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Chiral Chromatography

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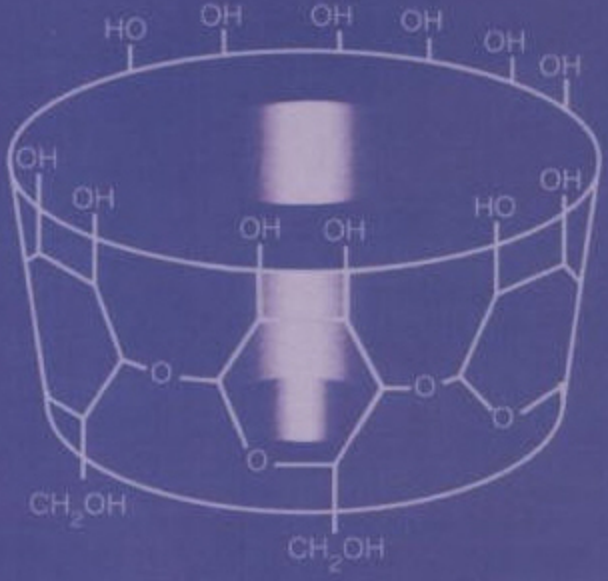

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Chiral Chromatography

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Preface

In the world of science, nothing invigorates the mind so much as to watch a concept develop from some small seed of discovery to a universally applicable technology. In the last four decades, chromatography, the once mysterious and very crude technique, has grown into a very sophisticated and reliable separation methodology. Over the last decade, the application of the accumulated knowledge to the once esoteric field of chiral separations has made impressive advances particularly in the last three years. The chromatography of enantiomers has required the preparation of highly structured phases, which had to be designed to have a direct bearing on the nature and the chemistry of the materials to be separated. New terms had to be introduced and defined, such as three point interaction and inclusion complexation to describe some of the new interactive mechanisms that were invoked. As will be apparent from this book, however, the basic chromatography terms and the physical chemical principles that determine that a chromatographic separation is possible remain the same. In dealing comprehensively with the subject of chiral chromatography, a significant amount of theory must be included, but we have tried to present this in a manner that explains the interactions that takes place and provides a rational direction that can be taken to solve practical problems.

Having been involved in chromatography for many years (our combined experience extending over three quarters of a century) we find it gratifying to see the very substantial increases in successful chiral separations that are currently published in the field. Starting from the pioneering work of Gil-Av (1966) and Bayer (1974) followed by that of Okamato and Pirkle and culminating with the recent, highly innovative phases, introduced by Armstrong, chiral chromatography has now reached a high degree of sophistication. The creation of the cyclodextrin phases and the introduction of the macrolytic antibiotics by Armstrong has brought new incentives to the field and many new areas of application.

This book has been written to serve both the novice in the field and the experienced chromatographer. In addition to giving detailed information on chiral separations, it also discusses the principles involved in chiral selectivity and, for those new to the technique, describes the fundamentals of a chromatographic separation and the essential apparatus needed to carry it out. To make the book as complete as possible we have included chapters on preparative chiral chromatography and some basic

information on chiral capillary electrophoresis and electro-chromatography. Finally, to help readers choose the correct phase system and operating conditions for their particular sample, we have incorporated experimental selection schemes for the four major chiral stationary phases that are commercially available.

THOMAS. E. BEESLEY
RAYMOND P. W. SCOTT
NOVEMBER 1998

Acknowledgments

In the preparation of any technical book, information must be gathered from a variety of sources. The authors would like to take this opportunity to thank the many journals who have granted us permission to reproduce diagrams from their publications, and those chromatography vendors who have supplied us with the technical details of their products. In particular, we would like to thank the companies Advanced Separations Technologies Inc. (ASTEC), Chromtech AB, Regis Technologies, Chiral Technologies, and Supelco for providing both technical data and permission to reproduce their product bibliographies. Special thanks are also due to Advanced Separations Technologies Inc. (ASTEC) who contributed laboratory facilities for data collection and to their staff members, Dr. Heng Liang Jin and Mr. B Buglio, who helped provide the chiral retention data that is used in the optimization procedures.

Finally, thanks are due to the production department of John Wiley and Sons for their careful review of the manuscript before publication. In particular we appreciate the efforts of Mr. Martin Tribe and his assistants for their much appreciated comments and advice.

THOMAS E. BEESLEY
RAYMOND P. W. SCOTT
OCTOBER 1998

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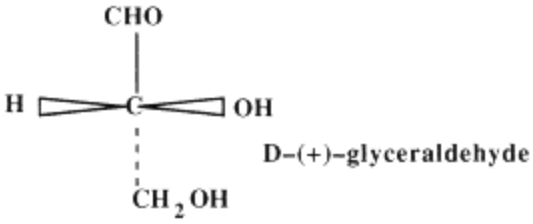
An Historical Perspective

Until relatively recently, interest in chiral chemistry has been largely academic and, as a consequence, has occupied a relatively minor position in the analytical chemistry syllabuses of most universities. Despite the emphasis that has been placed on the recent advances in chiral chemistry, optical isomers have been know for many years and were first identified by Biot [1] in the early 1800s, and their existence was established by the work of Pasteur [2] in 1848. Both van't Hoff [3] and Le Bel [4] proposed the existence of the asymmetric carbon atom and used it to explain the cause of optical rotation. However, it was Emil Fisher [5], who made the first serious attempts to relate the absolute stereochemistry of optical isomers and determined the configuration of (+)-glucose for which he received the Nobel prize. Fisher predicted that the (+)-isomer of glyceraldehyde was the D-isomer and arbitrarily assigned the stereochemistry as;



D–(+)–glyceraldehyde

Fisher's assumption was later proved correct by Bijovet [6] using X-ray crystallography. Thus, the foundations of chiral chemistry were

established. The study of stereochemistry progressed steadily, albeit relatively slowly, for some years. However, about 1980, there was a sudden increase in the commercial interest in chiral substances, particularly chiral drugs, and this interest proliferated very rapidly. This new enthusiasm was fostered by the discovery that the respective physiological activity of the isomers of a drug could differ radically and this was found to be true for many physiologically active compounds and, in particular, physiologically active biotechnology products. However, the major stimulation arose from the unfortunate birth defects initiated by one of the enantiomers of Thalidomide. This drug was manufactured and sold as a racemic mixture of N-phthalylglutamic acid imide. However, the desired physiologically activity was found to reside solely in the R-(+)-isomer and it was discovered, too late, that the corresponding S-(-)-enantiomer was teratogenic and caused serious fetal malformations.

The thalidomide disaster evoked the interest of all pharmaceutical manufacturers and also the drug regulatory committees. Research activity in the field of stereochemistry became almost frenetic. The United States Food and Drug Administration recommended that each isomer of all new drugs should be individually tested, forcing companies to address the possible problems associated with enantiomeric mixtures. The demand for enantiomerically pure drugs rose rapidly and a few years ago (1993), the world market in enantiomerically pure drugs exceeded $35 million, and of that total, nearly two thirds were cardiovascular and antibiotic drugs. The mandate to test the different enantiomers of a drug evoked the need for appropriate analytical procedures to separate and quantitatively assay them. A decade ago, there were very few effective techniques available.

In early 1980, few commercial stationary phases for either gas chromatography or liquid chromatography were available. Nevertheless, in 1966 Gil-Av *et al*., [7], had described the first chiral stationary phase for gas chromatography and in 1976 Sogah and Cram [8] introduced chiral crown ethers as stationary phases. In 1978, Harada *et al.* [9]

introduced the cyclodextrins as chiral separation agents and, in 1980, Armstrong [10] used the cyclodextrins as mobile phase additives for chiral separations by thin-layer chromatography. Today, the cyclodextrins are one of the more common chiral agents used in chromatography and electrophoresis for the separation of enantiomers, although other reagents, such as those based on cellulose, have found many areas of application. However, to appreciate the difficulties involved in the separation of enantiomers, and the mechanism by which they are selectively retained on chiral phases, some basic understanding of chiral chemistry is necessary.

A Short Introduction to Stereochemistry

Stereochemistry is not primarily germane to the subject of this book, and it is not intended to discuss the subject in any detail. Nevertheless, the basic concepts, definitions and conventions, currently used in stereochemistry, will be considered to help those less familiar with the subject to understand the separation technology that will be introduced and described in the subsequent chapters. Stereochemistry is the study of the three-dimensional structure of chemical compounds. Isomers of the same substance, that only differ in the spatial arrangement of their atoms are called stereoisomers. Certain stereoisomers that differ only in their capacity for rotating the plane of polarized light passed through them, are termed optically active, or chiral, and the isomers are called enantiomers. It follows, that as the subject of this book pertains to the separation of chiral substances, the method used for measuring optical activity needs to be briefly described.

Optical activity was originally measured by means of a polarimeter, the principle of which is depicted in figure 1.1. Light from an appropriate source passes through a polarizer, typically consisting of a set of crossed Nichol prisms, which produces a beam of plain polarized light usually polarized vertically. The vertically polarized light then passes through a sample tube containing the optically active substance. The light is rotated in the sample cell and transmitted with the plane of polarization turned through an angle, the magnitude of which is determined by the nature of the substance and its concentration in solution. The light then passes

through a second polarizer that is adjusted by rotation, until the angle of the transmitted light is again returned to the vertical.

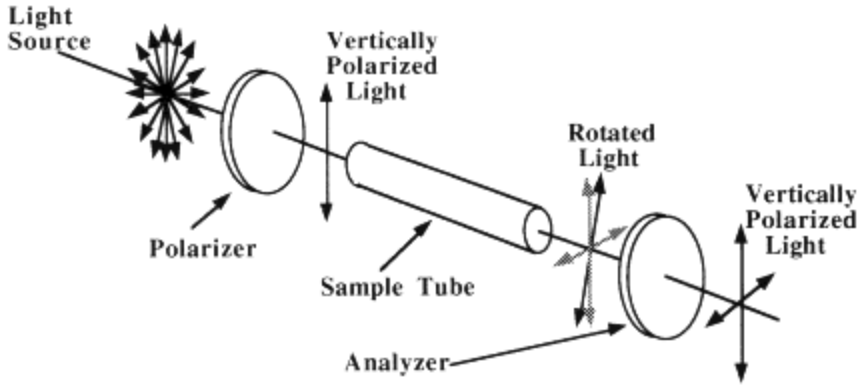


Figure 1.1
The Basic Polarimeter

The angle through which the second polarizer is rotated is called the optical rotation of the sample. Clockwise rotation of the polarized light by the sample is designated as (+) and anti-clockwise rotation as (-). At this point, it is necessary to mention some apparently conflicting nomenclature. Traditionally, the (+) isomer has been designated as *dextro* (*d*) and the (-) isomer as *levo* (*l*). However, as previously mentioned, Fisher, in his work, arbitrarily defined the (+) isomer of glyceraldehyde as the D-isomer. However, using the same notation, D-glutamic acid is actually (1) or (-). It is clear that considerable care must be taken when using the symbols (D), (L) and (*d*) and (*l*) and they can only be related to each other after the actual rotation has been experimentally measured.

The specific rotation of a substance, $[\alpha]$, is given by:

$$[\alpha] = \frac{\alpha}{l\,d}$$

where (α) is the measured rotation,

(l) is the length of the sample cell,

and (d) is the concentration of the sample (g/ml), (if the sample is pure, d=ρ=density).

This method is now rarely used and so the modern method of measuring optical activity needs to be briefly discussed. Light consists of a sinusoidally changing electric field normal to, and in phase with, a sinusoidally changing magnetic field. The plane of the electric vector in normal light, takes no particular orientation, but in plane polarized light, the electric vector is either vertically or horizontally polarized. If the electric vector transcribes a helical path, either to the right or left, the light is said to be *circularly polarized*. A linearly polarized beam of light, can be considered to be the resultant of two, equal-intensity, in-phase components, one left, and the other right, circularly polarized, or of two orthogonal linear components at ± 45°.

The differential absorption of these two ± 45° linear components in a medium is known as *linear dichroism*. If there is a differential velocity between the two ± 45° linear components, when passing through a medium (*i.e.* the refractive index of the medium to the two light components differ), then this is known as *linear birefringence*. In an analogous manner, the difference in the adsorption characteristics of a medium to the left and right circularly polarized light, is termed *circular dichroism* (CD). It follows, that the difference in refractive index of a substance to the two light components, is called *optical rotary dispersion* (ORD), sometimes reported as *specific optical rotation*.

CD spectra are usually measured as the differential absorption of left and right circularly polarized light, *i.e.* (A_L-A_R) and is usually reported as the differential molar extinction coefficient ($\Delta\varepsilon$), where:

$$\Delta\varepsilon = (\varepsilon_L - \varepsilon_R) = \frac{(A_L - A_R)}{cl}$$

where (l) is the length of the cell (cm),

and (c) is the molarity of solute

The basic apparatus that is used for measuring circular dichroism is shown in figure 1.2 It consists of a light source, a linear polarizer, a Fresnel rhomb that converts the linear polarized light to circularly

polarized light, a sample cell, and finally an appropriate light intensity measuring device.

The rotation of the linear polarizer ± 45°, to the appropriate Fresnel rhomb axis, induces the generation of left or right polarized light.

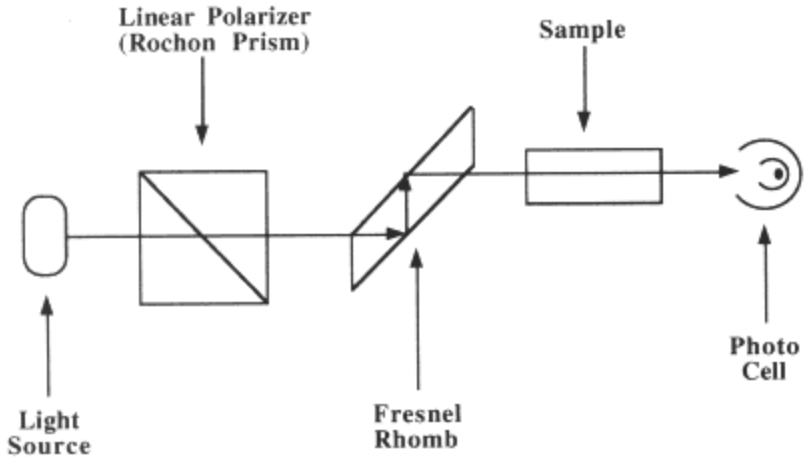


Figure 1.2
The Basic Apparatus for Measuring Circular Dichroism

The modern form of the CD spectrometer is shown in Figure 1.3.

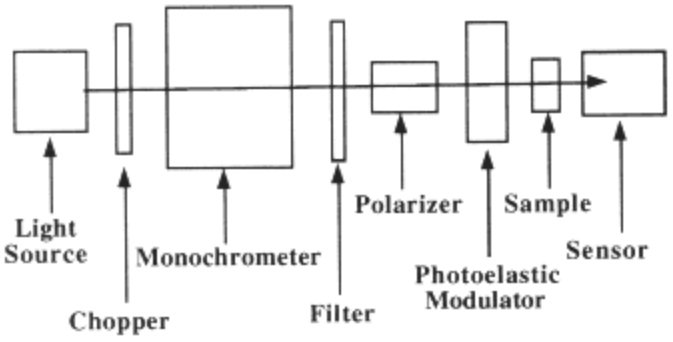


Figure 1.3
A Modern CD Spectrometer

Light from a broad emission source passes through a chopper, and then to a monochromator that allows light of a selected wavelength to be passed through a filter to the polarizer. The polarizer can be a Rochon prism and the polarized light would then be passed through a photoelastic modulator (Pockels cell), the function of which will be described below. The selected left or right circularly polarized light is then passed through the cell and the intensity of the transmitted light monitored by the sensor.

Polarization Modulation

The alternate production of left or right circularly polarized light, which is called polarization modulation, is an essential process for CD measurement. Now, a linearly polarized light beam can be said to be the resultant of two orthogonal, in phase, linear, light beams. Consider a block of isotropic fused silica that is rendered birefringent by pressure exerted along the (x) or (y) axis, as shown in figure 1.4.

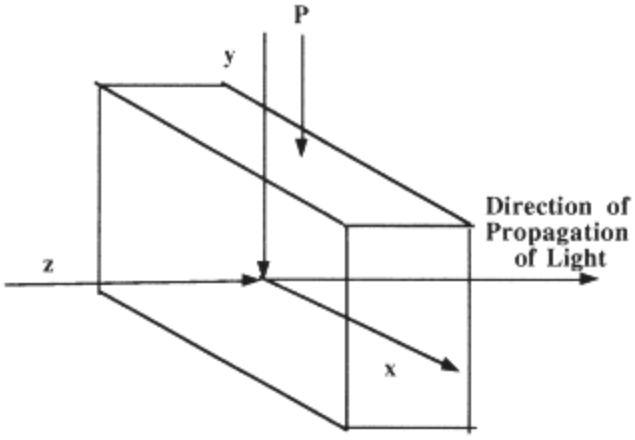


Figure 1.4
Polarization Modulation

Under these condition the refractive indexes (n_x and n_y) will differ, and:

$$n_x \neq n_y$$

If the light beam passing through the block is oriented with its resultant axis at 45° to the pressure axis, one of the components will travel faster

through the medium than the other. If $n_x > n_y$, then the (x) component of the light beam will travel more slowly. If the retardation is exactly ($\frac{\lambda}{4}$) the emergent light beam will be right circularly polarized.

If the retardation is ($-\frac{\lambda}{4}$ or $-\frac{3\lambda}{4}$, $-\frac{7\lambda}{4}$, etc.) then the emergent light beam will be left circularly polarized. This an example of a general principle which was first described by Brewster and given the term photo elasticity. Electro-optic modulizers, that are used in some modern instruments, operate in a similar way.

Practical Chiral Measuring Devices

A detailed description of a commercially available LC chiral detector will be given in the chapter 7. However, some general comments on the properties of chiral detectors would be appropriate here. Contemporary, chiral detectors are relatively insensitive and, consequently, there are no GC chiral detectors commercially available at this time. Capillary columns will only *function* with *very small charges* and these types of column must be employed for the great majority of chiral separations, in order to provide the necessary efficiency. Unfortunately, the sensitivity of chiral sensing systems, investigated so far, have been inadequate for use with GC capillary columns. In contrast, after considerable research and development, the sensitivity of LC chiral detectors has been improved to a level where (although still relatively insensitive) they can often be used satisfactorily with contemporary small particle LC chiral columns.

One commercially available detector is the PDR Chiral detector that is manufactured by PDR-Chiral Inc. The detector has a sensitivity of 25 micro degrees. The flow cell has a path length of 50 mm and a volume of 56 µl. This is very large compared with the normal UV sensor cell. which has a volume of 3 to 8 µl. Such a large volume will cause early peaks, from columns packed with particle 5 µm or less, to merge with consequent loss of resolution. Later peaks, however, will be detected

satisfactorily and, unfortunately, the large cell volume must be tolerated to achieve the necessary sensitivity. The cell is constructed of Teflon and fused silica and the laser light source has a wavelength of 675 nm. The PDR detector, at the time of writing this book, is claimed to be the, most sensitive chiral detector commercially available. Both positive and negative signals are produced, depending on the direction of rotation. This means that the isomers need not be completely resolved to obtains a quantitative estimation of individual enantiomers. An example of a separation monitored by the detector is shown in figure 1.5.

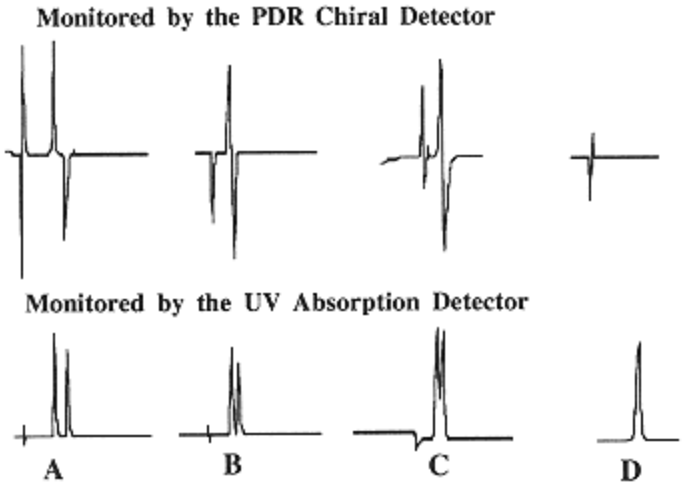


Figure 1.5
The Separation of the Enantiomers of Dihydrobenzoin at Different Resolution Monitored by the PDR Chiral Detector and the UV Detector Courtesy of PDP-Chiral Inc.

The separation was carried out on a A Cyclodextrin-1 2000 AC column. The column eluent was methanol/TEA(pH 4.4); separation A, 10/90 v/v, B, 30/70 v/v, C, 50/50 v/v and D pure methanol. The upper set of chromatograms were monitored by the chiral detector and the lower by a UV detector at 254 nm. It is seen that when the enantiomers are will separated (chromatogram (A)) the positive a negative peaks accurately

follow the profile produced by the UV detector. At the other extreme, (D), when there is no separation, the chiral detector gives no signal, as the rotation of the two isomers cancel each other out. Even when the resolution is poor, (C), the detector gives, misleading, but clear negative and positive peaks.

At this point, it is important to describe the various ways the enantiomeric content of a mixture of isomers is defined and measured. There are three definitions that the chiral chemist uses to describe a product purity.

1. *Optical Purity* which is given by:

$$\text{Optical Purity} = \left[\frac{(\alpha)}{\alpha_{max}}\right]100$$

where (α) has the meaning previously defined.

2. *Enantiomeric Excess* (e.e) which is given by:

$$\text{Enantiomeric Excess} = \left[\frac{(R-S)}{(R+S)}\right]100$$

Where (R) and (S) represent the proportion of the respective isomers in the mixture.

3. *Chromatographic Purity* which is given by:

$$\text{Chromatographic Purity} = \left[\frac{R}{(R+S)}\right]100$$

All three terms are used by chemists generally to describe the enantiomer content of a mixture, although the enantiomeric excess may possibly be the criteria more commonly used.

To return to the subject of chiral chemistry, optical rotation was first discovered by Jean Baptiste Biot in 1815 and, from his observations, some of the foundations of stereochemistry were laid down in the work

of Pasteur in 1848. In practice, Pasteur found that optically inactive sodium ammonium tartrate contained two kinds of crystals, one type of crystal being the mirror image of the other. He separated the two kinds of crystals by a somewhat laborious process using a lens and a pair of tweezers. He found that the two crystal types rotated plane polarized light differently, one to the right (that is clockwise), *dextro* (*d*) and one to the left, that is anti-clockwise), *levo*, (*l*), respectively. The reason for the existence of the two types of tartaric acid is complex and requires some detailed discussion.

The bonds associated with the carbon atom are assumed to take the positions of the apexes of a regular tetrahedron with the carbon atom situated at the center. Consider a carbon atom joined to four different atoms or groups (*e.g.* lactic acid) and the structure being placed in front of a mirror as shown in figure 1.6.

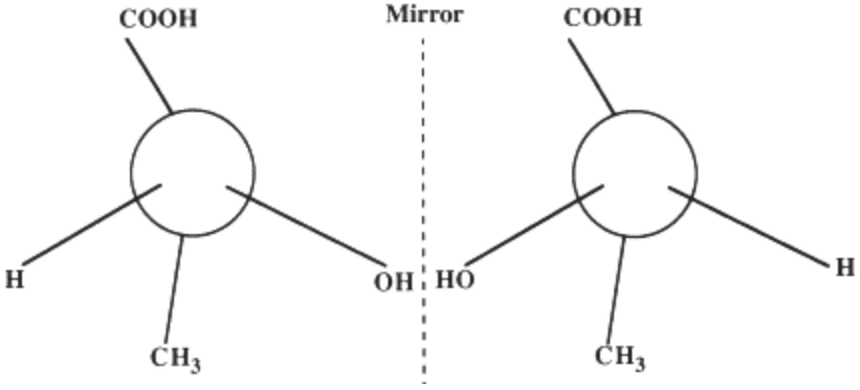


Figure 1.6
The Structure of Lactic Acid

It is seen that the molecules are not superimposable and are not interconvertable. It follows that they are different substances although they have the same chemical formulas. These types of isomers are optically active and these specific stereoisomers are called enantiomers. Other examples of enantiomers are 2-methyl-butanol and *sec*-butyl chloride shown in figure 1.7.

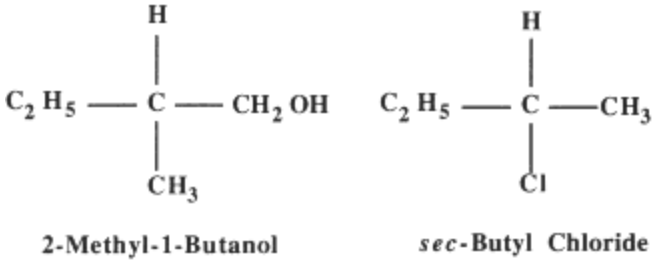


Figure 1.7
Structures of 2-Methyl-Butanol and *sec*-Butyl Chloride

The structures shown in figure 1.7 can be simplified to identify the chiral center, and to display the spatial orientation of the bonds from the chiral carbon, using the simple convention depicted in figure 1.8. By convention, the horizontal bonds attached to chiral atom are assumed to be coming out of the page, whereas the vertical bonds to the chiral atom are considered to be directed backward and behind the plain of the paper.

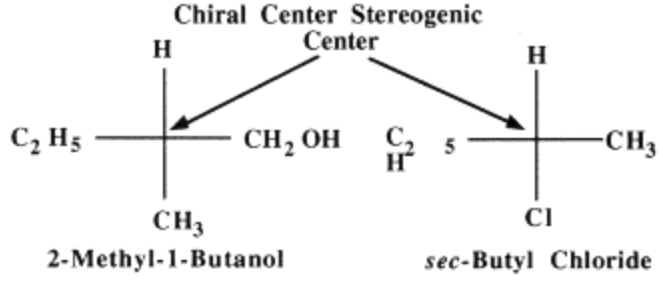
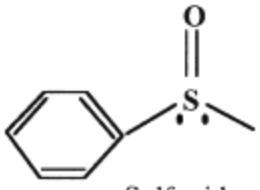


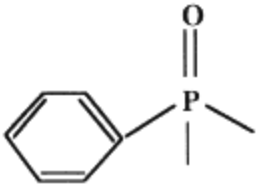
Figure 1.8
Structures of 2-Methyl-Butanol and *sec*-Butyl Chloride
Depicted in the Convention that Identifies the Chiral Center
and the Direction of the Bonds to the Chiral Carbon

Other elements can produce chiral centers besides carbon, although their importance in industry and in health studies is not as great. The sulfur atom can produce chiral centers for example the sulfoxides, sulfoximides, sulphonates and the sulfonium ion. An example of a chiral sulfur atom is given by the following sulfoxide structure.

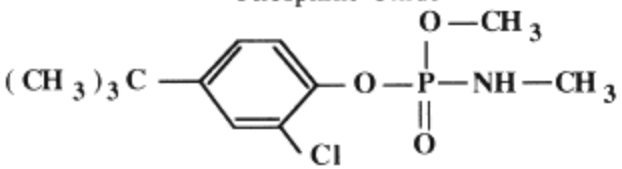


Sulfoxide

In addition, enantiomers based on the element phosphorous are also possible for example phosphine, phosphine oxide, phosphinates and the phosphonium ion.

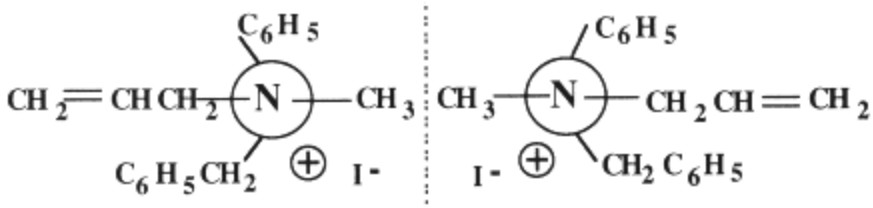


Phosphine Oxide



Crufomate (also called Ruelene or Montrel)

Similarly, there can be enantiomers based on nitrogen such as amine oxide and the ammonium ions.



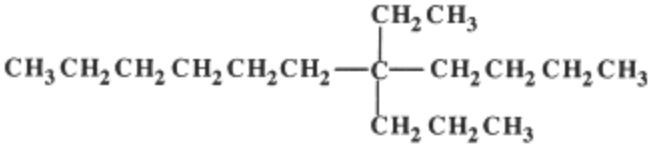
(+) and (-)-Methylallylphenylbenzylammonium Iodide

All enantiomers have identical boiling points, densities, refractive indices and other physical properties, except for their capacity to rotate the plane of polarized light. In addition, all enantiomers have identical chemical

properties *except* toward other enantiomers. Consequently, in a chiral environment, such as a biological system, specific reactions between enantiomers can be anticipated. For example, in some cases one enantiomer (*e.g.* the (+) isomer) will readily react with another enantiomer whereas the (-) isomer will not react at all. An example of this type of selectivity is afforded by the mold *penicillium glaucum*. Glucose (+) can be readily fermented and metabolized by *penicillium glaucum* yet (-) glucose is completely rejected. In a similar way (-) ephedrine is the active enantiomer of the drug (±) ephedrine but (-) ephedrine is not only inactive, its presence with the other isomer actually reduces the activity of the (+) isomer. A particularly striking example of the contrasting interactivity and physiological effect of different isomers are the enantiomers of carvone. Carvone (-) is the compound that gives the characteristic aroma to Spearmint oil, whereas (+) carvone, its enantiomer, is the essential characteristic constituent Caraway oil.

A mixture of the (+) and (-) enantiomers in equal proportions is called a racemic modification (racemate) and is optically *inactive*. The optical inactivity results from the rotation caused by one enantiomer canceling out that produced by its complementary enantiomer. The racemic modification is designated as (±) (*e.g.* (±) lactic acid). As the enantiomers of a substance have identical physical properties, they cannot be easily resolved employing the usual separation techniques such as fractional distillation. As a result, the isolation of optical isomers often pose difficult separation problems and it is usually necessary to resort to some very special techniques to achieve a satisfactory resolution: hence the *raison d'être* for this book.

The nature and structure of the substituent groups around the chiral atom determines the extent of rotation. Different groups of very similar types will make very small contributions to the overall rotation. For example, ethyl-*n*-propyl-*n*-butyl-*n*-hexyl methane, all containing hydrocarbon substituents, as shown below,



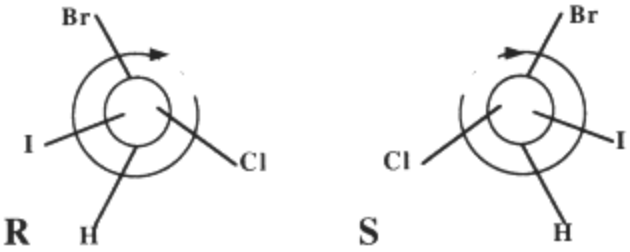
will have a calculated specific rotation of 0.00001° which is far lower than the present minimum level of measurement.

Configuration Specifications

In order to completely define the structure of an enantiomer using the information so far described, it is necessary to draw the structure of the molecule. This procedure can be clumsy and tedious and thus a system of symbols is necessary to provide the structural information in a concise and understandable manner. The use of the symbols (R) and (S) (*rectus* and *sinister*) has been proposed by three chemists, R. S. Cahn (the Royal Society of Chemistry), Sir Christopher Ingold (University College, London) and V. Prelog (Eidgenössiche Technische Hochschule, Zurich) to describe the structure of an enantiomer, and the format that they suggested has now been generally accepted. To define the structure two rational procedures must be carried out in two steps.

Step 1 Following a set of sequence rules (which will be given later) a sequence priority is assigned to the atoms or groups around the chiral atom. If there are only atoms attached to the chiral atom (*e.g.* CHClBrI) the priority will be in order of their atomic number (*i.e.* the priority sequence will be I, Br, Cl, H).

Step 2 The molecule is visualized so that the lowest priority atom or group points away from the viewer into the paper. Labeling each atom or group in order of their priority sequence, if the decrease in priority order is clockwise the substance is defined as (R) and if the decrease in priority sequence is anti clockwise it is defined as (S).

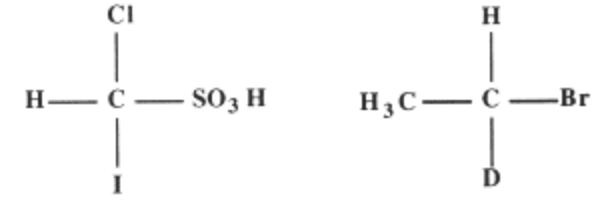


If both the conformation and the rotation are known then the compound can be specifically defined, *e.g.* (S)-(+)-*sec* butyl chloride. A racemic mixture of *sec*-butyl chloride would be designated as (R,S)-*sec*-butyl chloride. However, the designation of (R) and (S) should not be used in conjunction with (+) and (-) *unless it has been experimentally verified*.

In most cases, chemical *groups* are attached to the chiral atom, not single monovalent atoms and so it follows, that some sequence rules are necessary to determine the priority sequence. There are three rules that can be applied to identify the priority sequence.

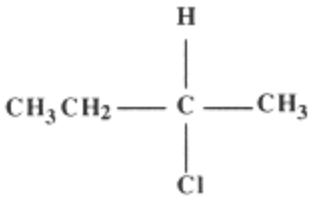
Rule 1. If all the atoms directly attached to the chiral atom are different, then the priority sequence is determined by the atomic number of each element.

e.g



Rule 2. If rule (1) can not produce a logical priority sequence then the next atom to that attached to the chiral carbon is used in a precisely similar manner.

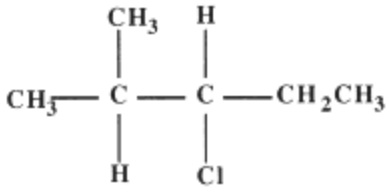
e.g



Priority Sequence
Cl, C_2H_5, CH_3, H

The priority sequence starts with the element with the highest atomic number, *i.e.* chlorine and the second is the ethyl group because the carbon has a carbon atom attached to it whereas the methyl has only hydrogen atoms attached to it. The remaining priorities follow the first rule, *i.e.* the methyl group and then hydrogen.

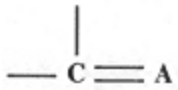
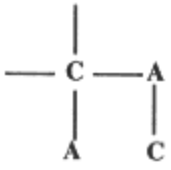
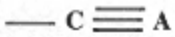
Another example illustrating the designation of the priority sequence is as follows,



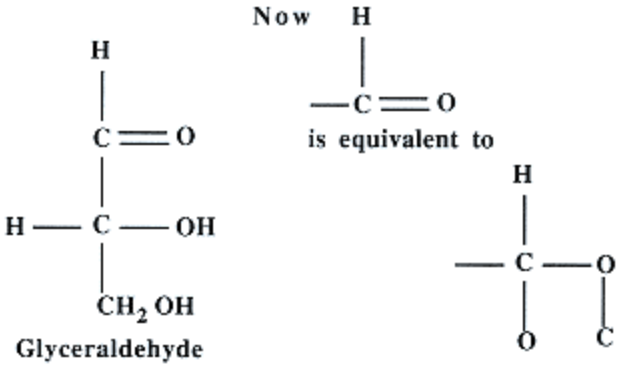
Priority Sequence
Cl, $(CH_3)_2$ CH, C_2H_5, H

Again, the chlorine atom, having the highest atomic number, has the highest priority followed by the isopropyl group which has *three* carbon atoms attached to the first carbon atom bonded to the chiral atom. The third priority is the ethyl group that has only *two* carbons attached to first carbon atom bonded to the chiral atom and the lowest priority position is obviously occupied by hydrogen.

Rule 3. Finally, it is necessary to consider the manner in which double bonds are dealt with in the priority sequence. If a double bond, or triple bond, is present, the specific atom(s) attached to the double bond are considered to be doubled or tripled respectively.

For example  **is considered equivalent to**  **and**  **is considered equivalent to** 

This concept can become quite complicated. Consider glyceraldehyde and the priority sequence of the groups around the chiral atom.

