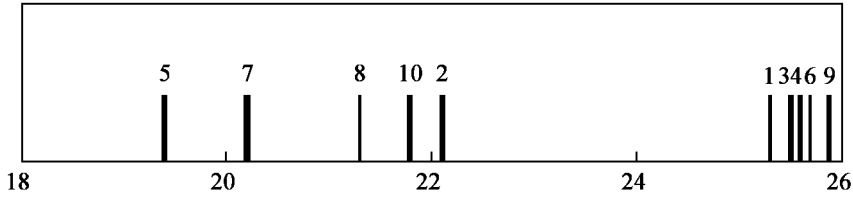

Fig. 13.2 Scatter plot of the data in Table 13.1.

numbers reported in Table 13.1, and then the plot in Fig. 13.2. It is very clear that, even in a very simple data set like this one (just ten samples, and only one variable) the information obtained by looking at the plot is superior and much more easily available than the information one can get by analysing the raw numbers. From the plot, it is very evident that the samples are clustered into two groups of the same size, the one at higher values being much tighter than the one at low values; much more time and effort is required when we want to get the same information from the table.

Let us now take into account a more complex data set, i.e. the one reported in Table 13.2, in which each object is described by two variables. The same data are plotted in Fig. 13.3. This bivariate data set, beyond showing once more that a

Table 13.2 Twenty samples described by two variables

Sample	Variable 1	Variable 2
1	21.2	32.5
2	16.2	21.0
3	13.1	21.7
4	11.6	21.3
5	20.8	29.9
6	10.4	20.6
7	19.5	26.8
8	9.8	25.2
9	15.2	31.2
10	12.0	26.0
11	17.6	28.5
12	24.0	30.0
13	17.8	33.1
14	15.0	24.0
15	11.0	24.2
16	24.8	25.3
17	12.8	23.3
18	26.5	30.6
19	22.9	27.5
20	9.7	22.8

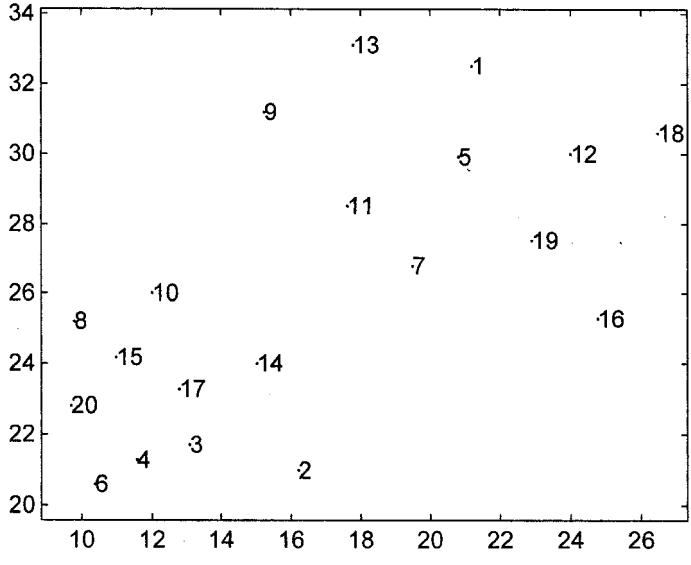

Fig. 13.3 Scatter plot of the data in Table 13.2.

plot is much more easily handled by the human brain than a data table, demonstrates that when dealing with more than one variable, the analysis of just one variable at a time can lead to wrong results. In this data set we have 20 samples, supposed to belong to the same population. When looking at the plot,

we realise that we are in a situation very similar to what we found with the univariate data set; the samples are split into two clusters of the same size, with the objects of the first one more tightly grouped than the objects of the second one. This conclusion cannot be reached when looking at one variable at a time, since neither of the two variables is able to discriminate between the two groups.

If we had a data set with three variables it would still be possible to visualise the whole information by a tridimensional scatter plot, in which the co-ordinates of each object are the values of the variables. But what to do if the variables are more than three? What we need therefore is a technique permitting the visualisation by simple bi- or tri-dimensional scatter plots of the majority of the information contained in a highly dimensional data set. This technique is Principal Component Analysis (PCA), one of the simplest and most used methods of multivariate analysis. PCA is very important especially in the preliminary steps of an elaboration, when one wants to perform an exploratory analysis in order to have an overview of the data.

It is rather common to have to deal with large data tables, in which, for instance, a series of samples is described by a number (p) of chemico-physical parameters. Examples of such data sets can be samples of olive oils from different origins described by their content in fatty acids and sterols, or samples of wines described by Fourier-Transformed Infra-Red (FT-IR) spectra. It is easy to realise how, especially in spectral data sets, p can be very high (> 1000); in such cases it would be impossible to obtain valuable information without the help of multivariate techniques.

From a geometrical point of view, we can consider a p-dimensional space, in which each dimension is associated to one of the variables. In this space each sample (object) has co-ordinates corresponding to the values of the variables describing it. Since it is impossible to visualise all the information at once, one should stay content with the analysis of several bi- or three-dimensional plots, each of them showing a different part of the global information.

It is also evident that not all possible combinations of two or three variables will give the same quality of information; for instance, if some variables are very highly correlated, then the information brought by each of them would be almost the same. If two variables are perfectly correlated, then one of them can be discarded, losing no information at all; in this way, the dimensionality of our space will be reduced from p to $p - 1$. If two variables are very highly correlated, then the elimination of one of them would produce only a slight loss of information, while the dimensionality of the space would be reduced to $p - 1$. So, one can deduce that the information contained in the 'lost' p^{th} dimension was well below the average of the information contained in the other dimensions.

It is quite apparent now that not all the dimensions have the same importance, and that, owing to the correlations among the variables, the 'real' dimensionality of our data matrix is somehow lower than p. Therefore, it would be very valuable to have a technique capable of concentrating on a few variables, and therefore on a few dimensions, the bulk of our information. This is exactly what

is performed by PCA; it reduces the dimensionality of the data and extracts the most relevant part of the information, placing into the last dimensions the non-structured information, i.e., the noise. According to these two characteristics, the information contained in very complex data matrices can be visualised in just one or a few plots.

From the mathematical point of view, the goal of PCA is to obtain, from p variables ($X_1, X_2, \ldots, X_p$), p linear combinations having two important features: to be uncorrelated and to be ordered according to the explained variance (i.e., to the information they contain). The lack of correlation among the linear combinations is very important, since it means that each of them describes different 'aspects' of the original data. As a consequence, the examination of a limited number of linear combinations (generally the first two or three) allows us to obtain a good representation of the studied data set.

From a geometrical point of view, what is performed by PCA corresponds to looking for the direction that, in the p-dimensional space of the original variables, brings the greatest possible amount of information (i.e., explains the greatest variance). Once the first direction is identified, the second one is looked for; it will be the direction explaining the greatest part of the residual variance, under the constraint of being orthogonal to the first one. This process goes on until the p^{th} direction has been found. These new directions can be considered as the axes of a new orthogonal system, obtained after a simple rotation of the original axes. While in the original system each direction (i.e., each variable) brings with it, at least in theory, $1/p$ of total information, in the new system the information is concentrated in the first directions, and decreases progressively so that in the last ones no information, only noise, can be found.

The global dimensionality of the system is always that of the original data (p), but, since the last dimensions explain only a very small part of the information, they can be neglected and one can take into account only the first dimensions (the 'significant components'). The projection of the objects in this space of reduced dimensionality retains almost all the information, which can now be analysed also in a visual way, by bi- or three-dimensional plots. These new directions, linear combinations of the original ones, are the Principal Components (PC) (or Eigenvectors).

With a mathematical notation, we can write:

$$\text{var}(Z_1) > \text{var}(Z_2) > \ldots > \text{var}(Z_p)$$

where $\text{var}(Z_i)$ is the variance explained by component i. Furthermore, since a simple rotation has been performed, the total variance is the same in the two systems of axes:

$$\sum \text{var}(X_i) = \sum \text{var}(Z_i)$$

The first PC is formed by the linear combination

$$Z_1 = a_{11}X_1 + a_{12}X_2 + \ldots + a_{1p}X_p$$

explaining the greatest variance, under the condition that

$$\sum a_{1i}^2 = 1$$

This last condition notwithstanding, the variance of Z_1 could be made greater simply by increasing one of the values of a. The second PC

$$Z_2 = a_{21}X_1 + a_{22}X_2 + \ldots + a_{2p}X_p$$

is the one having var(Z_2) as large as possible, under the conditions that

$$\sum a_{2i}^2 = 1$$

and that

$$\sum a_{1i}a_{2i} = 0$$

(this last condition assures the orthogonality of components one and two).

The lower order components are computed in the same way, always under the two conditions previously reported.

From a mathematical point of view, PCA is solved by finding the eigenvalues of the variance-covariance matrix; they correspond to the variance explained by the corresponding principal component. Since the sum of the eigenvalues corresponds to the sum of the diagonal elements (trace) of the variance-covariance matrix, and the latter corresponds to the total variance, one has the confirmation that the variance explained by the principal components is the same explained by the original data. It is now interesting to locate each object into this new reference space. The co-ordinate on the first PC is computed simply by substituting into equation $Z_1 = a_{11}X_1 + a_{12}X_2 + \ldots + a_{1p}X_p$ the terms X_i with the values of the corresponding original variables. The co-ordinates on the other principal components are then computed in the same way. These co-ordinates are named scores, while the constants a_{ij} are named loadings. By taking into account the loadings of the variables on the different principal components, it is very easy to understand the importance of each single variable in constituting each PC; a high absolute value means that the variable under examination plays an important role for the component, while a low absolute value means that it has a very limited importance.

If a loading has a positive sign, it means that the objects with a high value of the corresponding variable have high scores on that component; if the sign is negative, then the objects with low values of that variable will have high scores. As already mentioned, after a PCA the information is mainly concentrated on the first components. As a consequence of that, a plot of the scores of the objects on the first components allows the direct visualisation of the global information in a very efficient way; it is now very easy to detect similarity between objects (similar objects have a very similar position in the space) or the presence of outliers (they are very far from all other objects) or the existence of clusters. Taking into account at the same time scores and loadings it is also possible to interpret very easily the differences among objects or groups of objects, since it

is immediatly understandable which are the variables giving the greatest contribution to the phenomenon under study.

Now, let us see the application of PCA to a real data set. Seven variables describing the protein composition have been measured on 23 samples of peas, of different cultivars. Fifteen samples were from smooth pea cultivars, while eight samples were from wrinkled pea cultivars. The data are reported in Table 13.3. It could be interesting to check whether the protein composition of the smooth peas is different from that of the wrinkled peas. When looking separately at each of the seven variables, it can be seen that none of them completely separates the two categories. Therefore, one could say that, though some variables are on average higher in one category (e.g., the vicilin/legumin ratios are higher in the wrinkled peas), it is not possible to discriminate between smooth and wrinkled peas. As a consequence, one could look for different (and possibly more expensive to be determined) variables.

After a PCA (Fig. 13.4), it is instead evident that the information present in the seven variables is sufficient to clearly discriminate the two categories. Once more, it has to be pointed out that taking into account all the variables at the same time gives much more information than just looking at one variable at a time.

Now, let us go one step back and let us try to understand how this result has been obtained. At first, since the variables have different magnitudes and different variances, a normalisation has to be performed, in such a way that each variable will have the same importance. Autoscaling is the most frequently used normalisation: it subtracts from each variable the mean value, and divides the result by the standard deviation of that variable. After that, each variable will have mean = 0 and variance = 1. Table 13.4 shows the data after autoscaling.

The results of PCA are such that PC1 explains 30.3% of the total variance and PC2 23.6%. This means that the PC1–PC2 plots shown in Fig. 13.4 explain 53.9% of total variance.

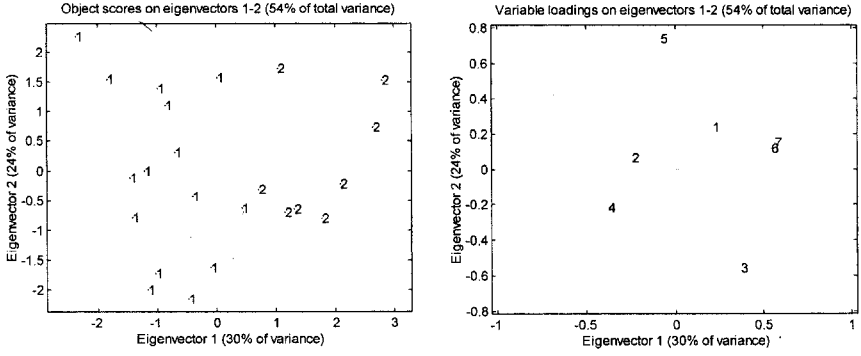

Fig. 13.4 PCA of the data of Table 13.3. On the left, the score plot of the objects (coded according to the category number), on the right the loading plot of the variables (coded according to the order in Table 13.3).

Table 13.3 Protein composition of peas (Gueguen and Barbot, 1988) (reduced data set). (a) 1 = smooth pea cultivars; 2 = wrinkled pea cultivars; (b) Laurell's technique; (c) ultracentrifugation

Object	Category (a)	Protein	Non-prot. material	Albumin	Globulin	Insoluble prot. fract.	Vicilin/ legumin (b)	Vicilin/ legumin (c)
1	1	219	20.7	24.3	55.7	20.0	2.2	2.0
2	1	273	30.2	12.3	61.0	26.6	1.3	1.5
3	1	255	17.8	19.3	53.8	26.9	1.5	2.0
4	1	262	30.2	13.1	63.2	23.5	1.6	2.3
5	1	242	20.8	20.8	52.6	26.5	0.8	1.3
6	1	235	16.1	23.2	60.8	16.0	0.8	1.4
7	1	272	14.9	17.9	62.1	19.9	0.8	1.3
8	1	235	24.5	25.1	59.6	14.9	0.8	1.4
9	1	225	22.0	25.0	58.8	16.1	1.9	1.8
10	1	195	20.0	15.1	58.6	26.2	2.1	2.1
11	1	181	18.7	16.1	65.4	18.4	2.7	3.2
12	1	236	16.6	20.0	57.0	23.0	1.2	1.6
13	1	261	22.1	19.2	63.7	17.0	1.3	1.6
14	1	244	21.9	19.6	65.0	22.2	1.8	1.9
15	1	239	32.1	27.9	58.0	14.1	1.6	1.6
16	2	263	19.8	21.9	59.4	18.6	2.5	2.5
17	2	263	20.3	22.8	60.3	16.8	2.9	2.8
18	2	309	18.5	24.6	58.5	16.8	2.2	2.5
19	2	241	16.7	24.0	58.6	17.3	2.5	3.7
20	2	241	19.3	24.6	55.6	19.7	3.2	3.2
21	2	292	21.3	20.0	54.6	25.3	2.0	3.0
22	2	287	21.2	21.5	54.7	23.7	4.3	3.3
23	2	278	20.0	23.1	55.6	21.3	2.5	4.7

Table 13.4 Autoscaled data

Protein	Non-prot. material	Albumin	Globulin	Insoluble prot. fract.	Vicilin/legumin (b)	Vicilin/legumin (c)
−1.040	−0.094	0.837	−0.871	−0.115	0.304	−0.326
0.777	2.042	−2.144	0.614	1.495	−0.727	−0.887
0.171	−0.746	−0.405	−1.403	1.569	−0.498	−0.326
0.407	2.042	−1.946	1.230	0.739	−0.383	0.010
−0.266	−0.071	−0.032	−1.739	1.471	−1.300	−1.111
−0.502	−1.128	0.564	0.558	−1.090	−1.300	−0.999
0.743	−1.398	−0.753	0.922	−0.139	−1.300	−1.111
−0.502	0.760	1.036	0.222	−1.359	−1.300	−0.999
−0.838	0.198	1.011	−0.002	−1.066	−0.040	−0.551
−1.847	−0.251	−1.449	−0.058	1.398	0.189	−0.214
−2.318	−0.543	−1.200	1.846	−0.505	0.876	1.018
−0.468	−1.015	−0.231	−0.507	0.617	−0.842	−0.775
0.373	0.221	−0.430	1.370	−0.846	−0.727	−0.775
−0.199	0.176	−0.331	1.734	0.422	−0.154	−0.439
−0.367	2.469	1.732	−0.227	−1.554	−0.383	−0.775
0.440	−0.296	0.241	0.166	−0.456	0.647	0.234
0.440	−0.184	0.465	0.418	−0.895	1.105	0.570
1.988	−0.588	0.912	−0.086	−0.895	0.304	0.234
−0.300	−0.993	0.763	−0.058	−0.773	0.647	1.579
−0.300	−0.409	0.912	−0.899	−0.188	1.449	1.018
1.416	0.041	−0.231	−1.179	1.178	0.075	0.794
1.248	0.019	0.142	−1.151	0.788	2.709	1.130
0.945	−0.251	0.539	−0.899	0.203	0.647	2.699

Table 13.5 Loadings of the variables on PC1 and PC2

	Protein	Non-prot. material	Albumin	Globulin	Insoluble prot. fract.	Vicilin/ legumin (b)	Vicilin/ legumin (c)
PC1	0.214	−0.239	0.370	−0.372	−0.080	0.546	0.563
PC2	0.237	0.066	−0.557	−0.219	0.739	0.115	0.151

Table 13.6 Scores of the objects on PC1 and PC2

Object	Category	Score on PC1	Score on PC2
1	1	0.425	−0.627
2	1	−2.358	2.264
3	1	0.006	1.576
4	1	−1.841	1.548
5	1	−0.858	1.100
6	1	−1.023	−1.735
7	1	−1.453	−0.119
8	1	−1.153	−1.998
9	1	−0.099	−1.623
10	1	−0.978	1.386
11	1	−0.404	−0.439
12	1	−0.700	0.304
13	1	−1.408	−0.784
14	1	−1.217	−0.004
15	1	−0.466	−2.148
16	2	0.714	−0.312
17	2	1.151	−0.705
18	2	1.304	−0.647
19	2	1.781	−0.806
20	2	2.085	−0.226
21	2	1.040	1.724
22	2	2.797	1.535
23	2	2.653	0.737

Table 13.5 shows the loadings of the variables on PC1 and PC2. From it, the loading plot in Fig. 13.4 is obtained.

From the score plot in Fig. 13.4 it can be seen that PC1 perfectly separates the two categories. By looking at the loading plot and at Table 13.5 it is possible to know which are the variables mainly contributing to PC1 (and therefore to the separation). Variables six and seven (the two vicilin/legumin ratios) have the loadings with the highest absolute values, both being positive. This means that these ratios are higher in the wrinkled peas (the objects of category two, being on the right side of the score plot, have higher scores on PC1) than in the smooth peas. Also albumin and globulin have high absolute value of their loadings on PC1, though having opposite sign (positive for albumin, negative for globulin).

This means that wrinkled peas have a higher content of albumin and a lower content of globulin. Table 13.6 reports the scores of the objects on PC1 and PC2.

As previously shown, the scores of an object are computed by multiplying the loadings of each variable by the value of the variable. As an example, let us compute the score of sample one on PC1 (since the autoscaled data have been used, these are the values that must be taken into account):

$$0.214 * (-1.040) + (-0.239) * (-0.094) + 0.370 * 0.837 + (-0.372$$
$$*(-0.871) + (-0.080) * (-0.115) + 0.546 * 0.304 + 0.563 * (-0.326)$$
$$= 0.425$$

13.3 Classification

In the previous section we could verify that the smooth and the wrinkled peas are indeed well separated in the multivariate space of the variables. Therefore, we can say that we have two really different classes. Let us suppose we now get some smashed peas (so that we cannot see if they are smooth or wrinkled) and we want to know which is their class. After having performed the chemical analyses, we can add these data to the previous data set, run a PCA and see where the new samples are placed. This will be fine if the new samples fall inside one of the clouds of points corresponding to a category, but what if they fall in a somehow intermediate position? How can we say with 'reasonable certainty' that the new samples are from a smooth or from a wrinkled pea? We know that PCA is a very powerful technique for data display, but we realise that we need something different if we want to classify new samples. What we want is a technique producing some 'decision rules' discriminating among the possible categories.

While PCA is an 'unsupervised' technique, the classification methods are 'supervised' techniques, since they must be told to which category each of the objects belongs. The most commonly used classification techniques are Linear Discriminant Analysis (LDA) and Quadratic Discriminant Analysis (QDA). They define a set of delimiters (according to the number of categories under study), in such a way that the multivariate space of the objects is divided in as many subspaces as the number of categories, and that each point of the space belongs to one and only one subspace. Rather than describing in detail the algorithms behind these techniques, I will focus on the critical points of a classification.

As I said earlier, the classification techniques use objects belonging to the different categories to define boundaries delimiting regions of the space. The final goal is to apply these classification rules to new objects that will be classified into one of the existing categories. The performance of the technique can be expressed as classification ability and prediction ability. The difference between 'classification' and 'prediction', though quite subtle at first glance, is instead very important and its underestimation can lead to very bitter deceptions.

Table 13.7 Example of the performance of a classification technique

Category #	Objects	Correct class	% correct class
1	112	105	93.8
2	87	86	98.9
3	21	10	47.6
Total	220	201	91.4/80.1

Classification ability is the capability of assigning to the correct category the same objects that have been used to build the classification rules, while prediction ability is the capability of assigning to the correct category objects that have not been used to build the classification rules. Since the final goal is the classification of new samples, it has to be clear that predictive ability is by far the most important score to be looked at.

The results of a classification method can be expressed in several ways. The most synthetic one is the percentage of correct classifications (or predictions). Note that in the following, only the term 'classification' will be used, but it has to be understood as 'classification or prediction'. This can be obtained as the number of correct classifications (independently of the category) divided by the total number of objects, or as the average of the performance of the model over all the categories. The two results are very similar when the size of all the categories is very similar, but can be very different if the size is quite different. Let us consider the case shown in Table 13.7. The very poor performance of category three, by far the smallest one, almost does not affect the classification rate computed on the global number of classifications, while it produces a much lower result if the classification rate is computed as the average of the three categories.

A more complete and detailed overview of the performance of the method can be obtained by using the classification matrix, by which also the categories to which the wrongly classified objects are assigned can be known (in many cases the cost of an error can be quite different according to the category the sample is assigned to). In it, each row corresponds to the true category and each column to the category to which the sample has been assigned. Going on with the previous example, a possible classification matrix is the one shown in Table 13.8. From it, it can be seen that the 112 objects of category one were classified in the following way: 105 correctly to category one, none to category two and seven to category three. In the same way, it can be deduced that all the objects of category three that

Table 13.8 Example of a classification matrix

Category	1	2	3
1	105	0	7
2	1	86	0
3	11	0	10

were not correctly classified have been assigned to category one. Therefore, it is easy to conclude that category two is well defined and that the classification of its objects gives no problems at all, while categories one and three are quite overlapping. As a consequence, to have a perfect classification more efforts must be made to better separate categories one and three. All this information cannot be obtained from just the percentage of correct classifications.

If overfitting occurs, then the prediction ability will be much worse than the classification ability. To avoid it, it is very important that the sample size is adequate to the problem and to the technique. A general rule is that the number of objects should be more than five times (anyway, no less than three times) the number of parameters to be estimated. LDA works on a pooled variance-covariance matrix; this means that the total number of objects should be at least five times the number of variables. QDA computes a variance-covariance matrix for each category; this makes it a more powerful method than LDA, but this also means that each category should have a number of objects at least five times higher than the number of variables. This is a good example of how the more complex, and therefore 'better' methods, sometimes cannot be used in a safe way because their requirements do not correspond to the characteristics of the data set.

13.4 Modelling

In classification, the space is divided into as many subspaces as categories, and each point belongs to one and only one category. This means that the samples that will be predicted by such methods must belong to one of the categories that have been used to build the models; if not, they will anyway be assigned to one of them. To make this concept clearer, let us suppose the use of a classification technique to discriminate between water and wine. Of course, this discrimination is very easy, and each sample of water will be correctly assigned to the category 'water' and each sample of wine will be correctly assigned to the category 'wine'. But what happens with a sample of orange squash? It will be assigned either to the category 'water' (if variables such as alcohol are taken into account) or to the category 'wine' (if variables such as colour are considered). The classification techniques are therefore not able to define a new sample as being 'something different' from all the categories of the training set. This is instead the main feature of the modelling techniques.

Though several techniques are used for modelling purposes, UNEQ (one of the modelling versions of QDA) and SIMCA (Soft Independent Model of Class Analogy) are the most used. While in classification every point of the space belongs to one and only one category, with these techniques the models (one for each category) can overlap and leave some regions of the space unassigned. This means that every point of the space can belong to one category (the sample has been recognised as a sample of that class), to more than one category (the sample has such characteristics that it could be a sample of more than one class)

or to none of the categories (the sample has been considered as being different from all the classes).

Of course, the 'ideal' performance of such a method would be not only to correctly classify all the samples in their category (as in the case of a classification technique), but also that the models of each category would be able to accept all the samples of that category and to reject all the samples of the other categories. The results of a modelling technique are expressed the same way as in classification, plus two very important parameters: specificity and sensitivity. For category *c*, its specificity (how much the model rejects the objects of different categories) is the percentage of the objects of categories different from *c* that have been rejected by the model, while its sensitivity (how much the model accepts the objects of the same category) is the percentage of the objects of category *c* that have been accepted by the model.

While the classification techniques need at least two categories, the modelling techniques can be applied also when only one category is present. In this case the technique detects if the new sample can be considered as a typical sample of that category or not. This can be very useful in the case of Protected Denomination of Origin products, to verify whether a sample, declared as having been produced in a well-defined region, has indeed the characteristics typical of the samples produced in that region.

The application of a multivariate analysis will greatly reduce the possibility of fraud. While an 'expert' can adulterate a product in such a way that all the variables, independently considered, still stay in the accepted range, it is almost impossible to adulterate a product in such a way that its multivariate 'pattern' is still accepted by the model of the original product, unless the amount of the adulterant is so small that it becomes unprofitable from the economic point of view.

13.5 Calibration

Let us imagine we have a set of wine samples and that on each of them the FT-IR spectrum is measured, together with some variables such as alcohol content, pH or total acidity. Of course, the chemical analysis will require much more time than a simple spectral measurement. It would therefore be very useful to find a relationship between each of the chemical variables and the spectrum. This relationship, after having been established and validated, will be used to predict the content of the chemical variables. It is easy to understand how much time (and money) this will save, since in a few minutes it will be possible to have the same results previously obtained by a whole set of chemical analyses.

Generally speaking, we can say that multivariate calibration finds relationships between one or more response variables y and a vector of predictor variables x. As the previous example should have shown, the final goal of multivariate calibration is not just to 'describe' the relationship between the x and the y variables in the set of samples on which the relationship has been

computed, but to find a real practical application on samples that in a following time will have the x variables measured.

The model is a linear polynomial ($y = b_0 + b_1x_1 + b_2x_2 + \ldots + b_Kx_K + f$), where b_0 is an offset, the b_k ($k = 1, \ldots, K$) are regression coefficients and f is a residual. The 'traditional' method of calculating **b**, the vector of regression coefficients, is Ordinary Least Squares (OLS). This method has anyway two major limitations, that make it inapplicable to many data sets:

- it cannot handle more variables than objects
- it is sensitive to collinear variables.

It can be easily seen that both these limitations do not allow the application of OLS to spectral data sets, where the samples are described by a very high number of highly collinear variables. If one wants to use OLS to such data anyway, the only way to do it is to reduce the number of variables and their collinearity through a suitable feature selection (see later).

When describing the PCA, it has been noticed that the components are orthogonal (i.e., uncorrelated) and that the dimensionality of the resulting space (i.e., the number of significant components) is much lower than the dimensionality of the original space. Therefore, it can be seen that both the aforementioned limitations have been overcome. As a consequence, it is possible to apply OLS to the scores originated by PCA. This technique is Principal Component Regression (PCR).

It has anyway to be considered that Principal Components are computed by taking into account only the x variables, without considering at all the y variable(s), and are ranked according to the explained variance of the 'x world'. This means that it can happen that the first PC has little or no relevance in explaining the response we are interested to. This can be easily understood by considering that, even when we have several responses, the PCs to which the responses have to be regressed will be the same.

Nowadays, the most favoured regression technique is Partial Least Squares Regression (PLS, or PLSR). As it happens with PCR, PLS is based on components (or 'latent variables'). The PLS components are anyway computed by taking into account both the x and the y variables and therefore they are slightly rotated versions of the Principal Components. As a consequence, the order by which they are ranked corresponds to the importance in the modelling of the response. A further difference with OLS and PCR is that, while the former must work on each response variable separately, PLS can be applied to multiple responses at the same time.

Because both PCR and PLS are based on latent variables, a very critical point is the number of components that have to be retained. Though we know that information is 'concentrated' in the first components and that the last components explain just noise, it is not always an easy task to detect the correct number of components (i.e., when information finishes and noise begins). Selecting a lower number of components would mean removing some useful information (underfitting), while selecting a higher number of components would mean incorporating some noise (overfitting).

Before applying the results of a calibration, it is very important to look for the presence of outliers. Three major types of outliers can be detected: outliers in the x-space (samples for which the x-variables are very different from that of the rest of the samples; they can be found by looking at a PCA of the x-variables), outliers in the y-space (samples for which the y-variable is very different from that of the rest of the samples; they can be found by looking at a histogram of the y-variable) and samples for which the calibration model is not valid (they can be found by looking at a histogram of the residuals).

The goodness of a calibration can be summarised by two values, the percentage of variance explained by the model and the Root Mean Square Error in Calibration (RMSEC). The former, being a 'normalised' value, gives an initial idea about how much of the variance of the data set is 'captured' by the model; the latter, being an absolute value to be interpreted in the same way as a standard deviation, gives information about the magnitude of the error.

As already described in the classification section and as pointed out at the beginning of this section, the goal of a calibration is essentially not to describe the relationship between the response and the x-variables of the samples on which the calibration is computed (training, or calibration, set), but to apply it to future samples on which only the cheaper x-variables will be measured. In this case too, the model must be validated by using a set of samples different from those that have been used to compute the model (validation, or test, set). The responses of the objects of the test set will be computed by applying the model obtained by the training set and then compared with their 'true' response. From these values the percentage of variance explained in prediction and the Root Mean Square Error in Prediction (RMSEP) can be computed. Provided that the objects forming the two sets have been selected flawlessly, these values give the real performance of the model on new samples.

13.6 Variable selection

Usually, not all the variables of a data set bring useful and non-redundant information. Therefore, a variable (or feature) selection can be highly beneficial, since from it the following results are obtained:

- removal of noise and improvement of the performance
- reduction of the number of variables to be measured and simplification of the model.

The removal of noisy variables should always be looked for. Though some methods can give good results even with a moderate amount of noise disturbing the information, it is clear that their performance will benefit when this noise is removed. So, feature selection is now also widely applied for those techniques (PLS and PCR) that in the beginning were considered to be almost insensitive to noise.

While noise reduction is a common goal for any data set, the relevance of the reduction of the number of variables in the final model depends very much on the kind of data constituting the data set, and a very wide range of situations are possible. Let us consider the extreme conditions:

- each variable requires a separate analysis
- all the variables are obtained by the same analysis (e.g., chromatographic and spectroscopic data).

In the first case, each variable not selected means a reduction in terms of costs and/or analysis time. The variable selection should therefore always be made on a cost/benefit basis, looking for the subset of variables leading to the best compromise between performance of the model and cost of the analyses. This means that, in the presence of groups of useful but highly correlated (and therefore redundant) variables, only one variable per group should be retained. With such data sets, it is also possible that a subset of variables giving a slightly worse result is preferred, if the reduction in performance is widely compensated by a reduction in costs or time.

In the second case, the number of retained variables has no effect on the analysis cost, and the presence of useful and correlated variables improves the stability of the model.

Intermediate cases can happen, in which 'blocks' of variables are present. As an example, take the case of olive oil samples, on each of which the following analyses have been run: a titration for acidity, the analysis of peroxides, a UV spectroscopy for ΔK, a GC for sterols and another GC for fatty acids. In such a situation, it is not the final number of variables that counts, but the number of analyses one can save.

The only possible way to be sure that 'the best' set of variables has been picked up is the 'all-models' techniques, by which all the possible combinations are tested. Since, with k variables, the number of possible combinations is 2^k-1, it is easy to understand that this approach cannot be used unless the number of variables is really very low (e.g., with 30 variables more than 10^9 combinations should be tested).

The simplest (but least effective) way of performing a feature selection is to operate on a 'univariate' basis, by retaining those variables having the greatest discriminating power (in the case of a classification) or the greatest correlation with the response (in the case of a calibration). By doing that, each variable is taken into account by itself, without considering how its information 'integrates' with the information brought by the other (selected or unselected) variables. As a result, if several highly correlated variables are 'good', they are all selected, without taking into account that, owing to their correlation, the information is highly redundant and therefore at least some of them can be removed without any decrease in performance. On the other hand, those variables are not taken into account that, though not giving by themselves significant information, become very important when their information is integrated with that of other variables.

An improvement is brought by the 'sequential' approaches. They select the best variable and then the best pair formed by the first and second and so on in a forward or backward progression. A more sophisticated approach applies a look back from the progression to reassess previous selections. The problem with these approaches is that only a very small part of the experimental domain is explored and that the number of models to be tested becomes very high in the case of highly dimensional data sets, such as spectral data sets. For instance, with 1,000 wavelengths, 1,000 models are needed for the first cycle (selection or removal of the first variable), 999 for the second cycle, 998 for the third cycle, and so on.

More 'multivariate' methods of variable selection, especially suited for PLS applied to spectral data, are currently available. Among them, we can cite Interactive Variable Selection (Lindgren *et al*., 1994), Uninformative Variable Elimination (Centner *et al*., 1996), Iterative Predictor Weighting PLS (Forina *et al*., 1999) and Interval PLS (Nørgaard *et al*., 2000)

13.7 Future trends

In future, multivariate analysis should be used more and more in everyday (scientific) life. Not so many years ago, experimental work resulted in a very limited amount of data, and the analysis of these few numbers was quite easy and straightforward. Nowadays, it is common to have instrumentation producing an almost continuous flow of data. One example is process control performed by measuring the values of several process variables at a rate of one measurement every few minutes (or even seconds). Another example is quality control of the final product of a continuous process, on which an FT-IR spectrum is taken every few minutes (or seconds).

In section 13.5 I cited the case of wine FT-IR spectra, from which the main characteristics of the product can be directly predicted. It is therefore clear that the main problem has shifted from obtaining a few data to the treatment of a huge amount of data. It is also clear that standard statistical treatment is not enough to extract all the information buried in them.

Many instruments have already some chemometrics routines built into their software in such a way that their use is totally transparent to the final user (and sometimes the word 'chemometrics' is not even mentioned, to avoid possible aversion). Of course, they are 'closed' routines, and therefore the user cannot modify them. It is quite obvious that it would be much better if chemometric knowledge were much more widespread, in order that the user could better understand what kind of treatment his data have undergone and eventually modify the routines in order to make them more suitable for his requirements. As computers become faster and faster, it is now possible routinely to apply some approaches requiring very high computing power. Two of them are genetic algorithms (GA) and artificial neural networks (ANN).

Genetic algorithms are a general optimisation technique, that has found good applicability in many fields, especially when the problem is so complex that it

cannot be tackled with the 'standard' techniques. In chemometrics it has been applied especially in feature selection (Leardi, 2000). GA try to simulate the evolution of a species according to the Darwinian theory. Each experimental condition (in this case, each model) is treated as an individual, whose 'performance' (in the case of feature selection for a calibration problem, it can be the explained variance) is treated as its 'fitness'. Through operators simulating the fights among individuals (the best ones have the greatest probability of mating and thus spreading their genome), the mating among individuals (with the consequent 'birth' of 'offspring' having a genome that is derived by both the parents) and the occurrence of mutations, the GA result in a pattern of search that, by mixing 'logical' and 'random' features, allows a much more complete search of complex experimental domains.

Artificial neural networks try to mimic the behaviour of the nervous system to solve practical computational problems. As in life, the structural unit of ANN is the neuron. The input signals are passed to the neuron body, where they are weighted and summed, then they are transformed, by passing through the transfer function into the output of the neuron. The propagation of the signal is determined by the connections between the neurons and by their associated weights. The appropriate setting of the weights is essential for the proper functioning of the network. Finding the proper weight setting is achieved in the training phase. The neurons are usually organised into three different layers: the input layer contains as many neurons as input variables, the hidden layer contains a variable number of neurons and the output layer contains as many neurons as output variables. All units from one layer are connected to all units of the following layer. The network receives the input signals through the input layer. Information is passed to the hidden layer and finally to the output layer that produces the response.

These techniques are very powerful, but very often they are not applied in the correct way. In such cases, despite a very good performance on the training set (due to overfitting), they will show very poor results when applied to external data sets.

13.8 Conclusion: The advantages and disadvantages of chemometrics

In one of his papers, J. Workman Jr. (Workman, 2002) very efficiently depicts the advantages and disadvantages of multivariate thinking for scientist in industry.

From the eight advantages of chemometrics he clearly outlines, I would like to underline the following ones:

1. Chemometrics provides speed in obtaining real-time information from data.
2. It allows high-quality information to be extracted from less resolved data.
3. It promises to improve measurements.

4. It improves knowledge of existing processes.
5. It has very low capital requirements – it's cheap.

The last point especially should convince people to give chemometrics a try. No extra equipment is required, just an ordinary computer and some chemometrical knowledge (or a chemometrical consultancy). It is certain that in the very worst cases the same information as that from a classical analysis will be obtained in a much shorter time and with much more evidence; in the great majority of cases, instead, also a simple PCA can provide much more information than what was previously collected. So, why are people so shy in applying chemometrics? Workman (2002) gives some very common reasons:

1. The perceived disadvantage of chemometrics is that there is widespread ignorance about what it is and what it can realistically accomplish.
2. This science is considered too complex for the average technician and analyst.
3. Chemometrics requires a change in one's approach to problem solving from univariate to multivariate thinking.

So, while chemometrics leads to several real advantages, its 'disadvantages' lie only in the general reluctance to use it and accepting the idea that the approach that has been followed over many years can turn out not to be the best one.

13.9 Sources of further information and advice

13.9.1 Books

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15

Meat and meat products

M. Lees and B. Popping, Eurofins Scientific Analytics, France

15.1 Introduction

Meat authentication is related in part to issues of definition. If a product is to be labelled correctly, meat must be defined both in relation to which parts of the animal are used and the presence of other constituents such as fat, water or connective tissue which are not defined as meat. Meat authentication is also linked to problems of adulteration. Adulteration usually involves substituting high value raw materials with cheaper ones. Adulteration problems include:

- substituting meat from one species for another
- adding poorer quality meat such as mechanically recovered meat (MRM)
- using cheaper proteins of vegetable origin, for example

This chapter looks at these authentication issues, beginning with species identification.

15.2 Species identification

This chapter discusses a number of analytical methods:

- electrophoretic techniques
- antibody techniques
- DNA techniques
- chromatographic techniques

15.2.1 Electrophoretic techniques

Electrophoretic techniques are frequently used in species identification as a specific spectrum of soluble protein bands is produced for each animal species. Identification involves the use of homogenous gels, concentration gradient gels, pH gradient gels or denaturants such as urea or detergents that dissociate the tertiary protein structure. The band patterns in a supporting gel are visualized by simple non-specific staining or by enzymological or immunological methods. (Patterson, 1985). There are a number of electrophoretic techniques available:

- polyacrylamide gel electrophoresis (Anon, 1988b)
- isoelectric focusing (IEF) (Malmheden, 1986)
- sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Zerifi *et al.*, 1991b and c)

A number of these methods have been used to identify meat species. IEF has been used to differentiate between species in raw meat (Skare *et al.*, 1969; Bauer and Kelner, 1989). It has given good results with beef, pork, mutton, lamb, horse meat and venison (Anon, 1988a). It has also been used to distinguish poultry, game, goat, buffalo, deer and antelope (Collins, 1986; Jemmi and Schlosser, 1991; King and Kurth, 1982; Gleeson *et al.*, 1983). SDS-PAGE can be used to differentiate meat from cattle, sheep, lamb, deer and rabbit (Kim, 1989).

Electrophoretic methods have also been applied to heated products but specificity can be poor in thoroughly cooked products. Quantitative results have been obtained for meat pie fillings and canned meat loaf which have been autoclaved at 115ºC for 40 min. This is possible by extraction in 8 M urea and 1% 2-mercaptoethanol at 18–20ºC for 16 h, followed by separation on gels containing 6% polyacrylamide (Guy *et al.*, 1973; Connell, 1973; Anon, 1988a; Hofmann, 1988). IEF has also been used to detect one species mixed in another at levels above 20% in heated products (Jemmi and Schlosser, 1991). SDS-PAGE has also been shown to differentiate between species, even in mixtures (Zerifi *et al.*, 1991a). If a large number of proteins are present, identification can be difficult as electrophoretic patterns are often complex and difficult to interpret. Positive identification in either of these techniques relies on the comparison of carefully selected sets of species-specific bands. This means that authentic control material is required for ultimate confirmation (Hofmann, 1989).

15.2.2 Antibody techniques

Antibodies bind to their targets (antigens) in a very specific manner. Different antibodies bind selectively to particular antigens such as serum albumin or muscle protein from a particular animal species. The effectiveness of immunoassays depends on selecting both a suitable antibody and antigen. Antibodies developed against thermostable muscle antigens have been used to differentiate species such as cattle, pig, horse, camel and buffalo (Gacheru *et al.*,

1994). Antibodies developed against soluble chicken proteins have been used to detect the presence of chicken in raw meat mixtures (Stevenson *et al.*, 1994; Martin *et al.*, 1991). Monoclonal antibodies have also been developed for this purpose as well as for identifying other species such as horse (Martin *et al.*, 1991; Garcia *et al.*, 1994).

As has been noted, the choice of antigen is critical to the effectiveness of a selected antibody. As an example, the use of an anti- (chicken IgG) antibody to detect chicken meat in heated meat mixtures has a detection limit of 5–10% (Rossmanith and Bauer, 1994). However, antibodies selected against adrenal (BE) antigens (rather than heated IgG antigens) improve the detection limit to 1% (Reddy *et al.*, 1990). Target antigens used for meat and meat products include blood or serum proteins, such as albumin, though muscle proteins, preparations of adrenals and sarcoplasmatic extracts have been used for species such as rabbit, goat and sheep. Reliability and sensitivity usually decreases with increased heating of samples, even when relatively stable proteins, such as myoglobulin (Hayden, 1979), adrenal preparations (Hayden, 1981) or troponin (Schweiger *et al.*, 1983), are used as target antigens.

Traditional antibody techniques include immunodiffusion and immuno-electrophoretic methods, and remain useful for routine analysis because of their relative simplicity and robustness. Such methods are based on commercially available species-specific antisera against bovine, ovine, porcine and equine antigens (Allsup, 1987). Examples include field tests (with overnight incubations) for identifying bovine and poultry meat down to 1%, detecting multiple adulterants (beef, poultry meat, pork, mutton, horse meat, venison) in a variety of meat products (raw whole, ground or emulsified products) (Cutrufelli *et al.*, 1987; Cutrufelli *et al.*, 1993).

The most sensitive assays have been established using the sandwich enzyme immunoassay format. Compared to the non-competitive assay format, utilising direct coating of the antigen to the solid phase, the sandwich enzyme immuno-assay avoids variation of the assay response due to variations in coating efficiency if different types of samples are analysed. The main advantages of enzyme immunoassays over immunodiffusion or immunoelectrophoresis procedures are greater sensitivity, reduced assay time (usually less than 4 hours), the need for small amounts of antisera, the possibility of obtaining quantitative results, and the potential for automation, thus allowing a large number of samples to be processed.

Like immunodiffusion methods, enzyme immunoassays are best suited for analysis of raw meat or mildly heated meat products. Enzyme immunoassays utilising blood or serum proteins, such as albumin, as the target antigen show limited suitability in testing heat-treated sample materials. A progressive loss in activity is observed with increased heat treatment due to denaturation of the antigen (Goodwin, 1992). On the other hand, a number of assays have been described based on antibodies against heat stable or heat treated antigens (Berger *et al.*, 1988; Patterson and Jones, 1989; Sawaya *et al.*, 1990; Sherikar *et al.*, 1993). Another problem is that the antigenic properties of blood and serum

proteins may change on storage (Griffiths and Billington, 1984). However, the main drawback of these methods is the need for more complex extraction steps. Antisera have to be treated by absorption of immunoaffinity procedures to provide sufficient specificity. A water or buffer solution extract of meat or meat products is used in many enzyme immunoassay formats. Enzyme immunoassays are discussed in more detail in Chapter 3.

15.2.3 DNA techniques

DNA methods are based on sequence-specific recognition and binding of probes or primers to unique DNA sequences. DNA recognition has a number of advantages over methods based on detection of proteins, notably its sensitivity. DNA withstands most sorts of food processing better than proteins. DNA methods are often applicable to heavily heat-treated foods such as canned meat products. The vast amount of information present in DNA also allows differentiation between species that are closely related. It is even possible to determine the sex of an animal or to assign an animal to a population and thereby resolve some questions related to the geographical origin of meat.

There are a number of different DNA techniques, including hybridisation-based methods, Polymerase Chain Reaction (PCR) and sequence analysis. A number of hybridisation procedures have been reported (Buntjer *et al.*, 1995, 1999; Hunt *et al.*, 1997; Janssen *et al.*, 1998) with a sensitivity of 1 to 5%. They require less skilled personnel to operate them than other DNA techniques. The chief limitation of hybridisation procedures are that they do not differentiate closely related species like sheep and goat, bovine species or deer species (Waye and Haigh, 1992).

Most DNA assays are based on PCR amplification which gives assays a high degree of sensitivity. Amplification of mitochondrial DNA has been used to differentiate animal species, though it can give misleading results in cases of species hybridisation (Verkaar *et al.*, 2001; Nijman *et al.*, 2003; Partis *et al.*, 2000). Some mitochondrial PCR methods are more sensitive to hybrids (Colombo *et al.*, 2000; Montiel-Sosa *et al.*, 2000). Detection of repetitive elements in a DNA sequence also allows more sensitive analysis of pork and beef (Calvo *et al.*, 2002a, 2002b; Guoli *et al.*, 1999). Other PCR techniques include PCR-sequencing which has been used to identify endangered or exotic meat species (Forrest and Carnegie, 1994), and PCR-RFLP (Restriction Enzyme Length Polymorphism) used to distinguish game and beef-producing species (Burgener and Hubner, 1998; Wolf *et al.*, 1999; Verkaar *et al.*, 2001). The choice between the available methods depends on the specific application and on considerations of convenience. Satellite hybridisation is suitable for screening many samples simultaneously, but has a limited flexibility. For PCR, mitochondrial DNA offers a better sensitivity than nuclear single-copy genes DNA. PCR-RFLP is the most simple and flexible method for detection of species-specific mutations. Sequencing of the PCR product and matching the sequence to the nucleotide database is more expensive, but is much less likely to

be disturbed by intraspecies variation. DNA methods are discussed in more detail in chapter 2.

15.2.4 Chromatographic techniques

Gas chromatography has been used to analyse fatty acid profiles in meat to identify differences in fatty acid composition between species and ages of animals, including pork, beef, chicken, horse and buffalo (Cantoni and Galli, 1992; De Brabander and van Hoof, 1991). A combination of High Performance Liquid Chromatography (HPLC) and SDS-PAGE has also been used to distinguish between species by analysing ratios of histidine dipeptides such as carnosine, anserine and balenine (Carnegie *et al.*, 1982, 1983, 1985).

A simple quantitative and sensitive method for the determination of chicken or turkey in a blend uses liquid chromatography (LC). The method involves extraction of water soluble proteins followed by injection into the LC system. Chicken and turkey chromatograms have major specific peaks which can be used for species identification (Ashoor *et al.*, 1988). This method can also be used with frozen chicken and turkey, but not with heat-treated samples. The method has a detection limit of 5% and does not suffer serious interference from the presence of other common meats such as beef or pork (Ashoor and Osman, 1988).

15.3 Meat content and adulteration

Overall meat content can be estimated by chemical analysis of nitrogen content (Lees, 1998). The method involves the determination of the nitrogen content of the sample using a standard method (ISO 937: 1978) and the calculation of the 'fat-free meat content' by application of an appropriate nitrogen factor. Nitrogen factors are the mean nitrogen content of cuts of raw meat of particular species expressed on a fat-free basis. A range of nitrogen factors have been published (Lumley, 1996). It is essential that corrections are made for sources of non-meat nitrogen (e.g. soya protein, rusk, etc.) and excess connective tissue (via determination of hydroxyproline). Fat content can be analysed using a standard method (ISO 1443: 1973), as can connective tissues via measurement of hydroxyproline (ISO 3496: 1994). The addition of water in meat can be determined on the basis of the Feder value (Pearson, 1976).

Mechanically recovered meat (MRM) is prepared by applying high pressures to bones to which residual meat is still attached. The process results in MRM containing foreign material. This is the basis for detecting the presence of MRM in meat products. SDS-PAGE has been used to detect MRM in admixtures with a detection limit of 5–10% for red meat and 25% for poultry meat (Savage *et al.*, 1995). Since heat treatment influences the protein pattern significantly, the method is less suitable for heat-treated samples. MRM can often be detected by the presence of bone and cartilage fragments, revealed by microscopy

(Panebianco and Giovanni, 1988). This approach has been evaluated in a number of mixtures containing MRM and hand-deboned meat, both raw and heat-treated (Pickering *et al.*, 1995a). The different protein composition of MRM and hand-deboned meat also provides the basis for using immunoassays to detect MRM. MRM can be differentiated from hand-deboned meat because the former contains reduced amounts of actin and myosin (Stevenson *et al.*, 1991; Pickering *et al.*, 1995b). One problem with this technique is interference from other meat product constituents like blood proteins, soya proteins and milk proteins.

Added blood in comminuted meat products can be detected by detecting haemoglobin using isoelectric focusing (Horn, 1991). This method can identify a blood content of less than 0.1%. The addition of 4–5% blood in frankfurter-type products has also been detected by rocket immunoelectrophoresis (Bremer *et al.*, 1989). Soy proteins in meat products can also be detected by SDS-PAGE and numerous kits are also available for the ELISA detection of soya, casein and gluten (Tantillo and Tiecco, 1988; Hugo, 1995; Hall *et al.*, 1987). Nuclear Magnetic Resonence (NMR) imaging has also been used to identify pure beef muscle adulterated by liver or kidney to a level of 10%. NMR is discussed in chapter 6.

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16

Milk and dairy products

F. Ulberth, European Commission, Belgium

16.1 Introduction: authenticity issues for milk and dairy products

The driving force behind any adulteration is to maximise revenues by either using a cheap ingredient to (partially) substitute a more expensive one, or to (partially) remove the valued component in the hope that the altered product passes undetected by the final user or consumer. Watering of milk or skimming off cream are good examples to illustrate the point, and these fraudulent operations have been practised for a long time. Dairy products account for a large share of the total value of agricultural production in the developed world. Major advances in agronomy, large-scale transport, processing, and the introduction of efficient distribution systems have resulted in increased technological complexity, a higher degree of globalisation and lower product prices. The economics of dairying are very complex and mostly governed by intricate guidelines and laws to balance production and demand, stabilise prices and protect local interests. A complicated market scheme for milk products is at the very heart of the European Union's Common Agricultural Policy (Rasmussen, 2003). Fraudulent malpractice creates unfair competition, leading to market distortions, which in turn may impact the local or even the international economy. Therefore, authentication of milk and dairy products is of primary importance both for consumers and manufacturers, and at all levels along the process chain.

Extension of a product with a cheap ingredient, also known as 'economic adulteration', usually does not carry a health hazard for consumers. This statement is not generally valid regarding adulterated milk and milk products. Consumers allergic to cows' milk may suffer severely if they ingest, e.g., ovine or caprine milk fraudulently extended with bovine milk or whey.

As a consequence most countries have set up a complicated legal framework to ensure proper consumer protection and to foster fair trade practices. Compositional product standards, codified by national as well as supranational authorities, e.g., FAO/WHO Codex Alimentarius, International Dairy Federation (IDF) and the European Commission (EC), represent an integral part of food legislation.

Product labelling is a vital instrument to inform consumers about the identity of a product, thereby obliging producers to conform to predefined product standards. Infringement of labelling regulations could lead to criminal charges in the courts. Therefore, analytical data used in court or in other disputes have to stand up to scrutiny. A large number of methods have been developed and standardised with a view to that particular purpose. They can be found in method manuals issued, e.g., by the IDF, the Association of Analytical Chemists International (AOAC Internat.), or the EC.

Most of the product standards refer *inter alia* to hygienic quality parameters, e.g., total plate count, number of various indicator micro-organisms, somatic cell count, etc., which are at the borderline of product authenticity and product safety. These issues are beyond the scope of this chapter and readers are referred to the respective literature (e.g., Robinson, 2002).

In general, authenticity issues fall into at least one of the following categories:

- non-compliance with legal requirements (product standards) such as
  - max/min content of water, solids-non-fat, and fat in certain dairy products (butter, cheese, yoghurt, etc.)
  - geographical origin of the product
- wrongful addition of certain ingredients of dairy or non-dairy origin such as
  - watering of milk
  - milk of different species
  - addition of non-dairy protein
  - altering the casein/whey protein ratio
  - addition of buttermilk or whey powder to milk powder
  - addition of vegetable or animal fats to milk fat
  - addition of reconstituted milk to fluid milk
  - non-authorised preservatives
- non-compliance regarding use of certain technological processes
  - heat treatment
  - cheese ripening
  - membrane technology.

Whatever type of fraud is perpetrated, authenticity testing relies either on a fundamental difference between the original and the adulterant, or on an intimate knowledge of their composition and possible ranges of compositional

variation. The former case is much more tractable than the latter. Detection of foreign proteins added to milk of a certain species by exploiting differences in their electrophoretic mobilities is an example where a fundamental dissimilarity is used to check the purity of the product. If no tangible differences exist, compositional data of authentic samples have to be gathered, taking into account all possible natural variations, e.g., due to breed, stage of lactation, production systems, geographical origin, etc. The authenticity of a product is confirmed when its compositional data fit into the data space represented by authentic samples. This type of testing usually depends on some form of statistical decision making procedure. Recently, chemometrics, the discipline concerned with application of multivariate statistical methods as well as those methods based on mathematical logic, to chemistry (Brown *et al.*, 1992), are increasingly applied to compositional data in order to solve authenticity and classification problems.

Milk represents a very complex physico-chemical system, where virtually all components present contribute information that is valuable for authenticity testing (Table 16.1). Clear cut distinctions of different principles are of course not always possible, e.g., heat treatment results in an increased formation of Maillard products which are derived from the reaction of proteins with reducing sugars (lactose).

Table 16.1 Analytes of indicative value for the detection of adulteration of milk and milk products

Milk component	Source of adulteration	Analyte(s)
Fat	Non-dairy fat or oil Buttermilk added to milk	Fatty acids Triglycerides Phospholipids Sterols Fat-soluble vitamins
Protein	Non-dairy proteins Milk of a different species Whey added to milk Heat load	Caseins Whey proteins Glucomacropeptide Casein bound-P Protein-N Denatured proteins
Lactose	Water Heat load	Freezing point Furosin Lysinoalanine HMF Glycosylated proteins
Minerals	Water	Freezing point

16.2 Detection and quantification of foreign fats

Milk fat (MF) is perhaps the most valued milk component and therefore has been the target of dubious manipulations for a long time. Traditional physico-chemical methods to verify the authenticity of MF, e.g., by determining the iodine value (a measure of the total unsaturation of a fat), Reichert-Meissel value (titrimetric determination of steam-volatile, water-soluble fatty acids (FA)), or Polenske value (titrimetric determination of steam-volatile, but water-insoluble fatty acids) are successful only in recognising massive adulteration of MF, or even its substitution by another fat (Collomb and Spahni, 1991). With the advent of gas-liquid chromatography (GLC) techniques, the classical fat values were substituted by the analysis of the complete FA spectrum.

Strategies to detect adulterated MF are based either on the concentration ranges of individual FA or the concentration ratios of two or more FA. A large number of different FA ratios have been proposed (Fox *et al.*, 1988; Hughebaert and Hendrickx, 1971; Muuse *et al.*, 1986; Toppino *et al.*, 1982; Ulberth and Rogenhofer, 1989; Younes and Soliman, 1986). The effectiveness of 19 such indices were compared using the FA composition of a large number of authentic MF samples (Ulberth, 1994). The addition of vegetable fats or oils was easier to detect than commingling of animal fats. Additions of coconut fat or linoleic acid rich vegetable oils (sunflower seed oil, corn oil, safflower oil, etc.) were traced down to a level of 2% by the ratios C12:0/C10:0 and C14:0/C12:0, and C18:2/C8:0, respectively. At a level of 10% commingling, palm oil and olive oil were detected by using C14:0/C18:2 and C18:2/C8:0. At 5% commingling 50% of the adulterated samples passed the test undetected. Tallow was particularly difficult to detect, the ratio C16:0/C14:0 being the most suitable. However, it was possible only to detect 15% of the cases where 5% tallow had been added to MF. If the information content of an FA chromatogram was exploited in a more efficient way, i.e., subjected to linear discriminant analysis instead of forming FA ratios, more than 95% of cases where either tallow, lard, olive oil or palm oil were added to MF at the 3% level, were correctly classified (Ulberth, 1994).

FA ratios have been used with success to discriminate between MF of different species (bovine, ovine and caprine milk). Iverson and Sheppard (1989) used the C12:0/C10:0 ratio to detect the addition of bovine to ovine or caprine cheese milk. For instance, the ratio for bovine MF averages around 1.16, while it is 0.46 for caprine and 0.58 for ovine MF. This ratio was employed to indicate the level of cows' milk in cheeses labelled as goats' or ewes' milk cheese. Other FA indicators, mostly based on the ratio of a medium-chain and a volatile, short-chain FA (e.g., C14:0/C8:0), have been proposed and they were summarised by Ramos and Juarez (1984). They also reported that the limit of detection for cows' MF in mixture with goats' or ewes' MF is 5–10%. Applying a more sophisticated methodology (pattern recognition techniques) to evaluate certain FA ratios (C14:0/C8:0, C14:1/C8:0 and C14:1/C16:1) an even more sensitive limit for the detection of cows' in ewes' milk cheese was established (Schwaiger and Vojir, 1995).

The difficulty of detecting fat of animal origin added to MF has led to the development of so-called triglyceride (TG) formulae for MF purity control. Originally, Timms (1980) described an approach using the information content inherent to the TG profile of MF and combined it with a multivariate evaluation of the results to allow the determination of non-MF, including animal depot fats, in MF down to a level of 5%. His basic idea was further refined by Precht and co-workers (Precht and Heine, 1986a, b; Precht, 1992a, b). Several collaborative studies organised by the EC demonstrated the general applicability of the approach (Precht, 1992c). It was adopted as a reference method for the detection of foreign fats in MF within the EC (Commission Regulation (EC) No 213/2001). Based on the TG profile of 755 different MF so-called S-values were derived by regression analysis. The S-values for authentic MF fluctuate within a certain range. If these limits are transgressed, the presence of a foreign fat can be assumed with a given level of statistical confidence. All types of foreign fats can be detected using the formula:

$$\begin{aligned} S = {} & -2.7575^{*}C26 + 6.4077^{*}C28 + 5.5437^{*}C30 - 15.3247^{*}C32 \\ & + 6.2600^{*}C34 + 8.0108^{*}C40 - 5.0336^{*}C42 + 0.6356^{*}C44 + 6.0171^{*}C46 \end{aligned}$$

For authentic MF the S-value for the 'total formula' fluctuates within a range of 95.68 to 104.32 (99% confidence level). Typical values for the limit of detection are 4.5–5.0% for vegetable fats (soybean oil, olive oil, palm oil, etc.) and 4.7% for lard and 5.4% for tallow. For a number of foreign fats (e.g. coconut fat, palm oil, lard, etc.) particular TG formulae have been developed, which are more sensitive and allow detection at a level of 2–3% adulterant.

The validity and applicability of the TG formulae have been confirmed by others (Collomb *et al.*, 1998a; Luf, 1988; Povolo *et al.*, 1999; Ulberth *et al.*, 1998; Van Renterghem, 1997). Although packed column GLC has been used to establish the TG formulae, certain types of capillary columns are fully equivalent and can be used without impacting the approach (Collomb *et al.*, 1998b; Molkentin and Precht, 1994; Ulberth *et al*., 1998). Besides multiple linear regression analysis, other ways of multivariate treatment of TG data to detect foreign fats were suggested (principal components analysis and partial least squares regression techniques, artificial neural networks), but have not found widespread acceptance (Collomb *et al.*, 1998c; Lipp, 1996a, b). Intensive lipolysis of MF, e.g., in (over)matured cheese, may lead to false positive results when the TG formulae are applied, highlighting the need for the development of special formulae taking into account fat degradation (Battelli and Pellegrino, 1994). The TG formula approach to verify the authenticity of bovine MF was recently extended to caprine MF (Fontecha *et al.*, 1998).

Sterol analysis is a straightforward way to detect vegetable fats added to MF, since phytosterols do not occur in MF in measurable amounts. Detection of β-sitosterol, stigmasterol, campesterol, etc., is taken as unequivocal proof that a vegetable fat is present. However, care has to be exercised in drawing correct conclusions as minor components (e.g., lanosterol) in chromatograms of the

unsaponifiable of MF may elute closely to β-sitosterol (Homberg, 1991). Currently, two methods standardised by IDF exist for the determination of sterols in MF; one is based on the difference in melting points of phytosteryl acetate and cholesteryl acetate (IDF Standard 32: 1965), the other on a GLC procedure (IDF Standard 54: 1970). A similar GLC procedure to detect β-sitosterol and stigmasterol was also described by the EC (Commission Regulation (EC) No 213/2001). As an alternative to the lengthy sample preparation described in those standards, a hyphenated LC-GC technique was introduced (Kamm *et al.*, 2002).

The limit of detection for the determination of β-sitosterol via LC-GC was found to be 2 mg/kg fat. This is considerably lower than detection limits of 40 mg/kg and 10 mg/kg, respectively, reported for the conventional procedures, (Homberg and Bielefeld, 1979). Considering the amount of β-sitosterol present in rapeseed oil (ca. 4000 mg/kg), this indicates that an addition of about 0.05% rapeseed oil to MF would be detectable. An adulteration at such a low level is of no practical concern. However, additions of only 1–2% vegetable oil will be detected with certainty. Even for palm oil, an example for a vegetable oil exhibiting only a relatively low content of β-sitosterol (200–400 mg/kg), the resulting limit of detection (0.5–1%) would be sufficient.

Misbranding of spreadable fats, which contain MF and suitable non-MF, is another problem area. Products introduced on the spreadable fats market within the EU must comply with Council Regulation (EC) No 2991/94. Up to now, no official method for the determination of the MF proportion in the fat blend has been specified. Usually, butyric acid (C4:0), which exclusively occurs in MF of ruminant animals, is used as a marker to estimate the amount of MF in the blend. A number of reliable methods have been suggested for the determination of C4:0 in spreadable fats by GLC (Molkentin and Precht, 1998a, b; Pocklington and Hautfenne, 1986; Ulberth 1998a, b) or by HPLC (Christie *et al.*, 1987). A major drawback of the approach is the natural variation of the C4:0 content in MF (Molkentin and Precht, 1997).

For a representative number of samples taken all over Europe the figures varied between 3.07 g and 3.75 g per 100 g MF, with a mean value of 3.42 g/100 g. The variations were due to differences in feeding regimen, lactation stage and breed. By using the average content of C4:0 in MF the proportion of MF in an unknown fat blend may thus deviate by up to $\pm$10% from the true value, without taking into account any additional analytical errors (Molkentin and Precht, 1998b). When a sample of the MF used for blend formulation is available for analytical testing, the performance of the method in terms of accuracy and precision can be improved. The EU Expert Group 'Milk and Milk Products' (Agriculture DG) has collaboratively tested the latter methodology in order to gain precision data. Reproducibility of the method was 1.7% MF for a mixture containing 25% MF, and 3.2% MF for a mixture containing 60% MF (Molkentin and Precht, 2000).

Alternatives to chromatographic techniques for MF authentication like differential scanning calorimetry (Bringer *et al.*, 1991; Coni *et al.*, 1994) and

infra-red spectroscopic techniques (Sato *et al.*, 1990; Laporte and Paquin, 1998) were proposed but have not found wide application. In particular, spectroscopic techniques would be highly welcome, since they do not need lengthy sample preparation, have therefore a high throughput, and are non-destructive testing methods.

16.3 Identifying milk of different species

In most countries producers of dairy products are required to label the milk type (bovine, ovine, caprine) used for manufacture. Since the production volume of ovine and caprine milk is much smaller and their supply varies to a considerable extent, an incentive for economic adulteration exists. Moreover, certain traditional products that are highly valued by consumers on a world-wide scale, like Mozzarella, Roquefort, Manchego, Pecorino or Feta cheese, are exclusively made from non-bovine milk. Hence, analytical methods are needed to check for the presence of cows' milk in products declared to be made solely from ewes', goats' or water-buffalos' milk. In some production areas the addition of goats' milk to ewes' milk used for the production of traditional cheese varieties could also be an issue.

Differences in the molecular make-up of milk proteins are the primary route to discriminate milk of different species. Various forms of electrophoresis, chromatography and immunochemistry are used as analytical tools to track down those differences. The protein-based methods may be supplemented by the analysis of the fat phase (FA ratios, TG profile) as described in section 16.2.

Separation of milk proteins by various forms of electrophoresis is one of most applied techniques in dairy products authentication. Early attempts focused on the higher electrophoretic mobility in polyacrylamide gels (PAGE) of the α_{s1}-casein of bovine milk as compared to ovine and caprine milk (Aschaffenburg and Dance, 1968; Foissy, 1976; Freimuth and Krause, 1968). A limit of detection of 1% of cows' in goats' milk was reported by those authors. As the caseins are partially degraded during cheese ripening, newly formed peptides obscure to a certain extent the region of the bovine α_{s1}-casein. As a result the sensitivity of the method drops, and data interpretation becomes much more difficult. Discontinuous electrophoresis of β-caseins was used as an alternative by Mayer and Hörtner (1992) for the determination of bovine caseins in milk and dairy products.

Due to its high separation efficiency, isoelectric focusing in thin polyacrylamide gels (PAGIF) has become a preferred technique for the separation of complex protein mixtures. Applying this technique Krause *et al*. (1982) made use of the γ-caseins, proteolytic breakdown products of β-casein, as an indicator for the detection of an admixture of bovine to ovine and caprine milk and cheese. Ovine and caprine milk cannot be distinguished by this technique. The addition of plasmin to cheese caseins in order deliberately to create γ-caseins and their subsequent separation by PAGIF greatly enhances the sensitivity of the method

(Addeo *et al.*, 1990a). It has been officially adopted for the control of cheese within the EU (Commission Regulation (EC) No 213/2001).

Evaluation is performed by comparing the protein patterns of the unknown sample with reference standards on the same gel. Detection of cows' milk in cheeses from ewes', goats' or water buffalos' milk and mixtures of ewes', goats' and buffalos' milk is done via the γ_3- and γ_2-caseins, whose isoelectric points range between pH 6.5 and pH 7.5. The limit of detection is less than 0.5% of cows' milk. The method is suitable for a sensitive and specific detection of native and heat-treated cows' milk and caseinate in fresh and ripened cheeses made from non-bovine milk but it is not suitable for the detection of milk and cheese adulteration by heat-treated bovine whey protein concentrates.

PAGIF of para-κ-caseins allows not only the detection of an addition of bovine milk to cheese from other species but also differentiation between cheese of ovine or caprine origin. However, in the case of ripened Roquefort cheese, a peptide migrating with cows' milk para-κ-casein was identified leading to a false-positive response (Addeo *et al.*, 1990b). Such difficulties were not noticed in an other study with Camembert made from milk of different species (Mayer *et al.*, 1997). The method can be extended to other dairy products. The addition of rennet to ewes' yoghurt to artificially produce para-κ-caseins and subsequent separation of the caseins by cationic PAGE allowed the detection of cows' milk down to 1% (Kaminarides and Koukiassa, 2002).

Whey proteins are not markedly altered by proteolysis during cheese ripening. In order adequately to exploit this feature, separation systems have been set up based on differences in electrophoretic mobilities of whey proteins, in particular of β-lactoglobulin (Addeo *et al.*, 1989; Amigo *et al.*, 1991; Rispoli *et al.*, 1991). When silver nitrate was used for staining, cows' milk at a level of 1% was detectable in various types of cheese (Amigo *et al.*, 1991). The drawback of the method is that heat treatment denatures whey proteins to a variable extent thus affecting the test results. For example, heating to 90ºC for 30 min. denatured the whey proteins and gave negative results when cows' milk was added to milk for cheese making (Amigo *et al.*, 1991).

The increased availability of commercial capillary electrophoresis instrumentation has led to an increased transfer of traditional electrophoretic assay formats to this novel technique. The high resolution power and the speed of analysis are its most attractive features. Both the casein fraction and the whey proteins can be analysed and used for authentication purposes. As is the case with traditional PAGE, α_{s1}-casein of bovine milk had the highest mobility among the different caseins by applying capillary zone electrophoresis (CZE) in an uncoated tube, which proved to be useful for the detection of cows' milk in goats' milk (Lee *et al.*, 2001). However, caprine para-κ-caseins and bovine β-casein were also found to be good markers for the presence of the milk of these species in Iberico-type cheese (Molina *et al.*, 2000). The differences between the CZE patterns of the casein fraction of bovine, ovine and caprine milk allowed identification and even quantification of the milk of each species in binary and ternary mixtures by multivariate regression analysis (Molina *et al.*, 1999). The

mean errors in prediction were lower than 3% in all cases. A similar chemometric approach was reported by Vallejo Cordoba (1998).

Whey proteins of different species were successfully separated by CZE and can also serve as authenticity indicators (Cartoni *et al.*, 1999; Cattaneo *et al.*, 1996; Recio *et al.*, 1995). The ratio of the corrected peak areas of bovine β-lactoglobulin B to ovine α-lactalbumin was linearly related to the amount of cows' milk present within a range of 0–20% (Cartoni *et al.*, 1999).

High-performance liquid chromatograph (HPLC) is another route to protein separation and was effectively used to determine individual milk proteins. Different chromatographic profiles are obtained for the proteins from different species. Ion-exchange as well as reversed-phase (RP) columns were applied to fractionate either the caseins or the whey proteins or total milk proteins. Bovine, ovine and caprine para-κ-caseins were base-line separated by cation-exchange HPLC and used for quantifying the mixture proportion of the milk types used for cheese making (Mayer *et al.*, 1997). Others used RP columns to separate primarily α-, β-, and κ-casein fractions of different species (Bordin *et al.*, 2001; Urbanke *et al.*, 1992; Veloso *et al.*, 2002). By analogy to the official EC method, Volitaki and Kaminarides (2001) added plasmin to the isolated caseins to intensify the γ-casein fraction and separated the mixture by RP-HPLC. The caseinomacropeptides of different species are separable by HPLC and can serve as markers (Lopez Fandino *et al.*, 1993). HPLC analysis of whey proteins is also of interest for species discrimination (Bobe *et al.*, 1998a; de Frutos *et al.*, 1991; de Noni *et al.*, 1996; Romero *et al.*, 1996).

When quantitative aspects regarding mixture proportions are considered, the different casein contents of bovine, caprine and in particular ovine milk used for cheese making have to be taken into account. Since ewes' milk has a much higher casein content than cows' milk, the resulting relationships are non-linear (Addeo *et al.*, 1990a, b; Mayer *et al.*, 1997).

Recently, new strategies for the structural analysis of milk proteins based on mass spectrometric technologies, in particular matrix assisted laser desorption-time of flight mass spectrometry (MALDI-TOF), have been developed. Owing to its speed and the minimum of sample preparation required the MALDI-TOF technique is very attractive. Using particularly α-lactalbumin and β-lactoglobulin as markers, addition of cows' milk to the milk of other species and to water buffalo mozzarella was easily detected by MALDI-TOF (Angeletti *et al.*, 1998; Cozzolino *et al.*, 2001; Cozzolino *et al.*, 2002; Fanton *et al.*, 1998).

Due to its excellent sensitivity and minimal sample preparation requirements immunochemical methods have found wide acceptance to discriminate milk of different species. Various assay formats and antibodies directed against different antigens have been described (Table 16.2). Many of the assays target bovine β-casein, as this fraction has the highest allergenic potential of all caseins (Anguita *et al.*, 1996a). The limit of detection for most of the assays is 0.1–1.0% depending on the assay format. If polyclonal antibodies were used, they were usually purified by affinity chromatography to eliminate cross-reactivity. Commercial test kits are now on the market and they have found wide application.

Table 16.2 Immuochemical assay formats used for the discrimination of milk of different species

Assay	Antibody	Antigen	Reference
Competitive indirect ELISA	Polyclonal	Bovine γ-caseins	Richter *et al.* (1997)
Competitive indirect ELISA	Polyclonal	Native and heat denatured bovine β-lactoglobulin	Beer *et al.* (1996)
Indirect ELISA	Monoclonal	Bovine β-casein	Anguita *et al.* (1995)
Indirect ELISA	Monoclonal	Bovine α_{s1}-casein	Rolland *et al.* (1993)
Indirect ELISA	Monoclonal	Caprine α_{s2}-casein	Haza *et al*. (1996)
Indirect ELISA	Polyclonal	Bovine caseinomacropeptide	Bitri *et al.* (1993)
Indirect ELISA	Polyclonal	Caprine whey proteins	García *et al.* (1994)
Sandwich ELISA	Monoclonal	Bovine β-lactoglobulin	Levieux and Venien (1994)
Sandwich ELISA	Polyclonal	Bovine caseins	Rodriguez *et al.* (1993)
Immunostick ELISA	Monoclonal	Bovine β-casein	Anguita *et al.* (1996b)
Western blotting	Monoclonal	Bovine β-lactoglobulin	Molina *et al.* (1996)

Immunological methods can fail when the targeted antigen is substantially degraded either by heating or proteolysis. DNA from somatic milk cells (mostly leucocytes) is suggested to persist in ripened cheese and may be amplified by polymerase chain reaction (PCR) and analysed for species discrimination. Plath *et al*. (1997) used primers encoding a partial sequence of the β-casein gene to detect the corresponding genomic DNA in milk and cheese. The PCR product from ovine or caprine β-casein DNA contained a specific restriction enzyme site that was not present in bovine β-casein DNA. After restriction enzyme analysis and subsequent separation of the fragments by PAGE, the undigested bovine β-casein fragment was detected as an additional band if cows' milk was present. A similar approach was described for the identification of water buffalo, bovine, ovine and caprine milk in cheese, based on amplification of a 359 bp fragment of the cytochrome-b gene and restriction fragment chain length polymorphism analysis (Branciari *et al.*, 2000). A single step PCR method with bovine-specific primers for a fragment of the cytochrome-b gene to detect cows' in goats' milk was described by Bania *et al.* (2001).

Genomic DNA was extracted from cheese and PCR double-stranded amplifications were conducted using various suitable sets of primers for species-specific DNA amplification to detect the milk source (bovine, ovine and caprine) in cheese (Calvo *et al.*, 2002). A duplex PCR was developed to identify cows' milk and buffalos' milk in cheese products, particularly in buffalo mozzarella cheese (Bottero *et al.*, 2002).

Mitochondrial (mt) DNA shows also species diagnostic sequence variations and, on top of that, the number of copies of mtDNA is much higher than genomic DNA. Cow-specific primers were designed to target the control region of mtDNA and the resulting PCR product of 413 bp separated by agarose gel

electrophoresis. Limit of detection of cows' milk in goats' cheese was less than 0.1% (Maudet and Taberlet, 2001).

A DNA-based technique which combines PCR, ligase chain reaction (LCR) and an enzyme immunoassay (EIA) to detect the presence of cows' milk in ewes', goats' and buffaloes' milk and corresponding cheeses was developed by Klotz and Einspanier (2001). It is based on subtle differences in the β-casein gene of cow, sheep, goat and buffalo species. DNA, extracted from milk or cheese samples, served as a template to amplify a universal β-casein PCR product. Subsequently, LCR with species-specific primers was performed using the PCR product as a template. LCR primers were labelled with biotin or digoxygenin for further sensitive detection by EIA. This screening technique allowed clear discrimination of cow species from sheep, goat and buffalo species in milk and cheese.

The sensitivity of the DNA-based methods is very high; limits of detection are reported to be better than 0.1% of the targeted species; however, quantification of mixture proportions seems to be difficult as the source of the DNA is somatic milk cells. It is well known that the somatic cell count is affected by a number of factors (number and stage of lactation, udder health) which are uncontrollable.

16.4 Other authenticity and traceability indices

16.4.1 Detection of non-milk proteins

Non-dairy proteins of vegetable or animal origin are generally cheaper than milk proteins and are sometimes added to extend the product (economic adulteration) or because of their functional properties. In particular, soy protein has good water holding and binding capacity and therefore can improve the texture of a product (e.g., soft cheese). A number of electrophoretic (Cattaneo *et al.*, 1994; Kanning *et al.*, 1993; Manso *et al.*, 2002), chromatographic (Cattaneo *et al.*, 1994; Espeja *et al.*, 2001) and immunochemical (Turin and Bonomi, 1994; Sanchez *et al.*, 2002) methods have been devised to detect the addition of non-milk proteins.

16.4.2 Watering of milk

Addition of water to a beverage is the epitome of food adulteration. Dilution with water alters the density of milk, the refractive index of the lactoserum and, most importantly, its freezing point. The thermistor cryoscopic determination of the freezing point of raw milk is probably the most widely and frequently applied technique for food authenticity testing. The freezing point of authentic raw milk varies only within narrow limits. Breed, stage and parity of lactation, feeding regimens, udder health, production region, season and milking time are seen as the most important factors influencing the freezing point (Buchberger, 1994, 2000; Rohm *et al.*, 1991; Wiedemann *et al.*, 1993). Mean values were

reported for different countries in a range between −0.5310 and −0.5209ºC (Buchberger, 1990; Coveney, 1993; Rohm *et al.*, 1992; Slaghuis 2001). The procedure for the determination of the freezing point has been standardised (IDF Standard 108B:1991; Commission Decision 91/180/EEC). Although the method is considered to be robust, a number of operating parameters may influence the test result and have to be controlled carefully (Rohm, 1993).

16.4.3 Alteration of the casein/whey protein ratio

The by-product of cheese making, rennet whey, is of low value but the volumes produced abound. Therefore, it is tempting fraudulently to add whey to other dairy products. Advances in membrane filtration technology opened up interesting possibilities to split skim milk into different protein fractions to give products with an added value on one hand and less valuable fractions on the other (Creamer *et al.*, 2002). Furthermore, protein standardisation of milk and milk products is now permitted provided that only milk components are added or removed and the ratio of casein to whey protein is not altered. Therefore, reliable methods are needed to check the protein composition of dairy products. The methods proposed are either indirect, e.g., they determine certain protein fractions not as such, but as an inherent characteristic feature, or direct using, e.g., electrophoretic or chromatographic methods to separate the protein mixture into individual components.

Whey proteins contain significantly more sulphur-containing amino acids (cysteine and cystine), and more aromatic amino acids (tryptophan, tyrosine and phenylalanine). Additions of whey proteins therefore increase those values as compared to the genuine product. The former can be determined by a modified ninhydrin reaction (De Koning and Van Rooijen, 1971) or by polarography (Mrowetz and Klostermeyer, 1976; Lechner and Klostermeyer, 1981). The limitation of the polarographic method, although very effective and reliable, is that it uses hazardous chemicals (methylmercury chloride). A very elegant way to determine the casein/whey protein ratio is the application of derivative spectroscopy. Second-order (Luf and Brandl, 1987) and even fourth-order derivatives (Lüthi-Peng and Puhan, 1999; Meisel, 1995; Miralles *et al.*, 2000) have been proposed. The method quantifies aromatic amino acid residues of milk proteins and is unaffected by other absorbing non-protein material in the sample solution.

The different content of protein bound phosphorous of casein and whey protein is the basis of an effective, though laborious testing principle that detects whey additions (Wolfschoon-Pombo and Furtado, 1989). Direct measurement of all relevant protein fractions by electrophoretic (Basch *et al.*, 1985; Meisel and Carstens, 1989; Miralles *et al.*, 2000) or chromatographic methods (Bobe *et al.*, 1998b; Bordin *et al.*, 2001) are more laborious but give a more detailed insight. The casein to whey protein ratio can be determined directly from the obtained chromatographic trace, after careful calibration using reference compounds.

The caseinomacropeptide (CMP), which results from the cleavage of κ-casein during renneting of milk, is a good indicator for the presence of rennet

whey. Two methods based on gel-filtration HPLC have been adopted by the EC (Commission Regulation (EC) No 213/2001) to check skim milk powder for the presence of rennet whey powder. Instead of UV detection, pulsed electrochemical detection of CMP, which was very sensitive and selective, was proposed (Van Riel and Olieman, 1995a). As an alternative to the HPLC procedures, CMP was determined by capillary electrophoresis (Recio *et al.*, 2000; Van Riel and Olieman, 1995b). Proteolytic activity, particularly from psychrotrophic bacteria, during cold storage of milk produces peptides similar to CMP, which may interfere with CMP detection. This could give rise to false-positive results (Martinez Penagos *et al.*, 1993; Recio *et al.*, 1996). Other, less often applied techniques to estimate the casein/whey protein ratio are photoacoustic spectroscopy (Doka *et al.*, 1999) and pyrolysis mass spectrometry (Schmidt *et al.*, 1999).

16.4.4 Heat load

The primary aim of heating milk is to ensure its microbiological safety and stability. However, heating milk profoundly alters the physico-chemical state of its components, leading primarily to the denaturation of certain vulnerable protein fractions (immunoglobulins, enzymes, whey proteins) and the formation of so-called browning products (Maillard reaction).

According to Council Directive 92/46/EEC, pasteurised milk is obtained by heat treatment, at least 71.7ºC for 15 seconds, or any other temperature/time combination producing an equivalent effect. Pasteurised milk has to show a negative reaction to the phosphatase test and a positive reaction to the peroxidase test. However, the production of pasteurised milk which shows a negative reaction to the perioxidase test is authorised, provided that the milk is labelled as 'high-temperature pasteurised'. The required tests (phosphatase and peroxidase test) have been standardised (Commission Decision 91/180/EEC); alternative, more rapid testing methods (e.g. Fluorophos®, Reflectoquant®) were also proposed (Berger *et al.*, 2001; Lechner, 1996).

Higher heat loads result from a number of other processes applied during manufacturing of dairy products, primarily UHT treatment, sterilisation, concentration by water evaporation and drying. Therefore, methods are needed that are (i) capable of discriminating between the severity of heat treatment applied, and (ii) to detect products with a high heat load, which have been added to other milk products (e.g., addition of dried milk to fluid milk).

The American Dry Milk Institute (ADMI) has standardised a turbidimetric method developed by Harland and Ashworth to distinguish between different heat loads (low-, medium-, high-heat) in skim milk powder (ADMI, 1971). In this test casein and heat-denatured whey proteins are precipitated with NaCl at neutral pH; the supernatant is then acidified to coagulate the native whey proteins and the resulting turbidity is taken as a measure for the content of non-denatured whey proteins. More recently, the amount of non-denatured, soluble whey proteins has been quantified directly by HPLC procedures (IDF Standard

178: 1996; Kneifel and Ulberth, 1985; Resmini *et al.*, 1989; Villamiel *et al.*, 2000). Immunochemical methods were also successfully employed to determine heat-denatured whey proteins (Jeanson *et al.*, 1999; Rosenthal *et al.*, 1999).

Methods based on the determination of native whey proteins are particularly suited to distinguish products with rather low heat load (pasteurisation conditions). The content of several Maillard reaction products like lactose isomerisation and protein-glycation are indicative for severe heat treatment. The two main markers are furosine and lactulose. Furfurals are another group of heat treatment indicators, which found wide application not only in the dairy industry (Albalá-Hurtado *et al.*, 1997; Ferrer *et al.*, 2000), but also throughout the food industry.

Furosine is an amino acid obtained by acid hydrolysis of glycosylated proteins, in particular of the lysine-lactose adduct formed in the Maillard reaction. It can be determined by HPLC (Hartkopf and Erbersdobler, 1993; Henle *et al.*, 1995; Nicoletti *et al.*, 2000; Resmini *et al.*, 1990) or by capillary electrophoresis (Corradini *et al.*, 1996). Furosine has been used not only to distinguish between different types of heat treatment (Clawin-Raedecker *et al.*, 2000; Pellegrino *et al.*, 1995; Villamiel *et al.*, 2000), but also to determine whether milk powder has been added to fluid milk or as an indicator for reconstituted milk (Ohta *et al.*, 2002; Van Renterghem and De Block, 1996).

Lactulose is formed by isomerisation of lactose during the heating of milk, and has been proposed as an analytical index to distinguish UHT from sterilised milk (Clawin-Radecker *et al.*, 1992); it is not found in pasteurised milk. A variety of methods were used for its determination: GLC (Martinez-Castro *et al.*, 1987), HPLC (Cataldi *et al.*, 1999; IDF Standard 147:1991), capillary electrophoresis (Soga and Serwe, 2000), enzymology (Amine *et al.*, 2000a; Kuhlmann *et al.*, 1991), colorimetry (Amine *et al.*, 2000b) and continuous-flow amperometry (Mayer *et al.*, 1996; Moscone *et al.*, 1999).

Another Maillard reaction product, i.e., lysinoalanine (LAL), was shown to be a sensitive indicator for heat treatment of milk and for addition of dairy-based substitutes rich in LAL (caseinates, etc.) to other milk products, in particular cheese (Faist *et al.*, 2000; Moret *et al.*, 1997; Pellegrino *et al.*, 1996). Maillard products have fluorescent properties and this feature was used for a very sensitive and rapid determination of the heat load, which was in good agreement with more established procedures (Birlouez-Aaragon *et al.*, 2002). Novel strategies for the estimation of heat load are the direct determination of glycosylated proteins, either by immunology (Pallini *et al.*, 2001), by HPLC (Pellegrino and Cattaneo, 2001), or by mass spectrometry (Cozzolino *et al.*, 2001).

16.4.5 Characterisation and denomination of the geographical origin

Products manufactured in a particular way in a specific geographical region have always found a following, although they usually command a higher price. Marketing of agricultural products has recently focused on promotion of

premium goods in affluent countries. Traditional cheese varieties like Camembert, Parmesan or Stilton are in high demand, and to protect their market legal instruments have been introduced in the EU (Council Regulation (EEC) 2081/92). To be eligible to use a protected designation of origin (PDO) or a protected geographical indication (PGI), an agricultural product or foodstuff must comply with strict specifications. On-site inspections by a control authority are currently the only accepted way to safeguard the PDO/PGI label, as reliable and validated analytical testing methods do not exist yet. The most promising approach seems to be to characterise the products by determining stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{16}O/^{18}O$) and subsequent application of mathematical pattern recognition techniques. It has been applied to the characterisation of the geographical origin of milk (Kornexl *et al.*, 1997), butter (Rossman *et al.*, 2000) and Pecorino Sardo cheese (Manca *et al.*, 2001). It was found that the feeding regimen, in particular maize silage, can influence the $^{13}C/^{12}C$ ratio, use of industrial fertilisers the $^{15}N/^{14}N$ ratio and the water supply the $^{16}O/^{18}O$ ratio of milk and milk products.

A number of chemical (fat content and pH value), biochemical (L- and D-lactate, and pyruvate), microbiological (lactobacilli and enterococci), colour and sensory parameters were investigated to discriminate between Emmental cheese of different origin (Pillonel *et al.*, 2002). Although some promising results have been obtained, the analytical approach is in its infancy and much needs to be done to give a reliable indication that verifies the origin of a product.

16.5 Conclusions

Detection of fraud is complicated by the fact that the quantities of certain indicators vary due to biological, climatic, agronomical and temporal factors. Moreover, processing can dramatically change the composition of minor constituents therefore too stringent specifications cannot be set by food inspection, as this will eventually increase the number of false-positive results. Since unscrupulous manufacturers or vendors have developed an excellent understanding of the underlying principles of detecting fraud, they have managed in many cases to tailor blends in a way that they comply with product specifications.

In many cases no fundamental differences, ideally the lack or presence of a product-specific component, between the genuine and the adulterated product exist. Consequently, purity criteria have to be empirically determined by analysing a wide array of genuine products and creating and regularly updating a database holding information about the concentration ranges of certain indicative components of the commodity concerned. In order to solve difficult cases more than one analyte has to be considered for detecting fraud. Likewise, a combination of different analytical techniques to determine dissimilar characteristics of a commodity (e.g., a combination of spectroscopic and chromatographic methods) could be more useful than relying on one single

methodology. Given the complexity of some problems, univariate statistics (measures of location and dispersion) have to be substituted by intricate statistical algorithms to aid in pattern recognition and classification of genuine and adulterated products. The merits of such procedures, though scientifically sound, are difficult to comprehend for those not familiar with advanced statistical data interpretation techniques, and might, therefore, find little acceptance in a court of law.

The challenge for food law enforcement agencies is to be a step ahead and to develop constantly new methods to get a better insight into the complex chemical mixture representing food, in order to identify a set of possible marker components for authentication purposes.

16.6 References

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17

Cereals

G. Downey, TEAGASC – The National Food Centre, Ireland

17.1 Introduction

This chapter reviews authenticity issues for the following cereals:

- wheat
- pasta
- rice

17.2 Wheat

Wheat authentication issues are mainly related to variety since the processing properties of different grain varieties differ significantly. Typical authentication problems include:

- grain contaminated with other cereals of similar appearance, e.g. rye, triticale
- grain contaminated with the wrong wheat species, e.g. durum wheat (*Triticum durum*) contaminated with common wheat (*Triticum aestivum*)
- wheat of a specified type contaminated with a different type, e.g. soft wheat containing hard wheat

A more recent problem is the use of genetically modified cereals. The detection of GMOs is discussed elsewhere in this book. This section discusses the following methods for identifying varieties of particular cereals:

- phenol and rapid tyrosinase tests
- electrophoretic and other techniques for identifying varieties by protein composition

- techniques identifying varieties by grain hardness
- digital imaging to distinguish varieties
- emerging technologies

17.2.1 Phenol and rapid tyrosinase tests

The phenol test measures the level of phenol oxidase enzymes in wheat bran layers. Whilst it cannot distinguish individual varieties, it can identify groups of varieties. This sorting by groups can be used to supplement visual inspection in identifying a potential problem (Downey, 1998). The rapid tyrosinase test is used to distinguish common wheat (*Triticum aestivum*) from durum wheat (*Triticum durum*) (Mahoney and Ramsey, 1992). It works on a similar principle to the phenol test but uses tyrosine as a substrate. It relies on the enzymes that oxidize the tyrosine being at very low levels in durum wheat compared to common wheat. It is a simple way of estimating contamination and can be used to analyse large samples.

Another test, based on high-performance liquid chromatography (HPLC), relies on polyphenol oxidase being present in common wheat and not in durum. This enzyme can be separated by electrophoresis and the technique can be used on individual grains, bulk ground wheat samples, semolina and flour. With bulk samples, 5% contamination of common wheat with durum wheat can be detected (Downey, 1998).

17.2.2 Electrophoretic and other techniques for identifying varieties by protein composition

A good marker for a variety is its protein composition which is unique to that variety (Frazier, 1992). This composition is determined genetically and is independent of environmental conditions. The protein composition of a particular wheat or flour sample can be characterised in terms of a profile of the gliadin proteins by polyacrylamide gel electrophoresis (ISO 8981:1993; ICC 143). Comparison of the gliadin profile generated with those from known standards can confirm whether a wheat sample is of the required variety (White and Cooke, 1992). It can also be employed in identifying material of unknown variety (Bietz and Simpson, 1992). The technique is not always able to identify components in a complex sample with certainty, but it is often possible to narrow the identification down to a small number of varieties. The degree of accuracy is very dependent on the varieties involved but, in any event, contamination at 5% or less is unlikely to be detected.

Separation of gliadins using capillary electrophoresis has enabled more precise differentiation of wheat varieties and promises to become a routine tool for wheat varietal identification (Marchylo *et al.*, 1992; Noni *et al.*, 1994; Lookhart and Bean, 1995a) and for prediction of quality (Bietz and Schmalzried, 1995). This technique gives excellent resolution of high molecular weight glutenin subunits that correlate with bread-making quality (Werner *et al.*, 1994).

The technique is also useful for rapid differentiation of oat and rice cultivars (Lookhart and Bean, 1995b).

As well as electrophoresis, Reversed Phase High Performance Liquid Chromatography (RP-HPLC) has also been used and in some cases has produced better resolution than electrophoresis. Given the relative cost and complexity of the procedure, HPLC is best viewed as a supplement to electrophoretic methods. There are also antibody-based ELISA kits available for the identification of common wheat in durum wheat samples. These generally work on a reaction to friabilin proteins present in common wheat but not in durum wheat. With suitable standards, it is possible to detect contamination down to 1% or less (Greenwell *et al.*, 1992).

17.2.3 Techniques identifying varieties by grain hardness

Most varieties tend to have characteristic values for grain hardness which, in pure samples, should fall within certain ranges. Results from these tests on bulk samples can help indicate if the variety is correct or if the wheat has the right characteristics. Absolute measures for hardness are based on the Particle Size Index of a meal ground and subsequently sieved under defined conditions (Hoseney, 1987). Automated methods have been developed, based either on Near Infrared Spectroscopy (NIR) (AACC 39-70) or on mechanical destruction (e.g. with the Perten Single Kernel Characterisation System 4100; AACC 55-31; FTWG 20). The latter measures the force necessary to crush a single kernel. In the case of the single kernel characterisation system, hardness is expressed in terms of a hardness index calculated from the mean value for 300 kernels. Both the NIR and mechanical systems require appropriate standards (e.g. those produced by the National Institute of Standards & Technology, Gaithersburg, MD 20899) in order to calibrate the equipment.

17.2.4 Digital imaging to distinguish varieties

A computer controlled laser scanning system capable of acquiring three-dimensional images of the surface of cereal grains has been developed to distinguish between wheat varieties, and also detect and differentiate sprouted and unsprouted grains. A combination of 14 features based on nine topographic images and five intensity images permits the system to classify 92–94% of kernels correctly. In particular, features that measure deformation of the germ end of the kernel are crucial to the discrimination process (Thomson and Pomeranz, 1991). Discriminant analysis based on fluorescence intensity, hardness and protein data has also been demonstrated to allow separation of wheat into proper classes for 100% of durum, hard red spring, club, soft red winter and soft white winter wheats and for 94% of hard red winter wheat (Irving *et al.*, 1989). These imaging techniques have been used both to distinguish wheat varieties and to identify different grain types such as wheat, barley and oats (Sapirstein, 1993).

17.2.5 Emerging technologies

Immunological methods are based on detecting differences by antibody reactions. Such methods have the advantages of simplicity, speed and low cost but have not yet been applied successfully in cereal analysis. The main problem lies in the relative insolubility of hordein proteins and the consequent difficulties in preparing specific antisera against them. Monoclonal antibodies represent the most promising immunological approach. Such antibodies react specifically to certain hordeins and do allow certain varietal distinctions to be made (Skerritt *et al.*, 1988).

DNA-based techniques using methods such as Restriction Fragment Length Polymorphism (RFLP) for barley variety identification have been developed (Bunce *et al.*, 1986; Ainsworth and Sharp, 1989; Graner *et al.*, 1990). Discrimination between durum wheat and common wheat via amplification of a durum-specific repeat sequence has been described by Bryan *et al.* (1998). Reports of PCR-based techniques for cereal variety identification have been published but there remain uncertainties about their accuracy and method of reproducibility (Weining and Langridge, 1991; Dweikat *et al.*, 1993; Munthali *et al.*, 1992).

17.3 Pasta

High-quality pasta is made from durum wheat. Authentication problems relate particularly to the potential substitution of durum what by cheaper wheat varieties. Regulations in a number of countries allow a maximum of 3% common wheat in durum wheat, indicating that any method for authenticating pasta must reach a high degree of precision. In addition to adulteration by addition of inferior wheat, it is also possible that cheaper ingredients may be added to high-value speciality products such as egg pasta, fortified products or saffron pasta. A number of techniques distinguishing durum and common wheat varieties are described in section 17.2. This section discusses other common methods.

17.3.1 Electrophoretic techniques to identify common wheat

A standard approach to identify common wheat is to detect the products of the D-genome, a group of chromosomes present in hexaploid wheats such as common wheat, but absent from durum wheat. Proteins such as albumins and gliadins are expressed by the genome and can be extracted by water or alcohol and then separated by Isoelectic Focusing (IEF) or Polyacrylamide Gel Electrophoresis (PAGE) (Resmini and Bernardi, 1976). PAGE can be used to quantify water-soluble enzymes such as polyphenol oxidases and esterases (Feillet and Kobrehel, 1974; Cooke *et al.*, 1986). PAGE has also been used to detect adulteration via separation and quantification of alcohol-soluble omega-gliadins (Kobrehel *et al.*, 1965; Burgoon *et al.*, 1985). As noted earlier, the

combination of high separation efficiency, short running times and choice of detectors has also attracted interest in capillary electrophoresis for the detection of proteins and peptides (Kuhr, 1990; Gonzalez, 1996).

Electrophoretic methods have some limitations, including cost and technical complexity (McCarthy *et al.*, 1990). The drying stage during pasta manufacture causes protein denaturation and enzyme inactivation, exacerbated by the use of higher temperatures to reduce drying times (Feillet and Kobrehel, 1972; Stroh, 1986). Environmental factors during wheat cultivation may also affect protein expression (Blumenthal *et al.*, 1993).

17.3.2 Other techniques to identify common wheat

Common wheat proteins can also be assayed using immunochemical techniques. Both water-soluble and alcohol-soluble kinds have been determined (Piazzi *et al.*, 1972; McCarthy *et al.*, 1992). High Performance Liquid Chromatography (HPLC) has been used to separate γ/B gliadins as a means of analysing common wheat in durum wheat semolina and subsequently extended to the determination of adulteration in dried pasta products (Barnwell *et al.*, 1994). The lipid sitosterol palmitate exists in high concentrations in common wheat and can be assayed by Thin Layer Chromatography (TLC) or HPLC (Berry *et al.*, 1968; Sarwar and McDonald, 1993; De Noni *et al.*, 1994). Mass spectrometry has also been applied to protein analysis (Burlingame *et al.*, 1992). As with electrophoretic techniques, high temperature drying reduces the effectiveness of techniques such as HPLC. DNA techniques have been proposed as a way of overcoming problems of denaturation of proteins during processing and the differential expression of particular proteins as a function of environmental growth conditions (Downey, 1998).

17.4 Rice

The main authenticity problem with rice is the potential substitution of a premium rice variety, notably Basmati, with a cheaper variety. A simple scheme of the Indian Agricultural Research Institute for identifying Basmati rice stipulates fine grain, mild to strong aroma (as measured by potassium hydroxide treatment), linear kernel elongation on cooking and non-slimy and non-splitting nature (Indian Agricultural Research Institute, 1980; Siddiq, 1982; Vaingankar and Kulkarni, 1988, 1989).

The Campden and Chorleywood Food Research Association (CCRA) has developed a number of tests for evaluating the authenticity of Basmati rice based on physical measurements using:

- image analysis
- Rapid Visco-Analysis (RVA)
- Near Infrared Spectroscopy (NIR)

Image analysis has given the most reliable results (Downey, 1998). More recently the Food Standards Agency has proposed a method based on DNA analysis using PCR (www.food.gov.uk).

17.5 References

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18

Herbs and spices

R. S. Singhal and P. R. Kulkarni, Institute of Chemical Technology, Mumbai, India

18.1 Introduction: quality and adulteration issues

Herbs and spices are added to processed food to enhance consumer appeal by imparting taste/flavour profiles which can make all the difference between the food being acceptable or not. Spices have been defined by various organizations. While the International Standards Organization (ISO) defines spices and condiments as 'vegetable products or mixtures thereof, free from extraneous matter, used for flavouring, seasoning and imparting aroma in foods', Rosengarten describes a spice as a product that enriches or alters the quality of a food to give it zest or pungency; a piquant or lasting flavouring; or a relish. The US FDA defines spices as 'any aromatic vegetable substance in the whole, broken or ground form, except for those substances which have been traditionally regarded as foods, such as onions, garlic or celery; whose significant function in food is seasoning rather than nutritional; that is true to name; and from which no portion of any volatile oil or flavouring principle has been removed.

The term 'spice' usually incorporates herbs. The distinction between the two is very fine. While herbs are defined as dried leaves of aromatic plants used to impart odour and flavour to foods, sometimes with addition of colour, spices are the dried parts of aromatic plants with the exception of leaves. It should be noted that leaves are traded separately from the rest of the plant parts. The classification of spices and the plant organ in which it is found are shown in Table 18.1. Besides imparting flavour to foods, herbs and spices also act as natural preservatives due to their antioxidant activity. A few of the typical antioxidants derived from spices are eugenol and gallates from cloves, flavonoids from marjoram and licorice, and carnosol and related compounds from rosemary and sage.

Spices are added to foods in several forms (Farrell, 1985). These include whole spices, ground spices, essential oils, oleoresins, and spice extracts (or dispersed spices). Essential oils are the principal but not the sole flavouring constituents of spices. Some essential oils are dry distilled and some are vacuum distilled, and some are expressed cold. Essential oils are sterile, free from extraneous matter, soluble in liquid fats/oils, emulsifiable in other liquid solutions, stable under good storage conditions and represent up to 98% savings in weight and storage space. Furthermore, the flavour quality is fairly uniform for a given variety and species of a spice, irrespective of the quantity present in the said spice.

Oleoresins, manufactured by solvent extraction of the ground spices, consist of essential oil, resins soluble in the organic solvent used for its extraction, and other related materials present in the spice as well as many non-volatile fatty acids. Extraction of essential oils and oleoresins is accomplished by steam distillation, extraction with hydrocarbon or chlorinated solvents, enzymatic treatment and fermentation, and supercritical fluid extraction. Oleoresins extracted with non-polar solvents are preferred in flavouring processed foods due to their heat stability, whereas the perfume industry prefers polar solvents since they are soluble in most perfume materials and do not deposit fatty materials in the bottles and containers.

Dry soluble spice or soluble spice flavourings contain a fixed amount of oleoresin dispersed on a predetermined amount of salt, sugar, dextrose, corn syrup solids or some other edible innocuous substance. It is blended completely until uniformly dispersed throughout the product. Liquid spice flavourings are blends of essential oils and oleoresins diluted to specific spice strength with a suitable solvent such as propylene glycol or glycerol; polysorbate 80 is added to make it water soluble and hydrocolloids to give an emulsion if desired. Encapsulated spice oils are prepared using microencapsulation techniques. An overview of the approaches for herbs and spices is shown in Table 18.2.

Spices, their concentrates, essentials and oleoresins are expensive additives to processed foods and form an important class of international commerce. They are vulnerable to admixture, adulteration and substitution because of their high cost. With the active international trade in spices and essential oils, it has been felt necessary to lay down quality specifications to ensure purity and also to check adulterations. In whole spices, adulteration is usually with an inferior variety, an immature dried material, other parts of the same plant, with other plant materials of similar appearance, and with exhausted spices. Spice powders may be admixed with powders of the above adulterants, other plant materials, grain flours, starch and even sawdust. The internationally recognized standards for spices include the specifications below.

18.1.1 Chemical quality

In terms of gross composition, chemical analysis includes:

- volatile oil content to indicate adulteration with foreign material, low quality or spent material

Table 18.1 Classification of some commonly used spices, the plant part used as a spice and their important flavour constituents

Classes	Spice	Plant part	Important flavour compounds
Hot spices	Capsicum (chillies) (*Capsicum annum, C. frutescens*)	Berries	Capsaicin, dihydro capsaicin
	Black/white pepper (*Piper longum*)	Berries	Piperine, S-3-carene, β-caryophyllene
	Ginger (*Zingiber officinale*)	Rhizome	Gingerol, shogaol, neral, geranial
	Mustard (*Brassica nigra, B. junceae*)	Seed	Allyl isothiocyanate
Mild spices	Paprika (*Capsicum annum*)	Fruits	Capsaicin
	Turmeric (*Curcuma longa*)	Rhizome	Turmerone, zingeberene, 1,8-cineole
	Coriander (*Coriandrum sativum*)	Seed	d-Linalool, C1—C14-2-alkenals
Aromatic spices	Allspice (*Pimenta diocia*) (pimento)	Berries	Eugenol, β-caryophyllene
	Cardamom (*Elettaria cardamomum*)	Fruit	α-Terpinyl acetate, 1,8-cineole, linalool
	Cassia	Bark	Cinnamaldehyde, eugenol
	Cinnamon (*Cinnamomum zeylanicum*)	Bark	Cinnamaldehyde, eugenol
	Clove (*Syzygium aromaticum*)	Buds	Eugenol, eugenyl acetate
	Cumin (*Cuminum cyminum*)	Seed	Cuminaldehyde, p-1,3-menthadienal
	Dill (*Anethum graveolens*)	Seed	d-Carvone
	Fennel (*Foeniculum vulgare*)	Seed	(E)-Anethole, fenchone
	Fenugreek (*Trigonella foenumgracecum*)	Seed	
	Mace (*Myristica fragrans*)	Aril	α-Pinene, sabinene, 1-terpenin-1-ol
	Saffron *(Crocus sativus*)	Pistil (female part of the flower)	Safranol
	Nutmeg (*Myristica fragrans*)	Kernel	Sabinene, α-pinene, myristicin
	Ajowan (*Trachyspermum ammi* L.) syn. *Carum copticum* Hiren	Seed	Thymol, γ-terpinene, *p*-cymene, β-pinene and α-pinene

Table 18.1 Continued

Classes	Spice	Plant part	Important flavour compounds
Herbs	Basil (*Ocimum basilicum*)	Leaf	Methylchavicol, linalool, methyl eugenol
	Bay (*Laurus nobilis*)	Leaf	1,8-Cineole (major component), α-pinene, β-pinene, myrcene, α-terpinene, *p*-cymene, linalool, γ-terpinene, β-caryophyllene, humulene
	Curry (*Murraya koenigii*) leaf	Leaf	α-pinene, β-pinene, d-sabinene, tauterpinene, α-terpinene, terpinen-4-ol, β-phellandrene, β-caryophyllene, sadinene, cadinol
	Dill leaves	Leaf	Limonene, *cis*-dihydrocarvone, *trans*-dihydrocarvone, carvone, dihydrocarveol
	Marjoram (*Origanum majorana*)	Leaf	*e*- and *t*-Sabinene hydrates, terpinen-4-ol
	Tarragon (*Artemisia dracunculus*)	Leaf	Methyl chavicol, anethole
	Thyme (*Thymus vulgaris*)	Terminal shoot, leaf	Thymol, carvacrol
Aromatic vegetables	Onion (*Allium cepa*)	Bulbs	Sulphur containing compounds originating from S-alk(en)yl cysteine sulphoxides
	Garlic (*Allium sativum*)	Bulbs	Precursors from alliin or S-allyl cysteine sulphoxide
	Celery (*Apium graveolens*)	Seed	*d*-Limonene, selinene, sedanolide, sedanoic anhydride

Refs: Singhal, Kulkarni and Rege, 1997; Peter, 2001; Polat and Otles, 1999; Nagalakshmi *et al*., 2000.

- moisture content or maximum moisture content set for each commodity for its stability and also as an indicator of weight, and hence price
- ash and acid insoluble ash to indicate added impurities and cleanliness.

Specifications for these parameters as laid down by the European Spice Association are as given in Table 18.3.

Table 18.2 An overview of the analytical tests for flavourants

- Natural plant materials
  - a) General tests: ash, crude fibre, extractive matter, volatile oil, extraneous matter and filth
  - b) Tests of limited application: starch content, microscopy, sieve analysis, undesirable plant parts or foreign matter
  - c) Specific test: curcumin in turmeric, gingerine in ginger, capsaicin and colour index in capsicum, piperine in pepper, etc.
- Essential oils
  - a) General tests: specific gravity, optical rotation, refractive index, solubility
  - b) Tests of limited application: boiling point, melting point, flash point
  - c) Instrumental: GC, IR, UV
  - d) Specific tests: acetals, alcohols, phenols, esters, etc.
- Oleoresins
  - a) General tests: volatile oil content, solubility, solvent residues
  - b) Specific tests: capsaicin, piperine, curcumin
- Dispersed spices
  - a) General tests: volatile oil, carrier base, constituents, extractives, microbial examination
  - b) Specific tests: same as oleoresins
- Synthetic chemicals
  - a) General tests
    - i) Liquid: specific gravity, refractive index, optical rotation, solubility, boiling point, flash point
    - ii) Solids: melting point, solubility, freedom from insoluble matter, congealing point
  - b) Specific tests: for purity – GLC, IR
- Vanilla extract
  - a) Specific gravity
  - b) Alcohol content
  - c) Glycerin content
  - d) Vanillin content
  - e) Total solids, ash
  - f) Neutral lead number
  - g) Acidity
  - h) Colouring matter
  - i) TLC, paper chromatography

18.1.2 Quality in terms of the content of the active constituent

In cases where active constituents responsible for taste and aroma are known, specifications may be laid down on the basis of their contents, although there usually is a wide range of their concentration in materials from different regions and of different varieties. Examples of such constituents are piperine content in

Table 18.3 Quality standards (% w/w max) for specific herbs and spices as laid down by various organizations

Product	Moisture	Ash	Acid insoluble ash	Volatile oil
Aniseed	12 (ISO)	9 (ISO)	2.5 (AFNOR)	1 (ISO)
Basil (BSI)	12	16	3.5	0.5 (ESA)
Bay (ISO)	8	7	2	1
Cardamom (ESA)	12	9	2.5	4
Cassia (ESA)	14	7	2	1
Celery seed (ISO)	11	12	3	1.5
Chilli (ISO)	11	10	1.6	–
Cinnamon (ESA)	14	7	2	0.4
Cloves	12 (ISO)	7 (ISO)	0.5 (ISO)	14 (AFNOR)
Corriander	12 (ISO)	7 (ISO)	1.5 (ISO)	0.3 (ESA)
Cumin (ESA)	13	14	3	0.5
Dill seed (ESA)	12	10	2.5	1
Caraway (ISO)	13	8	1.5	2.5
Fennel seed (ISO)	12	9	2	1.5
Fenugreek (ISO)	12	7	2	–
Garlic powder	7 (ESA)	6 (ESA)	0.5 (ISO)	–
Ginger	12 (ISO)	8 (ISO)	2 (ESA)	1.5 (ISO)
Mace	10	4	0.5	5
Marjoram (ISO)	12	10	2	1
Mint (ISO)	13	12	2.5	0.5
Mustard (BSI)	10	6.5	1	–
Nutmeg	12 (ESA)	3 (ISO)	0.5 (ISO)	6.5 (ESA)
Oregano (BSI)	12	10	2.5	1.5 (ESA)
Black pepper	12 (ESA)	7 (ISO)	1.5 (ESA)	2 (ISO)
Rosemary	10 (ISO)	8 (ESA)	1 (ESA)	1 (ISO)
Saffron (whole) (ISO)	12	8	1	–
Sage (ISO)	12	12	2	1.5
Tarragaon (ESA)	8	12	1.5	0.5
Thyme	12 (ISO)	14 (ISO)	12 (ISO)	1 (ISO)
Turmeric (whole) (BSI)	12	8	2	2.5

AFNOR Association Francaise de Normalisation
BSI British Standards Institute
ESA European Spice Association
ISO International Standards Institute
Ref: Peter, 2001

pepper; ASTA colour values, Scoville heat units and capsaicin content for capsicum; and curcumin for turmeric.

18.1.3 Cleanliness quality specifications

These are needed to take care of microbial contamination, insect infestation and fungal toxins, and include:

- Cleanliness: These give permitted amounts of extraneous matter/filth, visible mould count, insect infestation, excreta and insect damaged material.
- Microbial contaminants to indicate coliforms, salmonella, gross contamination as total plate count (TPC), visible mould contamination and freedom from pathogens.
- Mycotoxin levels, especially aflatoxin and ochratoxin.

18.2 Whole spices and spice powders

Raw spices are often contaminated with microorganisms, insect filth and undesirable adulterants. Although standards specify an absolute maxima of 10^6 for microbial count and total absence of *Salmonella*, counts in excess of 10^6 and presence of food pathogens such as *Salmonella* are often encountered. Decontamination of spices using ethylene oxide was prevalent until recently. However, with the banning of these chemical treatments, the only effective and safe alternative is irradiation.

Several countries have been irradiating spices on a commercial scale to counter both insect and microbial contamination. The use of high-pressure steam in highly specialized equipments is another method of control of microbial load. The European Spice Association has laid down the specifications of minimum quality of herbs and spices (Table 18.4). For many individual herbs and spices, cleanliness specifications laid down by the American Spice Trade Association in terms of whole insects, excreta, mould, insect infestation and extraneous matter could be a guideline. These standards (Table 18.5) are used within the United States. In the case of ground spices, apart from the quality specifications specified for the whole spices, an additional standard is the mesh size/particle size to indicate uniformity of particle size in the powder. Adulterants for individual herbs and spices and/or their powders are as shown in Table 18.6. Methods to detect some of these adulterants are described below.

18.2.1 Physical methods such as microscopy

This can be used to distinguish whole cardamom powder from cardamom seed powder as the presence of abundant pitted fibres, spiral cells of the vascular bundles, empty parenchymatous cells and scattered resin cells with brownish clumps in the former. Papaya seeds can be differentiated from pepper by the thick cell walls of the seed husks, its star-shaped cross-section, and the relative number of oil containing cells (Seidemann, 1994).

Microscopy has also been used to distinguish cassia and cinnamon. Cassia that is frequently used as an adulterant of cinnamon has much broader fibres (usually above 40 μm), while cinnamon has fibres that are usually under 30 μm. Similarly, cassia contains cork and larger starch grains. All these can be observed microscopically.

Table 18.4 Minimum quality specifications for herbs and spices as laid down by the European Spice Association

Specification	Limits
Extraneous matter	Herbs 2%; Spices 1%
Sampling	(For routine sampling) Square root of units/lots to a maximum of 10 (For arbitrary purpose) Square root of all containers
Foreign matter	Maximum 2%
Ash, acid insoluble ash and moisture content	As specified for individual herbs or spices
Packaging	As agreed between buyer and seller; if jute bags, then conformation to standards set by CAOBISCO Ref C502-51-sj of 20-02-95
Heavy metals	Should comply with national/EU legislation
Pesticides	In accordance with manufacturer's recommendations and good agricultural practices, and comply with the national/EU legislation
Treatments	Use of any EC approved fumigation in accordance with manufacturer's instructions, to be indicated on accompanying documents (irradiation not to be used unless agreed between the buyer and seller)
Microbiology	*Salmonella* – absent in (at least 25 g); Yeast and mould count 10^5/g target, 10^6 absolute maximum; *E. coli* 10^2/g target, 10^3 absolute maximum; Other requirements as per agreement between the buyer and seller
Off odours	Shall be free from off odour or taste
Infestation	Should be practically free of live/dead insects, insect fragements and rodent contamination as visible to the naked eye
Mycotoxins	*Aflatoxins* Should be grown, harvested, handled and stored to prevent or minimize the risk of contamination. For capsicum species, piper species, nutmegs, turmeric and ginger, the maximum permitted EC limits are total aflatoxin 10 ppb max.; aflatoxin B_1 – 5 ppb max. *Ochratoxin A* Should be grown, harvested, handled and stored to prevent or minimize the risk of contamination
Volatile oil	As specified for individual herbs or spices
Adulteration	Shall be free from
Bulk density, water activity and species	To be agreed between the buyer and seller
Documents	Should provide: details of any treatments the product has undergone; name of the product; weight; country of origin; lot identification/batch number; year of harvest

Ref: Peter, 2001

Table 18.5 Cleanliness specifications for herbs and spices as laid down by the American Spice Trade Association

Product	Whole insects, dead by count	Excreta, mg/lb		Mould, wt %	Insect infested, wt %	Extraneous matter, wt %
		Mammalian	Other			
All spice	2	5	5	2.0	1.0	0.5
Anise	4	3	5	1.0	1.0	1.0
Sweet basil	2	1	2	1.0	1.0	0.5**
Caraway	4	3	10	1.0	1.0	0.5
Cardamom	4	3	1.0	1.0	1.0	0.5
Cassia	2	1	1.0	5.0	2.5	0.5
Cinnamon	2	1	2.0	1.0	1.0	0.5
Celery seed	4	3	3.0	1.0	1.0	0.5
Chillies	4	1	8.0	3.0	2.5	0.5
Cloves*	4	5	8.0	1.0	1.0	1.0*
Coriander	4	3	10	1.0	1.0	0.5
Cumin seed	4	3	5.0	1.0	1.0	0.5
Dill seed	4	3	2.0	1.0	1.0	0.5
Fennel seed	See note 1 below			1.0	1.0	0.5
Ginger	4	3	3	3	3	1.0
Laurel leaves	2	1	10	2.0	2.5	0.5
Mace	4	3	1	2.0	1.	0.5
Marjoram	3	1	10	1.0	1.0	1.0**
Nutmeg (whole)	4	0	0	5	5	0.0
Oregano	3	1	10	1.0	1.0	1.0**
Black pepper	2	1	5.0	1.0	1.0	1.0
Poppy seed	2	3	3.0	1.0	1.0	0.5
Rosemary leaves	2	1	4.0	1.0	1.0	0.5**
Sage	2	1	4.0	1.0	1.0	0.5
Savory	2	1	10	1.0	1.0	0.5**
Sesame seeds	4	5	10	1.0	1.0	0.5
Tarragon	2	1	1.0	1.00	1.00	0.50**
Thyme	4	1	5.0	1.00	1.00	0.50**
Turmeric	3	5	5.0	3.00	2.50	0.50

* Less than 5% allowance by weight for unattached clove stems over and above the tolerance for other extraneous matter is permitted.
** Extraneous matter also includes other plant material such as foreign leaves.
[1] In case of fennel seed, if 20% or more of the sub-samples contain any rodent, other excreta or whole insects, or an average of 3 mg/lb or more of mammalian excreta, the lot must be reconditioned.

Ref: Peter, 2001.

Table 18.6 Some common adulterants of herbs and spices and/or their powders

Herb/spice whole/powder	Common adulterants employed
Asafoetida	Gum Arabic, colophony resin, red clay, gypsum, slices of potato; chalk, barley
Aniseed	Exhausted fruits; ground aniseed is sometimes adulterated with ground fennel which resembles it in odour, and is considerably cheaper
Chilli powder	Bleached pericarp, seeds, calyx and peduncle to increase bulk density; artificial dyes like sudan red, almond shell dust and dried red beet pulp
Black pepper	Low quality pepper, stems, chaff, and various foreign matter such as papaya seeds. Sometimes with fruits of *Lantana camara* or *Vitex attissima* Linn; seeds of *Mirabilis jalapa*
Small cardamom	Seeds from lower grades and also from large cardamom; decorticated or immature seeds; powder is adulterated with cereal/pulse flours and extracted ginger
Large cardamom	Seeds with seeds of *Amomum aromaticum*; unroasted coffee seeds
Cinnamon	Rougher, thicker and less aromatic bark from cassia and *C. tamala*. Powder adulterated with beechnut husks aromatized with cinnamaldehyde, sugar, ground walnut shells, *galanga* rhizome; adulteration of commercial cinnamon with pepper (can be detected by UV diode array and electrochemical detection
Turmeric powder	Starch from tapioca, arrowroot or cereal flour, husks, coal tar colours, lead chromate, volatile oil of other *Curcuma* species
Saffron	Extracted saffron, style of saffron flower, dyed berigonia syrups, honey, glycerine, oils, barium sulphate, calcium carbonate, gypsum, potassium hydroxide, starch, glucose, dried petals of sunflower or marigold or poppy flowers, stigmata from other *Crocus* species, pomegranate flowers, sandalwood dust, red coloured gelatine, organic colouring matter like martius yellow, fuchsin or picric acid
Mustard seed	Flour with linseed meal; whole black seeds with rapeseed, turnip seeds
Poppy seeds	*Amaranthus* seeds that closely resemble poppy seeds

Refs: Singhal, Kulkarni and Rege, 1997; Peter, 2001; Oberdieck, 1992; Kermasha, Goetghebeur and Dumont, 1994

18.2.2 Physical properties such as solubility

Cassia has higher quantities of mucilage that can be extracted in water, as compared to cinnamon. Hence, the content of cold water extractable mucilage can detect cassia (the adulterant) in cinnamon (the spice).

18.2.3 Chemical analysis of some active constituents

For instance, admixture of whole mustard seeds with rapeseed (*Brassica napus*) and turnip seed can be detected by hydrolysis of the thioglycoside. While mustard seed yields allyl cyanide, rapeseed and turnip seed yield butenyl cyanide and pentenyl cyanide respectively, in addition to allyl cyanide. The method is sensitive enough to detect 5% of the adulterants (Vangheesdaele and Fournier, 1977). Similarly, for garlic, the compliance markers are the various volatile non-polar sulphur-containing compounds, which have the potential to determine the sensory quality and authenticity in products such as garlic powder.

The pungency of alliums such as onion has been based on the determination of thiopropanal-S-oxide, the lachrymatory compound. The method requires extraction with hexane and then determination of the same spectroscopically or by GC or by HPLC. Determination of sulphinates as an indicator of pungency by derivatizing it by N-ethylmaeimide, followed by measuring the absorbance of the conjugate at 515 nm are the key steps in this determination (Thomas, Parkin and Simon, 1992). GC with ion trap MS of the semivolatile compounds from true cinnamon and cassia by headspace solid-phase microextraction could successfully distinguish between the two and also detect the presence of cassia in cinnamon (Miller, Poole and Pawlowski, 1996)

18.2.4 Chemical constituents in the adulterant

Amaranth seeds as an adulterant in poppy seeds can be detected by popping in a sand bath, wherein any amaranth seed puffs and shows its presence. It can also be quantitatively estimated by estimating the hydrocarbon squalene (Singhal and Kulkarni, 1990). Similarly, adulteration of white pepper powder with rice/maize starch can be detected not only from the decreased level of piperine, but also from the increase in the ratio of K : Ca (Archer, 1987).

A constituent present in papaya seeds, *Carica papaya* is a glucotropaeolin that gives benzyl isothiocyanate on hydrolysis. This has been made the basis for detecting papaya seeds that are commonly used as an adulterant of whole black pepper as well in the powder form (Curl and Fenwick, 1983). Extraction of the suspected sample with ethylene dichloride followed by spotting on TLC is reported to give a fluorescent spot at R_f 0.943 that proved to be a very promising marker for the presence of papaya seed powder in black pepper powder at a level as low as 2%. Characterization of this spot by GC/MS showed aldehydes such as *n*-nonanal, 2-decanal and *trans*-2-undecenal which are totally absent in black pepper, and hence could serve as marker compounds for this adulteration (Paradkar, Singhal and Kulkarni, 2001).

Turmeric or *Curcuma longa* in the powder form is often adulterated with other curcuma species such as *C. caesia*, *C. domestica* or *C. zedoaria*. In such cases, microscopic evaluation is difficult. Detection is based on the detection of constituents such as camphor and camphene in the adulterants. A rapid and sensitive TLC technique using a three-step colour sequence and having a sensitivity to detect 5% adulterant is described by Sen *et al*. (1974).

18.2.5 Use of molecular markers

An example of this approach has been recently reported with galangal (*Alpinia galangal*), which is used as a spice in Asian foods, and is often adulterated with other closely related *Alpinia* species. Sequence analysis in nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) regions showed ITS 1 to range from 177 to 178 base pairs, and ITS 2 to range from 225 to 234 base pairs. The size of the 5.8S coding region was determined to be 164 base pairs for all species. Pairwise sequence divergence and use of some of the molecular markers could distinguish *Alpinia galanga* and related species (Zhao *et al.*, 2001)

18.3 Essential oils

The essential oils may be adulterated synthetic components identical with those found in nature, essential oils from inferior parts of the plant or inexpensive plants with similar properties, ethanol, or even mineral and edible oils. The essential oils and their commonly used adulterants for some of the herbs and spices are as shown in Table 18.7. In the case of essential oils quality specifications and genuineness (i.e. freedom from any adulteration) could be established in the following terms:

- Certain **physical characteristics** such as optical rotation, solubility characteristics, molecular refraction, specific gravity, refractive index, congealing, melting or boiling range, evaporation residue and flash point are made the basis of the standards. Freezing point is also useful in some cases, as has been shown with peppermint oil; a value lower than 10.5 ºC is indicative of turpentine (Lu, 1994). This approach has not been widely studied with essential oils from herbs and spices.
- **Chemical properties** such as determination of acids, esters, alcohols, aldehydes and ketones, phenols, iodine number, etc., and some specific tests such as flavour tests and tests for halogens are also included in the standards.
- In a few cases, wherever possible, use of **marker compounds** is very useful. For instance, it is known that anise oil from *Pimpinella anisum* L. is frequently adulterated with the less expensive star anise oil from *Illicium verum*. Although being equally acceptable and interchangeable in use, oil from *P. anisum* is believed to be superior to *I. verum*. The detection of up to 5% 2-methylbutyryl ester of 4-methoxy-2-(1-propenyl)-phenol as the marker

Table 18.7 Some essential oils from herbs and spices and their commonly used adulterants

Essential oil/their origin	Adulterants employed	Method of detection
Allspice (*Pimenta diocia*) berry oil	Pimenta leaf oil, clove oil and its fractions	Gas chromatography
Bay leaf oil	Clove stem oil, clove leaf oil or their terpenic fractions	Difficult to detect
Anise oil (*Pimpinella anisum* L.)	Star anise oil	Presence of foeniculin, several terpene hydrocarbons and 1,4-cineole
	Fennel oil	Change in optical rotation
	Synthetic anethole	From a change in solidification point of +15ºC–+19ºC
	Turpentine oil, cedarwood oil, and copaiba and guryun balsam oils	No method reported as yet
Star anise oil (*Illicium verum*)	Addition of star anise leaf oil	From a lowering of congealing point, and of the anethole content
	Mineral oil or fatty oil	From the alteration in specific gravity, congealing point and solubility in 90% alcohol
Pepper (*Piper longum*) essential oil	Low priced and readily accessible terpenes and sesqui-terpenes, such as phellandrene, dipentene and caryophyllene	Difficult to detect, since these are natural components of the oil
	Mineral oil	Detected as turbidity in Holde's test, or by TLC as fluorescent spots under UV lamp
Cinnamon bark oil (*Cinnamomum zeylanicum* L.)	Leaf oil, cassia, resin, petroleum or oil of cloves; synthetic cinnamaldehyde	Detected by analysis of cinnamaldehyde or eugenol contents, and by measuring specific gravity
Marjoram (*Origanum majorana*) oil	Synthetic racemic alcohols and acetates	H-NMR using a chiral lanthanide shift reagent
Nutmeg (*Myristica fragrans*) oil	Adulteration of true nutmeg by other Myristica species, e.g., *M. malabarica*, *M. otaba*	Poor quality as judged by sensory analysis, since the adulterants are devoid of aroma
Clove (*Syzygium aromaticum*) bud oil	Clove stem oil and low-price clove leaf oil	Principal component analysis and stepwise discriminant analysis, particularly for eugenol, α-cubebene, 11-terpinyl acetate, (E)-α-bergamotene and caryophyllene

Table 18.7 Continued

Essential oil/their origin	Adulterants employed	Method of detection
	Clove terpenes, obtained as byproducts in extraction of eugenol from clove oil	Smaller quantities cannot be detected, but larger quantities can be detected by a decrease in eugenol content, refractive index and specific gravity
	Acetins	Detected by a high saponification number of the water soluble materials
	Terpineol, dibenzyl or dibenzyl ether	By odour of non-phenolic portions of the oil
Oil of wintergreen	Synthetic methyl salicylate	Slight lowering of optical rotation
Sage (*Salvia officinalis*) oil	American cedar leaf oil	No reports
Thyme (*Thymus vulgaris*)	Cresol isopropylation derivatives	GC, GC-MS and NMR, IR spectrophotometry
Turmeric (*Curcuma longa*) oil	Oil from other curcuma species such as *C. caesia* and *C. domestica*	TLC based on detection of camphor and camphene in the adulterants
Celery seed (*Apium graveolens*) oil	Chaff oil, terpenes chiefly *d*-limonene resulting from the concentration of sweet orange oil	No reports
Cumin (*Cuminum cyminum*) oil	Synthetic cinnamaldehyde	Excess can be detected by change in optical rotation; other techniques that may be of help are selected ion monitoring (SIM) or stable isotope ratio analysis (SIRA)
Caraway (*Carum carvi*) oil	Cumin oil, *d*-limonene obtained as byproduct of extraction of carvone or from orange oil	No reports

Refs: Ravid *et al*., 1987; Singhal, Kulkarni and Rege, 1997; Peter, 2001; Tateo, Salvatore and Nicoletti, 1992, 1993

compound by direct mass spectrometry is a rapid and reliable method by which the genuineness of anise oil can be ascertained.

- **Colourimetric analysis** of glycerol could indicate adulteration of essential oils with edible oils. So would the TLC analysis of the hydrocarbon fraction, and GLC/GC profile of the oil.