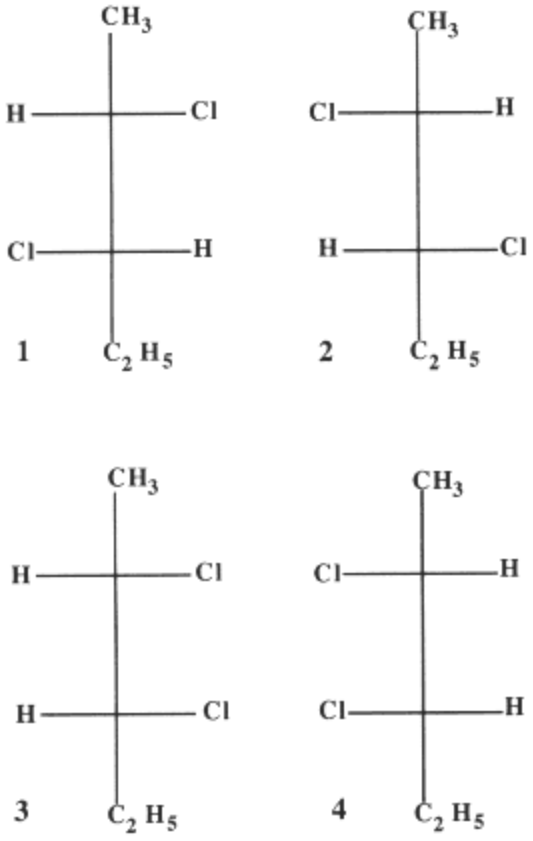


It can be seen that the O, O, H of the equivalent aldehyde group takes priority over the O, H, H, of the CH_2OH group Thus, the priority sequence of the groups around the chiral carbon is, OH, CHO, CH_2OH, H.

Diastereoisomers

If a stereoisomer contains more than one chiral center, it is said to be a diasteroisomer. Even when the diastereoisomer contains only two chiral centers the identification of the different structures can become very complex. Consider 2,3 dichloropentane that contains two chiral centers.

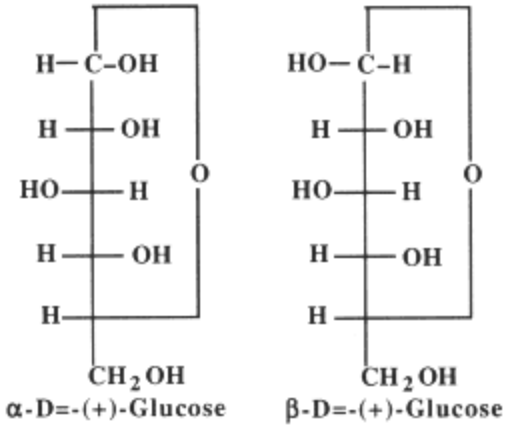
There are 4 apparent structures given below. Structure (1) is the mirror image of structure (2) but the two structures are not superimposable and thus structure (1) and (2) are enantiomers. Furthermore, structure (2) can not be converted to structure (1) by any form of rotation and thus both (1) and (2) are also optically active. In a similar way structures (3) and (4) are mirror images, not superimposable and not interconvertable by rotation, and thus they are enantiomers and optically active. However, structures (3) and (4) are not mirror images of structure (1) and thus they are not enantiomers, but are only diastereoisomers of structure (1). In a similar way structures (3) and (4) are not mirror images of structure (2) and thus these are also not enantiomers but are diastereoisomers of structure (2).



Diastereoisomers have different chemical properties and thus can often be resolved by conventional separation techniques. There are two other terms that need to be defined regarding diastereoisomers and they are *anomers* and *epimers*.

Anomers

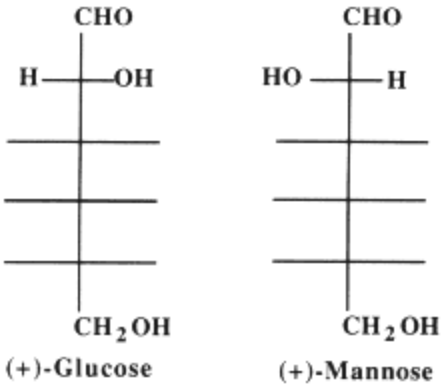
Consider the pair of aldoses, α-D-(+)-glucose and β-D-(+)-glucose depicted below. It is seen that the two diastereoisomers, α-D-(+)-glucose and β-D-(+)-glucose, only differ in the configuration of the (H) atom and the (OH) group about the *first* carbon atom, but have the same configuration about the rest of the carbon atoms.



A pair of diastereomeric aldoses that differ only in the configuration about the first chiral carbon are called *anomers*.

Epimers

Consider the structures of the aldoses (+)-glucose(+)-mannose shown below. It is seen, that the two diastereoisomers, (+)-glucose and (+)-mannose, only differ in the configuration of the (H) atom and the (OH) group about the *second* carbon atom but have the same configuration about the rest of the carbon atoms.



A pair of diastereomeric aldoses that differ only in the configuration about the second chiral carbon are called *epimers*. The more important nomenclature used in chiral chemistry is summarized in figure 1.9.

It should be emphasized that whereas all enantiomers are stereoisomers, not all stereoisomers are enantiomers

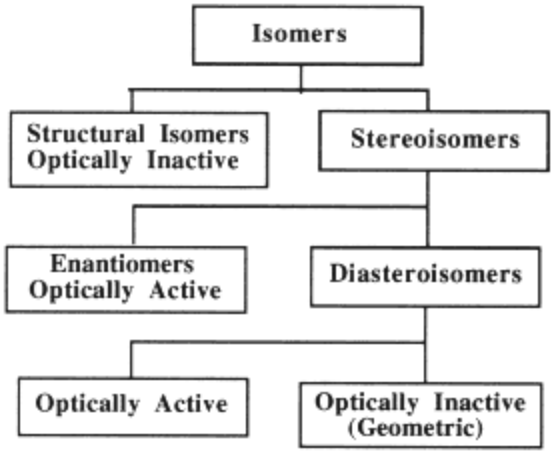


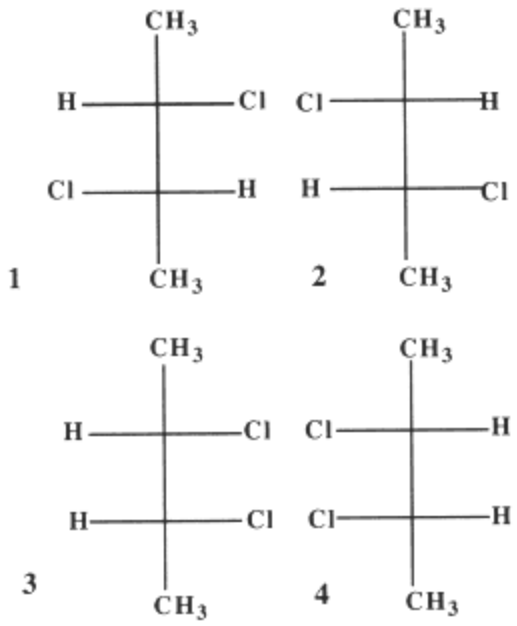
Figure 1.9
Nomenclature for Different Types of Isomers

In this book, chromatography and electrophoretic techniques are mainly applied to the separation of enantiomers, but the techniques are generally applicable to all types of isomers. In fact, the first complete resolution of

the *m* and *p* xylene was achieved by gas chromatography in 1958 using a packed column, 50 ft long.

Meso Structures

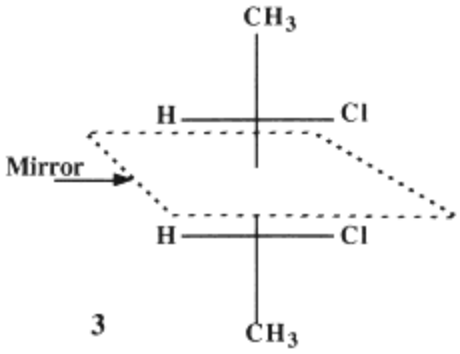
Consider the various possible structures of 2,3-dichlorobutane. It is seen that structures (1) and (2) are neither superimposable or interconvertable by rotation and thus, are enantiomers and both are optically active. Structures (3) and (4) are both superimposable and interconvertable and thus are neither enantiomers, or optically active, and are identical in every way. Although structures (3) and (4) are not chiral and cannot exist in enantiomeric forms, they contain, nevertheless *two chiral centers*. These types of stereoisomers are called *meso structures* or *compounds*. Defined somewhat differently, meso compounds are those where molecules are superimposable on their mirror images even though they contain chiral centers.



A meso compound is inactive because the rotation caused by one chiral center is exactly offset by the equal and opposite rotation produced by the other chiral center. It is often possible to recognize a meso structure

by the fact that one half of the molecule is the mirror image of the other half. This can be seen by redrawing structure (3) of 2,3-dichlorobutane. It is seen that the groups round one chiral carbon atom is the mirror image of the same groups round the second chiral carbon atom. This is clear in the diagram of 2,3-dichlorobutane, as constructed, the plane of the hypothetical mirror shows one chiral carbon to be the mirror image of the other.

As already stated, because enantiomers have exactly the same physical properties (with the exception of their capacity for rotating the plane of polarized light) they can be exceedingly difficult to separate. In fact, with the exception of chromatographic and electrophoretic techniques, the effective isolation of pure enantiomers can often only be achieved by exploiting the spatial differences between the respective isomers.



3

Separation Techniques for Chiral Chemistry

The separation problems associated with the synthesis of pure enantiomers can often be circumvented by forming the material from optically pure intermediates. This approach, however, only moves the problem of purification to a point higher in the synthetic chain. A more common solution is to use a biosynthetic procedure, and choose an organism (*e.g.* a yeast or bacteria) that will selectively cleave or chemically alter one enantiomer only.

There are also a limited number of mechanical separation procedures basically similar to that originally used by Pasteur to separate the different tartaric acids. Such methods usually require the use of special crystallization techniques, or the use of chiral additives such as cyclodextrin. These types of crystallization procedures, however, can be somewhat tedious to operate and not usually applicable. Nevertheless, a limited number of processes have been successfully established, where a concentrated racemic solution is carefully maintained at a critical temperature and then seeded with one isomer only. Under these conditions the crystals produced are almost entirely of that of a single isomer only. However, these are rather special cases and the field of application for mechanical separation methods, in general is rather limited.

The most successful method for separating enantiomers is to exploit the differential interactions that can take place as a result of their unique spatial orientation with those of a third enantiomer, which is introduced into the system to induct specific selectivity. The use of the special spatial arrangement of an enantiomer, to specifically interact with an enantiomeric reagent, and thus cause it to be selectively retained in a chromatographic or electrophoretic system, is currently the most direct and technically viable way to achieve the resolution of a wide variety of optical isomers. The extremely high efficiencies available from modern chromatographic and electrophoretic apparatus makes this approach by far the most effective for analytical purposes. The precise mechanism responsible for the selective retention of a specific enantiomer is very complex, but, nevertheless, can be explained on a rational basis and will be the subject of discussion in subsequent chapters.

For reasons given earlier, the separation and identification of stereoisomers in drugs, food additives, and food can be very important analytical problems and chromatography has been shown to be very effective in the analysis of such products. The use of gas chromatography for the separation of stereoisomers is not nearly so common as liquid chromatography, but nevertheless there are a number of very effective

optically active stationary phases that can be used in GC for the separation of volatile enantiomers. More recently the use of electrophoretic techniques has also been very successful for separating mixtures of enantiomers and examples of the use of the technique will be described in the later chapters of the book.

Synopsis

The optical activity of certain substances was first noted by Biot in the early 1800s and the existence of optical isomers was established by Pasteur in 1848. The concept of the asymmetric carbon atom helped van't Hoff and Le Bel to explain the existence of optical isomers and Emil Fisher in the late 1880s determined the configuration of (+)-glucose, for which he was awarded the Nobel prize in chemistry. Later, Bijovet confirmed the work of Fisher by X-ray crystallography. Work in the field of stereoisomers continued relatively slowly until about 1980 when the selective physiological activity of the different optical isomers of drugs became recognized. The additional impact of the Thalidomide disaster, forced work on stereoisomers to become a high priority. The United States Drug Administration mandated the testing of the optical isomers of all drugs that could exist in enantiomeric forms. This evoked a demand for analytical techniques, which were sadly lacking, despite earlier work on chiral stationary phases and reagents by Gil-Av, Harada *et al*. and others. The most common contemporary chiral agent for the separation of enantiomers is, without doubt, the cyclodextrins. Optical activity is measured as the capacity of the substance to rotate the plane of polarized light. Measurements are made by polarizing the light emitted from a source in an analyzer, and then passing it through the sample contained in a cell. The rotated light is allowed to pass through a second analyzer, which is turned until the plane of the light is the same as the incident on the sample cell. The angle the analyzer is rotated is taken as the rotation of the sample. This method is now rarely used. The modern method of measuring optical activity is by circular dichroism determinations. The circular dichroism is taken as the differential

absorption of left and right circularly polarized light and is usually reported as the differential molar extinction coefficient.

The enantiomeric content of a mixture is reported as either the *optical purity*, the *enantiomeric excess* or the *chromatographic purity.* Each determination measures the specific content of one enantiomer relative to that of the other. The type of stereoisomer can be identified by placing the structure in front of a mirror. If the molecules of the original and the image are not superimposable, then the specific stereoisomers are called *enantiomers*. If, in addition, the structures are not interconvertable (*i.e.* the image cannot be made identical to the original structure by rotation in the plane of the paper) then these types of isomers are also *optically active*. All enantiomers have identical physical properties (with the exception of their capacity for rotating the plane of polarized light) but may exhibit differing chemical properties in diverse chiral environments. One optical isomer may readily react with another enantiomer, whereas the other will not. An equal mixture of both optical isomers will be optically inactive and is called a *racemic* mixture. The type of atoms or groups around the chiral atom determines the extent of rotation. Similar hydrocarbon chains contribute very small increments to the overall rotation. The structure of the chiral substance can be defined as (S) and (R) but to determine which demands some rather complicated rules. Firstly, a sequence priority is assigned to the atoms or groups around the chiral atom. The molecule is then visualized so that the lowest priority atom or group points away from the viewer into the paper. If the decrease in priority order is clockwise the substance is defined as (R) and if the decrease in priority sequence is anti-clockwise it is defined as (S). The priority sequence is determined by a somewhat complex procedure established by the nature of the atoms attached to the chiral atom, if necessary, the nature of the atoms attached to them, and the presence of double or triple bonds. If a stereoisomer contains more than one chiral center it is said to be a *diasteroisomer*. Certain diastereoisomers have been given different terms, for example, in the case of aldoses if the diastereoisomers differ in the orientation of the atoms around the first carbon atoms then the stereoisomers are called *anomers*, if they differ in

the orientation of the atoms around the second carbon atom, they are called *epimers*. Diastereoisomers can assume a structure where the optical rotation of one chiral center exactly cancels that of the other; such structures are called *Meso Structures* and the substances are called *Meso Compounds*. Due to enantiomers having exactly the same physical properties, they are extremely difficult to separate by conventional methods such as distillation. Pure enantiomers are usually prepared by exploiting their unique spatial properties, where one isomer will react with another enantiomer, whereas the other will not. The most effective separation techniques that can used for the analysis of enantiomers are chromatography and electrophoresis. However, separation can only be achieved by using chiral agents in the separation process. Some of the most effective chiral agents that can be used for this purpose are the cyclodextrins, amino acids and their derivatives, proteins, linear and branched carbohydrates and the macrocyclic glycopeptides.

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Chapter 2—
The Mechanism of Solute Retention

Enantiomers of a single substance are essentially chemically identical and only differ in the spatial arrangement of chemical groups or atoms around a single atom, referred to as the stereogenic center or the chiral center(s). Such differences impart mostly very subtle physical chemical variations between the individual isomers and thus must be carefully exploited if a chromatographic separation of the individual enantiomers is to be achieved.

The parameters that will determine how well a pair of chemically similar substances are separated in any chromatographic system will be the relative retention of the two substances. The greater the retention difference between any pair of solutes, the better will be the resolution, and the farther apart they will appear in the chromatogram. Consequently, an algebraic expression for the retention volume of a solute will show how retention is controlled and thus how the separation can be achieved or improved. The chromatogram, which depicts the elution of the solutes from a column, is actually a graph relating the concentration of the solute in the mobile phase leaving the column to elapsed time. However, as the flow rate is constant, the actual relationship is a curve relating the concentration of solute in the exiting mobile phase to the *volume of mobile phase* passed through the column. It follows that an equation is required that will relate the concentration of the solute in the mobile phase leaving the column to the volume of mobile phase that has passed through it. In figure 2.1, a simple chromatogram shows the elution of a single peak. The expression f(v) will be the elution curve equation which will be derived using the plate theory.

Once the elution-curve equation is derived, and the nature of *f(v)* identified, then by differentiating *f(v)* and equating to zero, the position of the peak maximum can be determined and an expression for the retention volume (V_r) obtained. The expression for (V_r) will disclose those factors that control the magnitude of solute retention.

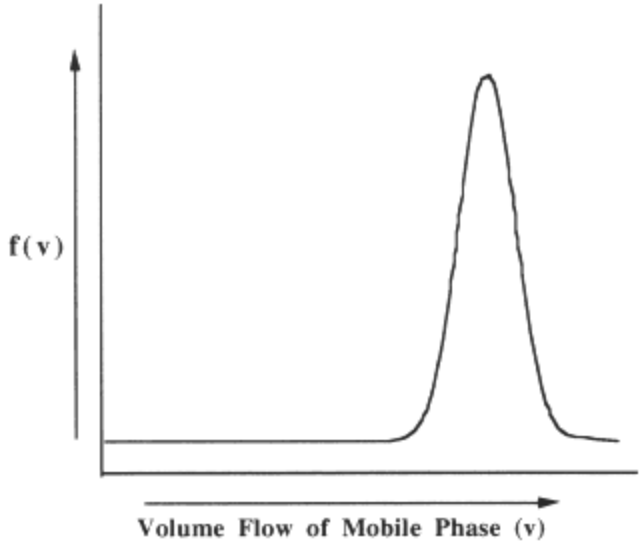

Figure 2.1
The Elution Curve of a Single Peak

The Plate Theory

The plate theory assumes that the solute is, at all times, in equilibrium with the mobile and stationary phase. Due to the continuous exchange of solute between the mobile and stationary phase as the solute progresses down the column, equilibrium between the phases, in fact, is *never* actually achieved. To accommodate this non-equilibrium condition, a technique originally used in distillation theory is adopted, where the column is considered to be divided into a number of cells or plates. Each cell is allotted a finite length, and thus, the solute spends a finite time in each cell. The size of the cell is such that the solute is considered to have sufficient residence time to achieve equilibrium with the two phases. Thus, the smaller the plate, the more efficient the solute exchange

between the two phases in the column and consequently the more plates there are in a given column. Said [1] developed the Martin plate concept [2], to derive the elution curve equation in the following way. Consider the equilibrium that is assumed to exist in each plate,

then:

$$X_S = KX_m \qquad (1)$$

Where, (X_m) and (X_s) are the concentrations of the solute in the mobile and stationary phases, respectively, and (K) is the distribution coefficient of the solute between the two phases.

It should be noted that (K) is defined with reference to the stationary phase; *i.e.* $K = X_s/X_{m,}$ thus the larger the distribution coefficient, the more the solute is distributed in the stationary phase. (K) is a dimensionless constant and thus in gas/liquid and liquid/liquid systems, (X_s) and (X_m) are conveniently measured as *mass of solute per unit volume of phase.* In gas/solid and liquid/solid systems an alternative method of measurement could be, *mass of solute per unit mass of phase.*

Equation (1) merely states that the general distribution law applies to the system and that the adsorption isotherm is linear. In fact, in both GC and LC, virtually all the solutes exhibit Langmuir type isotherms between the two phases which, over a wide concentration range, will certainly not be linear. However, at the extremely low solute concentrations employed in most chromatographic separations, that portion of the isotherm that is pertinent, and over which the chromatographic process is operating, will be very close to linear.

Differentiating equation (1):

$$dX_S = KdX_m \qquad (2)$$

Consider three consecutive plates in a column, the (p-1), the (p) and the (p+1) plates and let there be a total of (n) plates in the column. The three plate are depicted in figure 2.2. Let the volumes of mobile phase and stationary phase in each plate be (v_m) and (v_s), respectively, and the concentrations of solute in the mobile and stationary phase in each plate

be $X_m(p-1)$, $X_s(p-1)$, $X_m(p)$, $X_s(p)$, $X_m(p+1)$, and $X_s(p+1)$, respectively. Let a volume of mobile phase, dV, pass from plate (p-1) into plate (p), at the same time displacing the same volume of mobile phase from plate (p) to plate (p+1).

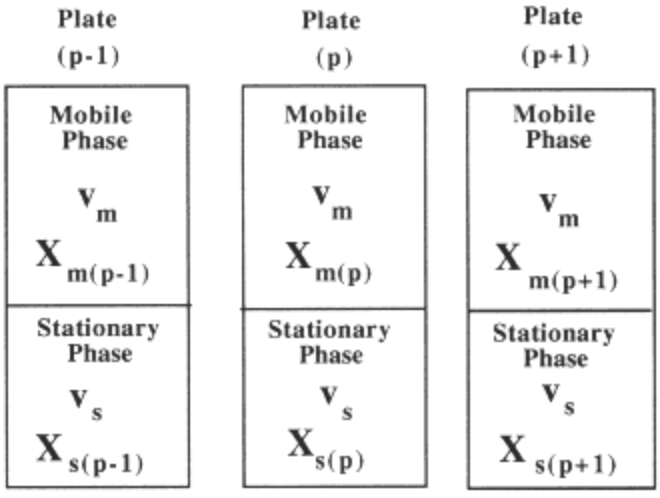

Figure 2.2
Three Consecutive Theoretical Plates in a Column

In doing so, there will be a change of mass (dm) of solute in plate (p) that will be equal to the difference in the mass entering plate (p), from plate (p-1), and the mass of solute leaving plate (p), and entering plate (p+1). Thus bearing in mind that mass is the product of concentration and volume, the change of mass of solute in plate (p) will be:

$$dm = (X_{m(p-1)} - X_{m(p)})dV) \qquad (3)$$

Now, if equilibrium is to be maintained in the plate (p), the mass (dm) will distribute itself between the two phases, which will result in a change of solute concentration in the mobile phase of $dX_m(p)$, and in the stationary phase of $dX_s(p)$.

Then:

$$dm = v_S\, dX_{S(p)} + v_m\, dX_{m(p)} \qquad (4)$$

Substituting for $dX_s(p)$ from equation (2):

$$dm = (v_m + Kv_S)\, dX_{m(p)} \qquad (5)$$

Equating equations (3) and (5) and rearranging:

$$\frac{dX_{m(p)}}{dV} = \frac{X_{m(p-1)} - X_{(p)}}{v_m + Kv_s} \qquad (6)$$

Now, it is mathematically convenient to effect a change of variable. The volume flow of mobile phase will now be measured in units of $(v_m + Kv_s)$, instead of milliliters. Thus the new variable (v) can be defined where:

$$v = \frac{V}{(v_m + Kv_s)} \qquad (7)$$

The function $(v_m + Kv_s)$, is termed the 'plate volume' and thus, for the present, the flow of mobile phase through the column will be measured in 'plate volumes' instead of milliliters. The 'plate volume' can be defined as that volume of mobile phase that would contain all the solute that is in the plate at the equilibrium concentration of the solute in the mobile phase. It is an important concept and is extensively used in different aspects of chromatography theory.

Differentiating equation (7):

$$dv = \frac{dV}{(v_m + Kv_s)} \qquad (8)$$

Substituting for dV, from (8) in (6):

$$\frac{dX_{m(p)}}{dv} = X_{m(p-1)} - X_{(p)} \qquad (9)$$

Equation (9) is the basic differential equation that describes the rate of change of concentration of solute in the mobile phase in plate (p) with the

volume flow of mobile phase through it. Thus, the integration of equation (9) will provide the equation for the elution curve of a solute eluted from any plate in the column. A simple, algebraic solution to equation (9) is given in Appendix 1, and the resulting elution curve equation for plate (p) is shown to be:

$$X_{m(p)} = \frac{X_o e^{-v} v^p}{p!}$$

Where ($X_{m(p)}$) is the concentration of the solute in the mobile phase leaving the (p)th plate

and (X_o) is the initial concentration of solute placed on the 1st plate of the column

Thus, the equation for the elution curve from the (n) th plate, which is the last plate in the column (that is, the equation relating the concentration of solute in the mobile phase entering the detector to volume of mobile phase passed through the column), is given by:-

$$X_{m(n)} = \frac{X_o e^{-v} v^n}{n!} \qquad (10)$$

Equation (10) is the elution curve equation from which much information concerning the chromatographic process can be educed. Equation (10) is a Poisson function, but can be shown that, if (n) is large, the function approximates very closely to a normal Error function or Gaussian function. In practical chromatography systems, (n) is always greater than 100, and thus, in linear chromatography, (the adsorption isotherm range is linear) all chromatographic peaks will be Gaussian or nearly Gaussian in shape.

The Retention Volume of a Solute

The retention volume of a solute is that volume of mobile phase that passes through the column between the injection point and the peak

maximum. It is therefore, possible to determine that volume by differentiating equation (10) and equating to zero and solving for (v)'

Restating equation (10):

$$X_{m(n)} = X_o \frac{e^{-v} v^n}{n!}$$

$$\frac{dX_{m(n)}}{dv} = X_o \frac{-e^{-v} v^n + e^{-v} n v^{(n-1)}}{n!}$$

$$= X_o \frac{-e^{-v} v^{(n-1)}}{n!} (n - v)$$

Equating to zero and solving for (v): n - v = 0, or v = n

At the peak maximum, (n) plate volumes of mobile phase have passed through the column. Now, remembering that the volume flow is measured in 'plate volumes' and not ml, the volume passed through the column in (ml) will be obtained by multiplying by the 'plate volume' ($v_m + Kv_s$). It follows that, the retention volume (V_r) is given by:

$$V_r = n(v_m + Kv_S)$$

$$= nv_m + nKV_S$$

Now, the total volume of mobile phase in the column, (V_m), will be the volume of mobile phase per plate multiplied by the number of plates *i.e.* (nv_m). In a similar manner, the total volume of stationary phase in the column, (V_S) will be the volume of stationary phase per plate multiplied by the total number of plates, i.e. $(nv)_s$,

Thus:

$$V_r = V_m + KV_S \qquad (11)$$

In practice, for an unretained peak eluted at the dead volume,(V_o):

$$V_o = V_{r(o)} + V_E$$

$$= V_m + V_E \qquad (12)$$

where, (V_E), is the extra column volume contained in the injection system, connecting tubes and detector cell. In many cases, (V_E), may be sufficiently small to be ignored, but for accurate measurements of retention volume the actual volume measured should always be corrected for the extra column volume of the system and equation (11) should be put in the form,

$$V_r = V_m + KV_S + V_E \qquad (13)$$

Returning to equation (13), it is now possible to derive and equation for the adjusted retention volume, (V'_r):

$$V'_r = V_r - V_o$$

Thus, from equations (12) and (13):

$$V'_r = V_m + KV_S + V_E - (V_m + V_E)$$

and:

$$V'_r = KV_S \qquad (14)$$

To avoid any confusion, it should be reiterated that although the stationary phase is assumed to have a volume and thus implies the stationary phase is liquid, by replacing the *volume* of stationary phase with *mass* of stationary phase, then the equations can be made appropriate for liquid/solid systems. However, as already stated, the units of concentration must also be redefined in the measurement of (K).

Consider the retention volume of two solutes (A) and (B):

$$V_{r(A)} = V_m + K_{(A)}V_S + V_E \qquad (15)$$

and:

$$V_{r(B)} = V_m + K_{(B)}V_S + V_E \qquad (16)$$

Furthermore, the corrected retention volumes will be:

$$V'_{r(A)} = K_{(A)}V_S \qquad (17)$$

and:

$$V'_{r(B)} = K_{(B)}V_S \qquad (18)$$

It follows that for the two solutes (A) and (B) to be separated:

$$K_{(A)}VS <> K_{(B)}VS \text{ and } K_{(A)}VS \neq K_{(B)}VS$$

Consequently either:

$$V_{S(A)} <> V_{S(B)}$$

or:

$$K_{(A)} <> K_{(B)}$$

It is clear that the magnitude of (K) and (V_s) determines the extent of separation. Thus, to achieve the separation of a mixture of solutes either, (1) the distribution coefficient (K) of all the solutes must be made to differ, or (2), the amount of stationary phase (V_s), available to each component of the mixture, must be made to differ, or (3), (which is the method frequently used to ensure the separation of enantiomers) is to make appropriate adjustments to both the values of (K) and (chirally selective) the values (V_s).

It follows, that having identified the primary factors that govern retention (*i.e.* the size of (K) and (V_s)), it is now necessary to recognize those parameters that control the magnitude of the distribution coefficient itself, and the volume of available stationary phase in a column. However, before discussing distribution coefficient control, two other retention parameters should be described, the capacity ratio of a peak and the separation ratio of a peak pair.

The Capacity Ratio of a Solute

The capacity ratio of a solute (k') was introduced early in the development of chromatography and was used as an alternative means of identifying a solute as the measurement eliminated the effect of flow rate. The capacity ratio was defined as the ratio of the distribution coefficient of the solute to the phase ratio (a) of the column. In turn, the phase ratio of the column was defined as the ratio of the volume of mobile phase in the column to the volume of stationary phase in the column.

i.e:

$$a = \frac{v_m}{v_s} = \frac{V_m}{V_s}$$

Thus:

$$k' = \frac{KV_s}{V_m}$$

and:

$$k' = \frac{V'_r}{V_m}$$

Note that (V_m) is the volume of mobile phase in the column and not V_o the total dead volume of the column.

Consequently, in practice:

$$k' = \frac{V'_r}{V_o - V_E} \qquad (19)$$

Both (V_m) and (V_S) will vary between different columns and may vary between different solutes, due to the differing exclusion properties of solid stationary phases and supports. Thus, some caution must be shown in comparing (k') values for the same solute from different columns and for different solutes on the same column.

In calculating (k'), the value taken in practice is often the ratio of the corrected retention distance (time), (*i.e.* the distance in centimeters on the chart, between the dead point and the peak maximum, or the time as measured by a computer) to the dead volume distance (time), (*i.e.* the distance in centimeters on the chart, between the injection point to the dead point on the chromatogram, or the equivalent time interval as measured by the computer). This calculation assumes the extra column dead volume is not significant which unfortunately, in many cases will <u>not</u> be true. It follows that (k') values calculated in this way will be in error and, unless corrected for extra column volume, should not be used for solute identification.

The Separation Ratio

As previously discussed, the capacity ratio of a solute, (k'), was introduced to develop a chromatographic measurement, simple to calculate, independent of flow-rate and one that could be used in solute identification. Although helpful, the capacity ratio is so dependent on the accurate measurement of extra column volume and on very limited solute

exclusion by the support and stationary phase, that it is less than ideal for solute identification. An alternative measurement, called the separation ratio (α). For two solutes (A) and (B), the separation ratio was defined as:

$$\alpha_{A/B} = \frac{V'_{r(A)}}{V'_{r(B)}} = \frac{K_{(A)}V_s}{K_{(B)}V_s} = \frac{K_{(A)}}{K_{(B)}} \qquad (20)$$

It is seen that the separation ratio is independent of all column parameters and depends only on the nature of the two phases and the temperature. Thus, comparing data from two different columns, providing the same phase system is used in each, and the columns operated at the same temperature, then any two solutes will have the same separation ratio on both columns. The separation ratio will be *independent* of the *phase ratios* of the two columns and the *flow rates.* It follows, that the separation ratio of a solute can be used more reliably as a means of solute identification.

A standard substance is often added to a mixture and the separation ratio of the substance of interest to the standard is used for identification. In practice the separation ratio is taken as the ratio of the distances in centimeters between the dead point and the maximum of each peak or, if computer data processing is employed, distances are replaced by the corresponding times.

The Thermodynamic Properties of the Distribution Coefficient

There are two ways of describing solute distribution between two phases, (1) by thermodynamical argument and (2) by molecular interactions. The thermodynamic argument is particularly important with respect to the separation of enantiomers, as the primary separation function in all chiral separations is largely entropic in nature.

Classical thermodynamics provides an expression that relates the change in *free energy* of a solute when transferring from one phase to the other as a function of the equilibrium constant which, in the case of

chromatographic retention, will be the distribution coefficient (K). The expression is as follows:

$$RT \ln K = -\Delta G_O$$

where (R) is the gas constant,

(T) is the absolute temperature,

and (ΔG_o) is the Standard Free Energy Change.

Now:

$$\Delta G_o = \Delta H_o - T\Delta S_o$$

where (ΔH_o) is the Standard Enthalpy Change,

and (ΔS_o) is the Standard Entropy Change.

Thus:

$$\ln K = -\left(\frac{\Delta H_0}{RT} - \frac{\Delta S_o}{R}\right) \qquad (21)$$

or,

$$K = e^{-\left[\frac{\Delta H_0}{RT} - \frac{\Delta S_0}{R}\right]}$$

It is seen that if the *standard entropy change* and *standard enthalpy change* for the distribution of any given solute between two phases can be calculated, then the distribution coefficient (K) and, consequently, its retention volume can also be predicted. Unfortunately, these properties of a distribution system are *bulk* properties, that include, in a single measurement, the effect of all the different types of molecular interactions that are taking place between the solute and the two phases. As a result it is often difficult to isolate the individual interactive contributions in order to estimate the magnitude of the overall distribution coefficient, or identify how it can be controlled. Nevertheless, there are a number of ways in which this can be done and, in any event, the thermodynamic approach can provide valuable information with regard to the nature of the distribution.

Rearranging equation (1):

$$\ln K = -\frac{\Delta H_o}{RT} + \frac{\Delta S_o}{R}$$

Bearing in mind:

$$V' = KV_S$$

$$\ln V' = -\frac{\Delta H_o}{RT} + \frac{\Delta S_o}{R} + \ln V_s \qquad (22)$$

It is seen that a curve relating In(V') to 1/T should give a straight line, the slope of which will be proportional to the standard *enthalpy* change during solute transfer. In a similar way, the intercept will be related to the standard *entropy* change and, thus, the dominant thermodynamic contribution to any specific distribution system can be identified from such curves. Such curves are called van't Hoff curves and an example of two van't Hoff curves relating log(V') against 1/T for two different types of distribution systems are shown in figure 2.3.

However, before discussing the contribution of enthalpy and entropy to the distribution coefficient and, thus, retention, the sign convention for the standard entropy, (ΔS) and the standard enthalpy, (ΔH) needs to be defined. When the solute molecule is held by molecular forces in the stationary phase its entropy has been reduced as, in the mobile phase, where the molecular interactions were weaker, it has a greater freedom of movement and could behave in a more random manner. It follows that as the entropy of the solute is reduced, the standard entropy change (ΔS) must be *negative*. As the molecule is held on the stationary phase by intermolecular forces, energy has been used during the interaction and thus the standard enthalpy must also be *negative*. If (ΔH) is negative then the first term in equation (22) will be positive in practical systems. Likewise, if (ΔS) is also negative then the second term in equation (22) will be found to be negative in practical systems. This means that the enthalpy terms and entropy terms oppose one another, the enthalpy term increasing retention and the entropy term reducing retention.

In figure 2.3, it is seen that distribution system (A) has a large enthalpy value $\left[\frac{\Delta H_o}{RT}\right]_A$ and a low entropy contribution $\left[-\frac{\Delta S_o}{R} + V_s\right]_A$. The

large value of $\left[\frac{\Delta H_o}{RT}\right]_A$ means that the distribution is *predominantly controlled* by *molecular forces.*

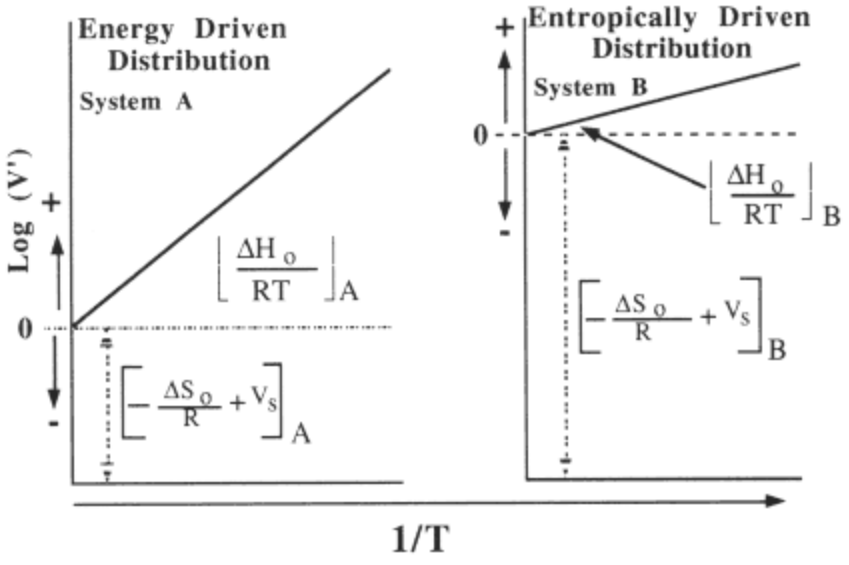

Figure 2.3
The van't Hoff Curves for Two Different Distributions Systems

The solute is preferentially distributed in the stationary phase as a result of the interactions of the solute molecules with those of the stationary phase being much greater than the interactive forces between the solute molecules and those of the mobile phase. Because the change in enthalpy is the major contribution to the change in free energy,

the distribution, in thermodynamic terms, is said to be "energy driven".