18.3.1 Spectroscopy

Spectroscopic analysis such as IR can indicate the presence of edible oils and paraffin oil in essential oils. Cottonseed oil, for instance, gives absorption bands characteristic of esters and unsaturated esters (at 1705–1720 cm^{-1}), acetates (at

1245 cm^{-1}, and the carbonyl group (at 1250–1170 cm^{-1}). Paraffin oil gives a broadened absorption band at 3000 cm^{-1} which characterizes the saturated and unsaturated hydrocarbons (Mostafa *et al.*, 1990b). Spectrophotometric scanning of aqueous saffron extract has also been shown to be useful to quantify its quality. For instance, crocins, bitter constituents such as picrocrocin and volatile fragrances such as saffranalal can be assayed as absorbance at 440 nm, 257 nm and 330 nm, respectively. HPLC with photodiode array detection (HPLC-DAD) coupled with mass spectrometry has been useful for determination of these constituents, and is the method of choice for the analysis of crocetin glucosides with one to five glucose units and differentiation of *cis/trans* isomers (Sujata, Ravishankar and Venkataraman, 1992; Tarantilis, Polissiou and Manfait, 1994; Tarantilis, Tsoupras and Polissiou, 1995). Headspace chromatographic methods and thermal desorption gas chromatography in line with mass spectrometry are particularly useful (Alonso, Salinas and Garijo, 1998; Alonso *et al.*, 1996 Tarantilis and Polissiou, 1997).

18.3.2 Selected ion monitoring

A technique that has been very useful in detecting the addition of synthetic flavour compounds is selected ion monitoring (SIM) of the impurities in the same way that is characteristic of the route used to prepare them. The absence of these impurities is an indication of the essential oil being natural (Frey, 1988). While a mass spectrometer scans over a range of trace compounds to obtain information on every compound in the mixture, SIM decreases the number of masses to be detected, and results in a 10–100 fold increase in detection sensitivity for a single compound. This can be best explained with an example. Synthetic cinnamaldehyde that is added to cassia oil contains phenyl pentadienal, benzyl alcohol and eugenol as impurities. These impurities can be quantified at levels as low as 0.55 parts of synthetic cinnamaldehyde in natural cassia oil (Zhu *et al*., 1996). This approach could be exploited to trace other similar fraudulent practices with other essential oils from herbs and spices.

18.3.3 GC/MS or IR spectroscopy

The addition of nature identical substances could also be identified by GC/MS or IR spectroscopy, which are both simple and rapid. A case in point is detection in high-value onion oil which could be checked for the onion furanone, 2-n-hexyl-5-methyl-3(2H) furanone (Losing, 1999).

18.3.4 Isotopic mass spectroscopy

Isotopic mass spectrometry, based on the measurement of $\delta^{13}C/^{12}C$, has been very useful in the authentication of saffron oils (Bigois *et al*., 1994), and for the adulteration of mustard oil with synthetic allyl isothiocyanate (Butzenlechner *et al.*, 1996). Similarly, site-specific natural isotope fractionation studied by NMR

(SNIF-NMR) combined with molecular isotope ratio determination by mass spectrometry (IRMS) can characterize linalool and linalyl acetate from chemical synthesis or extracted from natural oils. Using this technique, adulteration of cinnamon oils (and so also bitter almond oil) has been made possible by examining the site-specific deuterium content on benzaldehyde that can be used as a molecular probe. Distinction between synthetic (using benzal chloride and toluene), natural (kernels of apricots, peaches and cherries) and semi-synthetic (cinnamaldehyde extracted from cinnamon), wherein the transformation of cinnamaldehyde to benzaldehyde is exploited to identify the origin of the cinnamaldehyde. This method is reproducible, and can also serve to quantify the adulterants (Remaud *et al.*, 1997). This technique has an added ability to distinguish the components from different botanical and/or geographical origins. Another modification of this technique is the use of chirality as a criterion for differentiating between components of nature and nature-identical types (Werkhoff *et al.*, 1991). In this case, selectivity is achieved by using enantioselective capillary GC coupled with stable isotope ratio analysis (Hener *et al.*, 1992).

18.3.5 Enantiomeric purity

Use of enantiomeric purity of constituents can be an indication of its origin, and can be used to identify adulterants. For instance, carvone from essential oils of caraway, dill and spearmint can be detected by using this technique. While S(+)-carvone is detected in herb oils of caraway and dill, spearmint oils from various countries contain only R(−)-carvone (Ravid *et al.*, 1992). Similarly, a very high enantiomeric purity of R(+)-limonene of 99.1–99.5% is indicative of the purity of both dill and caraway essential oils, although limonene content itself could vary among the cultivars of these spices (Zawirska-Wojtasiak and Wasowicz, 2000).

18.3.6 Isotopic analysis

The use of isotopic analysis is a method to check for the presence of synthetic components in essential oils as has been shown with cinnamaldehyde. The presence of ^{14}C in cinnamaldehyde as the main natural constituent and its absence in the synthetic counterpart forms the basis of their distinction. Unfortunately, the technique has been overcome by addition of ^{14}C enriched cinnamaldehyde.

18.3.7 Enantiomers

A very interesting detection of synthetic components in commercial essential oils is based on differences in bioactivity of the enantiomers. For instance, (−)-α-pinene had a greater inhibitory activity on 18 of the 25 different bacterial species as compared to the (+)-α-pinene. Evaluation with 20 strains of *Listeria*

Table 18.8 Physical properties as quality standards for various essential oils

Spice	Specific gravity (20 °C)	Refractive index (20 °C)	Optical rotation (°) (20 °C)	Solubility characteristics	Other remarks
Asafoetida	0.906–0.973	1.493–1.158	−9 °0′ to +9 °18′		Sulphur content, 15.3–29%
Allspice (Pimenta berry oil)	1.024–1.055[a]	1.525–1.536	0 °32′ to 5 °0′	Soluble in 1–2 vols and more of 70% alcohol, occasionally with opalescence to turbidity on dilution	Phenol content, 65–89%
Pimenta leaf oil	1.026–1.065	1.530–1.540	Inactive to 5 °30′	Soluble in 1–2 vols of 70% alcohol	Phenol content, 65–96%
Bay oil	0.960–0.985[a]; in oils of lower quality as low as 0.951	1.506–1.520	Laevoratatory up to −2 °, seldom up to −3 °	Freshly distilled oils are soluble in usually in 1–2 vols of 70% alcohol; solubility decreases rapidly on storage	Phenol content, 57–60%; in oils of poor quality as low as 40%
Cardamom	0.923–0.941[a]; 0.917–0.947 at 25 °C	1.462–1.467	+22 ° to +41 °	Soluble in 2–5 vols of 70% alcohol; in all proportions in benzylalcohol, diethyl phthalate and fixed oil; insoluble in glycerine and propylene glycol; soluble in mineral oil with opalescence	Acid number up to Ester number, 12; unstable in the presence of strong acids and strong alkali
Cinnamon bark oil	1.020–1.030	1.568–1.535	−1 °0′ to 2 °10′	Soluble in 1–2.5 vols of 70% alcohol, occasionally opalescent to hazy	Aldehyde (calculated as cinnamaldehyde), 51.8–56%; Phenol (Eugenol), 14–18%

Cinnamon leaf oil	1.037–1.055[a]	1.529–1.535	−1°36′ to 0°40′	Soluble in 1.5 vols or more of 70% alcohol, sometimes with opalescence or paraffin separation	Aldehyde, up to 4%; Phenol, 77.3–90.5%
Cassia oil	1.055–1.070[a]	1.600–1.606	−1°0′ to +6°0′	Readily soluble in 1–2 vols of 80% alcohol, 2–3 vols of 70% alcohol	Aldehyde, 75–90%
Clove bud oil	1.043–1.068[a]	1.527–1.537	Up to 1°35′	1–2 vols or more of 70% alcohol with slight turbidity; freshly distilled in 2.5–3.0 vols of 60% alcohol	Eugenol, 78–95%, seldom up to 98%
Clove stem oil	1.040–1.067[a]; 1.048–1.056 at 25°C	1.531–1.538	Up to 1°30′	1–2 vols or more of 70% alcohol and 2.5–3.0 vols of 60% alcohol	Eugenol, 83–95%; in exceptional cases, higher
Clove leaf oil	1.032–1.067[a]; 1.036-1.046 at 25°C	1.531–1.539	−0°50′ to 1°53′	0.9 vols or more of 70% alcohol	Eugenol, 78–93%
Ginger	0.877–0.886[a]; oils with lower and higher specific gravities have been observed	1.489–1.494	−26°0′ to 50°0′; lower values observed for oil distilled from old roots stored for a long time	Only sparingly soluble in alcohol. Up to 7 vols of 95% alcohol required for solution which is not always clear. In 90% alcohol, the oils are generally, but not always completely soluble	Acid number, up to 2; Ester number, up to 15; Ester number after acetylation, 24–50
Mustard	1.014–1.030	1.527–1.529	Inactive	Soluble in 160–300 parts of water, 7–10 parts of 70% alcohol, 2.5–3.0 vols of 80% ethanol, in 0.5 vols of 90% ethanol, clearly miscible with ether, amyl alcohol, benzene and pet ether	Allyl isothiocyanate, 94%; boiling range at 760 mm, 148–154°C

Table 18.8 Continued

Spice	Specific gravity (20 °C)	Refractive index (20 °C)	Optical rotation (°) (20 °C)	Solubility characteristics	Other remarks
Nutmeg	0.859–0.868	1.469–1.472	+40 °48′ to +49 °48′		Acid number, 1.0–1.3; Ester number, 6.8–7.3
Mace	0.860–0.892	1.472–1.479	+21 °42′ to +41 °30′		Acid number, 1.5–6.2; Ester number, 2.8–12.8
Marjoram	0.886–0.902; 0.892–0.910[a]	1.470–1.476	+13 ° to +25 °0′	Soluble in 1–2.2 vols of 80% alcohol	Acid number, <1.4 2.8; Ester number, 10–86; Ester number after acetylation, 41–86.8
Oil of wintergreen	1.180–1.193[a]	1.535–1.536	−0 °30′ to −1 °30′	Clearly soluble in 6-8 vols of 70% alcohol	Ester number, 354–365; Ester content, calculated as methyl salicylate, 96–99%
Onion	1.047–1.098[a]	1.537–1.559	+1 °3′ to +3 °53′	Most oils not completely soluble in 10 vols of 95% alcohol. Occasionally soluble in 1–2 vols or more of 95% alcohol	Acid number 12.0–19.8; Carbonyl number, 9.8–15.1; Iodine number, 59.9–66.2
Pepper oil	0.873–0.916	1.480–1.499	−10 °0′ to +3 °	Not readily soluble in alcohol, usually soluble in 10–15 vols of alcohol; soluble in 3–10 vols of 95% alcohol	Acid number, up to 1.1; Ester number, 0.5–6.5; Ester number after acetylation, 12–22.4; Phellandrene test, usually strongly positive
Star anise	0.98–0.00	1.553–1.557	Up to 2 °; sometimes up to 0 °36′	Soluble in 1.5–3.0 vols of 90% alcohol	Congealing point, +14 to +18 °
Ajowan oil	0.919–0.930[a]	1.498–1.504	Up to 5 °0′	Soluble in 1–2 vols and more of 80% alcohol	Phenols, 45.0–57.0%
Coriander	0.870–0.885[a]	1.463–1.471	+8 °0′ to +13 °0′	Soluble in 2–3 vols of 70% alcohol	Acid number, up to 5.0; Ester number, 3.0–22.7

Dill	0.895–0.915[a]	1.481–1.491	+70 °0′ to +82 °0′	Soluble in 4–9 vols of 80% alcohol	Carvone content, 40–60%
Anise oil	0.980–0.990	1.552–1.559	Upto 1 °50′	Soluble in 1.5–3.0 vols of 90% alcohol	
Fennel seed oil	0.965–0.977[a]	1.528–1.539	+11 °0′ to +24 °0′	Soluble in 5–8 vols of 80% alcohol and in 0.5 vols of 90% alcohol	Congealing point, not below 5 °, and as high as 10 ° in good oils
Celery seed oil	0.872–0.891[a]	1.480–1.484	+65 °53′ to +76 °51′	Turbid in 90% alcohol	Saponification number, 25.1–47.6
Caraway seed oil	0.907–0.919[a]	1.484–1.488	+70 °0′ to +81 °0′	Seldom soluble in 70% alcohol, soluble in 2–10 vols of 80% alcohol; clearly soluble in equal vols of 90% alcohol	Carvone content, 50–60%
Parsley seed oil	1.043–1.110[a]	1.512–1.528	−4 °0′ to 10 °8′	4–8 vols and more of 80% alcohol	Acid number, up to 6; Ester number, 1–11; Ester number after acetylation, 4–20
Parsley herb oil	0.902–1.016[a]	1.509–1.526	+1 °16′ to +4 °30′	Soluble in 95% alcohol	Acid number, up to 1; Ester number, 5–14; Ester number after acetylation, 19–68
Cumin oil	Wide variation observed among different geographical regions				
	0.894–0.929[a]	1.491–1.507	+2 °55′ to +7 °[a]	2–11 vols in 80% alcohol	Cuminaldehyde, 16.00–62.70%
Curry leaf oil	0.9748 at 25 °C	1.5021 at 25 °C	+4.8 at 25 °C	Soluble in 80% alcohol with slight opalescence	Saponification value, 5.2; Saponification value after acetylation, 54.6; Acid value, 3.8

[a]at 15 °C

Refs: Singhal, Kulkarni and Rege, 1997; Peter, 2001

Table 18.9 Recommended iodine values for pure specimens of various essential oils and isolates

Essential oil/isolate	Recommended iodine value
Oil of ajowan, lab distilled*	232–265
Oil of fennel, lab distilled*	160–185
Oil of dill, lab distilled*	265–307
Oil of clove, lab distilled*	232–243
Oil of cinnamon leaf, lab distilled	46–52
Oil of black pepper, lab distilled	300–324
Oil of cumin seed, lab distilled	193–195
Oil of ylang**	175
Oil of lavidin (abrialis)**	167
Oil of parsely seeds, lab distilled*	248
Oil of spike lavender**	35
Oil of black jeera, lab distilled*	230
Oil of *Curcuma amada*, lab distilled*	266
Oil of *Piper longum*, lab distilled*	265
Oil of dry ginger, lab distilled*	185
Oil of *Pimpenalla anisum*, lab distilled*	296

* Samples collected from different places
** Samples procured from different companies
Ref: Kumar and Madaan, 1979

monocytogenes showed 19 to be inhibited to a greater degree by the (+)-isomer than the (−)-isomer. The results documented in the study indicated differing bioactivities of α-pinene enantiomers to be useful in indicating adulteration of essential oils (Lis-Balchin *et al.*, 1999).

Table 18.8 shows the physical properties as quality standards for various essential oils, and Table 18.9 shows the recommended iodine values for genuine essential oils from various herbs and spices. Their efficacy in detecting adulterants can be seen from Table 18.10. These physical properties should however be considered as presumptive tests and should be confirmed by other more specific analysis.

18.4 Oleoresins

Oleoresins may contain vegetable oils and solvents such as ethanol as extenders and solvent residues. In many cases, a two-stage extraction process is employed for the preparation of oleoresins. In the first stage, the oil is recovered along with the resins by solvent extraction, while in the second the oil is recovered by steam distillation followed by solvent extraction for recovering the oleoresin. The oil and the oleoresin are then blended to meet the required specifications. In the case of oleoresins and extracts, a limit has to be specified for solvent residues, especially if the solvent is toxic. The tolerances for all the solvent residues as per

Table 18.10 Critical region (borderline) for detection of adulterated oils by different adulterants[a]

Properties/ adulterants added (%)	Marjoram	Petit grain bigrade	Fennel
Specific gravity at 25 °C	0.9414	0.9082	0.9734
Ethanol	>10	>0.5	>15
Paraffin oil	>10	>0.5	>1
Cottonseed oil	>20	>5	>15
Refractive index at 25 °C	1.4452	1.4919	1.5198
Ethanol	>20	>0.5	>10
Paraffin oil	>0.5	–	>10
Cottonseed oil	>0.5	–	>10
Specific optical rotation	13.45	3.08	6.39
Ethanol	>5	–	>10
Paraffin oil	>5	>40	>10
Cottonseed oil	>5	40 only	>10.5
Ester number	45.16	193.4	17.22
Ethanol	>15	>5	>0.5
Paraffin	>20	>10	>0.5
Cottonseed oil	>0.5	–	>2.0

[a] Significant at 5% level
– Not detected
Ref: Mostafa *et al.*, 1990a

Part 21 CFR of the Federal Drug and Cosmetic Act are:

- acetone residues: max. 30 ppm
- ethylene dichloride residues: max. 30 ppm; if residues of other chlorinated solvents are also present, the total should be a maximum of 30 ppm
- methylene chloride residues: same as for ethylene dichloride
- isopropyl alcohol residues: max. 50 ppm
- methyl alcohol residues: max. 50 ppm
- hexane residues: max. 25 ppm.

The purpose of using an oleoresin very often dictates the specifications required of it. For instance, if colour is the main requirement of using an oleoresin from chillies, paprika oleoresin with more colour and pungency would be the specification. On the other hand, if pungency is the main criterion, capsicum oleoresin with less colour and high pungency would be desirable. In cases where both pungency and colour are desirable, red pepper oleoresin containing moderate amounts of both colour and pungency are suitable. The colour value is determined by measuring the absorbence of a 0.01% solution of an oleoresin in acetone and

measuring the absorbence at 258 nm. The absorbence is multiplied by 61,000 to convert to total colour units. The colour value specifications, as laid down by the Essential Oils Association for capsicum, red pepper and paprika oleoresins, are $< 4{,}000$, $< 20{,}000$ and 40,000–100,000, respectively.

The corresponding values for pungency as Scoville heat units are $> 4{,}80{,}000$, $> 2{,}40{,}000$ and 0, respectively. All these oleoresins may have similar solubilities in different solvents. Chilli, paprika and red pepper oleoresin have all to be soluble in benzyl benzoate, partly soluble in alcohol with an oily layer, soluble in fixed oils and insoluble in propylene glycol. Chilli oleoresins are very often adulterated with synthetic saturated acid vanillylamides such as pelargonic vanillylamide, which can be detected by GC of the saponified extract, or by TLC coupled to HPLC.

Similarly, although the yield of yellow colouring pigment, curcumin ([1,7-bis-(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione] varies among different turmeric varieties, and further yield of oleoresin varies from 3 to 15% depending on variety, it forms about one-third of a good quality oleoresin. Hence turmeric oleoresins are evaluated strictly on the basis of colour, expressed as colour value (cv) that is equivalent to ten times the specific extinction coefficient in ethanol at 422 nm. A specific extinction coefficient of 1,600 has been recommended as a reasonable yardstick for assay of cucumin. Further, this fits the HPLC data excellently (Verghese, 1999).

Ginger oleoresin is characterized by pungency that is attributed to gingerol. It is very frequently adulterated with capsicum and grains of paradise, both of which are added to give increased pungency. Turmeric is also usually added to restore the colour. Microscopic examination and TLC on 90% ethanol extract are reported to detect this adulteration.

One of the adulterants of black pepper oleoresin that shot to prominence during World War II was an oleoresin from the fruits of *Schnius molle* belonging to the family *Anacardiaceae*, and which had a strong peppery odour. This can be detected by the presence of glucose in the oleoresin sample. Preparation of the oleoresin from black pepper admixed with papaya seeds is a very strong possibility, since the admixture is often practised with whole seed and a powder form of the spices. Such malpractices could be detected using very simple indicators such as saponification value (SV), iodine value (IV) and the ratio of SV/IV. While the SV increased with an increase in the amount of the adulterant, IV decreased proportionately. Although piperine content could be used as an indicator, there are some inherent drawbacks in its use. These are mainly due to variations in piperine content as a function of variety, cultivar, season and geographical origin (Madan, Singhal and Kulkarni, 1996).

18.5 Testing for sensory quality and geographical origin

An important attribute of aromatic spices is their sensory quality. In cases where volatile compounds contributing to the characteristic aroma of the spice have

been well established, comparison of sensory and GC analysis data would be useful as a standard or criteria of purity. However, it should be remembered that such data on the sensory aspects of flavouring compounds are often vague, probably because of the inherent variability of the sensory response which require extensive training of the judges, and adequate replication and detailed statistical analysis of the observations. Measurement of the pungency of chillies as Scoville heat units has a few drawbacks such as quick desensitization of the human palate after the tasting more than a few samples, and the sensitivity of taster's palate to pungency. In a few cases, for instance, for chillies a correlation between organoleptic for pungency as Scoville heat units and total capsaicinoids has been developed. One ppm capsaicinoids has been considered to be equivalent to 16 Scoville heat units.

An organoleptic test preceded by a chemical test is useful in detecting the adulteration of ginger tinctures with capsaicin from capsicum and paradol from grains of paradise. The method is based on the fact that the pungent principles from the adulterants are affected marginally by alkali treatment, whereas the gingerol is rapidly decomposed with a resultant loss of pungency. This procedure is, however, dependent on the sensitivity of the taste buds of individuals.

18.5.1 Geographical origin and quality issues for herbs and spices

All natural products including herbs and spices and products derived therefrom, such as oleoresins and essential oils, show a wide variation in their quality. Hence, it is not surprising that admixture of a 'superior' variety with a comparatively inferior variety is commonly observed in international trade. Even simple analysis such as mineral constituents can vary widely, as has been shown for marjoram. In the case of saffron, the place of origin is very often falsified. The best-quality saffron are those harvested in La Mancha, traditionally regarded as 'Saffron Mancha' or 'Azafrán Mancha'. Saffron from different parts of the world is sold as saffron mancha. Authentication methods that can trace the geographical origin of such products are a challenge to food scientists. Information on such issues is scant in scientific literature, and is just beginning to emerge. The approaches that have so far been used are outlined below.

18.5.2 Looking out for a constituent(s) that can distinguish the geographical origin

Although physical properties such as specific gravity, refractive index, optical rotation, solubility characteristics and character-impact compounds vary widely in products of different geographical origin (as has been shown with cumin essential oil, in Table 18.9), it cannot be considered as appropriate to track down the origin, or for that matter even the traceability to the herb/spice in question.

In the case of nutmeg, GC, GC-MS and RP-HPLC analysis has shown essential oil from East India to be better than West India due to the presence of higher amounts of phenyl propanoids and terpenes, and myristicin (> 1% for West Indian oil as compared to up to 13.5% for East Indian variety). There is a huge variation in the components of essential oil obtained from different geographical origins, as has been shown by Lewis (1984). Three isomers of oximene, (E)-β-ocimene, (Z)-β-ocimene and (Z)-α-ocimene, have been recently identified in the Jamaican oils, and are absent in other oils. The levels of the monoterpenoids α-pinene (19.9%), β-pinene (18.8%) and terpinen-4-ol (17.8%) also differed between Jamaican oils and that from other regions indicating these compounds may be used as markers to distinguish between geographical origins of these oils (Simpson and Jackson, 2002).

Differentiation of rosemary oil of different geographical origins could be distinguished on the basis of GC/MS determination of natural constituents. While Spanish oils are rich in α-pinene (19.4–24.7%), 1,8-cineole (19.0–21.8%) and camphor (16.3–18.9%), the French oils contain α-pinene (19.9–35.1%), 1,8-cineole (5.3–24.8%) and bornyl acetate (1.2–14.3%). Moroccan oils are typically rich in 1,8-cineole (43.5–57.7%) (Chalchat *et al.*, 1993). The composition of curry leaf (*Murraya koenigii* L.) essential oils is a very strong function of the geographical origin. While the stocks from Northern India contained 70% β-pinene, that from Pant Nagar and Southern India contained 65.7% α-pinene and 53.9% β-caryohyllene respectively. Eastern India stocks were predominant in α-phellandrene (30.2%) and β-caryophyllene (24.2%) (Raina *et al.*, 2002).

The proportion of linalool has recently been shown to be useful in identifying the geographical origin as well as the species of cinnamon oils (Upadhaya, Kirihata and Ichimoto, 1994). Another instance where chemical composition can distinguish between the geographical origin of the spice is with dried leaves and fruits of *Myrica gale* which is used as a spice in soups and stews and as a flavouring in beer. The composition of sesquiterpenes is of use in distinguishing between the Scottish and Finnish samples of this spice. While the Scottish samples are rich in β-elemenone and germacrene, the Finnish samples are predominant in γ-cadinene (Svobada *et al.*, 1998). However, it should be remembered that chemical analysis is not always helpful in determining the geographical origin of essential oils as has been shown with sage essential oils (Lawrence, 1994, 1998).

18.5.3 Use of isotopic analysis

An on-line gas chromatography pyrolysis isotope ratio mass spectrometry that can bring out clear cut origin dependent differences of $^{2}H/^{1}H$ in E-2-hexenal and E-2-hexanol has demonstrated the potential of this technique in authenticity studies of flavour constituents in a natural matrix (Hor *et al.*, 2001). This approach needs to be investigated further with herbs and spices.

18.6 Future trends

It is evident that heavy research inputs are needed in order to establish the authenticity and traceability (botanical as well as geographical) of herbs and spices, and the products derived therefrom. The spice equivalence of products such as microencapsulated essential oils/oleoresins and liquid spice flavourings needs to be brought into the limits of legislation. A quick review of the literature indicates hardly any work in these areas, and is the need of the hour. Developments in analytical techniques, particularly the use of isotopic methods, needs to be looked into. A recent trend is the extraction of valuable essential oils by use of newer techniques such as supercritical carbon dioxide or the use of high-pressure technologies. Analytical methodologies that can confirm the use of these technologies are other challenging areas. This is due to the products of these technologies being more expensive than the conventional technologies, and hence the probability of malpractice in international trade.

18.7 References

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19

Identifying genetically modified organisms (GMOs)

B. Popping, Eurofins Scientific Analytics, France

19.1 Introduction

One of the most important fruits in Hawaii is the papaya. It was in the 1940s when the papaya ringspot potyvirus (PRSV) was discovered. This virus had the most devastating effect on the papaya plantations in Hawaii. In the 1950s large papaya plantations on Oahu island were virtually eliminated. The industry was forced to relocate to the Puna district on Hawaii island in the 1960s. The papaya industry thrived in Puna because of the ideal rainfall conditions, availability of land, and most importantly, because Puna was free of the papaya ringspot potyvirus. Even though the area infected with PRSV was only approximately 20 miles away from Puna, geographic isolation and diligent surveillance kept the virus from Puna and as a consequence 95% of the papaya in Hawaii were produced there by the 1980s. Unfortunately, PRSV was discovered in Puna in 1992 and had spread through the Puna district by 1994, again devastating the plantations.

In 1986 Powell showed that transgenic tobacco expressing a coat protein gene of Tobacco Mosaic Virus (TMV) was resistant to Tobacco Mosaic Virus and that the resistance was due to that expressed coat protein. Coat protein genes have been shown to be effective in preventing or reducing infection and disease caused by homologous and closely related viruses. Coat protein-mediated protection has been reported for tobacco mosaic virus, TMV, tomato mosaic virus, ToMV and cucumber mosaic virus, CMV.

What had worked for tobacco also worked for papaya. In 1998 Dennis Gonsalves at Cornell University, Richard Manshardt and Maureen Fitch at the University of Hawaii and the USDA, and Jerry Slightom at Upjohn Company developed in a collaborative programme the transgenic cultivars Sunrise and

Rainbow which were resistant to PRSV by expressing a coat protein of this virus. These varieties are now successfully grown on Hawaii in areas where PSRV is still present. This was just one example where modern biotechnology helped to solve agricultural problems.

Biotechnology began a long time ago. The earliest indications of biotechnology were the domestication and breeding of cattle, followed by the domestication of maize in Oxaca and Puebla, Mexico, at least 7000 years ago. Teosinte (*Zea mays mexicana* or *Zea mays parviglumis*), a grass-like plant, is the ancestor of what we today know as maize. The morphological differences between maize and its wild relative are substantial and dramatic.

It has been shown that relatively few genes are responsible for the large morphological differences. Research by John Doebley[1,2] has shown that as few as five genes may be responsible. In teosinte, the maize kernel is surrounded by a hard coating called a glume. The glume helps to protect the seed when going through the digestive tract of animals or when the seed is sitting in the ground during the winter. However, the tough glume is difficult to chew and digest by humans. Selection for a reduced glume made it easier to digest maize seed.

Modern field maize has a single stalk and ear per plant. This is not the case in teosinte, where many stalks or tillers are found per plant, and there are many inflorescences (female ears and male tassels) per plant. By concentrating energy resources into a single stalk and a single ear it was probably possible to create larger ears that were easier to harvest. The structure of the maize ear is very different from that of teosinte. Teosinte essentially looks like one row of seeds, although from a structural point of view it is two interleaved rows. In contrast, modern maize has a cob and as many as 20 rows.

All these modifications over the last 7000 years have been achieved by selective breeding and outcrossing, which is nothing less than changing the genetic make-up of a plant, nothing less than conventional biotechnology. Today maize provides much of the starch necessary for the world food supply. During its domestication, it was this starch that was probably most important to people. In tropical and subtropical environments, starch is limited which is most likely the reason why people focused their domestication efforts on crops like maize and manioc.

While the domestication of maize has taken several thousand years using conventional biotechnology in the form of out-crossing and cross-breeding, modern biotechnology is able to change the genetic information of an organism in a much shorter time, taking from development to marketing only an average of 12 years. Maize is now available in many varieties like sweetcorn, flint corn, dent corn, flour corn, popcorn and represents one of the largest crops in the world with 140 million ha grown worldwide.

One of the major agricultural threats to maize is the cornborer, an insect from the family of Lepidoptera. The larvae feed on the maize, causing the farmers and the maize industry enormous losses. It therefore became an obvious target for the first generation of transgenic crops.

19.2 Characteristics of transgenic crops

It is well known that spraying maize fields with an aqueous solution containing *Bacillus thuringiensis* (Bt) kills the larvae of the cornborer when feeding on the leaves grown on these fields. Death is caused by a toxic protein, the Bt toxin, also known as cryI (from crystallographic I, since the three-dimensional structure of this protein has been resolved using Synchroton X-ray analysis). The protein has a lethal effect on the insect by dissolving its gut. However, culturing bacteria is not an easy task and dropping tons of bacterial suspension on a field can significantly affect the microflora. As an alternative, the toxin could be 'inserted' into the plant itself so that feeding on the plants was still lethal but without the bacteria having to be sprayed onto these fields.

As the toxic protein came from a bacterium, the DNA sequence was rapidly analysed; however, there were several obstacles. Bacterial DNA is not normally expressed in plants since the right promoters, initiating the transcription of the DNA sequence into messenger RNA (mRNA) which is then translated into protein, are missing. Secondly, some plants do not transcribe (bacterial) sequences with a high A/T content.* Thirdly, a suitable terminator sequence needs to be found, signalling the end of the transcription sequence.

To express the Bt-toxin protein in plants a constitutive, strong promoter from the Cauliflower Mosaic Virus (CaMV), the P-35S, was linked to a modified Bt-toxin DNA sequence (cryIAb) with a higher G/C content followed by the nopaline synthase terminator (T-NOS) from *Agrobacterium tumefaciens*. However, this construct existed only in the laboratory and needed to be transferred into the plant to be integrated into the genome. Only this ensures that the offspring carries the same phenotype, i.e., the resistance to the cornborer by expressing the Bt toxin gene.

In the case of monokotyledonae† like maize, this is often done by shooting tiny gold particles coated with the DNA of interest at individual plant cells, using a high-pressure apparatus. The procedure is called ballistic transformation or microprojectile bombardment, the apparatus particle gun. Since the success rate of this procedure is limited, one needs to find a way of selecting the transformed cells. This is done by adding a selectable marker, in this case the ampicillin resistance gene (*bla* for β-lactamase), to the construct of interest (P-35S cryIAb T-NOS). After particle bombardment, the cells are grown on a medium containing ampicillin, and only those that carry the *bla* gene, and therefore the P-35S cryIAb T-NOS construct, will grow and are selected for the production of a transgenic variety.

* A/T = Adenine/Thymine, two of the four nucleotides used in the DNA, with Guanine/Cytosine (G/C) being the other two. A/T are paired by double hydrogen bonds while G/C are paired by triple hydrogen bonds.

† The number of cotyledons found in the embryo is the actual basis for distinguishing the two classes of angiosperms, and is the source of the names Monocotyledonae ('one cotyledon') and Dicotyledonae ('two cotyledons'). The cotyledons are the 'seed leaves' produced by the embryo. They serve to absorb nutrients packaged in the seed, until the seedling is able to produce its first true leaves and begin photosynthesis.

For dicotyledonae[†] like sugarbeets and potatoes, a different transformation method is typically used: the bacterium *Agrobacterium tumefaciens* itself is employed to carry the transgenic load into the plant. The pathogenic form of the bacterium naturally infects, transferring its own DNA into the plant and causing crown gall disease (CGD). The modified laboratory strain still transfers the DNA into the plant (in this case the transgenic construct) but without causing CGD. Here, as in the case of transgenic maize, the transformed cells are selected with a marker and then grown into fertile, transgenic plants.

19.2.1 Pros and cons of transgenic crops

Transgenic crops have certainly polarised opinions. While the European public, not least because of the campaigns of environmental pressure groups, became very concerned about transgenic crops, the American public, at least in the beginning, appeared to be comfortable with the idea of eating products containing transgenic material. Despite the marketing and educational campaigns of biotech companies, the public opinion did not change in Europe, and even in the US voices for labelling of transgenic crops are becoming louder. But what are the advantages and disadvantages of transgenic crops?

Clearly, from the point of view of the biotech companies, transgenic crops reduce the use of pesticides and insecticides on the fields and therefore contribute to less damage to the environment. Transgenic crops also provide a higher yield. Some can grow in arid parts of the world or adapt to other stress conditions that conventional crops cannot. According to the biotech industry, these characteristics can benefit poor nations and help to feed the world's population. However, 70% of all current field tests in Europe and the US relate to herbicide resistance rather than crop yield, according to a study released by the United Nations University Institute for New Technologies (UNU/INTECH).[3]

In the European Union and other countries, the public was concerned about transgenic crops for numerous reasons. Information about a complex topic like transgenic modification was limited or not understood. Food health and hygiene problems like bovine spongiform encephalopathy (BSE) and the death of several elderly people in Scotland as a consequence of food poisoning by *E. coli* created a climate of distrust. Therefore it was only natural that a new, poorly understood technology like genetic modification with perceived unpredictable impact on foodstuffs was rejected.

In addition to emotional reasons there were fears that the transgenic crop could escape into the wild, forming a kind of 'superweed', changing the face of nature completely. But there were also religious and ethical concerns about the use of transgenic crops. From today's perspective, without any long-term data on the influence of transgenic crops on our food production and the environment, it is difficult to judge either way. However, short-term data suggest that there is no adverse health effect to be expected from the consumption of transgenic food, nor any negative effect on the environment. But countries still have to take into account the anxieties and concerns of their

citizens. As a consequence, the European Commission introduced labelling regulations, not so much on the basis of health concerns but to allow the consumer to make an informed choice.

19.3 Labelling requirements

Labelling regulations for transgenic crops were first introduced in Europe. In 1998, the novel food regulation 259/98/EC required the labelling of novel foods if they are not 'substantially equivalent' to conventional foods in their composition, nutritional value, nutritional effects or the intended use of the food. From this regulation, the introduction of novel DNA or protein alone is not sufficient to require labelling of the food, especially if these proteins confer only herbicide tolerance or insect resistance. However, this labelling regulation applied only to foods which were brought to the market after the regulation came into force but not to foods which were already on the market. At the time, several food products already contained Bt 176 maize or Roundup Ready® (RR) soya that had normally fallen under this regulation. Therefore another regulation had to be generated for products containing those two transgenic varieties. While this was in preparation, several other varieties had been approved under the 258/97/EC, including Bt 11 and T25 maize.

The new regulation, specifically for Bt 176 and RR, was eventually passed in 1998 (1139/98/EC). It was now stipulated that a food was novel, and therefore subject to labelling, if either transgenic DNA or protein could be detected. This regulation did not give a threshold level and initiated a race for the lowest detection level between the laboratories being able to analyse for transgenic material. In addition, a labelling situation was created where food had to be labelled if transgenic maize of the variety Bt 176 could be detected (under 1139/98/EC) while labelling was not required if Bt 11 maize was detected (under 256/98/EC).

Industrial lobbyists complained about this extremely confusing situation and triggered the generation of two other regulations in 2000: the 49/2000/EC and the 50/2000/EC, of which one gave a threshold level while the other did not. The regulation 49/2000/EC, referring to the 1139/98/EC, specified that foods had to be labelled if the percentage of transgenic material in the ingredient exceeded 1%. This means that each individual ingredient of a composite food may contain 1% transgenic material per plant species. As an example, a food contains three ingredients: soya flour (50%), maize flour (30%) and wheat flour (20%). The soya flour contains 1% RR, the maize flour 1% Bt 176. In total, 100 g of this food product contain a total of 0.8 g transgenic material. This product does not need to be labelled as the percentage of transgenic material does not exceed a level of 1% per ingredient.

Another food, consisting of 95% maize flour and 5% soya (of which 2% are RR), despite the fact that 100 g of this product contains only 0.1 g of transgenic material requires labelling as the ingredient soya flour exceeds the threshold of

1%. Another issue is that 49/2000/EC relates to the 1139/98/EC which covers only RR soya and Bt 176 maize. So there is no regulation regarding how to deal with Bt 11 or any other variety which was notified under 258/97/EC in case the only difference is the new transgenic protein (or DNA). The second regulation, which entered into force at the same time as 49/2000/EC, was 50/2000/EC. Unlike 49/2000/EC, this regulation covered not only ingredients but additives, of which lecithin is a very important one. Unlike 49/2000/EC, this regulation does not set a threshold level, i.e., as soon as RR soya DNA is detected in the lecithin, the product requires labelling.

Clearly, these new regulations did not help to solve the confusion but rather contributed to it. Even the European Commission considered these regulations as reflecting 'lack of foresight' and 'piecemeal'. As a consequence, two new draft proposals have been submitted which are aiming to resolve the conflicts generated by the existing regulations on labelling of transgenic food. One proposal, COM 2001–425 final, was submitted to cover the labelling aspects for all approved transgenic material, the other, COM 2001–182 final, to deal with the traceability of transgenic products from farm to fork. The rules will improve the labelling and traceability of GMOs and products derived from GMOs, and will introduce a new procedure for regulating GM food and feed. Traceability entails the ability to follow products through the production and distribution chains. Under the new regulation, traceability is ensured by any person in the chain having to transmit and retain information that identifies, at each stage of the chain, from whom GM products have been received and to whom they have been made available.

In contrast to the labelling system currently in place, the proposal will require the labelling of all food produced from GMOs, irrespective of whether there is DNA or protein of GM origin in the final product. The proposal also introduces strict labelling requirements for GM feed. The current threshold level for labelling of 1% will be reduced in the new proposal to 0.9% for varieties approved for placing on the market in the EU, and to 0.5% for positively assessed but not yet approved varieties. For unassessed or unapproved varieties, the rejection threshold will remain at 0%.

While clear rules are set out in the EU for the assessment and authorisation of GMOs and GM food, the responsibilities are divided between Member States and the Community. It is therefore proposed to establish a 'one door, one key' procedure for the scientific assessment and authorisation of GMOs, GM feed and GM food, resulting in a centralised, clear and transparent Community procedure where an operator has to file only a single application. The scientific risk assessment will be carried out by the European Food Safety Authority.

On 29th November 2002 the EU Agricultural Council agreed on

- the obligation of manufacturers not to place products derived from or containing GM ingredients on the market unless authorised
- products to be labelled if the food is or contains a GMO
- the labelling requirement for products which are derived from or contain a GMO

- 0.9% threshold for labelling of products containing an EU approved GM material
- 0.5% threshold for labelling of EU-positively assessed GM material
- rejection of all unapproved or not (yet) positively assessed GM material (0% tolerance)
- labelling requirement of products derived from GM material, even if DNA or protein is no longer detectable (e.g., refined soybean oil).

On 10th December 2002, the EU Environmental Council agreed on

- the requirement of traceability of individual GMOs contained in food or feed
- the exemption of traceability for technically unavoidable adventitious contamination if the conditions for authorisation of the GM material are met.

19.4 Detection methods and traceability systems for GMOs

While, with the adoption of the new proposals, companies filing applications for placing on the market of transgenic organisms need to provide a robust detection method with its application, enforcement authorities and private testing laboratories currently have to rely on their own methods or on methods which have been ring-trialled and validated. Different methods are typically being used at different stages of the production chain. While at the farm level and in the grain elevators dip-stick ELISA tests (the same principle as a pregnancy test) are being used for reasons of speed and cost, these can no longer be used once the material is highly processed. This is due to the fact that the proteins tend to be too degraded in processed foods and are no longer recognised by the antibodies bound to the dip-sticks. Here, polymerase chain reaction (PCR) techniques are more applicable.

The principle of PCR is briefly described as copying a critical, unique DNA sequence. This technology can be used to identify species of animals or individuals (paternity analysis) as well as for the detection and identification of samples containing or consisting of genetically modified organisms (GMO). PCR, itself, however is only a qualitative technique. Since regulation 49/2000/EC states that labelling is required if more than 1% transgenic material is present, there was a need to transform the technology into a quantitative one. For a full quantitative analysis the so-called real-time PCR technology was developed.

19.4.1 ELISA

The ELISA tests (dip-stick and microtiter) are based on antibodies recognising their target protein. While the qualitative dip-stick assay can be used as a field test, the quantitative microtiter ELISA test is typically performed in a laboratory. However, ELISA tests are especially prone to cross-reactivity giving (false-)positive results for similar protein structures. Therefore these test systems

need to be thoroughly validated and the scope of the test specified. In the case of the Roundup Ready® microtiter ELISA, the validation was done in an interlaboratory study, organised by the European Commission Joint Research Center.[4] Other microtiter ELISA systems (MON810, StarLink) were later evaluated by the American Association of Cereal Chemists (AACC). Currently, there are a range of validated ELISA systems available which are predominantly used on unprocessed materials where time is the critical factor.

19.4.2 PCR

Since ELISA methods allow only the detecting of GMO protein in raw and lightly processed products, the methodological focus was clearly on PCR-based assays. As the first collaborative PCR study, the JRC organised in 1998 a ringtrial[5] for a so-called screening method for transgenic plants, detecting the 35S promoter from Cauliflower Mosaic virus and the NOS terminator from *Agrobacterium tumefaciens*, both of which sequences do not naturally occur in the plants' genome. Twenty-nine laboratories in 13 countries took part in this trial. It showed that detection was clearly possible at the 2% level for both elements in maize and soybeans but these screening methods had a significant drawback. Several cases of 'false-positive' detections were discovered which were due to the fact that the promoter and terminator sequences are often similar even across species, and if PCR conditions are not stringent enough, endogenous sequences from maize and soya were amplified with very similar amplicon sizes.[6] Despite this source of error, standard PCR detection of the 35S promoter and the NOS terminator are still very common in many laboratories.

Later ringtrials focused on the detection of specific elements like CP4-EPSPS, the gene coding for the bacterial enzyme which allows the plant survival when sprayed with the herbicide Roundup Ready®, or cryIA, the gene coding for the insecticidal *Bacillus thuringiensis* toxin, which is lethal for the cornborer, a common maize pest. However, all of these ringtrials were only qualitative but legislation 49/2000/EC introduced a labelling threshold of 1% for GMOs. Since protein detection by ELISA does not work in most highly processed foods and (standard) PCR is only qualitative, an alternative method had to be found. This method is called real-time PCR.

Apart from two primers which are required for the standard PCR, a third element that recognises the sequence between the primers is required, the so-called probe. The probe itself is fluorescently labelled and a signal is produced each time another copy of the amplicon is made during the PCR process. In the early phase of the PCR, the amplification is linear logarithmic and therefore allows calculation of the starting quantity of material by measuring the produced fluorescence. Using this procedure it is possible to determine the percentage of transgenic material relative to a plant species. This procedure carries a higher cost as the fluorescent probe is comparatively expensive. Nevertheless, laboratories providing high quality to their customers already apply this procedure for the qualitative analysis for higher specificity, reducing the risk of

false-positive results, and controlled sensitivity, reducing the risk of false-negative results.

Real-time PCR methods have also been validated on an international scale by the Federal Institute for Consumer Health and Veterinary Medicine (BgVV[‡]) in Germany. Three different real-time PCR machines were evaluated, using the same amplicon system to detect five different concentrations of Roundup Ready® soybean flour (0%–5%) and one sample of textured vegetable protein (TVP), the latter containing 2% transgenic material. It showed that the ABI Prism 7700 performed best in this particular validation study, having the lowest relative standard variation (RSD) in most cases. Clearly, ringtrials say very little about the performance of a particular laboratory. They only provide information on how a laboratory uses this one method. Often, laboratories have developed their own methods which are not externally validated to maintain a commercial advantage but some still outperform methods validated in a ringtrial. As one example, Eurofins Scientific developed a series of assays named GMO PLATINUM ASSAY in 2000, using advanced chemistry which improved – compared to existing technologies – specificity, robustness and sensitivity. Although these assays have been successfully validated between the three molecular biological laboratories of the group, and, more importantly, in numerous proficiency tests, details of the assay could not be disclosed to maintain commercial advantage.

To allow any kind of assay to compare with other methods, performance assessment schemes (also called 'proficiency tests' or 'PT') for GMO were set up by several institutions and organisations. The first scheme was fully funded by the UK Food Standards Agency (FSA) and organised by the Institute of Food Research (IFR) in Norwich, UK. This scheme showed that there were significant differences between the participating labs in terms of precision and accuracy. These differences became smaller over time but still remained significant.

In 2000, the Central Science Laboratory in York, UK, added GMO analysis to its already existing Food Analysis Performance Assessment Scheme (FAPAS®) scheme. The GMO scheme later acquired its own name, GeMMA, for Genetically Modified Material Analysis. This scheme was quite similar to the FSA scheme but participants had to pay for each round of tests. In mid-2002, GeMMA had run its 11th round with 54 laboratories participating worldwide. Any quantitative value produced by a laboratory gets a z-score associated with the result. The z-score is an indication of laboratory performance. The closer the z-score is to zero, the better the laboratory performs. Typically a z-score between −2 and +2 is considered satisfactory.

These performance assessment schemes are a pivotal quality-control tool to allow a client to make an assessment of the laboratory before submitting samples. Other quality control tools are laboratory accreditation schemes (e.g., ISO 17025). The reason these tools are so crucial is the fact that PCR analysis is

[‡] BgVV: Bundesamt für gesundheitlichen Verbraucherschutz und Veterinärmedizin.

often error prone and only well-trained and experienced laboratory staff can handle this procedure properly. Due to the high risk of cross-contamination, it is mandatory to separate pre-PCR, PCR and post-PCR processes. This requires substantial laboratory space which may not always be available. Other criteria are equally important: dedicated pipettes for each process, filter tips to prevent contamination from aerosols, UV light for decontamination. Only if all these aspects are taken into account and the laboratories obtain good proficiency testing results on a regular basis, showing they have thoroughly validated their methods, can it be assumed that their results are reliable and reproducible.[7]

19.4.3 Traceability of transgenic crops

With the new regulations expected to come into force in the not too distant future, traceability of transgenic crops is required. This will likely be a combination of paper-based audit trail and analytical tests at various points of the food production scheme. While the seed company typically performs seed-quality analysis in-house by PCR, the farmer will use dip-stick ELISA. After harvest, the products are transported from the farm to the grain elevator where dip-sticks are also used. From here, the commodity is shipped to the processors, e.g., ingredient producers. They typically have the incoming goods tested by ELISA or real-time PCR using a contract laboratory. From the ingredient producer, products are shipped to the food processor, who also has the incoming goods tested by a contract laboratory, using real-time PCR. The retailer displaying the finished products on the shelves also has the products tested to comply with the due diligence requirements.

From stage to stage, all products will be accompanied by appropriate documentation to certify their origin. As there is a significant probability to have cross-contamination if the same elevators, ships, trucks, barges and production lines are used for transgenic and non-transgenic material, the favoured option is the vertical product chain where transport and production lines for transgenic and non-transgenic materials are separated and a complete audit trail including analytical test points is available.

19.5 Future trends

There are certainly various scenarios for the future of transgenic crops. It is highly unlikely that transgenic crops will completely disappear as a consequence of consumer concern and green-rights activist campaigns. Currently, a great number of countries require labelling of transgenic crops, and a non-transgenic crop is a premium product. But as farmers learn to appreciate the benefits of transgenic crops, the area where transgenic crops have been grown has increased steadily over the last five years from 1.7 million hectares in 1996 to over 50 million hectares in 2001. It is expected that transgenic crops will dominate conventional crops in a few decades. But the transformed traits are changing

from the so-called input traits (insect resistance, herbicide tolerance) to output traits (enhanced levels of provitamin A, elevated levels of beneficial metabolites, reduced levels of unwanted substances).

These products will also require segregation from the main commodity lines and labelling but for a different reason. As some of the products in the pipeline are beneficial to health (e.g., high oleic acid levels), they might gain greater acceptance with the general public compared to the input traits and can be sold at a premium price. Once transgenic products that increase life-expectancy are on the market, there is little doubt about their acceptance. Another, less controversial application of transgenic crops is for mass production of proteins, e.g., antibodies for medical purposes. This field of 'green/red' biotechnology will certainly gain significantly more importance over the next few years.

From what we know today, transgenic crops are here to stay and can potentially bring significant benefits to the agricultural industry as well as to the consumer. But there are too few long-term data available to allow a prognosis on the environmental impact or the long-term effects on human health. Until we can be certain about this, there will be a need to trace and detect transgenic material.

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20

Wine authenticity

I. S. Arvanitoyannis, University of Thessaly, Greece

20.1 Introduction: traditional and novel methods for testing wine authenticity

Authenticity of foods and, in particular, of wine has been extensively investigated because wine is an easily adulterated product due to its strong chemical basis (high alcohol content, low pH) and its availability throughout the world (Medina, 1996). Meticulous and continuous controls are required to maintain the quality of wine. Authenticity is guaranteed by strict guidelines laid down by the responsible national authorities (e.g., Institut National des Appelations d'Origine in France) which include official sensory evaluation, chemical analyses, and examination of the register kept by the wine producer. Wine mobility in bulk containers within the European Community is also carefully controlled requiring transport documents which certify authenticity as defined by the EC directive 986/89. There is currently a great range of combined techniques employing group classification to identify wine authenticity. The introduction of new sophisticated techniques in conjunction with great consumer demands and expectation for safer products gives a tremendous impetus to food quality assurance.

Wine adulteration, mainly in terms of varieties and regions of origin (geographical) has been very widespread (Arvanitoyannis *et al.*, 1999). Therefore, apart from novel experimental techniques (GC-MS, ICP-MS, ^{13}C NMR, FT-IR and DNA among others) the need has emerged for a more comprehensive statistical data analysis. Mutivariate analysis comprising principal component analysis (PCA), discriminant analysis (DA), canonical analysis (CA), cluster analysis (CLA), has, in most cases, been effectively employed in wine differentiation and classification according to geographical origin (Arvanitoyannis *et al.*, 1999).

20.1.1 Traditional and novel methods for testing wine authenticity

Various methods have been developed over the years aimed at detecting wine authenticity. The classification of methods into old and novel or 'state of the art' is usually made for grouping purposes. One could classify as old methods those employed prior to the advent of the new-generation methods.

The former are determination of total soluble solids with hydrometry and/or refractometry, High Performance Liquid Chromatography (HPLC) of glycerol and ethanol, determination of alcohol with ebulliometric analysis, hydrometry, gas chromatography (GC) and enzymatic analysis (employment of nicotinamide-adenine dinucleotide (NAD)), determination of organic acid content by measuring the titratable acidity (titration of acids (tartaric, malic, citric, sulphuric, lactic, acetic) with base) and spectrophotometrically (UV region), determination of volatile acidity with steam distillation and titration or enzymatically (monitoring changes in the NADH concentration), determination of reducing sugars by chemical reaction of reducing sugars with copper (Joslyn, 1950), enzymatic method (McCloskey, 1978) and determination of phenolics with Folin-Ciocalteau method (Zoecklein *et al.*, 1990).

The latter could comprise the determination of the various phenolic compounds (non-flavonoid, volatile phenols, flavonoids, catechins, leucoanthocyanidins, flavonols, tannins, anthocyanins/anthocyanidins) with HPLC in conjunction with colour determination, determination of minerals (iron, copper, lead, antimony, magnesium, calcium, manganese, zinc, etc.) with Atomic Absorption Spectrophotometry (AAS) or ICP-MS (Zoecklein *et al.*, 1990). The employment of Fourier Transform Infra-Red (FT-IR) spectroscopy and Nuclear Magnetic Resonance (NMR) stand for two more recently applied methods for determination of geographical origin and variety, respectively. Furthermore, DNA methods were also employed toward authentication of varietal wines.

In an attempt to develop a technique for the identification of grape cultivars in commercial wines, a method for the extraction of DNA from must and experimental wines was adopted and optimal PCR conditions for the amplification of this DNA were established. DNA was analysed during the fermentation process for six cultivars. Expected profiles from these cultivars were obtained with DNA extracted from the solid parts during the fermentation process (Siret *et al.*, 2000). Capillary Zone Electrophoresis (CZE) was recently successfully applied to wines from the Canary Islands (Spain) in order to differentiate them (Pazourek *et al.*, 2000).

20.2 Analysis of minerals and trans-resveratrol

The analysis of minerals, and in particular Na, K, Ca, Mg, Mn, Li, Fe, Cu, Pb, has been extensively employed as one of the most promising methods either on its own or in conjunction with other methods for detection of wine authenticity (variety, geographical origin) (Kallithraka *et al.*, 2001a; Moret *et al.*, 1994;

Gomez-Plaza *et al.*, 2000; Galani-Nikolakaki *et al.*, 2000; Frias *et al.*, 2003; Baxter *et al.*, 1997). The majority of the studies were carried out in the Mediterranean countries (Spain, France, Italy and Greece) because of their great interest in wine authenticity due to their own production.

Latorre *et al.* (1994) used pattern recognition analysis (employing Li and Rb as key minerals) for differentiating successfully 41 wines from northwest Spain (between Rias-Baixas and non-Rias Baixas). Stroh *et al.* (1994) also reported the importance of lanthanides in determining the geographical origin of wines. Multi-element analysis of 112 Spanish and English wines with inductively coupled plasma mass spectroscopy (ICP-MS) unequivocally identified the region of origin of Spanish wines from three different regions. Complete differentiation (100%) of English and Spanish white wines also occurred whereas red and rosé wines were distinguished with 95% accuracy (Baxter *et al.*, 1997).

Frias *et al.* (2003) conducted with 100% sensitivity and specificity the classification of commercial wines from three Canary Islands (Spain) by determining their mineral contents with AAS and flame emission. Pena *et al.* (1999) studied 39 red wines from Galicia (NW Spain) in terms of their trace metal composition. An acceptable level of differentiation and classification of wine samples of *Ribeira sacra* and non-*Ribeira sacra* origin was found by applying several multidimensional techniques. Employment of the key metals (Li and Fe) resulted in a satisfactory level of correct classification between the two wine groups without, however, being able to exclude entirely the possibility of an incorrect classification. Employment of pattern recognition analysis with a greater number of attributes on the same 39 wines led to more accurate determination of origin (Rebolo *et al.*, 2000). Kallithraka *et al.* (2001a) showed that the mineral content of 33 Greek red and white wines varies substantially with their origin and can be effectively employed as a reliable indicator for differentiation of wines from various regions (north Greece, south Greece and the islands).

A comparison of levels of K (ppm) among wines from Greece, Spain and France showed that Greek and Spanish wines had almost similar levels of potassium (variation range ~4%) whereas the French wines contained substantially greater amounts of K (20–100% greater values). It is noteworthy that the highest K values were obtained when measurements were taken on the pressed grapes (Day *et al.*, 1994). Since K levels greatly depend on anthropological activity, the latter were found to gradually decrease, as already shown by other researchers (Table 20.1), whereas the levels for other minerals may increase or decrease depending on the particular kind of treatment and equipment employed (Day *et al.*, 1994; Latorre *et al.*, 1994).

The Na content of Greek wines was twice as high as the levels of Spanish and French wines. The Mg level of Greek wines was also found to be three times higher than that for French wines and can be successfully employed as another promising feature for differentiation. In the case of Fe, the Greek wines equally contained greater levels than the other wines but this can hardly be considered an advantage since it is well known that high levels of Fe and Cu can result in

Table 20.1 Mineral levels (mg/l) of red and white wines of various origins

		Minerals												
Red wine samples	Country of origin	Na	K	Ca	Li	Mg	Mn	Fe	Cu	Cd	Zn	Pb	Method	References
30	Spain	32.5±1.5	808±16	58±3.9	–	–	–	1.7±0.1	–	–	–	–	AAS	Gomez-Plaza *et al.*, 2000
9	Spain	–	–	–	–	–	–	–	130±9	1.5±0.4	280±15	75±6	Polarograph	Arcos *et al.*, 1993
45	Spain	120±25	880±130	82±3.8	14.6±1.7	87±9.5	0.72±0.06	3.1±0.3	0.29±0.02	–	0.44±0.06	–	AAS	Frias *et al.*, 2003
39	Spain	21±14	1075±200	–	35±11	–	2.4±0.2	3.3±0.2	–	–	0.27±0.06	–	AAS	Pena *et al.*, 1999, Rebolo *et al.*, 2000
3	France	–	–	–	32.5	–	–	7768	210	–	569.8	–	ICP-MS	Baxter *et al.*, 1997
21	Greece	63±12	838±20	1487±35	–	174±8	1.76±11	16.4±0.7	1.0±0.1	–	1.82±0.13	–	ICP	Kallithraka *et al.*, 2001a, b
30	Greece	–	–	–	–	–	1.15	7.5	0.4	0.003	1.5	0.23	AAS	Galani-Nikolopoulou *et al.*, 2002
25	France	–	1532	65.2	–	63.3	0.44	1.6	1.06	–	0.77	–	AAS	Day *et al.*, 1994
34	France	20.5	1253	76.7	–	53.3	0.62	–	–	–	–	–	–	Etievant *et al.*, 1988a, b
White wine samples														
59	Italy	25±0.5	7400±	97±21	13±3	–	–	–	–	–	–	–	Flame emission AAS	Moret *et al.*, 1994 1994
42	Spain	40±3.7	810±37	95±8.2	24±4.3	–	–	–	–	–	–	–	AAS	Latorre *et al.*, 1994
11	Greece	37.2±1.5	560±24	185±9	–	99.6±2.4	1.48±0.2	4.7±0.3	0.66±0.1	–	1.38±0.1	–	ICP	Kallithraka *et al.*, 2001a, b
35	Hungary	–	–	–	–	–	2.3	20.5	0.35	–	1.45	–	–	Muranyl and Papp, 1998

iron and copper casse (hazy and cloudy wines). Table 20.1 summarises all the studies reported in the literature about the determination of minerals in wine providing information regarding the method used, their content and country of origin.

20.2.1 Analysis of trans-resveratrol

Although trans-resveratrol is a constituent of many plant species, grapes and related products are their most important source. The therapeutic effect (beneficial action against atherosclerosis and coronary heart disease) of trans-resveratrol in conjunction with the high publication record are the main reasons for presenting this compound separately from the rest of the phenolic compounds.

A rapid and sensitive method was developed for the determination of this compound in wines. Trans-resveratrol was determined for 29 red Greek wines of appellation of origin. The concentrations found varied considerably (0.550 and 2.534 mg/l) and it was possible to classify the wines into five groups by employing both CLA and PCA (Kallithraka *et al.*, 2001b).

Trans-resveratrol was determined with Overpressured Layer Chromatography (OPLC) in 25 different Hungarian wines from the same wine-making region harvested in 1998. Although cluster analysis failed to provide clear-cut differentiation between white and red wines on the basis of their trans-resveratrol content, this was made possible with PCA. Implementation of PCA led to the formation of two distinct groups; perfect separation of red and white wines (Csomos *et al.*, 2002).

Capillary electrophoresis (CE) has been used by several researchers to determine the level of trans- and cis-resveratrol in wine samples with good sensitivity, speed and reproducibility. Most of the CE methods for measuring resveratrol in wine were able to reliably detect resveratrol at 0.2–1.0 μM levels. CE was shown to be effectively combined with Solid Phase Extraction (SPE) and/or Micellar Electrokinetic Chromatography (MEKC) for improved analysis of flavonoid compounds in wine (Gu *et al.*, 2000).

Pinto *et al.* (1999) showed that both resveratrol and its oxidised form obtained with hydroperoxidase activity are effective inhibitors of lipoxygenase activity. The fact that the maximun rate of resveratrol oxidation was obtained at a concentration of inhibitor at which dioxygenase activity was abolished, suggests that the dioxygenase and hydroxyperoxidase activities of lipoxygenase are independent.

Resveratrol levels reported in red US wines are below 1 mg/l (Lamuela-Raventos and Waterhouse, 1993; Seiman and Creasy, 1992) and much higher in Italian, French and Spanish wines (Jeandet *et al.*, 1993; Mattivi, 1993; Lamuela-Raventos *et al.*, 1995).

Goldberg and his coworkers (1994) analysed more than 1000 wines finding very little trans-resveratrol (typically < 0.1 mg/L) in white wines, whereas red wines had concentrations ranging from 0.1 to 12.0 mg/L with lowest

concentrations in wines from California, Australia and Italy and highest in wines from Oregon, Canada and from various regions of France.

Threlfall *et al.* (1999) studied the effects of grape variety, UV light exposure, enzyme addition, skin contact time and the fining agents, carbon and PVPP, on resveratrol levels of US wines. Enzyme addition was shown to increase resveratrol level in some wines. Skin contact time influenced the extraction of resveratrol from the skin in the red varieties whereas carbon addition decreased the resveratrol level. The addition of PVPP decreased substantially the resveratrol level of wine.

20.3 Analysis of phenols, volatiles and amino acids

20.3.1 Phenols

Epidemiological evidence shows that increased levels of fruit and vegetables in the diet reduce the risk of cancer and heart disease as well as cataracts, brain and immune dysfunction and stroke. Since phenolics are practically ubiquitous in plant material and may occur at high levels, it is likely that these compounds will play a major role in determining the antioxidant potential of foodstuffs (Steinmetz and Potter, 1991; Block *et al.*, 1992; Hertog *et al.*, 1995; Vinson *et al.*, 1998). Phenolics are important to wine and grape juice because they contribute to colour, flavour, oxidation and other reactions. The popularity of phenolics should mainly be attributed to their antioxidant properties which could be summarised as follows: metal-ion chelating action, hydrogen donation/radical scavenging, inhibition of radical generation, anticarcinogenic action, enzyme inhibition and specific receptor interactions (e.g., oestrogen receptors) (Parr and Bolwell, 2000). Three of the principal factors affecting the phenolic content of wines are the phenolic composition of the grape, the procedure used to make wine and reactions that take place during ageing (Blanco *et al.*, 1998). An overview of the phenolic compounds determined is given in Table 20.2.

The most widely employed method for phenol determination is HPLC analysis. Apart from HPLC, the solid phase extraction (SPE) approach prior to CE analysis was used by several investigators (Gu *et al.*, 1999) and resulted in cleaner and more concentrated samples. In addition to concentration, the procedure facilitated the identification of antioxidants by altering sample conditions and removing interfering materials. In general, the limit of detection for resveratrol determination in wine was lowered ten-fold with good recoveries (95–102%) using SPE. The capillary zone electrophoresis (CZE) method was shown to separate epicatechin, catechin, quercetin, myricetin, rutin, gentistic acid, caffeic acid, gallic acid and trans-resveratrol (Gu *et al.*, 2000).

Goldberg *et al.* (1999) determined the concentrations of selected phenols (catechin, epicatechin, quercetin, rutin, cis- and trans-resveratrol, and p-coumaric acid) in white wines of various countries with the aid of an HPLC method with a diode array detection. They found that climatic factors appearing to modulate polyphenol concentrations in red wines such as stress, fungal

Table 20.2 Phenolic contents (mg/l) of red wines from various countries

Country	Total phenols	Catechin	Epicatechin	Myricetin	Quercetin	Caffeic acid	Ferulic acid	References
Greece	1514	16.7	7.1	6.2	25.7	62.8	3.9	Kallithraka *et al.*, 2001a, b
Yugoslavia	–	94.8	53.2	–	–	–	–	Kovac *et al.*, 1992
Canada	–	72.2	38.8	–	2.0	5.5	1.7	Soleas *et al.*, 1997
Canada	1200	240	82.0	–	18.5	–	–	Goldberg and Soleas, 1999
Spain	–	–	45.5	–	16.0	–	–	Bonilla *et al.*, 1999
Spain	–	–	1.2	–	–	0.4	0.12	De la Presa-Owens and Noble, 1995
USA	2700	230	68	7.2	7.5	6.4	–	Frankel *et al.*, 1995
USA	–	80	31	–	2.2	–	–	Goldberg *et al.*, 1996
USA	–	152	–	59	7.3	–	–	Waterhouse *et al.*, 1999
USA	1000	32.6	40.3	–	–	–	–	Blanco *et al.*, 1998
Italy	–	–	–	–	–	2200	37	Cappiello *et al.*, 1999
Italy	–	160	–	–	8.1	–	38	Goldberg *et al.*, 1996
Italy	–	37	23	–	8.0	–	–	Goldberg *et al.*, 1996
France	–	67.3	31.3	–	3.4	–	–	Goldberg *et al.*, 1996
Australia	–	39.8	34.1	–	8.2	–	–	Goldberg *et al.*, 1996

pressure and sunlight do not seem to be important for white wines. The intrinsic properties of the individual cultivars or clones together with regional differences in enological practices may be the most important factors.

Phenolic composition of 92 wine vinegars coming from different wines from the south of Spain was determined with HPLC. Phenolic content was shown to effectively classify and predict the membership of samples according to employed treatment method or geographical origin of substrate wine (Garcia-Parilla *et al.*, 1997). Teissendre and Landrault (2000) analysed the catechins and procyanidins in an attempt to show their effectiveness *in vitro* as powerful inhibitors of LDL oxidation and platelet aggregation. It was found that phenolic intake is ten times higher from red wines (400.2 mg/person/day) than for white wines (44.1 mg/person/day). Catechin monomers can represent 40% of total catechins. Moreover, it was shown that the consumption of wine by humans leads to an increase in the antioxidant capacity of plasma.

Liquid Chromatography-Mass Spectroscopy (LC-MS) with capillary scale particle beam interface was used to detect 18 phenolic compounds in Italian red wine samples. This technique allowed reproducible, library searchable electron ionisation spectra at only 1 μl/min. mobile phase flow-rate for a sensitive detection of the analytes in complex matrices. The method makes use of a narrow-bore, reversed-phase packed capillary column for sample separation. Detection limits were in the low picogram range for most compounds. Sensitivity and response linearity were evaluated for eight phenolic acids, which are often encountered in red wines. The phenolic compound composition was outlined in two red wines obtained using different ageing processes (Cappiello *et al.*, 1999).

Pellegrini *et al.* (2000) determined the total phenol content of eight commercial Italian *vini novelli* (young red wines) from different geographical origins with regard to their antioxidant activity. The average flavanol content (424.7 ± 121.3 mg/L catechin equivalents) and the total antioxidant activity (16.8 ± 3.8 mmol/L Trolox equivalents) of *vini novelli* were higher than the corresponding values (382.7 ± 174.5 mg/L catechin equivalents) and 12.3 ± 3.3 mmol/L Trolox equivalents found for aged wine. This experiment revealed that ageing and not wine-making technique is the main factor influencing the antioxidant activity of red wines.

Sato *et al.* (1996) analysed 31 wine samples differing in their origin for production and vintages for total phenolic content. The polyphenol content of red wine ranged from 735.9 to 2858 mg/L and that of white wine was in the range 259.4–720.5 mg/L.

20.3.2 Volatiles

Wine is a hydroalcoholic solution containing hundreds of compounds that come from grapes or result during wine-making and storage. Several of these compounds affect wine aroma which, besides being a parameter of quality, act as a 'fingerprint' for each wine variety. The sulphur compounds occurring in

wines are classified in five different families according to their structure: thiols, sulphides, polysulphides, thioesters and heterocyclic compounds. The presence of most of them imparts an unpleasant odour to wine (Mestres *et al.*, 2000).

Fifty-two young monovarietal Spanish red wines were analysed with HRGC-MS to obtain quantitative data on 47 odorants previously identified as potential aroma contributors by olfactometric techniques. Thirty-three odorants were present in the wines at concentrations higher than their corresponding odour thresholds. These included ethyl-octanoate, β-damascenone, ethyl hexanoate, isovaleric acid and isoamyl acetate as the most important, which together with isoamyl and β-phenylethyl alcohols, fatty acids, 2,3-butanedione and ethyl butyrate are always found at concentrations higher than their odour thresholds. In some cases the ethyl esters of isobutyric and isovaleric acids, β-ionone, methionol, isobutyric acid, ethyl cinnamate, ethyl dihydrocinnamate, γ-nonalactone, eugenol, c-3-hexanol, geraniol, guaiacol, 3-isobutyl-2-methoxy-pyrazine, 4-ethylguaiacol, acetoin and t-whiskylactone were at concentrations high enough to be odour-active (Ferreira *et al.*, 2000).

Rosillo *et al.* (1999) used a dynamic head space analysis with GC-MS for determining the volatiles in grapes and classifying some *Vitis vinifera* varieties. This method permits the analysis of the volatile fraction of a wine by purging with an inert gas followed by thermal desorption and gas chromatography. Application of cluster analysis to the volatiles resulted in three groups, one for white grapes, one for Monastrell, Tempranillo and Cabernet Sauvignon, and the other for Dyer Grenache. Hexyl acetate, benzyl alcohol, phenylethyl alcohol and benzaldehyde were the four discriminant variables for group differentiation.

Analysis of 41 volatile compounds carried out with GC-MS on 60 white Spanish wines of three varieties, different wineries and vintage year, in conjunction with PCA, revealed that some higher alcohols can help the separation of wines according to winery and vintage year (de la Presa-Owens *et al.*, 1995b). Forty-four odour active compounds were quantified in Scheurebe and Gewurtztraminer wines. Calculation of odour activity of odorants showed that differences in odour profiles of both varieties were caused by cis-rose oxide in Gewurtztraminer wine and by 4-mercapto-4-methylpeptan-2-one in Scheurebe wine. These compounds are suitable indicators for the determination of flavour differences, and can lead to wine authentication (Guth, 1997).

Chatonnet *et al.* (1992) studied the ethylphenols which stand for important aromatic compounds of red wines. It was shown that these volatile phenols are responsible for the 'phenolic', 'animal' and 'stable' off-odours found in certain red wines. The results reported show that the synthesis of high quantities of ethylphenols found in the 'phenolic' red wines can occur during the ageing of wines having normally completed their alcoholic and malo-lactic fermentations.

Simulant wines containing aminoacids, sugar, water, and yeast nutrients were fermented by *Saccharomyces cerevisiae* and the volatile composition of the fermented media was analysed with GC. Significant differences were found in the levels of some important volatile compounds (ethanol, ethyl acetate, acetic acid, higher alcohols) and some of their acetates (methionol, isobutyric acid,

ethyl butyrate and hexanoic and octanoic acids). The levels of some of the volatiles were well correlated with the aromatic composition of wines made with grapes of the same varieties (Hernandes-Orte *et al.*, 2002).

2,6,6-Trimethylcyclohex-2-ene-1,4-dione (TMCHD, a norisoprenoid) and diacetyl (caramel descriptors) was reported for the first time in fortified wines from the Douro demarcated area of Portugal. Olfactory Gas Chromatography (GC-O) of a volatile wine extract, previously isolated with preparative gas chromatography, indicated the presence of a zone containing an intense honey descriptor. The targeted odour compound was identified by GC-MS, GC-O and Kovats index. Quantitative analysis using a selected characteristic ion (*m/z* 96) indicated that young Douro fortified wines from the 1997 vintage contained up to 4 μg/L TMCHD. The wine volatile diacetyl was identified as a strong contributor to the sweet caramel aroma descriptor often associated with port (Rogerson *et al.*, 2001).

Kotseridis *et al.* (2000) made an attempt to detect and identify the potent odorants with the caramel odour of Merlot and Cabernet Sauvignon wines with Gas Chromatography/Olfactometry. Two odorant zones with this odour resulted in identification of 4-hydroxy-2,5-dimethylfuran-3(2H)-one (HDMF) and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methylfuran-3(2H)-one (HEMF). Aroma extraction dilution analysis (AEDA) method showed a higher dilution factor (FD) for HDMF in the Merlot wine extract than in the Cabernet Sauvignon extract. Conversely, HEMF was found to produce the caramel perception in the Cabernet Sauvignon wines (as well as in the Merlot wines), but not to differentiate the Merlot from the Cabernet Sauvignon wines.

Free and potential aromas of eight Spanish varieties of grapes were established using a single extraction. The potential aroma was determined with two procedures: direct analysis of glycosides and indirect analysis of glycons released by hydrolysis. Non-terpenyl compounds (alcohols and aldehydes of six carbon atoms) were the most abundant aroma substances in the considered Spanish grapes. Moreover, aroma components were quantitatively affected by the maturation index. Differences due to varietal and climate characteristics were clearly shown (Lopez-Tamames *et al.*, 1997).

Seeber *et al.* (1991) analysed musts and wines from 31 growing areas in a rather small geographical region of Italy. Musts were analysed with amino acid analyser and the results obtained were treated with multivariate statistics. Close relationships emerged among the volatiles and some of the amino acids determined. Table 20.3 provides an overview of the analysis of volatiles (methodology employed, compounds detected) of wines and distillates.

20.3.3 Amino acids

Free amino acids can characterise grape varieties when studied along with other variables in multivariate analysis (de la Presa-Owens, 1995a). Etievant *et al.* (1988b) showed that the amino acid of grapes is dependent upon the fertilisation and climatic conditions and on duration of skin maceration in the must.

Table 20.3 Analysis of volatiles of wines/distillates

Country	Number of samples/ varieties	Method	Volatiles determined	Reference
Spain	Many (Review)	Solid phase microextraction (SPME), Dynamic Headspace	thiols, sulphides, polysulphides, thioesters and heterocyclic compounds	Mestres *et al*., 2000
Spain	52/4	GC-MS	ethyl-octanoate, β-damascenone, ethyl hexanoate, isovaleric acid and isoamyl acetate as the most important, which together with isoamyl and β-phenylethyl alcohols, fatty acids, 2,3-butanedione and ethyl butyrate, ethyl esters of isobutyric and isovaleric acids, β-ionone, methionol, isobutyric acid, ethyl cinnamate, ethyl dihydrocinnamate, γ-nonalactone, eugenol, c-3-hexanol, geraniol, guaiacol, 3-isobutyl-2-methoxy-pyrazine, 4-ethylguaiacol, acetoinand t-whiskylactone	Ferreira *et al*., 2000
Spain	NA/4	Dynamic Headspace GC-MS	hexanol, 3-hexen-1-ol, (trans) 2-hexenal, linalool, geraniol, benzyl alcohol, phenylethyl alcohol, 1-pentanol, 1-heptanol, 1-octanol, 1-octen-3-ol, ethyl hexanoate, hexyl acetate, ethyl heptanoate, ethyl decanoate, ethyl dodecanoate, isobutyric acid, hexanoic acid, heptanoic acid, octanoic acid, benzaldehyde, decanal, limonene	Rosillo *et al*., 1999
Spain	60/3	GC-MS	ethyl propionate, isobutyl acetate, ethyl butyrate, isoamyl acetate, isoamyl acetate, butanol, limonene, ethyl hexanoate, hexyl acetate, isoamyl isovalerate, cis-3-hexen-1-ol acetate, 3-methyl-1-propanol, ethyl lactate, hexanol, 3-ethoxy-1-propanol, cis-3-hexen-1-ol, trans-2-hexen-ol, ethyl octanoate, cis-furan linalool oxide, octyl acetate, 2,3-butanediol, butyrolactone, ethyl decanoate, isoamyl decanoate, isoamyl octanoate, diethyl succinate, α-terpinol, isoamyl butyrate, linalool, octanol, nerol, citronellol, ethyl dodecanoate, ethyl mirystate, 2-pentanol, geraniol, benzaldehyde, hexanoic acid	De la Presa-Owens and Noble, 1995

France	NA	GC-MS	4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol, 4-vinylguaiacol	Chatonnet *et al*., 1992
Korea	23 (whiskey and brandy)	Capillary column GC-MS	lactic acid, glycoloic acid, oxalic acid, malonic acid, capric acid, succinic acid, lauric acid, myristic acid, malic acid, palmitic acid, tartaric acid, stearic acid	Park *et al*., 1999
Spain	Six lots per must	GC	ethanol, ethyl acetate, ethyl propionate, isobutyl acetate, ethyl butyrate, isobutanol, isoamyl acetate, acetic acid, propanoic acidisobutyric diethyl succinae, methionol, hexanoic acid,	Hernandes-Orte *et al*., 2002
Portugal	19/1	GC	2,6,6-trimethylcyclohex-2-ene-1,4-dione, diacetyl (caramel descriptors)	Rogerson *et al*., 2001
France	9/2	GC/ Olfactometry GC/MS	4-hydroxy-2.5-dimethylfuran-3(2H)-one (HDMF) and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methylfuran-3(2H)-one (HEMF)	Kotseridis *et al*., 2000
Spain	32/8 (grape cultivars)	GC-MS	35 free volatile compounds and 36 bound compounds released by enzyme hydrolysis and glycoside form	Lopez-Tamames *et al*., 1997
Spain	32/2 regions	GC (SPI)-MS	40 volatile compounds were identified and quantitatively determined	Garcia-Jares *et al*., 1995

Amino acids were very effectively employed for separating champagnes from sparkling wines in a study of 110 wines. Champagnes are richer than sparkling wines in all amino acids, except arginine, because of the second fermentation in the bottle and long contact with lees (Tusseau *et al.*, 1996).

Palma *et al.* (1995) analysed the amino acid composition (proline, hydroxyproline, arginine, ornithine, alanine, serine, glycine, valine, leucine, asparagine, threonine, isoleucine, methionine, lysine, tyrosine, phenylalanine, histidine, ethanolamine) of 34 French red wines. However, only proline, hydroxyproline and ethanolamine emerged as the descriptors leading to effective grouping.

De la Presa-Owens *et al.* (1995a and 1995b) reported the determination of free amino acids and ethanolamine for the characteristic Spanish white wines from the Penedes region. Asparagine, proline and lysine proved to be the most important compounds for distinguishing the varieties on the basis of their geographical origin.

In another study, Hernandes-Orte *et al.* (2002) analysed 11 amino acid compositions imitating the characteristic amino acid profile of 11 different grape varieties. A multiple linear regression study produced good models for most of the odorants for which the level was related to the must amino acid composition. Partial least squares regression models confirmed that amino acid composition explains a high proportion of the variance in the volatile composition; the by-products of fatty acid synthesis are related to threonine and serine, the level of β-phenyl ethanol is closely related to phenylalanine and the level of methionol is linked to methionine content.

Soufleros *et al.* (2003) found that the amino acid concentrations of Greek white wines were within the range reported for other European white wines. The results indicate the influence of grape variety, geographic location and vintage on the amino acid composition of wine. The type of fermentation also had an impact on the concentration of certain amino acids. Within all wine samples tested as a group, arginine and γ-amino butyric acid were the most abundant amino acids followed by lysine, alanine, glycine, asparagine, leucine and ethanolamine (Table 20.4).

20.4 The use of NMR, FT-IR and sensory techniques

20.4.1 NMR

Proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopic methods were applied in the differentiation of 53 German white wines from the regions Rheinhessen, Rheingau, and Mosel-Saar-Ruwer. The robustness of these differentiations was tested by leaving out one sample at a time and replacing it into the test as a test sample. Small variations in the baseline were successfully treated with the aid of a pre-processing algorithm called partial linear fit (Vogels *et al.*, 1993).

Table 20.4 Analysis of amino acids of wines

Country	Number of samples/varieties	Method	Amino acids selected for classification	Reference
France	110/2 (Champagne/sparkling wine)	Amino acid analyser	All except for arginine	Tusseau *et al.*, 1996
France	34/	HPLC	Proline, hydroxyproline, ethanolamine	Palma *et al.*, 1995
Spain	60/3	HPLC with diode array UV visible after derivatisation	Asparagine, proline, lysine	De la Presa-Owens and Noble, 1995a and De la Presa-Owens *et al.*, 1995
Spain	33/11	HPLC	Threonine, serine, phenylalanine, methionine	Hernandes-Orte *et al.*, 2002
Italy	31 (73 musts)/NA	Amino acid analyser	Glutamic acid, aspartic acid, proline, leucine, alanine, serine	Seeber *et al.*, 1991
Greece	42/7	HPLC with fluorescence detector	Arginine, methionine, γ-amino butyric acid	Soufleros *et al.*, 2003

Kosir *et al.* (2001) applied SNIF-NMR and IRMS (isotope ratio mass spectroscopy) to 50 white wines from the three main wine-producing regions of Slovenia. It was shown that the separation of wines according to geographical criteria and authenticity is very good when the coastal region is compared to the continental ones. In the case of enrichment of wines with beet sugar the separation is improved by application of PCA and cluster analysis.

1D and 2D ^{1}H and ^{13}C homo- and hetero-nuclear magnetic resonance analysis (large signal suppression methods) was employed for characterising anthocyanins and amino acids of ten Slovenian white wines. It was shown that the use of NMR signals of seven amino acids gives a good separation of wines according to vine variety and geographical origin. The reliable ^{1}H and ^{13}C NMR assignment of the signals of anthocyanins in glucoside form is used for the identification of various fractions in Liquid Chromatography analysis of anthocyanins (Kosir and Kidric, 2002).

In a recent study, Brescia *et al.* (2002) used NMR analysis for detecting the geographical origin of 41 red wines supplied from various wine-makers from the Apulia region (Italy). NMR spectroscopy emerged as the most advantageous technique because of the rapidity with which information can be obtained about a large number of compounds and of the smaller amount of sample required for analysis. It was suggested that the use of ^{13}C NMR, though less sensitive than ^{1}H NMR, could be a promising approach because of its minor signal overlapping and a chemical shift range 20 times larger than for protons. This allows the choice of a considerably larger number of signals for statistical analysis, thus permitting a more efficient fingerprinting of the wines. A synoptical table of implementation of NMR in wine authentication is given in Table 20.5.

20.4.2 Fourier Transform Infra Red (FT-IR)

Gallignani *et al.* (1994) applied FT-IR for the direct determination of ethanol in alcoholic beverages. The method was based on first-order derivative FT-IR measurements between the peak at 1052 cm^{-1} and the valley at 1040 cm^{-1}, which are present in aqueous solutions and alcoholic beverages. This method provided accurate results in the determination of ethanol in alcoholic beverages without requiring any previous chemical treatment of the sample. The limit of detection corresponds to 0.025% (v/v).

Roussel *et al.* (2003) showed that high-level multi-sensor fusion significantly improved the white grape must variety classification with regard to individual discrimination. The fusion procedure is not based on the combination of signals, but on the class assignments provided individually by each sensor. Although olfaction does not seem to be the best approach toward discriminating grape varieties, the adjunction of the aroma sensor identity declaration slightly improved FT-IR and UV spectral classification efficiency. The effective fusion method leads to a significant improvement in grape variety discrimination.

Implementation of FT-IR spectroscopy (in the region between 800 and 1200 cm^{-1}) on the extracted polymeric materials of Portuguese white wines

Table 20.5 Use of nuclear magnetic resonance to wine analysis

Country	Number of samples/ regions	Technique	Method details	Reference
Germany	53 white wines/3	^{1}H, ^{13}C	Pre-processing algorithm called partial linear fit	Vogel *et al.*, 1993
Slovenia	50 white wines/3	^{2}H, % $^{13}C/^{12}C$	SNIF-NMR, Isotope Ratio Mass Spectroscopy (IRMS), study of grape must	Kosir *et al.*, 2001
Slovenia	10 white wines/3	^{1}D and $^{2}D^{1}H$, ^{13}C	Study of anthocyanins	Kosir and Kidric, 2002
Italy	41 red wines/3	^{1}H	NMR in conjunction with inductively plasma atomic emission spectrometry	Brescia *et al.*, 2002

revealed that this technique can be effectively used to characterise white wine polysaccharide composition. It was possible to identify the wine-making process involved (must clarification and/or maceration) and its influence on the amount and kind of wine polysaccharides. Finally, the results showed that it is possible to use the FT-IR combined with multivariate techniques for an in-depth characterisation of white wine polymeric fractions (Coimbra *et al.*, 2002).

Palma and Barroso (2002) recorded FT-IR spectra in an attempt to differentiate and classify wines and brandies during their storage as well as for classification of distilled drinks from various producing countries. Ethanol and sugars proved to have a high response in the IR spectra. Therefore, samples with different ethanol and sugar content are bound to have different spectral zones to be used as fingerprints in characterisation studies.

20.4.3 Sensory evaluation

Bakker and Arnold (1993) showed that when using a panel of tasters, minimal training was needed to carry out sensory profiling when the tasters were not required to develop or agree usage of a consensus set of terms. Analysis of the data revealed that all three transformations done with GPA, translation, rotation/reflection and scaling, were highly significant. It was also found that the use of analytical colour measurements could be of considerable use in the assessment of the sensory characteristics of wines related to colour, having a good predictive value.

Le Fur *et al.* (2003) made an attempt to correlate both sensory and combined headspace gas chromatography-olfactometry (GC-O) analyses. Wines tested by sensory analyses and the headspace samples analysed by GC-O were described with a heterogeneous vocabulary distributed into nine overall classes of descriptors. It was found that dynamic headspace analysis induces a distortion with respect to sensory data, which systematically affected the perception of both spicy and herbaceous characters of wines. Gawel *et al.* (2001) showed that astringency in red wine can manifest itself in many subtle yet complex forms, and that tasters can be effectively trained to reproducibly discriminate and rate the intensities of astringent sub-qualities elicited by young dry red wines.

Fischer *et al.* (1999) employed descriptive analysis in order to investigate the sensory properties of commercial Riesling wines from two vintages, five wine estates and six vineyard destinations within the viticulture region Rheingau. Based on the number of significant F-ratios among ten odour and four orally perceived attributes, vintage and wine estate proved to have a similar impact to vineyard designation. Since PCA revealed substantial variations, even within the same vineyard designation, the authors claim that a classification system focusing on geographic origin would be rather confusing for consumers. The results of a study carried out by Carlucci and Monteleone (2001) disclosed that a step-by-step approach to analysing sensory data (fixed ANOVA model on raw and scaled data) can eventually be suitable for validating the sensory profile of typical regional food products. The intensity of sensory descriptors does not

distinguish amongst the products. Furthermore, the results were not affected by assessor discrepancy and so can be referred to the typical sensory profile of young Aglianico red wine (Table 20.6).

Vannier *et al.* (1999) employed a fixed choice profile technique to detect sensory differences by qualitatively and quantitatively characterising gustatory and olfactory properties of over 56 Champagne wines. The original 64 attributes were reduced to a working set of 19 objective attributes showing a good range of scores, low incidence of zeros and no hedonic aspects. The trained panellists' repeatability as well as their discriminative efficiency were estimated. In a recent study, Douglas *et al.* (2001) demonstrated that Riesling wines from two Canadian terroirs are distinctly different by applying univariate and multivariate statistics.

In Table 20.6, a summary is given of the sensory analysis methods (i.e. blind test, attributes, intensity scale) employed on red and white wines of various countries by trained panellists.

20.5 Data analysis

PCA implementation of all instrumental and sensory data of wines did not show any major differences among either red or white wine categories, despite their different geographical origins. On the other hand, the PCA of anthocyanins and sensory analysis resulted in the effective classification of red wines into two groups: north Greek and south Greek (Peloponnese and islands) wines. Therefore, anthocyanins emerged as the crucial factor in terms of red wine classification, whereas minerals (ions) and phenols did not allow any valid clustering of wines (Kallithraka *et al.*, 2001a).

Application of multivariate analysis (PCA, CLA, LDA) to phenolics resulted in satisfactory classification of different wine vinegars (Table 20.7). Attempts to differentiate vinegars were based on the kind of employed raw material or on the process involved (Guerero *et al.*, 1994). Seven significant factors were used for PCA and with these factors, 76% of total variance was explained. Cluster analysis was applied for searching natural grouping among the samples. Thus, the data matrix was subjected to a hierarchical agglomerative cluster analysis of cases. A dendrogram (tree diagram) was obtained, taking the Euclidean distance as metric and the Ward method as an amalgamation rule. These two methods have assumed knowledge of the number of classes. Some variables were selected for the classification according to manufacture and some others for the classification according to geographical origin with the use of LDA.

Sivertsen *et al.* (2001) studied two Chilean wines made from the Cabernet Sauvignon grape stored in bottles at four different temperatures for a period of 44 months. During storage they were evaluated by sensory descriptive and chemical analyses. Different sensory profiles were obtained for the wines stored at different temperatures. Wines were stored at various temperatures to determine if it was possible to predict the shelf-life of wines stored at optimal temperatures. However, no valid conclusions were deducted.

Table 20.6 Sensory analysis of red and white wines by trained panellists

Country	Wine/no. of samples	Method of analysis	Number of judges	Number of attributes examined/scale	Reference
Greece	21 red and 12 white/33	Randomised presentation	10 staff members	7/continuum and unstructured (from none to extreme)	Kallithraka *et al*., 2001a
Portugal	39 port wines/78	Blind	7 male	9/0–9	Bakker and Arnold, 1993
France	6 Chardonnay wines	4 sessions/selection of terms	14 (5 males, 9 females)	144/9 classes	Le Fur *et al.*, 2003
Australia	72 red wines	Panellists' ability assessment	14 tasters with 5 years' experience	24/4 classes Astringency vocabulary development	Gawel *et al.*, 2001
Germany	20 Riesling wines/ 5 estates	Reference standards prepared daily	9 enology students	10 odour attributes/4 oral attributes intensity ratings scored on 10 cm unstructured scale	Fischer *et al.*, 1999
Italy	16 young wines south Italy wines/4 regions	Preliminary sessions (10 + 6)	8 food science students	25 initial attributes reduced to 15 based on citation frequency. The intensity was rated on a 10 cm unstructured scale	Carlucci and Monteleone, 2001
Canada	14 Riesling wines/ 2 regions	Initial training session	10 students and staff members	15 sensory attributes (10 aroma, 2 taste and 3 other attributes). The intensity was rated on a 10 cm unstructured scale (anchored with terms none, moderate and intense at 1, 5 and 9 cm)	Douglas *et al.*, 2001

Chile	16 samples (2 wines, 4 temp., 2 replicates)	Six preliminary training sessions	11 employees	15 sensory attributes (2 visual, 8 aroma, 5 attributes for evaluation of storage effect). Intensity scale ranging from low intensity to high intensity on a 15 cm distance	Sivertsen *et al.*, 2001
Canada	60 samples/3 sites	Randomised coded samples	11–15 agriculture Canada staff	8 attributes. Intensity recorded on a 5 cm unstructured line scoresheet	Reynolds *et al.*, 1996
France	56 Champagne wines	Two preliminary sequences of 12 and 6 sessions	18 volunteers	64 attributes reduced to 19 objective attributes. Fixed choice profiling. Intensity measured on an unstructured ratio scale from 0 to 10	Vannier *et al.*, 1999
France	9 wines/2 varieties	Two weeks' training (4 sessions)/Triangular test	17 students	20 attributes reduced to 12. Intensity measured on a scale from 0 to 5 (no and highest perception, respectively)	Kotseridis *et al.*, 2000

Table 20.7 Phenolic compounds used for classification of wines

Country	Wines	Phenols employed	Method	Reference
Greece	White and red	Resveratrol, gallic acid, myricetin, quercetin, catechin, vanillic acid, caffeic acid, epicatechin, p-coumaric acid, ferulic acid	HPLC	Kallithraka *et al.*, 2001a & b
Spain	Red	Genticic acid, p-coumaric acid	Capillary zone	Pazourek *et al.*, 2000
Spain	Red	Caftaric acid, coutaric acid, catechin epicatechin, delphidin, peonidin, petunidin malvidin	HPLC-UV detector	Gomex-Plaza *et al.*, 2000
Italy	Red	Total phenols, total flavanoids	HPLC	Pellegrini *et al.*, 2000
California (USA)	White and red	Gallic acid, catechin, caftaric acid, cyanidin, epicatechin, myricetin, quecetin, malvidin	HPLC	Ftankel *et al.*, 1995
Canada	White and red	Gallic acid, caffeic acid, p-coumaric acid vanillic acid, ferulic acid, gentisic acid	GC-MSD	Soleas *et al.*, 1997
Italy	Red	Gallic acid, gentisic acid, vanillic acid, caffeic acid, p-coumaric acid, frulic acid, salicylic acid, cinnamic	LC-MS capillary scale particle beam	Cappiello and Famiglini, 1999
Italy, France California (USA), Australia, France	Red	Catechin, epicatechin, cis-polydatin, trans-polydatin, trans-resveratol, rutin, quercetin	HPLC-diode array detector	Goldberg *et al.*, 1996
France	Red	Anthocyanidins	HPLC	Etiévant *et al.*, 1988a
Italy		Procatechuic acid, trans-coumaric acid	HPLC	De la Presa-Owens and Noble, 1995
Spain		Anthocyanidins	Spectrometry	Almela *et al.*, 1993
Spain		Coutaric and caftaric acid, procyanidins	HPLC	Mayer *et al.*, 1997

Table 20.8 Classification of wines according to geographical origin, variety and ageing by applying several multivariate analysis methods

Classification of wines according to	Determining propert	Multivariate analysis	References
Geographical origin	*1. Instrumental*		
	a. elements		
	K, Na, Ca, Mg, Rb, Li	PCA, CDA	Etiévant *et al.*, 1988b
	Li, Na, K, Rb, Zn, Fe, Mn, Ni, Co	LDA, KNN, PCA	Pena *et al.*, 1999
	Li, Na, K, Rb, Zn, Fe, Mn, Ni, Co	LDA, KNN, PCA, SIMCA	Rebolo *et al.*, 2000
	Li, Rb	LDA, KNN, SIMCA	Latorre *et al.*, 1994
	Ba, St, Rb, Li, $(DH)^{o}_{w}$, $(DH)_{I}$, $(DH)_{II}$	ANOVA, CDA	Day *et al.*, 1995
	Na, K, Rb, Cs, Cr, Fe, Co, Zn, Ag	CLA	Siegmund and Bachman, 1978
	Mn, Li	MLRA, KNN, SIMCA	Gonzales-Larraira *et al.*, 1987
	B, P	PCA	Baldi *et al.*, 1984
	Li	PCA	Médina and Van Zeller, 1984
	Pb isotopes	DA	Médina 1996
	Lanthanides	PCA	McCurdy *et al.*, 1992
	b. volatiles		
	total volatiles	LDA, KNN, SIMCA, PCA	Rebolo *et al.*, 2000
	total volatiles	PCA	Médina and Van Zeller, 1984
	Alcohols, acids, esters, terpenes, miscellaneous	PCA, CLA, SIMCA, KNN	Garcia-Jares *et al.*, 1995
	total volatiles	CLA, DA	Forcen *et al.*, 1992
	Decanoic acid	PCA	Médina and Van Zeller, 1984
	Polyalcohol content	CLA, MANOVA, LDA	Antonelli *et al.*, 1997
	c. Phenols		
	Procyanidin B2	PCA	Médina and Van Zeller, 1984
	Anthocyanin, flavonol aglycones, glucosides	PCA, DA	Gomez-Cordoves *et al.*, 1995
	total phenols	LDA	Palma *et al.*, 1995
	(hydroxymethyl) furaldehyde, tyrosol, caffeoyltartaric acid, syringaldehyde, vanillic acid, caffeic acid, gallic acid ethyl ester, vanillin	LDA	Garcia-Parilla *et al.*, 1997
	total phenols	PCA, CDA	Sivertsen *et al.*, 1999
	Epicatechin, caffeic acid, p-coumaric acid, ferulic acid	LDA, KNN, SIMCA, PCA	Rebolo *et al.*, 2000

Table 20.8 Continued

Classification of wines according to	Determining propert	Multivariate analysis	References
Geographical origin (Continued)	*d. Amino acids*		
	Proline, total nitrogen	CLA, DA	Forcén *et al.*, 1992
	Threonine, serine, phenylalanine	PLS	Hernandes-Orte *et al.*, 2002
	Arginine, methionine, γ-amino butyric acid	DA	Soufleros *et al.*, 2003
	Proline, hydroxyproline, ethanolamine, total nitrogen	PCA, CDA	Etievant *et al.*, 1988a
	e. FT-IR	PCA	Coimbra *et al.*, 2002
		PCA	Palma and Barroso, 2002
	f. NMR	PCA	Vogel *et al.*, 1993
		CLA, PCA	Kosir *et al.*, 2001 and Kosir and Kidric, 2002
		PCA, CLA, DA	Brescia *et al.*, 2002
	2. Sensory evaluation	PCA, CDA	Sivertsen *et al.*, 1999
		PCA	Kallithraka *et al.*, 2001a & b
		GPA	Le Fur *et al.*, 2003
		PCA	Gawel *et al.*, 2001
		PCA	Fischer *et al.*, 1999
		PCA, LDA	Seeber *et al.*, 1991
		PCA	Vannier *et al.*, 1999
Varieties	*1. Instrumental*		
	a. elements		
	Na, K, Ca, Mg	KNN, DA	Moret *et al.*, 1994
	Na, K, Ca, Mg, Fe, Cu, Zn, Mn, Sr, Li, Rb	CLA, PCA, SIMCA	Frias *et al.*, 2003
	b. Volatiles		
	Hexanol	PCA	Thurin, 1984
	total volatiles	PCA, CLA, SIMCA, KNN	Garcia-Jares *et al.*, 1995
	total volatiles	PCA, DA	Almela *et al.*, 1993
	Ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl laurate, isobutyl acetate, isoamyl acetate, hexyl acetate, phenylethyl acetate	PCA, SLRA	Ferreira *et al.*, 1995

	c. Phenols		
	s-Glutathionyl-caftaric acid, epicatechin, trans-coumaric acid, trans-ferulic acid, trans-coumaric/trans-caftaric ratio, total caftaric, trans- & cis-caftaric acid, coutaric acids	ANOVA, PCA	De la Presa-Owens and Nobel, 1995
	Procyanidin B3	PCA	Thurin, 1984
	total phenols	PCA, ANOVA	De la Presa-Owens *et al.*, 1995
	Anthocyanins	PCA, DA	Almela *et al.*, 1993
	Coumaric acid, caftaric acid, procyanidin	PCA	Mayen *et al.*, 1997
	Isoquercetin, coumaroyltartaric acid, (hydroxymethyl) furaldehyde, tyrosol, caffeoyltartaric acid, p-hydroxybenzoic acid, gallic acid, p-coumaric, isoquercetin	LDA, BPANN	Garcia-Parilla *et al.*, 1997
	d. Amino acids		
	Proline, serine, ornithine, citrulline, arginine	PCA	Millery *et al.*, 1984
	e. Colour parameters		
	L*, a*, b*	PCA, DA	Almela *et al.*, 1993
	2. Sensory evaluation	ANOVA, PCA	De la Presa-Owens and Nobel, 1995
		PCA	Fischer *et al.*, 1999
		PCA	Douglas *et al.*, 2001
		ANOVA, LSD	Kotseridis *et al.*, 2000
Ageing	*a. Elements*		
	b. Volatiles		
	Trans-2-hexenol, isoamyl acetate, ethyl hexanoate	ANOVA, PCA	De la Presa-Owens *et al.*, 1995a
	c. Phenols		
	Gallic acid, vanillic acid, caffeic acid, syringic acid, vanillin, syringaldehyde, ferulic acid, coniferyl aldehyde, furfural, p-hydroxybenzaldehyde	LDA, KNN, PLS, SIMCA	Mangas *et al.*, 1997
	total phenols	LDA	Galvez *et al.*, 1995
	d. Amino acids		
	Glycine, tyrosine	ANOVA, PCA	De la Presa-Owens and Nobel, 1995
	e. Sensory analysis	ANOVA, PCA, PLS	Sivertsen *et al.*, 2001

Changes in phenolic compounds during accelerated browning in white wines from two Spanish varieties were evaluated by using PCA. Two variables produced the best differentiation between the wines of the two varieties studied (Mayen *et al.*, 1997). Several lots of Chardonnay and Grenache blanc grapes were treated with pomace contact and hyperoxidation prior to classification. Variations in the chemical and sensory properties were examined by principal component and factor analyses. PCA was performed on the compositional and browning capacity data to illustrate the relationships among the analytical variables and the wines. Three principal components were shown to be the most significant ones accounting for 87% of the total variance. The PCA of the sensory data showed that pomace contact was beneficial to Chardonnay wines and detrimental to Grenache wines (Cheynier *et al.*, 1991).

Rebolo *et al.* (2000) found that the composition data of Galician red wines with a certified brand of origin (CBO) involving metals, volatile and phenolic compounds were used to differentiate between samples corresponding to other nearby brands. Pattern recognition techniques (KNN, LDA, PCA) applied to a chemical dataset were able to extract the required information for obtaining a satisfactory classification of the wine samples according to their geographical origin. Application of classification procedures with only delphinidin, Li, Rb, Fe and epicatechin as selected features indicated good performance in terms of classification and prediction for CBO Ribeira Sacra wines and produced a more successful assignation than when the entire set was employed.

Eleven major elements were used to characterise sweet and dry wines of El Hierro, La Palma and Lanzarotte islands (Canary Islands, Spain). Grape maturity at harvesting for elaboration influences the final mineral levels, thus sweet wines from La Palma, elaborated with over-mature grapes, have higher levels than the dry ones. The results obtained show that the mineral content is similar to that of other wines with the exception of sodium whose content in Canarian wines is higher than in other wines (Frias *et al.*, 2003). All the metals presented differences in content according to island of origin. Cluster analysis showed that Canarian wines could be grouped in accordance with the island of origin and ripening state of grapes. LDA showed very good recognition.

Moret *et al.* (1994) studied the chemometric multiyear experimentation within the frame of characterisation and classification of wines by chemical variables. Analysis of 273 samples (five typical Venetian white wines) was followed by implementation of multivariate statistics (KNN, SIMCA and canonical variate). The enological characteristics of different clones of Monastrell were studied by Gomez-Plaza *et al.*, 200). Table 20.8 is a very long and comprehensive table summarising the multivariate analysis method employed and the determining property of wine.

20.6 Conclusions

Recently great progress has been made both in terms of novel methods (fast, accurate) for detecting wine authenticity and in the implementation of pattern

recognition techniques for grouping purposes. In most cases, grouping was successful and resulted in a very high classification. Among the various attributes analysed are minerals, colour, phenols, volatiles, amino acids, etc. Generally speaking, pattern recognition or multivariate analysis methods stand as a powerful and promising tool for detecting the authenticity of wine in terms of its variety and geographical origin.

20.7 References

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Part III

Traceability

21

Traceability in food processing: an introduction

C. Morrison, Youngs Bluecrest Seafoods Ltd, UK

21.1 Introduction: the key objectives of traceability

Traceability and systems associated with the ability to link a finished food product with its ingredients and processing have always formed a key part of any good manufacturing practice and quality assurance scheme. Without such schemes in place, it would be very difficult to substantiate any on-pack marketing claims, for example 'Organic'; to provide due diligence in the event of a Public Enforcement challenge or to minimise the quantities involved in the event of a recall. There has been significant growth in both global sourcing and manufacturing, along with centralisation of food production into specialist sites, in recent years. As a result, large volumes of raw materials and ingredients will be sourced from suppliers around the world and converted into equally large volumes of finished product for global distribution. In these circumstances, traceability is especially important to the manufacturer, whether international, national or regional. It is also clear that, in order to be proactive in meeting ever-increasing consumer demands for clear labelling and transparency, traceability will gain in importance.

Traceability schemes will always need to go beyond any legislative requirements. This, in turn, will lead to improved process controls and good manufacturing practices (GMP). The absolute need for a fully documented traceability system within the food chain has never been stronger, building from the late 1980s through to the millennium. Two issues in particular, the BSE crisis, followed by the genetically modified organisms (GMO) debate, can best illustrate this.

Initial loss of confidence of consumers in British beef arising from the BSE crisis was further exacerbated by the extended ban on all exports. This very

much centred around issues of lack of adequate government-controlled cattle traceability schemes. British retailers tackled this problem for their home markets by instigating their own traceability systems and assured labelling schemes, which were heavily promoted in advertising and in-store leaflet campaigns. The Meat and Livestock Commission (MLC) also established a national traceability scheme for beef and beef products,[1] which could be translated into on-pack quality claims for manufacturers and retailers participating in the scheme. While BSE was fundamentally a food safety issue, it is now widely perceived that the GMO debate is one of consumer choice, transparency and ethical labelling. Traceability is an indispensable aspect, whether to meet developing labelling legislation within the EU in this area, or to justify labelling claims of 'GM-free'.

In consideration of the way in which two recent problems affect the food industry, and the globalisation of manufacturing and supply, it is possible to identify some key objectives required from a traceability scheme.[2]

- Compliance with international and national traceability coding legislation as appropriate.
- Development of agriculture traceability schemes for commodities and 'organics'
  - ability to make marketing claims
  - ability to make health and other claims.
- Definition and control of ingredients
  - comprehensive specifications
  - ability to avoid the need for negative claims e.g. 'may contain nut traces'.
- Improved process control and GMP
  - comprehensive process control specifications
  - comprehensive final product standards – improved quality and consistency
  - due diligence systems enhanced
  - ability to minimise recall losses.
- Improved consumer perception
  - transparency
  - confidence
  - loyalty.

21.2 Traceability coding

The legal obligations of traceability coding are to facilitate free movement of foodstuffs across borders and to ensure correct product identification in the event of a Public Enforcement recall for health and safety reasons. The manufacture and/or the owner of the food product will need to ensure that the quantity of product involved in a recall is kept to a minimum. For example, a 'lot' is a batch of foodstuffs produced under essentially the same conditions. This can be translated, for a long shelf-life product, as the minimum durability

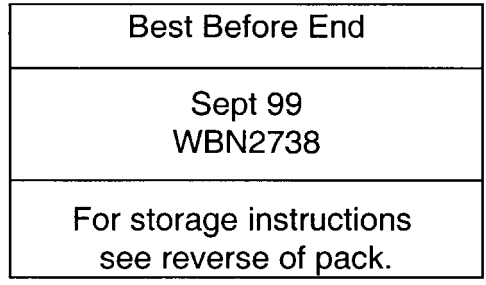

Fig. 21.1 A batch code.

code of 'Best Before End' – June 2001, i.e. a month's production as described above. It follows, therefore, that supplementary coding further defining the batch is advisable.

Just as for the lot mark, or durability code, a batch code should be clearly legible and easy to find, particularly for consumers when reporting a problem to the product manufacturers. This is equally applicable to holders of stock, in the event of a recall or withdrawal, in order to ensure only affected product is returned and sales of unaffected stock continue. Typically, the code will be close to the durability code as illustrated in Fig. 21.1. In this example:

Best Before End	Sep99	is a 12 month shelf-life, i.e. produced during October 1998
W		is producing factory identifier
B		is the specific production line
N		is hour code
273		is Julian calendar day
8		is last digit of year 1998

Traditionally, coding equipment had to be set up by placing individual letters and numbers into a printing block. This meant that codes could probably be changed only once per day or per shift. New technology inkjet and laser systems are now able to encode and print significant amounts of information, including the durability code and the production codes, down to the minute if required. Similarly, the outer distribution cases into which packed product is placed, can be batch coded to the hour as can the pallet on which the cases are stacked. It is therefore possible to locate a problem to a very specific batch, thereby minimising the number of recalled items, providing, of course, data on the issue is available. There is no point in having this end-of-line fine detail, of course, if it is not paralleled by an equivalent level of sophistication backwards through the production process, and all the way back to the raw materials and packaging materials and forwards through the distribution chain.

A computerised traceability system will accomplish this speedily, although it is perfectly possible to provide the same information through a paperwork system. In the event of a recall, however, a computerised system will generate in hours what might take many people days to accomplish.

21.3 Components of traceability systems

Production control systems contain details of each product recipe at a number of levels starting with the individual ingredients, each identified by a unique code (see Fig. 21.2). They may then track intermediate operations or recipe levels, e.g. preparation of a spices batch, through to the sauce batch, a cooked chicken piece through to combination and completion of the product by depositing in a foil tray, the placing of trays into cartons, then distribution cases and finally stacking on a pallet. Such systems are driven by a sales forecast, from which the production plan is determined along with product batch sizes, standard yields and the purchase order plan for ingredients. In order for such a system to work, it requires access to complete details of unique product specifications, ingredients, packaging components and approved suppliers. Ideally the specification system should be linked directly to the management control system. In this way any approved changes flow automatically through the system for information from a specific date so that there can never be any question about what was used on or about the changeover date.

Many processes within the total operation may also be computerised and records of results could also be integrated into the main system. Figure 21.3 outlines a product traceability flow sequence for a process from raw materials receipt to delivery to distribution store. In this instance, all of the information concerning the raw material receipt is contained within a hand-held computer. Records of the intake, such as order number, followed by quality attributes, such as weights, are entered directly into the hand-held computer. Comparison with standards is made automatically and the pass/fail can then be downloaded into the main system. It is also possible to apply bar coding to this system so that samples can be sent to laboratories with unique codes. Where microbiological or analytical release is required, this could again be completed via the laboratory's Laboratory Management Information System (LIMS), or equivalent system using the bar code reference applied to the samples for analysis, again with results loaded directly into the main system.

A similar approach can be applied within, for example, batch weighing of ingredients. It is possible to apply a bar code to each ingredient linked to its unique computer code. As each is weighed on a computerised weighing system, a bar code sticker is generated for the container according to the recipe weight. The components are then bar-scanned into the next stage of the process, so ensuring that no item is left out. This principle applies for all process records right through to final on-pack coding and quantities delivered to store. Typically, for legal compliance, records have to be kept for a minimum of three years, so it can be envisaged how much archive space has to be provided for a large production unit using a paperwork recording system compared to an electronic system as described.

An area of traceability not yet described is the on-pack bar coding required by many retailers. The system used is known as 'Article Numbering and Symbol Marking' and is administered by the International Article Numbering Association.[3]

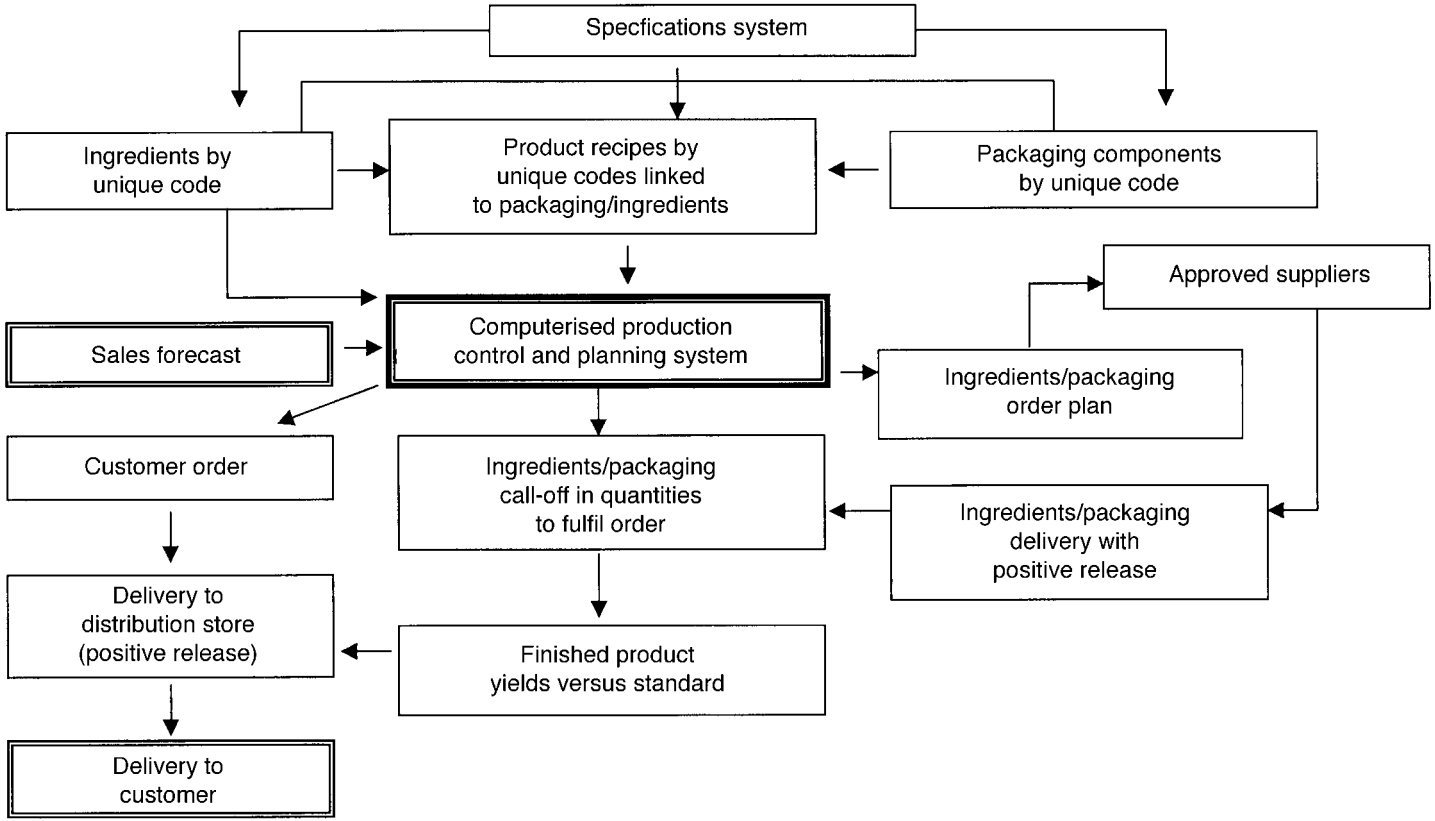

Fig. 21.2 A typical computerised production and management control system.

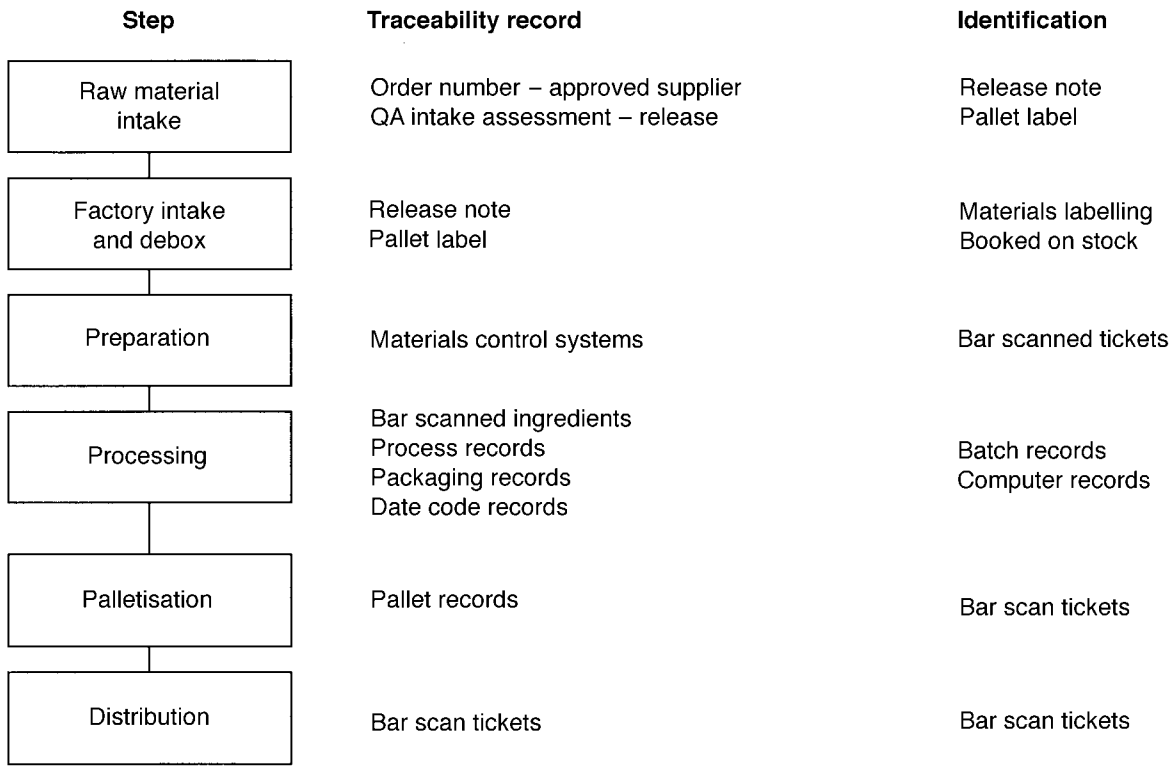

Fig. 21.3 Product traceability flow.

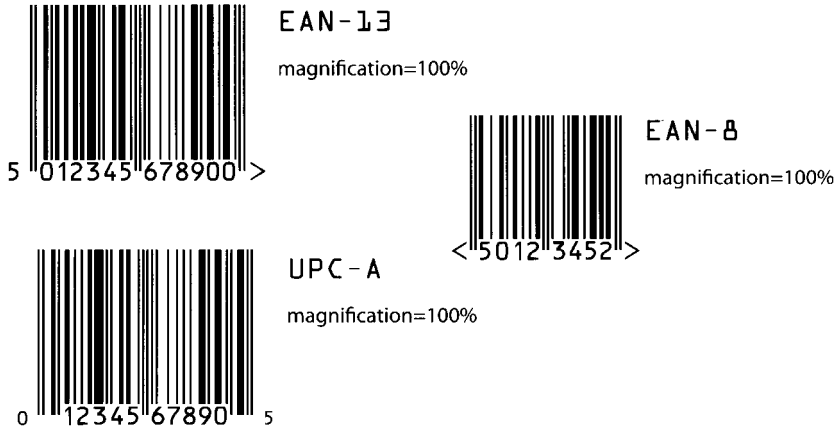

Fig. 21.4 Bar code symbols (source: Bar Coding: recommendations for best practice (conclusions of 'MR BIG' (the major retailers Bar code Integrity Group)), published by the Institute of Grocery Distribution – November 1997 ISBN: 1 898044 40 6).

In Europe a 13-digit EAN (originally European, but now international Article Number) bar code has been applied (EAN-13), while in the United States and Canada a 12-digit UPC code (UPC-A) is used. The 12-digit code can be read in Europe but the 13-digit code will become the standard. An 8-digit code (EAN-8) may also be used when a retailer has its own brand as this set of numbers is unique to the retailer (see Fig. 21.4).

The 13 digits of EAN-13 correspond to the thick, thin and spacing characteristics of the bar code which is electronically scanned. The first two digits refer to the administration association issuing the bar code, followed by five digits for the company, five digits for the unique product description and the final digit is for checking purposes.

For product packed into distribution cases, i.e. traded units, there are two different coding systems in use:

- ITF-14 which is a basic bar code symbol, which identifies the product and number of sales units per case.
- EAN-128 which is designed to encode supplementary data as well as item identification such as:
  - use-by/best before dates
  - measurements
  - batch and serial numbers.

EAN-128 is seen as a more flexible and integrated system than ITF-14 or EAN-13. It is gaining greater acceptance across Europe and is a standard which UK retailers require suppliers to meet. Finally, for pallets of traded units in distribution, there is an ANA code based on EAN-128, known as the Transport Unit Bar Code Symbol.

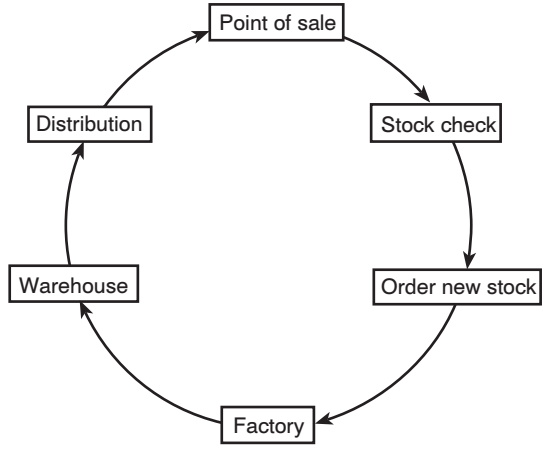

Fig. 21.5 Bar codes in the supply chain (source: Bar Coding: recommendations for best practice (conclusions of 'MR BIG' (the major retailers Bar code Integrity Group)), published by the Institute of Grocery Distribution – November 1997 ISBN: 1 898044 40 6).

It can be seen from the preceding descriptions of bar coding that a high level of traceability can be achieved already through this system and this will improve with time. Already some major manufacturing companies are able to link directly into Point of Sale Scanning Information at retailer store level (see Fig. 21.5). Within the UK, retailers, through the Major Retailers Bar Code Integrity Group, are currently embarked on a project in conjunction with the Article Numbering Association (UK) Ltd, known as 'Mr Big'. The purpose of this initiative is that 'Total quality in the use of EAN bar code symbols is achieved when the right place reads correctly every time'. The project has issued best practice guidelines covering such issues as the presentation, size, positioning and print quality of bar codes.

21.3.1 Use of care lines on product packaging in traceability

The care line concept has developed rapidly over the past seven years and is now used by many major manufacturers on their brands. Previously, a Consumer Guarantee of Satisfaction would be included on the pack along with the address of consumer services department to contact in the event of a problem. The care line offers a much more proactive and interactive communications route for consumers. It can be suggested on-pack, for instance, that in addition to phoning on quality satisfaction matters, they also seek more information about other products, for instance through recipe cards. Most of all, it enables a gentle interrogation of the consumer to establish the actual batch codes and traceability information, since very often, when responding by post mail, they will send the bar code only, which whilst identifying the product exactly, will not identify the batch at this time.

21.4 Using traceability systems when problems arise

The ability to recognise and respond rapidly to a problem is essential in any management system. This allows the impact and potential losses to be contained to a minimum. Quality systems such as ISO 9000[4] require regular management review of complaints and problems together with records and corrective actions taken. Any due diligence defence or mitigation would also require evidence of a similar nature. Most larger food manufacturers have a consumer services department, which together with the technical department, will handle consumer quality, safety issues and interactions with the customers and Public Enforcement bodies such as governmental bodies, e.g. Food Standards Agency (FSA) MAFF, Department of Health (DH), Environmental Health (EHO) and Trading Standards (TSO) in the UK. Other contacts may also come into the company head office via employees, or in the event of threats, via the police, media or extortionists.

To determine the scope of any reported complaint, a recording system and analytical process is required, which would encompass investigation, rapid report back and risk assessment where necessary. Such a system is outlined in Fig. 21.6, which is based on a consumer services department as the central function, initially co-ordinating the complaints received and responding back to the complainant, or in the case of serious complaints, instigating further investigation which may result in the involvement of the internal crisis management team. Within the EU the implications of Food Safety Regulations, General Product Safety Regulations and product liability legislation will have to be considered in these circumstances.[5–7]

21.4.1 The trace

Where the crisis management team is called together and, after assessing the available information, the team instigates a recall, there will be two important tasks to carry out: identifying all affected stock and communicating the recall (see Fig. 21.7). Stock identification is essentially a collation of information, based on date code information, of the affected product, through to the implicated process or ingredient as appropriate (see Fig. 21.8). Once this information has been collated and customers likely to be holding stock identified, a decision as to how far to 'widen the net' to include codes either side as a safeguard needs to be taken. Nothing could dent customer or consumer confidence more than to have to instigate a second recall later for the same problem.

21.4.2 Communications

During the run-up to the recall, a recall notice will have been planned. First and foremost within the notice will be the clear details of product identification, i.e. all of the on-pack traceability features, followed by details of the problem and recommendations of actions to take:

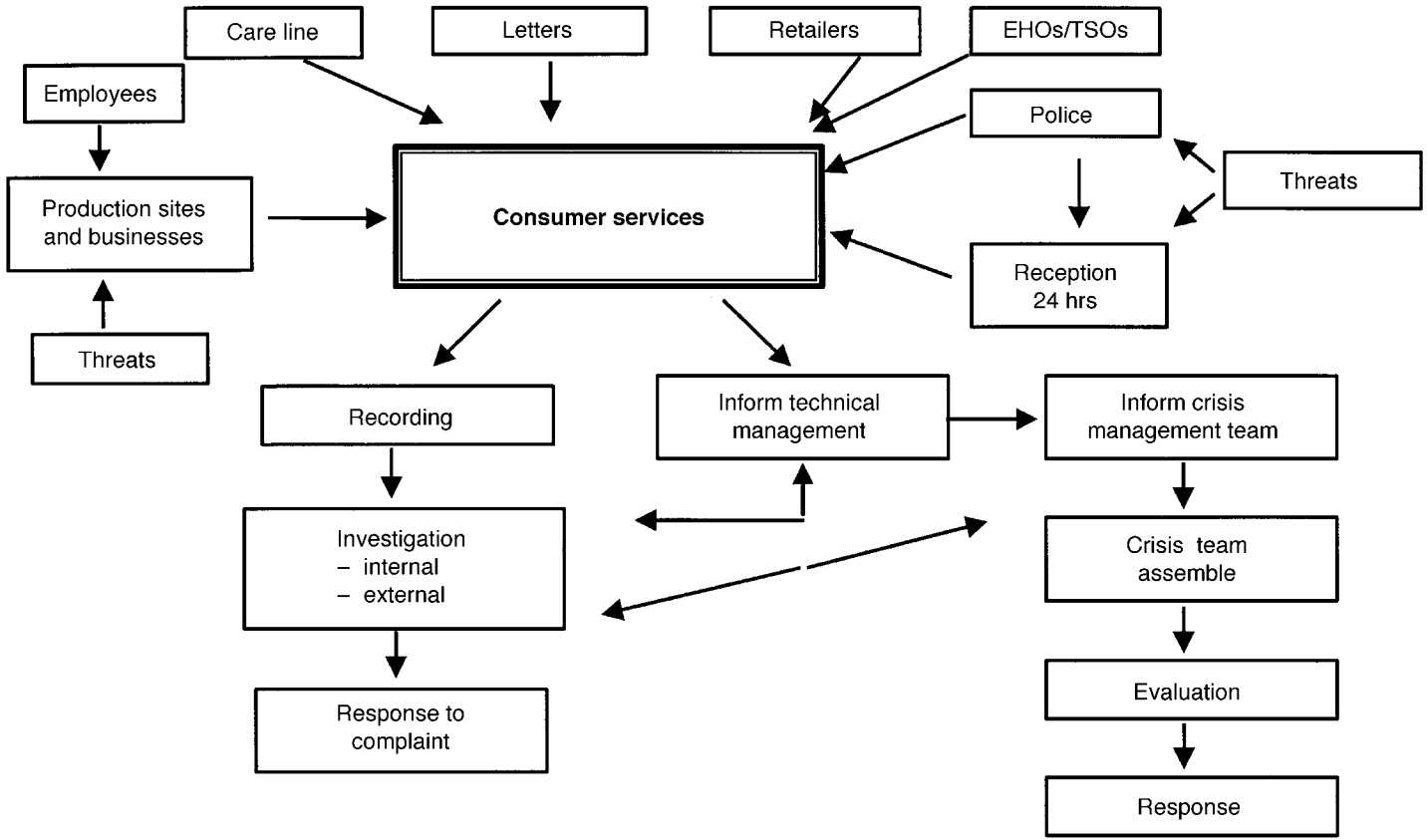

Fig. 21.6 Flow diagram for co-ordination of complaints.