In contrast, it is seen that for distribution system (B) there is only a small enthalpy change $\left[\frac{\Delta H_o}{RT}\right]_B$, but in this case a high entropy contribution

$\left[-\frac{\Delta S_o}{R}+V_s\right]_B$. This means that the distribution is *not* predominantly controlled by molecular forces. The entropy change reflects the loss of randomness or freedom that a solute molecule possessed when transferring from one phase to the other. The more random and the greater freedom the solute molecule has, to move in a particular phase, the greater its entropy in that phase. In system (B), the large entropy change indicates that the solute molecules are more restricted, or less random, in the stationary phase than they were in the mobile phase. Because the standard entropy is negative, this loss of freedom is responsible for a *reduced* distribution of the solute in the stationary phase and, thus, diminished solute retention. Inasmuch as the change in entropy in system (B) is the major contribution to the change in free energy,

the distribution, in thermodynamic terms, is said to be "entropically driven".

Chiral separations, or separations dominated by size exclusion are examples of entropically driven systems. For example, one enantiomer may fit more closely to the stationary phase surface, or interact more proximately with the stationary phase molecules, and thus its freedom will be more restricted, and its movements less random. However, chromatographic separations need not be predominently "energetically driven" or "entropically driven"; in fact, very few are. In most cases, retention has both "energetic" and "entropic" components which, by careful adjustment, can be made to achieve very difficult and subtle separations. For example, if, as a result of its unique configuration, one enantiomer can interact more close with the surface, and in doing so come closer to an energetically interacting group, both the enthalpy and entropy of the distribution will be changed. As a consequence, the separation of one isomer from its corresponding enantiomer will be achieved by both energetic and entropic contributions to the standard free energy of distribution. The energetic contribution would, however, be a direct result of the *primary entropic* difference between the two enantiomers.

It is interesting to note that in the majority of distribution systems met in gas chromatography, the slope of the van't Hoff curves are positive and the intercept negative. The negative value of the intercept means that the standard entropy change of the solute has resulted from the production of a less random and more orderly system during the process of distribution. More important, this entropy change *reduces* the magnitude of the distribution coefficient. This means that the greater the forces between the molecules, the greater the energy (enthalpy) contribution, the larger the distribution coefficient and the greater the retention. In contrast, any reduction in the random nature of the molecules or an increased amount of order in the system, reduces the distribution coefficient and ***attenuates*** the retention. Thus, in the majority of distribution systems met in gas chromatography, the enthalpy and entropy changes oppose one another in their effect on solute retention. In fact, there is considerable parallelism shown between the standard entropy and standard enthalpy of a series of solutes for a given distribution system.

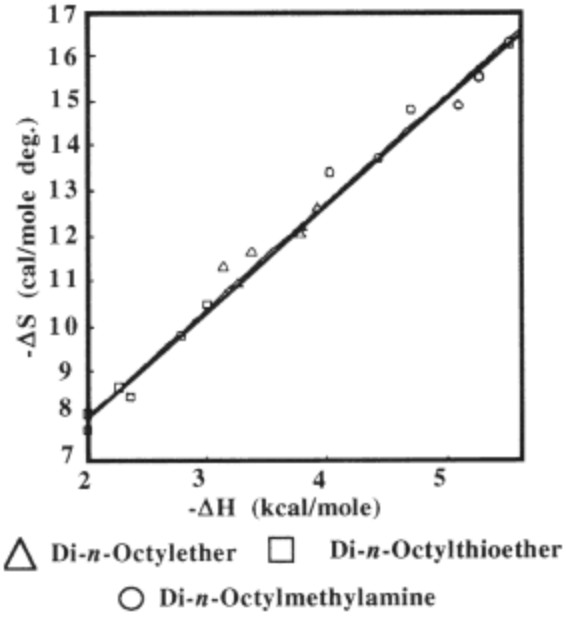

Figure 2.4
Graph of Standard Free Entropy against Standard Free Enthalpy for and Ether, Thioether and Amine.

This relationship between entropy and enthalpy has been reported many times in the literature. An example of a graph relating (ΔH) to (ΔS), produced by Martire and his group [3–7] is shown in figure 2.4. From a theoretical point of view this relationship is to be expected. Any increase in enthalpy indicates that more energy is used up in the association of the solute molecule with the molecules of the stationary phase. This means that the intermolecular forces are stronger and thus the stationary phase molecules hold the solute molecules more tightly. In turn, this implies that the freedom of movement, and the random nature of the solute molecule, are also more restricted which results in a larger change in standard entropy. It follows that, unless other significant retentive factors are present, any increase in standard enthalpy is usually accompanied by a corresponding increase in standard entropy. It should also be noted that if a particular enantiomer suffers a reduction in freedom or randomness, relative to that of another in the distribution system then, providing the energy changes are the same for both isomers, the isomer that experiences the greater entropy change will be eluted *first.*

In figure 2.5, curves are shown relating Log(k') against 1/T for two pairs of enantiomers. The data has been curve fitted to a linear function and thus the enthalpy and entropy contributions are extracted as the slope and intercept of each curve.

Thus:

$$\mathrm{Log}(k'_1) = \frac{\Psi_1}{T} - \phi_1 \quad \text{and} \quad \mathrm{Log}(k'_2) = \frac{\Psi_2}{T} - \phi_2$$

where ($\psi 1$) and ($\psi 2$) are the slopes of the curves for enantiomer (1) and enantiomer (2),

and ($\phi 1$) and ($\phi 2$) are the intercepts of the curves for enantiomer (1) and enantiomer (2).

When $k'_1 = k'_2$ Then:

$$\frac{\Psi_1}{T} - \phi_1 = \frac{\Psi_2}{T} - \phi_2 \quad \text{or} \quad T = \frac{\Psi_1 - \Psi_2}{\phi_1 - \phi_2} \qquad (23)$$

It is seen that if the enthalpies and entropies differ for two enantiomer pairs, there will always be a temperature where they elute coincidentally and can not be separated. From the curves and intercepts given in figure

2.5, the temperature for coincident retention of the two phenyl ethanol enantiomers is 432°K or 159°C and for the methylpiperidine enantiomers is 433°K or 160°C which agrees excellently with the curves shown in figure 2.5

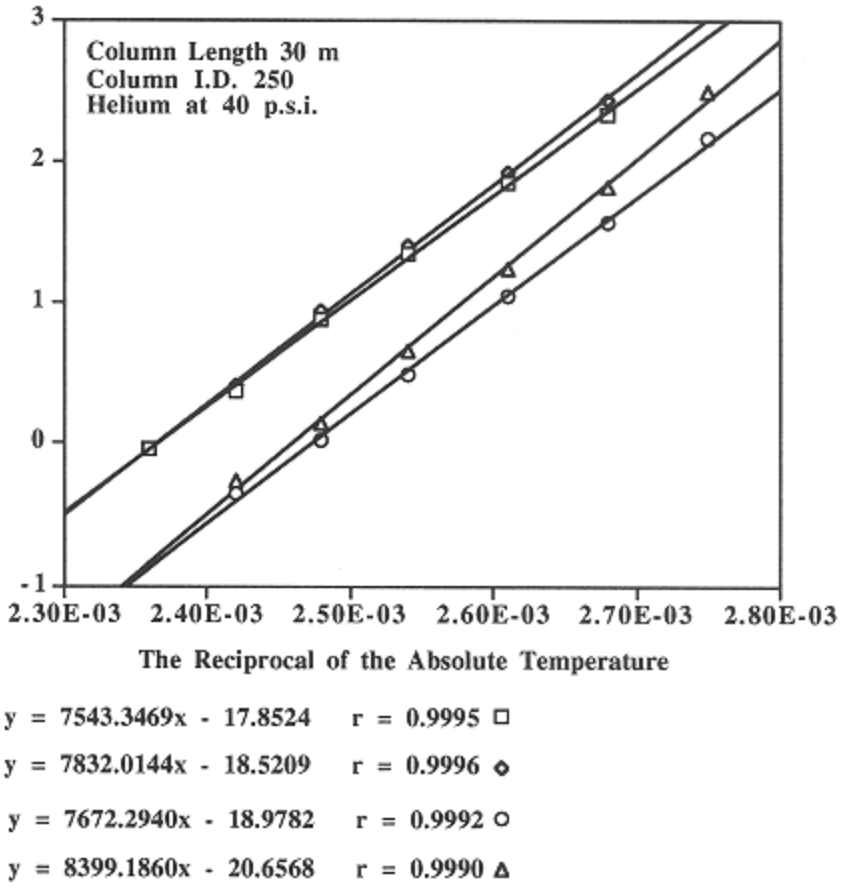

Figure 2.5
Curves Relating Log(k') against 1/T for Two Pairs of Enantiomers
Data Generated by Heng Liang Jin of ASTEC INC.

Now, assume that in order to separate a pair of enantiomers, a separation ratio of (α) is required. Assuming, $\psi 1 \neq \psi 2$ and $\phi 1 \neq \phi 2$, it

is now possible to calculate the temperatures at which a separation ratio of (α) can be realized.

Now:

$$\alpha = \frac{k'_1}{k'_2}$$

Thus

$$\ln(\alpha) = \ln(k'_1) - \ln(k'_2)$$

$$= \left(\frac{\Psi_1}{T} + \phi_1\right) - \left(\frac{\Psi_2}{T} + \phi_2\right)$$

$$= \frac{\Psi_1 - \Psi_2}{T} + (\phi_1 - \phi_2) \qquad (24)$$

Therefore:

$$T = \frac{\Psi_1 - \Psi_2}{\ln(\alpha) + (\phi_2 - \phi_1)}: \qquad (25)$$

Equation (25) allows the temperature to be calculated at which the separation ratio between the solutes would be (α).

From equation (24):

$$\alpha = e^{\frac{\Psi_1 - \Psi_2}{T} + (\phi_1 - \phi_2)} \qquad (26)$$

Thus, the separation ratio that will be obtained for the solute pair can be calculated for any temperature. This equation will be used in chapter 5 to calculate the minimum column length necessary to complete the separation in the minimum time.

The Availability of the Stationary Phase

The volume of stationary phase with which the solutes in a mixture can interact (V_s in equation (11) is not simply related to the *total* volume of stationary phase in the column. The stationary phase available to the solute will depend on whether the stationary phase is a porous solid, having pores of size commensurate with the molecular dimensions of the solutes, or whether the stationary phase is chiral and can offer greater

depth of interaction to one enantiomeric solute, than to the another. If the stationary phase is contained in, or on, a porous solid, for example, some solutes (by way of illustration those of smaller molecular size) can penetrate and interact with more stationary phase than larger molecules which are partially excluded. As a consequence, the retention will be partly controlled by size exclusion. If, however, the stationary phase has *chiral* components, then the amount of stationary phase with which any solute can interact will also depend on the chirality of the solute molecule and how closely it can fits to the chiral surface and can interact with neighboring dispersive or polar centers.

In size exclusion chromatography, retention is not *exclusively* controlled by the size of the solute molecule, it will still be controlled by the magnitude of the molecular interactions between the solute molecules and those of the two phases. *Only* if the magnitude of the forces between the solute and both phases is the *same* will retention depend solely on the pore size distribution of the stationary phase. The larger molecules, being partially or wholly excluded, will elute first and the smaller molecules elute last. It is also important to appreciate that, even if the dominant retention mechanism is controlled by molecular forces, if the stationary phase or supporting material has an appropriate porosity, *exclusion will still play a part in retention.* There are a number of different media available for exclusion chromatography, the two most common being silica gel and macroporous polystyrene divinylbenzene resins.

It should also be pointed out that in LC many chiral stationary phases are bonded to porous supports such as silica. Consequently, in a chiral separation, molecular forces, exclusion and chiral selectivity may all contribute to the retention of a particular enantiomer.

There are a number of ways of effecting the separation of a mixture of enantiomers and these will be discussed later in this book. In LC, many racemic mixtures can be separated using ordinary reverse phase columns by adding a suitable chiral reagent to the mobile phase. If the material is

adsorbed strongly on the stationary phase then selectivity will reside in the stationary phase, if the reagent is predominantly in the mobile phase then the chiral selectivity will remain in the mobile phase. Examples of some suitable additives are, camphor sulphonic acid (8) and quinine (9). Chiral selectivity can also be achieved by bonding chirally selective compounds to silica, in much the same way as a reverse phase. An example of the use of this type of stationary phase is afforded by the use of bonded cyclodextrins in the separation of some Warfarin isomers, an example of which is shown in figure 2.6. The column was 25 cm long and 4.6 mm in diameter packed with 5 micron CYCLOBOND 1. The mobile phase was approximately acetonitrile/methanol/glacial acetic acid/triethylamine:90/10/0.2/0.2 v/v/v/v. It is seen that an excellent separation has been achieved with the two isomers completely resolved.

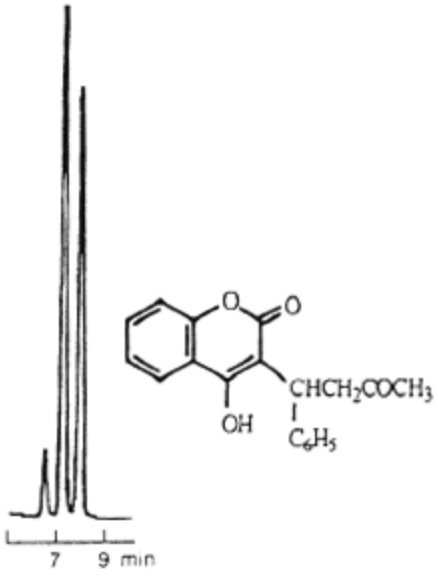

Figure 2.6
The Separation of Warfarin Isomers on a
CYCLOBOND Column
Courtesy of ASTEC Inc.

This separation is an impressive example of an entropically driven distribution system where the normally random movements of the solute

molecules are restricted to different extents, depending on the spatial orientation of the substituent groups. These retentive mechanisms will be discussed in detail in later examples.

Synopsis

In order to obtain an expression for the retention volume of a solute, the equation for the elution curve of a substance must be educed. The elution curve equation is derived from the plate theory, which premises that the column consists of a number of theoretical plates in which equilibrium between the solute and the two phases is assumed to occur in each plate. A mass balance is applied to a plate, and from this the differential equation for the change of concentration of the solute in the plate, with the flow of mobile phase through it, is obtained. The integration of this equation provides the elution curve equation. Differentiating this equation and equating to zero discloses an expression for the retention volume of a solute, which is shown to depend on the distribution coefficient of the solute between the two phases, and the volumes of stationary and mobile phase in the column. Having obtained an expression for the retention volume of a pair of solutes, then it is shown that their separation will depend on the relative magnitudes of their distribution coefficient with respect to the stationary phase, and the relative amount of stationary phase available to the two solutes. If the retention volume is corrected for the column dead volume, an expression for the capacity ratio and separation ratio can be derived, both of which can also be used for solute identification. By thermodynamic argument, the distribution coefficient can be separated into enthalpic and entropic components, both of which contribute to retention. An increase in the standard enthalpy of distribution contributes to an increase in the distribution coefficient, and a consequent increase in retention. An increase in the standard entropy of distribution usually contributes to a decrease in both the distribution coefficient and retention. The standard free enthalpy and standard free entropy correlate in the majority of GC separations. It can be shown that if the enthalpy and entropy of two solutes differ, then there is a particular temperature at which the two solutes coelute. It can also be shown that the separation ratio of the two

solutes increases as the temperature diverges from the temperature of coelution. There will be two temperatures at which any two solutes exhibit a specific separation ratio and these temperatures can be calculated from the standard enthalpies and standard entropies of the two solutes. In practice either temperature may be outside the practical temperature range over which the column can be operated and so only one temperature may be practically useful. The retention of a solute is directly related to the volume of stationary phase available and this is rarely the same as the *total amount* of stationary phase present in the column. The available stationary phase is limited by the porosity of the support (only those molecules small enough to enter the pores can interact with the stationary phase) and the chiral characteristics (spatial arrangement) of the stationary phase. Only those molecules having the appropriate spatial arrangement will achieve close interaction with the stationary phase molecules or surface. Conversely, those solute molecules of opposite chirality will be partially hindered from close interaction with the stationary phase or surface, and experience reduced interaction. Retention depends on (1), the magnitude of the interactive forces between the molecule and the two phases, (2) the change in the random nature of the solute molecule when it transfers from one phase to the other, and (3) the availability of the stationary phase, which will depend on the exclusion properties of the support and the chiral nature of the surface.

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Chapter 3—
Molecular Interactions

In order to learn how to achieve the selectivity required to resolve a pair of enantiomorphs, the mechanism of retention must be fully understood. This means that the molecular forces that control retention must be defined, their mode of action identified, and their effect on the distribution coefficient (K) examined. The magnitude of (K) depends on the relative affinity of the solute for the two phases. Consequently, the stationary phase must be chosen to interact strongly with the solutes to achieve a separation (*i.e.* the intermolecular forces between solute and stationary phase must be relatively large). In contrast, the interactions between the solute molecules and the mobile phase should be chosen to be relatively weak, to allow the stronger forces to dominate in the stationary phase and produce the required retention and selectivity. This will naturally occur in GC, as the probability of interaction (collision between solute and gas molecules) is very small compared with that in a liquid, and due to the small mass of the mobile phase molecules, the strength of any interactions that do occur will be extremely weak. This will not be true in LC, and the mobile phase must be chosen so that the *type* of interactions that take place with the solute will be weaker than those that take place between the solute and the stationary phase. This will become clearer when the different types of molecular interactions are understood.

All intermolecular forces are electrical in nature. Although gravitational and magnetic forces between molecules may exist, they are many orders of magnitude weaker than the electrical forces and for the purposes of

solute retention in chromatography can be ignored. There are three different basic types of intermolecular forces, *dispersion forces, polar forces* and *ionic forces*. All interactions between molecules are composites of these three different types of molecular force although, individually, they can vary widely in strength.

Dispersion Forces

Dispersion forces were first described by London [1], and for this reason were originally called 'London's dispersion forces'. However, over the years London's name has been dropped and they are now simply referred to as 'dispersion' forces. They arise from charge fluctuations throughout a molecule resulting from electron/nuclei vibrations. They are a statistical effect and a little difficult to appreciate. Many years ago Glasstone [2] gave a good description of dispersion forces and suggested that;

> *"although the physical significance probably cannot be clearly defined, it may be imagined that an instantaneous picture of a molecule would show various arrangements of nuclei and electrons having dipole moments. These rapidly varying dipoles when averaged over a large number of configurations would give a resultant of zero. However, at any instant they would offer electrical interactions with another molecule resulting in interactive forces".*

Dispersion forces occur between all types of molecules but are typically, and specifically, those that occur between hydrocarbons and are responsible for the fact that hexane, at S.T.P., is a liquid boiling at 68.7°C and is not a gas. In biotechnology and biochemistry, dispersive interactions are often referred to as 'hydrophobic' or 'lyophobic' interactions, apparently because dispersive substance such as the aliphatic hydrocarbons do not dissolve readily in water. The interpretation of the biochemical terms for molecular interactions will be discussed later.

The theoretical treatment of molecular interactions is extremely complicated and the mathematics can become cumbersome. In the

following discussion, certain simplifying assumptions are made that are only approximate and, thus, the expressions given below, for both dispersive forces and polar forces, will not be exact. Nevertheless, they will be sufficiently precise to allow the parameters that control the different types of interaction to be identified.

As a first approximation, the interaction energy, (U_D), involved with dispersive forces has been calculated to be:

$$U_D = \frac{3h\nu_o\alpha^2}{4r^6}$$

where (α) is the polarizability of the molecule,

(ν_o) is a characteristic frequency of the molecule,

(h) is Plank's constant,

and (r) is the distance between the molecules.

The dominant factor that controls the dispersive force is the polarizability (α) of the molecule, which, for substances that have no dipoles, is given by:

$$\frac{D-1}{D+2} = \frac{4}{3}\pi n\alpha$$

where (D) is the dielectric constant of the material,

and (n) is the number of molecules per unit volume.

If (ρ) is the density of the medium and (M) is the molecular weight, then the number of molecules per unit volume is Nρ/M where (N) is Avogadro's number.

Thus:

$$\frac{4}{3}\pi N\alpha = \frac{(D-1)}{(D+2)}\frac{M}{\rho} = P$$

where (P) is called the molar polarizability.

It is seen that the molar polarizability is proportional to M/ρ, the molar volume; consequently, dispersive forces (and thus "hydrophobic" or "lyophobic forces") will be related to the 'molar volume' of the interacting substances. A diagrammatic representation of dispersive interactions is shown in figure 3.1. Dispersive interactions occur where there is no localized charge on any part of the molecule, just a host of fluctuating, closely associated charges that, at any instant, can interact with instantaneous charges of an opposite kind situated on a neighboring molecule.

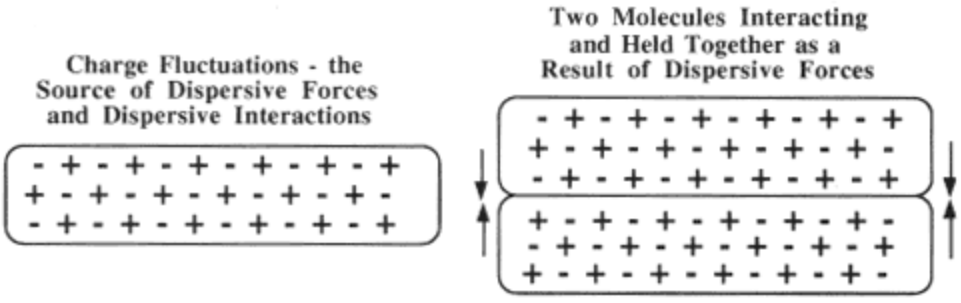


Figure 3.1
Dispersive Interactions

Polar Forces

Polar interactions arise from electrical forces between localized charges residing on different parts of the molecule that result from permanent or induced dipoles. Polar interactions cannot occur in isolation, but must be always be accompanied by statistically generated dispersive interactions. Polar interactions can vary widely in strength, depending on the magnitude of the charges on each dipole. The forces can be very strong and result in molecular associations that approach, in energy, that of a weak chemical bond. Examples of such instances are 'hydrogen bonding' and in particular the association of water with itself. Conversely, very

weak polar interactions can occur such as those arising between induced dipoles.

Dipole-Dipole Interactions

The interaction energy (U_p) between two dipolar molecules is given, to a first approximation, by:

$$U_P = \frac{2\alpha\mu^2}{r^6}$$

where (α) is the polarizability of the molecule,

(μ) is the dipole moment of the molecule,

and (r) is the distance between the molecules.

The energy is seen to depend on the square of the dipole moment, which can vary in strength over a wide range of values. Unfortunately, the numerical value of the dipole moment, taken from bulk measurements of dielectric constant over a range of temperatures, does not always give an indication of the strength of any polar interactions that it might have with other molecules. Water, for example, an extremely polar molecule, has a dipole moment of only 1.76 Debyes. In a similar manner the dipole moment of methanol, another extremely polar substance, has a dipole moment of only 2.9 Debyes. Unusually, low values for the dipole moments of strongly polar substances can result from electric interaction between the two dipoles due to molecular association and/or from internal electric field compensation, that can occur when more than one dipole is present in the molecule. For example, water associates strongly with itself by very strong polar forces or 'hydrogen bonding' which reduces the net dipole character of the associated molecules, when determined from *bulk* external electrical measurements made on the substance. Methanol also associates strongly with itself in a similar manner. In many cases, the electric field from one dipole opposes that from the other, resulting in a reduction in the *net* field as *measured externally.* Consequently, *bulk* properties would not reflect the true value for the dipole moment of the individual dipoles. Another molecule,

however, approaching a water or methanol molecule would experience the uncompensated field of the single dipole and interact accordingly.

An example of the internal compensation of two dipoles affecting the magnitude of the overall apparent dipole moment, as calculated from bulk measurements of dielectric constant, is the low dipole moment of dioxane, 0.45 Debyes. Compared with a dipole moment of 1.15 Debyes for diethyl ether, which theoretically should be about half that of dioxane, it is seen that there is strong internal compensation between the dipoles from each of the ether groups. Again, however, another molecule, approaching one ether group of the dioxane molecule would experience the uncompensated field of that single dipole and, again, interact accordingly. Consequently, dioxane although having the low dipole moment of 0.45 is still a very polar material that is miscible with water. These examples, clearly indicate that considerable caution must be taken when attempting to relate the magnitude of polar interactions with the dipole moments of the molecules concerned.

Now, the *polarizability* of a substance that contains no dipoles is likely to give an indication of the *strength of the dispersive* interactions that might take place with another molecule. In contrast, due to possible self-association or internal compensation, the *dipole moment* of a substance, determined from bulk dielectric constant measurements, will *not* always give an indication of the *strength of any polar interaction* that might take place with another molecule. A diagrammatic impression of a dipole-dipole interaction is shown in figure 3.2.

The dipoles interact directly as would be expected, but it is important to appreciate that behind the dipole-dipole interaction is the dispersive interaction from the charge fluctuations on both molecules. The net molecular interaction will, therefore, be a combination of both. Dispersive interactions are the only interactions that can occur in the absence of any other. All other types of interaction, polar and/or ionic, will occur in conjunction with dispersive interactions. Examples of some substances that have permanent dipoles and exhibit polar interaction with other molecules are alcohols, esters, ethers, amines, amides, nitriles, etc.

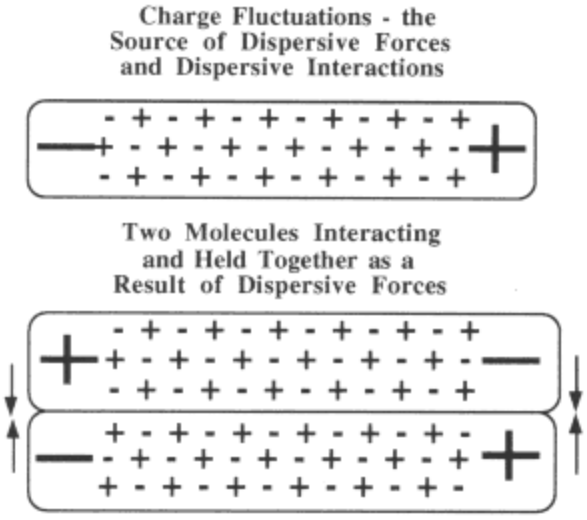


Figure 3.2
Polar Interactions: Dipole-Dipole-Interactions

Dipole-Induced-Dipole Interactions

Certain compounds, such as those containing the aromatic nucleus and thus (π) electrons, are polarizable. When such molecules come into close proximity with a molecule having a permanent dipole, the electric field from the dipole induces a counter dipole in the polarizable molecule. This induced dipole acts in the same manner as a permanent dipole and the polar forces between the two dipoles result in interaction between the molecules. Aromatic hydrocarbons are typically polarizable compounds and an example of their separation using induced dipole interactions to affect retention and selectivity will be given later. A diagrammatic impression of a dipole-induced dipole interaction is shown in figure 3.3. Just as dipole interactions must take place coincidentally with dispersive interactions, so are induced dipole interactions always accompanied by dispersive interactions. Thus, aromatic hydrocarbons can be retained and separated solely on the basis of purely dispersive interactions, for example in GC using a *n*-alkane stationary phase. Alternatively, they can be retained and separated by combined induced-polar and dispersive interactions using a polyethylene glycol stationary phase. Molecules need

not exhibit one type of polarity only. Phenyl ethanol, for example, will possess both a dipole as a result of the hydroxyl group and be polarizable due to the aromatic ring. More complex molecules can have many different interactive groups.

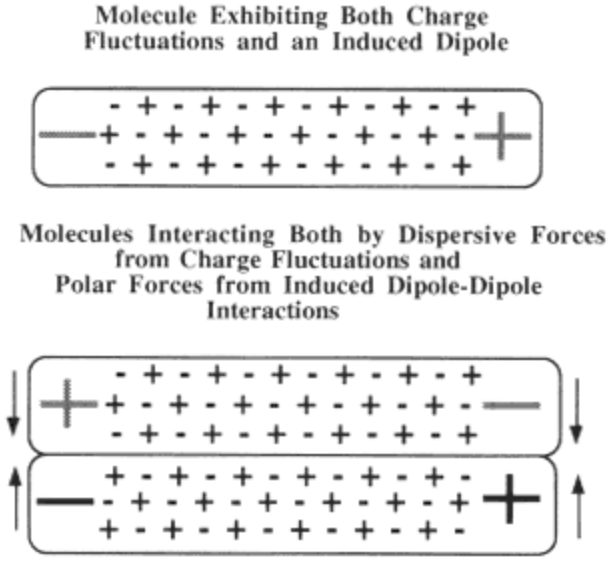


Figure 3.3
Polar Interactions: Dipole -Induced Dipole Interactions

Ionic Forces

Polar compounds, although possessing dipoles, have no net charge on the molecule. In contrast, ions possess a net charge and consequently can interact strongly with ions having an opposite charge. Ionic interactions are exploited in ion exchange chromatography, where the counter ions to the ions being separated are situated in the stationary phase. In a similar manner to polar interactions, ionic interactions are always accompanied by dispersive interactions and usually, also with polar interactions. Nevertheless, in ion exchange chromatography the dominant forces controlling retention usually result from ionic interactions. Ionic interaction is depicted diagramatically in figure 3.4. A molecule can have many interactive sites comprised of the three basic types, dispersive,

polar and ionic. Large molecules (for example biopolymers) can have hundreds of different interactive sites throughout the molecule and the interactive character of the molecule as a whole will be determined by the net effect of all the sites. If the dispersive sites dominate, the overall property of the molecule will be dispersive which the biotechnologists call "hydrophobic" or lyophobic".

If dipoles and polarizable groups dominate in the molecule, then the overall property of the molecule will be polar, which the biotechnologists call "hydrophilic" or lyophylic". These terms are not based on physical chemical arguments, but have evolved largely in the discipline of biology. They have important significance to biologists and biochemists and therefore deserve some discussion.

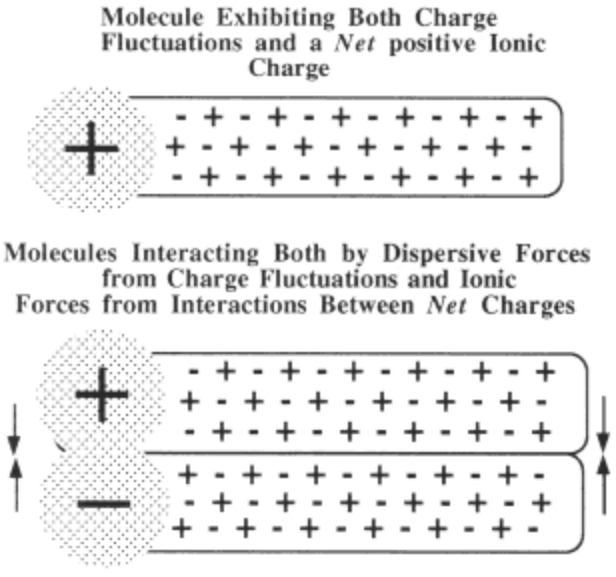


Figure 3.4
Ionic and Dispersive Interactions

Hydrophobic and Hydrophilic Interactions

The term "hydrophobic" is a most unfortunate term in that it implies some form of molecular repulsion, which, of course, outside the van der Waals radii of a molecule, is impossible. The term "hydrophobic force",

literally meaning "fear of water" force, is an alternative to the well-established term, *dispersive* force. The author is not aware of how the use of this word for molecular interactions originated, but it may have been provoked by the immiscibility of a dispersive solvent such as *n*-heptane with a very polar solvent such as water. The reason that *n*-heptane and water are immiscible is *not* because water molecules *repel* heptane molecules. They are immiscible because the forces between two heptane molecules and the forces between two water molecules are much greater than the forces between a heptane molecule and a water molecule. Thus, water molecules and heptane molecules interact *very much more strongly* with *themselves* than with *each other*.

Water has, in fact, a small but finite solubility in *n*-heptane, and *n*-heptane has a small but finite solubility in water. Although *water-water* interactions and *hydrocarbon-hydrocarbon* interactions are much stronger than *water-hydrocarbon* interactions, the latter does exist and are sufficiently strong to allow weak solution to take place. However, despite the strong interactive force between hydrocarbon and hydrocarbon, at normal temperatures, a small fraction will randomly acquire sufficient kinetic energy to break the association and part. Thus, in a *saturated solution* of any hydrocarbon in water, equilibrium occurs when the *probability* of two hydrocarbon molecules *colliding and interacting* will be equal to the thermodynamic *probability* of another pair gaining sufficient energy to *part*. As the latter is very small, so must the saturated concentration of heptane in water (which determines the probability of collision) also must be small. Hence the very low mutual solubility of water and hydrocarbons. The term "hydrophilic force", literally meaning "love of water" force, appears to have been introduced merely as the complement to "hydrophobic". It is equivalent to the term polar, and polar solvents are hydrophilic solvents because they interact strongly with water or other polar solvents.

The reasons for the introduction of the terms "lyophobic" (meaning fear of lye) and "lyophilic" (meaning love of lye) are a little more obscure and to some extent are irrelevant, as they are essentially alternatives to the terms hydrophobic and hydrophilic. The terms originated in the early

days of the soap industry when soap was prepared by boiling a vegetable oil with an alkaline solution obtained from leaching 'wood ash' with water. The alkaline product from the wood ash was a crude solution of sodium and potassium carbonates called "lye". On boiling the vegetable oil with the lye, the soap (sodium and potassium salts of long-chained fatty acids) separated from the lye due to the dispersive interactions between the fatty acid alkane chains and were thus called "lyophobic". It follows that "lyophobic", from a physical chemical point of view, would be the same as "hydrophobic", and interactions between hydrophobic and lyophobic materials are dominantly dispersive. The other product of the soap-making industry was glycerol, which, being strongly polar, remained in the lye and was consequently termed "lyophilic". Thus, glycerol mixes with water because of its many hydroxyl groups, is very polar and hence is a "hydrophilic" or "lyophilic" substance.