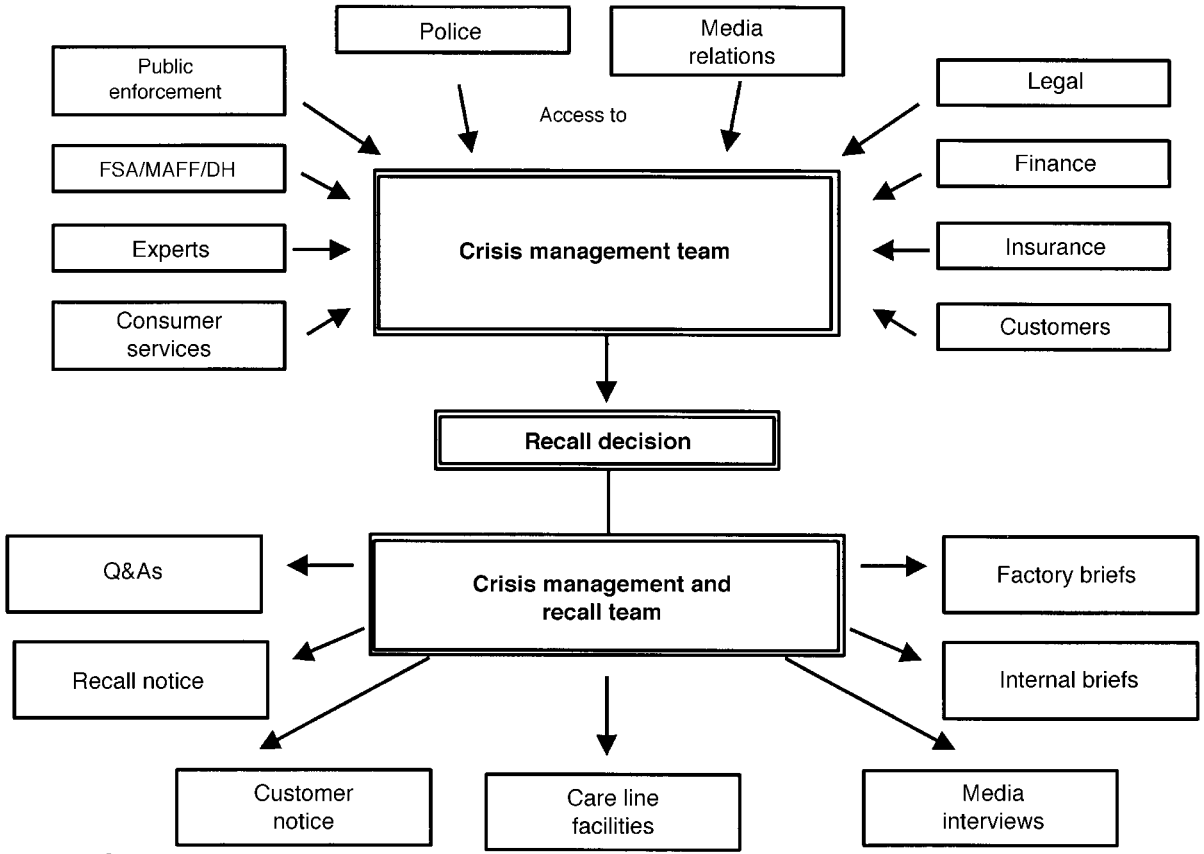

Fig. 21.7 Crisis management.

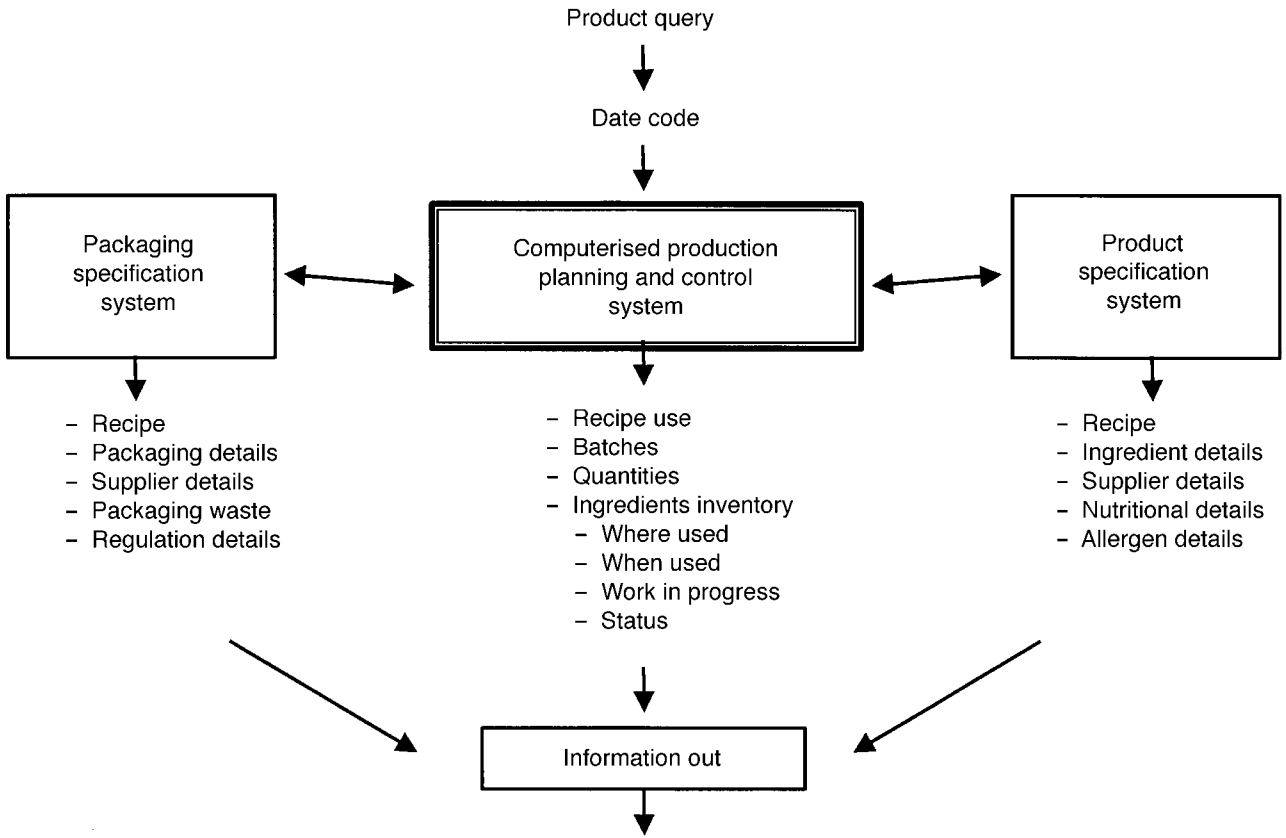

Fig. 21.8 Traceability and computerised management systems.

- full product description – brand name, pack size
- identification codes – computer codes, bar codes, batch codes, best before dates
- features to distinguish from similar products
- nature of problem
- reassurance and steps to take if product already consumed
- how to return/dispose of product
- how to claim
- potential links to other products/issues
- potential links to previous company's issues.

21.5 Summary

The key objectives for a comprehensive traceability scheme have been described.

- In the EU laws requiring clear on-pack batch 'lot' marking and durability codes have been strengthened or introduced over the past 10 years. This has served to increase the free movement of foodstuffs and trade, but also to facilitate product recalls for safety reasons.
- Manufacturers and brand owners have generally improved their traceability schemes well beyond legal requirements. This has been both to minimise losses in the event of a recall, and also as part of increasingly efficient management control systems. This has been aided by advances in modern technology and computerised systems.
- Consumer demands for greater transparency in labelling, coupled with more sophisticated products, products with particular nutritive, environmental, or ethical attitudes, are resulting in a proliferation of label claims, necessitating ever more authenticity verification, in turn placing increasing demands on traceability.

21.6 References

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4. British Standards Institution, ISO9000, *Quality Management and Quality Assurance Standards*: *Part 1 – Guidelines for selection and use, 1994; Part 2 – Generic guideline for the application if ISO9001, ISO9002 and ISO9003*, 1997, London.

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22

Developing traceability systems across the supply chain

A. Furness and K. A. Osman, Centre for Automatic Identification and Intelligent Systems, Technology Innovation Centre, UK

22.1 Introduction

Traceability is now emerging as a 'watch-word' for consumer and regulatory confidence with respect to food quality, food safety and the infrastructure for producing, processing and delivering food products from the point of origin to the point of sale. Various definitions have been derived for traceability, including a European Union (EU) General Food Law Regulation definition in which traceability is defined as: 'the ability to trace and follow a food, feed, food-producing animal or substance through all stages of production and distribution' (EU Regulation Food Law: 8/5/01). An International Standards Organisation (ISO) definition is also to be found that defines traceability as: 'the ability to trace the history, application or location of an entity by means of recorded information' (ISO 8402:1994). The ISO definition, whilst more general in terms of the traceable entity, draws attention to the importance of recorded information that is essential for satisfying traceability requirements.

The need for traceability has arisen primarily from consumer and government concerns over food safety, hygiene and authenticity. These concerns range from the human risks associated with animal-borne pathogenic organisms, such as salmonella, listeria, clostridium and E-coli O157 through agents causing bovine spongiform encephalitis (BSE) to those risks perceived for genetically modified (GM) vegetables, cereals, fruits and GM influenced foods in general. As a consequence of national needs and sectoral responses for traceability, sensitised by developments in global trade and consumer demands, a number of traceability guidelines have been developed. Notably these guidelines include the Traceability of Fish – Application of EAN.UCC Standards (EAN International); Traceability of Beef Guidelines (EMEG); Fresh Produce

Traceability Guidelines (EAN International); Traceability in the Supply Chain (GENCOD EAN France) and Traceability Implementation (EAN.UCC project). In response to initiatives such as these and to the burgeoning requirements for legislative compliance, increasing numbers of representative bodies for food sector supply chains are deriving and implementing traceability systems. With varying degrees of supply chain coverage and an emphasis on particular foodstuffs such as fish, meat and wine, such systems can satisfy some traceability requirements for these products but cannot readily be expanded to encompass other products. As an increasing number of supply chains attempt to develop sector-specific traceability systems, problems will inevitably occur where the ability to connect across different supply chain boundaries will be essential to provide traceability of multi-ingredient food products. A fruit pie, for example, will have a multiplicity of ingredients stemming from many different supply chains.

Complexities will arise with respect to the range and variation in the accessible information required for traceability. They will also arise with respect to incompatibilities in systems' structures for transferring and accommodating appropriate information. While food safety and food quality may be seen as the primary drivers for traceability, other needs can and will be recognised for which traceability systems will be required. Food authentication, quality assurance, label verification, shrinkage (product loss) management, process development and consumer support are further areas in which traceability can be seen to be necessary, each characterised by particular functional needs that will increase overall complexity. This also draws attention to a system requirement for being able to distinguish and satisfy particular traceability functions using a common traceability system structure – separating traceability functions from system requirements.

It has to be recognised that the food production and supply industry is highly integrated and of global significance in terms of trading reach and trading opportunities. Harmonisation of traceability systems is required in order to avoid the almost inevitable chaos that could ensue through lack of compatibility and effective management of complexity. This becomes increasingly significant as traceability systems are structured to make more and more use of information and communications technology (ICT) and the opportunities for powerful and effective information transfer across communication networks such as the Internet.

Aspects of commonality and moves towards exploiting existing standards for numbering and identification are, fortunately, evident in many of the systems under development. So too are developments in electronic data communications for business support purposes, offering potential for integration into traceability systems. Despite this attention to legacy, the need is still evident for deriving a generic approach to traceability that can handle cross-supply chain interaction and provide a logical framework for harmonisation and systems compatibility. Any approach would also have to support a growing list of traceability functions and allow effective management of supply chain complexity.

Underpinning this generic approach is a deeper understanding of the nature of traceability and the factors influencing the need to implement traceability functions. Firstly, it is important to consider further why traceability is required, including the factors influencing the implementation of traceability systems. Food safety is the primary reason for traceability and various functional components can be distinguished. The UK Food Agency has identified the following functional roles for traceability within the food supply chain:[1]

- **Food safety incidents** – requiring robust traceability to facilitate rapid response to breakdowns in food safety, allowing remedial actions, such as product withdrawals and recalls to be initiated for the purposes of protecting public safety.
- **Food residue surveillance programmes** – using traceability systems to facilitate food sampling at appropriate points throughout the food supply chain, testing for residues, such as pesticides, and mapping to establish where in the supply chain excessive residue levels may have occurred.
- **Risk assessment from food exposure** – where a traceability system can facilitate access to information concerning foods or food ingredients that may have significance with respect to food safety.
- **Enforcement of labelling claims** – using traceability to help resolve allegations of false labelling and help to determine supply chain integrity with respect to food claims.
- **Fraud** – wherein effective traceability, regular audit and reconciliation measures can assist in preventing fraud and theft of food items.
- **Food wastage** – where traceability and associated quality control systems can be applied to speed up and improve food distribution processes and reduce food wastage.
- **Meat hygiene** – where traceability can help enforce and support meat hygiene in processing and handling of food within supply chains.

Other reasons for traceability, often associated with safety, include compliance with food legislation, quality assurance of food producing and handling processes, authentication of food items, process and supply chain development and consumer services in respect of food products. Each of these factors constitutes a traceability function requiring access to relevant, function-defined, item-related information. By suitable partitioning and coding of information into sets, the traceability functions may be more readily accommodated and linked to a common traceability structure.

A significant feature of a traceability system *per se* is that it should be continually available for traceability, yet is used only as and when required. As a stakeholder development requiring capital expenditure and expenditure to maintain, a traceability system may be viewed as an imposition, adding little or no value to the processes concerned. Where then is the incentive to implement

[1] Traceability in the Food Chain, UK Food Agency, Food Chain Strategy Division (Paper Note 02/02/01), 14.02.2002.

traceability? Legislation is clearly a driving influence in this respect, but hardly seen as an incentive, unless perceived with respect to consumer confidence. Having to comply with regulatory demands rather forces the issue. However, through appropriate consideration of process structure and functionality, traceability systems may be devised that not only fulfil the traceability requirement, but also add value to the processes concerned. Such developments invariably make use of item-attendant data carriers that allow automatic identification and data gathering and facilitate improvements in process efficiencies and quality. Such developments may also be seen to align with quality practice. The ISO 9000 series of Quality standards, ISO 9001 in particular, distinguishes traceability as a requirement for compliance. It requires that a product be traceable from a current stage of existence back through all its stages of manufacture or production by means of either paper-based or computer-based records, accurately and promptly produced for the purpose. By adding value to the processes an incentive-based approach to traceability can be promoted.

22.2 Accommodating multi-functional traceability requirements

In gaining further insight into the generic approach to accommodating multi-function traceability requirements, it is necessary to distinguish further between the traceability **system** and traceability **functions**. A traceability system is required to provide an unambiguous, uninterrupted means of physically tracing and tracking an item, and/or its constituent components, through and between the inter-linking nodes of a supply chain. A node is distinguished as a point in the chain in which the item is handled or processed in some way. To achieve such a system it is necessary for the item or constituent items concerned to be appropriately identified and that the identifiers provide linkage to the relevant item information that is stored remotely. The need for the linkage is to facilitate access to the item-specific information which in turn can be used for satisfying a traceability or process support function.

Traceability systems may be paper-based or structured to exploit the benefits of information and communications technology (ICT). While not losing sight of the need for paper-based alternatives it is logical to structure systems on technology that can provide more and more efficient and speedy traceability support. To achieve a fully integrated, harmonised approach to traceability requires effective identification of all items within supply chains; from raw materials, through product items, packaged product, logistical units to palletised units; with appropriate coded linkage and communication paths to appropriately coded information sources for satisfying the needs of the various traceability functions. Flexibility must be provided through partitioning of information. In some cases information may also accompany the item or items concerned. In general the quantity of information required to satisfy a traceability need will be

too large to be carried on or with the item. Moreover, various items of information may be required to satisfy a range of process support and traceability requirements. Generally, information concerning items will be held in appropriate databases. To accommodate the diversity and intrinsic complexity of the information requirements, information partitioning and item identification needs, it is necessary to determine the areas of commonality that exist with respect to traceability systems and derive a strategy and a framework that can exploit the features concerned.

All traceability systems, irrespective of supply chain items, industry affiliation and functions supported, can be seen to exhibit common structural features:

- **Item identification**, unambiguous and linkable for accommodating processing and handling in the supply chain.
- **Item-attendant and/or item-associated information** appropriate to nodal transforms and transactions and any inter-nodal events that have a bearing upon traceability.
- **Process-based information** relating and linked to items processed or handled in the supply chain.
- **Communication** links to allow access and exchange of information.

Based upon these features of commonality it is possible to construct a generic structure for a traceability system characterised by a vertical item and data flow (up and down the supply chain) facility and a transverse (supply chain inter-nodal) data gathering, data flow and storage facility. The vertical structure would be minimalist in the sense that it would exploit the simplest of item identifiers and data carriers and provide the necessary code identifier transfer and access links to transverse information stores to support the traceability requirements. The transverse structures accommodate the wealth of item information required for traceability and process support purposes, but appropriately coded into information sets to serve the respective traceability or process functions. The next step in defining a generic framework for traceability is to define the means by which the vertical and transverse components can be inter-linked.

22.2.1 Vertical and transverse inter-linking

The essential features of the generic framework, for a single node in a supply chain, depicting the vertical and transverse structure are illustrated in Fig. 22.1. It is significant in respect of the core item and process entities to distinguish essential information (possibly object-orientated) for more efficient access and routeing to additional information. By including appropriate data carrier support for items between nodes the facility can also be provided for better control and performance of inter-nodal events. As a consequence, further information may be generated that must be carried with or associated with items or communicated directly to appropriate nodal information management systems by mobile

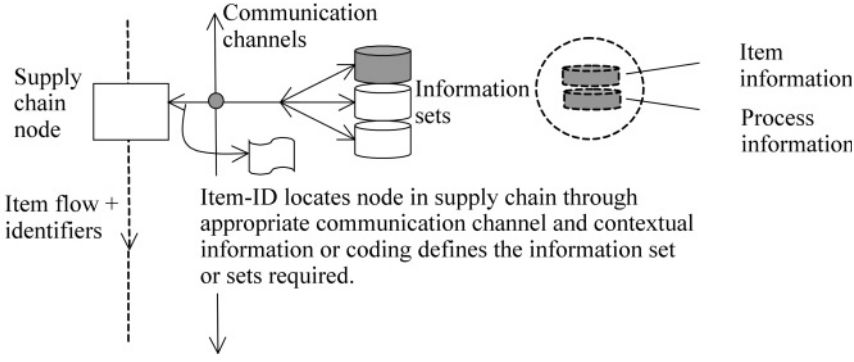

Fig. 22.1 Vertical and transverse inter-linking in the supply chain.

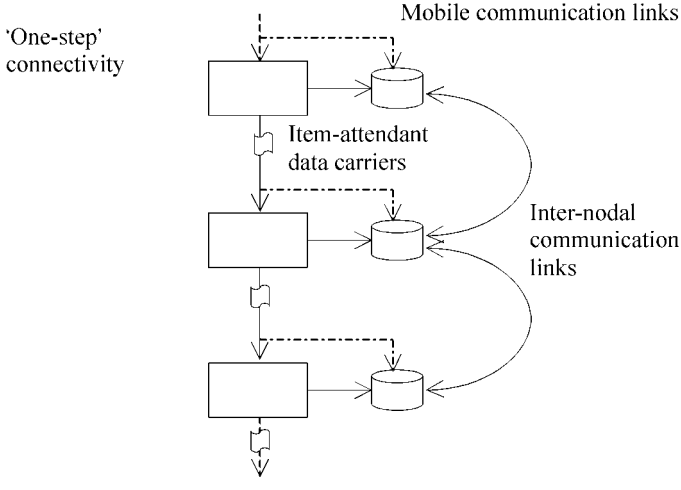

Fig. 22.2 One-step connectivity in a traceability system.

communications (Fig. 22.2). These 'one-step' links not only support linkage for traceability purposes, they can also support the flow of information between adjacent nodes for the purposes of added-value process functionality, transaction and supply chain management. The 'one-step' look-forward, look-back structure, appropriately interfaced and extended, may also facilitate first layer, node-to-node ('daisy-chain') communication linking along the supply chain. The information storage facilities and associated information management systems (IMS) may be viewed as traceability control and information support points.

Within a minimalist traceability structure little if any information is conveyed with the item, the principal function of the item-attendant or item-accompanying data carriers being to provide item identification, which is structured and sufficient in form to allow linking and access to information stored in local or linked databases through appropriate communication channels. In some cases

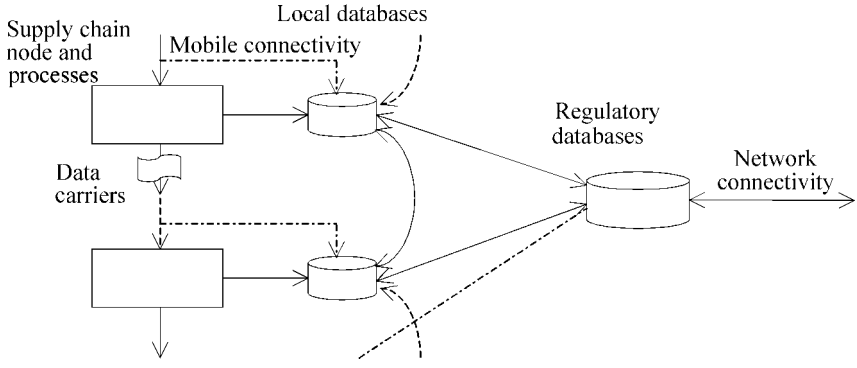

Fig. 22.3 Linking databases in a traceability system.

the need may be seen for item-attendant, machine-readable, portable data files that allow specific information to accompany the item, the nature and extent of the information depending upon item and nodal-specific needs. For example, a consignment of perishable goods may be accompanied by a data file that acts as a shipping manifest, containing information on both shipment contents and the dispatch time, destination and consignment handling details. This approach ensures that critical information is immediately available with the item as and when required, circumventing problems that might otherwise occur due to breakdown of communication links to remotely held data.

In some cases the amount of information stored in nodal databases could be substantial, particularly where a number of process and traceability functions have to be satisfied. This will almost certainly require the use of large relational databases and, in some instances, linkage with larger, national or other scheme-related databases. This introduces a further layer in the traceability infrastructure. To allow access to these databases and management systems for traceability purposes requires a network infrastructure (including use of the Internet) with appropriately authorised access control and communication protocols (Fig. 22.3).

In defining an infrastructure of this kind for traceability it is necessary to identify standard data structures, identifiers and both interface and communications protocols to meet the needs of different supply chains, with connectivity in and between supply chains and across national boundaries. The identifiers must include coding structures for location, information sets and access control as well as item identifiers.

22.2.2 Location requirements

Being able to identify the nodes, and any other item- and information-handling points in the supply chain, is an essential requirement for engineering a generic, fully integrated traceability system capable of handling appropriately coded and

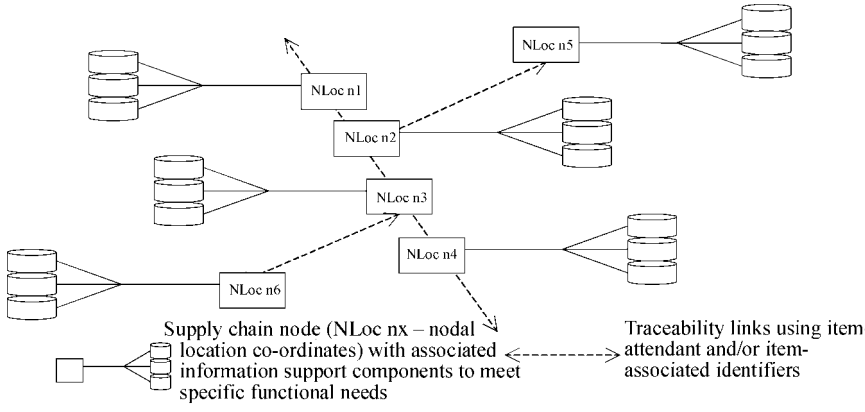

Fig. 22.4 Integrating location requirements into a traceability system.

accessible information sets (Fig. 22.4). Various agencies can be recognised for implementing location-based identification, including the EAN.UCC Global Location Number (GLN) and Global Positioning Systems (GPS) defined location codes. Both provide the facility for assigning a code to a particular legal, functional or physical, fixed positional location.

22.3 Item-specific data capture

At an origination node in a supply chain, or where new food items or ingredients enter the chain, it will generally be necessary to establish primary identification of food entities concerned for the purposes of authentication and onward identification. The primary identification techniques are many and varied, and are selected for use depending upon the food entity concerned. Typically techniques include DNA methods, protein and metabolic fingerprinting, NMR spectroscopy and a wide range of analytical techniques for determining food components, as described in other chapters of this book. From a traceability standpoint such techniques provide the means of individually identifying a particular food item on the basis of its intrinsic features, providing a biological 'foot-print' or profile that can be appropriately stored and accessed as required. On this basis any food entity, such as a batch of wheat, an animal, an item of fruit, vegetable or spice, entering the supply chain can be identified individually or by category. Linked by the primary identification is likely to be further information concerning the item that is held and this forms an accessible information set. To access this information remotely by electronic means may require an additional code or identifier.

Any other identifier used within the supply chain is secondary, and generally involves a 'unique' item, batch or other item-linked number or alpha-numeric string being assigned and attached to a selected item for the purposes of

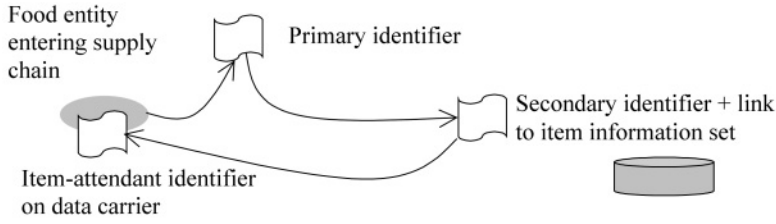

Fig. 22.5 Item-attendant identifiers in a traceability system.

traceability. Ideally, the secondary identifiers should be linked through the assigned coding structure to the primary identifier. In some cases the item-specific, feature-based, primary identifier will be coded to distinguish item type. Legislation is in prospect concerning coded identification of genetically modified organisms (GMO),[2] wherein specific identification codes will be assigned to authorised genetic transformations or modifications to be marketed or used in food products. A further aspect of secondary identification is the assignment of an identification code that can be used to facilitate on-going identification of the item and its handling in subsequent parts of the supply chain. For an ICT supported traceability system it is necessary for the identifier to be machine readable and ideally supported by an appropriate numbering and identification standard (such as the EAN.UCC system, considered later) (Fig. 22.5).

A larger part of the vertical traceability structure will inevitably be achieved using item-attendant identifiers, most probably using standard EAN.UCC numbering and identifier structures. Particular information will relate to items entering and items leaving nodal points along the supply chain. A node must have a link and knowledge of the appropriate item information at one nodal level above and one level below the node concerned in order to satisfy the lowest demand for traceability. The one-step above and one-step below node-to-node links should be seen as constituent links for traceability purposes, with traceability identifiers on item-attendant data carriers being used to achieve linkage. Node-to-node communication links will add to the robustness of linkage for traceability purposes, providing a degree of protection against link failure which would otherwise compromise the traceability chain.

To achieve the necessary harmonisation and intra-operability of traceability systems will require the use of standardised approaches to item identification, information handling and communications. Fortunately, there exists a legacy of standards that go a long way towards achieving the harmonisation and intra-operability required.

[2] Proposal for a Regulation of the European Parliament and the Council concerning traceability and labelling of genetically modified organisms and traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC.

22.4 The EAN.UCC coding system

A significant and widely used system for secondary item-attendant identification is the EAN.UCC System[3] of numbering and identification. The system also extends to identification of locations and services, and has already been applied in practical traceability systems and promoted through guidelines specifically directed at traceability.[4] Six standard numbering structures presently comprise the EAN.UCC System. These structures and their components are discussed below.

22.4.1 Global Trade Item Number (GTIN)

Formerly known as the Article Number, the GTIN is used to positively identify trade items. The trade item is recognised as any entity, product or service for which there is a need to retrieve pre-defined item-attendant data at any point within a supply chain. The definition embraces both items and items accommodated in different forms of packaging, for example multi-packs or case level products.

The GTIN itself is defined as a 14-digit number from which a family of four unique numbering structures is derived. The full 14-digit number system, known as the EAN/UCC-14 structure, is used to identify trade items that do not cross a retail point of sale. The structure comprises a leading (left side) 'indicator' digit (or logistical variant) followed by 12 digits for the identification of items. The final digit is a check digit, derived from the other 13 for the purposes of providing a level of error detection. The indicator digit allows each user to increase the numbering capacity when seeking to identify similar trade units accommodated in different packaging configurations. The number 9 in this position is reserved for identifying outer cases of items of continuously variable measure, usually weight.

EAN/UCC-13

The GTIN structure without the indicator digit represents a 13-digit number known as the EAN/UCC-13 structure. This and two other truncated structures are used to identify items by type at point-of-sale. The EAN.UCC-13 structure effectively comprises three parts: a company prefix number, an item reference number and a check digit. The company prefix number is made up of an EAN.UCC two or three prefix issued by one of the world-wide network of EAN member organisations to decentralise the administration of identification numbers. Each organisation has a characteristic number: 50 for the UK for example, 00 to 09 for the USA and Canada, 549 EAN-Iceland, 93 EAN-Australia and so forth, covering almost 100 countries. The company part of the number is assigned to the user by the numbering organisation or by the UCC,

[3] EAN – International Numbering Association, UCC – Universal Code Council.
[4] Traceability in the Supply Chain – From Strategy to Practice, GENCOD EAN France (2001).

depending upon whom the user approaches, and can be 7, 8 or 9 digits in length depending upon the user's numbering requirements. The prefix together with the company number form the company prefix number. Within the EAN/UCC-13 structure 12 digits comprise the company prefix number and the item reference. Consequently, the more digits assigned to the company number the fewer are available for item reference, the latter being assigned by the user to distinguish particular items or products. For example:

- If the company number is 5012345, five digits are available for the item reference, allowing 100,000 items to be distinguished.
- If the company number is 50123456, four digits are available for the item reference, allowing 10,000 items to be distinguished.
- If the company number is 501234567, three digits are available for the item reference, allowing 1,000 items to be distinguished.

It is the system user's responsibility to ensure that the item reference number is selected to achieve a completed number that is unique for the item type or service being numbered. A check digit forms the final part of the structure and is calculated (automatically within system software) according to a specified algorithm and used to check for errors.

UCC-12 structure

The UCC-12 structure is similar in function and form to that of the EAN-13 structure, comprising a UCC company prefix, item reference and check digit. It is used within the USA and Canada for item type identification at the point of sale.

EAN/UCC-8 structure

The EAN/UCC-8 structure is essentially used for in-store item identification in which a truncated prefix can be used, together with an item reference and a check digit. The numbers so distinguished provide unique identification when processed in a 14-digit data field, zeros being used to fill the leading (left) field positions as appropriate for the EAN/UCC-13, UCC-12 and EAN/UCC-8 structures. It is the field format for GTIN used in all business transactions and EDI messaging supporting the standard.

Although the EAN.UCC system provides a number of structures for identification purposes it is insufficient for achieving unique identification of each and every individual item that crosses the point of sale. Other identifiers are required to achieve this level of granulation. One such scheme is being proposed by the MIT Auto-ID Center and considered by EAN.UCC. Known as the electronic product code (ePC), this scheme is based upon a 96-bit structure, but has still to be endorsed and promoted as a standard for item identification.

22.4.2 Serial Shipping Container Code (SSCC)

The Serial Shipping Container Code is an 18-digit code used to identify logistical units – shipping containers or transport units – the additional digits

over the GTIN being to accommodate larger item reference numbers and supplementary information. The leading digit (extreme left), called an extension digit, effectively increases the capacity of the SSCC and is used to qualify the application of the code by assigning values 0–9 in the data field according to specified rules. The remainder of the structure consists of an EAN.UCC company prefix, serial number and a check digit.

22.4.3 Global Location Number (GLN)

The Global Location Number provides a unique numbering system for locations. The Global Location Number (GLN) of EAN.UCC identifies business or organisational entities such as:

- **legal entities**: whole companies, subsidiaries or divisions such as supplier, customer, bank, forwarder and so forth
- **functional entities**: a specific department within a legal entity, e.g., accounting department
- **physical entities**: a particular room in a building, e.g., warehouse or warehouse gate, delivery point, transmission point.

Each location is allocated a world-wide unique identification number. Those GLNs are reference keys for retrieving information from databases such as postal address, region, telephone and fax numbers, contact person, bank account information, delivery requirements or restrictions.

The identification of locations by GLN is required to enable an efficient flow of goods and information between trading partners through EDI messages and other electronic data exchange systems, physical location marking and routeing information on logistic units. The use of GLN provides companies with a method of identifying locations, within and outside their company, that are:

- **unique**: with a simple structure, facilitating processing and transmission of data
- **multi-sectoral**: the non-significant characteristic of the EAN.UCC numbers allows any location to be identified and consequently any business regardless of its activity
- **international**: location numbers are unique world-wide. Moreover, the international network of EAN.UCC Numbering Organisations, covering about 100 countries, provides support in the local language.

22.4.4 Global Returnable Asset Identifier (GRAI)

The Global Returnable Asset Identifier is used to identify reusable entities such as containers and totes, which are normally used for the transportation and storage of goods. The structure is essentially 14 digits in length and accommodates the EAN/UCC-13 structure and an optional facility for adding a serial number up to 16 digits in length.

22.4.5 Global Individual Asset Identifier (GIAI)

The Global Individual Asset Identifier is used to identify uniquely an entity that is part of an inventory within a given company. The number, which accommodates the EAN.UCC company prefix, together with a variable length individual asset reference, is up to 30 digits in length.

22.4.6 Global Service Relation Number (GSRN)

The Global Service Relation Number is an 18-digit code comprising an EAN.UCC company prefix and a service reference used to identify the recipient of services from a service provider. It is not therefore identifying a particular person or legal entity but a relationship or action that requires an identification point for accommodating transaction data, for example.

22.4.7 Areas of application

These numbering structures effectively service five areas of application, namely the identification of trade items (item type), logistical units, assets, locations and service relations. While each may have value in structuring traceability systems the need remains for a standard, an extension of the EAN.UCC system that can support greater granularity in identifying individual items both within the supply chains and at point of sale. Batch codes are often incorporated into product labels but only in human readable form. For further traceability support the need can be seen for at least machine-readable batch code additions to food labels. Such coding is feasible using low-cost printable composite codes.

22.5 Data carrier technologies

Because of the diversity to be found in supply chains, a generic framework for traceability not only requires a standard approach to numbering and identification, but also requires a range of identifier carrier technologies that can provide flexibility in defining traceability systems. A range of item-attendant technologies can be identified for this purpose. Moreover, technologies may also be identified that can add further functionality and offer opportunities for engineering innovative solutions to supply chain problems. The item-attendant technologies may be grouped as follows:

- **data carrier technologies** – including linear bar codes, two-dimensional (multi-row bar code and matrix codes) and composite codes, contact and non-contact magnetic data carriers, contact memory and radio frequency identification (RFID) data carriers
- **location and locating technologies** – exploiting GLN by carriers including RFID and EANCOM, and active RFID real time locating systems (RTLS) and global positioning systems (GPS) locating technologies

- **communication technologies** – including wireless local area network (WLAN) technologies
- **sensory technologies** – exploiting at the item level developments in sensory and telemetry technologies
- **security technologies** – embracing a range of technologies for fraud prevention and security at packaging level.

Presently the data carrier technologies are the most significant for traceability purposes.

The simplest and most commonly used of the data carrier technologies is the linear bar code, where the data is encoded as a series of narrow and wide black bars and light spaces that can be readily printed onto paper, cardboard, plastic and other substrates by a variety of methods including thermal-transfer, direct-thermal, inkjet and dot-matrix printing. Linear bar codes are generally read by illuminating with a red light source, either by raster scanning a single-spot laser across the symbol or by illuminating with red light emitting diodes and using a charge-coupled device (CCD) detector, demodulating the reflected light to retrieve the modulated response and further decoding this to extract the data. A wide range of scanning devices is available, but all have a 'line-of-sight' constraint, i.e., the bar code must be visible so that it can be illuminated and scanned.

An extension of the linear (1D) bar code is the 2D bar code, either in the form of a multi-row or matrix symbol. PDF417 is a multi-row symbology that is commonly used to encode shipping details, bills of lading and so forth. Such symbols generally facilitate a large data payload in excess of 1,500 characters (Fig. 22.6). The low cost realisation of 2D codes renders them attractive for portable data file applications. For traceability purposes they may be used both to carry identifiers and item-assisted information. As such they can be useful in supply chain processes for real-time labelling and item tracking, particularly where items are being separated or combined with other items (e.g. ingredients for a food product). The printability of 2D codes using a variety of printing methods provides considerable flexibility for tackling in-chain labelling. Using inkjet technology 2D codes can even be printed onto foodstuffs, providing the ink is safe to use, is regulatory acceptable and the surface is suitable for accepting a printed symbol.

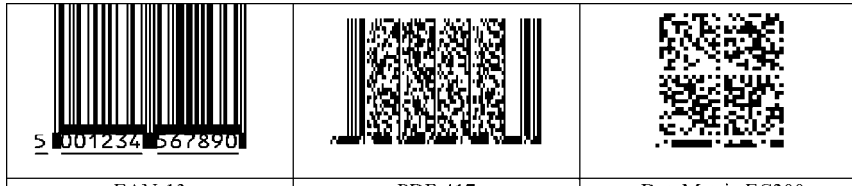

Fig. 22.6 Types of bar code.

An important feature of these data carriers is error control (error detection and error correction). Linear bar codes are typically printed with a human readable form and if for any reason the bar code cannot be decoded, a human operator can key in the human readable data. These also incorporate a check digit, which allows any decode errors to be detected. If a decode error is identified or the bar code is physically damaged, a human operator can key in the human readable text. Clearly for 2D bar codes, this is insufficient, so robust error detection and correction schemes have been developed. These allow errors up to a certain proportion of the payload to be both identified and corrected, at the expense of requiring more error correction code words to be incorporated into the symbol. Composite code structures are also available that exploit a combination of linear and multi-row bar coding.

22.5.1 EAN.UCC bar code data carriers and application identifiers

A number of bar code data carrier standards (symbologies) have been adopted as EAN.UCC standards for carrying system numbers and identifiers. The capability of being able to use the numbering structures in data carriers that can also allow further data to be added and distinguished in a standardised way offers considerable flexibility in supporting item-attendant data handling and process improvement/innovation. The data carriers adopted for EAN.UCC system applications, that are now well established, presently comprise linear bar code symbols supported by the following standard symbology specifications (the rules that determine how a bar code is structured):

- EAN.UPC symbologies including UPC-A and UPC-E, EAN-13, EAN-8. These are symbologies specifically designed for omni-directional scanning at point-of-sale retail outlets and constitute the standard for use on items scanned in this way. The symbols may also be used on other trade items.
- Interleaved Two-of-Five (ITF) symbology for symbols carrying identification numbers on trade items not for scanning at retail outlets. The symbology is particularly suited for printing directly onto corrugated fibre-board and similar substrates. However, in contrast to EAN.UCC standard symbologies, the ITF is not exclusively licensed.
- UCC/EAN-128 symbology, a particular variant of Code 128, is exclusively licensed to EAN.UCC as the symbology supporting systems applications in which the system numbering and application identifiers are exploited. It is a variable length, alpha-numeric symbology offering considerable flexibility for identifying and handling item-attendant data. The symbols are not intended to be scanned at point of retail but within other areas of supply chain and industrial activity.

A reading device for EAN/UCC symbologies usually carries the facility to generate, on reading a bar code symbol, a symbology identifier to be transmitted along with the element string as a means of distinguishing between the different EAN.UCC data structures and those of other bar code symbologies. Such

Extract from the list of EAN application identifiers (AIs)

AI	Encoded data content	Format
00	Serial Shipping Container Code	n2 + n18
01	Global Trade Item Number (GTIN)	n2 + n14
10	Batch or Lot Number	n2 + an..20
13	Packing Date (YYMMDD)	n2 + n6
15	Minimum Durability Date (YYMMDD)	n2 + n6
21	Serial Number	n2 + an..20
30	Variable Count	n2 + n..8
310x	Net Weight in kg	n4 + n6
400	Customer's Purchase Order Number	n3 + an..30
410	'Ship to – Deliver to' EAN.UCC GLN	n3 + n13
421	'Ship to – Deliver to' Postal Code with 3-digit ISO Country Code	n3 + n3 + an..9
...		

n – numeric; an – alpha numeric

Fig. 22.7 Examples of application identifiers in the EAN.UCC system.

facilities are of course important for achieving automatic processing of data, particularly for transactions and EDI message formatting. The EAN.UCC system also defines a range of over 90, two, three and four digit application identifiers (AIs) and so provides a framework for supporting the identification of application measures (Fig. 22.7). AIs are also available to identify features such as logistics units expressed as a serial shipping container code (SSCC), batch and lot numbers, serial numbers, production and packaging dates to name but a few. A data format is specified for each AI to indicate the number and disposition of numeric and alpha characters. The AI to denote the identification of a logistic unit comprising the SSCC is 00, having the format n2+n18, two digits (n) for the AI and 18 for the SSCC. An example of an alpha-numeric AI is for a lot number (AI = 10) having the format n2+an..20, denoting two digits for the AI and up to 20 alpha-numeric (an) characters.

The AIs for measures are grouped into metric and non-metric trade item measures and metric and non-metric logistic item measures for parameters such as weights, lengths, areas and volumes. While numbering and identifiers constitute the means whereby traceability can be implemented, functional information sources with appropriate access codes are required to exploit the traceability expedient. Also required is a vehicle for partitioning information according to process and communication needs.

More recent additions to the EAN.UCC printable range of data carriers are the reduced space symbologies (RSS) and composite symbologies, examples of which are illustrated in Fig. 22.8. These symbologies have not yet achieved the impact that their potential offers but provide a useful extension to the printable data carriers available to support traceability. The RSS symbologies (which in effect comprise a group of RSS limited, stacked and expandable codes) are

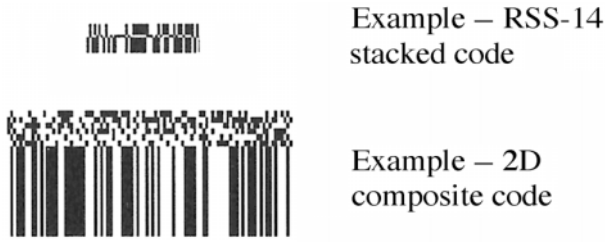

Fig. 22.8 Examples of reduced space EAN.UCC data carriers.

essentially high-density linear bar codes designed to accommodate the EAN.UCC system numbering, encoding up to 14 digits in a very small 'footprint'. The expandable RSS symbols can accommodate additional data. The composite symbols comprise a linear (UPC/EAN, RSS or EAN128) symbol paired and in some cases data linked to a 2D symbol and printed immediately above the linear component. The 2D component is either a PDF417 or MicroPDF417 symbol, with data capacities ranging from 56 to 2,361 digits according to choice.

22.5.2 Radio-Frequency Identification (RFID)

Bar codes have two potential limitations relating to the line-of-sight constraint and the fixed nature of the data payload. Radio waves can, however, penetrate commonly used packaging materials such as paper, cardboard, plastic, and in conjunction with suitable semiconductor 'chips' can be configured to provide RFID data carriers or 'tags'. Such tags can be read from and written to using an appropriate radio-frequency carrier and modulation scheme and in the case of passive or battery-less tags, the RF carrier also powers the tag.

Although RF tags therefore appear to offer a number of advantages over bar codes, their use introduces other complexities and constraints that must be recognised. Firstly, RF tags containing semiconductor chips will always be more expensive than bar code technology, and it is difficult to see the business case for replacing bar codes on low-cost items. Secondly, the EAN.UCC system is a standard that is usable world-wide, with users in China for example being confident that they can scan an EAN bar code printed in the UK because the EAN.UCC standards define both the numbering system and the data carrier. RFID currently has five frequency bands that are used, but not all frequencies are usable world-wide and there are also regional differences in the allowable maximum power levels. ISO is standardising the air-interface for different tag frequencies under ISO 18000 parts 1–6 and work is also under way through ISO to define data content identifiers and data syntax for RFID tags. Also being pursued with respect to the ISO 18000 series of standards developments is a structure aligned to accommodating EAN.UCC systems requirements (GTAG).

It is clear that RFID has great potential in many applications where line-of-sight or read-write data is required. For example, the animal tagging standards ISO 11784/5 define the code structures and technical concepts for the tagging of

animals using low-frequency RFID tags. For many animals an ear-tag is used but for cattle, bolus tags, which lodge within the rumen, can also be used.

22.6 Linking item-attendant data and database information

Wireless communications and the Internet enable remote access to previously inconceivable quantities of information. Given our newfound freedom to communicate between remote data sources, the 'licence plate' approach to accessing information within a traceability system may be viewed as the single vehicle for accessing data held remotely. Every entity would be reduced to a unique number and all data relating to this entity would be held in a single database or a series of distributed and linked databases. All the required information would be accessible and updatable from any point at which the unique identifier can be entered to query the remote servers. Unfortunately this is not always a practical proposition.

When considering the appropriate use of connectivity in a given application there is a need to establish the balance between item-attendant data payloads and accessibility of data from remote databases through the available connectivity. It is often essential to guarantee a predictable response time to a remote request for information and to be able to safeguard the application against failure due to disruption or loss of connectivity. Where there is an opportunity or a requirement to exploit connectivity and access data remotely but performance constraints (such as a predictable and short transaction time) counter-indicate the use of direct information access, consideration should be given to the local caching of data or information in a suitable data carrier on the item itself. This will reduce significantly the demands on remote host bandwidth and reduce problems due to loss of connectivity or slow response times. By recognising the capabilities for using connect, cache and carry, independently or in combination with connectivity, considerable flexibility can be realised in distributed solutions for the management of item related data.

As with the data carriers for satisfying the vertical inter-linking needs to achieve traceability through use of a standardised approach to identification, so too there is a need for a standardised approach to data exchange and the accessing of information. A significant feature of any traceability system is the facility for communication and information exchange. Electronic data interchange (EDI) has for some time been applied as a fast and reliable means of achieving electronic, computer-to-computer exchange of information between trading partners with a supply chain legacy based upon the use of the EANCOM® language (a subsystem of the EDIFACT (Electronic Data Interchange for Administration, Commerce and Transport)). Some of the 47 message structures provided in this standard have relevance to traceability, including shipping notice (DESADV), product information (PRODAT), receiving notice (RECADV), transportation status (IFTSTA) and inventory status (INVRPT).

The advent of the Internet and mobile data communications have provided important new dimensions for communications and information exchange that can be readily applied for traceability purposes. They emerge as timely vehicles for helping to accommodate the developments in globalisation of trade and the growing diversity in supply chain structures, and developments are now in prospect for exploiting XML (eXtensible Markup Language) as a facility for supporting traceability communications.

22.7 The FOODTRACE project

FOODTRACE is a fifth framework European Concerted Action project, the aim of which is to establish a generic framework for harmonised supply, and cross-supply, chain traceability. The primary need[5] to be accommodated was a technology-independent, but technologically supportable, identification scheme for achieving traceability. This would allow developing countries the facility to specify traceability systems that allow migration from rudimentary supported systems to the technologically supported systems achievable in the more developed countries. The essence of such a scheme resides in agreement upon numbering and identification schemes, of which the EAN.UCC system is internationally recognised and, moreover, is supported by adopted technology for carrying data. However, it was recognised that the EAN.UCC legacy, although a core component of such a framework, would undoubtedly require further development to accommodate needs arising from developments in handling various aspects of system infrastructure development, including enhanced granulation of item identification and coding structures to allow identification of supply chains, primary identifier profiles, information sets and access control for multi-function traceability.

22.7.1 Coding and access strategy

A significant work item within FOODTRACE was to define the overall information and access strategies for the generic framework. In defining a 'vertical' minimalist structure for using licence-plate coding links for and between supply chains the need was seen for coding that links the 'vertical' structure to the 'lateral' nodal structures for storing supply chain information relevant to the particular supply chain and the various traceability functions that need to be supported.

The data structures being proposed for use within the supply chain traceability systems for inclusion in item-attendant data carriers will comprise a set of item identification, information identifiers and access codes, including:

[5] Primary need – that need which if unfulfilled compromises the target solution even though all other needs may be taken into consideration within the framework.

- **Supply chain coding** – codes to identify particular supply chains.
- **Nodal location coding** – codes to identify supply chain nodes and inter-nodal locations where significant processing of food items occurs. It is envisaged that these codes will use or be based upon the EAN.UCC numbering and identification system for location coding.
- **Item identification coding** – codes that will use or be based upon the EAN.UCC numbering and identification system. The need to extend this system is being considered to allow identification of individual food items, from ingredients to products and product variants. Each component in the realisation of products will need to be identified and linked to the parent source or sources of constituent parts and any immediately previous nodal coding.
- **Information set coding** – codes to distinguish nodal information sets corresponding to particular traceability and support functions. It is envisaged that these codes will feature as possible extensions to the EAN.UCC numbering and identification system application identifiers.
- **Access coding** – codes to allow access to information sets, with a default level to general information within sets and priority levels to control access to particular components of information.
- **National location registers** – to register at national level supply chain and nodal location codes, together with traceability function codes. It is envisaged that such registers would be accessible through the Internet and eventually offer international linkage for traceability purposes.
- **Label-based product identifiers** – machine-readable coding for information access through the national location registers, allowing access to general information. It is envisaged that the label coding would include the item identification, supply chain code and the last nodal location code. By reading the code the user would be linked to the last node through which the product came and to the general information relevant to that item. The register site would support access to other information in the supply chains through use of appropriate access and traceability function codes. This would allow traceability investigators with the appropriate access codes to access particular traceability information stored in nodal-based information sets along the supply chain. Similarly, commercial information may be accessed by means of priority access codes. Item information would be accessible at any node within the supply chain using item and access codes to constituent information sets.

By way of example, a label upon an item taken at the point of sale or consumption would allow access to a National Location Register or last node at which the item was realised, wherein general information on the item could be obtained, together with last nodal location at which the item was realised if not included in the label. Access to the nodal location would provide information on what went into the product or how it was processed. It would also identify the nodal locations corresponding to each of the component parts of the item or the

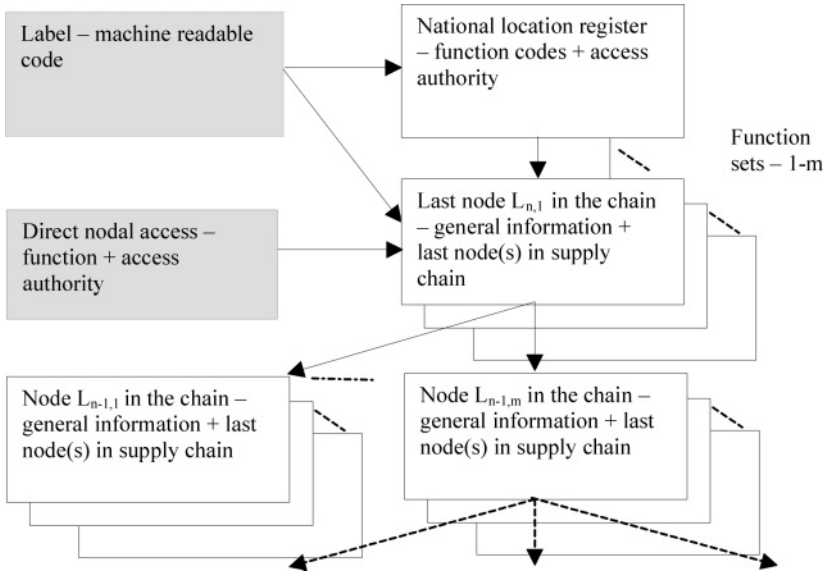

Fig. 22.9 The structure of the FOODTRACE system.

processes linked to this stage in the supply chain. Access to other nodes within the chain would similarly provide constituent and process information corresponding to the item or items concerned. The level of available information would be determined by the access codes available to the investigator and the function information sets supported. Rapid access tooling would allow rapid listing of constituents and locations corresponding to products produced and transferred through the chain (Fig. 22.9).

22.7.2 The need for a universal data appliance protocol

In the absence of a universal data carrier or a range of standards-supported data carriers there is a need for a universal protocol and interface platform to accommodate the diversity of data carrier and other item attendant technologies available for realising traceability systems. The disparate nature of the technologies and the associated products present problems in interfacing different products to different software systems. A common protocol to accommodate these differences is therefore seen as an essential requirement for optimising supply chain systems. Moreover, the need is seen for a flexible protocol that can accommodate the consequences of change and facilitate migration, as appropriate, to more advanced systems and systems intelligence.

The need for a universal method of integrating or connecting different item-attendant or associated appliances into systems is already being seen and accommodated through proprietary developments. From a generic traceability standpoint it is important to consider the need for an industry-wide standard. The basis for this assertion resides in the need to specify:

- a common interface between back-end enterprise software and a disparate range of item-attendant data collection devices
- forward compatibility for new data collection devices and support technologies
- plug and play capabilities with respect to bar code, RFID and other automatic identification and data capture devices
- remote network management with optimised network reliability.

Developments in the XML-based approach to messaging may be seen as a highly extensible solution in this consideration, supporting the transfer of multiple elements in a single message.

Other features include:

- simple command structure that allows easy configuration and administration
- automatic recognition and registration of new devices on the network supporting a unique level of interoperability with different data collection devices
- flexible and extensible device facility that enables all the relevant characteristics of the devices on the network to be automatically described, including properties, methods and events
- automated monitoring of device availability and status
- alarm events to indicate problems with device functionality.

Herein lies the facility for a fully integrated traceability infrastructure.

22.8 Conclusions

The FOODTRACE generic framework, based upon the considerations outlined earlier, is essentially an attempt to provide a holistic approach to traceability of open systems by providing a significant degree of harmonisation and inter-operability but without sacrificing privacy of information and confidentiality where such is required.

Achieving a generic framework for harmonised traceability within and across supply chains is an important requirement. The approach being adopted through FOODTRACE distinguishes the minimalist vertical and lateral infrastructure and strategic, standardised coding of items, supply chain nodes, information sets and access systems. A significant legacy is in place to assist this process, including standards for numbering and identification and electronic data exchange. By building upon this legacy the opportunity is seen for realising the aim of the FOODTRACE initiative. Moreover, by appropriate attention to legacy and migration strategy the framework can be developed to accommodate existing traceability systems and future requirements with respect to traceability functions and emerging legislation through information set coding and access structures. Through developing and applying appropriate process methodology the traceability infrastructure may be developed in a way that adds value to

supply chain processes with attendant benefits with respect to food production, distribution and the reduction of waste.

The on-going deliverables of FOODTRACE will comprise a set of guidelines for assisting supply chain developers in structuring traceability systems to fulfil their individual requirements. Being generic the framework offers a schema for developing solutions that are supply chain specific yet offer interoperability as and where required, together with scope for process and supply chain innovation.

23

Developing and implementing an effective traceability and product recall system

M. Dillon and M. Thompson, Humber Institute of Food and Fisheries, UK

23.1 Introduction

Traceability of raw materials and food products has long been recognised as an important aspect of food safety. As an example, the UK 1990 Food Safety Act explicitly states, 'that food, not only for retail sale but throughout the *food chain*, must not have been rendered injurious to health; be unfit; or be so contaminated, whether by extraneous matter or otherwise, such that it would be unreasonable to expect it to be eaten'. The Act forms part of a framework of UK food legislation which includes:

- The Food Safety Act 1990
- UK Food Safety (General Food Hygiene) Regulations 1995
- Food and Environment Protection Act 1985
- Consumer Protection Act 1987
- Water Resources Act 1991
- 93/43/EEC General Food Hygiene Directive
- Health and Safety at Work Act 1974
- The Poisons Act 1972
- Control of Pollution Act 1974
- Control of Pesticides Regulations 1986
- Control of Substances Hazardous to Health Regulations 1988 (COSHH)
- Environment Act

The importance of product recall and traceability in food manufacturing and catering has been made even more pertinent by the introduction of EC regulation 0178/2002 which lays down the standards for the implementation

of European Food Law to Member States. This emphasises the need for improved Supply Chain Management and the need to protect food chains from terrorist attack in light of the incidents in America on 11th September 2001. The regulation requires traceability at all stages of the supply chain from the feed given to animals, for example, to the consumption of the final product by the consumer. It also integrates traceability into the implementation of wider food safety management systems such as HACCP. The dioxin incident in Belgium, the BSE and Foot and Mouth outbreaks in the UK have highlighted the importance of identifying and dealing with hazards early in the supply chain (IQPC 2000). The importance of tracing raw materials has also been emphasised by consumer concerns over genetically-modified organisms.

The introduction of internationally-recognised standards for food safety via multilateral trade agreements now has an increasing impact on issues such as traceability. The Sanitary and Phytosanitary (SPS) Agreement is a mechanism through which the World Trade Organisation (WTO) allows this process of standardisation in food safety to happen. Harmonisation, or equivalence, of legislation means that the systems which operate in the UK, for example, can be mirrored with suitable controls anywhere else in the world, allowing increased market access and movement of goods. The World Health Organisation (WHO) and Food and Agriculture Organisation (FAO) established the Codex Alimenterius Commission (CAC) in 1961 to protect the health of consumers, ensure fair practices in food trade, and produce common standards and codes of practice for adoption by international governmental and non-governmental organisations. To support the General Agreement on Tariff and Trade (GATT) agreement of 1997, the implementation of internationally agreed standards and guidelines allows the World Trade Organisation (WTO) to impose fines upon countries who fail to comply. The SPS agreement was devised to ensure common minimum standards of food safety. These standards have, in turn, influenced national legislation and the policies of national bodies such as the Food Standards Agency in the UK. The food industry in most countries is now required to:

- Hold accurate records of all ingredients within a product;
- Demonstrate procedures which provide traceability of ingredients and products;
- Provide Product Recall and Emergency Incident Procedures;
- Ensure that the ‘due diligence’ and precautionary principles apply.

Traceability of food is now an important aspect of a ‘due diligence’ defence, for example in providing courts with copies of product recall procedures and records. Simply ‘passing the buck’ back down the supply chain is widely seen as an insufficient defence in assessing whether a business has been negligent in its food safety responsibilities.

23.2 Building traceability in the supply chain: an example

The supply chain involved in producing a particular food is often complex. Figure 23.1 describes key steps and systems required in a relatively simple example: the production of fresh herbs. It summarises:

- key inputs into production
- the principal process stages, including distribution
- the relevant control points (CPs) which can be used to minimise potential hazards
- critical control points (CCPs) within a HACCP system which represent essential steps in eliminating or preventing food safety hazards
- relevant documentation detailing CP and CCP procedures
- relevant legislation (in the UK)

Figure 23.2 demonstrates the procedures used in selecting CPs and CCPs in this process, using the CODEX Decision Tree (Dillon and Griffith 2001) and Critical Path Analysis (Lockyer and Gordon 1995). As an example, the identification of a chemical Maximum Residue Limit (MRL) as a CCP at the harvest stage reflects the fact that this is the sole point where control can be exercised over a significant potential hazard for consumers. Prior to this, the application of fertiliser and pesticide chemicals is controlled by CPs governing exposure limits for the operator.

Legislation such as EC Regulation 0178/2002, 93/43/EEC General Food Hygiene Directive, Health and Safety at Work Act 1974 and the Food Safety Act 1990 require records to show that effective food safety systems have been established and are being correctly operated. These records used in this HACCP-based safety management system provide the basis for an effective treaceability system. As an example, those harvesting the crop will be responsible for the application of fertilisers and pesticides, particularly to 'hot spots' (e.g. weed proliferation) in the field which requires specific treatment. Application of chemicals is governed by agreed procedures and a system for recording chemical treatments as they are undertaken. These records allow 'hot spots' in individual fields to be both managed effectively and monitored. This in turn will allow the crop to be dispatched in compliance with regulatory and customer specifications with any problems quickly traced back to a particular 'hot spot'. Although this example considers only one section of the supply chain, the effective management of the whole chain will require common agreement on standards and protocols amongst stakeholders.

23.3 Key elements in a traceability system

A company should always use legislative, trade association or accepted industry practice as the standard for setting up and verifying a traceability management system (TMS). In particular:

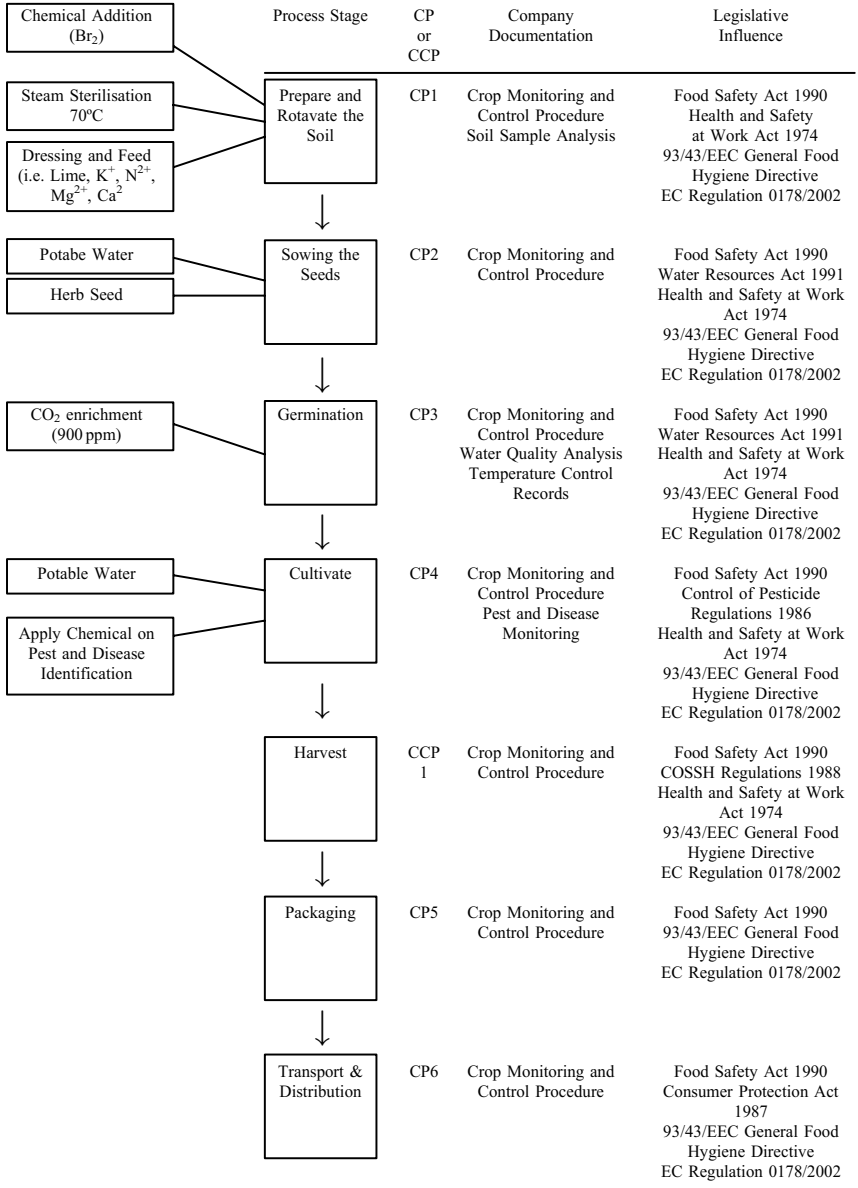

Fig. 23.1 A generic HACCP plan of fresh herb production.

- The business must have established procedures to trace all raw materials of a finished product by lot marking or batch code, and to identify the location of packaged product during distribution, to allow withdrawal or recall of any product retrospectively found to be out of specification.

Process Step	Hazard	Preventative Measure	Decision Tree Questions						
			1	1a	2	3	4	CCP?	CP?
Prepare the Soil	Glass	Visual inspection and random analysis	Y		N	Y	Y	N	Y
	Chemical	Soil analysis and extend preparation period	Y		N	Y	Y	N	Y
	Pest and Disease (P & D)	Treat with chemical application or biological control	Y		N	Y	Y	N	Y
Sowing the Seed	Chemical	Seed analysis	Y		N	Y	Y	N	Y
	P & D	Seed treatment	Y		N	Y	Y	N	Y
	Glass	Visual inspection			N	Y	Y	N	Y
Germination	P & D (Water)	Treat with chemical application or biological control	Y		N	Y	Y	N	Y
	Environment Control Lost	Monitoring records and readjust to required limits	Y		N	Y	Y	N	Y
	Chemical	Chemical application log	Y		N	Y	Y	N	Y
Cultivation	Chemical	Chemical application log	Y		N	Y	Y	N	Y
	P & D (Water)	Treat with chemical application or biological control	Y		N	Y	Y	N	Y
Harvest	Glass	Remove defective product and quarantine crop	Y		N	Y	Y	N	Y
	Chemical MRL	**Extend harvest interval**	**Y**		**Y**			**Y**	**N**
	Chemical OEL	**Extend harvest interval**	**Y**		**Y**			**Y**	**N**
	P & D	Treat with chemical application or biological control	Y		N	Y	Y	N	Y
Packaging	Glass	Remove effected packaging	Y		N	Y	Y	N	

Fig. 23.2 CCP justification: CODEX decision tree (Dillon and Griffith 2001).

- The business must ensure that a recall system, detailing key personnel, out of hours contacts and responsibilities for decision making is fully documented.
- The business must test the traceability system to prove relevant controls work, and test annually the product recall mechanism to ensure control remains effective.

The responsibility for the TMS should be divided amongst a multi-disciplinary team, including:

- Technical Manager/Quality Assurance (QA) Manager/Director
- Production Manager/Director
- IT Management
- Distribution and Transport Personnel
- Company Secretary/Director or Chief Executive

Raw material specifications must be established with suppliers and reviewed on a risk basis. Raw material suppliers should be audited frequently to ensure that their own quality and safety systems are adequate to meet raw material specifications. The Technical/QA Manager should hold all Product Specifications, inspection methods and results. An authorised member of the production team should inspect and authorise all incoming raw materials according to agreed procedures. These procedures may involve checking deliveries against order and delivery note details, visual or other types of inspection or testing, and posting a record of the delivery.