From a classical physical chemical point of view, these alternative terms describing molecular interactions are somewhat impertinent, and confusing, and perhaps should be avoided by those not involved in the biological sciences. Nevertheless, as they are extensively employed in biotechnology to describe the interactive character of a molecule, their meaning needs to be appreciated. The use of a more general term to describe the interactive property of a biomolecule can be understood if one considers the character of a biopolymer, for example a polypeptide. The peptide will contain a large number of different types of amino acids, each having different interactive groups. All will exhibit polar interactions with the carbonyl and amide groups but each amino acid will contribute its own unique interactive character to the peptide. An attempt to illustrate the interactive character of two polypeptides is made in figure 3.5. The molecular dimensions in figure 3.5 have no significance and the spatial arrangements are imaginary; the intent is solely to illustrate how the overall interactive character of a large molecule is made up from the individual contributions from the different chemical groups. The net character, resultant from all the groups, can be dispersive (hydrophobic) or polar (hydrophilic), which will determine their overall interaction with their environment. As an example, the peptide (A) might be considered to be the hexapeptide:

leucine-alanine-phenylalanine-leucine-alanine-phenylalanine

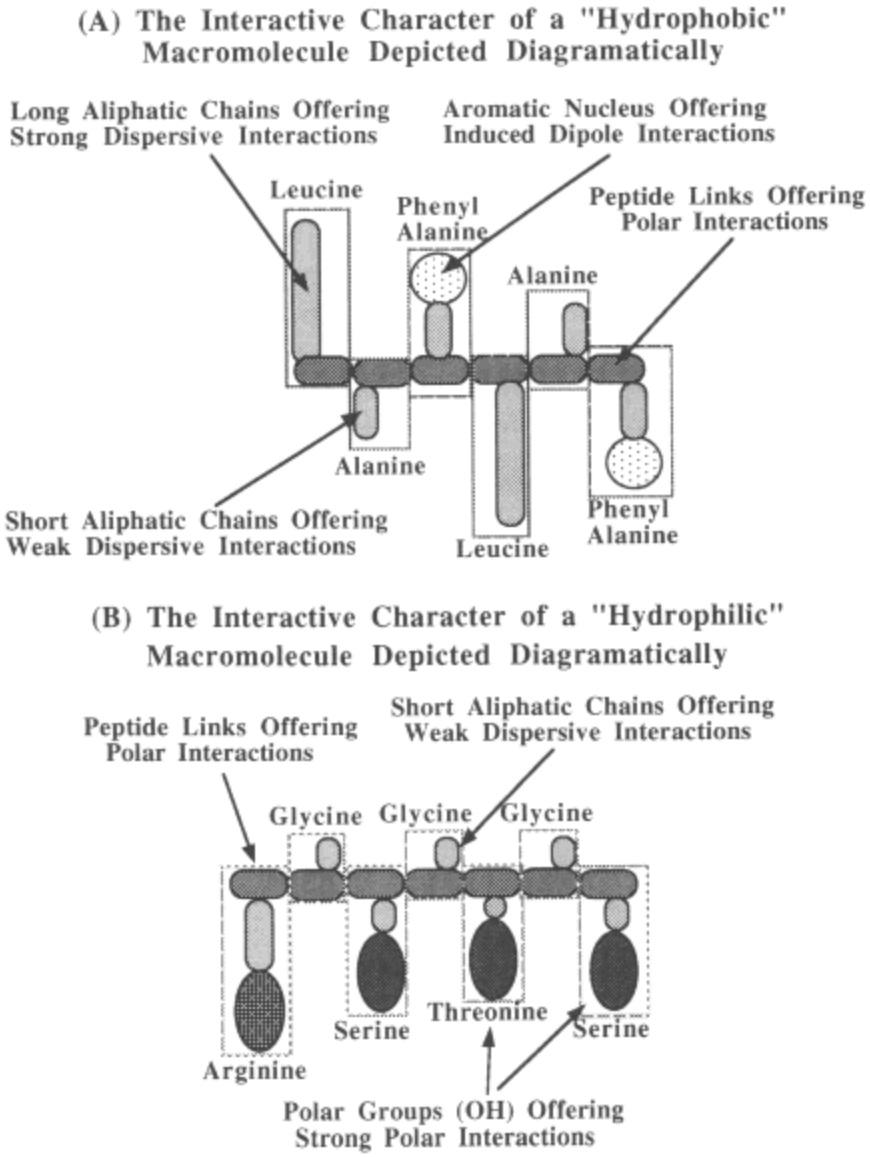


Figure 3.5
Examples of Hydrophobic and Hydrophilic Molecules

It is seen that there is a preponderance of aliphatic chains present: long chains contributed by the two leucine fragments, short chains contributed by alanine fragments. The only polar groups are the peptide bonds themselves and the two aromatic rings from the phenylalanine fragments that could produce induced dipoles. It follows that the overall character of the hexapeptide is likely to be dispersive and thus, the peptide would probably be defined as *hydrophobic* or *lyophobic* in nature.

In contrast, the lower peptide (B) might be considered to be the heptapeptide,

arginine-glycine-serine-glycine-threonine-glycine-serine

or of similar composition. It is seen that there is now a preponderance of polar groups present, hydroxyl groups contributed by the two serine and threonine fragments together with the peptide bonds themselves. The very short aliphatic chains contributed by glycine fragments (CH_2 groups) will only provide weak dispersive interactions. The arginine also provides potential ionic interactions with the free amine group. It follows that the overall character of the heptapeptide is likely to be polar, and thus the peptide would probably be defined as *hydrophilic* or *lyophilic* in nature.

The terms hydrophilic and hydrophobic are more often used to describe the overall interactive character of a large molecule as opposed to the individual group interactions. Nevertheless they are basically alternative terms that have been adopted to describe a predominance of polar and dispersive interactive properties in a molecule respectively.

Molecular Interactions in Mixed Phases

A phase of specific polarity can be simulated by mixing two substances having different polarities to provide the desired composite interactive properties. This procedure was examined in considerable detail by Purnell who published a number of papers describing the properties of

mixed stationary phases in GC (Purnell *et al*. [3], Laub and Purnell [4] and Laub [5]).

These workers examined the effect of mixed stationary phases on solute retention in GC during the late 1970s and early 1980s. They found that, for a wide range of binary mixtures, the corrected retention volume of a solute was linearly related to the volume fraction of either one of the two phases. These results produced considerable controversy which still persists in many contemporary academic circles. The major point of argument arose from the fact that as the corrected retention volume, and thus the distribution coefficient, was linearly related to the volume fraction of either phase, this meant the distribution coefficient was *linearly* controlled by the volume fraction of the stationary phase component and not exponentially related to it. This does not seem to be surprising, as the volume concentration will control the probability of interaction and thus if the concentration is doubled, the probability of interaction will be expected to double and also the distribution coefficient. However, this linear relationship only holds for binary mixtures. It will be seen that for ternary mixtures, that result from associations of the stationary phase components with one another, the relationship breaks down.

In terms of an expression for solute retention, the results of Purnell and his co-workers can be given as follows:

$$V'_{r(A)} = K_A \alpha V_S + K_B (1-\alpha) V_S$$

where $V'_{r(AB)}$ is the retention volume of the solute on a mixture of stationary phases (A) and (B),

(K_A) is the distribution coefficient of the solute with respect to the pure stationary phase (A),

(K_B) is the distribution coefficient of the solute with respect to the pure stationary phase (B),

(V_S) is the total volume of stationary phase in the column

and (α) is the volume fraction of phase (A) in the stationary phase mixture.

That is

$$V'_{r(AB)} = \alpha V'_A + (1-\alpha)V'_B \qquad (13)$$

where (V'_A) is the retention volume of the solute on the same volume of pure phase (A)

and (V'_B) is the retention volume of the solute on the same volume of pure phase (B)

Rearranging equation (13):

$$V'_{AB} = \alpha(V'_A - V'_B) + V'_B \qquad (14)$$

This simple relationship is depicted in figure 3.6.

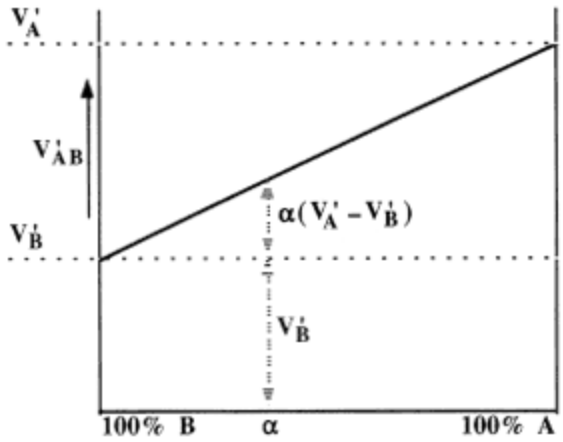
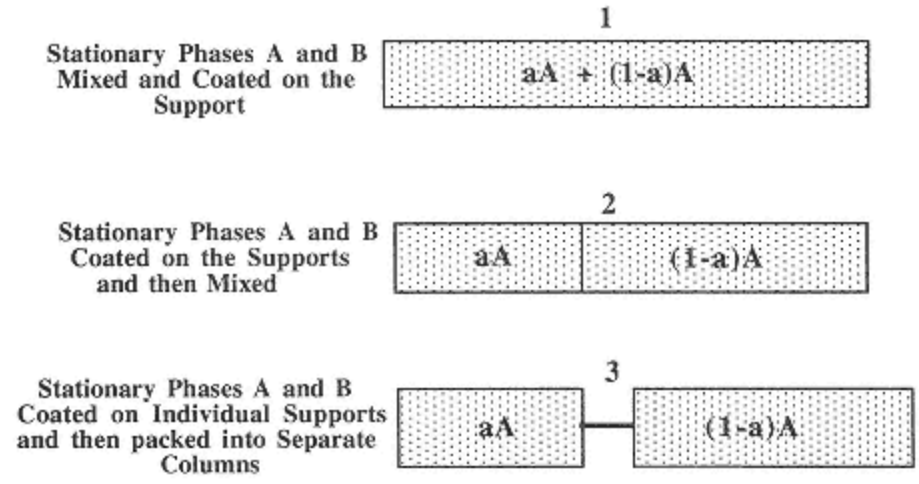


Figure 3.6
Graph of Corrected Retention Volume against Volume Fraction of Stationary Phase

Purnell experimentally further validated the above equation by the following three experiments.

First, the two fractions of stationary phase were mixed, coated on some support, and packed into the column; second, each of the two fractions were coated on separate aliquots of support and then the coated supports

mixed and packed in a column; third, each fraction was coated on a support and the appropriate quantity packed into separate columns and the columns joined in series.



(Purnell experimentally demonstrated that the retention volume of a given solute was the same on all three column systems)

Figure 3.7
Alternative Methods for Combining Volume Fractions of Stationary Phase in GC

The results verified that the same retention volume for a given solute was obtained on all three column systems. The results from these experiments are depicted in figure 3.7.

As already discussed, this simple relationship, unfortunately, was found not to be universal and broke down where strong association occurred between the two phases [6,7]. If there is strong association between the individual components (A and B) of the mixed phase *e.g.*,

$$A + B \rightleftharpoons AB$$

then the stationary phase would no longer be a binary mixture but would also contain the associate of the two phases as a third component. Thus,

the simple linear relationship between retention and volume fraction of one component of the mixture would not be expected to hold.

The simple, linear relationship between volume fraction of one component of a binary mixture and the retention volume of a solute, where there is only weak interaction between the individual components, again, is to be expected. The volume fraction of each phase will determine the probability that a given solute molecule will interact with a molecule of that phase, in much the same way as the partial pressure of a solute in a gas, determines the probability that a solute molecule will collide with a gas molecule. For those phase systems that give a linear relationship between retention volume and volume fraction of stationary phase, it is clear that the linear functions of the distribution coefficients could be summed directly, but their logarithms could *not*. The results of Purnell indicate that when there is little, or only weak, interaction between the components of a binary mixture used as a stationary phase, the solute retention or distribution coefficient is linearly, *not* exponentially, related to the stationary phase composition.

It would seem that stationary phases of intermediate polarities can easily be constructed from binary mixtures of two phases one, strongly dispersive and one strongly polar, provided that the two components do not interact strongly with each other. Furthermore, if there is interaction between the components, then, in GC, the procedure of Purnell can be used, using two columns connected in series each containing the appropriate quantity of stationary phase. As the individual stationary phase components are kept apart, no phase interaction can occur and the simple relationship between retention and volume fraction of stationary phase will hold. This procedure has not been used extensively in commercial columns, although it is probably the easiest and most economic method of fabricating columns having carefully adjusted intermediate polarities. This technique might be particularly useful for the separation of certain chiral isomers where an appropriate stationary phase comprising a pure substance proved difficult to identify and molecular interactions between the individual components of the

stationary phase did not allow the expected chiral selectivity to be realized.

The same linear relationship between mobile phase composition and retention was noted by Katz *et al.* [7] for binary mixtures in LC. However, in LC the situation is more complicated, and if strong association occurs between the mobile phase components, the relationship becomes nonlinear. Unfortunately, the technique of using the two columns procedure of Purnell is impractical for mixed mobile phases in LC. Furthermore, as the solvents most commonly used are water, methanol, acetonitrile and tetrahydrofuran, and all form strong associates, the use of the linear relationship demonstrated by Katz *et al.* is severely limited. An example of the linear relationship between volume fraction of one component of two binary mixtures and retention is shown in figure 3.8. The linear relationship is clearly demonstrated and it is seen that the distribution coefficient (which controls retention) can be adjusted to any selected value by choosing the appropriate mixture of the two solvents.

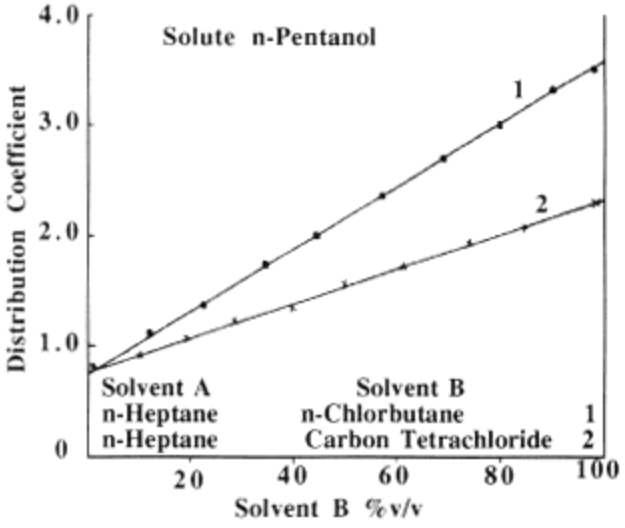


Figure 3.8
Graphs Relating Distribution Coefficient to Solute Retention for n-Pentanol

If one solvent is replaced by a chiral additive, retention will not be linearly related to additive concentration unless the additive is present at extremely low concentrations where the adsorption isotherm is linear. The effect of chiral additives on solute retention will be discussed below. Unfortunately, the linear relationship also breaks down completely when strong molecular association takes place between the components of the binary mixture. The mobile phase now becomes a ternary mixture, the composition of which is not simply related to the original concentrations of the unassociated solvents in the mixture. An example of such curves are given in figure 3.9.

It should be pointed out, that although the relationship between distribution coefficient and methanol concentration is not linear, neither is it logarithmic; a graph of log (distribution coefficient) against methanol concentration gives a shallow curve. In order to obtain an explicit function for the retention of a solute in terms of the original methanol concentration, it is necessary to determine the equilibrium constant for the association of water and methanol. This was carried out by Katz *et al.* [7] using refractive index and density data for different methanol/water mixtures and an expression for the retention of the solute was proposed.

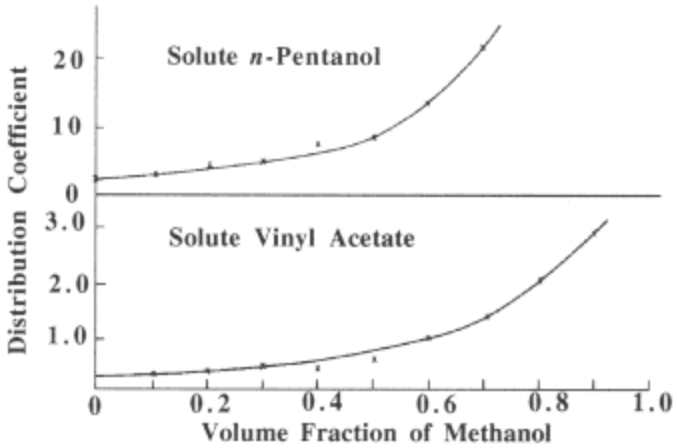


Figure 3.9
Graphs Relating Distribution Coefficient to Methanol Concentration

Chiral Additives

In liquid chromatography, chiral additives are frequently used with the mobile phase, and their direct effect on the differential retention of a pair of enantiomers can be very complex and difficult to calculate (although not impossible if sufficient data is available). The addition of an additive to the mobile phase changes both the interactive character of the mobile phase and the stationary phase, as the additive is distributed between the two phases in exactly the same way as a solute. The additive, however, is present at much higher concentrations than the solute and thus the isotherm is not linear. In order to understand the effect of an additive to the interactive nature of the stationary phase in LC, it is necessary to develop the Langmuir Isotherm which will provide an equation that describes the surface coverage by the chiral agent, in terms of its concentration in the mobile phase.

Consider 1 cm^2 of stationary phase surface carrying an adsorbed layer of chiral agent, at a concentration (C_s) $g.cm^{-2}$, in contact with a liquid containing (C_m)g of chiral agent per cm^{-3} of the solvent mixture. Let the molecular weight of the chiral agent be (M), and the area covered by the chiral agent molecule when adsorbed on the surface, be (S). This situation is depicted in figure 3.10.

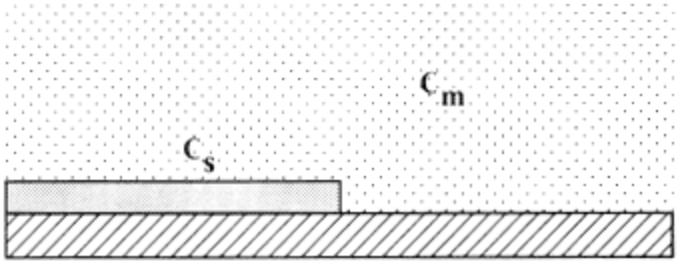


Figure 3.10
The Adsorption of a Chiral Reagent on the Surface of a Solid Stationary Phase

Assuming a mono-layer of solvent is formed on the surface, the area of exposed surface (ψ), is given by:

$$\psi = 1 - \frac{C_S}{M} N S \qquad (14)$$

where (N) is Avogadro's Number

Now the number of molecules (N_1), leaving the surface will be proportional to the concentration of adsorbed molecules and a constant (β), where (β) is that fraction of the adsorbed molecules that acquire sufficient kinetic energy to overcome the molecular forces holding the molecules to the surface, *i.e.*:

$$N_1 = \beta C_S$$

The number of molecules striking and adhering to the exposed surface (N_2), will be proportional to the concentration of chiral agent in the mobile phase, the unexposed area of surface and another constant (α). It follows that,

$$N_2 = \alpha \left(1 - \frac{CS_S}{M} N S \right) C_m$$

Now, under equilibrium conditions, the number of molecules leaving the surface will equal the number of molecules striking the surface and adhering,

i.e.;

$$N_1 = N_2$$

Thus,

$$\alpha C_m - \alpha C_m \frac{NS}{M} = \beta C_S$$

and:

$$C_S \left(\beta + \alpha \frac{NS}{M} C_m \right) = \alpha C_m$$

or;

$$\frac{\alpha}{\beta + \alpha \frac{NS}{M} C_m} = \frac{C_S}{C_m} = K \qquad (15)$$

where (K) is the net, effective distribution coefficient of the chiral agent between the stationary phase and a solution of the chiral agent in water at a concentration (C_m).

In practice, this means that if a neat sample of chiral agent is injected on to a column as a solute, when the mobile phase consists of an aqueous solution of the agent, at concentration (C_m), then, the magnitude of its retention volume will be determined by the distribution coefficient (K);

$$K = \frac{1}{g + \frac{NS}{M} C_m} \qquad (16)$$

where $g = \beta/\alpha$ = the desorption-adsorption coefficient of the solvent.

Now, if the reverse phase is considered to be packed into a column and operated with a mobile phase having a solvent concentration of (C_m) in water, the corrected retention volume, (V'_r), is given by the equation derived from the Plate Theory:

$$V' = K\varphi$$

where (φ) is the total chromatographically available surface area of the reverse phase in the column.

Substituting for (K) from equation (16),

$$V' = \frac{\varphi}{g + \frac{NS}{M} C_m}$$

or:

$$\frac{1}{V'} = \frac{g}{\varphi} + \frac{NS}{M\varphi} C_m \qquad (17)$$

It is seen that equation (17) shows a linear relationship between the reciprocal of the corrected retention volume and the concentration of solvent in the mobile phase. Consequently, if retention data is measured over a range of solvent concentrations, employing the chiral agent itself as the solute, a linear relationship will be obtained by plotting (1/V') against (C_m) and from the intercept and slope of the graph, values for

the (φ), (g) and (K) can be calculated. For example, if the intercept and slope of a curve relating (1/V') to (C_m) is (A) and (B) respectively,

Then:

$$\frac{A}{B} = \frac{gM}{NS} \quad \text{or:} \quad g = \frac{ANS}{BM} \qquad (18)$$

and:

$$\varphi = \frac{g}{A} \qquad (19)$$

Restating equation (17):

$$\frac{1}{V'} = \frac{g}{\varphi} + \frac{NS}{M\varphi} C_m$$

Now, as, $V' = V_r - V_o$, then, substituting for (V') in equation (17):

$$\frac{1}{V_r - V_o} = \frac{g}{\varphi} + \frac{NS}{M\varphi} C_m \qquad (20)$$

To aid in algebraic manipulation equation (7) can be put in the simple form:

$$\frac{1}{V_r - V_o} = A + BC_m \qquad (21)$$

where, as stated before:

$$\frac{g}{\varphi} = A \quad \text{and} \quad B = \frac{NS}{M\varphi}$$

Now, when the solvent is chromatographed as the solute in pure water as the mobile phase, (i.e. $C_m = 0$, and $V_r = V_r(0)$), then:

$$\frac{1}{V_{r(0)} - V_o} = A$$

Re-arranging:

$$V_o = \frac{AV_{r(0)} - 1}{A} \qquad (22)$$

Substituting for (V_o) from equation (21) into equation (20):

$$\frac{1}{V_{r(n)} - \frac{A V_{r(0)} - 1}{A}} = A + BC_{m(n)} \qquad (23)$$

Where ($V_{r(n)}$) is the retention volume of the chiral additive, when chromatographed as a solute in a mobile phase containing the additive at a concentration ($C_{m(n)}$). Equation (23) relates the *retention volume* of the additive (as opposed to the *corrected retention volume*) to its concentration in the mobile phase and thus, *the need to determine the column dead volume is eliminated*, and any errors associated with its measurement removed.

It is seen that the pertinent constants for the adsorption of a chiral reagent on the surface of an LC stationary phase can easily be measured. The effect of change of chiral agent concentration on surface coverage can then be calculated and then its effect on the relative retention of a pair of isomers assessed.

Synopsis

The retention of a substance is determined by the magnitude of its distribution coefficient between the two phases and hence the type, and strength of the interactive forces between the solute and phase molecules. There are basically three types of intermolecular forces, dispersive forces, polar forces and ionic forces all of which are electrical in nature. Dispersive forces arise from transient, randomly generated dipoles that are manifest throughout each molecule. Polar interactions arise from permanent or induced dipoles in the molecules and ionic interactions from permanent net charges on the molecule (*i.e.* ions). Dispersive interactions are related to the molar polarizability of the substance and, providing there is no internal or external field compensation, polar interactions are related to the dipole moment of the substance. In the field of biotechnology, dispersive interactions are termed hydrophobic and polar interactions hydrophilic. Large biopolymers that have predominantly dispersive groups are called hydrophobic in nature whereas those with predominantly polar groups are called hydrophilic in

nature. Providing there is no strong interaction between the components, the distribution coefficient and thus, the retention of a substance, is linearly related the volume fraction of either phase component. If there is a strong interaction between the components, then a ternary mixture is formed, and the simple linear relationship breaks down. Chiral additives to an LC mobile phase cause a layer of the reagent to be adsorbed onto the surface of the stationary phase according to the Langmuir equation. Using the Langmuir equation, and retention data for the chiral reagent itself from mobile phases carrying a range of reagent concentrations, the constants of the equation can be calculated. Having determined the Langmuir constants, the retention of a given chiral solute can be calculated at different reagent concentrations.

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Chapter 4—
The Basic Gas Chromatograph for Chiral Chromatography

The chromatographic instrumentation necessary for the satisfactory separation of a mixture of stereoisomers is essentially the same as that used in general GC analyses, with the exception that the columns and/or packing need to be specially prepared to provide chiral selectivity. The modern GC instrument can be fitted with a wide range of ancillary equipment designed for special analyses, for rapid sample processing or for high sensitivity, etc. However, those parts of the apparatus that are essential for normal use are shown in figure 4.1. The chromatograph can be considered to consist of five chromatographic components and four data processing and display units.

The chromatographic components comprise a number of gas supplies and controls which provide the carrier gas for the column and any other gases that may be necessary, *e.g*., hydrogen, and air or oxygen for a flame ionization detector (FID). These gases pass through a set of flow controllers (usually under computer control) and thence, either to the sampling device, or to the appropriate detectors. The injection device can take various forms, depending on the type of column that is to be used. The sample passes directly from the injection device (and if necessary through a splitter) directly into the column which is situated in an oven. The oven temperature is thermostatically controlled, and is fitted with a

temperature programmer so that its temperature can be changed linearly at chosen rates, over selected temperature limits during the chromatographic development. The column mobile phase (carrier gas) then passes to the detector which is situated in its own thermostatted oven and the concentration of solute in the exiting carrier gas continuously monitored. A range of different detectors is usually available and their output is acquired and processed by the dedicated computer system. In the early chromatographs, the output from the detector passed to a potentiometric recorder and the analysis was carried out by making measurements directly on the chromatogram.

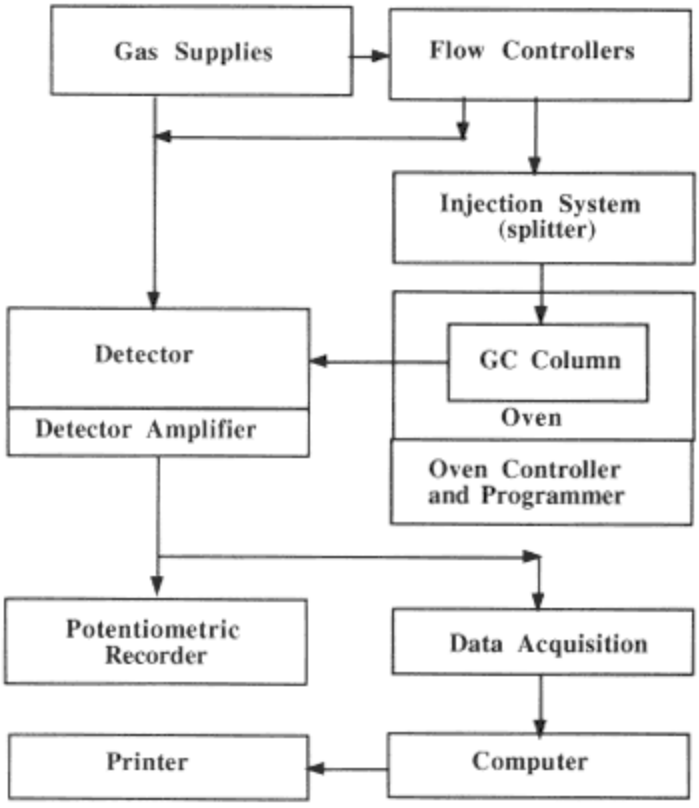

Figure 4.1
The Basic Gas Chromatograph

This simple procedure is still carried out to some extent, but the modern gas chromatograph includes a computer which acquires the data,

processes it using standard software, and then reports the results on an appropriate printer. The data from the detector is first digitized, then acquired by the computer and stored on disk. At the end of the run, the data is retrieved, processed, the report constructed and then printed out. The individual units will now be described in detail.

Gas Supplies

Originally, the gases were almost exclusively supplied from compressed gas cylinders. These were fitted with appropriate reducing valves that supplied gas to the instrument's gas controllers at the pressure recommended by the manufacturer (usually about 50 psi). Many contemporary instruments, however, are fitted with gas generators that may provide nitrogen or oxygen from the laboratory compressed air supply. Alternatively, hydrogen and oxygen can be produced by an electrolytic process which is claimed to be safer and less expensive to operate. From a chromatographic point of view, there is little to choose between the two types of gas supply.

Early models of gas chromatographs were fitted with pressure controllers to ensure that a constant pressure was applied to the head of the column. Unfortunately, applying a constant pressure to a column does not ensure a constant flow of carrier gas though the chromatographic system under all conditions of operation. For example, in the majority of GC analyses, a technique called temperature programming is frequently employed, where the temperature of the column oven is continuously raised during the development of the separation. This ensures that those substances in the mixture which would be strongly retained, are accelerated through the column reducing the analysis time. Now, as the temperature of a gas is raised, its viscosity increases, and thus at a constant inlet pressure, the flow rate will fall. The reduction in flow rate will depend on the temperature program limits and to a certain extent on the temperature gradient. To obviate this change in flow rate, modern gas chromatographs are fitted with *mass* controllers which ensures a constant mass of carrier gas through the column per unit time irrespective of the system temperature. If a constant mass-flow is achieved, then the

volume flow measured at atmospheric temperature and pressure at the end of the column is also constant. It follows that reliable, absolute retention data can be obtained with the use of a mass flow controller providing the appropriate pressure correction is applied [1]. Ancillary gas supplies that are required by specific detectors are also fitted with mass-flow controllers to facilitate detector stability. A diagram of a mass flow controller showing its mode of action is shown in figure 4.2.

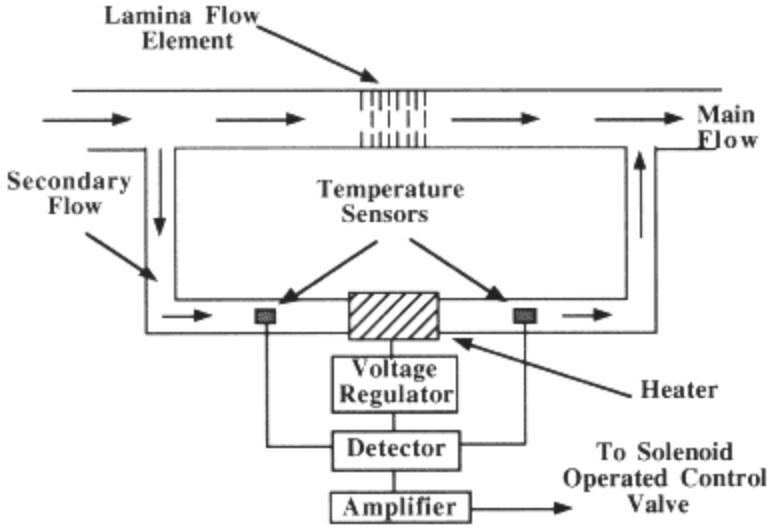

Figure 4.2
The Mass Flow Controller
Courtesy of Porter Instrumentation Company Inc.

The operation of the Porter mass-flow controller is a follows. The sensing system utilizes a by-pass tube with a heater situated at the center. Precision temperature sensors are placed equidistant up stream and down stream of the heater. A proprietary baffle system in the main conduit creates a pressure drop that causes a fixed proportion of the flow to be diverted through the sensor tube. At zero flow both sensors are at the same temperature. When there is flow, the down stream sensor is heated producing a differential temperature across the sensors. As the temperature of the gas will be proportional to the product of mass flowing and its specific heat, the differential temperature will also be

proportional to the mass-flow rate. The voltage generated from the differential sensor signal is compared to that of a set voltage and the difference is used to generate a signal to actuate a valve controlling the flow. Thus, a closed loop control system is formed that maintains the mass-flow rate set by the reference voltage. These devices can be made extremely compact, are highly reliable and, afford, accurate control of the carrier gas flow rate, irrespective of gas viscosity changes due to temperature programming. As the device is basically electronic it can easily be controlled by the chromatograph computer. It follows, that the mass-flow controller also allows the flow rate to be changed or stopped during injection or during a separation if so desired and, in fact, can also be used for flow programming. Flow programming is an analogous technique to that of temperature programming and is used to accelerate the late eluting peaks through the column and thus reduces the analysis time. Flow programming might be an alternative technique to choose if the late eluting solutes were thermally labile. Mass-flow control by the computer is used in special sampling and development procedures [2].

Sampling Devices

There are two basic types of injection systems used in GC, the sample valve and the sample syringe. The sample valve is a rotary valve system that interposes the sample, contained in a sample loop, in line with the carrier gas flow, so that it is swept by the carrier gas onto the column. In GC, sample valves are almost exclusively used for gas samples and are not necessary for the majority of chiral chromatography analyses. Liquid samples or solutions are placed on the column by means of a injection syringe and silicone septum. There are two types of syringe injection systems, those that are used for packed columns, and those that are used for capillary columns. Today about 80% of all GC analyses are carried out using capillary columns, but for the sake of completion, the syringe system that is used with packed columns will also be described.

Injection Systems for Packed Columns

A diagram of an injection system commonly used with packed columns is shown in figure 4.3. The syringes used for injecting the sample are

calibrated, and can vary in size from 1 µ1 to 50 or even 100 µ1. The most commonly used syringe sizes are 1 µ1 and 5 µ1. The sample must be placed on the column without interrupting the carrier gas flow rate and to do this, a septum type injector is used. The septum injector consists of a silicone rubber disc sandwiched between two metal discs with guide holes for the syringe needle The metal discs are arranged to compress the silicone so that after the needle has pierced the septum, and the injection made, the needle can be withdrawn, and the compressed silicone rubber disc expands into the hole making a gas type seal. If correctly fabricated, the system will permit many hundreds of injections before a leak can be detected.

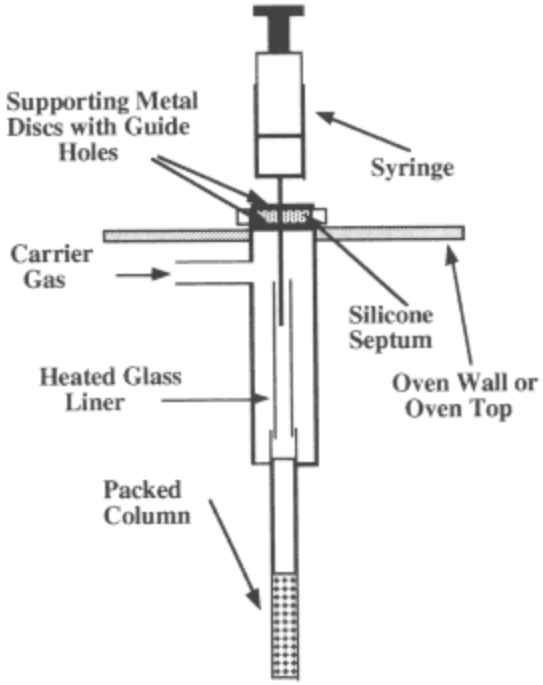

Figure 4.3
Injection System for Packed Column

After passing through the septum the needle discharges the sample into a heated glass liner, where it is vaporized and then swept onto the column by the carrier gas. This glass liner is called a "flash heater" and may cause thermal decomposition of certain samples. Alternatively, if the

heater is turned off, and a longer needle is used that can reach the column packing, then the sample can be discharged directly into the packing, at the column temperature. This procedure is called 'on-column' injection. Under these circumstances, even under temperature programming conditions, the sample is never heated above the temperature at which it elutes from the column.

Injection System for Small Diameter Capillary Columns

Small diameter capillary columns require very small samples, often only a fraction of microgram in size and as this sample size is too small for practical injection syringes, a split-flow system must be used. In effect, the sample is vaporized into a gas stream and a fraction of the gas stream (and consequently a fraction of the sample) is allowed to pass through the column. A diagram of a split-flow injector is shown in figure 4.4.

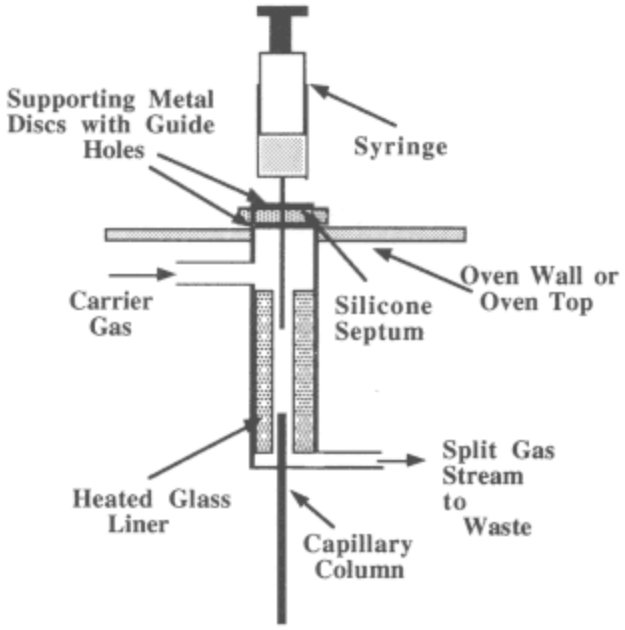

Figure 4.4
The Split-Flow Injector

The split-flow injector is very similar to the packed column injector except that only part of the carrier flow passes to the column, the rest exits to waste. By varying the exit flow-impedance, the split-ratio can be adjusted over a wide range. Without this type of split injection system, the small bore capillary columns would be virtually impossible to use. However, because of the waste of sample and the relatively small mass range obtainable from small bore columns, the large bore capillary column was introduced.

Injection Systems for Large Bore Capillary Columns

Large bore capillary columns can be considered as those that are sufficiently wide to permit a hypodermic needle to enter (*viz*. 0.052 in. I.D.) and thus allow on-column injection. A diagram of an injection system for large columns is shown in figure 4.5.

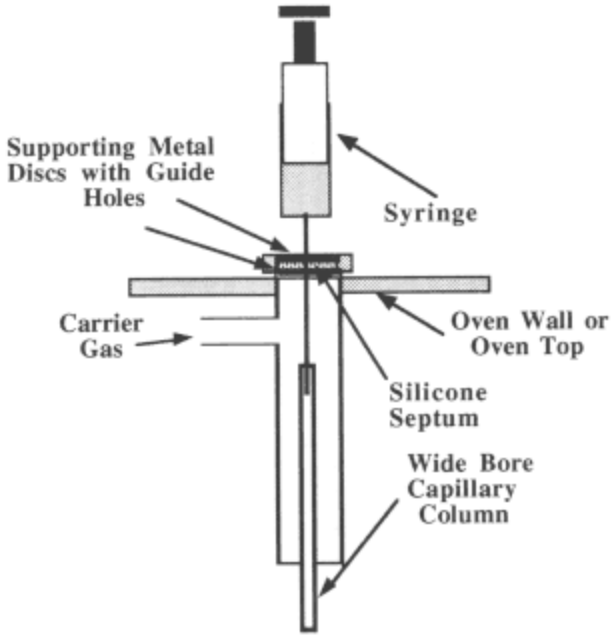

Figure 4.5
The Large Bore Capillary Column Injector

It is seen that the injector is very similar to the packed column injector except that the needle now penetrates into the open end of the column and

can discharge its contents directly onto the column walls. Unfortunately this type of injector has a number of disadvantages. When the sample is injected into the column, it breaks up into separate parts, as bubbles form along the first part of the column. This causes the sample to be deposited at two or more locations along the tube, as the solvent evaporates. When the separation is developed, each local concentration of sample acts as a separate injection and produces a large number of multiple peaks in the chromatogram.

One solution suggested that might solve this problem was the 'retention gap method' of injection. Stationary phase is removed from the first few meters of the column and the sample is injected into this section of the column. If the sample splits and bubbles are formed, the separate droplets would still vaporize in the normal way. As there is no stationary phase present, the solutes will all travel at the same speed as that of the mobile phase (carrier gas) until they reach a coated section of the column where they will be absorbed into the stationary phase. Thus all the sample will accumulate at this point. This technique is usually carried out in conjunction with temperature programming, always starting the program at a fairly low temperature, which facilitates the accumulation of all the solutes at the point where the stationary phase coating begins. The starting temperature is often chosen as the boiling point of the sample solvent. The sample solvent, itself, is chosen to have as low a boiling point as possible, and certainly below the boiling point of any of the sample components. The temperature program is initiated and the solutes are eluted through the column in the normal way. The success of this method depends on there being a *significant difference* between the boiling points of the sample solvent and those of the components of the sample. Retention-gap devices are available, from most venders, in various lengths with different wetting characteristics. They can be used to apply up to 10 µ1 samples per 5 meter length, for capillary tubes having diameters down to 125 µm.

Another effective procedure, called "solute focusing" involves more complex equipment. The oven system must be designed to have two consecutive, independently heated and cooled zones, at the column inlet.

Initially both zones are cooled and the sample is injected into the first zone. Sample splitting, almost inevitably occurs and the carrier gas removes the solvent which is eluted through the column. The first zone is then heated while the second zone is kept cool. The solutes from the first zone are evaporated and pass through the zone and condense and accumulate at the beginning of the cooled second zone where all the sample is now focused. The second zone is now heated and the separation developed in the usual manner. This procedure is more flexible than the "retention gap method" but both the apparatus and the procedure is more involved. Sample splitting does not occur in packed columns and it follows that, if the sample is amenable to separation in packed columns, then the packed column may be the column of choice if high accuracy and precision are required. However, today, packed columns represent only 15% of the GC market.

Automatic Injection Systems

Most contemporary gas chromatographs used for routine analysis include an automatic sampling device. This involves a transport mechanism that may take the form of a carousel or some type of conveyor system. The transporter carries a series of vials, that usually contain alternate sample and washing solvent. The sampling mechanism is quite complicated, and a complex sequence of operations are carried out that are usually controlled by a microprocessor. The syringe is washed with solvent, rinsed with sample, reloaded with the sample, moved by the transport mechanism to the column injector and finally the contents are discharged into the column. After the separation is complete, the next cycle commences with the next syringe washing procedure. In routine analytical laboratories, there may also be sample preparation, such as extraction, concentration, derivatization procedures etc., all carried out by microprocessor controlled robots.

The Column Oven and Temperature Programmer

Theoretically, the column oven should be capable of operating over a fairly wide temperature range (*e.g.* from 5°C to 400°C). In practice, however, the maximum oven temperature needed is often no more than

250°C, particularly when chiral stationary phases are being used, as many of them tend to deteriorate at higher temperatures. In the same way, initial temperatures below 50°C are rarely necessary. The oven should have a very strong circulating fan to ensure an even temperature throughout the oven. The temperature in any part of the oven should be stable to ± 0.5°C. Under isothermal operation, the column temperature should be constant to ± 0.2°C. The oven needs to have adequate space (1–2 cu. ft.) so that it can contain more than one column and some switching valves if so desired in order to provide facilities for multidimensional chromatography.

The temperature programmer (hardware and software) usually has a range of linear gradients available, that extend from 0.5°C/min. to about 20°C/min. Some GC systems include certain nonlinear programs such as logarithmic and exponential, but the vast majority of GC analyses can be effectively accomplished using solely linear programs. The program rate can be changed at any time in the chromatographic development or intermittent isothermal periods can be inserted where necessary in the program. The temperature programming limits are usually the same as those of the oven (*viz*. 5°C to 400°C). All connections between the column and the detector, that pass though the column oven wall to the detector oven, are supplied with their own heater so that no part of the conduit can fall below the column oven temperature. A cool spot in the conduit will cause condensation which can result in broad and distorted peaks.

Detectors

There are a large number of GC detectors available but the majority of GC separations are monitored by the flame ionization detector (FID), the nitrogen phosphorus detector (NPD), the electron capture detector (ECD) or the katherometer detector (or Hot Wire Detector). The latter is almost exclusively used in gas analysis and rarely used in chiral chromatography and so will only be briefly described here. Furthermore, the FID is used in probably 90% of all chiral analyses. However, before describing the construction and function of each detector the subject of detector specifications needs to be discussed.

Detector Specifications

There a number of different specifications for GC detectors and the subject is important and extensive. However, detectors are only peripheral to the subject of this book, and so detector specifications will only be briefly considered here; more detailed information can be obtained from *Chromatography Detectors* [3].

Detector Linearity

If a solute enters the detector at concentration (c), a linear detector will give the following response:

$$V = Ac$$

where (V) is the voltage output of the detector,

and (A) is a constant

Unfortunately, no practical detector is precisely linear, although the response can tend to true linearity. An alternative method for defining linearity is to assume, that for a nearly linear detector, the response could be expressed by the following simple relationship:

$$V = Ac^{\alpha} \qquad (1)$$

where (A) is a constant,

and (α) is a constant called the *response index*.

It is seen that a truly linear detector will have a response index (α) equal to unity and the numerical value of (α) will provide an accurate measure of the proximity of the detector response to strict linearity. The real merit of (α), is that if its numerical value is known, it can also be used to correct for any non-linearity that might exist and thus improve the accuracy of the analysis.

Equation (1) can be expressed in the form:

$$\text{Log}(v) = \text{Log}(A) + \alpha\text{Log}(c) \qquad (2)$$

It is seen that a numerical value for (α) can be easily obtained from an experimentally determined set of values for (V) and (c), from the slope of the curve relating log (V) to log(c); an example is given in figure 4.6.

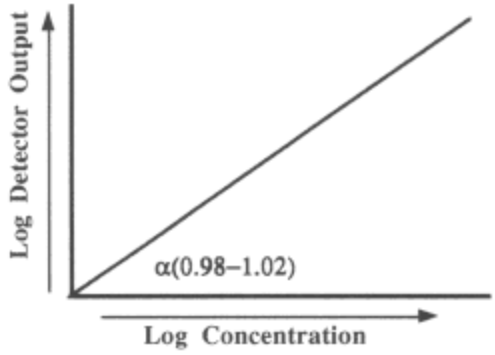

Figure 4.6
Graph of Log (V) against Log (c)

Data can be acquired by injecting samples of differing concentrations on to the column and measuring the respective area or height of the peaks obtained. For most practical purposes, true linearity can only be assumed if the response index (α) lies between 0.98 and 1.02 as shown in figure 4.6. If the response index is outside this range, then it will be necessary to use the numerical value of (α) to correct for the non-linearity.

Linear Dynamic Range

The *linear dynamic range* (D_L) of a detector is that range of solute concentration over which the numerical value of the *response index* falls within defined limits. For example, the *linear dynamic range* of a detector such as the FID might be specified as

$$D_L = 1 \times 10^{-10} \text{ to } 1 \times 10^{-5} \text{ g/ml } (0.98 < \alpha < 1.02)$$

The *dynamic range* of a detector (D_R), is that range over which the detector continues to respond to changes in solute concentration and is *not* the same as its linear dynamic range. The dynamic range may extend from 1×10^{-10} to 1×10^{-3} g/ml. The use of a detector outside its linear

dynamic range, is restricted to preparative chromatography, and today, GC is rarely employed for preparative purposes.

Detector Noise Level

Detector noise is the trace resulting from random changes in detector output in the absence of solute vapor. The detector noise is extremely important and determines the ultimate detector sensitivity or minimum detectable concentration. There are three types of noise, *short term noise, long term noise* and *drift*. These noise sources combine to give the *total noise* of the detector. The different types of noise are depicted in figure 4.7. Short term noise is the term given to baseline perturbations that have a frequency that is *significantly higher* than those of the eluted peaks. It is not often a serious problem as it can be easily removed by appropriate noise filters without significantly affecting the profiles of the peaks. Its source is usually electronic.

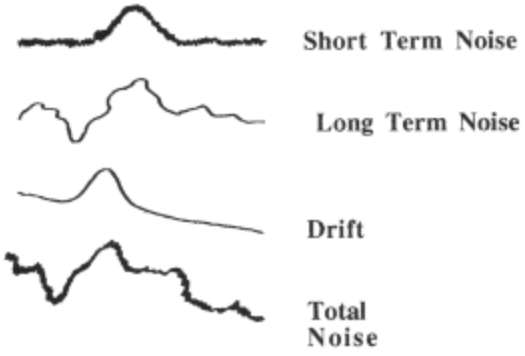

Figure 4.7
Different Types of Detector Noise

Long term noise is the term given to baseline perturbations that have a frequency that is *similar* to that of the eluted peak. Unfortunately, it is indiscernible from very small peaks in the chromatogram and is thus the most detrimental. It cannot be removed by electronic filtering without affecting the profiles of the eluted peaks. It is evident, in figure 4.7, that the peak profile can easily be discerned above the high frequency noise but is lost in the long term noise. Long term noise usually arises from temperature, pressure or flow-rate changes that occur in the detector sensing cell.

Drift is the result of baseline perturbations having a frequency that is significantly larger than that of the eluted peak. Drift is usually due to either changes in ambient temperature, changes in carrier gas flow-rate or column bleed. As a consequence, baseline drift often becomes very significant at high temperatures. In general, the sensitivity of the detector should never be set above the level where the combined noise exceeds 2% of the full scale deflection (F.S.D.) of the recorder (if one is used), or appears as more than 2% F.S.D. of the computer simulation of the chromatogram. Detector noise is measured as the maximum amplitude of the combined short and long term noise taken over a period of about 15 min..

Detector Response

Detector response can be defined as the voltage output for unit change in solute concentration. The detector response (R_c) can be determined by injecting a known mass of the chosen solute (m) onto the column and measuring the response from the dimensions of the peak. Assuming the concentration of the solute at the peak maximum is twice the average peak concentration, then the detector response is given by:

$$R_c = \frac{h\,w\,Q}{sm}$$

where (h) is the peak height,

(w) is the peak width at 0.607 of the peak height,

(Q) is the flow rate in ml/min,

and (s) is the chart speed or printer record equivalent.

Inevitably, the response of a detector will differ between different solutes and between different detectors. *Ipso facto*, the response of two detectors of the same type and geometry can only be compared under similar conditions.

Detector Sensitivity or the Minimum Detectable Concentration

Detector *sensitivity* or the *minimum detectable concentration* is the minimum concentration of an eluted solute that can be discerned

unambiguously from the noise. If a peak is considered decisively identifiable, then the ratio of the signal to the noise has been customarily taken as two. Although, this ratio originated from electronic theory, it is realistic, and any peak having a signal-to-noise ratio of less than two is seriously obscured by the noise and almost impossible to identify. Thus, the minimum detectable concentration is that concentration of solute in the mobile phase that provides a signal equivalent to twice the noise level.

Unfortunately, the concentration that will provide a signal equivalent to twice the noise level will usually depend on the physical properties of the solute used for measurement. Consequently, the detector sensitivity, or minimum detectable concentration, is quoted in conjunction with the solute that is used for measurement.

Thus, for a concentration sensitive detector, the detector sensitivity (X_D) is given by:

$$X_D = \frac{2N_D}{R_c} \quad (g/ml)$$

where (ND) is the noise level of the detector

GC detectors exhibit a wide range of sensitivities. At one extreme the katherometer has a sensitivity of about 1×10^{-6} g/ml and at the other, the electron capture detector can detect eluents at levels of 2×10^{-13} g/ml.

Pressure Sensitivity

The pressure sensitivity of a detector is one factor that determines the long term noise. It is the change in detector output per unit change in sensor-cell pressure. This specification is not critical for chiral chromatography as the katharometer (the detector most sensitive to pressure changes) is rarely used. The maximum pressure that the detector can tolerate is also important, particularly when multicolumn systems are employed and the detector is at the inlet pressure of the second column.

Flow Sensitivity

Flow sensitivity is measured as the change in detector output for unit change in flow rate through the sensor cell. The response of the FID is virtually unaffected by flow rate changes and, in fact, only responds to the *mass of solute passing through it per unit time*. In contrast, the katherometer is very sensitive to changes in flow rate and requires to be operated with a reference cell to compensate for any fluctuations in column rate.

Temperature Sensitivity

All GC detectors are thermostatted in their own oven, consequently GC detectors are relatively insensitive to changes in ambient temperature. In some circumstances detectors sensitivity to temperature changes can be important but not in chiral chromatography.

The Flame Ionization Detector

The FID, invented by Harley and Pretorious [4], and separately by McWilliams and Dewer [5], evolved from the Heat of Combustion Detector developed by Scott [6]. The FID detector employs hydrogen as the combustion gas which is mixed with the column exit gas (which may be helium, hydrogen or any other appropriate gas) and burnt at a small jet situated inside a cylindrical electrode. A potential of a few hundred volts is applied between the jet and the electrode and, when a carbon containing solute is burnt in the jet, the electron/ion pairs that are formed are collected at the jet and cylindrical electrode. The current is amplified and fed to a recorder or to the A/D converter of a computer data acquisition system. A diagram of the basic FID is shown in figure 4.8. During the process of oxidation, oxidized or partially oxidized fragments of the solute are formed in the flame, which are thought to generate electrons by thermionic emission. The electrons and ions are collected by a potential of 110 or 200 volts applied between the jet and the electrode. The background current (ions and electrons from the hydrogen flame

alone) is very small (1-2 × 10^{-12} amperes) and consequently, the noise level is also commensurably minute (about 10^{-14} amperes).

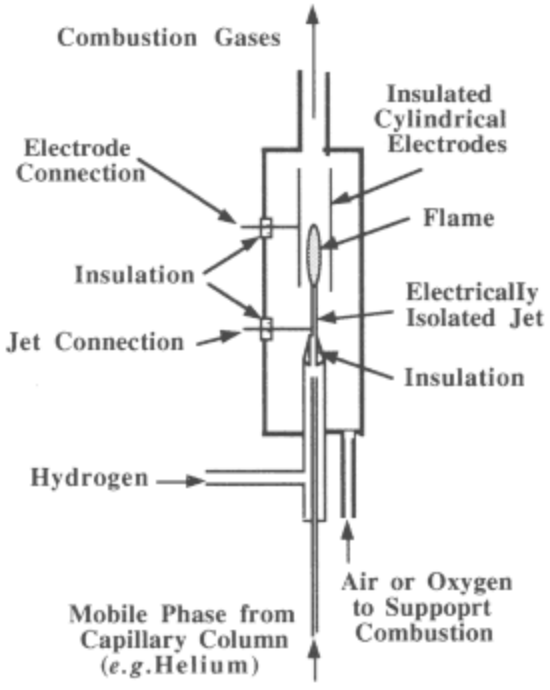

Figure 4.8
The Flame Ionization Detector

The ionization process is not very efficient, only 0.0018% of the solute molecules produce ions, (about two ions or electrons per 10^5 molecules). Nevertheless, because the noise level is very small, the minimum detectable mass of n-heptane is only 2 × 10^{-12} g/s. At a column flow rate of 20 ml/min, this is equivalent to a minimum detectable concentration of about 3 × 10^{-12} g/ml. The detector responds to *mass per unit time* entering the detector, not *mass per unit volume*, consequently, the response is almost independent of flow rate. It follows that the FID can be used very easily with capillary columns. Although the column eluent is mixed with the hydrogen prior to entering the detector, the diluting effect has no impact on the sensitivity. The FID detects virtually all carbon containing solutes, with the exception of a limited number of

small molecular compounds such as carbon disulfide, carbon monoxide, etc. In fact, due to its diverse and comprehensive response, it is considered a universal detector.

The Nitrogen Phosphorus Detector (NPD)

The nitrogen phosphorus detector (NPD), is a highly sensitive but specific detector. It gives a strong response to organic compounds containing nitrogen and/or phosphorus. Although it appears to function in a similar manner to the FID, in fact, it operates on an entirely different principle. A diagram of an NPD detector is shown in figure 4.9.

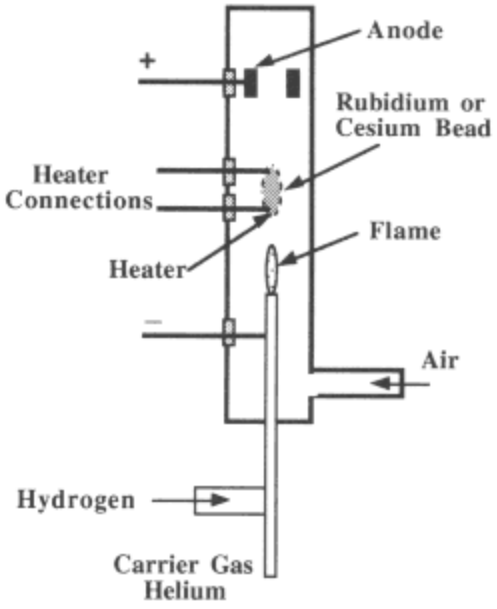

Figure 4.9
The Nitrogen Phosphorus Detector

The NPD sensor is a rubidium or cesium bead contained inside a small heater coil. The helium carrier gas is mixed with hydrogen and passes into the detector through a small jet. The bead, heated by a current passing through the coil, is situated above the jet, and the helium-hydrogen mixture passes over it. If the detector is to respond to both

nitrogen and phosphorus, then a minimum hydrogen flow is employed to ensure that the gas does not ignite at the jet. In contrast, if the detector is to respond to phosphorus only, a large flow of hydrogen can be used and the mixture burned at the jet. A potential is applied between the bead and the anode. The heated alkali bead emits electrons by thermionic emission which are collected at the anode and thus produce an ion current. When a nitrogen or phosphorus containing solute is eluted, the partially combusted nitrogen and phosphorus materials are adsorbed on the surface of the bead. This adsorbed material reduces the work function of the surface and, as consequence, the emission of electrons is increased which raises the current collected at the anode. The sensitivity of the NPD is about 10^{-12} g/ml for phosphorus and 10^{-11} g/ml for nitrogen).

Unfortunately, the performance deteriorates with time. Reese [7] examined the function of the NPD in great detail. The alkali salt employed as the bead is usually a silicate, and Reese showed that the reduced response was due to water vapor from the burning hydrogen, converting the alkali silicate to the hydroxide. At the operating temperature of the bead, the alkali hydroxide has a significant vapor pressure and consequently, the rubidium or cesium is continually lost during the operation of the detector. Eventually, all the alkali is evaporated, leaving a bead of inactive silica. This is an inherent problem with all NP detectors and, as a result, the bead needs to replaced fairly regularly if the detector is in continuous use.

The Electron Capture Detector

The electron capture detector consists of a small volume cell which contains a low energy β-ray source, usually ^{63}Ni. The detector can be operated in two ways, either with a constant potential applied across the cell (the DC mode) or with a pulsed potential across the cell (the pulsed mode). In the DC mode, hydrogen or nitrogen can be used as the carrier gas and a small potential (usually only a few volts) is applied across the cell that is just sufficient to collect all the electrons available and provide a small standing current. If an electron capturing molecule (for example a molecule containing a halogen atom which has only seven electrons in

its outer shell) enters the cell, the electrons are captured by the molecule and the molecules become charged. The mobility of the captured electrons are much reduced compared with the free electrons and thus the electrode current falls dramatically. There are some disadvantages to the use of the DC mode of detection arising from the variation of electron energy with applied potential. The electron capturing properties of molecules vary with the electron energy, and so the specific response of the detector to different molecules will depend on the applied potential

In the pulsed mode, a mixture of 10% methane in argon is usually employed and the electron capturing environment is quite different. The electrons generated by the radioactive source rapidly assume only thermal energy and, in the absence of a collecting potential, exist at the source surface in an annular region about 2 mm deep at room temperature and about 4 mm deep at 400°C. A short period square wave pulse is applied to the electrode collecting the electrons and producing a base current. The standing current, using 10% methane in argon is about 10^{-8} amp with a noise level of about 5×10^{-12} amp. The pulse waveform is shown in figure 4.10. During the inactive period of the waveform, electrons having thermal energy only will be come readily attached to any electron capturing molecules present in the cell and produce negatively charged ions. The negative ions quickly recombine with the positive ions and thus become unavailable for collection. Consequently the signal received will constitute a reduction in the standing current.

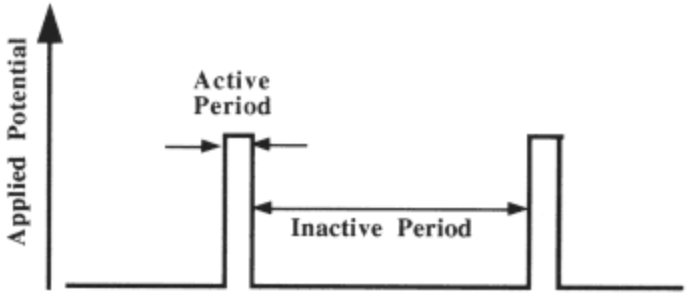

Figure 4.10
Waveform of Electron Capture Detector Pulses

The period of the pulsed potential is adjusted such that relatively few of the slow negatively charged molecules have time to reach the anode, but the faster moving electrons are all collected. During the "off period" the electrons re-establish equilibrium with the gas. The three operating variables are the pulse duration, pulse frequency and pulse amplitude. By appropriate adjustment of these parameters, the current can be made to reflect the relative mobilities of the different charged species in the cell and thus exercise some discrimination between different electron capturing materials.

A diagram of an electron capture detector is shown in figure 4.11. There are a large number of different detector designs, but the basic electron capture detector consists of a small chamber 1 or 2 ml in volume with two metal electrodes. The electrodes may be formed by concentric cylinders or by metal discs separated by a suitable insulator. The cell contains the radioactive source, usually electrically connected to the conduit through which the carrier gas enters and to the negative side of the power supply. A gauze "diffuser" is sometimes connected to the exit of the cell and to the positive side of the power supply. The electrode current is monitored by a suitable amplifier.

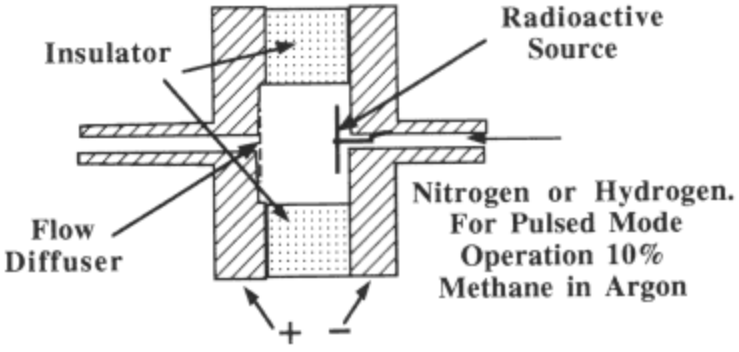

Figure 4.11
The Electron Capture Detector

The electron capture detector is extremely sensitive, probably the most sensitive GC detector available (*ca.* 10^{-13} g/ml) and is widely used in the detection and analysis of halogenated compounds, in particular,

pesticides. It will function using helium, argon or argon/methane mixtures as carrier gases.

The Katharometer (Thermal Conductivity or Hot Wire) Detector

One further detector should be mentioned that is commonly used for preparative chromatography employing packed columns. It is relatively insensitive (a feature that is necessary in preparative chromatography) but is generally not practical for use with capillary columns. It would probably be the detector of choice for preparing significant quantities of an enantiomer by preparative GC.

The katharometer detector [sometimes spelt catherometer and often referred to as the *thermal conductivity detector* (TCD) or the *hot wire detector* (HWD)] is the oldest GC detector still in use and commercially available. Although it is a relatively insensitive detector, it has survived largely as a result of its catholic response and use in preparative GC and gas analysis. It is simple in design and requires minimal electronic support and, as a consequence, is also relatively inexpensive. The precise manner in which it functions is not known as it responded to changes in both the *thermal conductivity* or the *specific heat* of the column eluent. It is possible that one or the other property may dominate in any particular system depending on the operating conditions employed, but the relationship is not simple.

The katharometer consists of a filament carrying a current which is situated in the column exit gas and, under equilibrium conditions, the heat generated in the filament is equal to the heat lost and consequently the filament assumes a constant temperature. The filament is made from a metal that has a high temperature coefficient of resistance and, at the equilibrium temperature, the resistance of the filament and thus the potential across, it will be constant. The heat lost from the filament will depend on both the thermal conductivity of the gas and its specific heat, both these parameters will change in the presence of a different gas or solute vapor. This causes change in resistance and a change in potential

across the filament which is amplified and monitored by a suitable recorder, or an appropriate data acquisition system.

As the detector filament is in thermal equilibrium with its surroundings, and the device actually responds to the heat lost from the filament, the katharometer detector is extremely *flow* and *pressure* sensitive. Consequently, all katharometer detectors must be carefully thermostatted and must be fitted with reference cells to help compensate for changes in pressure or flow rate.

There are two types of sensor, the "in-line" cell where the column eluent actually passes directly over the filament and the "off-line" cell where the filaments are situated away from the carrier gas stream and the gases or vapors reach the sensing element by diffusion. Due to the high diffusivity of vapors in gases, the diffusion process can be considered as almost instantaneous. A diagram of an in-line katharometer is shown in figure 4.12.

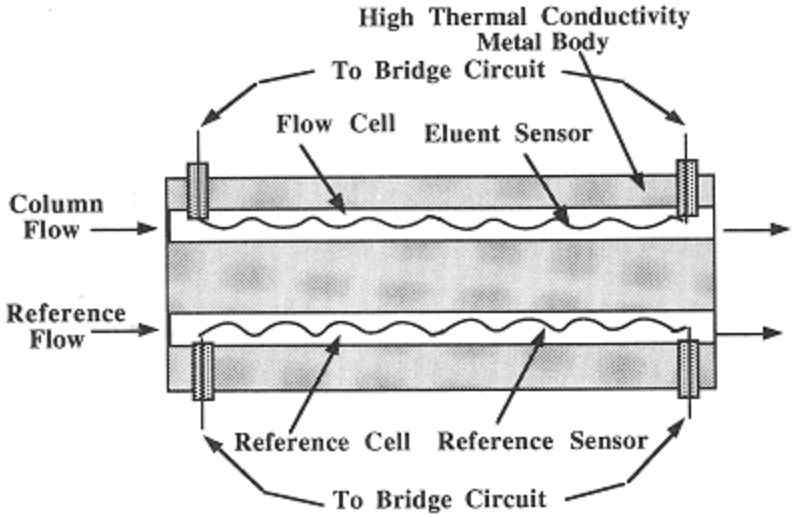

Figure 4.12
The Katharometer Sensor Cells (In-Line Cell)

The filament is usually tungsten or platinum, as both metals have reasonably high temperature coefficients of resistance and at the same time are very inert and unlikely to interact chemically with the column

eluent. Both filaments are situated in the arms of a Wheatstone Bridge, and a suitable current is passed through the filaments to heat them significantly above ambient temperature. The sensors and their conduits are installed in a high thermal conductivity metal block, which is thermostatted by means of a separate oven. This is necessary to ensure temperature stability. The out-of-balance signal, caused by the presence of sample vapor in contact with the sensor, is amplified and fed to a recorder or computer data acquisition system. For maximum sensitivity hydrogen should be used as the carrier gas, but to reduce fire hazards, helium can be used with very little compromise in sensitivity. The sensitivity of the katherometer is only about 10^{-6} g/ml (probably the least sensitive of all GC detectors) and has a linear dynamic range of about 500 (the response index being between 0.98 and 1.02). This detector can be used in most GC analyses that utilize packed columns and where there is no limitation in sample availability. The device is simple, reliable, and rugged and, as already stated, relatively inexpensive.

Data Acquisition and Processing

The acquisition and processing of data from chiral separations involves exactly the same type of equipment as that used in general GC analysis. The output from the detector is passed to a scaling amplifier that provides an output suitable for the digitizing range of the analog-to-digital converter. The digitized signal is then acquired by the computer and stored on disc. At the same time, some on-the-fly processing usually takes place. The output is continually printed out, thus providing a chromatogram in real time. In addition, the peak maximum is usually identified by a simple differentiating routine and the retention time printed out at the peak maximum peak maximum. At the end of the analysis, the peak areas are measured, response factors applied and a quantitative analytical report printed out. This represents the basic data processing package but other calculations and reports can be generated by different software. Often the beginning and end of the integration of each peak area is marked on the chart, corrected retention times, capacity ratios and sometimes separation ratios are also calculated and given in the report. Banks of retention data may be available on disk together with a

simple search routine to identify and label each solute. Different types of baseline correction may also be available and specific types selected to suit the particular chromatographic conditions. Once the detector output data is acquired and stored on disk, any chromatographic parameter can be calculated and reported, providing the necessary software is available or can be written by the user.

Synopsis

The basic gas chromatograph consists of five chromatographic components and three or four display and data processing units. The gas supply may be taken from compressed gas cylinders or gas generators. Nitrogen and oxygen can be generated from the laboratory compressed air by selective diffusion and cleaning and hydrogen and oxygen generated by electrolysis. The flow of gas to the detector and/or column is managed by mass-flow controllers actuated by the computer. Thus, the column can be flow programmed if so desired. A sample valve is used to place gas samples on the column and a hypodermic syringe and silicone rubber diaphragms used for placing liquid samples on the column. In packed columns, the sample can be discharged into a flash heater or directly into the actual column packing. Samples are placed on a capillary column using a similar device but with a split-flow, so that only a small portion of the sample passes onto the column. If large diameter tubular columns are used, the sample can be placed directly in the column. However, to prevent multi-peak formation, techniques such as the "retention gap injection" and "solute-focusing" may need to be used. The GC column oven temperature is controlled to ± 0.2°C and can maintained or programmed over a temperature range extending from 5°C to 400°C at program rates ranging from about 0.2°C per min to about 20°C per min. The detector is situated in its own thermostatted oven. Detector specifications should include, linearity, linear dynamic range, response, sensitivity or minimum detectable concentration, pressure sensitivity, flow sensitivity and temperature sensitivity. The most common detectors used in GC are the FID followed by the NPD and the ECD. The katharometer, another fairly popular detector, is used

mostly for gas analysis or as an option for preparative separations. Carbon containing molecules in the FID generate carbon fragments during combustion which emit electrons by thermal emission. These electrons are collected by an electrode system by a potential gradient of about 100–200 volts. The FID has a base current of about $1–2 \times 10^{-12}$ amperes, a noise level of less than 10^{-14} amperes and the minimum level of detection is about 2×10^{-12} g/s. The NPD detector is similar in form to the FID except that the hydrogen containing the solute vapor passes over a heated cesium or rubidium silicate bead. The heated bead emits electrons by thermal emission which are collected by a suitably placed electrode. When a nitrogen or phosphorus compound is present in the eluent gas, the partially combusted material is adsorbed on the surface of the bead and reduces the work function of the surface. This results in a increase in electron emission and an increase in current. If the detector is to respond to both nitrogen and phosphorus, a minimum flow of hydrogen is used that does not ignite. If a large flow of hydrogen is used, which burns, then the detector will only respond to phosphorus. The sensitivity is about 10^{-12} g/ml for phosphorus and 10^{-11} g/ml for nitrogen. Due to the formation of cesium or rubidium hydroxide from the presence of water, the alkali is continually lost at the bead temperature and thus the bead has a limited life and must be occasionally replaced. The ECD consists of a small volume cell containing a strong source of low energy β particles that ionize the gas in the cell. The electron current is collected across two electrodes under a relatively small applied voltage. In the DC mode, hydrogen or nitrogen is use as the carrier gas and all the electrons are connected providing a standing current. When an electron capturing substance enters the cell, some electrons are captured, and the large charged molecules move slower than the electrons and the current falls. In the pulsed mode, a 10% methane in argon mixture is use as the carrier gas. A pulsed voltage is applied to the electrodes and the free electrons are collected at every pulse. When some electrons are removed by electron capturing molecules, then the quantity of mobile electrons falls, and the pulse of current is reduced. This detector is probably the most sensitive GC detector (*viz.* 10^{-13} g/ml) but is selective for only those compounds that can capture

electrons. The katharometer detector is a low sensitivity device and can not be used with capillary columns, but is the detector of choice for packed columns used in the GC preparative isolation of an isomer. It functions by measuring the resistance of a wire situated in the column eluent. When a solute is eluted, the thermal conductivity and the specific heat of the carrier gas changes, which causes a change in the heat lost by the filament, resulting in a change in filament temperature and a consequent change in resistance. In data processing, the output of the detector is first scaled appropriately, digitized and the digitized signal acquired by a computer and then stored on disk. The output is printed in real time providing a chromatogram of the separation, which usually includes the location of peak maxima and the measurement of retention time. At the end of the separation, peak areas are measured, scaled according to their response factors and a quantitative report printed out.

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Chapter 5—
Gas Chromatography Chiral Stationary Phases and Columns

Although efforts were made to separate chiral substances in the late 1950s, the use of GC to separate enantiomers was not successfully started until the mid 1960s, at which time, the main developments in the general technique of GC had been completed and the methodology was well established. There were two reasons for this early lack of concern for chiral separations. Firstly, there was the lack of interest shown in the separation of chiral substances generally (the importance of their different physiological activities had, at that time, not been fully disclosed). Secondly, it was found very difficult to obtain adequate selectivity between such closely similar substances to effect a separation.

To achieve a separation between two substances, thermodynamics has shown that their standard free energy of distribution must differ. As the difference between enantiomers are solely spatial and not structural, any separation must be achieved by primarily changing the relative standard *free entropy* contribution to the standard free energy of each isomer. It will be seen later that this does not exclude a significant contribution from a change in free enthalpy as well, but the primary effect must be entropic in order to realize the corresponding change in free enthalpy. This will be better understood when actual separations are discussed. Thus, in order to obtain some selectivity between enantiomers, the structure of the stationary phase must be such that one isomer will fit

more closely to the stationary phase molecules (or surface) than the other(s). It is clear, that one way of achieving selectivity, would be to use a chiral stationary phase.

Early Days in Chiral GC

Early work in the field was frustrated by the small separation ratios that were obtained between the different optical isomers. Much effort was expended in the late 1950s on the separation of the amino acid optical isomers, but they were fruitless, and some successes claimed in the literature [1,2] could not be reproduced [3,4]. The first really successful separation of the amino acids by a GC separation procedure was obtained by Gil-Av *et al*. [5], and was reported in 1966. Gil-Av *et al*. immediately recognized that the small separation ratios between the isomers that were generally achievable demanded the high resolving power of the open tubular column, if a separation was to be realized. These workers also realized that if the separation was to be achieved in a reasonable time, then relatively high operating temperatures would also be necessary.