As raw materials are processed to become products, they need to be tracked by:

- Product code
- Pallet number
- Batch number

A database containing the above details should be maintained and accessed by the Technical and Production Manager to ensure a product can be traced through the system using these codes, maintaining product specifications and analysis results per product code and batch number. Raw materials and ingredients used should be included with the product code. Product destination and shipping codes should also link to product codes. All packs being dispatched should be assigned product codes and labelled with key information specified within the contract and product specification. The pack codes should match the product codes.

To ensure that a product may be recalled effectively in an emergency, the following information should be recorded:

- time and date of incident
- nature of the incident
- product affected
- what happened to any contaminant
- list of relevant tests and analyses carried out
- analysis reports

It is also essential to collect the following product information:

- Product code
- Number of cases affected
- Batch number
- Best before/use by date
- Pallet number

Using this information the emergency recall team should contact the appropriate company and ask for them to remove and recall all relevantly coded products back to the factory or source of origin of the problem.

23.4 Verifying control

Verification should ensure that:

- appropriate procedures are in place
- review systems and procedures are adequate for factory controls
- staff training is provided
- monitoring and challenge systems are designed to enable effective product recall

Verifying the effectiveness of a business' record-keeping and supply chain design is vital to provide evidence for a due diligence defence. An effective traceability system requires the following elements:

- Management Control
- Corrective Action Procedures
- Information Technology
- Verification Systems
- Factory and Operational Control
- Supplier Assurance
- Product Labelling

Table 23.1 provides an overview of the elements which should be considered when reviewing a traceability system, in the form of an audit checklist.

Testing and confirming the suitability and design of a given process is vital to ensure products are safe and suitably processed. The methodology described for validating the controls will involve theoretical evaluation of design, simulated checks of potential controls and factory and industry experience involving observations. Theoretical evidence and other information sources are used as a basis for validating effective design of controls.

Level 1: **Theory and Analysis** – identification of key data related to legislation, reference sources or previous experience or traditional methods related to the plan, PRP or CCP.

Table 23.1 Example content of verification of traceability control checklist

No.	Standard/Element/Requirement	Look At	Look For
1.0	Management Control Documented system for allocating roles and responsibility to ensure traceability and product recall systems operate effectively	• Product and Traceability ID Manual/ Procedures • Significance Determination Procedures • Legislation	• Scope of Manual and Procedure – RM, GMO, Allergen • Linkage to Other Policy Statements, i.e. Allergen Free • Risk Assessment and Determination Justification • Emergency Recall Procedure Responsibility • Procedures Linked to Key Legislation, i.e. EU/ UK GMO, Labelling, Allergens, Pathogens • Copies of Key Legislation Available on Site
1.1	Management Review	• Review of Traceability	• Responsibility and Frequency
1.2	Contract Review	• Traceability Specification • Product Specification • Raw Material Specification	• Supplier and Finished Product Coding • Supplier Agreements • Customer Agreements
1.3	Traceability Responsibility	• Organisation Chart • Job Descriptions • Resource Planning and Budgeting	• Responsibility Allocated • Roles Clearly Defined Against Procedure • Budgets and Targets with Management Review
1.4	Training and Experience	• Company Training Plan • Records of Staff Training and Experience	• Training Records and Procedures • Evidence of Qualification, Training and Experience • Appropriate Labour Resource for Appropriate Tasks
1.5	Recall Manual	• Product Recall Manual Contents • Emergency Recall Procedure • Records • Scope of Recall – Target Contaminant	• Key Contents and Actions • Emergency Recall Team Experience • Records of Mock Recall and Actual Frequency • Designed According to Product and Raw Material
1.6	Risk Assessment	• Significance Determination • Logic Identification of Hazards and Risk	• Scope of Determination • Consideration of all Hazards • Risk Assessment and Justification
1.7	Traceability and Product ID to Finished Product Recipe	• Traceability and ID Procedure	• Scope of Procedure • Coding of all Raw, Intermediate and Finished Products • Supplier Agreement

Level 2: **Significance Analysis** – the use of methods and rationale for the use of identifying key data against the plan, CCP or PRP.

Level 3: **Predictive Techniques** – the appropriate modelling and statistical analysis for predicting the effectiveness of the investigated element.

Level 4: **Technical and Trials Analysis** – use of mock assessments, secondary/support methods to ensure the theoretical data is sufficiently accurate and precise to recall product or trace raw material at any point in the chain.

This activity is based upon the HACCP validation approach to demonstrate design of the system is adequate. The use of HACCP provides an effective management tool for the management of hazards and risks, for establishing critical control points within the supply chain (IQPC 2000).

23.5 Conclusions

Integration of the whole supply chain from field to fork needs input from each element. This requires feedback from the point of sale back through the chain. Dillon, Ogier and Thompson (2000) discuss the importance of building the missing link between the producer and the retailer. They demonstrate that effective feedback and continuous improvement can provide improved benefits for the product. These are not just economic, in terms of achieving best price through matching supply and demand, but for the issues of product quality, customer feedback and effecting trials for improving existing and future product carriage and consistency. Companies must be able to demonstrate a quality assured chain from 'field to fork'. Thus, the raw material must be able to be treated as a final product, even if it is used as an ingredient later in the supply chain and is additionally treated.

1. Evidence to demonstrate control within the supply chain must be available, to show understanding, awareness and control of the supply chain.
2. Supply chain management is an increasingly evolving issue and control is necessary to allow product recall in a crisis. Integration of the supply chain provides economic benefits and can be used to focus quality product improvement.
3. Currently many supply chains have several steps. There is a need to minimise the number of process operations to afford easier supply chain management. How this is achieved by industry requires economic evaluation to justify improved supply chain performance versus increased control.
4. The legislative pressures upon a given supply chain imply that members of the chain show 'due diligence' through the transfer of food products from one part of the chain to another.
5. HACCP should be adopted as a foundation for Supply Chain Management within the Food Chain. HACCP provides an effective building block and

useful management tool for assessing hazards and risks. It must be noted that HACCP as a management system should not be used alone, but itself integrated with existing management systems.

23.6 Sources of further information and advice

To find more information about traceability and product recall issues try using the websites given below:

www.foodstandards.gov.uk	UK Food Standards Agency Homepage
www.europa.eu.int	European Union Homepage
www.codex.org	Codex Alimentarius Commission Homepage
www.wto.org	World Trade Organisation
www.sofht.org.uk	Society of Food Hygiene and Technology Homepage
www.fao.org	Food and Agricultural Organisation Homepage
www.who.org	World Health Organisation Homepage
www.fao.org/ag/aga/agap/frg/feedsafety/feedsafety.htm	FAO Food and Feed Safety Gateway
www.iafi.org	International Association of Fish Inspectors
www.foodsafety.gov	US Govenment Food Safety Information
www.cieh.org.uk	Chartered Institute of Environmental Health

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24

Traceability in fish processing

E. Larsen, Danish Institute of Fisheries Research

24.1 Introduction: the fish sector

The fish sector is nearly always regarded as a separate sector in food processing with special working conditions. The product is perishable and its shelf life is limited. This has served as an excuse for not introducing traceability management and documentation systems with the same speed as other food producing sectors (Jónsdóttir *et al.*, 2000). The main reason given is that catches of a large number of small items, each with its own characteristic composition, are difficult to track. Different species of fish may be caught together. Even when species are separated, fish are handled in bulk by batch or lot, making it hard to track an individual item.

The world production of fish has been increasing the last 50 years as shown in Fig. 24.1. The most dramatic increase has happened in the aquaculture sector, which now is regarded as the only sector where future increase can be obtained. The wild caught fish sector is more or less exploited to the maximum. The fish processing sector can be divided into main groups:

1. the processing of wild caught fish and
2. the processing of farmed fish.

There are combinations between the two main groups, e.g. catching small wild tunas and keeping them in big net enclosures, feeding them for long periods before selling them at the right time to get the highest price. The processing of wild caught fish is normally divided in two main areas:

- processing of demersal (being or living near the sea-bottom) non-shoaling lean fish species
- pelagic (belonging to the upper layers of the open sea) shoaling fat fish species.

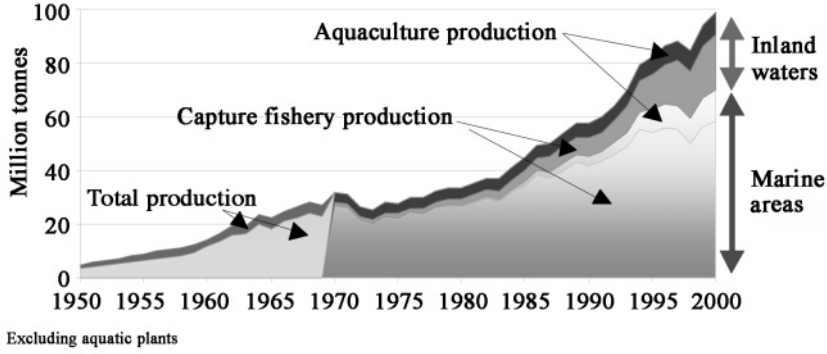

Fig. 24.1 Developing countries inland and marine fishery production. Total: 1950–2000; Capture/Aquaculture: 1970–2000. (FAO 2002).

The demersal fish species include such important species as the codfishes and the flatfishes. The catches of these fish normally consist of a variety of smaller and bigger fish, representing several year classes. Even if the fishes are caught in designated spawning areas such as the Norwegian Lofoten for cod, or the Southern Atlantic for hake, the individual fishes represent different sizes and conditions, before or after spawning, males or females. This demands a sorting process according to size or weight, which can be done in the fishing vessel or when landed. These operations often conflict with keeping track of the catching time and area, two important pieces of information for traceability systems. In very general terms the demersal fish species are iced in boxes on board the fishing vessel and then handed over to the next link in the chain when landed. In some hake, saithe and pacific pollack fishing operations the catches are processed and frozen on board the fishing vessel. These three groups of fishes have to be considered as semi demersal fish species because they shoal periodically.

Pelagic fishes include such important species as herring, sardines, mackerel and tuna. The catching of these fish species normally consists of large catches of fish that belong to the same year class. However, though more uniform, there are still important variations. As an example, Larsen *et al.* (1997) showed that there where significant differences in the fat content of herring caught in the North Sea and the Skagerrak. Fishing large catches makes it impossible to track each individual fish. Moreover, processing is normally based on making large quantities of the same product from bulk raw materials, such as cured herring, sardines in oil or smoked mackerel. Tuna is an exception, because it is such a big fish with a particularly high value compared to the other fatty fishes. This had made tuna one of the first fish that has been labelled individually so the buyers can trace the fish back to the catching vessel. Other fish that have had individual traceability for some time include wild caught Atlantic and Baltic salmon, because of a very strict quota system, which is based on the number of fishes and

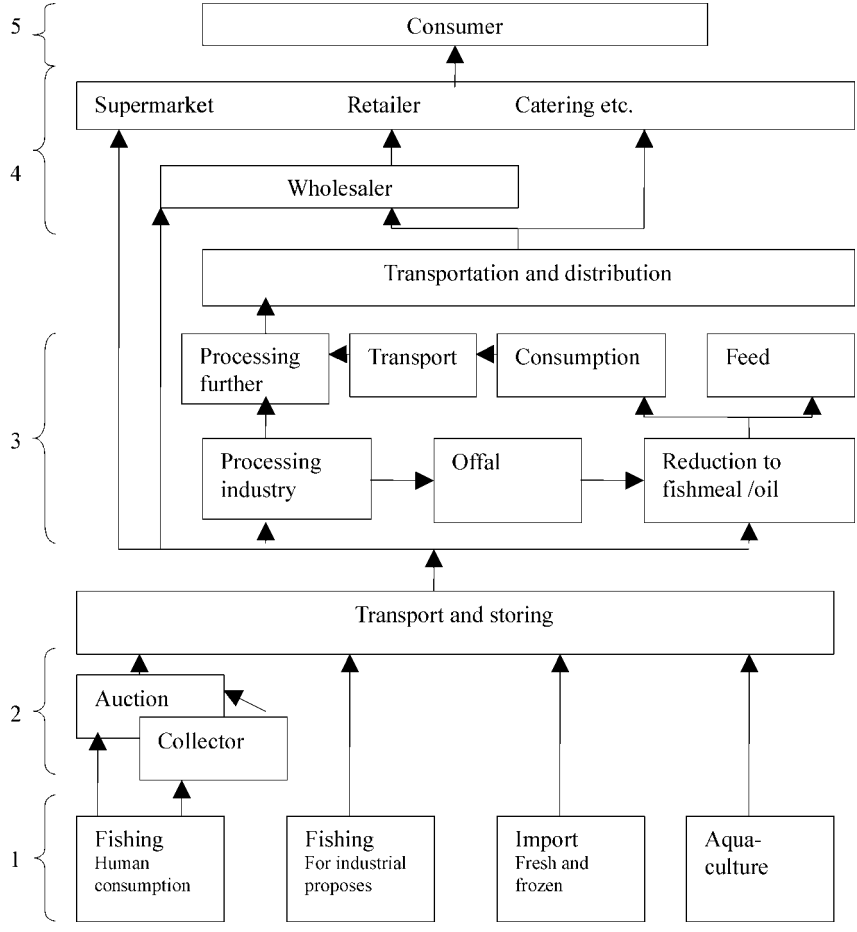

Fig. 24.2 Flow diagram of the Danish fish sector, divided into five main areas: 1) primary producers, 2) first sale, 3) processing, 4) final sale and 5) consumption. Transportation is not regarded as an area here.

not on the amount of fishes in tons as in other fisheries. To illustrate the complexity of the fish sector, Fig 24.2 shows the product flow of a typical fishing, processing and exporting country. Every box in this figure will represent a link in the chain from catch to the consumer. The ownership of the raw material may change several times during this flow diagram.

There are several definitions of traceability. The best known is in the ISO 9000 definition:

> Traceability is the ability to trace the history, application or location of that which is under consideration. When considering a product, traceability can relate to, 1) the origin of the product, 2) the processing

history and 3) the distribution and location of the product after delivery. (ISO, 2000)

There is a simpler definition from EU regulations:

> The ability to trace and follow a food, feed, food producing animal or substance, through all stages of production and distribution.

It would be in place to define track and trace. It is now more or less understood that tracking is done forward, e.g. from the producer to the end user, sometimes described as downstream. Tracing is done backwards from the end user to the primary producer, which can be described as upstream. This is not an official definition, but that is how the words are used in the fish sector (Børresen *et al.*, 2003).

Each chain or step is the production process consists of links representing the different stakeholders in the chain (Moe, 1998). An example of a chain could be the production chain in a factory. In broad terms the raw material starts at the factory gate where it is inspected, then placed in storage, taken out to be processed through a specific number of unit operations, ending up in the final storage. When the product is sold it joins the wider supply chain.

24.2 Recent legislation in Europe and the rest of the world regarding traceability

The two first years of the 21st century have seen an ongoing dispute particularly between the European Union and the United States of America. In 2001 the European Commission approved a memorandum requiring traceability and labelling for genetically-modified foods (Clapp, 2001 and 2002). One other reason for this dispute is the difference in defining the concept of traceability. The Americans prefer the concept 'traceback', used in illness outbreak investigations and food recalls. Whilst Americans emphasise traceback in the case of threats to the general health of the public, the Europeans want a system where the consumers are able to make their own choice of the origin of the product before buying it. EU legislation on traceability in the fish sector came into action in January 2002 (Councils decision no. 104/2000 17 December 1999), (Commission no. 2065/2001). From that date every fish product for purchase needs to be labelled with the following information:

- the product's commercial name according to a national list of names,
- the method of production, e.g. fishing in saltwater, freshwater or aquaculture
- the catching area (see Fig 24.3).

From 1 January 2005 full traceability and recall procedures will come into force.

The driving force behind the introduction of traceability in the food sector has been a combination of recent food scandals and the authorities' wish to protect the health of the consumers by reducing the risks from the food that they eat.

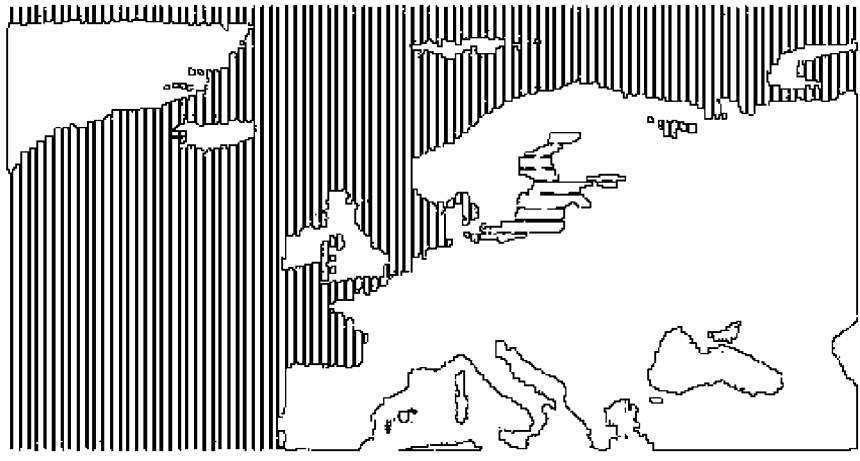

Fig. 24.3 Catching areas used when selling fish in the EU. The vertical striped area is the north-east Atlantic, the horizontal striped area is the Baltic Sea and the area without stripes the Mediterranean. By permission from Marusa Design.

Food scandals, such as BSE (bovine spongiform encephalopati) in cattle and high dioxin levels in chicken production coming from illegal use of transformer oil, are well known. The fish sector has had its own scandals. Consumption of shellfish and in particular raw shellfish is a well-known source of foodborne disease (Liston, 1990). Microbial health hazards in fish products and their control are discussed by Huss (1997) and Gram and Huss (2000). Parasites in fish products have caused many outbreaks of illnesses. The most important of the nematode zoonotics of marine fish are members of the family Anisakidae. The best known member of this family is *Anisakis simplex*, which uses herring, cod, mackerel and salmon as hosts (Williams and Jones, 1994). In 1987 three live nematodes in a herring fillet were shown in a television programme in Germany. This caused a dramatic fall in fish consumption. As an example, Danish fish exports to Germany dropped by 25% the next year and it took three years before the export value reached the previous level. This incident led to new legislation for curing herring and revised GMP (good manufacturing practice) procedures recommending freezing of herring fillets for more than 24 hours at −20º (Larsen, 2002).

24.3 Traceability systems in use today

Traceability systems in food producing are not new. It has long been mandatory to have a batch number on the final product, which can be traced back in the process to the raw material and the ingredients that have been used. This batch or lot number may cover a few minutes' production or several months' output in some cases. This has led to the development of more accurate systems, either

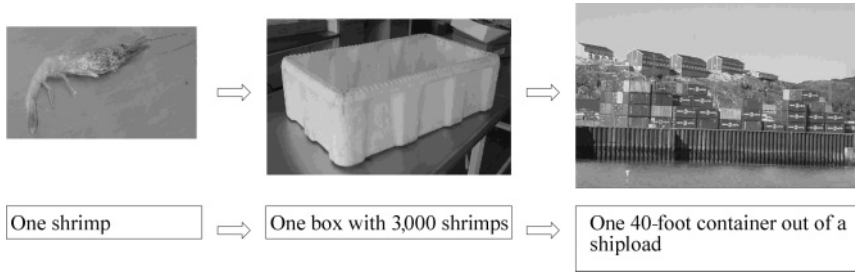

Fig. 24.4 Different examples of traceable units. By permission of T. Børresen.

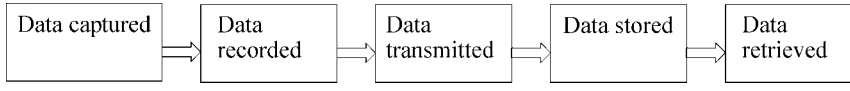

Fig. 24.5 Illustration of data flow in a process.

paper based or electronic/IT based (Larsen, 2001). Figure 24.4 shows an example from the shrimp processing industry of different traceable units. The three pictures in Fig. 24.4 also serve as an example of the different reasons to recall a product. A single shrimp can have too high a content of undesirable chemical substances, such as PCBs or dioxins. In this example the production manager knows how many boxes of shrimps have been used in this particular day's production and can quarantine the relevent batch. This batch may represent 25% of a container sitting on the quayside to be shipped of to the buyer who is packing the shrimps for onward dispatch to the retailer. If one of the containers arrives with a serious defect, all the shrimp blocks from this container batch might be recalled. In each case the traceable unit is different, but each is linked back through the production chain to a single item caught at a particular time and place.

Fig. 24.5 illustrates the complicated procedure of handling data from the production chain. There are now commercially available IT systems that can help solve the data handling problem (Larsen, 2001). There are different programs that are designed to take care of the HACCP system and at the same time can be used as a traceability program. There are also IT systems developed for handling the processing of the product, e.g. keeping records of where and when a certain raw material is needed during production, and at the same time keeping a traceability record. The problem is less getting a software system that can handle your data than that many of these software programs are overlapping or aren't able to communicate. Modern production needs different software systems to communicate. There are several ways to do this, but nearly all programs can work on the same IT platform, e.g. Microsoft software. Another issue is whether the data should be distributed on several computers/servers or should it be kept on one central server, a portal solution? The last solution makes it easy to control who has access to the server. If your product needs to be traceable from outside the production site, so that other links in the chain from

producer to end user can 'see' how and when a specific product was processed, your IT architecture should allow this to happen.

24.4 External traceability systems: how to generate data and inform other links in the chain

There are two approaches to handling data across the supply chain. Either everybody in the supply chain has access to data from all the other links in the chain or at least more than two links (an open approach), or every link is only responsible for pulling data from the previous link and pushing data to the next link in the chain (a closed approach). This last model is called the 'pull-push system', and for the moment the EU Commission is in favour of this system (Clapp, 2001). To discuss the benefit and drawback of the two systems, one has to take into consideration the 'ownership' of the data. Information is an asset. To have an open system of traceability one has to have a common agreement on what kind of information is needed and can be shared. Three voluntary 'open' standards for the fisheries sector have been agreed in a EU Concerted Action project, 'Tracefish' (2002). In November 2002 representatives from the fishing sector agreed that two of the standards, the one for wild caught fish and the one for farmed fish, could be the future standard for the kind of information needed in a traceable system. The 'Tracefish' concept is an electronic system of chain traceability. The specifications of the system have been made as a CEN (Comité Européen Normalisation) Workshop Agreement. The key element in a traceability system is labelling of each unit of goods traded, independent of the status of manufacturing, with a unique ID (identification number). The ID can change but each link shall keep track of the IDs of the raw material or products that they receive and link them to the new IDs. The method of identifying the units of goods traded is based on the EAN.UCC system (unique global identification system), which is a well-known system throughout the world. An example of the identified links from the farmed fish chain is given in Fig. 24.6.

Two examples of the open approach to traceability indicate a Danish system that operates with an Internet solution and a British system that is built up around a weight and grading system. Both systems operate with barcodes as the main carrier of information. The Danish system, 'Info-fish' (Frederiksen *et al.*, 2001), has been developed as a demonstration model for a fresh fish chain. 'Info-fish' is built on research with fishing vessels in the North Sea, developing better handling systems, on-board packing of fish in boxes with labels holding information on catching date, ground, fish species, weight, etc. (Frederiksen *et al.*, 1997). Today it is possible for buyers at auctions in Danish ports to buy fish per box, where all the information needed is given on the label, both as text and as a barcode.

The next step was to create a solution that gave every link in the production chain access to this information, from the fisherman to the consumer. This can be done by using labels thoughout the chain. However, in modern distribution

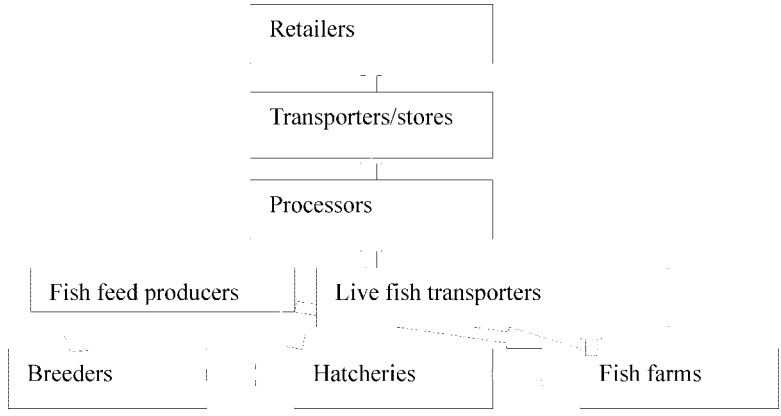

Fig. 24.6 Farmed fish distribution chain. The chain does not necessarily always consist in the sequence shown.

channels goods such as fresh fish are repacked several times. New labels have to be printed each time and glued to the new box. This proved difficult given the wet and cold environment and the many handling operations. It was decided that the transfer of data between the different links in the chain should be done over the Internet. To do this XML (eXtensible Markup Language) was chosen. This enabled the different links to synchronise their own systems by exchanging not just data but also the enabling underlying data structure for processing information. (Frederiksen, 2002). The next step was to find software that could make it possible for the different IT systems in each link to 'talk' together. Microsoft's Biztalk server was chosen because it was able to 'translate' the different software languages in the use in the different link to XML and, at the same time, was capable of dealing with secure and stable communication between the links. The result of the 'Info-fish' project was that Web-based communication was possible. In the UK, the company Nesco Weighing Limited is an active partner with the Seafish Authorities in introducing traceability in the first links in the fresh fish chain. It has, for example, proved possible to identify the exact position of the fishing vessel using the GPS signals from satellites and include this information in the label that is attached to each fish box.

24.5 Farmed fish – the difference between conventional and organic production

It seems evident that the production of farmed organic fish can be controlled much more than wild caught fish (section 24.6). However, the feed for farmed fish is usually manufactured from other fish. It is possible to raise, e.g., salmon on a diet of vegetable origin, but it creates problems with the eating quality of the product not to mention the ratio between the feed given and gained weight of the fishes. The ratio is more than three times better using a more balanced diet of

fish proteins and fish oil compound vegetable proteins and oils. This raises the issue of the traceability of the fish used for producing fishmeal and fish oil. In organic production a certain degree of sustainability must be incorporated in the standard for producing organic farmed fish. Traceability here is essential in documenting the origin of the feed.

The fish used for fishmeal and oil are normal small fatty fish, such as sand eels, sardines and capelin. Large fishing vessels operating huge catching gears that can hold many tons catch these fishes. The vessels' storage facilities are normally tanks where the catch is kept until landing, where it is pumped into fishmeal factories. Here the catch is stored in large tanks and processed continuously to separate the fish protein from the fish oil. Keeping track of the catch is difficult. The traceable unit is normally defined as the production quota for one day which may be several hundred tons of products from diverse sources.

The three major ingredients in fish feed are fishmeal, fish oil and a vegetable source of meal and oil, preferably soybeans. In conventional fish feed there does not have to be any guarantee that the feed is free of fish meal/oil coming from non-sustainable fish species or that the soybeans are not genetically-modified. Organic fish feed need such guarantees. Considering that feed production is based on continuous pumping of the materials through pipes, cookers and extruders, it is not hard to see that separating conventional and organic feed production demands major delays and cleaning procedures in the different productions' units, and up to as much as one day's production may be lost. More advanced traceability systems might reduce this logistical and economic barrier to the production of organic fish feed.

The other aspect that has to be discussed is the use of offal from the filleting fish industry. This practice is favoured in organic aquaculture because it is a more sustainable alternative to catching wild fish. The British Soil Association organic label demands that at least 50% of fish meal and fish oil used in organic aquaculture has to be from offal from the filleting industry. The requirement increases the demands on traceability systems in aquaculture.

24.6 Attitudes towards traceability in the fish sector

This section is based on the statement that representatives from the sector have expressed in the two EU financed Concerted Actions, 'Fish Quality Labeling and Monitoring' FAIR PL98-4174 and 'TraceFish' QLK1-2000-00164. Starting from the top of the fish chain consumers have expressed the view that traceability of fish products would be of value, especially in circumstances where there is doubt of the authentication of the product (Larsen *et al.*, 2003). There is a profound difference between the attitudes between the countries in Europe of how this traceability should be managed. The Nordic countries believe that a government-managed system will best ensure traceability and origin of the product. In the UK and the southern part of Europe, industry and certification bodies are believed to be the most credible managers of traceability systems.

The next link in the chain is the retailer. Retailers are definitely in favour of traceability. The more information retailers can get about fish products the better, as a means of ensuring the supply and quality of their products. The processing industries have traditionally waited for the retailers to demand traceability and develop standards such as the TraceFish standard for wild caught fish and farmed fish. Fishermen cannot be regarded as a homogeneous group because there are huge differences between the different fisheries. In fishing for tuna fishermen can easily mark each individual fish with the necessary data, but fishing pelagic fish such as herring makes it more difficult to mark smaller units than a box of fish. Giving away the exact position where the catch has been made is not part of the tradition of the fishermen.

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25

Safety and traceability of animal feed

S. Notermans and H. Beumer, TNO Nutrition and Food Research, The Netherlands

25.1 Introduction

Feedstuffs play an important role in maintaining the health of production animals and therefore of humans. In relation to food safety, the slogan 'healthy animals, healthy humans' is often used to demonstrate the clear relationship that exists between the health status of animals and that of human beings. Experience has shown that the transmission of diseases from domestic animals to man can be prevented effectively only by improving the health care of the animals themselves. It is even more of a challenge to prevent the transmission of zoonotic agents because, as the human population has increased, there has been a concomitant increase in the number of production animals. Factors involved in disease control include the availability of safe feedstuffs, husbandry practices, immunisation and the use of antimicrobials and other veterinary drugs. Strategies that have been explored to control foodborne human pathogens include the administration of selected microbial cultures to piglets and day-old chicks in order to establish a balanced gut microflora and increase colonisation resistance. In the case of ruminants, attempts have been made to reduce carriage of *Escherichia coli* O157 by using special dietary formulations. However, neither of these approaches to gut flora manipulation has been entirely successful.

In this chapter, a general outline is presented of measures for the production of safe animal feed. This is based on the setting of so-called 'feed safety objectives' which make use of principles that relate to animal health, animal welfare, legal aspects of farm practices and human food safety objectives for products of animal origin. Then, following a short historical review, there is a particular emphasis on the types of feed and animal feeding systems used in relation to feedborne animal diseases caused by infectious and chemical agents

and on the relationship between animal feed and zoonotic foodborne diseases. In addition the influence of feed on animal welfare is discussed.

To produce safe animal feed, a pro-active control system is advocated. This approach has been very successful in relation to human food and involves the use of 'good manufacturing practices' (GMP) and the 'hazard analysis critical control point' (HACCP) concept as the main tools. However, it has been shown that the HACCP system has certain shortcomings. To counteract these shortcomings, product traceability and hazard early-warning systems have been developed and are also described in this chapter.

25.2 Requirements for safe feed production

In the production of acceptable, safe food for human consumption, a generic framework for setting food safety criteria has been gradually established and is presented in Fig. 25.1. This framework is based on an internationally agreed food safety policy which comprises certain general rules. Examples of these are that carcinogens should be absent from food, and for extraneous chemicals the aim should be to follow the ALARA principle, which means that, levels 'as low

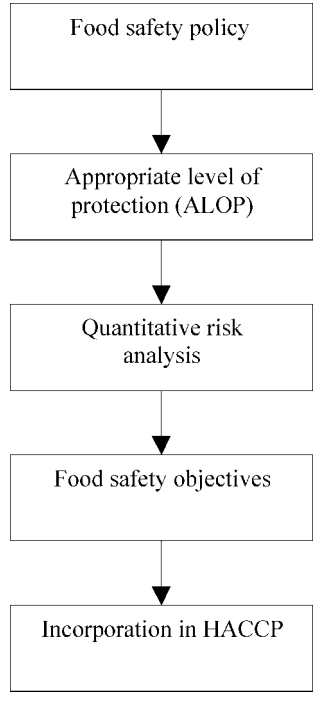

Fig. 25.1 Generic framework of current food safety systems as developed for chemical (additives, pesticides, etc,) and for microbiological agents (based on Hathaway, 2001).

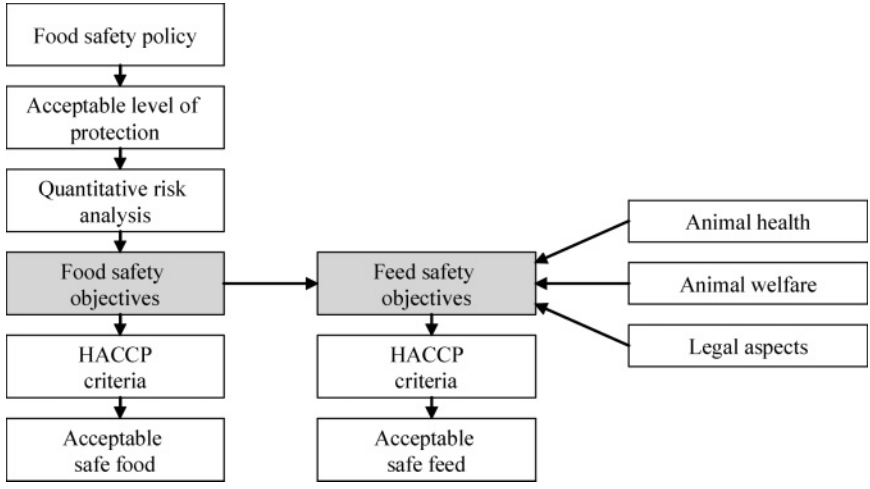

Fig. 25.2 Conceptual approach for production of safe feed. For explanation see text.

as reasonably achievable' are required. Also a suitable level of protection has been agreed. For most chemicals, concentrations below the no-effect level, including an uncertainty factor, are considered to provide 'an appropriate level of protection' (ALOP).

The risk assessment process used is primarily directed to assessing the characteristics of potentially hazardous agents and to carrying out an exposure assessment. The risk management procedure, which follows the requirements of the Codex Alimentarius Commission (CAC), results in the final food safety objectives. These must be incorporated in the relevant food production process by using GMP and the HACCP concept. Meeting food safety objectives for products of animal origin makes demands on the control of feed given to the animals involved. Thus, feed manufacture must allow for products of animal origin to meet food safety objectives. This conceptual approach for the production of safe feed is presented in Fig. 25.2. The starting point is the food safety objectives for chemical contaminants and zoonotic disease agents, which are essential to produce acceptable, safe food products for humans. Among the requirements of food safety objectives are maximum levels of particular chemical contaminants in feed and the number of feed-transmissible zoonotic disease agents. In addition to the feed safety objectives, animal health and welfare as well as legal aspects of farm practices need to be taken into account. As is the case for human food, feed safety objectives involve the production process and product and storage criteria, as specified in the HACCP system.

Human health risks

The human health risks from foods of animal origin include zoonotic agents and toxic chemicals which may be present in animal feed and transmitted via the animal to human food products. The relevant zoonotic agents include bacteria,

parasites, viruses and prions. Some of these agents do not affect the health of the animals or even their production rates. Thus the animals are 'silent' carriers of human pathogens. Examples include *Campylobacter jejuni* in chicken (Kapperud *et al*., 1993; Jacobs-Reitsma *et al*., 1995), *E. coli* O157:H7 in cattle (Chapman *et al*., 1997) and *Salmonella typhimurium* in pigs (Ishiguro *et al*., 1979). The existence of the carrier state may easily escape attention.

Hazardous substances that may be transferred from animal feed to food products of animal origin include mycotoxins, toxic plant materials and certain chemical residues present in raw feed materials. As far as human health is concerned animal passage may lead to dilution or even destruction of the substance in question. This needs to be taken into account in setting feed safety objectives.

Animal diseases

Feed may also contain infectious agents and toxic substances that can cause disease in animals but not in humans. An example is the virus responsible for foot and mouth disease (FMD) which is characterised by fever and blister-like lesions on the tongue and lips, in the mouth, on the teats, and among the feet. Other examples, which are also transmissible to humans, are anthrax caused by *Bacillus anthracis* and botulism caused by neurotoxins produced by *Clostridium botulinum*. Also bovine spongiform encephalopathy (BSE) and other transmissible spongiform encephalopathies (TSE) can be considered as infectious agents that might be transmitted by feed. As far as toxic and deleterious components are concerned, two classes can be recognised. The first class are inherent and naturally occurring constituents of an animal feed such as plant toxins and other metabolites. The second class is made up of substances that are present as extraneous contaminants, e.g., heavy metals or occur in feed due to mishandling or other factors, e.g., mycotoxins.

Animal welfare

The suitability of the feed is an important part of animal welfare. For example, it should be appropriate for the animal in question and cause no adverse effects. It must be available in sufficient quantities to satisfy hunger at all stages of production and readily accessible to all individuals.

Legal aspects

Finally, animal feed needs to meet certain legal requirements. These relate to animal health and welfare and zoonotic disease agents and there are also criteria that cover environmental aspects, trade protection, etc.

25.2.1 Historical aspects

Modern stock-farming practices must take account of food quality and safety, control of animal health and welfare and environmental issues. However, these aspects of farming have been recognised for a very long time. In The

Netherlands, for example, stock-farming has been a major economic activity for centuries and has been predominantly based on export. In the Dutch province of Friesland the number of beef cattle increased between 1700 and 1800 from 55,000 to 110,000. This number was equivalent to the size of the human population in Friesland (Faber, 1972). During the 18th and 19th centuries, the Dutch export of cattle increased from 11,000 to 146,000 animals (Priester, 1991). As early as 1600, Dutch cattle were being fed linseed cake imported from Britain and even from British colonies in America. Due to this practice of supplementing the diet, the animal population became much greater than was sustainable by feeding only home-grown crops.

Animal health

Although livestock production was extensive in the past in many parts of Europe, the animals were susceptible to many diseases. Rinderpest, which was present in Europe in the 18th century, killed hundreds of thousands of cattle. In 1770, in the Dutch Province of Holland, 160,000 cattle died, some 70% of the total stock. Similarly, 160,000 cattle were killed by Rinderpest in the province of Friesland (Faber, 1972). In that period, the United Kingdom lost 500,000 cattle and the German Bundesland East-Friesland 700,000 animals. These disasters were not limited to Europe and Rinderpest killed thousands of animals in other parts of the world. At the end of the 19th century, 5.5 million cattle died of Rinderpest in Africa alone. As a consequence, human society became completely disrupted (Pearce, 2000; Murphy *et al*., 1999). Deaths among cattle and human beings were so enormous in Africa that an elderly Masai said 'the corpses of cattle and people were so numerous and so close together that vultures had forgotten how to fly'. Also, due to FMD outbreaks in The Netherlands in 1894, 1897, 1907 and 1911 as many as ten thousand farms were affected (Bieleman, 2001). Combating animal diseases started after the end of the 19th century when, among others, Jenner and Pasteur discovered preventive vaccination.

Animal welfare

For much of the history of stock-farming, animal health did not have a high priority. In fact, when animal production intensified during the late middle ages, animal health declined considerably (Davids, 1989). A well-known example is the so-called 'case' calves (box calves) which were introduced in the 18th century (Bieleman, 2000). Also, at that time animal breeding was focused on the production of meat and many mistakes were made. Exotic breeding animals died from pneumonia, fell into the marshy earth or succumbed to malnutrition (Bieleman, 2000). There were similar problems in moving cattle long distances. Due to a lack of any transport vehicles the animals became completely exhausted. Losses of a quarter to one-third of the herds were no exception after travelling on foot from Denmark to Amsterdam or London (Verstegen and Hanekamp, 2001).

Product quality

The poor quality and safety of products of animal origin were due to a lack of proper scientific knowledge. Also, following liberalisation in many European countries and abolition of the crafts (guild, master and warden of crafts), the quality of food products further declined. Spoilage of food became an enormous problem, especially for products of animal origin. Also corruption was widespread. The problems were solved only during the first part of the 20th century (van Otterloo, 2000) when quality control systems became established.

25.3 Risks from animal feed

Feedborne hazards lead to diseases of man and animals caused by the consumption of products of animal origin. Feedborne animal diseases may be caused either by infectious agents or those producing intoxications. Human diseases caused by animal feed are predominantly due to zoonotic agents, but the hazards may include pre-formed toxins as well. In this section the most relevant types of animal feed are described followed by feedborne animal diseases. Subsequently feedborne zoonotic diseases are described. Finally, feedborne human intoxications are discussed.

25.3.1 Types of feed material

A feed ingredient is a component part or constituent of the final mix. Ingredients can include various types of grain, milling byproducts, added vitamins, minerals, fats/oils, and other nutrients. They may be raw or processed and used for manufacturing compound feedingstuffs. The final product may also contain certain additives to enhance health or performance in recipient animals. In addition there are 'dietetic' feedingstuffs which meet specific nutritional requirements and are used, for example, for cats and dogs with diabetes.

In theory, animal feeds provide a practical outlet for plant and animal byproducts that are unsuitable for human consumption. However, such byproducts are not always acceptable, especially abattoir waste and offal, and official bodies in many countries have produced lists of permitted feed ingredients that exclude them. This chapter considers animal feeds that have been processed in one way or another, for example by heating, fermentation, acidification or drying, thus excluding natural, unrestricted grazing. Although products of aquaculture are a major source of food protein and represent one of the fastest growing sectors of food production, these are not considered in detail.

25.3.2 Feedborne animal diseases

Feed ingredients are regularly subject to contamination from diverse sources, including environmental pollution and the activities of insects and micro-organisms. This is in addition to endogenous toxins associated with some of the

plant materials used. A well-known example is gossypol which may be present in cotton seed, and can be toxic to non-ruminants or immature ruminants. Many of these contaminants cause adverse health effects such as infections and intoxications in the animals and may be transmissible to humans. There are many infectious disease agents which are transmitted by feed to animals. Some of them are discussed briefly here as examples and an indication given of their control by the application of GMP and HACCP in the production process.

Foot and mouth disease (FMD)

FMD is a devastating disease of livestock and is characterised by fever and blister-like lesions on the tongue and lips, in the mouth, on the teats, and among the feet. All species of cloven-hoofed animals are susceptible and the disease is extremely contagious. FMD is endemic in many areas of the world, including several countries in Africa, as well as in Asia and South America. In 2001, the disease was confirmed in the United Kingdom, France, The Netherlands, the Republic of Ireland, Argentina and Uruguay. The United Kingdom reported 2,030 cases of FMD, slaughtered almost four million animals, and took seven months to control the disease outbreak. Financial losses as a result of FMD can be considerable. There are direct losses due to deaths in young animals, loss of milk and meat and a decrease in growth performance. The costs associated with eradication or control can be high and, in addition, there are indirect losses due to the imposition of trade restrictions. The disease is caused by an Aphthovirus member of the family *Picornaviridae*. Very few human infections have ever been described, despite regular exposure of farm personnel and others to infections in livestock (Bauer, 1997; Armstrong *et al*., 1967). Cases that have been reported have been mild and self-limiting, with no human to human transmission being evident, and FMD is not transmitted to humans through the food chain. Therefore, the disease is not a threat to public health.

The virus can easily be controlled by immunisation. Due to its extremely contagious character control of the virus by other means, such as bio-security, is difficult. FMD virus can survive for long periods of time in dark and moist conditions. Therefore, non-processed feed is considered to be an important route for transmission of the virus. The virus is easily killed by mild heat treatment, and is completely inactivated during pasteurisation at 75 ºC for a few seconds. The FMD virus is also extremely sensitive to pH. At pH values above 9 and below 6 the virus is rapidly destroyed. For this reason, acidification of feed either by fermentation or adding, e.g., citric acid, is effective.

Trichinellosis

Trichinellosis is cause by the nematode *Trichinella spiralis* which parasitises the intestinal tract of mammals, particularly pigs. The larvae encyst in the tissues, particularly the muscles which act as a source of infection for humans who consume raw or partially cooked meat. The clinical manifestations include fever, muscle pain, encephalitis and myocarditis. Death occurs only rarely. The cysts can be transmitted by non-processed feed. Treatments like freezing at

−18 ºC for 20 days or heat treatment cause inactivation. Effective cooking of raw meat and table scraps before feeding to farm animals will eliminate transmission. This is also the case when traditional rendering temperatures are used.

Bovine spongiform encephalopathy (BSE)

BSE is a disease of cattle which was first recognised in Great Britain in 1986. Cases have since occurred in many other countries. However, a high incidence was observed only in the United Kingdom – particularly in parts of Great Britain. BSE was first identified by pathologists at the Central Veterinary Laboratory (CVL), Weybridge, in November 1986 following examination of the brains from two cows which were independently submitted by Veterinary Investigation Centres in Kent and Devon (Wells *et al*., 1987; Fraser *et al*., 1988).

It is believed now that the disease is caused by a natural protein which folds in the wrong way and then causes other similar proteins to adopt a similar shape. The new form gradually accumulates and spreads. It can transmit disease from animal to animal at least experimentally and from contaminated animal tissue to humans (Bruce *et al*., 1997). These proteins are called prions. The complete exclusion of mammalian meat and bone meal (MBM) from all farm animal feed since 1988 resulted in a gradual decrease in the incidence rate of BSE (see Table 25.1).

Since the disease has an incubation period of about five years, it was expected that action taken in 1988–90 would take some years to show through.

Table 25.1 Cases of BSE in cattle in the United Kingdom. A clear decrease is observed some 5 years after the ban on animal meat and bone meal in 1988

Year	Number
1987 and before	442
1988	2,469
1989	7,137
1990	14,181
1991	25,032
1992	36,682
1993	34,370
1994	23,945
1995	14,302
1996	8,016
1997	4,312
1998	3,179
1999	2,274
2000	1,355
2001	1,113
2002*	695

* Per October 2002.

Studies revealed that traditional rendering of animal offal contaminated with BSE prions was not sufficient to inactivate their infectivity in test animals (Fraser *et al*., 1988). Therefore, the European Commission decided in 1993 to use a higher standard for the treatment of animal waste (133 ºC, 3 bars of pressure for 20 minutes). In 1994, the EU Commission also introduced a ban on the feeding of mammalian meat and bone meal to cattle, sheep and goats.

Botulism

Botulism is caused by neurotoxins produced by the bacterium *Clostridium botulinum*. These toxins are some of the most lethal substances known to man. *C. botulinum* produces eight different toxins, which are designated as types A, B, C1, C2, D, E, F, and G. Each of these toxins affects different species of animals and they are usually found in different environments. The most common source of botulism in cattle is through the ingestion of toxin-contaminated feed. Dead animals, such as chickens, that may be inadvertently picked up in hay and silage during farming activities, can act as an ideal medium for the organism to grow in and produce toxin. This is particularly true for type C botulism.

Small-grain silage (particularly from oats, barley and rye) is commonly involved in type B outbreaks. In 1976 and 1977 outbreaks of botulism, followed by the death of many affected animals, occurred in about 30 dairy farms in The Netherlands. The disease was caused by ingestion of spent brewer's grain that contained proteolytic *C. botulinum* type B cells and the associated toxin (Breukink *et al*., 1978; Notermans *et al*., 1981). Also, the toxin was detected in 4.5% of cattle fed with the spent brewer's grain. Although this material has been fed to animals for long periods, no cases of botulism have been reported either before or after the incident in question. After heat extraction of the malt, the remaining part of the grain is stored for a short period at the brewery and then transported to dairy farms. At these farms, the material is stored under anaerobic conditions for the making of silage. During storage, a fermentation process normally occurs and the pH falls, thus preventing spoilage. In this special case, it was observed that the pH did not drop sufficiently, probably because there was insufficient fermentable sugar.

How *C. botulinum* type B contaminated the product has not become clear. It was observed at the brewery that the storage tank for the grain was not completely closed and the organism may have been introduced by birds. Nevertheless, *C. botulinum* can be naturally present in malt in low numbers and the heat resistant spores are likely to survive the extraction process. During this episode and for a period of ten years afterwards, no human cases of botulism were reported in The Netherlands.

Mycotoxicoses

Many mycotoxins are harmful when consumed by animals. The toxins can accumulate in maturing corn, cereals, soybeans, sorghum, peanuts, and other feed crops in the field and in grain during transportation (Christensen, 1982). The toxins may be produced during storage under conditions favourable for the

Table 25.2 Major mycotoxins, feed products affected and possible effects on animals (source: myhttp://www.aces.edu/department/grain/ANR767.htm)

Mycotoxins	Feeds affected	Possible effects on animals
Aspergillus toxins		
Aflatoxins	Cereal grains, peanuts, soybeans	Hepatoxic, carcinogenic, reduced growth rate, haemorrhagic enteritis, suppression of immunity to infection and decreased productivity.
Ochratoxins	Cereal grains	Toxic to kidneys and liver, abortion, poor feed conversion, reduced growth rate and reduced immunity to infections.
Sterigmatocystin	Cereal grains	Toxemic and carcinogenic.
Tremorgenic toxin	Cereal grains, peanuts, soybeans	Tremors and convulsions.
Penicillium toxins		
Patulin	Cereal grains, apple products	Haemorrhages of lung and brain, oedema and toxic to kidneys.
Fusarium toxins		
Zearalone	Cereal grains	Hyperestrogenism, infertility, stunting and even death.
Deoxynivalenol	Cereal grains	Feed refusal by swine, cats and dogs, reduction in weight gain.
Trichotecenes	Cereal grains	Severe inflammation of gastrointestinal tract and possible haemorrhage, oedema, vomiting and diarrhoea; infertility, degeneration of bone marrow, slow growth and sterility.
Fumonisins	Corn	Leucoencephalomalacia in horses.
Ergot toxins	Cereal grains	Vasoconstriction and loss of extremities (tails, ears, feet, etc.).
Ergovaline	Fescue	Abortion and reduced weight gain.

growth of the toxin-producing fungus or fungi. The effects in domestic animals include allergic reactions, reproductive failure, unthriftiness, loss of appetite, feed refusal, suppression of the immune system, decreased feed efficiency, and mortality (Hesseltine and Mehlman, 1977) (Table 25.2).

Although the adverse effects of feeding mouldy feeds were long known by producers of red meat animals and poultry, a specific mycotoxin was not implicated. An outbreak of 'Turkey X disease' in Great Britain in 1960 was traced to contaminated peanut meal from Brazil. Aflatoxin was indicated as the cause of death for more than 100,000 young turkeys and some 20,000 ducklings, pheasants, and partridge poults. This problem stimulated modem research on mycotoxins and the ecology of mycotoxin-producing fungi. Some of the most common mycotoxins and associated fungi are given in Table 25.2. Aflatoxin B_l, which may be formed in cereals, sorghum, peanuts, and other oil-seed crops, is one of the most potent naturally occurring animal carcinogens (Shotwell, 1977). If sensitive young animals regularly consume between 50 and 100 mg of

Table 25.3 Examples of food of animal origin which may be naturally contaminated with mycotoxins (source: FAO report, 1997)

Mycotoxin	Occurrence	Maximun level reported in μg/kg
Aflatoxin B_1	Eggs	0.4
	Pig liver	0.5
	Pig muscle	1.04
	Pig kidney	1,02
Aflatoxin M_1	Cow's milk	0.33
Ochratoxin A	Pig liver	98
	Pig kidney	89
	Sausages	3.4
Zeralenone	Pig liver	10
	Pig muscle	10

aflatoxin B_1 per kg of feed, the result can be fatal liver cancer; in older or mature animals, though, the effects may be only minor. All species of animals appear to be susceptible, although susceptibility varies greatly from species to species and is due to different ways of metabolising the mycotoxins.

So far little information is available on the occurrence of mycotoxin residues in animal products intended for human consumption. Corn with 100 mg of aflatoxin per kg can be fed to nonlactating animals without adverse effects and without passing harmful amounts of aflatoxin or aflatoxin metabolites to human beings via the edible parts of the animals. Lactating cows consuming feed containing 20 mg/kg or less of aflatoxin will have less than 0.1 mg of aflatoxin per kg of milk. Some examples of mycotoxin residues that occur in animal tissues are presented in Table 25.3.

Three genera of fungi – *Aspergillus*, *Penicillium* and *Fusarium* – are the ones involved most frequently in cases of mycotoxin contamination in corn, small grains, and soybeans (Table 25.2). Toxin production by these moulds depends on factors such as temperature, a_W, the quality of the grain and its suitability for storage. Other factors include a high moisture content, physical damage to the kernels, and the extent to which storage fungi have invaded the seed. Fungi may grow well under a given set of conditions but not necessarily produce mycotoxins. Although *A. flavus* flourishes on many crop plants, it does not produce the same amount of aflatoxin on all of them. For example, the fungus produces much more aflatoxin on peanuts than on soybeans, although it grows equally well on both crops (Shotwell, 1977).

Pelletising feeds may eliminate fungi present in the stock but will not reduce or eliminate aflatoxin present in any of the ingredients. Recently, the addition of binding agents such as hydrated sodium or calcium aluminosilicate and bentonite clays to corn has been shown to decrease the effects of aflatoxin when fed to swine. These compounds probably work by non-specific binding to the mycotoxin and reducing the rate of passage through the gut. Although not

specifically approved for the purpose, various products that have this ability are approved as binding or anti-caking agents.

The diseases caused by the most relevant mycotoxins are presented in Table 25.2. Aflatoxins may cause vaccines to fail, increase birds' susceptibility to disease, and result in suppression of natural immunity to the infection (Elissalde *et al*., 1989). The animals then become susceptible to infection by bacteria such as *Salmonella* and to various viruses and other infectious agents commonly found around the farm, feedlot or poultry house. Normal healthy animals would ward off such agents.

Zearalenone and zearalenol are produced almost exclusively by *Fusarium* species that contribute to the ear and stalk rot that occurs in the ears of corn and on the heads of cereal grains. When consumed by swine at more than 0.1 to 5 mg toxin per kg body weight, these compounds cause the estrogenic syndrome, which is characterised in females by a swollen and edematous vulva with enlarged mammary glands and in young males by a shrinking of the testes. Young gilts may show uterine prolapse. The financial loss to farmers comes about primarily through poor reproductive performance.

Feeds that contain 1 mg of deoxynivalenol per kg may result in significant reductions in feed consumption and weight gain by swine (Bergsjø *et al*., 1992). Vomiting is rather uncommon in field cases because usually pigs will not eat enough of the contaminated feed. Clinical signs and lesions in affected swine included feed refusal, a few instances of vomiting, lack of weight gain, poor feed efficiency, failure of mature sows to return to oestrus, reduced efficiency, high mortality of nursing pigs, intestinal tract inflammation, and acute diarrhoea in young pigs. Dairy cattle and poultry are relatively insensitive to the dietary concentrations of deoxynivalenol likely to be found in feeds.

Apparently all domestic animals are susceptible to injury by dietary intake of trichotocenes such as T-2, HT-2, and diacetoxyscirpenol (DAS) in the region of a few mg/kg. In poultry, feed contaminated with 1 to 3.5 mg/kg of T-2 and 0.7 mg/kg of HT-2 (a closely related toxicant) may produce lesions at the edges of the beaks, abnormal feathering in chicks, a sudden and drastic drop in egg production, eggs with thin shells, reduced body weight gains, and mortality. The same feed given to turkeys results in reduced growth, beak lesions, and less immunity to infection. T-2 and DAS in cattle feed results in unthriftiness, decreased feed consumption, slow growth, reduced milk production, and sterility. An outbreak of haemorrhagic bowel syndrome and death of some animals can occur in herds of cattle and swine. In swine, infertility with some lesions in the uteri and ovaries result from consumption of feed contaminated with 1 to 2 mg/kg of T-2 toxin. As with most other mycotoxins, the only control is to avoid use of contaminated feeds.

Equine leucoencephalomalacia occasionally occurs in horses, mules, or donkeys foraging corn left standing in the field after harvest or when fed grain or screenings heavily infected with *F. moniliforme*. The toxins, fumonisin B_1 and B_2, are produced only by certain strains of *F. moniliforme*. This toxicant is also carcinogenic in laboratory tests. Ochratoxin A, produced primarily by members

of the *Aspergillus ochraceus* group and a number of species of *Penicillium*, especially *P. viridicatum*, has been found in some samples of feed grains. Frequently, citrinin is produced simultaneously by these same fungi. In the field, however, injury from ochratoxin poisoning has occurred chiefly (or only) in poultry and swine. Listlessness, huddling, diarrhoea, tremors, and other neural abnormalities are sometimes encountered in broiler flocks. Ochratoxin damage to the kidneys of swine is characteristic enough to be called 'porcine nephropathy,' which is recognisable in commercial slaughtering.

Intoxications by other components

In addition to mycotoxins and naturally occurring toxic constituents in plants, there are toxic and deleterious substances that are extraneous contaminants of industrial origin. These can be increased to abnormal levels in animal feed through mishandling or other factors. The most significant hazards to human health are those chemicals that accumulate in animal tissues, are excreted in milk or become incorporated in eggs. Examples of substances that attract international attention are the polychlorinated biphenyls (PCBs), dioxins and furans and certain pesticides like DDT (Dichloro-Diphenyl-Trichloroethane). Fish and fish by-products that are used to make fish meal and oil, and are ingredients in feed, may be sources of contamination in the food chain. In 1998/1999 a survey was conducted by the Canadian Food Inspection Agency (www.ispection.gc.ca/english/anima/feebet/dioxe.shtml). The purpose of the survey was to determine the levels of dioxins and furans, PCBs and DDT in fish meals, fish feeds and fish oils. The results of that study are presented in Table 25.4.

Table 25.4 Dioxin-furan and PCB contamination of fish products (source: http://www.ispection.gc.ca/english/anima/feebet/dioxe.shtml)

Country of origin (samples)	Mean dioxins and furans TEQ* (ng/kg) in fish feed/meal (minimum-maximum levels)	Mean total PCB (μg/kg) in fish feed/meal (minimum-maximum levels)	Mean value for DDT (μg/kg) in fish feed/meal (minimum-maximum levels
Fish meal and fish feed based on whole weight content			
Canada (14)	1.0 (0.11–3.73)	30.7 (1.5–74.3)	21.1 (ND**–51.0)
United States (7)	1.1 (047–1.71)	16.5 (0.2–29.5)	23.3 (ND–52.0)
Other countries (3)	0.15 (ND–0.23)	8.3 (0.6–12.7)	
Fish oil based on fat/lipid content			
Canada (2)	9.9 (7.1–12.7	130.7 (121.2–140.3)	25.5 (ND–44)
South Africa (4)	3.7 (0.2–11.5)	151.2 (188–201.2)	
Other countries (3)	9.1 (7.9–10.8)	230.3 (101.2–441.9)	

* Results presented in toxicity equivalents (TEQs).
** Not detected.

The results indicate that dioxin-furan and PCB levels in fish feed and fish meal would not to be expected to result in fish products with dioxin-furan or PCB levels above the Canadian guidelines for these chemical contaminants. These are a maximum level of 20 ng TEQ for dioxin and furan and 2.0 mg for PCBs per kg product. The same applies to fish oil. Also the levels of DDT were far below the maximum Canadian limit of 5.0 mg per kg product. Other contaminants include heavy metals such as lead, mercury and cadmium. In a Dutch survey carried out in 1998 (http://www.agralin.nl/kap/kap98/kap98_4.html) median levels were less than 1% of the cadmium limit of 1 mg per kg product for soybean husks, soybean forage, maize gluten feed, palm kernel husks and citrus pulp. Cadmium levels in mineral mixes also complied with the limit set for cadmium.

Another group of contaminants includes veterinary drugs that are administered via animal feeds. If the concentration used is high or withdrawal periods are not properly observed, foods of animal origin may contain residues that exceed established maximum residue limits (MRLs), such as those established by the CAC, and there may be a potential risk to human health. The problem can be avoided by applying good veterinary practices (GVP) and good manufacturing practices (GMP) in medicated feed production.

25.3.3 Zoonotic feedborne diseases

Epidemiological analysis of foodborne human diseases in The Netherlands, for example, shows that the majority of cases of food-related gastro-enteritis involve bacterial infections contracted from foods of animal origin. The data are presented in Table 25.5, which presents the estimated food attributive factor for each of the main pathogenic organisms as well as the total average incidence rate in the general population. The latter is expressed as incidents per year per million of the population.

For the Dutch situation, the working group of experts of the Health Council of the Netherlands estimated that up to 75% of foodborne diseases are transmitted through products of animal origin (Health Council of the Netherlands, 2000). The figure is based on the assumptions of experts. Viruses are not believed to be of animal origin. As far as parasites are concerned, a small but unknown proportion may be caused by these organisms, although this may differ from country to country. The most relevant organisms involved in foodborne diseases transmitted via products of animal origin include *Campylobacter* spp., *Salmonella enterica*, *Clostridium perfringens*, *Staphylococcus aureus*, *Bacillus cereus*, pathogenic *Escherichia coli* and *Yersinia enterocolitica*. All these organisms may originate from animal feed. The two most important organisms are described below.

Campylobacter spp.

The species of greatest concern is *Campylobacter jejuni* which is a Gram-negative, slender, curved, motile rod. It is a micro-aerophilic organism, which

Table 25.5 Occurrence of gastro-enteritis (and other diseases) in humans caused by pathogenic organisms transmitted by food. Adopted from Health Council of the Netherlands (2000)

Causative agent	Estimated food attributive factor	Average incidence rate in the general population/year/10^6 population	
		Total	Caused by products of animal origin
Bacteria			
Campylobacter spp.	>0.9	20,000*	15,000
Salmonella enterica	>0.9	8,000*	6,000
Clostridium perfringens	1	3,000*	2,250
Staphylococcus aureus	1	3,000	2,250
Bacillus cereus	1	3,000	275
pathogenic E. coli, other than O157	0.1–0.5	3,000*	675
Shigella	0.1–0.5	370	80
Yersinia enterocolitica	>0.9	350*	260
Escherichia coli VTEC O157	0.5–0.9	<70	<40
Listeria monocytogenes	>0.9	<7	<5
Clostridium botulinum	>0.9	<1	<1
Viruses			
Adenovirus 40/41	Presumptive**	2,000*	0
Astrovirus	Presumptive**	2,000*	0
Rotavirus	Presumptive**	6,500*	0
Noroviruses	0.1–0.5	20,000*	0
Parasites			
Cryptosporidium parvum	Unknown***	2,000*	Unknown but small
Cyclospora spp.		?	Unknown but small
Entamoebe histolytica	Presumptive**	1,200*	Unknown but small
Giardia lamblia	Unknown***	2,000*	Unknown but small

* Estimates based on sentinel and population studies.
** Transmission by food is only assumed.
*** Transmission is unknown.

means that it has a requirement for reduced levels of oxygen. It is relatively fragile, and sensitive to environmental stresses (e.g., 21% oxygen, drying, heating, disinfectants, acid conditions). The organism is especially sensitive to drying. Its main habitat is the intestinal tract of warm-blooded animals, especially the caecum of chickens, where $>10^6$/g *C. jejuni* may be present. The organism does not multiply at temperatures <30 ºC. Before 1972, when methods were developed

for its isolation from faeces, the organism was believed to be primarily an animal pathogen, causing abortion and enteritis in sheep and cattle. Surveys have shown that *C. jejuni* is the leading cause of human bacterial diarrhoeal illness, not only in The Netherlands but in many other countries, including the United States. Although *C. jejuni* is not usually carried by healthy people, it is often isolated from normal cattle, chickens, birds and even flies. Sometimes it is present in non-chlorinated water sources, such as streams and ponds.

C. jejuni frequently contaminates raw chicken meat. Surveys show that 20 to 100% of retail chicken meat is contaminated. This is not overly surprising in view of the high level of intestinal carriage. Raw milk is also a source of infection. However, cooking chicken properly, pasteurising milk, and chlorinating drinking water will kill the bacteria. There are several routes by which animals can become colonised by *C. jejuni.* Giving animals untreated water is a common source. Contamination may also occur by feeding green crops since these may become contaminated by birds that shed *C. jejuni* in their faeces. *Campylobacter* has been isolated very rarely from animal feeds like silage, cereals, or dry compound feeds. This is in accordance with the above indicated characteristics of the organism to dry conditions and susceptibility to environmental stress.

Salmonella enterica

The organism is a rod-shaped, motile bacterium, non-spore forming and Gram-negative. *Salmonella* is primarily present in the intestinal tract of animals. Various serotypes may be present and an overview of the most common serotypes in production animals is presented in Table 25.6. The same serotypes have also been found on products originating from these animals. Where feed is the source, epidemiological data show clearly that the route of transmission is animal feed → product of animal origin → human infection (Crump *et al*., 2002). The organism can be present in raw meat and poultry, raw seafood, eggs, milk, dairy products, frog legs, yeast, coconut, sauces, salad dressing, cake mixes, cream-filled desserts, cream toppings, dried gelatine, peanut butter, cocoa, and chocolate.

Table 25.6 The most important *Salmonella* serotypes (in order of decreasing importance) found in production animals in the United Kingdom (1985–1995)

Cattle	Calves	Pigs	Sheep	Poultry
S. Typhimurium	*S.* Typhimurium	*S.* Typhimurium	*S.* Typhimurium	*S.* Enteritidis
S. Dublin	*S.* Dublin	*S.* Derby	*S.* Arizona	*S.* Typhimurium
S. Gold coast	*S.* Gold coast	*S.* Kedegou	*S.* Dublin	*S.* Mbandaka
S. Agona	*S.* Agona	*S.* Gold coast	*S.* Montevideo	*S.* Senftenberg
S. Enteritidis	*S.* Enteritidis	*S.* Give	*S.* Derby	*S.* Thompson
				S. Virchow

Source: Report of CVL, New Haw, Addlestone, Surrey, KT15 3NB. Tel. + 44 1932 341111, Fax + 44 1932 349983

Table 25.7 Presence of *Salmonella* in animal feed and ingredients of animal feed

a. Presence of *Salmonella* in processed animal feed in the United Kingdom.

Year	Feed imported into UK	Feed produced in the UK
1995	7.2%	2.0%
1993	14.4%	2.3%
1991	22.0%	3.5%
1989	39.0%	5.2%

Source: Report of CVL, New Haw, Addlestone, Surrey, KT15 3NB. Tel. + 44 1932 341111, Fax + 44 1932 349983.

b. Presence of *Salmonella* in processed animal feed in Belgium.

Year	Number of samples tested	Feed produced in Belgium
1995	217	9.7%

Source: Report of Fierens and Huyghebaert (1996). *Int. J. Food Microbiol*. 31, 301–309.

c. Summary of contamination data for feed ingredients in UK feed mills.

Ingredients sampled Origin	Number	Number of samples	Number *Salmonella*-positive *n* (%)
Plant products	32	816	59 (7.2)
Animal products	6	147	8 (5.5)
Total	38	963	67 (7.0)

Source: MAFF Survey 1992.

Salmonella is a ubiquitous organism. It survives for long periods in natural environments and is resistant to, e.g., dry conditions. As a consequence it can be present in animal feed and raw materials used in animal feed. An overview of the presence of *Salmonella* in feed ingredients and processed feed is presented in Table 25.7. In the past, contaminated animal feed undoubtedly has contributed to the colonisation of food animals and subsequent contamination of their environment, which, in turn, may have contributed to infections in other animals. In the 1960s, fish meal and meals derived from slaughter-animal by-products contained a large variety of *Salmonella* serotypes and were frequently imported into European countries. Now that the significance of *Salmonella* contamination is so well recognised, greater efforts have been made to reduce the contamination of these products.

In an epidemiological study carried out in Denmark, an assessment was made of factors that contribute to the risk of *Salmonella* contamination in poultry flocks (Angen *et al*., 1996). The study revealed that the following factors (in

order of importance) contributed significantly to the contamination of poultry with *Salmonella*:

- origin of the day-old chicks
- company delivering the feed
- number of flocks present on the farm
- season of the year (more positive flocks in autumn)
- presence of *Salmonella* in previous flock(s).

Although several routes of transmission can be identified, animal feed is still thought to be a major source of *Salmonella* infection in poultry flocks. Therefore, efforts continue to be made to reduce the incidence of *Salmonella* in feeds given to poultry and other animals. There is no lack of knowledge or literature on processing methods and control measures for this purpose (Beumer, 1996; Beumer and Van der Poel, 1997). In the United Kingdom, there has been some success in reducing the *Salmonella* content of feed and data for the period 1989–1995 show a steady decrease in *Salmonella* contamination (Table 25.7a).

25.3.4 Animal welfare and feed

Feed plays an important part in animal welfare, as mentioned previously. Also the composition of feed is important. Feeding of pregnant sows on high-fibre diets appeared to reduce feeding motivation and thus improved the welfare of animals (Ramonet *et al*., 1999). Supplying calves with straw-cecal pellets is considered beneficial for the physiological aspects of welfare in veal calves (Morisse *et al*., 1999), and judicious use of disease-preventing additives also contributes to animal welfare. However, unrestricted use of antimicrobials to compensate for poor husbandry is detrimental.

A major obstacle to the judicious use of feed additives is the lack of unbiased information on their efficacy and safety for farm animals in the scientific literature (Kan *et al*., 1998). Welfare requirements alone make considerable demands on feed safety and feeds should not contain any infectious agents (bacteria, parasites and viruses) that could cause illness and discomfort in the animals. Toxic components such as naturally occurring plant toxins, anti-nutritional factors and mycotoxins should be controlled at levels that would avoid harm to the animals. Balanced formulations, well-controlled production technologies and quality and safety management in the feed industry should ensure that all nutritional requirements can be satisfied. However, feeds and feeding systems may also contribute to keeping animals under conditions that allow more natural behaviour and thereby reduce stress associated with high-performance animal production.

Drinking systems also have clear effects on animal health and welfare. For example, it was demonstrated by Turner *et al*. (1999) that aggression at the drinkers was greater for large groups of growing pigs when drinker allocation was restricted. Laitat *et al*. (1999) compared a ‘tube-type’ feeder, by which a

mixture of meal and drinking water could be given to weaned pigs, with another type of feeding system where drinking and eating were separated activities. It was observed that feeding behaviour and hence welfare were influenced by the type of feeder used, especially with large numbers of animals. The tube-type feeder resulted in a 'more relaxed' response. For lactating cows, it was observed that grooming, which is a behavioural need, increased significantly when feed cows were not locked up and had free access to feed (Bolinger *et al*., 1997).

25.4 Control systems to manage risks: GMP and HACCP

The traditional approach to controlling the safety of animal feed is based largely on practical experience, education and training of personnel, inspection of production facilities and operations, and testing of the finished product. End product testing is usually an integral part of the overall control programme. However, leaving aside questions regarding the accuracy and reproducibility of the methods used, it is clear that testing of feed is of limited value without a sound sampling plan. To overcome the problem, a book on sampling was produced by the International Commission on Microbiological Specifications for Foods (ICMSF, 1974). The book gives details of statistically based sampling plans for the microbiological examination of different types of food. Although the book gives an excellent account of the various sampling plans, it also reveals the limitations of testing for pathogenic organisms that may be infrequent, low in number and unevenly distributed throughout the test batch, especially when complete absence is the only acceptable result.

Table 25.8 demonstrates the probability of acceptance of a lot with defective samples. If it is assumed that 5% of samples are contaminated with *Salmonella*, and ten from the batch are examined, there is a probability of 0.60 (60%) that the organism will not be detected. If, instead of ten samples, 60 are examined, for the presence of *Salmonella* there is still a 5% probability that the lot will be declared *Salmonella* free. Since the contamination is not homogeneous, the probability of detection is even worse. This is also the case for undesirable chemical substances, which are only rarely present. Thus, testing to ensure that

Table 25.8 Probability of acceptance of lots containing indicated proportions of defective sample units (ICMSF, 1974)

% Defective samples	Number of sample units tested from the lot				
	3	10	20	40	60
2	0.94	0.82	0.67	0.61	0.30
5	0.86	0.60	0.36	0.23	0.05
10	0.73	0.35	0.12	0.08	<
20	0.51	0.11	0.01	<	
40	0.22	0.01	<		

the target pathogens and toxic substances are absent from the batch requires uneconomically large numbers of samples, with no guarantee that their absence can be established. However, pro-active control programmes for the production of safe animal feed have yet to be developed and controls are mostly based on an empirical approach. They are sometimes an over-reaction to unexpected problems. A successful pro-active control programme is now in use for the production of safe food for human consumption. The programme is essentially based on the application of two basic systems, GMP and HACCP.

25.4.1 GMP

One of the first quality assurance systems developed by the food industry was that involving the application of GMP, as a supplement to end-product testing. GMP is considered now as a prerequisite for safe food production and has been used for many years to ensure the microbiological and chemical safety and quality of food. The establishment of GMP is the outcome of long practical experience and attention to environmental conditions in the food plant, e.g., requirements for plant layout, hygienic design of equipment and control of operational procedures. GMP is now being introduced into the feed industry for the production of safe animal feed. This measure is supported by the FAO report on animal feeding and food safety (1997) which recommends that GMP is followed at all times in the production of animal feed. Thus specific control measures are given for identified hazards, which include TSEs, biological agents, veterinary drugs, agricultural chemicals and mycotoxins.

However, the GMP concept is largely subjective and its benefits are only qualitative. Also, it has no direct relationship to the safety status of the product. For these reasons, the concept has been extended by introducing the HACCP system, which seeks, among other things, to avoid reliance on testing of the end product as a means of controlling food safety. As indicated previously, such testing may fail to distinguish between safe and unsafe batches of food and is both time-consuming and relatively costly.

25.4.2 HACCP system

The HACCP concept is a systematic approach to the identification, assessment and control of hazards in a particular food operation. It aims to identify problems before they occur and establish measures for their control at stages in production that are critical to ensuring the safety of the food. Control is pro-active, since remedial action is taken in advance of problems occurring.

In a review of the historical background, Barendsz (1995) and Untermann *et al*. (1996) described the development of the HACCP approach, which began in the 1960s. The concept arose mainly from a collaboration between the Pillsbury Company, the US Army Natick Research and Development Laboratories and the US National Aeronautics and Space Administration. The original purpose was to establish a system of safe food production for use in human space travel. At that

Table 25.9 The seven principles of the HACCP system (CAC, Committee on Food Hygiene, 1997)

Principle	Activity
1 Conduct a hazard analysis	List all potential hazards associated with each step, conduct a hazard analysis, and consider any measures to control identified hazards
2 Critical control points (CCPs)	Determine critical control points (CCPs)
3 Critical limit(s)	Establish critical limits for each CCP
4 Monitoring	Establish a system of monitoring for each CCP
5 Corrective actions	Establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control
6 Verification procedures	Establish procedures for verification to confirm that the HACCP system is working effectively
7 Documentation and record keeping	Establish documentation concerning all procedures and records appropriate to these principles and their application

time, the limitations of end-product testing were already appreciated and therefore more attention was given to controlling the processes involved in production and handling rather than the products themselves. When first introduced at a congress on food protection (Department of Health, Education and Welfare, 1972), the concept involved three principles: (i) hazard identification and characterisation; (ii) identification of critical control points (CCPs) and (iii) monitoring of the CCPs.

Many large food companies started to apply HACCP principles on a voluntary basis, and in 1985 the US National Academy of Science recommended that the system should be used throughout the industry. Further support came from the ICMSF (1988), which extended the concept to six principles by adding the specification of criteria, corrective actions and verification (see Table 25.9). In 1989, the US National Advisory Committee on Microbiological Criteria for Foods added a further principle, the establishment of documentation concerning all procedures and records appropriate to the principles and their application. Use of the HACCP system was given a further international dimension by the Codex Alimentarius Commission (CAC) which published details of the principles involved in 1991 and their practical application (CAC, Committee on Food Hygiene, 1991). In 1997, the CAC laid down the 'final' set of principles and clarified the precise meaning of the different terms (CAC, Committee on Food Hygiene, 1997):

- General principles of food hygiene (Alinorm 97/13, Appendix II)
- HACCP system and guidelines for its application (Alinorm 97/13A, Appendix II)

- Principles for the establishment and application of microbiological criteria for foods (Alinorm 97/13A, Appendix III).

The full HACCP system, as described in Alinorm 97/13, is shown in Table 25.9. The document also gives guidelines for practical application of the HACCP system. By 1973, the FDA had made the use of HACCP principles mandatory for the production of low-acid canned foods (FDA, 1973) and, in 1993, the system became a legal requirement for the production of all food products in the European Union (Directive 93/43). Despite widespread usage, the present HACCP concept still has some weak points. One of them is the definition of a hazard. This is not defined as 'an agent with the potential to cause an adverse health effect', as is usual in risk assessment, but as 'an unacceptable contamination, growth and/or survival by micro-organisms of concern' (ICMSF, 1988), which is more restrictive and does not cover all possible hazards.

Another weakness arises from the definition of a CCP. It is stated that a CCP is a location, practice or procedure where hazards can be minimised (ICMSF, 1988; International Association of Milk, Food and Environmental Sanitarians (IAMFES, 1991)) or reduced to an acceptable level (Bryan, 1992; Alinorm 97/13). In both cases, these are qualitative objectives and may lead to differing interpretations. It was Notermans *et al*. (1995) who first made a plea to use the principles of quantitative risk assessment for setting critical limits at the CCPs (process performance, product and storage criteria). It was their opinion that only when the critical limits are defined in quantitative terms can the level of control at CCPs be expressed realistically. At the International Association of Food Protection (IAFP) meeting in 2001, Buchanan (2001) also favoured the use of these principles and suggested that food safety objectives should encompass end-product criteria, which are related to the criteria used in processing.

25.4.3 From feed safety objectives to HACCP criteria

A critical control point in a feed production process can be defined as a location, practice or procedure where hazards can be minimised or reduced to an acceptable level. Therefore, the identification of CCPs in a feed production process is an important step in the control of such hazards. There are various means for controlling potentially hazardous bacteria and chemical agents in feed production processes. Suitable control of hazardous chemical agents can be achieved by setting appropriate criteria for raw materials. Hazardous micro-organisms can be inactivated by, e.g., heating or irradiation, while acidification of feed and use of controlled storage conditions, etc., may also be of value. In some cases stabilisation of microbial levels, i.e., prevention of growth, may be sufficient, for example, for toxin-producing organisms such as *Clostridium botulinum*, which only produces toxin during multiplication. Stabilisation can be achieved by adjusting the formulation to give a low a_W, pH, etc. Such measures not only stabilise bacterial populations, but can also reduce the numbers of any pathogens present.

In summary, there are several options to produce safe animal feed. They comprise:

- setting requirements (criteria) for the raw materials used
- setting criteria for processing (e.g., heat treatment)
- composition of the feed material (e.g., pH, a_W)
- setting storage conditions.

For the relevant possibilities, criteria need to be set in advance. For example, if a dry feed product is being produced from wet raw materials and *Salmonella* could be present, a drying process should be defined that effectively kills *Salmonella*. If an acidified feed is required a pH range should be selected that does not allow multiplication of any hazardous organisms. For feed with a limited storage life, storage conditions need to be established, including temperature and maximum storage time. If there are no CCPs in the production process, they should be introduced for control purposes.

GMP and HACCP are now designed to meet the criteria set. The role of GMP is to ensure that hygienic equipment is used, that well-trained personnel are involved in the production process, that re-contamination is avoided, etc. HACCP is the managerial tool in assuring that the chosen criteria are met. Finally, verification that GMP and HACCP work as planned needs to be introduced. The approach described above is given schematically in Figure 25.3. Firstly, feed safety objectives are set. These feed safety objectives are translated into criteria for raw materials, processing operations, product composition and storage conditions. In setting criteria for CCPs, the use of risk analysis can be considered. Risk analysis transforms the largely qualitative HACCP system into a fully quantitative one. This is possible if CCPs are defined as operations (practices, procedures, processes, etc.) at which control should be exercised to achieve the criteria established (for further information see Notermans *et al*., 2002).

25.4.4 Shortcomings of the control system

Increasingly, recall actions involving food and feed result from the failure of our safety control systems. The recall of poultry meat contaminated with dioxin in Europe after broiler chickens had been given dioxin contaminated feed (van Larebeke *et al*., 2001), the presence of nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether), a herbicide with carcinogenic properties in German grain (see: http://www.fsai.ie/rapid_alerts/alerts/an_2002_215.htm) and the contamination of pig feed with medroxyprogesteron acetate in Belgium and The Netherlands (see: http://www.defra.gov.uk/animalh/int-trde/prod-ex/cins/2002/ape02–22.htm) are examples.

Following these problems, politicians as well as journalists preferably exaggerate the situation by using terms like scandals, failing safety systems and crisis. However, it should be recognised that recalls are the cornerstone of our feed and food safety systems. In the HACCP system recalls are part of the 'corrective actions'. These actions are necessary when monitoring indicates that a particular

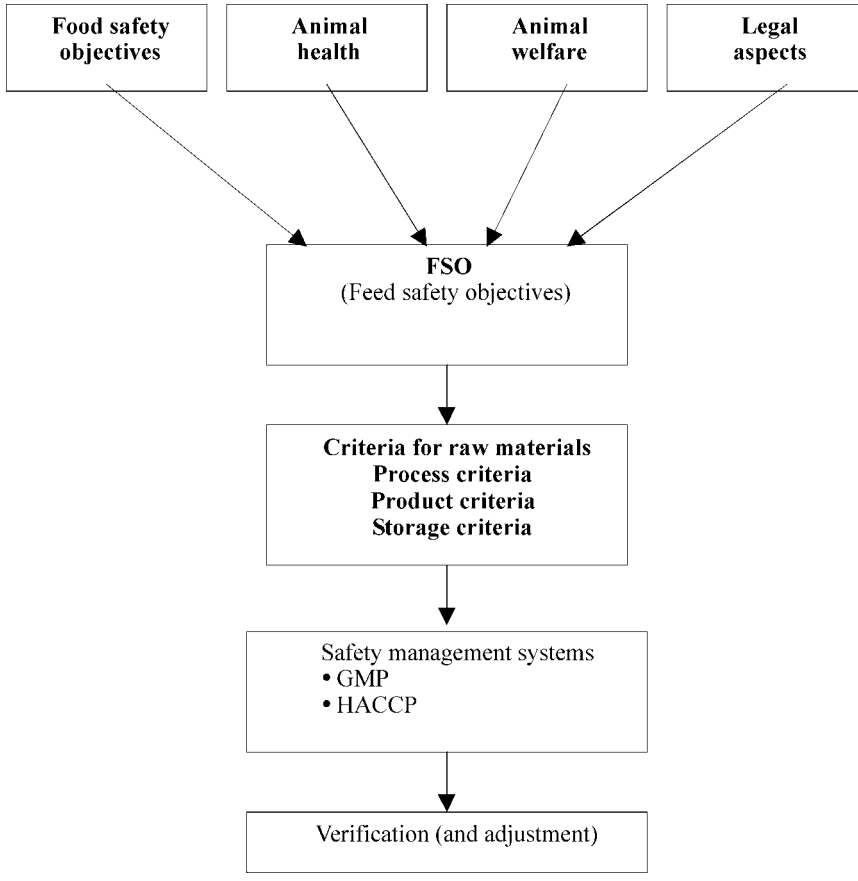

Fig. 25.3 Schematic presentation of the manner in which microbiologically safe feed is/should be produced and the role of risk assessment and risk management to establish food safety objectives and feed safety objectives. For further explanation see text.

CCP is not under control. If a failure is apparent only after the product has been sold a recall action prevents the anticipated health problem in either humans or animals. Incidentally, GMP and HACCP do not, in themselves, prevent specific health problems and corrective actions are necessary. Such situations may occur, for example, when unknown hazards appear or in the case of rare hazardous events. The same may apply where malicious practices or carelessness have occurred.

Unknown hazards

GMP and HACCP are largely based on knowledge collected in the recent past. The system may be inadequate if new, unknown hazards occur or when a hazard re-emerges. Recent examples are the internal contamination of eggs with *S.* Enteritidis and contamination of beef burgers with *E. coli* O157. Before 1980, *E. coli* O157 was unknown as a cause of foodborne diseases and after 1980 *S.*

Enteritidis re-emerged as a disease. A newly discovered chemical hazard is acrylamide which arises from the heating of certain food products. Once these 'new' hazards have been characterised they will be introduced into the GMP and the HACCP concept for relevant applications.

Rare hazards

There are many types of micro-organisms and even more chemical substances that can have an adverse effect on human and animal health. Hazards that occur only very rarely are not usually included in GMP and the HACCP concept. Control of these hazards is mostly carried out at national level by general measures on the part of government. Dioxin is such an example, and the government took general measures to reduce the probability that this toxic substance would spread in the environment. One of these measures was to prohibit the production of food and feed in a given area after contamination of that area had been established.

Corrupt practices

Corrupt or malicious practices are activities which are forbidden by law. A recent example was deliberate use of sugar waste which was contaminated with the hormone medroxyprogesteron acetate as a raw animal feed material. Malicious practices would include terrorist activity, for example, in the case of deliberately induced food poisoning.

Carelessness

Although GMP and HACCP aim to prevent human failings they sometimes fail themselves because of carelessness. Errors or failings occur when staff are stressed by pressure of work or temporary and poorly trained people or unmotivated personnel are employed to do the work. It is clear that control may be lost. The GMP and HACCP concept takes this into consideration by corrective action. If a problem is recognised before distribution, the product can be retained until the corrective action has been carried out. In the case of a product that has already been sold then a recall is necessary. For a successful recall, the product in question must be traced rapidly. Therefore an adequate system of traceability is an additional requirement in the production of safe food and feed. Since recalls may be very costly for the producer an early warning system to reduce the probability of a recall would be beneficial.

25.5 The role and requirements of traceability systems

From section 25.4.4, it is clear that even in the best-managed animal feed production industry, an issue involving the safety of a feedstuff may occur. This is apparent from the feed-related scares of recent years. Therefore, it is important that feed businesses assume that a safety issue will arise and therefore plan ahead. These plans must be tested periodically to ensure that they are

comprehensive and would serve to move an unsafe product out of the distribution chain.

The main objective of traceability is to minimise any adverse health effects by a quick and complete recall. The recall should cover lots of feed that do not meet the safety criteria, lots that are known to have caused adverse effects and others that are suspected of being capable of such effects (precautionary principle). For an adequate recall it is necessary that all feed products and all of the ingredients used in producing the feed are traceable at all stages of production, processing and distribution. This needs to be carried out within a short period of time.

One of the first considerations is to define a lot. Ideally, a lot is a quantity of feed produced and handled under uniform conditions. In practice, this usually means material produced within a limited period of time. The less uniform the conditions of production, the shorter that period of time should be. To apply such a principle implies knowledge about the uniformity of conditions. Under GMP, a lot number is a code that allows identification of the feed in relation to some aspect of production, the kind of formulation, or a time interval in the production schedule. In the commercial sense a lot is a quantity of product supposedly produced from identical ingredients, processed under the same conditions and usually on a particular production line.

The principles involved in developing a traceability system, as described here, are largely based on the Guidance Note No. 10 entitled *Product recall and traceability* published by the Food Safety Authority of Ireland (2002). For the development of a traceability system, feed businesses should first of all define the scope of the traceability that can be achieved. It is necessary that businesses' activities, from primary production on the farm and throughout the supply chain, should have a traceability system that runs seamlessly from one activity to the next. Traceability needs to be composed of the following elements:

- *supplier traceability*: traceability of suppliers and their products entering the business in question
- *process traceability*: traceability of feedstuffs through the supply chain
- *customer traceability*: traceability of feedstuffs to the immediate customer.

Attention must be given to the interface between the three areas above to ensure that the traceability system is seamless.

Supplier traceability

Each processor should be able to ensure that all ingredients entering the premises are traceable to individual suppliers. Important elements are:

- Each incoming unit of ingredient should carry a means of tracing its source of supply and history.
- Goods inwards documents for each delivery should record all information necessary to maintain traceability from the supplier. Relevant information includes:

– name of the supplier
– any supplier batch code
– delivery date
– confirmation of acceptance
– number of units
– lot number
– reference to any in-house quality control records associated with the delivery.

Process traceability

Each feed production business should be able to ensure that any batch of feedstuff can be traced back to the ingredients and primary packaging used in its manufacture. The important elements in process traceability are:

- A product batch must be identified.
- To ensure that a product batch is a true batch, it must be separated by a clean break from other product batches that use the same equipment. Where carry-over is likely, feed businesses should conduct studies to define the likely extent of carry-over, which should be documented.
- A unique batch code identifier should always be applied to it.
- Each and every saleable unit in the product batch should be coded.
- Internal documentation should accompany the product batch.
- The traceability codes of ingredients and primary packaging used for a product batch should be recorded and associated with the product batch code.
- Processing and quality records should contain all the necessary information relating to the processing conditions, ingredients used, storage conditions, etc., to allow traceability from the finished product. Examples of relevant information are:
  – product name
  – product batch code
  – date of production
  – time of start and end of the production
  – reference to any in-house quality control records
  – reference to any in-house process control records.

Each wholesale or retail feed business should at least be able to ensure that feedstuffs handled on site are traceable to the supplier at all times.

- Traceability information accompanying products entering a wholesale or retail business should be maintained throughout any handling operations that may occur on the premises.
- Customer traceability for all processors and wholesalers involved in business-to-business trade should be maintained.

Customer traceability

This is the third step in the development of a traceability system. The important elements are:

- A list of all immediate customers, details of the products they purchase and full contact details should be held by the feed business. The list must be updated regularly.
- Any documentation accompanying the product at the point of sale should contain all the information necessary for traceability to be maintained through the distribution chain. Examples of necessary information are:
  – name, address and contact details of the customer
  – name, address and contact details of the distributor
  – container code of the transport vehicle.
- A full list of the products being purchased by the customer with details of each product should be held. Examples are:
  – product name
  – product batch codes.

A responsible individual should be in place to deal with product that is rejected by the customer for feed safety reasons. If returned, the rejected product must be quarantined pending investigation and maintained separately from product cleared for release.

25.5.1 Difficulties encountered

There are many difficulties in establishing a traceability system for animal feed. In the case of bulk delivery of ingredients into large-scale storage and production facilities, it may not be possible to ensure that only ingredients from a single batch have been used. The delivery dates, identification of prior storage facility and weight or volume of the delivery may be the only way of checking this point. Thus, it is necessary to trace the contents of a bulk storage facility through the entire production process.

For feed production in particular, a clear separation between individual batches of finished feed is not always possible. The main reason is that carry-over of ingredients is very likely to occur to some extent. Another aspect is that, in cases of emergency, traceability needs to be carried out rapidly. In an industrial setting this can be done only if use is made of well-established computer systems that are designed for traceability purposes.

Bottle-necks in traceability may concern the internal organisation of the feed production facility, the production process and the product itself. Organisation-related difficulties comprise:

- character and size of the facility
- exchange of information between different parts of the production chain
- level of knowledge with respect to feed safety and traceability
- feed industry co-operation.

Character and size of the facility

Inevitably, there are companies of different nature and size. These will involve differences in degree of automation and availability of information and a

varying ability to implement and maintain the traceability system. Small and medium enterprises will find the requirements more costly than large enterprises. It might even be that the cost of a traceability system for them will be greater than the profits. This can be avoided partly by developing common codes of practice for traceability which provide clear instructions and utilise standardised computer software. This approach may be combined with the development and introduction of a HACCP system.

Information exchange in the production chain
The timing and the procedures for product transfer at the different stages are not well established. This applies especially to situations in which companies are using different control systems that may not always be adequate for product identification. The problem can be solved only by using identical electronic systems for transferring data between links. This is possible only with good co-operation and clear arrangements.

Level of knowledge with respect to feed safety and traceability
If the level of company knowledge is limited, buyers may make excessive demands on suppliers with respect to products and information, and they do not bear costs incurred. The converse is also possible if buyers do not make sufficient demand. Co-operation between parties involved in feed production may overcome this problem, especially through co-ordination of feed safety control and traceability. Introduction of an auditing system is advisable.

Difficulties relating to the production process and product comprise both general and chain-specific problems. General difficulties include:

- labelling of products and deciding the size of a lot
- separation of lots and stock management
- mixing of feed, carry-over of ingredients from one batch to another and reprocessing of returned material.

Chain-specific difficulties comprise:

- complexity of the process and product
- import and export.

Labelling of products and lot size
Problems may arise if the product cannot be physically labelled. This applies among others to dried and liquid bulk products which are common in feed production. In such cases, accompanying documents are necessary for the identification of the products. It may also be necessary to limit the lot size, especially where continuous processes are used.

Separation of lots and stock management
Separation of lots is necessary especially when there are different product formulations. However, strict separation is difficult when dealing with a variety

of products and production processes, and even the processing facilities themselves may contribute to the problem. Thus, stock management will become a problem and the first-in first-out system is difficult to realise. A solution may be to assess carefully the level of mixing between lots and make judgements about valid lot separation.

Mixing, carry-over and material returned for reprocessing
This applies especially to factors related to separation of lots. Examples are the use of external/internal returns, storage of bulk products in silos and tanks which have not been emptied and carry-over of ingredients where there has been no cleaning between the production of different lots. These difficulties are controllable but they often require a restructuring of a large part of the production facility.

Complexity in the process and product
Additional complicating factors that may be encountered are priority orders, automatic dispensing of additives, re-use of materials, product recycling and the use of by-products and waste materials. Procedures need to be developed that take all these aspects into consideration, including a quantitative analysis of their impact.

Import and export
Import of bulk feed products from third countries usually involves very large quantities transported by sea. Not all of the cargo may have come from the original producer. In addition, the materials could have undergone several periods of storage and transhipments. A complicating factor is that, almost certainly, the cargo will have changed hands. It will take time before importers develop a reliable traceability system. In the case of exports, a change in product destination can easily occur, although the exporter may know the purchaser of the product. For both imported and exported products, traceability may be delayed and in some cases even be impossible. The situation will improve only if international co-operation is increased and traceability is given a high priority.

25.6 Future trends: hazard early warning systems

Feed-related incidents have demonstrated that hazards can lead to crises when not detected in time. In the case of dioxin contamination of oils and fats, the animal feed and human food sectors experienced adverse affects. These include loss of reputation and consumer confidence, with significant economic losses. Another consequence is that regulatory authorities are obliged to take strong measures to prevent future crises and minimise possible adverse health effects by introducing crisis management procedures to re-establish consumer confidence. In practice, crisis management has now been adopted by the European Commission and several control systems have been introduced. These

systems are all curative and preventive measures are still needed to avoid crisis situations. Such preventive systems must be based on providing early information.

In the production of human food several information systems have been developed during the past ten years. The first included use of internet-based information on (regional) foodborne disease outbreaks and contaminants of raw food materials. One example is Enternet which is a European information system for foodborne disease outbreaks. Another example, which deals with the quality of raw agricultural foods and feed products, is the Dutch Programme for the Quality of Agricultural Products (http://www.agralin.nl/kap/report/kapuk1.html). In this programme, residues of substances such as PCBs, cadmium and aflatoxin in animal feed are monitored and the information made available via the internet to interested parties. Now, many other countries are developing similar monitoring systems. Another hazard early-warning system, termed EMPRESS, has been set up by FAO and deals with animal diseases (http://www.fao.org/waicent/faoinfo/agricult/aga/A7_en.htm). It is concerned with promoting the effective containment and control of major epidemic and newly emerging livestock diseases. This involves their progressive elimination on a regional and global basis through international co-operation in early warning, rapid reaction, enabling research and co-ordination. It includes the Global Rinderpest Eradication Programme (GREP) and projects on diagnosis, strategic epidemiological analysis and vaccine development for major epidemic diseases such as FMD, Rinderpest, and African Swine Fever (ASF) (http://www.fao.org/waicent/faoinfo/agricult/aga/programs_en.htm).

In The Netherlands, the Product Board Animal Feed has introduced a quality assurance programme that includes an Early Warning & Response System (EWS) and is presented on internet page http://www.pdv.nl/english/kwaliteit/. The purpose of this system is to identify and eliminate any potential hazards for animals and consumers that may arise in spite of preventive quality assurance. EWS was developed together with the Dutch research organisation TNO. The EWS network for animal feed is based on parties involved in the animal feed (and human food) production chain. Firstly, the necessary information sources are identified. The members include the links in the chain, interested organisations like the Dutch Association of the Animal Feed Industry, veterinary practitioners and additional information sources like research institutes and database systems. Veterinary practitioners play an important role in identifying the occurrence of hazards on farms. Secondly, a central EWS Information & Communication Centre (ICC) has been set up at the Product Board Animal Feed to cover the animal feed sector. Members of the EWS network are obliged to report all unusual situations when a problem cannot be handled by the organisation itself or when there is likely to be a negative impact on other links in the animal feed and food production chain.

The ICC receives information from all parties involved in the EWS network. It convenes the EWS Team that assesses the reported information on the basis of evaluation protocols and takes appropriate measures, either corrective or

preventive, in the animal feed and human food production sector. The EWS Team includes decision-makers and is responsible for the dissemination of information to relevant parties. The aim of the EWS Team is to take adequate measures before an issue becomes controversial and requires authoritative crisis management. Commitment among the members of the EWS network and confidentiality of information must be ensured. Suitable protocols and assessment methods are needed to handle the information adequately and to set appropriate measures.

Establishing an EWS network could result in the ICC being overwhelmed with information. Therefore, adequate filters are needed to sort and assess the information. The first step in this is the one person or organisation that identifies the information signal and evaluates its relevance. Unknown hazards are identified using indicators. Protocols have been developed to streamline the information. Additional tools to facilitate the assessment of information, e.g., risk profiling, are being developed by TNO. All members of the EWS network should record their own information in order to forecast possible problems and analyse trends.

25.7 Abbreviations

ALARA	As low as reasonably achievable
ALOP	Appropriate level of protection
ASF	African swine fever
a_w	Water activity
MBM	Meat and bone meal
BSE	Bovine Spongiform Encephalopathy
CAC	Codex Alimentarius Commission
CCP	Critical Control Point
ICC	Information & Communication Centre
DAS	Diacetoxyscirpenol
DDT	Dichloro-Diphenyl-Trichloroethane
EU	European Union
EWS	Early warning & response system
FAO	Food and Agricultural Organisation of the United Nations
FDA	Food and Drug Administration
FMD	Foot and mouth disease
GMP	Good manufacturing practice
GREP	Global Rinderpest eradication programme
GVP	Good veterinary practice
HACCP	Hazard analysis critical control point concept
IAMFES	International Association of Milk, Food and Environmental Sanitarians
IAFP	International Association of Food Protection
ICMSF	The International Commision on Microbiological Specifications for Foods

ICC	Information & communication centre
MRL	Maximum residue limits
PCBs	Polychlorinated biphenyls
TEQ	Toxicity equivalent
TSE	Transmissible Spongiform Encephalopathies

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26

Geographic traceability of cheese

L. Pillonel and J. O. Bosset, Federal Dairy Research Station of Liebefeld, Switzerland

26.1 Introduction

Food authenticity is a broad analytical challenge including many different types of misrepresentations (Lees, 1998). Determination of the geographic origin is one aspect which is receiving more attention with the increasing mobility and the low transport costs of our society. Food goods are travelling around the world whenever it is economically attractive. This traffic has its negative side which has been highlighted by recent food scandals (BSE, dioxin, etc.). As a consequence, European consumers have become aware, and more afraid, of globalisation in the food sector. It is not surprising that organic foods, protected designation of origin (PDO) products and other labels continuously gain in popularity. A category of consumers is looking for traditional, regional, hand-made products which are supposed to be safer because they are more natural. If one wants to keep consumers' confidence in such labels, the early detection of any kind of fraud must be made possible.

In the dairy sector, the control of the geographic origin is an important factor for PDO cheese and for cheese types produced in different countries and sold under the same name. Those countries, which originated a particular type of cheese, may wish to differentiate their product from legal copies made abroad (e.g., Emmental in Switzerland, Camembert in France, Mozzarella in Italy). Many investigations have already been carried out to characterise the Swiss (e.g., Bosset *et al*., 1992 and 1997, Bachmann *et al*., 1997, Bütikofer *et al*., 1998), the French (e.g., Berdagué *et al*., 1990a, b), the German (e.g., Kielwein, 1975) and the Austrian (e.g., Jaros *et al*., 1996) Emmental, but few were dedicated to the analytical traceability of the geographic origin of a cheese sample. Rohm (1992) carried out a regional classification of Emmental cheese in

western Austria based on some chemical parameters. Picque *et al*. (2002) used infra-red spectroscopy to differentiate three French Emmental cheeses. Bosset *et al.* (1998) and Collomb *et al.* (1999) found significant differences in the amount of terpenes and conjugated linoleic acids (CLA) in Gruyère cheese between a lowland and a highland production zone.

Manca *et al.* (2001) was able to distinguish between Pecorino Sardo, Siciliano and Pugliese cheese using stable isotope ratios and free amino acids ratios. Grappin *et al.* (1999) could correctly discriminate the origin of 20 Comté cheeses made in five different cheese plants using some physico-chemical variables, microbial counts and sensory characteristics. Sensory analyses have been applied to differentiate Parmigiano-Reggiano (Zannoni *et al*., 1991 and 1994) and Grana Padano cheese (Moio and Addeo, 1998) from their imitations. Muir *et al.* (1997) showed differences in flavour and texture between raw milk farmhouse Cheddar and Cheddar cheese made from pasteurised milk. However, no differences were found between the different countries of production.

The French Emmental 'Grand-Cru' was characterised by a more intense flavour described as fruity, salted, pungent and sweet and by a weaker rancid odour than in the other French Emmental (Berdagué *et al*., 1990b). Monnet *et al*. (2000) positively correlated the type of soil (edaphic parameters) with the sensory quality of 20 Comté cheeses from the French Jura. The influence of altitude on Abondance cheese flavour was investigated by Bugaud *et al*. (2001).

Switzerland manufactures less than 10% of European Emmental. This cheese type is not a strictly defined product and is offered to consumers under various forms: foil vs. rind ripened, stainless steel vs. copper vat, with vs. without additives, ripening time from six weeks up to more than twelve months, raw or pasteurised milk, etc. The Swiss authorities started a project to protect consumers against mislabelling of cheese according to their geographic origin. Emmental has been used as case study to develop an authentication strategy usable for other cheese types or food products.

26.2 Approaches to identifying geographical origin

To solve authenticity problems, many analytical methods have been proposed (e.g., Ashurst and Dennis, 1998, Cordella *et al*., 2002). Various laboratories propose powerful techniques such as isotope ratio mass spectrometry, infra-red spectroscopy or DNA analysis for the authentication of food products. However, these techniques are almost always considered separately. For a given problem, only one is used, eventually delivering several parameters that may be combined by multivariate analysis. In the problematic of geographic traceability and especially by complex matrices such as found in cheese, a single method may rarely be sufficient to recognise an origin. It might be useful to first get an overview of all possible analytical means and then choose the best among them.

Few authors tried to combine different techniques and evaluate the discriminating potential of their results. Lehtonen *et al*. (1999) differentiated

brands of distilled alcoholic beverages by means of chemical composition, ultraviolet and visible absorption and pH values. Investigations by gas chromatography, hydrogen- and carbon-isotope ratio measurements made possible a discrimination between spirits of different fruit origins (Bauer-Christoph *et al*., 1997). A classification of Austrian cheese was achieved by combining different chemical parameters (Rohm, 1992). Manca *et al.* (2001) used stable isotope ratios and free amino acids to distinguish between various Pecorino cheeses. Rebolo *et al.* (2000) used trace elements, volatile compounds and phenols to differentiate between various Galician wines. Different combinations of mineral-, polyphenol- and anthocyanin-content as well as sensory analyses were investigated for geographical classification of Greek wines (Kallithraka *et al*., 2001).

The current project is intended to bring another strategy in authentication issues with a more global approach. Instead of going straight to typical geographic indicators, several different kinds of analytical methods were investigated to check their ability as indicators of origin, carrying out a proper screening test. The project has been planned over three years. In a first step, only 20 Emmental cheese samples from six regions were investigated using more than 30 analytical methods. According to the results obtained, the best discriminating methods were selected for the remainder of the project. In the second step, about 90 samples from winter and summer production will be investigated with the selected methods. The last part is dedicated to the elaboration of a mathematical model for the determination of origin and its validation with new samples. This chapter presents a selection of the methods carried out in the first step, while the second step is in progress.

26.2.1 Sample selection

Table 26.1 summarises the geographic origin, the date of manufacture and the ripening time of the 20 Emmental cheeses selected from six regions. Three samples were chosen from each region (except for Switzerland with six and Finland with two samples). Three regions were adjacent to Switzerland (Savoie (F), Allgäu (D), Vorarlberg (A)). The geographic and climatic proximity of these regions, as well as the similar technologies used, allow one to test the robustness of the methods (intra-variability within a region vs. inter-variability between regions). The two remaining regions (Bretagne (F) and the central region of Finland) were quite distant from Switzerland. The corresponding cheeses will be indicated as ‘Allgäu’, ‘Bretagne’, Finland’, ‘Savoie’, ‘Switzerland’ and ‘Vorarlberg’. Taken together, these six countries accounted for approx. 77% of European Emmental production.

The cheese samples were supplied directly by the cheese factories or were collected with the help of a national dairy research centre. They were chosen with different ripening times (2.5–4 months) according to availability in the stores of the corresponding regions. For most regions, the ripening time is shorter than three months. In Switzerland, however, a four-month old

Table 26.1 Origin, date of manufacture and ripening time of the 20 cheese samples investigated

Abbreviation	Region (country)	Number of samples	Date of manufacture	Ripening time (months)
AL	Allgäu (D)	3	25.12.2000	4
BR	Bretagne (F)	3	20.02.2001	2.5
CH	Switzerland (CH)	6	26.12.2000	4
FI	Middle Finland (FI)	2	04.02.2001	3
SA	Savoie (F)	3	05.02.2001	3
VO	Vorarlberg (A)	3	02.02.2001	3

Emmentaler is still considered very young. We limited our investigations to young Emmental because there the risk of confusion is the highest.

26.2.2 Methods investigated

Very different methods, from standard gross chemical composition to radioactivity, were tested for their discriminant potential. At the end of the screening test, the methods were classified into three groups according to their level of importance (Tables 26.2–4). The first group contained the methods not adequate to determine the authenticity of the cheese samples because the differences were either not significant enough or the dependence on the season was too strong. They were not discussed in the current text. For more details see

Table 26.2 Methods which will no longer be used in the future

Methods/Parameters	Reason for the refusal	Pillonel *et al.*
Biogenic amines	Strong variation within a region	2003b
OPA	Same information as N-fractions	2003b
Propionic acid bacteria	No significant differences	2002a
Enterococci, facultative hetero-fermentative and salt tolerant lactobacilli	Poor significant differences	2002a
Water, alkaline phosphatase, citrate, vitamin A and E	No significant differences	2002a
Rheology	No significant differences except for penetration depth	2003b
Colour measurement	Strong dependence on the season	2002a
Fat chemistry, triglycerides, free fatty acids	Time-consuming, strong dependence on the season	2002b
Radioactive compounds	Poor significant differences, high cost	2003c
ICP-MS	Time consuming, lack of reference	2003c

Table 26.3 Selected methods/parameters for the follow-up of the project

Methods	Separated	Not separated	Pillonel *et al.*
Infraredspectroscopy[1]	All regions		2002c
Electronic nose	All regions[2]		2003a
Obligate heterofermentative Lb.	CH[2]	All others	2002a
Lb. helveticus	CH	All others	2002a
pH-value, L-lactate, succinate, pyruvate, N-fractions, fat, sodium chloride	VO, FI, BR, CH, AL[2], SA[2]		2002a, 2003b
Volatile short-chain acids	BR, FI[2]	AL, CH, SA, VO	2002a
AAS (major elements)	BR, FI, CH[2], VO[2], AL[2]	SA	2003c
IRMS (stable isotope ratios)	FI, BR, SA	CH, VO, AL	2003c

AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

Regions within brackets are not separated from one another.
[1] A linear discriminant analysis was applied on the spectra.
[2] A clear tendency of grouping was observed for these regions. They are, however, not perfectly separated.

Table 26.4 Promising but time-consuming methods

Methods	Separated	Not separated	Pillonel *et al.*
HPLC peptide profile	FI, (CH + VO), (BR + SA)	AL	2003b
SDS-PAGE	(BR, FI)	all others	2003b
Volatile compounds	CH, AL, VO, SA, FI	BR	2003a
Free amino acids	CH, VO, BR, AL, FI	SA	2003b

AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

Regions within brackets are not separated from one another.

the references quoted in Table 26.2. The methods listed in Table 26.3 have been selected as most promising for the remainder of the project. The methods from the third group (Table 26.4) delivered interesting results but were time consuming. These methods could be exploited if the ones used from the third group do not allow for sufficient separation.

To evaluate the potential of each parameter to discriminate between the origins of the cheeses, a difference test on the mean value of each region was calculated (Fisher's test). Furthermore principal component analysis (PCA) was carried out to visualise the natural groupings generated by selected analytical parameters. Trained classification techniques such as linear discriminant analysis were explicitly not used because they require a larger dataset to deliver reliable results.

26.3 Analytical methods: primary indicators

Parameters allowing the determination of the geographic origin are called indicators (of origin). Primary indicators are directly related to the geographic origin. In the case of cheese, compounds acting as primary indicators are transferred from the forage and the water consumed by the herd into the milk and finally the cheese. Primary indicators are not influenced by cheese making and depend only on cows' feeding. The forage or a part of it may, however, come from a far region, leading to a difficult interpretation of the results.

26.3.1 Stable isotope ratios

The technique of the stable isotope ratios is the most used for the determination of the origin of food products. All analyses were carried out on a casein fraction obtained through successively freeze-drying, degreasing and acidification of the cheese samples (Manca *et al*., 2001). The results of the four ratios $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{87}Sr/^{86}Sr$ are presented in Table 26.5.

The proximity of the ocean was responsible for the significantly higher $\delta^{2}H$ values in 'Bretagne'. The samples were depleted in ^{2}H in the Alpine regions and in Finland (high latitude) due to the faster raining out of the heavier isotope. Furthermore, Bretagne was the single region where maize, a C-4 plant, was fed during the winter months. Samples from this region showed consequently by far the highest $\delta^{13}C$ value. The significantly lower $\delta^{15}N$ value obtained in 'Savoie' compared to 'Bretagne' and 'Finland' was probably relating to the more extensive use of the pastures in this pre-alpine region.

The stable isotopic ratios presented above all undergo seasonal variations. Isotope ratios of strontium provide means by which regions may be subdivided further according to their respective geological conditions, independently of the season. ^{87}Sr is produced from ^{87}Rb by radioactive β-decay, whereas abundance of primordial ^{86}Sr remains virtually constant in a given rock. Old acidic rocks such as granite show the highest ratios, mafic and carbonate-rich rocks the lowest. Supplementation with imported forage (maize, concentrates, etc.) can, of course, modify the results. The mean $\delta^{87}Sr$ values lay between -1.64 and -1.00 for all regions except for 'Finland' with a mean value of +4.85‰. Finland was the single investigated region covered homogeneously with an old acidic rock bed, causing this significantly higher ratio.

A PCA of these four parameters led to a clear separation of the regions Bretagne, Savoie and Finland (Fig. 26.1). The remaining geographically close regions showed too few differences for these parameters to be significantly differentiated. More details on the interpretations of various isotopic ratios may be found in Chapter 7 of this book.

26.3.2 Major and trace elements

Major and trace elements were investigated by atomic absorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS). The

Table 26.5 Stable isotope ratios as well as major and trace elements (μg/g) in the 20 investigated Emmental cheeses

Analytes	ANOVA	Region (n=) AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Stable isotope ratios (‰)[1]													
$\delta^{13}C$	***	**−25.2^{C}**	0.29	**−17.8^{A}**	0.77	**−24.8^{C}**	0.52	**−26.38^{D}**	0.05	**−23.9^{B}**	0.35	**−24.9^{BC}**	0.14
$\delta^{15}N$	***	**5.3^{AB}**	0.37	**6.4^{A}**	0.72	**5.8^{AB}**	0.70	**6.4^{A}**	0.33	**3.8^{C}**	0.16	**5.1^{B}**	0.20
$\delta^{2}H$	***	**−121.0^{BC}**	1.1	**−102.0^{A}**	4.1	**−122.0^{C}**	1.9	**−132.0^{D}**	6.3	**−115.0^{B}**	1.8	**−122.8^{C}**	0.57
$\delta^{87}Sr$	***	**−1.6^{B}**	0.42	**−1^{B}**	1.6	**−1.2^{B}**	0.39	**4.9^{A}**	0.70	**−1.1^{B}**	0.26	**−1.6^{B}**	0.24
Elements by ICP-MS[2] (μg/g)													
Barium	*	**0.89^{ABC}**	0.07	**0.61^{BC}**	0.06	**0.90^{AB}**	0.14	**1.03^{AB}**	0.40	**0.54^{C}**	0.13	**1.01^{A}**	0.18
Gallium	*	**0.020^{AB}**	0.003	**0.013^{B}**	0.001	**0.019^{AB}**	0.004	**0.022^{AB}**	0.007	**0.012^{B}**	0.003	**0.022^{A}**	0.004
Iod	***	**0.01^{C}**	0.00	**0.07^{BC}**	0.01	**0.10^{AB}**	0.05	**0.17^{A}**	0.03	**0.06^{BC}**	0.01	**0.02^{C}**	0.00
Molybdenum	***	**0.11^{B}**	0.03	**0.07^{C}**	0.00	**0.15^{A}**	0.01	**0.07^{BC}**	0.00	**0.08^{BC}**	0.02	**0.07^{BC}**	0.01
Rubidium	**	**1.29^{A}**	0.47	**0.54^{B}**	0.18	**0.44^{B}**	0.17	**0.81^{AB}**	0.05	**0.78^{AB}**	0.32	**1.15^{AB}**	0.18
Elements by AAS (μg/g)													
Calcium	***	**9496.0^{B}**	153.0	**10498.0^{A}**	209.0	**9472.0^{B}**	193.0	**8929.0^{C}**	136.0	**10020.0^{A}**	357.0	**9345.0^{BC}**	134.0
Copper	***	**9.0^{AB}**	2.0	**<1.0^{C}**	–	**8.5^{B}**	1.6	**13.1^{A}**	2.7	**8.0^{B}**	1.3	**4.0^{C}**	1.4
Magnesium	**	**385.0^{A}**	11.0	**390.0^{A}**	4.0	**348.0^{B}**	18.0	**358.0^{AB}**	21.0	**365.0^{AB}**	13.0	**336.0^{B}**	11.0
Manganese	***	**0.28^{B}**	0.02	**0.40^{A}**	0.03	**0.26^{B}**	0.03	**0.30^{AB}**	0.08	**0.27^{B}**	0.07	**0.21^{B}**	0.02
Potassium	*	**931.0^{AB}**	120.0	**953.0^{AB}**	63.0	**837.0^{B}**	77.0	**800.0^{B}**	12.0	**977.0^{A}**	38.0	**868.0^{AB}**	3.0
Sodium	***	**2475.0^{A}**	329.0	**1700.0^{AB}**	630.0	**1316.0^{B}**	217.0	**2561.0^{A}**	39.0	**1482.0^{B}**	375.0	**1316.0^{B}**	355.0
Zinc	***	**43.5^{C}**	1.3	**47.8^{A}**	2.5	**42.2^{C}**	0.8	**47.1^{AB}**	0.5	**44.4^{BC}**	1.5	**41.3^{C}**	0.7

Key: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.
Production sites: A > B > C > D (= significantly different contents) or AB = A and B overlap by using a univariate discriminant analysis.
[1] $-\delta‰ = 1000\ [R_{sample} - R_{standard}]/R_{standard}$, where R represents the ratio of the higher mass over the lower mass isotopes.
[2] The results of the ICP-MS are semi-quantitative.

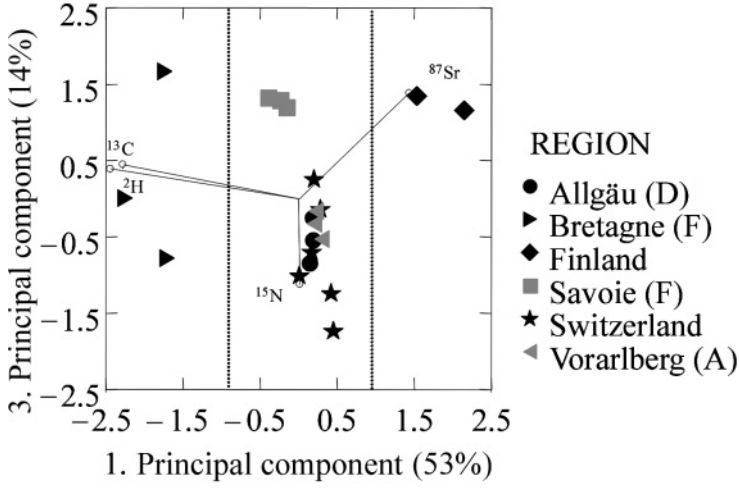

Fig. 26.1 Principal component analysis of the parameters $\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$, $\delta^{87}Sr$. Clear separation of the groups 'Finland', 'Savoie' and 'Bretagne' from the other regions.

results for the 20 cheeses are shown in Table 26.5. Seven elements were analysed using AAS. The calcium content from the two French regions (SA and BR) was the highest, that from Finland the lowest. This may be explained by the fact that no curdle washing was applied in France whereas Finland had the highest washing rate with up to 26% water addition. Further, the regions Bretagne and Finland had in common that cheese is manufactured in stainless steel vats instead of the traditional copper vats. The copper content was therefore the lowest in 'Bretagne'.

In 'Finland', the values were biased by the addition of copper sulphate into the milk during manufacture. Differences obtained for magnesium and potassium were less significant than for the other elements and more difficult to interpret. Manganese and zinc were found at highest concentrations in 'Bretagne' followed by 'Finland'. These higher concentrations could be explained by a different diet fed to the cows (type of concentrates). Sodium contents correlated well with chloride concentrations ($r = 0.95$, Pillonel *et al.*, 2002a), confirming its origin as sodium chloride from the salt bath.

Using ICP-MS, two further elements showed significant differences ($P \leq 0.001$) between the regions of origin. The iodine content was significantly higher in 'Finland' than in all other except 'Switzerland'. Molybdenum was interesting for separating 'Switzerland' from the other regions as it had significantly higher values. By combining the concentrations of molybdenum and sodium, 'Switzerland' was clearly differentiated. Furthermore, the geographically close regions 'Allgäu' and 'Vorarlberg' were separated one from the other (Fig. 26.2).

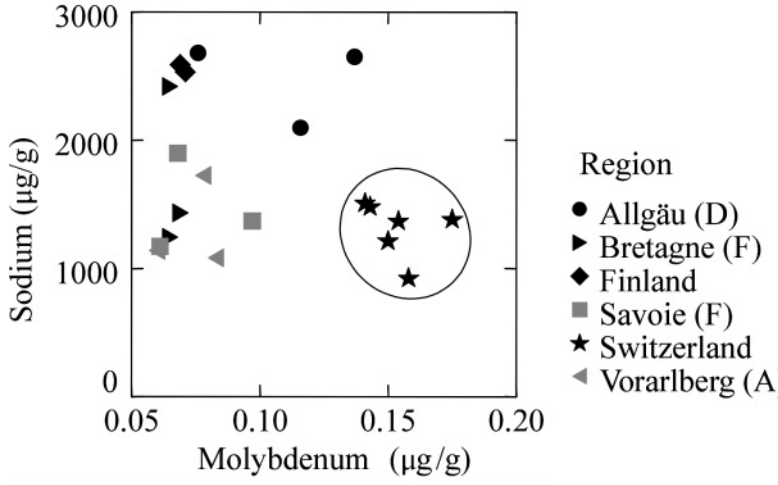

Fig. 26.2 Concentration of molybdenum and sodium by ICP-MS and AAS respectively. Separation of Emmentaler Switzerland™ from all other samples. 'Vorarlberg' and 'Allgäu' are distinctly separated one from the other using these two parameters.

26.4 Analytical methods: secondary indicators

Secondary indicators do not depend entirely on the geographic origin but mainly on the technology used for the transformation of a product, i.e., the milk used. Cheese making is related to local, regional or national traditions leading to differences within a cheese type. Starters, heating temperature of the curd and ripening time are some of the manufacturing parameters typical for a defined region and lead to chemical, physical or microbial secondary indicators.

26.4.1 Proteolysis

The enzymatic degradation of casein into peptides and amino acids is a very complex process involving different proteases and peptidases. Not only ripening time but also microorganisms present in raw milk or added as starters play a key role. The following parameters of the proteolysis were investigated: total nitrogen (TN), water soluble nitrogen (WSN), non-protein nitrogen (NPN), casein fraction by SDS gel electrophoresis, peptide pattern of the water soluble peptide fraction by RP-HPLC and free amino acids by HPLC.

Table 26.6 summarises the results of the nitrogen- and casein-fractions where significant differences were found between the regions. 'Bretagne', followed by 'Finland', showed the lowest NPN and WSN values as would be expected from such young cheeses. 'Allgäu' and 'Savoie', ripened 4 and 3.5 months respectively, showed the highest values for these two parameters. The values for 'Switzerland' lay in the middle. This Emmental cheese, aged for four months, is still considered to be very young in Switzerland. In fact, the proteolysis of 'Switzerland' is deliberately slowed down to allow a ripening time

Table 26.6 Nitrogen- and casein-fractions in the 20 Emmental cheese samples investigated

Analytes	ANOVA	Region (n=) AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Nitrogen fractions													
TN (g/kg)	*	**44.1**^{AB}	1.7	**45.03**^{AB}	0.72	**44.42**^{AB}	0.85	**45.5**^{AB}	1.3	**46.20**^{A}	0.77	**42.9**^{B}	1.6
WSN (g/kg)	***	**11.2**^{A}	1.1	**6.4**^{C}	1.7	**9.35**^{B}	0.41	**8.31**^{BC}	0.51	**10.72**^{AB}	0.99	**8.77**^{B}	0.38
NPN (g/kg)	***	**7.37**^{A}	0.65	**3.98**^{D}	0.90	**5.86**^{BC}	0.55	**6.19**^{ABC}	0.58	**7.20**^{AB}	1.07	**5.42**^{CD}	0.37
Casein fractions													
Para*K*	*	**8.87**^{A}	0.85	**6.60**^{B}	0.56	**8.02**^{AB}	0.83	**7.05**^{AB}	0.92	**7.33**^{AB}	0.58	**7.87**^{AB}	0.55
$\gamma 1$	*	**12.40**^{A}	0.96	**8.4**^{B}	1.7	**11.1**^{AB}	1.4	**9.6**^{AB}	2.6	**10.13**^{AB}	0.87	**9.57**^{AB}	0.23
$\gamma 2$	**	**7.1**^{A}	1.0	**3.73**^{B}	0.80	**6.53**^{A}	0.69	**6.0**^{A}	1.2	**5.83**^{A}	0.32	**5.93**^{A}	0.81
$\gamma 3$	*	**3.46**^{ABC}	0.76	**2.00**^{C}	0.10	**4.23**^{A}	0.88	**3.05**^{ABC}	0.78	**3.93**^{AB}	0.72	**4.0**^{AB}	1.2
β	**	**14.4**^{C}	1.4	**21.5**^{A}	1.6	**16.8**^{B}	1.5	**17.6**^{AB}	2.7	**17.1**^{B}	1.6	**18.1**^{AB}	1.4
α_{s2}	**	**n.d.**^{B}	–	**2.7**^{A}	2.4	**n.d.**^{B}	–	**n.d.**^{B}	–	**n.d.**^{B}	–	**n.d.**^{B}	–
α_{s1}	***	**8.7**^{BC}	1.5	**20.6**^{A}	1.4	**6.68**^{C}	0.96	**19.70**^{A}	0.28	**11.7**^{B}	1.8	**10.5**^{B}	1.7
α_{s1}-I	**	**19.5**^{ABC}	1.8	**15.7**^{C}	1.8	**22.3**^{A}	1.8	**16.9**^{BC}	2.2	**19.47**^{ABC}	0.92	**21.1**^{AB}	2.1
X3	***	**8.00**^{A}	0.66	**4.23**^{C}	0.60	**7.1**^{AB}	1.2	**5.70**^{BC}	0.14	**6.60**^{AB}	0.17	**5.53**^{BC}	0.23

Key: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, * p = 0.05, ** p = 0.01, *** p = 0.001.

Production sites: A > B > C > D (= significantly different contents) or AB = A and B overlap by using a univariate discriminant analysis.

of up to 12 months and more, leading to a very characteristic cheese. In the other regions considered for this study, a fast maturation is preferred for economic reasons.

In cooked cheeses such as Emmental, the chymosin is denatured extensively during manufacture and αs1 degradation is more likely due to enzymes of the lactobacilli present in cheese (Olson, 1990; Chamba, 2000). This type of proteolysis is, however, relatively slow. The low αs1 level in 'Switzerland' and 'Allgäu' can therefore be explained by the longer ripening time in these regions and/or by a higher protease activity of the lactobacilli present. Unlike chymosin, the endogenous milk enzyme plasmin is not damaged by the high temperature used in cooked cheese. The plasmin activity is even elevated in 'high cook' cheese varieties such as Emmental. The enzyme degrades αs2 and β casein. αs2 commonly disappears in Emmental after 45 days of ripening (Chaillet, 2002). The degradation of the β casein leads to the fractions γ1 to γ3. Once again 'Allgäu' and 'Switzerland' showed the typical values of longer ripened cheeses.

Sixteen free amino acids (relative amounts, Table 26.7) showed a significant difference between at least two groups. A principal component analysis (PCA) of the parameters asparagine, glycine, lysine, phenylalanine and proline allowed the separation of 'Switzerland' from the other groups (Fig. 26.3). 'Bretagne' was easily differentiated by methionine and glutamine. 'Finland' showed a very specific peptide pattern with peaks not appearing in the other cheeses (data not shown). Further it is known that peak 14 (Chopard *et al*., 2001; unknown structure) is typically smaller in the presence of *Lb. helveticus.* Switzerland was

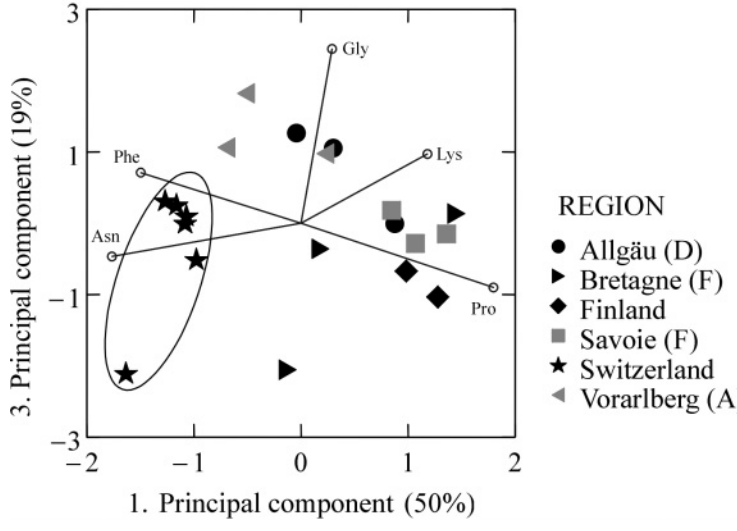

Fig. 26.3 Principal component analysis of the relative concentration of asparagine, glycine, lysine, phenylalanine and proline (total free amino acids = 100%); separation of the regions 'Switzerland' and 'Vorarlberg'.