Gil-Av *et al*. argued that the separation of the antipodes on a suitable asymmetric stationary phase would involve reversible association between the enantiomers and the asymmetric stationary phase molecules. The two antipodes would form diastereoisomeric interaction with somewhat different interatomic distances. Hence, there would be different polar, dispersive and/or steoric interactions between substituents situated round the asymmetric centers of the solute and stationary phase molecules, respectively. Such differences would effect the standard free energy of distribution and the magnitude of the distribution coefficients of the enantiomers. Gil-Av *et al*. used *N*-TFA-D-isoleucine lauryl ester and *N*-TFA-L-isoleucine lauryl ester as the stationary phase which were coated on the walls of a capillary column 100 m long, 250 µm I.D. and was shown to have an efficiency of about 98,000 theoretical plates. The samples of the derivatized amino acids were injected with a split ratio of 1:100. The separation was carried out isothermally at 90°C and the analysis time was just over 4 hours. The results obtained for the

separation of some derivatized amino acids on both the (L) and (D) column are shown in figure 5.1.

The chromatograms are shown broken at specific intervals to contract the chromatogram for clear presentation.

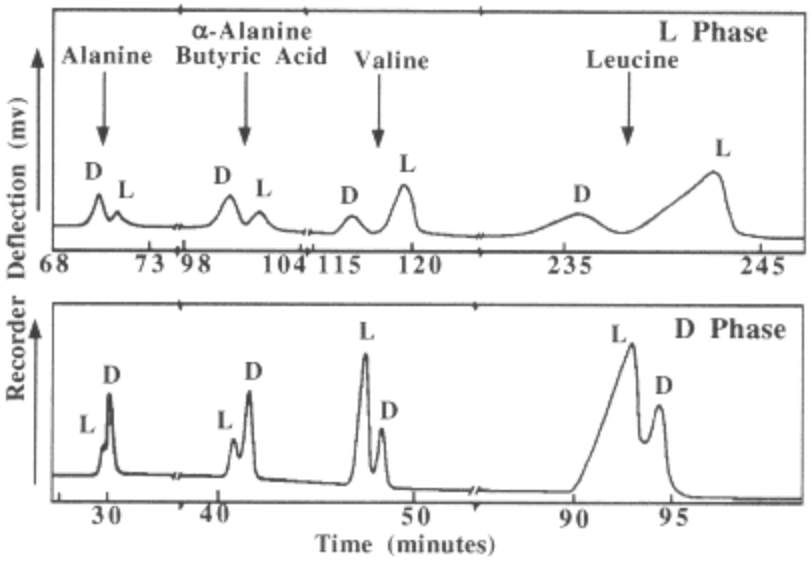


Figure 5.1
The Separation of the Enantiomers of *N*-TFA -(±)-
α-Amino Acid Isopropyl Esters

It is seen that the separation on the (L) phase appears to be slightly better than that on the (D) phase. It was also noted, that the (L) isomers eluted first on the (L) phase and the (D) isomers eluted first on the (D) phase. Thus, a given isomer would appear to interact more closely with its opposite stationary phase isomer and consequently be more retained. This would also imply an increase in standard free entropy change, which would also be accompanied by an even greater change in standard free enthalpy and consequently, greater retention. The disadvantage of the separation system was the very long analysis times which was caused by the relatively low operating temperature. Low operating temperatures

were essential due to the inherent poor temperature stability of these types of stationary phase.

Consequently, despite this relatively early successful application of the technique, the use of GC for the separation of enantiomers was still rather slow to develop. The delay, as already stated, was largely due to the temperature instability of the early stationary phases, which, at the elevated temperatures necessary, resulted in their racemization and consequent loss of selectivity. Although the chiral lability of the enantiomer solutes themselves can hardly be avoided, the key to the use of GC techniques to separate enantiomers proved to be the development of thermally stable chiral stationary phases.

Nevertheless, although the solution to the problem of chiral separations by GC was apparent, a thermally stable stationary phase was still not developed for over a decade. It was not until 1977 that Frank, Nicholson and Bayer [6] produced a chiral stationary phase by the co-polymerization of dimethylsiloxane with (2-carboxypropyl)methoxy-silane and L-valine-*t*-butylamide which proved to be far more thermally stable than those of Gil-Av. Frank *et al*. showed that by using polysiloxanes, the separation of most amino acids could be carried out in a much reduced analysis time. The polysiloxane phases had a much lower volatility and higher stability than previous chiral stationary phases and could be used up to temperatures of 175°C. It is clear that the shorter retention times were directly a result of the separation being carried out at a much higher temperature which, in turn, was due to the greater thermal stability of the polysiloxane phases.

The columns used were 20 m long, 300 µm I.D. and were pretreated with colloidal silicic acid to stabilize the film of stationary phase. The columns were coated with 0.15% solution of the stationary phase in chloroform using the static method of coating (the different methods of coating capillary columns will be described later). Prior to use, each column was conditioned at 175°C in a stream of hydrogen. The

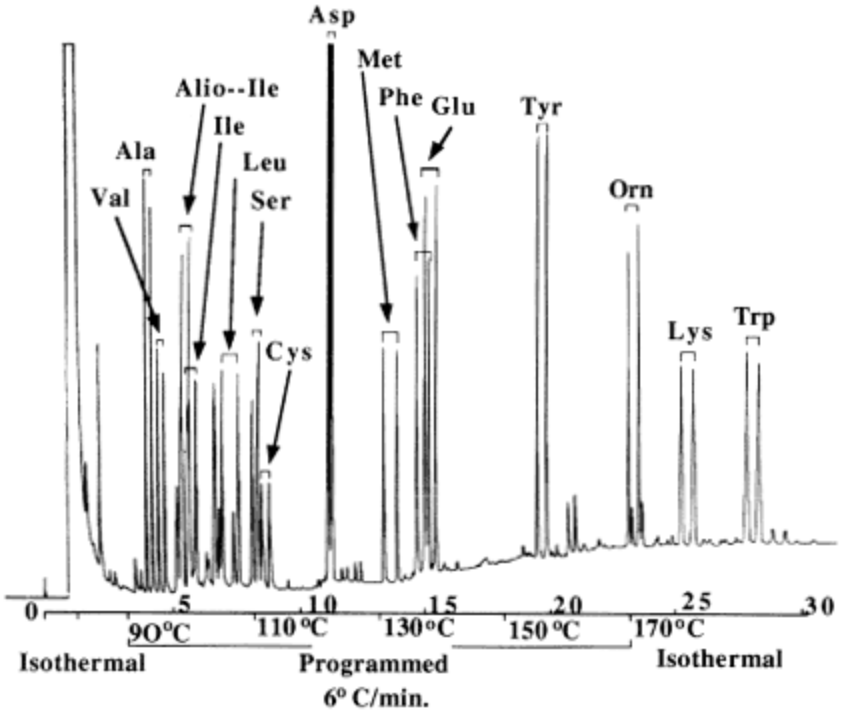


Figure 5.2
The Separation of 17 *N*-Pentafluoropropionyl D,L-Amino Acids on the Stationary Phase *N*-Propionyl-L-Valine *t*-Butylamide Polysiloxane
Courtesy of Supelco

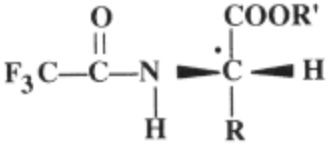
It is seen that the separation is completed in about 30 min., a much faster analysis than that of Gil-Av. It was found that the minimal operating temperature was 90°C, but the column could be programmed up to 175°C without column deterioration. As a result of the introduction of the chiral polysiloxane stationary phases by Frank *et al.*, the development of chiral GC gained momentum.

Chiral Stationary Phases for Gas Chromatography

Today, there are a number of effective chiral stationary phases suitable for GC, some based on amino acid derivatives, some on polysiloxane polymers and some, probably the more popular, based on cyclodextrin derivatives. Very few chiral columns, if any, are packed, the vast majority being open tubular columns having various lengths and an I.D. that ranges from about 250 µm or 320 µm There has, however, been a recent trend to smaller diameter columns (*cf.* 125 µm) to achieve higher efficiencies and even shorter analysis times.

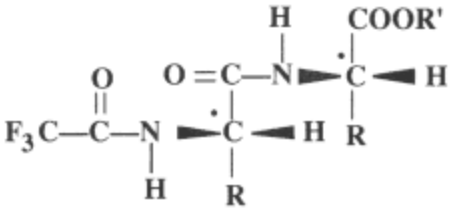
Small Molecule Stationary Phases

The first effective chiral stationary phase, as already referred to in chapter 1, utilized derivatized amino acids to provide chiral selectivity [7] and this was achieved as early as 1966. These types of stationary phases however, had very limited temperature stability and the optimum temperature for separation was often in excess of that at which the stationary phase was stable. The first relatively stable stationary phase, as already mentioned, was introduced by Bayer [6], who combined the derivatized optically active component of the stationary phase in a polysiloxane gum. Nevertheless, a number of small molecular weight materials were used as stationary phase in the early days of chiral gas chromatography. The first type introduced by Gil-Av are shown below.



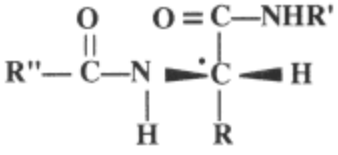
where R = *sec*-butyl and R' = dodecyl.

Despite the long alkyl chain introduced by Gil-Av, the phase lacked temperature stability and could not be used much above its melting point as excessive column bleeding occurred. In an attempt to increase the operating temperature dipeptide phases were examined [8,9]. An example of the type of dipeptides employed are as follows:



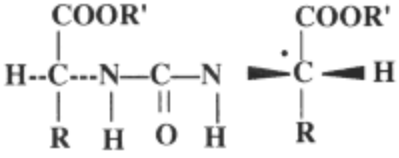
where R = isopropyl and R' = cyclohexyl

The dipeptide materials were, indeed, less volatile and, as a result of having two chiral centers, increased the chiral specificity (the probability of interaction with a chiral center was approximately doubled). The first separation of racemic glutamic acid was achieved using the dipeptide *N*-TFA-L-phenylalanyl-L-leucine cyclohexyl ester as the stationary phase. Tripeptides were also examined, but it was found their high melting points severely restricted their effective use as GC stationary phases. It was found that only the N-terminal amino acid contributed to high chiral selectivity [10,11], the C-terminal amino acid appeared to merely provide another amide group. As a result, the following structure was investigated which contains two thermostable amide groups.



where R = isopropyl, R' = *tert*-butyl, R" = undecyl ($C_{21}H_{43}$)

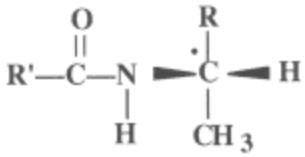
This dipeptide phase showed great promise and could be used satisfactorily at temperatures up to 200° C. The urea type phase, *N,N'*-carbonyl-*bis*(*L*-valine isopropyl ester) depicted as follows:



where R = R' = isopropyl

has been used very successfully for the separation of N-TFA amines [8]. However, its restricted temperature range of between 80°C and 100°C severely limited its area of application.

The presence of one imide function and one asymmetric center appears to suffice for the separation of N-acyl-α-amino acid esters, α-methyl-α-amino acid esters and α-methyl and α-phenyl carboxylic esters [12]. An example of this type of stationary phase is as follows,



where R = *l*-naphthyl and R' = undecyl

Chiral Polysiloxane Stationary Phases

The low thermal stability and relatively high volatility of the stationary phases discussed so far, limits their versatility, *e.g.* the separation of all the amino acids of biochemical interest in a single analysis. To allow high temperature operation, Bayer and his coworkers [6], synthesized a stationary phase that consisted of a chiral agent attached by an amide linkage to a carboxyl group of a polymer matrix of dimethylsiloxane or (2-carboxypropyl)-methylsiloxane. They combined the chiral selectivity of *L*-valine-*t*-butylamide with the high thermal stability and low volatility of the polysiloxanes. This stationary phase was eventually made available commercially as Chirasil-Val, which could be used over the temperature range of 30°C to 230°C. This material would allow the separation of all the racemic protein amino acids to be separated in one chromatogram taking only about 30 min.

An example of the separation of the *N*-(O,S)-pentafluoro-propanoyl-isopropylesters of the amino acids is shown in figure 5.3. It is seen that

the column bleed is quite manageable at 185°C and that a clean separation of all the enantiomers is achieved.

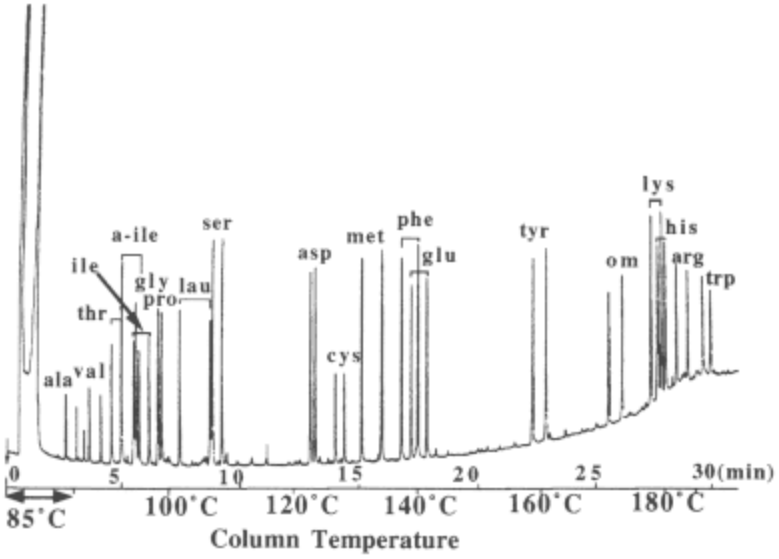
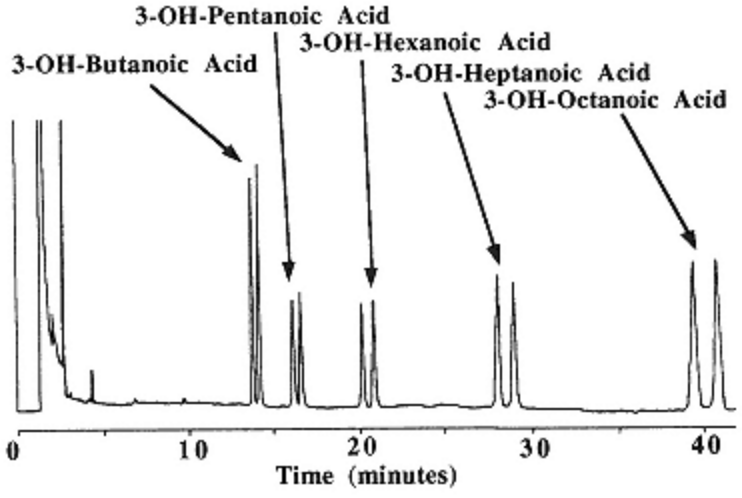


Figure 5.3
The Separation of the Protein Amino Acids as
N-(O,S)-Pentafluoro-Propanoyl-Isopropyl Esters on Chirasil-Val

Chirasil-Val can be used to separate many other chiral mixtures including aryl glycols, drug metabolites, 2- and 3-hydroxy carboxylic acids [13],, 2-halo carboxylic acids [14], and many underivatized substances such as alcohols, diketones and hydroxy lactones [15].

Chiral polysiloxanes can also be prepared from OV-225 (a well established polar GC stationary phase) by converting the cyano group into Chirasil-Val type polymers. Saeed *et al*. [16], hydrolyzed the cyano groups from OV-225 and the carboxy groups were coupled with *L*-valine-*t*-butylamide though the amino group. König *et al*. [17], produced a modified form of the same polymer containing (*R*)- or (*S*)-α-phenylethylamine as an additional chiral component. This latter stationary phase became known as XE-60-*L*-valine-*S*-α-phenylethylamide. In figure 5.4, the material is used to separate racemic β-

hydroxy acids as their *O*-(isopropyl-carbamoyl)isopropylamides. It is seen that the separation was complete in 40 minutes and the enantiomer pairs were well resolved, the (L) isomers being eluted first.



Column 25 m long, carrying XE-60-L-valine-*S*-α-phenylethylamide, operated at 180°C

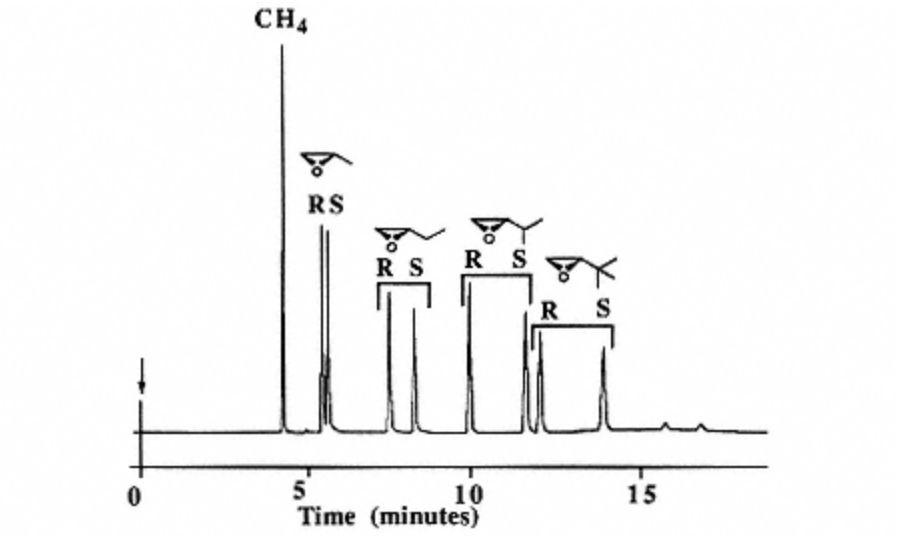
Figure 5.4
The Separation of Some β-Hydroxy Acids as Their
O-(Isopropyl-Carbamoyl)Isopropylamides
Courtesy of Angew. Chem. (ref.17)

König *et al*. [18] reduced the cyano groups of OV-225 to the amine and coupled it with *N*-acyl-L-Valine via the carboxyl group. This material was also applied to a wide variety of chiral separations.

Chiral Metal Chelating Stationary Phases

The resolution of chiral unsaturated hydrocarbons, ethers, ketones, etc., was sometimes found to be very difficult using the techniques previously discussed. It was thought that the interaction of such materials with optically active organometallic agents might aid in this type of separation. The validity of this concept was proved early in the

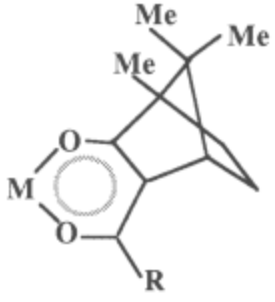
development of chiral GC by Schurig and Gil-Av in 1971 [19], using dicarbonylrhodium(I)-3-trifluoroacetyl-(1R)-camphorate to separate the racemic mixture of 3-methylcyclopentene coated on a stainless steel tube.



Glass Column 42 m long, 0.25 mm I.D. coated with the manganese complex in OV-101 and operated at 40°C

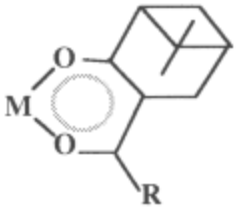
Figure 5.5
The Separation of the Enantiomers of Some Alkyloxiranes
Courtesy *J. Chem. Soc.* (ref. 19)

A number of other metal chelates have been investigated and a manganese chelate [20] was used to successfully separate the enantiomers of some alkyloxiranes. The structure of the complex was as follows:

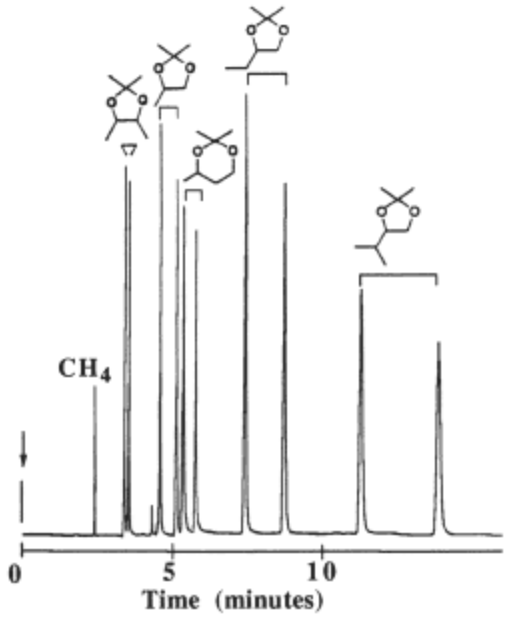


The metal and the attached group is given by: R = -CF_2 CF_2CF_3 and M = Mn. The separation that was obtained is shown in figure 5.5.

One final example of a separation by a chelation interaction is that of aliphatic diolacetonides. The chelating material had the following basic structure:



The chelate was incorporated in OV 101, a dispersive polysiloxane resin. The separation obtained is shown in figure 5.6.



Column 40 m long, 0.25 mm I.D. and operated at 80°C.

Figure 5.6
The Separation of Some Diol Acetonides
Courtesy of Anal. Chem. (ref. 20)

Cyclodextrin Chiral Stationary Phases

Despite the early successes with the polysiloxane based stationary phases carrying peptide chiral selection agents, the chiral selectors used in contemporary chiral GC are largely cyclodextrin based. The cyclodextrins are produced by the partial degradation of starch followed by the enzymatic coupling of the glucose units into crystalline, homogeneous toroidal structures of different molecular size.

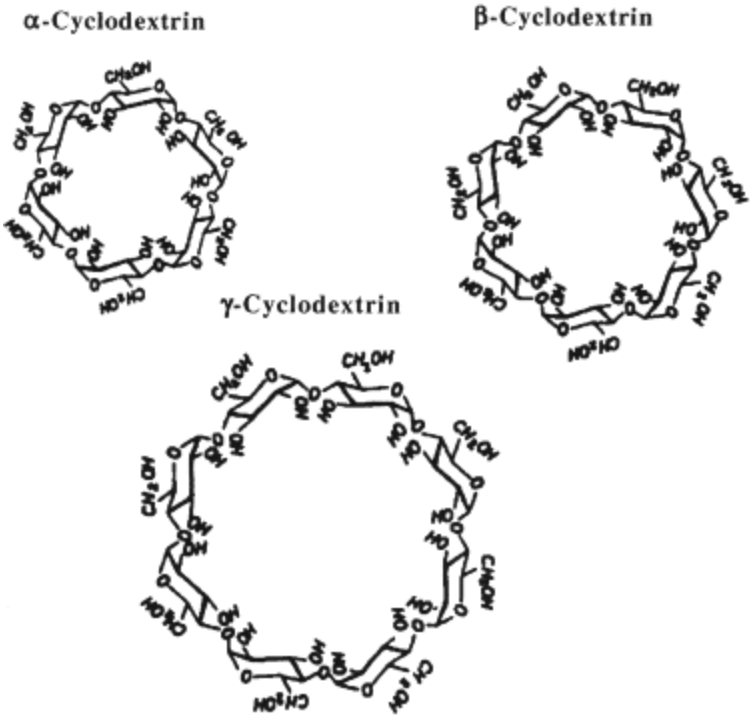


Figure 5.7
The Molecular Structure of α, β, and γ Cyclodextrins
Courtesy of ASTEC Inc.

Three of the most widely characterized are the *alpha*-, *beta*- and *gamma*-cyclodextrins and have been shown to contain 6 (cyclohexamylose), 7 (cycloheptamylose) and 8 (cyclooctamylose) glucose units, respectively.

These cyclic, chiral, torus shaped macromolecules contain the D(+)- glucose residues bonded through α-(1–4)glycosidic linkages. The arrangement of the glucose units in the three different forms of cyclodextrin are shown in figure 5.7.

The mouth of the torus-shaped cyclodextrin molecule has a larger circumference than at the base and is linked to secondary hydroxyl groups of the C_2 and C_3 atoms of each glucose unit (see figure 5.8).

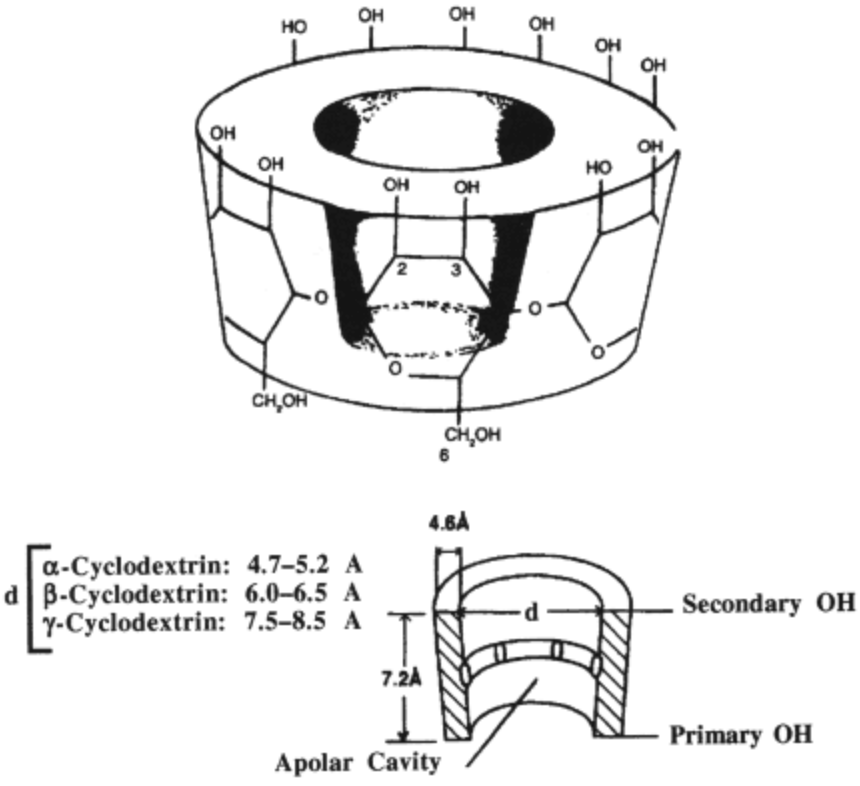


Figure 5.8
A Molecular Model of Cyclodextrin
Courtesy of Supelco

The primary hydroxyl groups are located at the base of the torus on the C_6 atoms. As these hydroxyl groups are free to rotate, they partially

block the base aperture. The size of the cavity increases with increasing number of glucose units (figure 5.7). The secondary hydroxyl groups can be reacted with appropriate reagents to introduce further interactive character to the cyclodextrin molecule. The very effective chiral characteristics of the cyclodextrins structures arise from the many chiral centers they contain, for example, β-cyclodextrin has 35 stereogenic centers. They are probably the most effective stationary phases presently available for the GC separation of stereoisomers.

When the α, β, or γ cyclodextrins are derivatized, the hydroxyl group on the 2-position reacts first. However, the derivative is still size selective and interaction will be determined by the size and functional groups contained by the interacting molecule. For example, very small molecules would be separated on the derivatized α-cyclodextrin whereas the large molecules would be separated on the derivatized γ-cyclodextrin, the derivatized β-cyclodextrin being selected for intermediate sized solute molecules. In contrast, if the 3-position hydroxyl group is derivatized all the cyclodextrins completely loose their size selectivity. Derivatizing the 6-hydroxyl position has little or no effect on chiral selectivity but does enhance the loading capacity of the stationary phase. This position is used for anchoring the cyclodextrins to silica gel in the preparation of LC stationary phases.

The α-, β- or γ- cyclodextrins that have been permethylated must be dissolved directly in appropriate polysiloxanes mixtures, and coated on the walls of a glass or fused quartz open tubular columns. The underivatized cyclodextrins, and those that have not been permethylated, can be coated directly onto the walls of the column. Many alcohols, diols, carboxylic acids, alkanes and cycloalkanes can be separated directly on such columns without derivatization. To improve the thermal stability of the mixed stationary phase, a phenylpolysiloxane can be included in the coating material. The presence of phenylpolysiloxane can also improve its stability towards oxidation at elevated temperatures, as cyclodextrin, being basically a sugar, is very susceptible to oxidation at high temperatures. Some methysiloxane, however, must be present to increase

the solubility of the cyclodextrin in the polymer matrix. The chiral activity can be further augmented by bonding other chirally active groups onto the secondary hydroxyl groups of the cyclodextrin (see again figure 5.8). Unfortunately, much of the chemistry used to derivatize these cyclodextrin compounds is proprietary and so synthetic details can not be given.

Some derivatized cyclodextrins can be extremely vulnerable to degradation, if they come in contact with water vapor. It is therefore imperative that all carrier gases are completely dry and that careful steps are taken to ensure that any sample that is placed on the column is also completely dry. Again, due their sugar characteristics, the cyclodextrins are very susceptible to oxidation by traces of free oxygen in the carrier gas consequently, oxygen free gasses must be used. The use of α-, β- and γ-cyclodextrin and a variety of their derivatives for the separation of enantiomers has been extensively studied for GC by Armstrong *et al*. [20], Li *et al*. [21] and Schurig and Novotny [22].

Some examples of specific derivatized cyclodextrin stationary phases have been described in the literature. In one product, the positions 2 and 6 are alkylated (pentylated) which provides very dispersive (hydrophobic) centers that can interact effectively with any alkyl chains of the solutes. Subsequent to the pentylation of the 2 and 6 positions, the 3 position hydroxyl group can then be trifluoroacetylated producing a very different stationary phase. This stationary phase has a wide field of application, the derivatized γ-cyclodextrin being more selective than the β material. It has been successfully used for the separation of the widest range of compound classes, from very small to very large molecules. Another stationary phase involves the substitution of the cyclodextrin hydroxyl groups with pure 'S' hydroxypropyl groups followed by permethylation. This reduces the size selectivity of the material but at the same time introduces more polar (hydrophilic) groups. The β material has a greater chiral selectivity than the α or γ phases. This material provides a good general purpose column. It is clear that there are many possibilities for derivatizing the cyclodextrins to provide unique

interactive character; there are a large number commercially available at this time and many more are likely to be synthesized in the future. Other cyclodextrin stationary phases will be discussed when the applications of chiral GC are considered.

Factors Controlling Selectivity

The chemical structure and the stereoisomerism of a stationary phase are not the only factors that control chiral selectivity. There are two other major factors that also affect selectivity and they are the stationary phase loading on the column and the operating temperature. The effect of these two variables on the retention ratio of a pair of enantiomers has been reported by Supelco and their data is shown in figure 5.9.

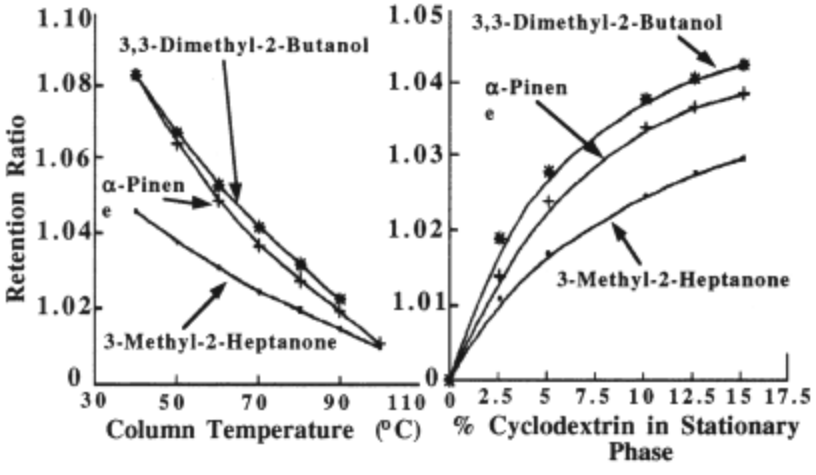


Figure 5.9
Curves Showing the Relationship between Retention Ratio and Stationary Phase Loading and Temperature
Courtesy of Supelco

The retention ratio of a pair of enantiomers increases steadily with the amount of cyclodextrin in the stationary phase mixture. This increase tends to flatten above 15%. The effect of temperature is even more interesting and it is clearly seen that temperature is a major factor controlling selectivity. Reducing the operating temperature from 100°C

to 70°C increases the retention ratio of the isomers of α-Pinene from about 1.015 to about 1.035. However, the reduced temperature will also cause an increase in retention time and thus a longer analysis time. The situation becomes even more confused, when it is realized that as the retention ratio has been increased, an excess of theoretical plates is now available and the column can be shortened. This, in turn, will reduce the retention and analysis times. The net advantage or disadvantage of raising or lowering the column temperature to control analysis time must be assessed quantitatively, and this will be carried out when open tubular columns are considered.

Open Tubular Columns

Most contemporary open tubular columns are made from fused quartz although, in some applications, stainless steel columns or even soft glass can be successfully used with equivalent performance. However, fused quartz columns are perceived as "state of the art" columns and are highly satisfactory for use in chiral GC. Quartz must be drawn at extremely high temperatures and are produced using argon arc heating systems. During the drawing procedure, the outside skin of the tube cools more rapidly than the inside causing stresses in the outside skin. These stresses allow the adsorption of water on the surface to initiate cracks in the fused quartz and render it extremely friable. To eliminate this physical shortcoming, the outside of the capillary tube is coated with a polyimide polymer, immediately after drawing. The layer of polyimide prevents contact with water and thus stabilizes the tube (Patent held by HP Inc. US 4,293,415). As a result of their mechanical strength, the original, rigid soft glass capillary tubes do not need coating to prevent fracture but thin, flexible soft glass tubes do, unless very carefully annealed.

The internal surface of an open tubular column usually requires deactivation and/or cleaning before it can be coated with stationary phase. Deactivation procedures used for commercial columns are seen by many to be "shrouded with mystery" and the procedures are usually considered as highly proprietary. Although some unique or special samples may, indeed, need select and complete column deactivation,

many samples, including those for chiral analysis, can be analyzed on columns that are deactivated by a simple clean-up procedure.

A simple column preparation program for silica and soft glass columns would first entail an acid wash. The column is filled with 10% w/w hydrochloric acid, the ends sealed and heated to 100°C for 1 hour. It is then washed free of acid with distilled water and dried. This procedure is thought to remove traces of heavy metal ions that are believed to cause adsorption effects. The column is then filled with a solution of hexamethyldisilazane, sealed, and heated to the boiling point of the solvent for 1 hour. The reagent reacts with any surface hydroxyl groups that were formed on the fused silica or glass surface during the acid wash and, in effect, chemically 'blocks' them from chromatographic availability. It may be advantageous to employ a polar or semipolar reagent as opposed to the dispersive silicone, if the column is to be subsequently coated with a polar stationary phase. The column is then washed with the pure solvent, dried at an elevated temperature in a stream of pure nitrogen and will then be ready for coating.

Open tubular columns can be coated internally with the appropriate stationary phase dissolved in a suitable solvent, or, alternatively, with monomeric materials that are subsequently polymerized to form a relatively rigid polymer film on the column walls. There are two basic methods for depositing the stationary phase as a surface film on a capillary column, dynamic coating and static coating, the latter being the most commonly used.

Dynamic Coating

Dynamic coating is carried by placing a plug of solvent containing the stationary phase at the beginning of the column. The strength of the solution, among other factors, determines the thickness of the stationary phase film. In general, the film thickness of an open tubular column ranges from 0.10 µ to about 0.75 µ. As an estimation, a 5% w/w of stationary phase in the solvent will provide a stationary film thickness of about 0.5 µ. However, this can only be considered very approximate as

the film thickness will also be determined by the nature of the surface, the solvent and the stationary phase. Furthermore, each of these three factors can have a very significant effect on the coating efficiency and consequently the eventual film thickness. The coating procedure is depicted in figure 5.10.

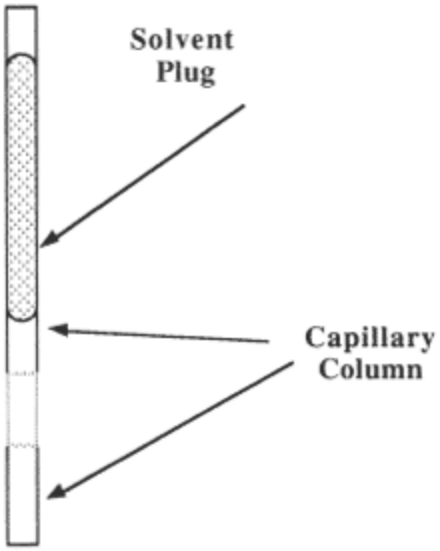


Figure 5.10
The Dynamic Coating Procedure for an
Open Tubular Column

A plug of stationary phase solution is run into the front of the column (sufficient solution should be added to fill about 10% of the column length) and a gas pressure is applied to the front of the column so that the plug velocity through the column is about 3 mm/second. After the plug has passed through the column, the gas flow is continued for about an hour. It is important not to increase the gas flow too soon, otherwise the stationary phase solution adhering to the walls of the tube is displaced forward in the form of ripples, which produces a very uneven film. After an hour, the flow rate is increased and the column stripped of solvent by evaporation. This procedure requires some practice to provide

evenly coated columns of the desired film thickness, but the thickness of the stationary phase film can be controlled, and is probably the simpler of the two coating procedures to use.