Table 26.7 Free amino acid ratios and peak 14 from peptide profile in the 20 Emmental cheese samples investigated

Analytes	ANOVA	Region (n=) AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Total free amino acids (g/kg)	*	**28.2^{A}**	7.1	**17.2^{B}**	5.2	**19.8^{AB}**	3.0	**22.5^{AB}**	7.3	**28.7^{A}**	4.4	**19.3^{AB}**	2.0
Alanine (%)	ns	**3.08**	0.58	**3.84**	1.3	**3.34**	0.17	**3.71**	0.84	**3.26**	0.49	**3.03**	0.21
α-Amino butyric acid (%)	ns	**1.49**	2.10	**0.13**	0.01	**0.18**	0.12	**0.06**	0.02	**0.20**	0.11	**0.06**	0.06
Arginine (%)	ns	**0.10**	0.04	**0.04**	0.01	**0.84**	0.83	**0.86**	0.15	**0.08**	0.03	**0.08**	0.03
Asparagine (%)	***	**3.08^{BC}**	1.17	**4.63^{AB}**	1.95	**7.06^{A}**	0.62	**1.35^{C}**	0.11	**2.51^{BC}**	1.22	**4.77^{AB}**	1.8
Aspartic acid (%)	*	**2.91^{A}**	1.54	**1.27^{AB}**	0.80	**1.78^{AB}**	0.42	**0.16^{B}**	0.00	**0.96^{B}**	0.16	**1.35^{AB}**	1.00
Citrulline (%)	*	**0.89^{B}**	0.39	**2.14^{AB}**	0.52	**3.31^{A}**	0.83	**2.43^{AB}**	0.92	**2.10^{AB}**	1.10	**2.45^{AB}**	0.55
γ-Amino butyric acid (%)	*	**0.40^{A}**	0.34	**0.30^{AB}**	0.28	**0.05^{B}**	0.04	**0.01^{AB}**	0.01	**0.05^{AB}**	0.04	**0.03^{AB}**	0.02
Glutamine (%)	***	**2.30^{B}**	0.55	**4.25^{A}**	0.67	**1.94^{B}**	0.58	**3.20^{AB}**	0.74	**2.81^{B}**	0.26	**2.43^{B}**	0.52
Glutamic acid (%)	ns	**18.38**	0.54	**16.99**	0.24	**18.19**	1.1	**17.63**	0.62	**18.23**	0.41	**19.03**	0.64
Glycine (%)	*	**2.03^{AB}**	0.07	**2.03^{AB}**	0.14	**1.90^{B}**	0.15	**1.83^{B}**	0.01	**1.97^{AB}**	0.02	**2.16^{A}**	0.01
Histidine (%)	**	**0.35^{B}**	0.36	**2.81^{A}**	0.09	**1.84^{A}**	0.32	**3.24^{A}**	0.17	**2.20^{A}**	1.8	**1.87^{AB}**	0.41
Isoleucine (%)	**	**5.12^{A}**	0.72	**3.87^{ABC}**	0.65	**2.86^{C}**	0.28	**3.77^{ABC}**	0.66	**4.50^{AB}**	0.78	**3.53^{BC}**	0.73
Leucine (%)	**	**12.14^{B}**	1.2	**12.19^{B}**	0.84	**13.90^{A}**	0.53	**11.87^{B}**	1.3	**11.94^{B}**	0.59	**13.87^{A}**	0.55
Lysine (%)	*	**12.58^{A}**	0.34	**10.82^{B}**	1.6	**11.34^{AB}**	0.43	**12.69^{A}**	0.26	**12.69^{A}**	0.07	**11.60^{AB}**	0.35
Methionine (%)	***	**2.50^{A}**	0.11	**1.95^{B}**	0.09	**2.44^{A}**	0.07	**2.57^{A}**	0.03	**2.28^{A}**	0.22	**2.45^{A}**	0.09
Ornithine (%)	*	**5.09^{A}**	0.31	**3.63^{AB}**	0.49	**2.47^{B}**	1.0	**2.99^{AB}**	1.7	**4.33^{AB}**	1.8	**3.73^{AB}**	0.82
Phenyalanine (%)	***	**6.26^{AB}**	0.72	**5.33^{C}**	0.18	**6.96^{A}**	0.35	**6.06^{BC}**	0.34	**5.95^{BC}**	0.09	**6.53^{AB}**	0.29
Proline (%)	***	**6.28^{BC}**	2.74	**9.44^{AB}**	1.00	**4.05^{C}**	0.35	**9.98^{AB}**	0.78	**10.21^{A}**	0.74	**5.27^{C}**	2.4
Serine (%)	ns	**2.10**	0.10	**2.20**	0.47	**1.82**	0.20	**2.35**	0.38	**1.80**	0.37	**1.98**	0.28
Threonine (%)	ns	**2.90**	0.07	**2.57**	0.44	**2.77**	0.08	**3.16**	0.34	**2.83**	0.28	**2.97**	0.07
Tryptohphan (%)	*	**0.34^{B}**	0.05	**0.37^{B}**	0.10	**0.41^{AB}**	0.04	**0.53^{A}**	0.07	**0.45^{AB}**	0.09	**0.39^{AB}**	0.00
Tyrosine (%)	ns	**2.21**	0.65	**2.42**	0.37	**2.76**	1.0	**2.48**	0.99	**1.30**	0.57	**2.63**	0.72
Valine (%)	***	**7.49^{AB}**	0.20	**6.80^{C}**	0.16	**7.78^{A}**	0.19	**7.08^{BC}**	0.00	**7.34^{AB}**	0.40	**7.77^{A}**	0.13
Peptide pattern													
Peak 14 (arbitrary unit)	***	**5707^{BC}**	3774	**2554^{C}**	1246	**14755^{A}**	3873	**955^{C}**	585	**683^{C}**	58	**9649^{AB}**	1641

Key: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.
Production sites: A > B > C > D (= significantly different contents p = 0.01) or AB = A and B overlap by using a univariate discriminant analysis.

the single investigated region where *Lb. helveticus* was not used in cheese starters. In consequence, the concentration of this peptide was significantly higher in 'Switzerland' than in the other cheeses (Table 26.7). A PCA of the three parameters αS1, X3 (peptide with unknown structure migrating before αS1) and peak 14 led to a clear separation of 'Bretagne', 'Finland' and 'Savoie' (figure not shown).

26.4.2 Volatile compounds

The origins of volatile compounds found in cheese are diverse and can be classified into two groups: the first one contains native volatile compounds already present in milk which are not transformed during cheese manufacturing while the second group includes components produced in the cheese itself during manufacture or maturation. The former compounds are a function of the forage and the second ones of the manufacturing technology.

Two analytical techniques were tested for the discrimination of the origin using volatile compounds, gas chromatography (GC) and mass spectrometry-based electronic nose. Table 26.8 summarises the compounds with significantly different concentrations using GC. Some of them seemed highly specific to a given region: hexan-1-ol and propyl ester of acetic acid were present only in 'Allgäu'; 3-methylbutylester of acetic acid occurred only in 'Allgäu' and 'Savoie'; 'Savoie' showed significantly higher concentrations of propan-2-ol and pentan-2-ol than in the cheeses from the other regions. 'Finland' and 'Vorarlberg' showed some similarities: 2,3,4-trimethylpentane, 2,2-dimethylheptane as well as two different octenes were detected only in cheese from these two regions. It must be pointed out that only the sample FI1 and not FI2 contained the latter compounds. Furthermore, 'Vorarlberg' was the only region where oct-1-ene and a further alkene were found. It was therefore very easy to separate 'Allgäu', 'Vorarlberg', 'Savoie' and 'Finland' one by one from the other regions using their specific volatile compounds. The concentration of butan-1-ol was significantly higher in 'Bretagne'. This region may be separated from the others using the concentrations of butan-1-ol and hexanal. 'Switzerland' was the single region where butan-2-ol was not detected in any of the samples. Combining the concentration of butan-2-ol, butan-2-one, 3-hydroxybutanone and an octene by PCA, a separated group including 'Switzerland' was formed (Fig. 26.4). A tendency of grouping by the other regions (except 'Finland') was also observed on the latter figure. This technique is however expensive and very time consuming.

A promising alternative is offered by electronic noses because of their rapid screening capacity. An MS-based electronic nose with static headspace injection was used. Considering only the regions Bretagne, Vorarlberg and Finland, 100% separation was achieved by PCA. By separating Swiss from non-Swiss samples, respectively 90 and 91% correct classification was achieved. The future use of a preconcentration step instead of a direct static headspace injection should enhance the results.

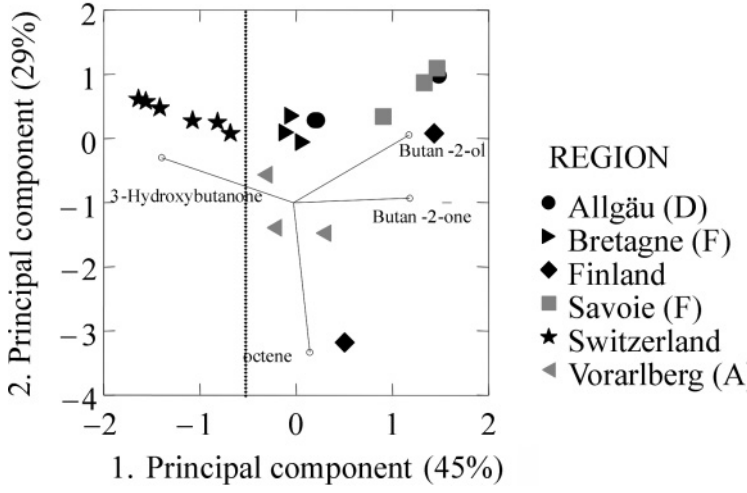

Fig. 26.4 Principal component analysis using the concentrations of butan-2-one, 3-hydroxybutanone, butan-2-ol and octene; the group 'Switzerland' is perfectly separated.

26.4.3 Organic acids and further parameters

The results of various further parameters showing significant differences are listed in Table 26.9. Volatile short chain acids were analysed by GC. 'Finland' and 'Switzerland' showed the highest, 'Allgäu' and 'Bretagne' the lowest propionic acid concentrations. The intensity of the propionic fermentation is also expressed by the diminution of lactate. Both parameters were highly negatively correlated ($r = -0.94$). No lactate and only traces of pyruvate were found in 'Finland', making it easy to differentiate. Succinate and L-lactate concentrations were very interesting for separating 'Switzerland' from the other regions. The butyrate content was the highest in 'Bretagne' and partly in 'Finland', most probably because cows were fed with silage. 'Vorarlberg' showed a significantly higher fat content than all other regions except 'Allgäu'. Sodium chloride and pH-value, both cheap and fast measurements, showed differences between the groups as well.

26.4.4 Microbiology

Enterococci, facultative heterofermentative lactobacilli, propionic acid and salt tolerant bacteria did not show very interesting variations between regions of origin. The major differences were found in the concentration of obligate heterofermentative lactobacilli (OHL) which was significantly lower in 'Switzerland'. The longer ripening times encountered in this region force the manufacturers to use starters with a low content of OHL because these microorganisms may be involved in late fermentation problems. *Lactobacillus helveticus* is a microorganism with a strong proteolytic activity. It is commonly used in all investigated regions except Switzerland for shortening the ripening

Table 26.8 Concentration of volatile compounds showing significant differences (ANOVA, p = 0.05) using purge and trap gas chromatography (arbitrary units)

Analytes	Retention Index[1]	ANOVA	Region (n =) AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
			x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Ketones & aldehydes														
Propan-2-one[a]	466	*	**78.0**^{B}	29	**341.0**^{AB}	230	**572.0**^{A}	260	**552.0**^{AB}	200	**280.0**^{AB}	370	**89.0**^{B}	52
Butan-2,3-dione[a]	556	*	**20.0**^{AB}	10	**60.0**^{A}	29	**35.0**^{AB}	17	**31.0**^{AB}	7	**30.0**^{AB}	7	**12.0**^{B}	3
Butan-2-one[a]	567	***	**44.0**^{BC}	27	**41.0**^{BC}	9	**21.0**^{C}	7	**118.0**^{A}	68	**74.0**^{AB}	13	**21.0**^{BC}	10
3-Hydroxybutanone[b]	683	***	**u.d.l.**^{B}	–	**3.4**^{B}	2.6	**18.7**^{A}	7.9	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–
Hexanal[a]	780	***	**24.0**^{AB}	10	**4.8**^{C}	1.6	**26.9**^{A}	8.2	**30.4**^{A}	2.7	**7.3**^{C}	2.4	**13.2**^{BC}	1.9
Alcohols														
Propan-2-ol[a]	481	***	**131.0**^{BC}	64	**167.0**^{B}	80	**62.0**^{C}	35	**38.0**^{C}	19	**291.0**^{A}	56	**66.0**^{BC}	11
Propan-1-ol[a]	535	*	**530.0**^{A}	310	**148.0**^{B}	42	**143.0**^{B}	94	**115.0**^{B}	51	**271.0**^{B}	140	**144.0**^{AB}	120
Butan-2-ol[a]	583	***	**83.0**^{AB}	53	**21.0**^{BC}	20	**u.d.l.**^{C}	–	**7.0**^{BC}	6	**116.0**^{A}	55	**18.0**^{BC}	23
2-Methylpropanol[a]	616	*	**11.8**^{AB}	2.5	**4.3**^{B}	2.5	**7.8**^{AB}	3.9	**7.1**^{AB}	2.4	**13.5**^{A}	2.1	**6.5**^{AB}	3.8
Butan-1-ol[a]	652	**	**33.0**^{B}	18	**143.0**^{A}	61	**19.0**^{B}	21	**52.0**^{B}	62	**18.0**^{B}	4	**24.0**^{B}	32
Pentan-2-ol[a]	685	***	**100.0**^{B}	75	**45.0**^{B}	38	**11.0**^{B}	7	**u.d.l.**^{B}	–	**291.0**^{A}	92	**23.0**^{B}	7
2-Methylbutanol[a]	725	*	**77.0**^{AB}	12	**61.0**^{AB}	9	**61.0**^{B}	25	**100.0**^{AB}	51	**129.0**^{A}	58	**43.0**^{B}	13
Hexan-1-ol[a]	852	***	**8.6**^{A}	3.7	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–
Esters														
Butanoic acid ethyl ester[a]	695	**	**54.0**^{BC}	30	**23.0**^{C}	10	**169.0**^{AB}	66	**122.0**^{ABC}	30	**216.0**^{A}	110	**82.0**^{ABC}	25
Acetic acid propyl ester [a]	697	***	**25.0**^{A}	14	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–
Acetic acid 3-methylbutylester[b]	859	**	**5.1**^{A}	2.6	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**u.d.l.**^{B}	–	**3.3**^{AB}	1.1	**u.d.l.**^{B}	–

Hydrocarbons													
Pentane[a]	499	**	**14.9^{A}**	1.7	**9.2^{B}**	4.5	**8.2^{B}**	0.9	**9.6^{AB}**	2.1	**10.8^{AB}**	2.1	**6.9^{B}**	1.0
2,3,4-Trimethylpentane[b]	756	**	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**9.0^{AB}**	11	**u.d.l.^{B}**	–	**12.0^{A}**	5.7
Toluene[a]	761	**	**12.6^{B}**	2.3	**12.0^{B}**	2.8	**10.6^{B}**	3.7	**25.0^{AB}**	22	**11.0^{B}**	3.6	**37.0^{A}**	13
3-Methylheptane[a]	776	*	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**6.0^{A}**	2.7	**6.6^{A}**	8.0	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–
2,2-Dimethylheptane[b]	787	**	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**7.9^{AB}**	9.7	**u.d.l.^{B}**	–	**10.9^{A}**	4.9
Oct-1-ene[b]	790	***	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**22.1^{A}**	9.4
Oct-?-ene[b]	803	*	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**7.3^{A}**	8.9	**u.d.l.^{B}**	–	**5.8^{AB}**	2.5
Alcene (C8H16)[b]	808	***	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**5.5^{A}**	2.2
Oct-?-ene[b]	813	*	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**u.d.l.^{B}**	–	**5.2^{A}**	5.9	**u.d.l.^{B}**	–	**4.9^{A}**	2.1

Key: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.
Production sites: A > B > C > D (= significantly different contents p = 0.01) or AB = A and B overlap by using a univariate discriminant analysis.
[a] Confirmed by comparing retention indices.
[b] Identification with MassLib.
[1] SPB1 chromatographic column.
u.d.l. = under the detection limit.

Table 26.9 Gross chemical composition, alkaline phosphatase and vitamins in the 20 Emmental cheese samples investigated

	ANOVA	Region (n=) AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s
Fat (g/kg)	***	**322.0^{AB}**	15	**300.0^{C}**	7.9	**318.0^{B}**	7.4	**307.0^{BC}**	2.5	**306.0^{BC}**	7.7	**342.0^{A}**	6.3
Sodium chloride (g/kg)	**	**6.43^{A}**	0.83	**4.37^{AB}**	1.58	**3.43^{B}**	0.41	**5.60^{AB}**	1.13	**3.60^{B}**	1.04	**3.57^{B}**	0.98
pH-value	***	**5.73^{AB}**	0.05	**5.57^{C}**	0.05	**5.63^{BC}**	0.06	**5.73^{AB}**	0.02	**5.79^{A}**	0.08	**5.65^{BC}**	0.03
L-Lactate	***	**45.0^{A}**	13	**47.0^{A}**	15	**23.8^{B}**	4.4	**0.00^{C}**	0.00	**27.0^{AB}**	9.5	**45.7^{A}**	4.5
D-Lactate	***	**59.0^{A}**	29	**48.7^{AB}**	7.6	**21.8^{CD}**	6.2	**0.00^{D}**	0.00	**24.3^{BCD}**	9.9	**35.0^{ABC}**	2.7
Succinate	***	**6.7^{ABC}**	4.4	**8.6^{AB}**	3.4	**3.2^{C}**	0.48	**13.1^{A}**	1.3	**12.5^{A}**	1.9	**4.9^{BC}**	3.3
Pyruvate	**	**2.8^{CD}**	3.9	**8.2^{AB}**	2.7	**6.1^{ABC}**	1.5	**0.20^{D}**	0.00	**8.9^{AB}**	0.12	**7.4^{BC}**	1.9
Formate (C1)	*	**4.59^{AB}**	0.38	**3.55^{B}**	0.60	**4.04^{AB}**	0.32	**3.59^{B}**	0.19	**4.04^{AB}**	0.17	**4.90^{A}**	0.76
Acetate (C2)	**	**39.0^{BC}**	9.9	**32.3^{C}**	7.0	**44.6^{AB}**	3.6	**50.7^{A}**	0.8	**51.8^{A}**	3.3	**40.2^{ABC}**	2.7
Propionate (C3)	***	**23.9^{C}**	17.1	**30.4^{C}**	5.7	**59.5^{A}**	7.8	**69.9^{A}**	0.7	**53.2^{AB}**	3.1	**36.9^{BC}**	6.5
Butyrate (C4)	***	**0.91^{B}**	0.18	**4.88^{A}**	0.65	**0.94^{B}**	0.19	**1.49^{B}**	1.41	**0.86^{B}**	0.08	**0.92^{B}**	0.04
Capronate (C6)	*	**0.302^{A}**	0.008	**0.211^{AB}**	0.091	**0.318^{A}**	0.100	**0.095^{B}**	0.010	**0.239^{AB}**	0.026	**0.280^{AB}**	0.069
Total volatile acids	***	**68.7^{C}**	26.7	**71.5^{C}**	13.1	**109.6^{AB}**	11.7	**126.0^{A}**	1.3	**110.3^{AB}**	6.0	**83.4^{BC}**	9.5

Key: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.
Production sites: A > B > C > D (=significantly different contents p = 0.01) or AB = A and B overlap by using a univariate discriminant analysis.

time. Traces of *Lb. helveticus* may be found in 'Switzerland' as natural milk contaminant but the concentrations were at least one order of magnitude lower than in the other regions (see also sections 26.4.1–26.4.3).

26.4.5 Infra-red spectroscopy (IR)

The advantages of IR measurements are well known, they are rapid, cheap, non-destructive and multi-parametric. In the dairy sector, IR was almost exclusively used for the measurement of main components such as fat, protein, moisture, lactose and urea. IR found however application for identification/authentication of various products such as meat, honey, coffee and fruit juice as well as Emmental cheese (Picque *et al.*, 2002). For details or references on the technique, see Chapters 6 and 7.

Three different sample preparations were tested on three different analytical instruments within the project: (i) grated cheese using near IR diffuse reflection (NIR), (ii) cheese slices using middle IR attenuated total reflection (MIR/ATR) and (iii) water dispersion of cheese spread and dried on a polyethylene card using MIR Transmission. All techniques allowed a promising discrimination of the regions of origin applying linear discriminant analysis (LDA) on principal component scores. Trained classification techniques such as LDA are necessary in IR technology because of the large number of parameters (wavelengths) delivered. The results are consequently only trends which will have to be confirmed later with more samples. MIR Transmission allowed 100% separation by Swiss and non-Swiss samples. Using NIR and MIR/ATR, samples from the six regions were correctly classified.

26.5 Conclusion

The first step of a three-year project to differentiate Emmental cheeses from different regions has just been completed. Many analytical methods have been tested on 20 Emmental cheeses from six European regions and their potential for discrimination has been evaluated. For each parameter, a difference test on the mean value of each region was calculated. Principal component analysis (PCA) was also carried out to visualise the natural groupings generated by selected parameters. According to those results, two spectroscopic methods were selected for the remainder of the project; infra-red spectroscopy and MS-based electronic nose.

Further selected chemical parameters were as follows: pH-value, L-lactate, succinate, pyruvate, N-fractions, fat, sodium chloride and volatile short-chain acids. Investigation on the following elements will also be carried out using atomic absorption spectroscopy: calcium, sodium, magnesium, zinc, copper, manganese and molybdenum. Finally the isotope ratios $^{2}H/^{1}H$, $13C/^{12}C$, $^{15}N/^{14}N$ and $^{87}Sr/^{86}Sr$ delivered promising results and will be investigated too. Trends obtained with these selected methods will be, in the next step, checked with many more samples both from winter ($n = 90$) and summer ($n = 80$) production.

The larger database obtained will also make possible the use of powerful trained classification techniques such as linear discriminant analysis, partial least squares regression or artificial neural network. In the end, it should be possible to elaborate a mathematical model for the determination of origin.

26.6 References

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27

Advanced DNA-based detection techniques for genetically modified food

A. Holst-Jensen, National Veterinary Institute, Norway

27.1 Introduction

In recent years many countries including the European Union (EU) have introduced regulations for the release and use of genetically modified (GM) organisms (GMOs) and their derived products. These regulations commonly distinguish between authorisation of the GMOs and labelling of products derived from the GMOs. The authorisation procedure is usually meant to assure the safety of the GMO and its derived products, whereas labelling is meant to assure purchasers of products a freedom to choose between GM and non-GM products. A consequence of these regulations is that methods to assure compliance with the legislations are needed. To be able to distinguish between authorised and non-authorised GMOs, the methods must be able to identify the (source of) GMO (derived) material. The identity of a GMO is given by a unique transformation event, which is referred to in all official documents and notifications. Labelling requirements usually means that at the species level, the product must be labelled if the GMO content calculated on an additive basis exceeds a given threshold. The present food-labelling threshold in the EU (European Commission, 2000) is 1%. If a product contains three GM-maize derivatives, then the total GM-maize content ($\sum$GM-maize) = content of GM-maize1 + content of GM-maize2 + content of GM-maize3, e.g., 0.6% + 0.4% + 0.4% = 1.4%. This means that methods to identify and quantify the amount of each GMO must also be available.

The process of genetically modifying an organism means to add, remove or modify a piece of genetic material (DNA). With present day technology this can be done within certain limitations, depending on the type of organism to be modified and the type of modification to be performed. In general,

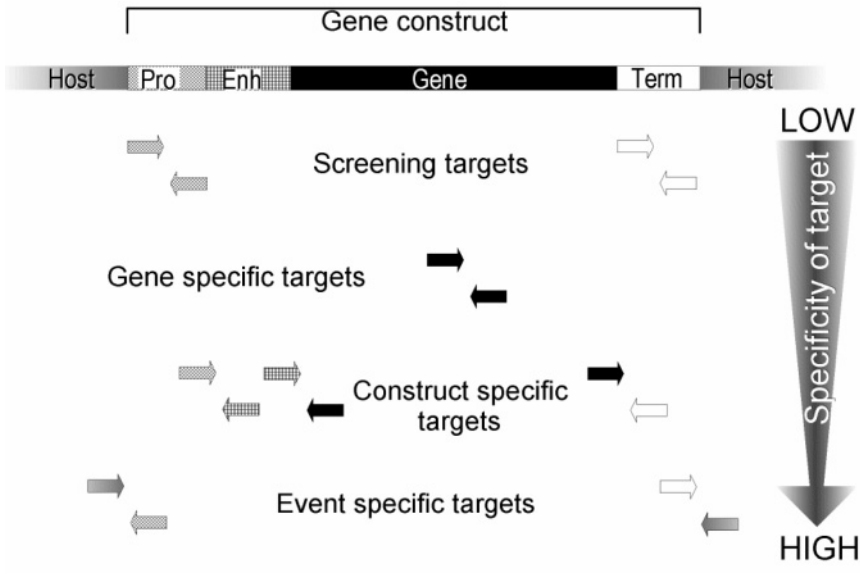

Fig. 27.1 Schematic structure of an integrated gene construct and its insertion locus in the host genome. Abbreviations: Host = host genome flanking the insert (insertion locus), Pro = promoter element, Enh = enhancer element, Gene = the gene of interest (responsible for the functional modification of the organism), Term = terminator element. Each element or junctions spanning two elements represent typical targets for PCR-based GMO analysis. The specificity and fitness for purpose varies significantly, i.e., from screening purposes to event-specific identification and quantitation purposes.

microorganisms are easily modified, animals somewhat more complicated, whereas plants are the most difficult organisms to modify. The simplest modifications are insertions of new DNA, whereas substitutions of single nucleotides are the most difficult ones to perform. Yet, when we talk about GMOs we usually mean GM plants. This is because the use of GM microorganisms is limited to closed (contained) systems, minimising their presence in final products, and because no GM animals are yet on the market. To reflect the current status, a GMO will in the following refer to a plant whose genome has integrated a new piece of DNA (an insert). The insert contains a combination of genetic elements usually referred to as a gene construct. A gene construct is composed of at least three elements, i.e., a promoter, a gene and a terminator (Fig. 27.1). In addition to these three elements, enhancers and cloning vector elements are often present in the insert. At present it has not been possible to control in detail the transformation of plants, and an important consequence of this is that if the same insert is repeatedly integrated into the genome of the same recipient organism, it will never integrate in the same position of the recipient genome. In other words, the junction between the recipient genome and the integrated insert is unique for each transformation event. The Cauliflower Mosaic Virus (CaMV) 35 S promoter (P-35S) and terminator (T-35S) elements,

as well as the *Agrobacterium tumefaciens* nopaline synthase terminator (T-Nos) in particular, but also some enhancer elements and genes, are elements found in several of the gene constructs used to transform different GMOs (Fig. 27.2).

27.2 Issues in detecting genetically modified organisms (GMOs)

The first generation of DNA-based GMO detection methods were qualitative screening PCR methods, targeting elements shared by several GMOs (see review in Holst-Jensen *et al.*, 2003). Later, PCR methods targeting genes or construct-specific junctions, as well as quantitative competitive and real-time PCR methods, were developed (see Holst-Jensen *et al.*, 2003). Transformation event-specific PCR methods have only been available for a few years (Zimmermann *et al.*, 2000; Berdal and Holst-Jensen, 2001; Taverniers *et al.*, 2001; Terry and Harris, 2001; Holck *et al.*, 2002; Hernandez *et al.*, 2003; Rønning *et al.*, 2003; Windels *et al.*, 2003).

Reliable identification and quantitation is highly dependent on specific targets for PCR, in particular on the uniqueness of the DNA sequence of the amplicon and its copy number relative to haploid genome copies. Screening targets are neither unique nor their copy number invariant among all GMOs. As a matter of fact they may even be present in non-GM materials (Wolf *et al.*, 2000). Screening methods are useful to assess the potential presence of GMO derived DNA, but can only exceptionally be used to verify or quantify the GMO content. Genes or internal junctions of constructs may be unique, and the copy number of the targets may be invariant. But several genes and constructs have been used more than once in the process of developing commercial transformants, and genes and constructs may also be used again in the future. Event-specific integration junctions, however, are always unique, and their copy number is always one in a haploid GM genome. These targets are therefore believed to represent the most reliable targets for any identification and quantitation method available today.

However, even with event-specific integration junctions a few uncertainties have to be considered. First of all, there is always a risk of allelic instability of a target sequence, e.g., substitutions within the target sequence may invalidate primers or probes. Secondly, homozygous and heterozygous GMOs contain two and one copy of the event-specific integration junction per diploid genome, respectively. Thirdly, different parts of tissues of different plants can be haploid, diploid and triploid, respectively, and the contribution of DNA from each of the parents can vary accordingly. For example, in maize, the seed coat, embryo and endosperm of a seed have quite different genetic background (see Fig. 27.3). Fourthly, hybridisation between two GMOs, e.g., T25 maize and Mon810 maize, will yield offspring containing the event-specific integration junctions of both GMOs (gene stacking), and this offspring will normally be indistinguishable from its two parents unless the analysed DNA is isolated from a single plant

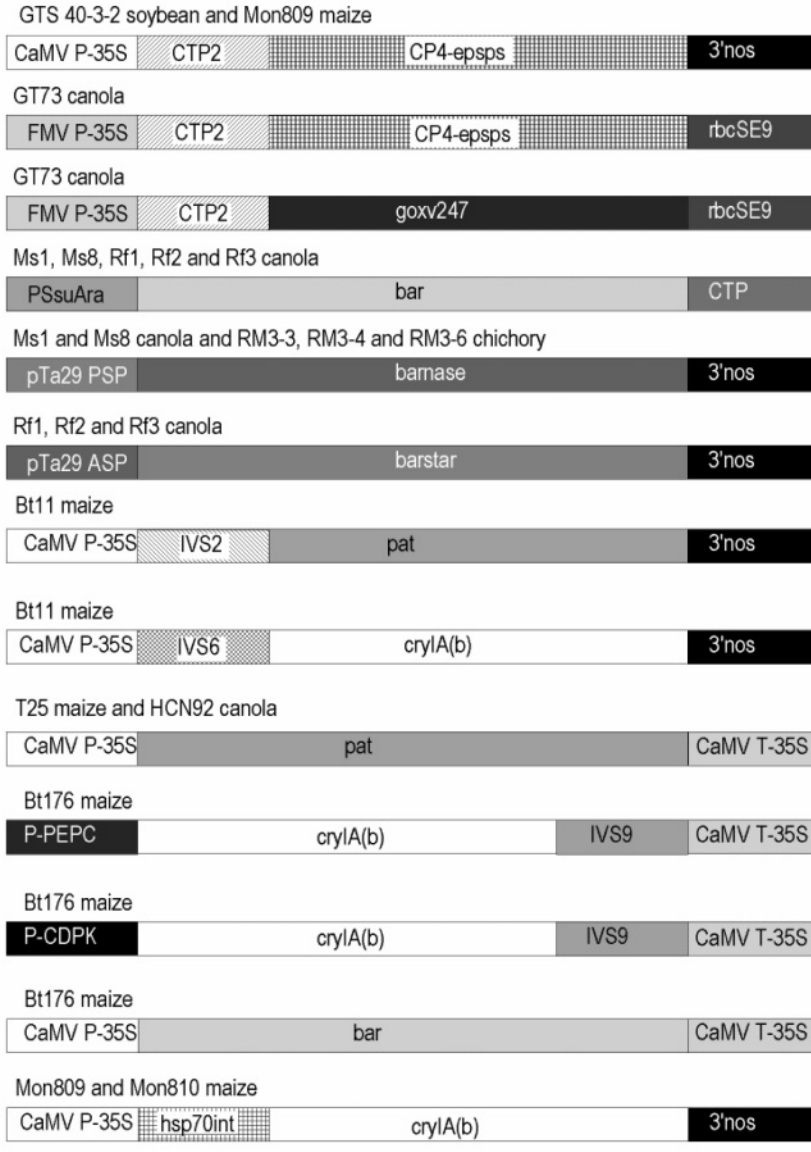

Fig. 27.2 Schematic structure of the eukaryotic gene constructs used to transform GMOs authorised in the European Union (source: AgBios database, March 12, 2003). If the same promoter was used in different gene constructs, this will be seen as promoters with the same colour. Similarly for enhancers, genes and terminators. Note that although the same elements may appear in different constructs, they may differ at the sequence level.

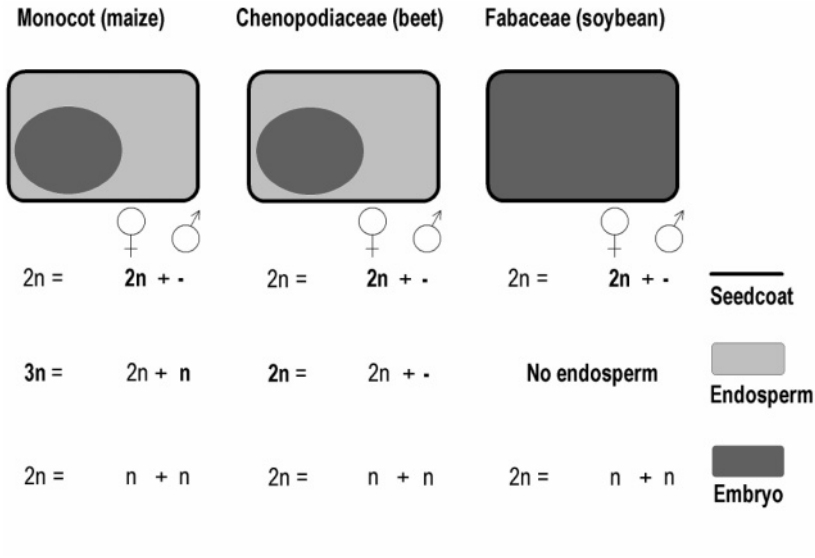

Fig. 27.3 Variation in ploidy level between seed coat, endosperm and embryo of maize (*Zea mays*), soybean (*Glycine max*) and sugar beet (*Beta vulgaris*). Figure adapted from Marc De Loose.

specimen. This clearly represents a problem if a gene stacked GMO requires separate authorisation relative to its parents, such as is the case in the EU.

27.2.1 Improving DNA extraction

A low concentration of DNA means that few copies of any DNA are present. If a GMO is present at a low concentration in the matrix, e.g., 0.5%, then a low DNA concentration could mean that GMO derived DNA is absent in the template DNA used in the PCR. A concentration of 0.5%, however, is certainly above the limit of detection (LOD) and quantitation (LOQ) expected by most purchasers of GMO tests. Therefore a high template DNA concentration is recommendable to achieve a satisfactory LOD and LOQ. Impurities in the DNA may inhibit the PCR, leading to false negative or erroneous quantitative estimates. Highly degraded DNA may not be amplifiable, because a successful PCR requires intact target DNA copies.

GMO testing is performed on processed and composite products as well as on raw materials. Processing often implies DNA degrading, DNA modifying or DNA removing techniques, such as grinding, heating, chemical treatment and fractionation. As a consequence, it may be difficult to obtain pure and concentrated DNA of sufficient size to be amplified with PCR.

Along with the starting material, the template DNA is therefore the single most critical factor determining the LOD and LOQ of PCR-based methods. Despite this,

there has been surprisingly little focus on improving and validating DNA extraction procedures for GMO testing. This may in part be explained by the significant variation between apparently homologous products, e.g., corn flakes or starches. At present (January 2003) no DNA extraction method has been fully validated according to harmonised international protocols for method validation (Horwitz, 1995; Thompson *et al.*, 2002), although a modified CTAB protocol has been partly validated (Van den Eede *et al.*, 2000a). As focus has shifted towards automation of extraction protocols, there now appears to be little incitement for developing or modifying conventional protocols such as the CTAB method (Murray and Thompson, 1980). Variation between individual samples to be analysed, however, represent a serious problem for automation, particularly evident with processed composite food products. For serial extraction of DNA, e.g., from raw soybeans in a production plant, or from maize flour, establishment of standardised and validated extraction methods is possible, and these could probably also be automated. Significant differences in performance of one method in comparison with another are known to be linked with specific matrices. A first step would therefore be to systematically test the protocol(s) and modifications of the protocol(s), to identify the domain(s) and limits of application of the protocol(s). Validation could be performed for each method with a limited set of matrices and samples, but validation data would under no circumstances be fully applicable to any other matrix, which of course is a serious limitation.

27.2.2 Reference genes for species identification and quantitation

With many composite and processed products, there is a risk of failing to extract amplifiable or sufficient amounts of DNA derived from the target species, i.e., the species to which the GMO belongs. It may therefore be useful to perform an initial PCR to verify the presence of target species derived DNA. Furthermore, by quantitation of this DNA, e.g., using real-time PCR, it becomes possible to determine whether the amount is sufficient to meet specific LOD and LOQ requirements, requested by the purchaser of the GMO analysis (cf. Berdal and Holst-Jensen, 2001; Holst-Jensen *et al.*, 2003).

For these purposes, it is common to use a PCR targeting a gene believed to be ubiquitously present in the target species, in a stable number of copies per haploid genome, and without allelic variation within the species, while at the same time being absent or having a distinguishable genotype in all non-target species.

A number of PCR methods with associated reference genes for GMO testing purposes have been published, e.g., the soybean lectin gene (Meyer *et al.*, 1996), the maize hmg, invertase and zein genes (Zimmermann *et al.*, 1998; Ehlers *et al.*, 1997; Vaïtilingom *et al.*, 1999), and the rape seed AccCoA gene (Hernandez *et al.*, 2001). However, the use of these genes is often based on very limited investigations, e.g., using only a few cultivars of the target species, a few non-target species, and without examining the copy number per haploid genome. Consequently, further studies to assure the applicability of these genes as well as alternative genes and genes for additional species are needed.

27.2.3 Characterising specific GMOs

Developing a PCR method requires prior knowledge of the sequence of the target DNA. Consequently, the DNA sequence of the event-specific integration junction is needed to be able to develop event-specific PCR methods as described above. Whereas the DNA sequence of the gene construct is usually included in the official dossiers from the applicant company, the insertion locus is only rarely known and described in these dossiers. Furthermore, the sequence information in the dossiers is usually confidential, made available only to competent authorities. More limited, publicly available and unrestricted information can be found in non-confidential parts of the dossiers and in databases (e.g., Agbios, 2003). As a minimum to be able to proceed, prior knowledge of PCR primer sites inside an integrated part of the gene construct used to transform the GMO is necessary. This will allow for further characterisation of the DNA sequence upstream and downstream of the primer site(s), using a range of different PCR strategies (Liu *et al.*, 1995; Siebert *et al.*, 1995; Hartl and Ochman, 1996; Theuns *et al.*, 2002). However, difficulties may arise, e.g., due to lack of uniqueness of primer sites, tandem repeated sequence motifs, and inverse duplicate and/or multiple inserts containing the same primer site. The source of DNA used for characterisation is also a critical factor. If the DNA is extracted from a matrix such as maizeflour where the DNA may originate from several GMOs, then problems are more likely to be faced than if the DNA is originating from a single plant specimen.

27.3 Developing improved GMO detection methods

On a worldwide level the commercialised and authorised GMOs largely outnumber the GMOs for which a specific detection and/or quantitation method is published. In consequence this means that there is an urgent need for methods targeting additional GMOs. Even if a method is available, the method may not be applicable, e.g., some construct-specific methods are applicable for single ingredient matrices but not for composite products. Furthermore, as the number of detectable GMOs increases, the need for more efficient, rapid and cost effective tests increases.

27.3.1 Access to materials and sequence data

To be able to develop methods, reference materials are required in addition to sequence data. Reference materials should preferably be certified (CRMs; e.g. the IRMM-4XX series, IRMM 2003). An alternative is material delivered directly from the producer of the GMO, but such materials need to be checked for consistency prior to use as reference materials. However, the IRMM series hitherto only comprised four GMOs, and the producers have been very reluctant to distribute their GMO materials, for various reasons. Consequently, alternative sources of material may have to be explored, e.g., contaminated seeds and grains for feeds. Of course there are uncertainties associated with alternative sources of

materials, and the most important of these have been discussed elsewhere (Holst-Jensen *et al.*, 2003).

27.3.2 Some problems faced by method developers

Once the sequence data are made available for an event-specific integration junction, primer and probe design is initiated. A number of unexpected problems were faced by the teams developing the first event-specific PCR methods. Zimmermann *et al.* (2000) reported that the insert of Bt11 maize was integrated in a tandemly repeated approximately 180 bp knob specific motif. This created difficulties with respect to primer design, and later these problems have been corroborated by observations by Rønning *et al.* (2003) who further had to struggle with the problem while designing TaqMan probes for real-time quantitative PCR. Windels *et al.* (2001) reported an unexpected duplication of an internal part of the gene construct (partial EPSPS gene) appended to one end of the integrated construct in RoundupReady® soybean (line GTS 40-3-2). This duplication was not reported in the official notifications from the developing company (Monsanto), and the observation clearly demonstrated the importance of carefully assessing the copy number of the target sequence for the PCR method for quantitation. Other recent observations include integration in a presumably mobile Huck-1 or Huck-2 retrotransposon element (SanMiguel *et al.*, 1996; EMBL/GenBank accession numbers AF 050438-40) in T25 maize (Yves Bertheau, INRA, France, pers. com.), and duplicate integration in sequences homologous to maize chloroplast sequences (but not located in chloroplasts) in StarLink maize (line CBH 351; Windels *et al.*, 2003).

Development of four different assays for real-time PCR-based detection and quantitation of GTS 40-3-2 (Berdal and Holst-Jensen, 2001; Taverniers *et al.*, 2001; Terry and Harris, 2001) also led to a number of interesting observations regarding the sensitivity of methods possibly associated with different types of real-time probes. For example, the log-linear phase of reactions using FRET probes (LightCycler probes) was only 2–3 logs, whereas with TaqMan probes it was 5–6 logs. This may be of particular importance when the background noise in the analyses of some samples is high, since noise forces the fluorescence level of the baseline to increase, i.e., reducing the number of logs between the baseline and the endpoint level of the PCR (Fig. 27.4).

27.3.3 Limits of detection (LOD) and quantitation (LOQ)

The LOD and LOQ of a method may be expressed on the basis of copy numbers or relative values (percentages). Until recently, the LOD and LOQ of GMO testing has consistently been reported as relative values on the basis of positive and correct results using DNA extracted from CRMs, e.g., 0.1% GTS 40-3-2 flour (IRMM 410R). This is mainly due to the fact that practically all GMO regulations refer to the relative GMO content. However, because it is often of interest to analyse samples of more processed and composite materials, relative

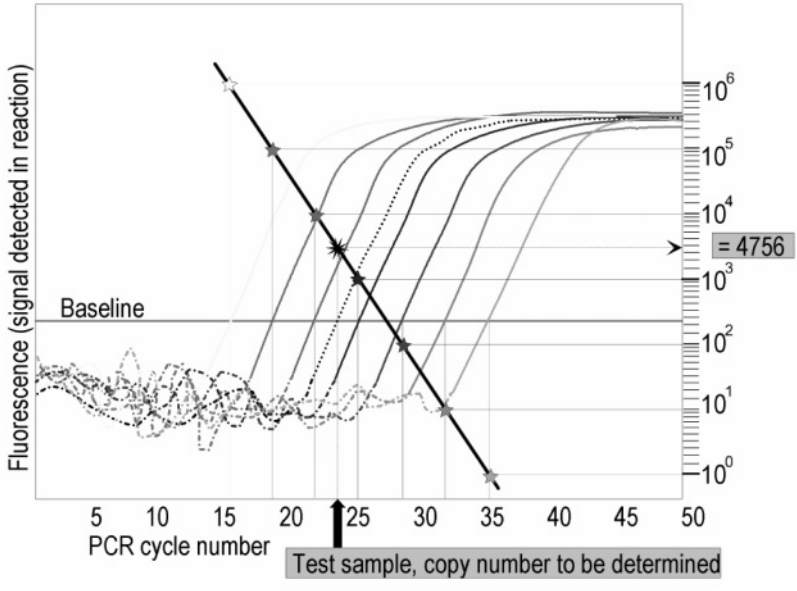

Fig. 27.4 Calibration curve quantitation. A series of calibrants are analysed in parallel with the test sample (black dotted graph). Each calibrant contains a predetermined number of copies of the target sequence. A regression line (black diagonal line, the calibration curve) is produced as the function of the copy number of the calibrants on one axis (rightmost vertical axis) and the corresponding Ct-values (number of cycles required to reach a defined threshold level of fluoresence [baseline]) on the other axis (horizontal axis). Stars corresponding to each calibrant are used to indicate the points producing the calibration curve. The copy number present in the test sample can be determined by the reverse procedure, reading the copy number corresponding to the observed Ct-value from the standard curve. The baseline must always be defined to exceed the background fluorescence (bumpy broken lines). In the present example, the calibrants contain a 10-fold dilution series of copies from 10^6 to 1 copy, and the copy number is estimated to be 4,756 in the test sample.

LODs and LOQs determined on the basis of CRMs may not be applicable, e.g., if only a small fraction of the material is composed of soya, and/or this fraction is processed (e.g., a protein extract).

27.3.4 Alternatives for quantitation using PCR

Whereas a copy number determination can be done directly by comparison to a calibration curve using a series of calibrants with known copy numbers, Fig. 27.4, a relative determination is done indirectly by comparing results of two PCRs, one targeting the GM-specific sequence, and the other targeting a reference gene. A relative determination using real-time PCR can either be done by comparing the number of PCR cycles required to reach a certain amount of

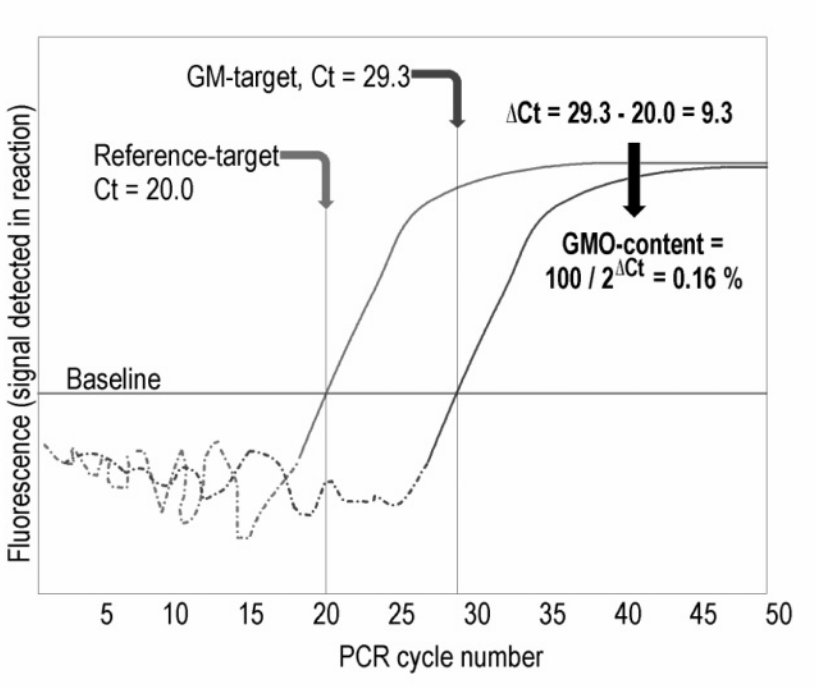

Fig. 27.5 Quantitation with the ΔCt-method. The number of PCR cycles (Ct) required to reach a defined threshold of fluorescence (the baseline) is determined for a reference target (Ct_{ref}) and a GM target (Ct_{GM}), using the same dilution of the template DNA. The difference in Ct (ΔCt) is determined: $\Delta Ct = Ct_{ref} - Ct_{GM}$. Under the assumption that the number of copies of the target is doubled for each cycle in the PCR, it is possible to estimate the relative ratio between the reference and GM targets using the formula: $\%GMO = 100 \times \frac{1}{2^{\Delta Ct}}$.

copies of the amplicons (ΔCt method, see Fig. 27.5) under the assumption that the two amplicons are amplified with the same efficiency (each cycle yielding the same relative increase in number of copies of the amplicon), or by comparing the estimated initial copy numbers of each of the two amplicons from calibration curves using known concentrations of the template DNA (calibration curve method, Fig. 27.4). Quantitative competitive PCR is largely being replaced by less labour intensive real-time PCR, and will therefore not be discussed further here. The interested reader may find more information elsewhere (Anklam *et al.*, 2002; Holst-Jensen *et al.*, 2003).

There are several advantages of using calibration curves in comparison to the ΔCt method. First of all, the validity of the assumption of equal amplification efficiency of the GM and reference gene amplicon is questionable. Therefore it is preferable to use a method that does not rely on this assumption. Secondly, calibration curves allow for determination of the LOD and LOQ for each analytical sample, whereas the ΔCt method will only allow for determination of

the LOD and LOQ of the method relative to a reference material, e.g., a CRM. Thirdly, calibration curve methods are much easier to validate, since these can be validated for a single target sequence, whereas ΔCt methods have to be validated targeting both GM and reference gene-specific sequences simultaneously.

27.3.5 Method validation and the analytical procedure

The analytical procedure can be seen as a series of interdependent steps, where the results of one step are used as input for the next step. Sampling ideally delivers homogeneous and representative material. A normal assumption of homogeneity and representativity of the sample is only valid, however, if the sample is really homogeneous and representative. Empirical studies of homogeneity of soybean lots are currently being conducted by members of the European Network of GMO Laboratories (ENGL), coordinated by the EC Joint Research Centre, Institute for Health and Consumer Protection, Ispra, Italy (see http://engl.jrc.it and http://biotech.jrc.it/kelda.htm). Further processing, e.g., grinding of the material to reduce the original sample to a laboratory sample, is also assumed to deliver a homogeneous and representative material. This assumption may be invalid if, for example, particle size varies among seed coat, endosperm and embryo (cf. Fig. 27.3), and small or large particles are overrepresented in the laboratory sample. The DNA extracted from the laboratory sample is the basis for the PCR analysis. Analysis of a DNA sample of high quality (no detectable inhibitory effect on PCR) with a high concentration of DNA of a given species of interest and being representative of the original lot, shall produce correct estimates of the GM content for the species of interest with a valid PCR method. Thus with a valid sampling, a valid preparation of the laboratory sample, a valid DNA extraction and a valid PCR method, the final analytical result (estimate) is also valid. Unfortunately, it is difficult to validate all the individual methods of the procedure, e.g., the sampling. As a consequence the final result may end up being incorrect. The challenge lies in developing and validating methods at all steps of the analytical procedure. It has been natural to start with the PCR methods, because the measurement uncertainty introduced by the PCR is relatively easy to estimate and it is easy to run a sufficient number of PCR trials to meet with strict statistical requirements. In contrast it is extremely difficult, costly and labour intensive to obtain representative samples from large heterogeneous lots. The next step where it should be possible to determine the introduced measurement uncertainty is through validation of DNA extraction methods. At present, there is a debate on how DNA extraction methods should be validated. Thus, there is still a long way to go before we can claim that the analytical results are valid for the entire analytical procedure. But we can claim that they are valid for DNA samples, and with valid DNA extraction methods we will also be able to claim that the results are valid for the laboratory samples.

27.4 Future trends in detecting GMOs in food

Sampling is the starting point of all GMO testing schemes. Present GMO sampling schemes are often very similar to sampling schemes for mycotoxin testing. Mycotoxins and mycotoxin producing fungi are only rarely distributed homogeneously in contaminated lots, due to local variation in temperature, gas exchange, water conditions and inoculum in storage containers, fields and soils, as well as patchy damage by insects (causing secondary damage by fungi). Similarly, GMO contamination from pollen influx, contaminated seed lots, from single fields or from remains in storage and transport containers, is likely to be distributed heterogeneously. Sampling schemes need to take potential heterogeneous distribution of the target analyte into consideration. The more heterogeneous the lot, the more of the lot needs to be sampled to obtain a statistically representative sample. The lot size is often very large, and several hundred kg of a single kernel lot may have to be sampled. Assessment of the heterogeneity of lots of different commodities may provide a better basis for development of sampling schemes fit for the purpose. While the problems may seem minor when considering liquid commodities (e.g., milk, oil), considerable problems are associated with typical agricultural commodities such as kernels (soybeans, dried maize), and the problem becomes very complex once each sampling unit is large (e.g., potatoes or watermelons) or when processed products (e.g., canned foods, pizza) are considered. A tool to assess the effect of heterogeneity in kernel lots has recently been developed and the potential effects discussed (Paoletti *et al.*, 2003), and ongoing research in Europe will provide the first empirical data on the heterogeneity of imported soybean lots (http://biotech.jrc.it/kelda.htm). The results of these studies may also be relevant for other product types where solid sampling units are distributed spatially.

Because the lot sample is too large to be analysed directly, it is usually ground and homogenized, and a subsample (the laboratory sample) successively taken. This process may add or remove heterogeneity. Grinding may produce particles of different size and origin (cf. Fig. 27.3). Studies are therefore needed to assess the matrix-specific effects of different protocols for production of laboratory samples. However, the necessary analytical tools to conduct such studies are not yet available, i.e., tools to assure that observed variation is coming from the production of laboratory samples rather than from downstream analytical steps (e.g., DNA extraction and PCR).

27.4.1 DNA extraction

The first draft European and international standards for nucleic acids extraction in the context of GMO detection in foodstuffs were recently presented by the European Committee for Normalisation (CEN) and the International Standardisation Organisation (ISO). Unfortunately, none of the included methods have been subject to careful assessment of their domains of application and matrix limitations. This and several other challenges listed in the preceeding

sections of this chapter led to the initiation of the research project 'Reliable, standardised, specific, quantitative detection of genetically modified foods' (acronym QPCRGMOFOOD; http://www.vetinst.no/Qpcrgmofood/Qpcrgmofood.htm). In relation to DNA extraction, three of the most widely used methods (CTAB, SDS and G2 buffer based) formed the basis for examining the effects of a range of modifications to determine the domain of application and matrix limitations of the methods and modifications. On the basis of the results, selected matrices and associated matrix-specific methods were subjected to a small-scale ring trial to assess their robustness and transferability, and successively validated in a collaborative trial according to an internationally harmonised protocol, with the objective of providing fully validated methods for standardisation. Future research projects are expected to focus on yield (quantity), quality (size and degree of shearing/fragmentation), and purity (absence of substances other than DNA, e.g., PCR inhibitors) in relation to specific matrices. For these purposes, suitable reference materials (matrices) will have to be developed, and we also need specific analytical tools to determine the absolute and relative yields from a specified quantity of reference material, the effects on DNA quality of the extraction method, and to assess the purity of the resulting extract (specific removal of known non-target substances). Improving DNA extraction methods is of course also highly relevant for research in other fields of food and feed analysis such as detection of pathogens and potential allergens.

27.4.2 Reference genes

PCR methods targeting reference genes for soybean, maize, rapeseed and tomato have been published. PCR methods targeting reference genes for all other crop plants have yet to be published. Several candidate genes were examined for these four and other crop plants (potato, sugarbeet, sunflower, rice and wheat) within the Qpcrgmofood project. For each candidate gene, at least 20 lines of the species were included. As far as possible, the lines were selected to represent the phylogenetically most distantly related and geographically most separated origins available. This was done to increase the likelihood of detecting both copy number and allelic variation within the target species. Several candidate genes were discarded on the basis of observed copy number and/or allelic variation. The specificity of each PCR method was also assessed against approximately 20 common non-target food plant species. Reference gene-specific methods may serve not only as reference for GMO quantitation, but also as very useful tools for determining the practical LOD and LOQ of GMOs (even in the absence of GMOs). Unfortunately, reference genes may be associated with specific features of interest for breeders. Lectins, for example, are toxic protective substances produced by plants. Breeding including the use of gene technology may increase (improve protection) or decrease (avoid potential toxic effects on animals) lectin production in soybeans, and this may potentially invalidate the soybean lectin gene as a suitable reference gene (at least for

quantitative PCR). This gene is presently the only widely used reference gene for soybean. Future reseach projects are likely to include development of PCR methods for additional reference genes to function as backup or complements to existing PCR methods. Multiplex methods to detect reference genes of many plant species will improve the ability to test composite food products, and may also be suitable for detection of potential allergens in foods. Quantitative PCR methods for reference genes are also among the analytical tools necessary for validation of DNA extraction methods. For validation of reference gene-specific PCR methods, suitable reference materials must be developed. These materials could be purified genomic DNA, in which case the haploid genome size and the quantity (weight) of DNA of the material should be known. Alternatively, cloned plasmids may be used, provided that it can be demonstrated that the amplifiability of the reference material is the same as the amplifiability of the reference gene in commercial products.

27.4.3 GMO-specific methods

Target sequences

Only a few promoters and terminators are available for construction of functional gene constructs that can be transformed into plants. One consequence of this is that practically all GMOs can be detected on the basis of screening PCR. In addition, the limited selection of promoters and terminators may allow for development of semispecific multiplex PCR assays, based on the combination of a shared forward primer specific for, e.g., P-35S, with more specific reverse primers, e.g., for the CryIA(b), PAT and BAR genes (construct specific) or a part of the host genome (event specific). The use of multiplex consensus PCR and array-based detection and identification of GMO derived amplification products is explored in the research project 'New technology in food sciences facing the multiplicity of new released GMO' (Acronym: GMOCHIPS; http://www.gmochips.org), as well as in the Qpcrgmofood project. Unfortunately, lack of sequence information obtained from specific GMOs is often restricting the reliability of screening targets. Minor modifications of natural DNA sequences may be intentionally introduced to produce synthetic sequences with slightly altered characteristics and function, but may also lead to mismatches between primers/probes and target sequences. Detailed characterisation of the integrated and flanking DNA from each GMO is undoubtedly the best source of information to assure the specificity of primers and probes. In the Qpcrgmofood project, DNA isolated from GMOs of various origin (certified reference materials, ground seeds and contaminated seed lots) was sequence characterised for development of event-specific PCR methods. A side effect of this work was the production of updated genetic maps and better knowledge about the processes of integration of new sequences into the genomes of a number of plant species. In the future, legislation in the EU will require that the GMO developer shall provide the complete sequence data, genetic maps and specific detection and quantitation methods (most likely event

specific), as well as reference materials (European Commission, 2001; article 31(2) and annex IV A7). It should be noted, though, that access to sequence data and possibly also detection and quantitation methods may be restricted to competent authorities and their associated laboratories. Future research projects on method development are therefore likely to focus primarily on multiplexing, and on unauthorised/unknown GMOs (i.e., where no sequence data and methods are provided by the GMO developer).

Multiplex methods

Hitherto, multiplexing of GMO detection has been limited by the ability to coamplify multiple target sequences by PCR. A number of multiplex assays have been developed in the Qpcrgmofood and Gmochips projects, and other multiplex assays are used commercially by testing laboratories. The maximum number of targets that can be coamplified is claimed to be around 20, and none of the assays have been validated according to internationally harmonised protocols, for various reasons (costs, need for special laboratory apparatus, inconsistent results, lack of reference materials, etc.). Together with competitive effects and the wide range of genomes that GMO sequences may be associated with (increased by extensive crossbreeding of GM lines with locally adapted non-GM lines) this points in the direction of looking for alternative methods that do not depend on PCR. DNA arrays, with their potential for spotting of up to several thousand different specific probes on a single slide, are often discussed in this context. However, analysis of the resulting patterns would be similar to fingerprinting analysis, i.e., a multitude of signals must be analysed for covariance and signal intensity to obtain indicative answers, would require sophisticated statistical software, and in the foreseeable future the results would probably have to be confirmed by, e.g., event-specific quantitative PCR.

Quantitative methods

Real-time PCR has replaced double competitive PCR as the preferred quantitative PCR-based technology for several reasons: it is faster; the quantitative results are produced without the need for errorprone pipetting and image analysis; the risk of carry-over contamination is minimized by the lack of post-PCR pipetting; and the production and calibration of competitors is not required. However, even with the recent development of event-specific real-time PCR methods, in particular within the Qpcrgmofood project, quantitation methods are still wanting for the vast majority of GMOs authorised on a world-wide scale. Since (at least on a worldwide scale) the speed with which new GMOs are developed and authorised is continuously increasing, the gap between available methods and the number of authorised GMOs is expected to grow, until the GMO producers start to provide specific and reliable detection and quantitation methods together with their new products (GMOs). This is exactly what will be required in the future by EU legislation (European Commission, 2001; article 31(2) and annex IV A7). However, the reliability of PCR-based quantitation is limited also by PCR itself. Exponential amplification over 30–50

cycles (typical number of cycles used in quantitative PCR assays for GMOs) imply that any systematic error can be exponentially amplified, with potentially severe consequences for a quantitative result. Furthermore, quantitative PCR can only be done for one GMO per reaction vessel; no functional quantitative multiplex assay has yet been published, and certainly not validated in a collaborative trial according to an internationally harmonized protocol (Horwitz, 1995). The costs of quantitative analyses therefore become prohibitive with increasing numbers of GMOs on the market and in the products. Alternatives to conventional real-time PCR are explored. These include assays with end-point quantitation such as capillary analysis (Burns *et al.*, 2003) and semiquantitative hybridisation arrays (Rudi *et al.*, 2003), as well as PCR independent methods.

27.4.4 Reference materials

Reference materials serve a number of purposes. They may be used as calibrants for specific methods, or as reference matrices for a range of methods, and they may be produced with the objective of satisfying highly divergent performance criteria. In the context of GMOs, the term reference material is often interpreted as certified reference material (CRM). However, CRMs have only been produced from ground kernel materials and only for four of the GMOs authorised on a worldwide scale (IRMM-41X series for GTS 40-3-2 [RoundupReady] soybean, Bt176, Bt11 and Mon810 maize; IRMM, 2003). Other reference materials include ground maize and soybean kernels, flours and processed food products prepared for method validation studies (Van den Eede *et al.*, 2000a; 2000b), and proficiency tests (GeMMA, 2002), cloned plasmids for calibration of real-time PCR assays (Taverniers *et al.*, 2001; Kuribara *et al.*, 2002), genomic DNA extracted from leaves (Berdal and Holst-Jensen, 2001; Holst-Jensen *et al.*, 2003) and commercial DNA size standards (Berdal and Holst-Jensen, 2001). Present day CRMs have been produced on a weight:weight basis and were meant to function for both protein and DNA-based detection methods. However, they suffer from a number of weaknesses, e.g., uncertain relationship between weight and analyte copy numbers for GM and reference, they are unfit for determination of the absolute and practical LODs/LOQs, and for calibration of detection methods on absolute copy number basis. New types of CRMs will certainly be required in the future, and may include CRMs for calibration of DNA quantity (weight), reference and GM target copy numbers (DNA calibrants), for evaluation of DNA extraction (matrix specific), and for method validation and proficiency tests. In addition, a wide range of other reference materials may have to be developed as new technologies become available, e.g., DNA arrays may require special reference materials.

27.4.5 Method validation

Method validation is time and resource consuming. Normalisation of analytical methods may be required to avoid conflicts, e.g., between exporters and

importers, and require that methods are validated according to internationally harmonised protocols. Hitherto, it has been common practice to validate GMO detection methods by sending out a set of samples containing or prepared from CRMs, i.e., from flour produced from grains. With such an approach the LOD and LOQ are determined and therefore only valid for a specific matrix, and are determined to be the lowest relative quantity that is detected and quantified with an acceptable level of reproducibility. The dynamic range of the method is determined to include the lowest and highest relative concentrations that yield the correct results with acceptable uncertainty and reproducibility, and the validation includes both DNA extraction and PCR. Consequently, the dynamic range is limited to a small range of relative concentrations, the absolute LOD/LOQ is not determined, and the validity of a PCR method may be compromised by low quality, yield and purity of DNA extracts. In the Qpcrgmofood project this was avoided by separation of validation studies for DNA extraction methods and PCR methods, using purified DNA samples for the latter. This allowed for determination of the dynamic range of qualitative and quantitative PCR methods as the range between the lowest and highest number of copies of the target molecule that was detected/quantified reliably. The dynamic range therefore includes relative concentrations of 0–100%, and the validity of a PCR method is matrix independent but template DNA dependent. Thus, provided that template DNA is of sufficient quality and purity, the number of copies of a target sequence is the factor that is subject to testing, and a relative GM quantity is estimated by comparison of the number of GM and reference copies. With the limited number of reference gene-specific PCR methods available, conventional validation of GMO detection and quantitation methods includes repeating the validation of a reference gene-specific PCR method in every GM-specific PCR method validation study. Using purified DNA, and validating PCR methods on the basis of PCR target copy numbers by comparison with calibration curves, would allow for independent validation of GM- and reference-specific PCR methods, and would reduce the cost and workload of any validation study by almost 50%.

In the future, partly as a consequence of the EU legislation (European Commission, 2001), we should expect a much larger number of validation studies focusing not only on PCR methods but also on validation of the entire analytical procedure. One possible scenario is an upstream validation strategy, using downstream validated methods, e.g., validated PCR methods for reference genes to validate DNA extraction methods, validated DNA extraction methods to validate methods for production of laboratory samples, etc.

27.4.6 Unauthorised and unknown GMOs

Presence of authorised and non-authorised GMOs usually lead to quite different reactions from competent authorities. However, authorisation is given by competent authorities at the national level, and GMOs authorised in, e.g., the USA may or may not be authorised in, e.g., the EU. With today's worldwide

trade, contamination with unauthorised GMOs cannot be completely avoided and is regularly reported. One consequence of this is the recent proposal to introduce in the EU separate thresholds for labelling of adventitious contamination with GMOs authorised in the EU (0.9%) and authorised outside but not within the EU (0.5%). Authorisation includes extensive risk evaluation, and the rationale behind this proposal is that once a GMO is authorised by a competent authority, even if it is outside the EU, it is considered safe. Unfortunately, it cannot be excluded that GMOs can enter the food chain without prior authorisation of this type. Methods to detect unauthorised GMOs are presently limited to the use of screening or gene-specific methods that may give indicative evidence of the presence of unauthorised GMOs. However, any GMO that does not contain any of the screening elements or genes for which a detection method is already available would go undetected. Such unknown GMOs may at least theoretically pose a significant risk. Future GMO research is therefore expected to focus on development of better tools for detection, characterisation and identification of unknown GMOs. This challenge is not like searching for a 'needle in a haystack' but may be compared to searching for a 'needle-like object in the field'. For this purpose, PCR is unlikely to have sufficient power, and the solution may lie in the combined use of micro arrays, extensive data collection and sophisticated bioinformatic tools and databases. Although, preparations for research projects to cope with this challenge have been launched, the work is still in its infancy.

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