Static Coating

Static coating is carried out by filling the entire column with a solution of the stationary phase, sealing one end, and applying a vacuum to the other. As the solvent evaporates, the plug front retreats back down the tube leaving a coating on the walls. A diagram of the static coating procedure is shown in figure 5.11.

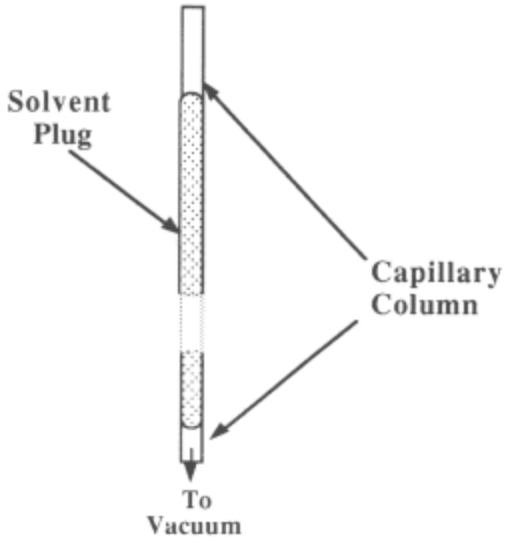


Figure 5.11
The Static Method for Coating Open Tubular Columns

Column Regeneration

One of the more popular cyclodextrin columns is derivatized with trifluoroacetic acid. If follows, that in the presence of water, the material will be hydrolyzed and the stationary phase will loose its chiral selectivity. This will occur if the carrier gas is not completely dry and/or

if the samples injected on the column contain water. Loss of column efficiency is a symptom of general column degradation and regeneration will not be possible and column replacement will be necessary. However, loss of chiral selectivity accompanied by little or no loss of efficiency, usually indicates that the column can be regenerated.

The column can be regenerated using the apparatus shown in figure 5.12. The column is first heated to 150°C for 30–60 min. The apparatus shown in figure 5.12 is then assembled.

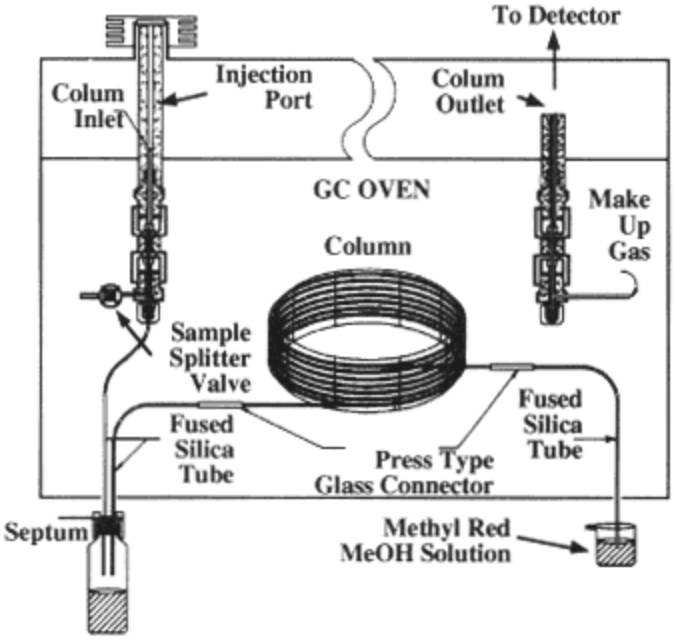


Figure 5.12
Column Generation Procedure
Courtesy of ASTEC Inc.

Fused silica tubing is quite flexible and can be allowed to pass through the joint between the oven door and the oven wall, and the oven door carefully closed without damaging the column. The injection port is connected to the vial, using a 2 m length of capillary tubing and arranged so that it dips into the headspace above the trifluoroacetic anhydride. The

headspace of the vial is then connected to the capillary column with a second piece of capillary tubing. Connections can be made with Teflon tubing. The end of the capillary column is then connected to the beaker containing a solution methyl red in methanol in such a way that the carrier gas from the column bubbles through the methyl red solution. A pressure is applied to the column and adjusted until a good flow of bubbles passes through the indicator solution. After about 20–30 min, the indicator changes from yellow to red. The anhydride in the exit gas should be adsorbed by allowing it to bubble through a 1 M solution of caustic soda. The oven is then programmed up to 150°C at 5°C per min. and held at 150°C for 30 min. The tubing is disconnected, the apparatus dissembled and the column is reassembled in the normal manner. Finally, the column is again programmed from 40°C to 150°C at 5°C/min and held at 150°C for 45 min. The column should now be regenerated and ready for use.

Capillary Column Design and Choice

In any GC analysis, the column must achieve two goals, the solutes of interest must be separated, and the analysis must be completed in as short a time as possible. Stereoisomers often have very small retention ratios and thus demands are made both on the column performance and the selectivity of the stationary phase. In order to consider these goals and how they can be realized it is necessary to resort to GC column theory. Purnell [23] derived an equation that gave the column efficiency (n) necessary to separate a pair of solutes having a separation ratio (α) where the first of the pair is eluted at (k').

$$n = 16 \frac{(1+k)^2}{k^2(\alpha-1)^2} \qquad (1)$$

Using equation (1), curves can be constructed relating efficiency required to achieve a separation to separation ratio for a series of (k') values. The calculated curves are shown in figure 5.13. It is seen that the efficiency required to achieve the separation increases dramatically as the

retention ratio is reduced. More important it is seen that higher efficiencies are needed when the solutes are eluted at low capacity ratios (k'). It follows that the optimum operating conditions that are necessary to achieve a particular separation rapidly may be quite difficult to recognize. In order to identify a rational approach to column optimization, a little basic capillary column theory must be employed. The Golay equation [8] that describes the variance per unit length (H) of an open tubular column is as follows:

$$H = \frac{2D_m}{u} + \left[\frac{\left(1 + 6k' + 11k'^2\right) r^2}{24\,(1 + k')^2 D_m} + \frac{k'^3 r^2}{6\,(1 + k')^2 K^2 D_s} \right] u \qquad (2)$$

or:

$$H = \frac{B}{u} + Cu \qquad (3)$$

where:

$$B = 2D_m$$

and:

$$C = \frac{\left(1 + 6k' + 11k'^2\right) r^2}{24\,(1 + k')^2 D_m} + \frac{k'^3 r^2}{6\,(1 + k')^2 K^2 D_s}$$

Taking the ratio (ρ) of the second term to that of the first:

$$\rho = \frac{4k'^3 D_m}{\left(1 + 6k' + 11k'^2\right) K^2 D_s} \qquad (4)$$

Now it has already been shown that:

$$k' = \frac{K v_s}{v_m} \quad \text{or} \quad K = \frac{k' v_m}{v_s}$$

Now:

$$\frac{v_m}{v_s} = \frac{\pi r^2}{2\pi r d_f} = \frac{r}{2d_f}$$

Thus;

$$K = \frac{k' r}{2d_f}$$

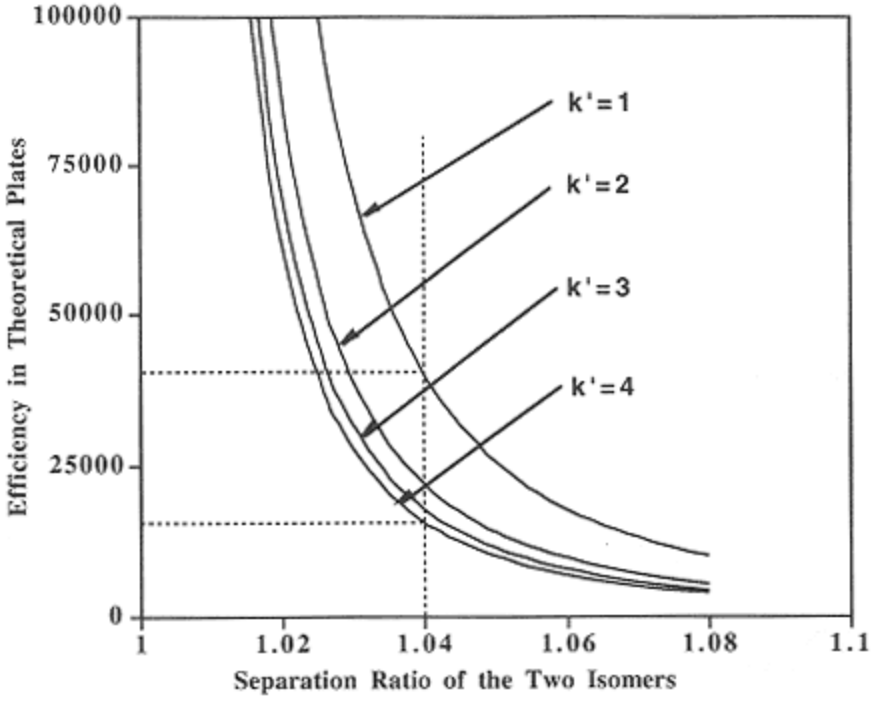


Figure 5.13
Graph of Efficiency Necessary for Resolution against Retention Ratio

Substituting for (K) in equation (4):

$$\rho = \frac{16\,k'\,d_f^2 D_m}{\left(1 + 6k' + 11\,k'^2\right) r^2 D_s} \qquad (5)$$

Consider a column 250 μm I.D. carrying a film 0.2 μμ thick, and as in GC, D_m ranges from (1–4) $\times 10^{-1}$ cm^2/s and D_s from (1–4) $\times 10^{-5}$ cm^2/s

Then:

$$\frac{D_m}{D_s} \cong 10^4$$

Substituting for $\frac{d_f^2}{r^2}$ and $\frac{D_m}{D_s}$ in equation (5):

$$\rho = \frac{0.102\,k'}{\left(1 + 6k' + 11\,k'^2\right)}$$

It is seen that for all values of (k'), the second function for (C) will only be a small percentage of the first function and thus can be ignored.

Consequently:

$$C = \frac{\left(1 + 6k' + 11\,k'^2\right) r^2}{24\,(1 + k')^2 D_m}$$

Differentiating equation (2) with respect to (u):

$$\frac{dH}{du} = -\frac{B}{u^2} + C$$

Thus:

$$H = H_{min}, \quad \text{then,} \quad -\frac{B}{u^2} + C = 0$$

and:

$$u_{opt} = \sqrt{\frac{B}{C}} \,.$$

Now:

$$H_{min} = \frac{B}{u_{opt}} + Cu_{opt.}$$

$$= \frac{B}{\sqrt{\frac{B}{C}}} + C\sqrt{\frac{B}{C}}$$

or:

$$H_{min} = 2\sqrt{BC}$$

Again substituting for (B) and (C):

$$H_{min} = 2\sqrt{(2\,D_m)\left(\frac{\left(1 + 6\,k' + 11\,k'^2\right)r^2}{24\,(1 + k')^2 D_m}\right)}$$

$$H_{min} = r\sqrt{\left(\frac{\left(1 + 6k' + 11k'^2\right)}{3(1+k')^2}\right)}$$

Thus the length (1) of column required is nH_{min} which from equation (1) is given by:

$$l = \frac{16(1+k')r}{k^2(\alpha-1)^2}\sqrt{\left(\frac{\left(1 + 6k' + 11k'^2\right)}{3}\right)} \qquad (6)$$

Now, from equation (26) in chapter 2, the separation ratio (α) of a solute pair and the capacity ratio (k') of the first eluted solute is given by:

$$\alpha = e^{\frac{\psi_1-\psi_2}{T} + (\phi_1-\phi_2)} \qquad (7)$$

and

$$k' = e^{\frac{\psi_1}{T} + \phi_1} \qquad (8)$$

where (ψ1) and (ψ2) are the standard free enthalpies divided by (RT) of the solute pair,

(ϕ1) and (ϕ2) are the standard free entropies divided by (R) of the solute pair,

and (T) is the absolute temperature.

Substituting for (α) and (k') from equations (7) and (8) in equation (6)

$$l = \frac{16(1+e^{\frac{\psi_1}{T}+\phi_1})r}{k^2(e^{\frac{\psi_1-\psi_2}{T}+(\phi_1-\phi_2)}-1)^2} x \sqrt{\frac{\left(1+6e^{\frac{\psi_1}{T}+\phi_1}+11\left(e^{\frac{\psi_1}{T}+\phi_1}\right)^2\right)}{3}} \qquad (9)$$

Employing a simple computer program and equations (7), (8) and (9), the values of (α), (k') and (L) can be calculated over a range of temperatures. The results obtained are shown in figure 5.14.

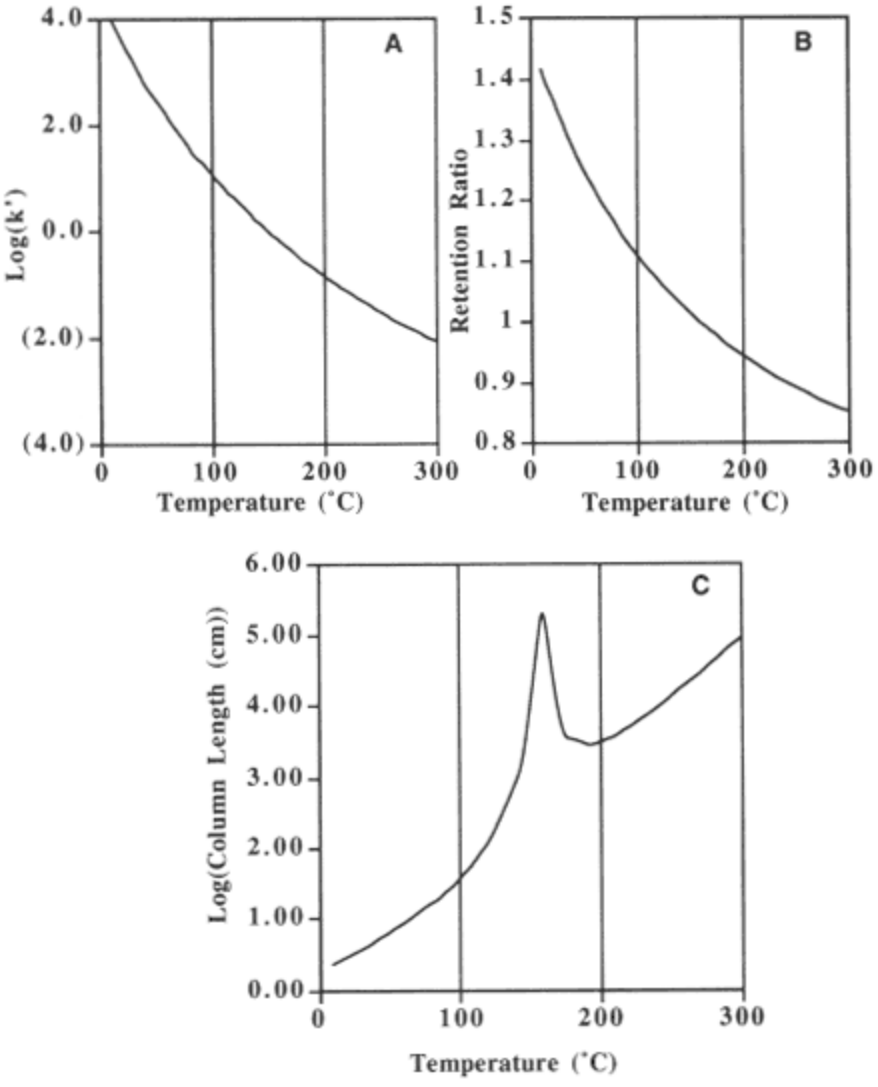


Figure 5.14
Graphs of Separation Ratio, Capacity Ratio and Minimum Column Length against Temperature

Referring to figure 5.14(C), it is seen that the minimum column length required to separate the phenyl ethanol enantiomers exhibits a curious relationship with column temperature. It should be noted that assuming all the columns are operating at their optimum velocity, the column length will be approximately proportional to the analysis time (time for the elution of the second component). At low temperatures, the column length and analysis time is exceedingly small, the column length being about 2 cm. Although the separation could theoretically be accomplished with a column of 2 cm long, due to both the very high value for (k') of the first peak and the retention ratio of the pair being relatively large, other factors would preclude its use. A column 2 cm long would have an impossibly small loading capacity and thus strain the performance of even the most sensitive detectors. In addition, it would be impossible to place a small enough charge on the column, even with the most sophisticated capillary column sampling techniques.

As the temperature rises, the (k') value and the retention ratio decreases resulting in an increase in column length and an increase in analysis time. When the temperature has reached 120°C the column length is 1 m, and with great experimental care, this column could probably be satisfactorily used with the most sensitive detectors and with a split injection procedure. As the temperature rises still further, the temperature of coelution is approached (where the elution order is reversed) and the retention ratio becomes very small and exceedingly long columns (1000 m) would be necessary to effect a separation. This would be accompanied with proportionally long analysis times. In fact, at the temperature of coelution, the column length and the analysis time would be infinitely large. It is interesting to see that the effect of further increase in temperature results in an increase in the retention ratio (a ratio of 0.91 is equivalent to 1.1 after the elution order is reversed) and thus the minimum column length is reduced. However, the (k') values also falls as the retention ratio increases and eventually the effect of the very small (k') value will dominate the demand for theoretical plates. Thus, although the retention ratio continues to increase, the minimum column length also increases accompanied by a proportional increase in analysis time. It is seen that although the various effects can be explained

on a theoretical basis, the relationship between column length and analysis time is complex and each separation will need to be assessed on its own characteristics. It should be emphasized that although the curves shown in figure 5.14 are generally of a form that will be exhibited by all enantiomeric pairs, they are specific to the enantiomers of phenylethanol. They are, thus, unique to that substance and the numerical values shown will digress considerably between different enantiomeric pairs.

Stationary Phases for the GC Separation of Chiral Substances

The vast majority of chirally selective stationary phases employed in GC are based on α, β or γ-cyclodextrin and a range of derivatives have been introduced that are designed to enhance the separation of enantiomers of different types and classes of compounds. The derivatized cyclodextrins are usually physically incorporated into appropriate polysiloxanes which are then coated on the column walls or onto an inert support. In certain stationary phases, the polysiloxane contains a proportion of phenyl groups which renders the mix more thermally stable and thus, can be used at higher temperatures. The level of the cyclodextrin component in the stationary phase varies between about 8% and 15% depending, to a large degree, on the solubility of the cyclodextrin based material in the particular polysiloxane which can be enhanced by introducing sufficient methylsiloxane into the polymer mix.

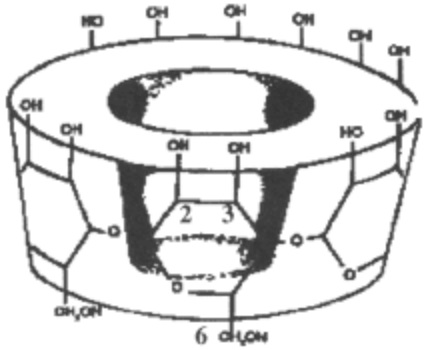


Figure 5.15
The Cyclodextrin Molecule

As already discussed, there are two parts of the cyclodextrin molecule that can be derivatized, but only one part significantly effects its chiral selectivity. Referring to the cyclodextrin molecule in figure 5.15, the OH group on the 6-position is usually alkylated and as the group is free to rotate, it blocks the base aperture to the cone and thus the 6-position offers no chiral selectivity. In addition, the alkylation of the 6-position can increase the solubility of the cyclodextrin in the polysiloxane matrix.

Groups on the 2 and 3-positions, however, can strongly effect selectivity. The enantiomer that fits closest to the chiral centers will have much greater interaction with groups substituted on positions 2 and/or 3 and thus by choosing appropriate groups, specific chiral selectivity can be introduced for particular solute types. It should be remembered, however, that derivatization in the 3-position removes the size selectivity of the cyclodextrin molecule. Groups with interactive character ranging from strongly polar(hydrophilic) to highly dispersive(hydrophobic), together with all the subtle combinations between, can be inserted, offering an almost limitless range of unique selectivities.

The permethylated β-cyclodextrin has been shown to have broad chiral selectivity based largely on polar (hydrophilic) interactions and in some cases inclusion/size selectivity. This material is thermally stable up to temperatures of 230°–250°C and appears to have the potential to separate over 30% of the GC chiral separations published to date.

The dimethyl derivatives of β-cyclodextrins have a similar temperature stability to the permethylated products and also exhibits broad chiral selectivity. There is some size selectivity but dispersive (hydrophobic) interactions appear to dominate the retention mechanism.

The stationary phases described above represent the more popular types presently used in chiral GC. There is, however, still a wide range of alternative cyclodextrin derivatives to be synthesized and evaluated and it is certain that many more stationary phases based on the chiral selectivity of cyclodextrin will be developed in the future. Nevertheless, the stationary phases mentioned above will serve to separate most chiral

mixtures, albeit some analysis times may be long, and some may need very high efficiencies to achieve the separation.

Synopsis

The first effective GC chiral separation was by Gil-Av, who resolved the enantiomers of some derivatized amino acids using the lauryl ester of a derivatized isoleucine as the stationary phase. The stationary phase was not very temperature stable. It was some years later that Bayer also used a derivatized amino acid as the stationary phase but this was incorporated in a polysiloxane that stabilized the material up to a temperature 175°C. Subsequently a number of polysiloxane stationary phases containing chiral agents were examined, including peptides, dipeptides and ureides. For example the cyano group on OV-225 was hydrolyzed and coupled with L-valine-t-butylamide to produce a chiral polysiloxane. In this way, the Chiral-Val stationary phase was produced which is still in fairly common use today. Organometal compounds containing rhodium or manganese chelated to chiral groups were also used to produce unique chiral selectivity in polysiloxane stationary phases. Today, the most important chiral stationary phases used in GC are the derivatized cyclodextrins. The cyclodextrins are produced by the partial degradation of starch followed by the enzymatic coupling of the glucose units into crystalline, homogeneous toroidal structures of different molecular sizes. Three of the most widely characterized are *alpha, beta* and *gamma*- cyclodextrins which contain 6, 7 and 8 glucose units, respectively. Cyclodextrins are, consequently, chiral structures and the beta-cyclodextrin has 35 stereogenic centers. Selectivity varies with the temperature, the amount of stationary phase on the column and the type of derivatization used. Open tubular columns are produced by the static or dynamic methods of coating, the static method of coating being the superior method providing more even coating, but is the more lengthy process. Cyclodextrins derivatized with trifluoroacetic acid, or similar reagents, are extremely vulnerable to hydrolysis by water. Thus, all procedures must be carried out under strictly anhydrous conditions. In some cases, the column can be regenerated, using an appropriate apparatus, by passing vapor of the derivatizing agent through the column

and subsequently removing the excess at elevated temperatures. There is a minimum column length that can be used to separate a pair of enantiomers and this will depend on the retention ratio of the pair and the capacity factor of the first eluted peak. At the minimum column length, the separation will be completed in the minimum time. As the column temperature approaches that of coelution, the necessary column length becomes extremely high and, at the co-elution temperature will, in fact, be infinite. After the temperature is raised above the coelution temperature, the retention ratio again increases rapidly and initially the minimum column length is reduced. However, the capacity ratio also decreases with increased temperature which will also necessitate the use of higher efficiencies. Thus, after the initial fall in minimum length immediately after the temperature of coelution, the critical length increases again. Although this effect of temperature on column length is general, the actual temperature range over which it occurs will be unique to each enantiomer pair. The contemporary cyclodextrin stationary phases may be derivatized in one or more of the three hydroxyl positions 2, 3, or 6. They are incorporated with polysiloxanes which are then coated on the wall of the column. The polysiloxanes may contain phenyl groups to improve temperature stability and if the cyclodextrin is permethylated a significant amount of the methylsiloxane must present to provide adequate solubility. Derivatization of the 6 position with alkyl groups also increases the solubility of the cyclodextrin material in the polysiloxane. Derivatization with alkane groups, propyl, butyl, pentyl, etc. on the 2, 3-positions introduces strong dispersive (hydrophobic) interactions with the solutes. In contrast the introduction of polar groups on the 2,3-positions will increase the polar (hydrophilic) interactions with the closest associate enantiomer. By careful choice of the molecular structure of the derivatizing reagent, very subtle mixtures of dispersive and polar interactions can be arranged that can be appropriate for the separation of specific types of chiral mixtures. All three cyclodextrins (α, β, and γ) can be derivatized, but it is frequently found that one specific cyclodextrin provides the greater selectivity for specific compounds.

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Chapter 6—
Chiral Gas Chromatography Applications

The Basic Principles of Chiral Selectivity

Gas chromatography has been used to analyze a wide range of volatile mixtures containing chiral substances. However, it is not only important to be aware of some of the typical areas of application, but also to comprehend the reason for column choice and to understand the interactive mechanism involved in the separation. Those new to the technique would be wise to bear in mind the comment by Einstein, "First order effects are usually simple". Furthermore, all separations can only be *enthalpically* and *entropically* driven and in GC only two types of interaction can effect the separation, those resulting from dispersive forces and those arising from polar forces (in analytical GC ionic materials will not be volatile). Thus, all chiral separations result primarily from one enantiomer (the solute), due to its spatial structure, making closer contact with another enantiomer (the stationary phase), and, as a result, interacting more strongly with its neighboring atoms or groups. In addition, for certain stationary phases (*e.g.* the cyclodextrins), inclusion of the solute molecule inside molecular apertures in the stationary phase structure can also occur, providing a second type of entropic contribution to retention. These two effects will constitute the essential *entropic* contribution to the separation. Thus, primarily, a stationary phase must be chosen such that one of a pair of enantiomeric

solutes will come closer to the stationary phase interactive moieties than the other.

However, the molecular size of the stationary phase material is large and the solute molecules will randomly interact with many parts of it that are not chiral. Thus, statistically, only a relative few of the total solute/stationary phase interactions will be with the chirally selective parts. Consequently, the differential interactions between the two enantiomers will be small and the relative retention difference limited. Thus, the stationary phase needs to possess as many chirally active groups as possible to increase the probability of selective interaction, and this explains the advantage of the cyclodextrin stationary phases which possess many chiral centers. It also follows, that the more chiral agent there is present in the stationary phase, the greater the number of chiral centers and the greater the chiral selectivity. At the extreme, the largest chiral selectivity will be achieved if the stationary phase consists entirely of the chiral agent. Certain cyclodextrin derivatives are crystalline (permethylated), or solid (2,3-dimethylsilyl), and need to be dispersed in polysiloxane polymers in order to be coated onto the walls of the column. This results in the concentration of chiral centers being reduced and a consequent reduction in retention and chiral selectivity. Other derivatives such as (2,6-diphenyl, 3-trifluoroacetyl-hydroxy propyl) are amorphous and can be coated directly onto the column, all giving higher selectivity and shorter analysis times.

Nevertheless, there is a limit to the number of chiral centers that can be introduced into the stationary phase and consequently, a limited probability of selective interaction must be tolerated. It follows, that in order to increase the chiral selectivity, the only recourse is to increase the energy of interaction between the solute molecule and those groups surrounding the chiral center when the statistical opportunity of interaction does occur. This will be the *enthalpic* contribution to the selectivity. However, the atoms or groups on the solute molecule that can interact with the stationary phase will differ from one solute to another. It follows that a range of different stationary phases will be required

from which to choose the one most appropriate for the particular enantiomeric pair that is to be separated. The choice will be dispersive groups for dispersive interactions (hydrophobic interactions), polarizable groups for induced dipole interactions (sometimes called π electron interactions) or polar groups for polar interactions (dipole-dipole interactions or hydrophilic interactions). From the point of view of synthetic chemistry, alkyl groups or chlorinated alkyl groups, attached to the neighboring atoms of the chiral center would introduce, or enhance, dispersive interactions. The aromatic nucleus, associated with the chiral center would introduce polarizability to the chiral environment and hydroxyl groups would introduce strong polarity. It should be pointed out, that induced dipole interactions can work two ways. A strong polar group on the stationary phase can result in induced dipole interactions with a polarizable group on the solute. Conversely, a strong polar group on the solute can provide induced dipole interactions with a polarizable group on the stationary phase.

Summarizing, the separation of enantiomeric pairs is achieved by the close selective interaction of one enantiomer with a stationary phase chiral center, resulting in stronger molecular interactions between the enantiomer and the neighboring groups or atoms round the stationary phase chiral center.

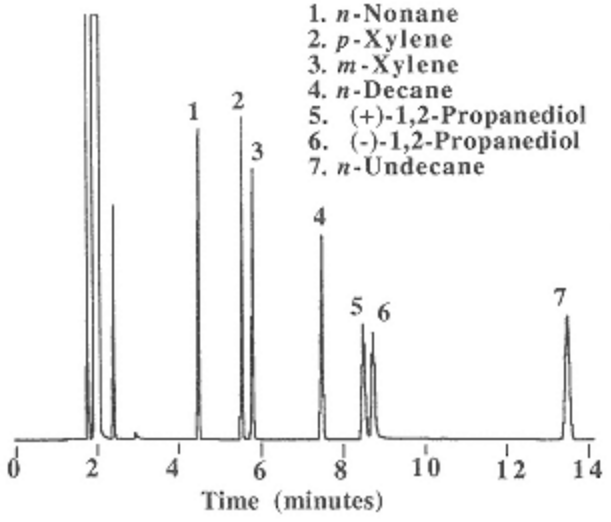
Thus, chiral selectivity is controlled firstly, by the probability of interaction with the stationary phase chiral center which is determined by the number of chiral centers available and secondly, by the strength of the interacting forces which is determined by the nature of the interacting atoms or groups.

Test Mixtures

It is clear, that the properties of the different chiral stationary phases available will differ considerably. In addition, due to the complex nature of many of the synthetic procedures involved, the products have the potential of varying from batch-to-batch. It follows, that test mixtures are needed to quality control the stationary phases and columns and also

to demonstrate the nature of the stationary phase. Such test mixtures should reveal the general chromatographic properties of the stationary phase as well as its capacity for separating enantiomeric pairs. The latter point is important, as most application samples contain many other compounds than those of a chiral nature and all, or most, will require resolution.

An example of a chromatogram of a test mixture used by Supelco to demonstrate the chromatographic characteristics of their α-DEX column is shown in figure 6.1. The stationary phase is claimed to have a high shape selectivity for positional isomers (*e.g.* xylenes, menthols, cresols etc.) and the small internal cavity of the permethylated α-cyclodextrin gives it a rigid character and unique chiral selectivities.

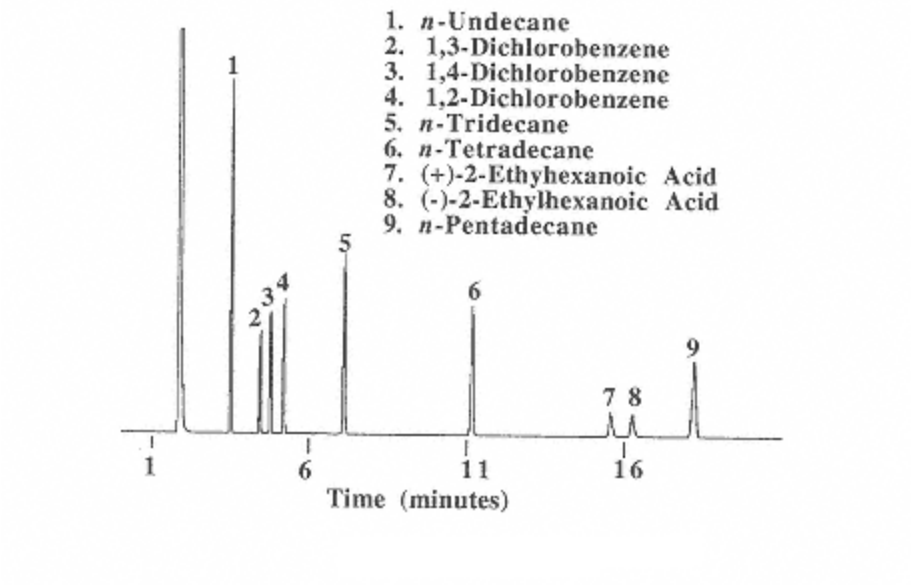


The α-DEX™ column was 30 m long, 0.25 mm I.D., carrying a film of stationary phase 0.25 µm thick. The temperature was 90°C and the helium flow velocity was 30 cm/s.

Figure 6.1
Chromatogram of a Test Mixture for a Permethylated
α-Cyclodextrin Stationary Phase
Courtesy of Supelco

The use of the different solutes illustrates the retentive character of the stationary phase. The relative elution times of the normal paraffins

(which are solely retained by dispersive interactions with the stationary phase) indicate a moderate dispersive character, but it is interesting to note that the propane diols are eluted between the C10 and C11 hydrocarbons. This indicates that the C3 chain alcohol is retained by polar forces to an almost equivalent extent as the C11 hydrocarbon retained by dispersion forces only. This implies a fairly strong overall polar character to the stationary phase. It is also seen that the *m* and *p* xylenes are well separated showing good spatial selectivity and the separation of diol enantiomers good chiral selectivity. As the two enantiomers are strongly polar it would also appear that the greater retention of the (-)-1,2-propane diol was due to polar interactions between the OH groups with the neighboring polar moieties on the α-cyclodextrin. Another example of a test mixture used, in this case, for examining a Supelco γ-DEX column is shown in figure 6.2.



The γ-DEX™ column was 30 m long, 0.25 mm I.D. carrying a film of stationary phase 0.25 µm thick. The column temperature was 125°C and the helium flow velocity 30 cm/s.

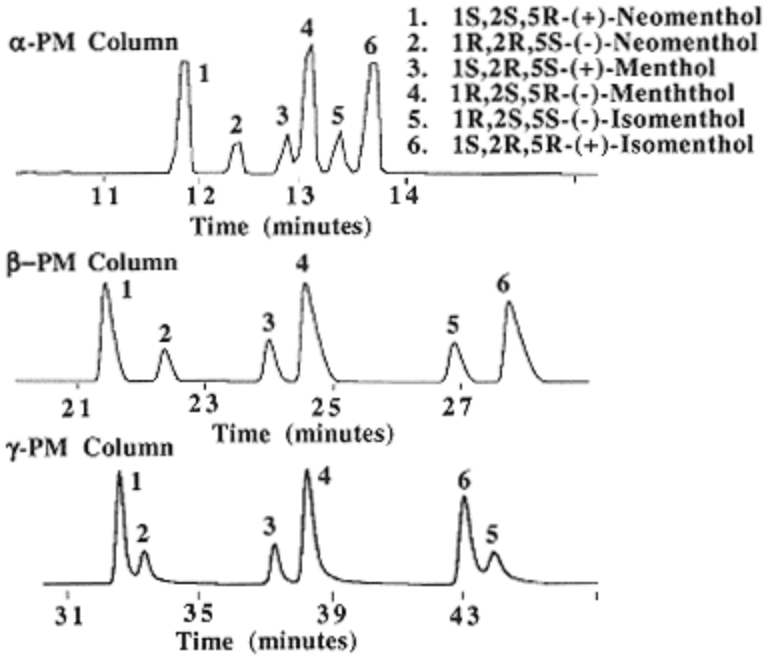
Figure 6.2
Chromatogram of Test Mixture for a γ-Permethylated Cyclodextrin Stationary Phase
Courtesy of Supelco

The permethylated γ-DEX column has the largest cavity relative to the corresponding α and β-cyclodextrins. In general, this material is said to be less selective to different enantiomers, but this will depend on the nature of the solute. However, it is more suitable for the resolution of larger molecules. The composition of the test mixture again provides information on the retentive mechanism of the stationary phase. The elution of *n*-pentadecane in only about 17 minutes at a temperature of only 125°C indicates that the dispersive contributions by the stationary phase to retention are relatively small. In contrast, the C8 ethylhexanoic acids are eluted just before the *n*-pentadecane showing the very strong polar character of the phase. The isomeric dichlorobenzenes demonstrate a good separation of the spatial isomers and an effective separation is also achieved between the two enantiomers. Again, the separation of the dichlorobenzenes indicates that the presence of strong *induced* polar interactions between the aromatic nuclei of the solutes, and the strong polar groups of the stationary phase, can provide more than adequate selectivity. In a similar manner, the strong polar interactions that occur between the carboxyl group of the enantiomer that penetrates closest to the stationary phase, and the neighboring polar groups of the interactive site, provides the necessary selective retention to separate it from its slightly more excluded enantiomer.

Elution Reversal of Enantiomers

Before discussing actual application examples, the nature of the interaction between a chiral solute and a chiral stationary phase needs to be considered further and in particular, elution order. If the stationary phase contains a substance with a particular spatial orientation, then *either* of a pair of enantiomeric solutes may be able to penetrate closest to the phase and, although chiral selectivity may be anticipated, the elution order of the enantiomeric pair will be unknown. In addition, the chiral substance used as the stationary phase will not be the same as the solutes and so can not predictively interact more closely with one specific enantiomer. It is important to understand a (*d*)-isomer in the stationary phase will not determine that the (*l*) or (*d*) isomer of the solute will be

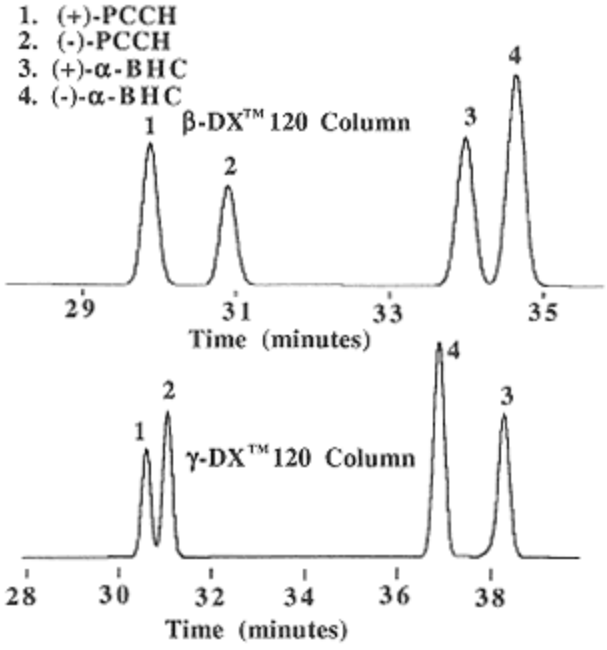
eluted first. An example of enantiomeric reversal is shown in figure 6.3. The separation of a mixture of chiral substances is shown separated on three different stationary phase all cyclodextrin based.



The columns were 30 m long, 0.25 mm I.D. carrying a film of stationary phase 0.25 µm thick. The column temperature was 110°C and the helium flow velocity 30 cm/s.

Figure 6.3
Chromatogram of a Chiral Mixture on Three Different Cyclodextrin Based Stationary Phases
Courtesy of ASTEC Inc.

All three stationary phases are permethylated cyclodextrins and for the most part the separations obtained from each are very similar. However, it is clear that there is a reversal in the order of elution for the isomenthol isomers on the γ-PM stationary phase. It is also seen, that this phase shows distinctly less selectivity for the other enantiomeric pairs. This reversal of elution is also demonstrated in figure 6.4.



The columns were 30 m long, 0.25 mm I.D. carrying a film of stationary phase 0.25 µm thick. The column temperature was 160°C and the helium flow velocity 30 cm/sec.

Figure 6.4
Chromatogram of a Chiral Mixture on Two Different Cyclodextrin Based Stationary Phases
Courtesy of Supelco

Both stationary phases were also permethylated cyclodextrins and again, the separations obtained from each are very similar. However, it is clear that there is a reversal in the order of elution for the α-benzylhexachloride (α-BHC) isomers on the γ-DEX stationary phase. There are still greater differences to note. The separation of the first pair of enantiomers on the β-DEX column, which has the tighter structure, is much greater than that on the looser structured γ-DEX column. This means that the (+)-PCCH enantiomer could approach more closely the stationary phase molecules, experience greater interactions and thus greater retention. However, in contrast, the larger solutes show the

opposite effect. Not only is the elution order reversed, but the separation ratio is now significantly larger for these solutes eluted from the γ-DEX column. It is also interesting to note that the peaks from the more open γ-DEX were less wide and the column more efficient. This may also indicate that the more open structure allows a more rapid exchange between the phases, and smaller band dispersion. In physical chemical terms, the resistance to mass transfer term in the Golay equation is smaller, leading to less peak dispersion, and a more efficient column.

An elution order change appears most likely to be due to a shift in the interactive mechanism. Evidence supporting this conclusion arises from the dramatic increase, or decrease, in retention, and the change in the loading capcity of the stationary phase, that often accompanies an elution order reversal. For example, assume a given elution order was the result of inclusion, where the atoms or groups round the chiral center interacted *dispersively* with neighboring groups on the stationary phase. Under these conditions, the most retained isomer would be the result of excess dispersive interactions over those of the other isomer. Now, assume the cavity size is reduced so that inclusion can not take place, or is significantly reduced. As dispersive groups would no longer be available for selective interaction, the groups around the chiral center would need to interact with different neighboring groups which would now be *polar*. Depending upon the particular orientation of the dispersive and polar groups round the chiral center of the solute, this could result in the opposite enantiomer experiencing stronger polar interaction, and would then become the most retained. *Ipso facto*, there would be a elution order reversal.

Selectivity Characteristics of the Different Cyclodextrins

Despite the fact that the cyclodextrins differ by only one glucose unit, the selectivity of the cyclodextrins show very different characteristics both between the cyclodextrins themselves and between the solutes that are separated on them. They will differ in chiral selectivity, absolute retention and in the efficiency the column can produce. The difference has to do

with the size of the toroidal cavity and the strength of the interaction that can take place within, and without, the cavity. There are two different entropic mechanisms by which the cyclodextrins can resolve enantiomers. One mechanism involves inclusion in the cyclodextrin cavity, and subsequent interaction with the chiral centers, and the other involves interaction with the chiral centers external to the cavity. If inclusion is required to effect a separation, then all three cyclodextrins may be appropriate as they have cavities of different sizes; if only external interactions are required to achieve a separation, then only the γ-cyclodextrins might be suitable. This is true for the use of the cyclodextrins in both GC and LC.

In addition, certain functional groups will readily enter the cavity whereas other will not. Chiral centers with functional groups that are largely dispersive such as aromatic and saturated rings, the halogens and also nitro, sulfo, and phospho groups appear to readily enter the cavity, whereas amines, aldehydes, ketones, acids and esters appear to be inhibited and more readily interact with chiral centers external to the cavity. However, if a stereogenic center contains a functional group that allows inclusion, but there are no appropriate groups with which the peripheral groups of the chiral center can interact, then there will be little of no chiral selectivity although there may be significant retention.

It is still extremely difficult to predict the retention characteristics for a given pair of solutes on a particular derivatized cyclodextrin phase. Nevertheless, it is often possible to estimate the size of the cyclodextrin that is most likely to be appropriate and, under certain circumstances, the most likely derivative that should be used. In contrast, the separation can often be explained in some detail, after the event. A large accumulation of experimental data has allowed a degree of predictability of functional group interactions (enthalpic contributions) with certain stationary phase structures.

The inclusion properties of the cyclodextrins can sometimes help in deciding which cyclodextrin to use as opposed to the best derivative. This

is illustrated by the separations shown in figures 6.5 and 6.6. The three chromatograms shown in figure 6.5 were obtained from the separation of the four isomers of bis-trifluoro-acetylated-3-aminomethyl-3,5,5-trimethyl-cyclohexanol on the three 2,6-dipentylated α, β and γ-cyclodextrins. It is seen that the separations are vastly different. The γ-cyclodextrin gives strong retention but poor enantiomeric selectivity.

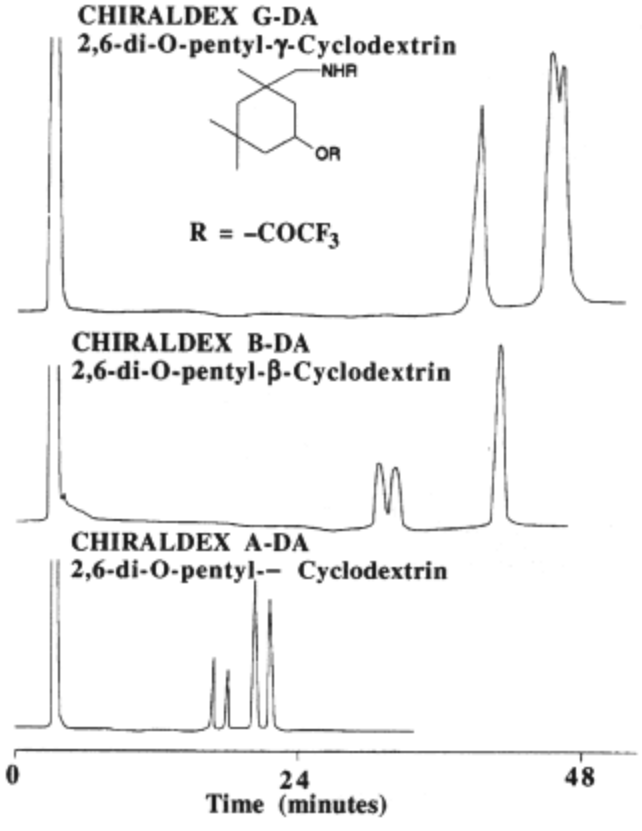


Figure 6.5
The Separation of the Four Enantiomeric Isomers of Bis-Trifluoroacetylated-3-Aminomethyl-3,5,5-Trimethyl-Cyclohexanol
Courtesy of ASTEC Inc.

This implies that the solutes entered the cavities but the cavity fit was insufficient to provide access to interacting groups that would lead to

chiral selectivity. It is also seen that as cavity penetration was taking place, the exchange rate was relatively slow and lower efficiencies were realized. The β-cyclodextrin shows improved selectivity, but for the other enantiomeric pair but the same problems obviously existed. Retention is slightly less and the column efficiency improved indicating faster solute/phase transfer, but cavity penetration produced no improved chiral selectivity. It is clear, that by far the best separation is obtained on the α-cyclodextrin. Excellent chiral selectivity is realized for both enantiomeric pairs and the column efficiency is high, indicating fast solute/phase transfer rates. Indeed, not only is a vastly improved separation achieved, but its is also completed in less time. This might have been anticipated on the basis that solute inclusion was not to be expected and that external surface interactions would produce the required chiral selectivity.

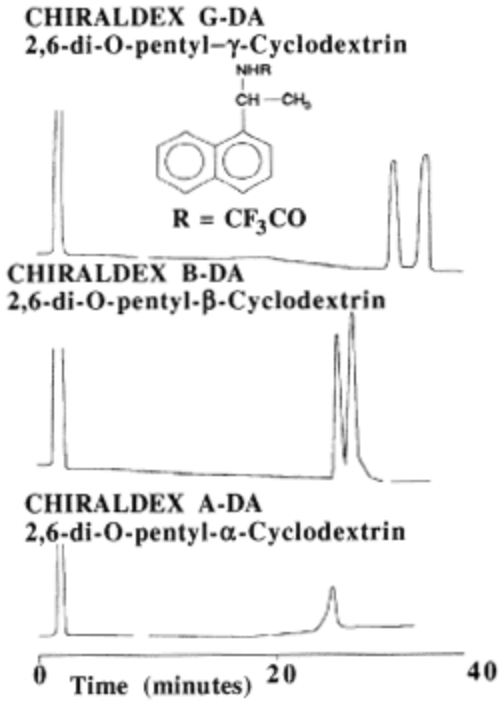


Figure 6.6
The Separation of the Enantiomers of
Trifluoro-acetylated 1-(1-Naphthyl)Ethylamine
Courtesy of ASTEC Inc.

At this point, it is important not to assume that the α-cyclodextrin is necessarily the better chirally selective stationary phase. Consider the separation of the enantiomers of trifluoro-acetylated 1-(1-naphthyl) ethylamine depicted in figure 6.6. The stationary phases are exactly the same as those in figure 6.5 and are presented in the same order. However, it is clear that the opposite situation exists, and there is little or no peripheral chiral selectivity and thus the α-cyclodextrin exhibits no chiral selectivity as the solutes cannot enter the cavity to provide chiral selectivity. In addition, it is also obvious that the solutes enter the cavities of the β- and γ- cyclodextrins, and the groups associated with the chiral centers of the solute can interact strongly with groups on the cyclodextrin to provide good enantiomeric selectivity. It is seen that β-cyclodextrin again occupies an intermediate position in chiral selectivity and it is now the γ-cyclodextrin with the larger cavity that provides the necessary resolution of the enantiomers. It is also seen that, although the efficiency is still not good, (exchange rates are relatively small) the retention is large and so the analysis time may also be relatively long.

Although examples, similar to those given in figures 6.5 and 6.6, can be found, in general, difficulties remain in trying to identify the best type of cyclodextrin to be used for an hitherto unknown separation. The situation is further complicated by the different derivatives that can be formed from each cyclodextrin, each giving it an interactive character that can range from largely dispersive to strongly polar. It begins to become more evident that the selection of the best phase for a given separation will rely heavily on experiment and experience. Different stationary phases must be tried to identify the one that will provide the desired chiral selectivity and at the same time provide the analysis in the minimum time. This choice must be made bearing in mind the chemical size and structure of the solute in relation to the cavity size of the cyclodextrin that is to be used. If the solute is largely dispersive (hydrophobic) then a cyclodextrin derivative that is also dispersive might be appropriate. Conversely, a polar solute might interact more strongly with a polar (hydrophilic) or polarizable group associated with the cyclodextrin. Unfortunately, the situation becomes even more

complicated if the solute requires to be derivatized to render it sufficiently volatile and stable to be separated by GC. The derivative group also will contribute new interactive characteristics to the solute molecule.

The Effect of Solute Derivatization on Chiral Selectivity

Consider the separation of the isomers of 2-butanol, on the (2,6-di-*O*-pentyl-3-trifluoroacetyl-γ-cyclodextrin) depicted in figure 6.7. This cyclodextrin derivative is designed to provide strong polar interactions round the chiral centers of the cyclodextrin, so that the isomer that approaches closest to the chiral phase, experiences the maximum polar forces to selectively retain it in the column.

It is seen that the type of the derivative that is used can have a profound impact on the chiral selectivity that is attained. It is also seen that the elution order of the enantiomers is determined by the type of derivative. The (R) isomer is eluted first with the trichloroacetyl and the trifluoroacetyl derivatives. In contrast, The (S) isomer is elute first in the case of the acetyl derivative. The acetyl derivative gives the best chiral selectivity and is eluted relatively rapidly. The reason for the difference may be spatial in that, the halogen atoms will make the derivative groups more massive than the acetyl groups. Also, the dispersive interactions with halogens, not surprisingly, increase with the size of the halogen atom. Consequently, the trichloracetyl derivatives are retained to a much greater extent than the trifluoroacetyl derivatives. It is interesting to note that the chiral selectivity shown by the trichloracetyl derivatives are somewhat less than the shown by the trifluoracetyle derivatives. This indicates that the chiral selectivity was dominantly polar and the large chlorine atoms hindered the close association of the isomers with the surface. The combined effect of the cyclodextrin structure and the type of derivative that is used can render the separation problem very complex indeed.

The separation of different derivatives of racemic 1-methoxy-2-aminopropane on two cyclodextrins are shown in figure 6.8. The two

cyclodextrins are the 2,6-pentylated α cyclodextrin and the ((S)-2-hydroxypropyl methyl ether)-β-cyclodextrin.

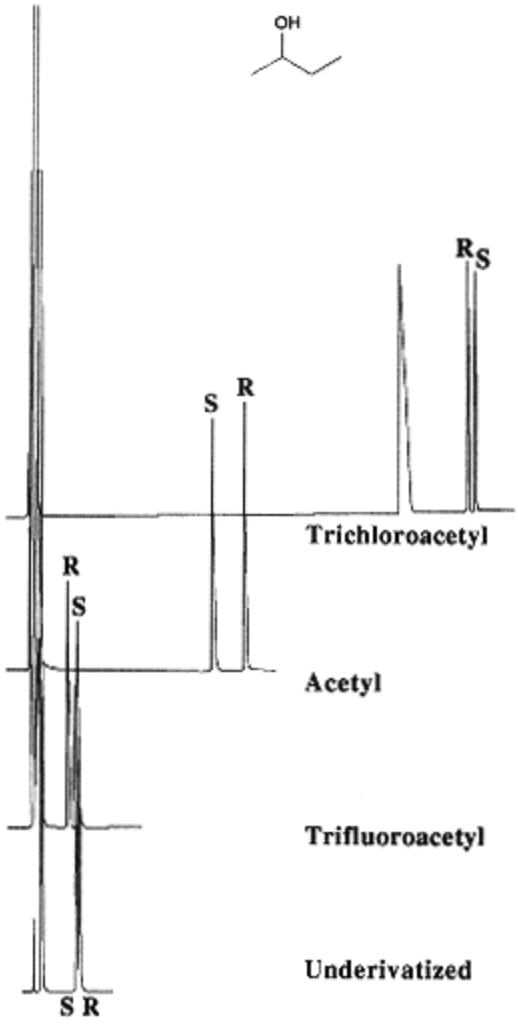


Figure 6.7
Effect of Derivatizing Agent on Chiral Selectivity
Courtesy of ASTEC Inc.

The three derivative groups that are used are the acetyl, the monochloroacetyl, the trichloroacetyl and the trifluoroacetyl. The eight alternative separations shown in figure 6.8 indicate that the possibilities

range from no selectivity at all, poor efficiency and long retention times to the separation carried out on the (S-2-hydroxy propyl methyl ether) with the monochloroacetyl derivative that gives good resolution, reasonable efficiency and a 12 min analysis time. It is clear that to predict the best derivative, the ideal cyclodextrin structure, and the most effective cyclodextrin derivative is virtually impossible without prior knowledge of the separation or separation type.

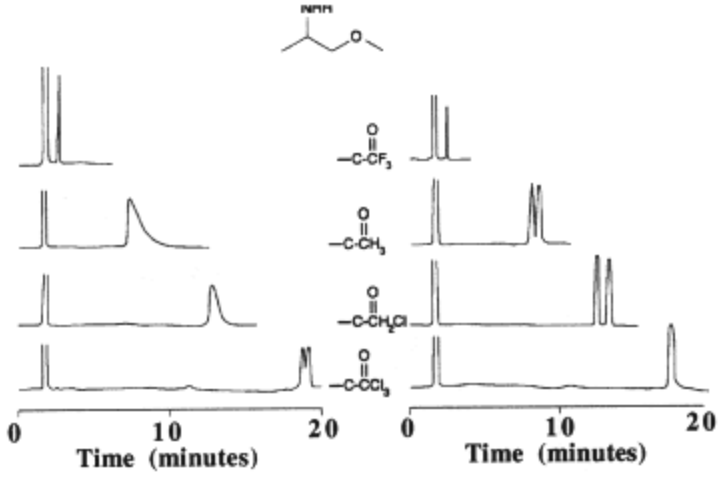


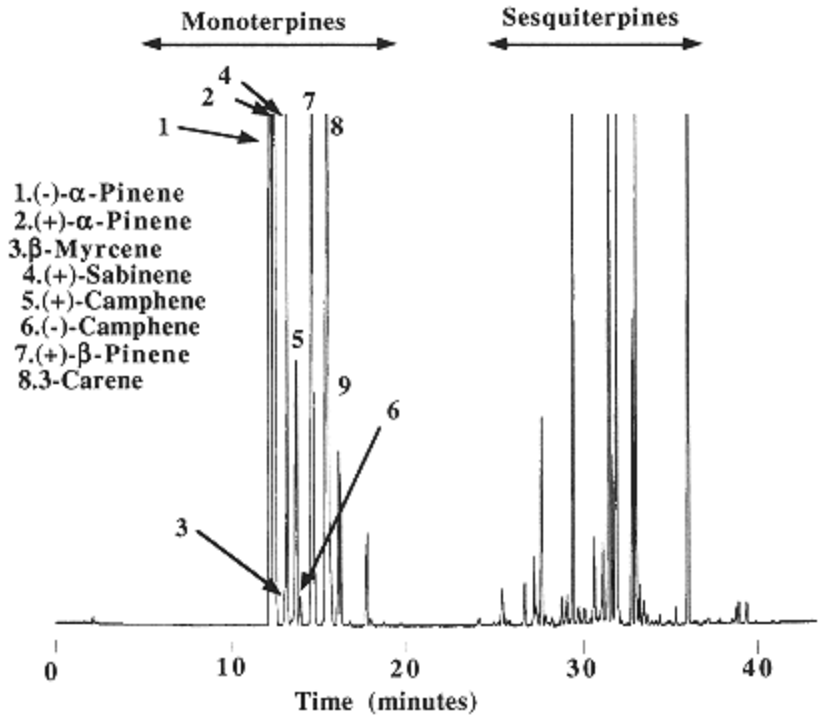
Figure 6.8
The Combined Effect of Derivatizing Cyclodextrin Structure on Chiral Selectivity
Courtesy of ASTEC Inc.

It follows, that, although some logical predictions can be made based on inclusion or exclusion entropic contributions, the optimum system must be determined largely by experiment and, ideally, adequate retention data should be readily available for reference.

Chiral Separations of Essential Oils

Essential oils contain many chiral compounds and, as already mentioned, one enantiomer may be entirely responsible for a particular taste or

odor, whereas the complementary enantiomer has an entirely different olfactory effect. It follows that the use of chiral chromatography is probably the most useful technique for the analysis of essential oils. A chromatogram of the essential oil vapor from White Pine leaves is shown in figure 6.9.



The columns were 30 m long, 0.25 mm I.D., carrying a film of stationary phase 0.25 µm thick of β-DEX™. The column was programmed from 40°C to 220°C at 4°C/min. The helium flow velocity was 35 cm/s.

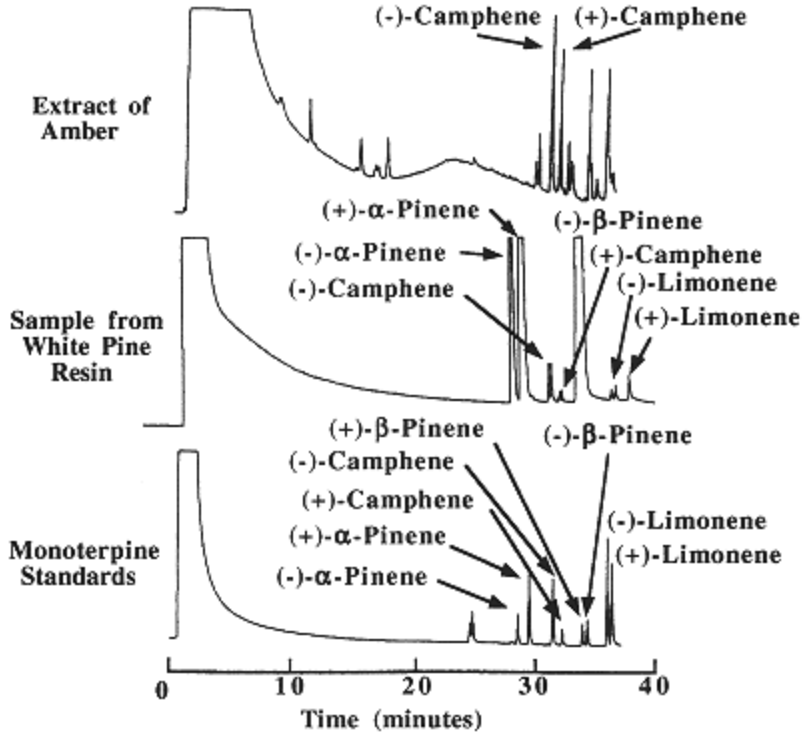
Figure 6.9
Chromatogram of the Essential Oil From White Pine Leaves
Courtesy of Supelco

The sample was taken by head space analysis from 0.5 g of pine leaves contained in a 7 ml vial. A solid state extraction procedure was

employed. A glass fiber coated with a polysiloxane film was exposed to the sample vapor at 40°C for 20 minutes. Using a special applicator the fiber was withdrawn from the sample vial and placed in a unique capillary column sample device. The fiber was then heated to 250°C for one minute and the vapors passed onto the column using a split injector with a 100:1 split. The column used was 30 m long 0.25 mm I.D. and carried a film of β-DEX 0.25 µm thick and was programmed from 40°C to 220°C at 4°C/min. Helium was used as the carrier gas at a velocity of 35 cm/s.

It is seen that the sample is broadly separated into two groups, the monoterpines and the sesquiterpines. The enantiomers of α-pinene and camphene are shown to be cleanly separated. As these compounds contain *no polar groups,* the chiral selectivity must be based entirely on differential dispersive interactions with the derivatized cyclodextrin. It should be noted that whereas the (-)-α-pinene is the first eluted enantiomer of α-pinene it is the (+)-camphene that is the first eluted of the camphene enantiomers. This again demonstrates that at present there is no rational procedure for predicting the order of elution of an enantiomeric pair.

Chiral gas chromatography has been used in a similar manner to identify the essential oil content of amber, a fossilized pine resin. The terpenoids are used as biological markers (biomarkers). Such markers can provide information on many fossil fuels including their source, maturity, migration and biodegredation. An example of some results from such an examination obtained by Armstrong *et al*. [1] is shown in figure 6.10. Chiraldex B-PM is also a permethylated β-cyclodextrin and due to its dispersive groups can provide significant dispersive interactions with polar solutes such as the terpenes. The amber fossils may be tens of millions years old but it is seen that the terpenes still exist in the material. The increased camphene content of the amber is thought to have formed over time by the dehydration of borneol. It is also interesting to note that after exceedingly long periods of time, the individual racemates are still present.

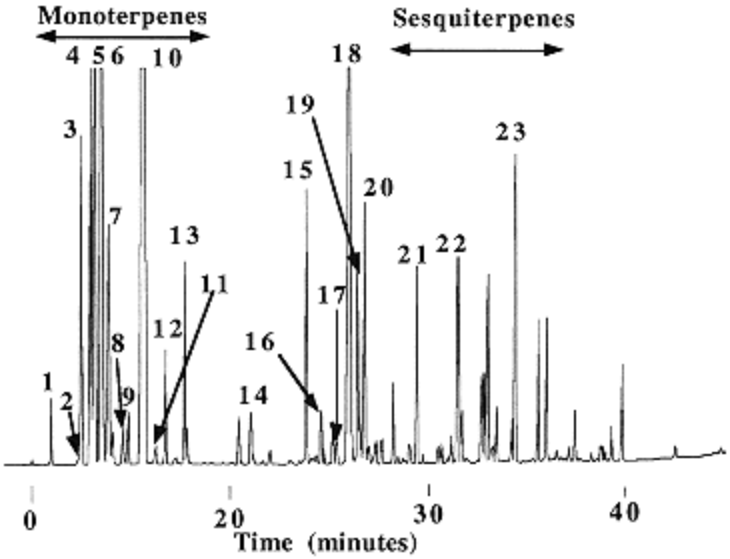


The column was 30 m long, 0.25 mm I.D., carrying a film of Chiraldex B-PM, 0.25 µm thick. The column was held at 40°C for 10 min and then programmed to 85°C at 2°C/min. Helium was used as the carrier gas at an inlet pressure of 10 p.s.i.

Figure 6.10
Chromatograms of the Essential Oils in Amber
Courtesy of Armstrong (*Chirality*, **8**(1996)39)

Further work by Armstrong *et al.* [2] extended the use of the derivatized cyclodextrins to the examination of biomarkers. Another example of the use of chiral chromatography for essential oil analysis is depicted in figure 6.11. The sample was taken from the head space over 0.5 g of Juniper leaves contained in a 7 ml vial. The essential oil vapor was sampled in the manner described previously, employing a quartz fiber coated with a polysiloxane resin. The fiber was exposed to the vapor for 20 min at 40°C and the sample desorbed in the column sampling system at 250° for 1 min. It is seen that two enantiomeric pairs were separated,

both were hydrocarbon type and thus the chiral selectivity was achieved solely by dispersive interactions.



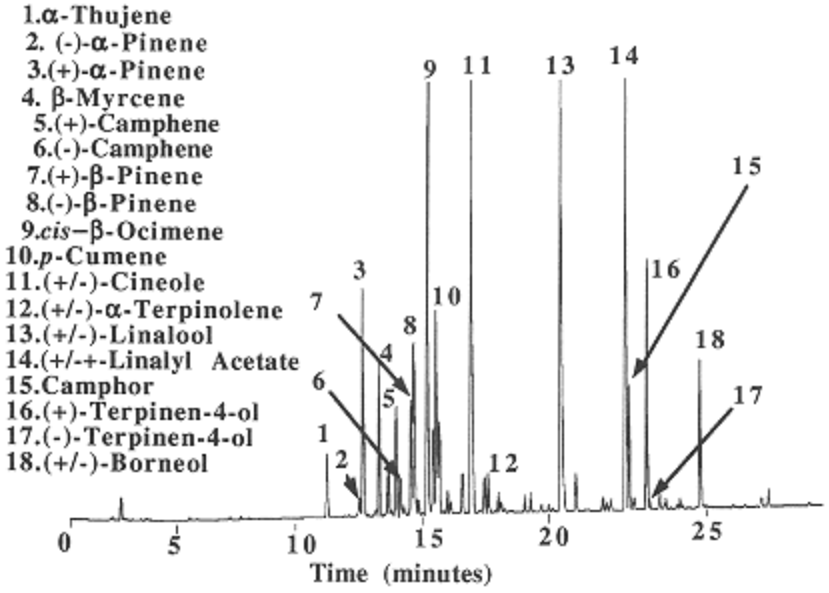
1 α-Thujene	7 (+)-Camphene	13 Terpinolene	19 Copanene
2 (-)-α-Pinene	8 (-)-Camphene	14 Linalool	20 (-)-β-Cadinene
3 (+)-α-Pinene	9 α-Terpinene	15 Camphor	21 Caryophylene
4 Tricyclene	10 (+)-Limonene	16 Terpinen-4-ol	22 γ-Cadiene
5 β-Myrcene	11 β-Phellandrene	17 Borneol	23 Gemacrene D
6 Sabinene	12 γ-Terpinene	18 Bornyl Acetate	

The columns were 30 m long, 0.25 mm I.D., carrying a film of stationary phase 0.25 µm thick of β-DEX™. The column was programmed from 40°C to 220°C at 4°C/min. The helium flow velocity was 35 cm/s.

Figure 6.11
Chromatogram of the Essential Oil From Juniper Leaves
Courtesy of Supelco

However, the overall character of the β-DEX phase is polar so the more polar materials are eluted much later in the chromatogram. There are no enantiomer pairs in the sesquiterpene group although single enantiomers are present. Nevertheless, if there were enantiomer pairs in this group then the major interaction that would effect their separation would be polar.

Another interesting separation that demonstrates the different type of chiral selectivity that can occur with the same stationary phase but with different enantiomer pairs is shown in figure 6.12.



The columns were 30 m long, 0.25 mm I.D., carrying a film of stationary phase 0.25 µm thick of β-DEX™. The column was programmed from 40°C to 220°C at 4°C/min. The helium flow velocity was 35 cm/s.

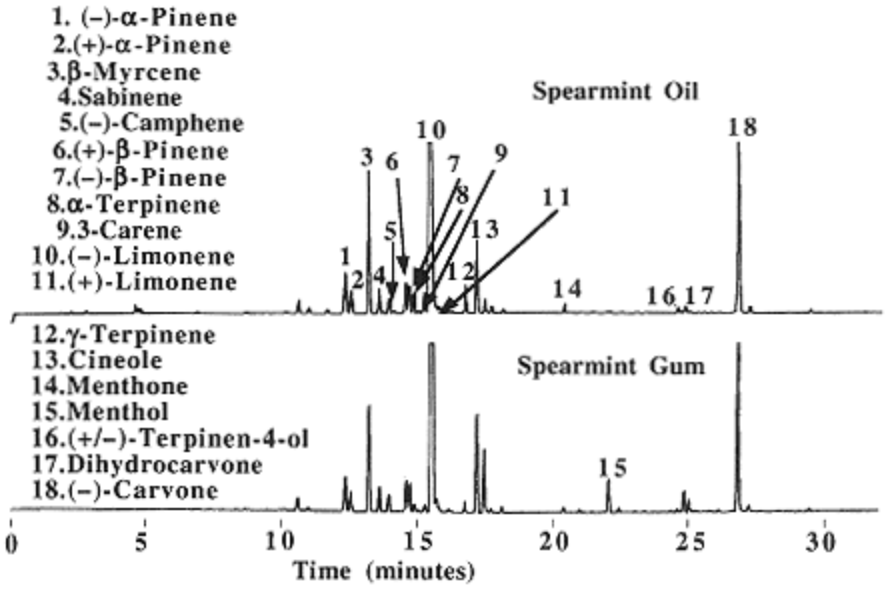
Figure 6.12
Chromatogram of Lavender Oil
Courtesy of Supelco

The sample is of the head space taken from lavender oil. The sampling procedure was the same as that described previously except that the head space was sampled at 30°C for only 30 s. The stationary phase was again a β-DEX which is largely polar in nature but with some dispersive interactive capability. As would be expected the hydrocarbon type monoterpenes are eluted early as the dispersive interactions are relatively weak. In contrast, the more polar materials appear late in the chromatogram as a result of the strong polar interactions of the stationary phase. In addition, the chiral selectivity of the phase for the

monoterpenes, which will depend entirely on the relative dispersive interactions with the stationary phase, is adequate but not great. This reflects the fact that the β-DEX permethylated cyclodextrin has relatively weak dispersive interactive capacity. However, the separation of the enantiomers (+)-terpinen-4-ol and (-)-terpinen-4-ol is also relatively small, and this small selectivity is for substances that are significantly polar and thus should provide a greater separation. This shows that, as a result of its unique spatial arrangement, the (-)-α-pinene can approach closer to the interacting cyclodextrin structure than the (-)-terpinen-4-ol. This is an example where the polarity of the phase is favorable to an increased chiral selectivity, but the spatial arrangement of the interacting groups of the solute can not take full advantage of it. The entropic contribution of the distribution is not great enough for the potentially increased enthalpic opportunities to be realized.

Essential oils have been of great interest to man since time immemorial and have been used as food enhancers in the form of flavors, as aphrodisiacs in the form of perfumes and as medicines in the form of inhalants and additives in emollients. The interest today is no less than it has ever been, albeit, somewhat more commercial. The difference is that now the importance of the chirality of the oil components is understood. Consequently, much of the work carried out previously on essential oils is being repeated, using chiral chromatography to identify the optical isomers present and their olfactory characteristics. Some of the more commercially important flavors are those of tea, coffee, a number of fruits, peppermint, spearmint and a range of herbs and spices. The flavor components of coffee, tea and cocoa have recently been investigated by Stalcup *et al*. [3], using Chiraldex B-TA, Chiraldex G-TA and Chiraldex B-PH. The Chiraldex B-TA has a β-cyclodextrin base with the 2,6-hydroxyl groups pentylated and the 3-position hydroxyl groups trifluoroacetylated. The material is strongly polar but still is highly selective for halogenated compounds. The Chiraldex G-TA is a similar product to the Chiraldex B-TA but has a γ-cyclodextrin base and thus has a more open structure that can accommodate larger molecular weight solutes. The Chiraldex B-PH has a β-cyclodextrin base that has hydroxy

propyl groups attached significantly increasing the polarity of the phase. Chiral chromatography is not used solely as an investigatory analytical tool but also for quality control and monitoring product stability. An example of its use in product monitoring is shown in figure 6.13.



The columns were 30 m long, 0.25 mm I.D., carrying a film of stationary phase 0.25 μm thick of β-DEX™. The column was programmed from 40°C to 220°C at 4°C/min. The helium flow velocity was 35 cm/s.

Figure 6.13
Chromatogram of Spearmint Oil and Spearmint Gum by Headspace Analysis
Courtesy of Supelco

Figure 6. shows chromatograms of the head space from an original sample of spearmint oil and that from the headspace over the spearmint gum product. Samples were taken in the manner described previously using solid phase extraction. It is seen that the different enantiomers can be very easily monitored so that any racemization that might take place

during processing can be quickly identified. As a particular enantiomer is often largely responsible for the unique taste and olfactory quality of any food flavor, it is very important to assure that the processing necessary to prepare the food does not reduce the level of the critical enantiomers by either direct loss, or by some racemization process.

A final interesting application, which perhaps should not be classed as an essential oil but, nevertheless is similar in form, is the use of chiral chromatography to examine sex pheromones, and in particular, that of the Scarab beetle, *Anomala cuprea.* This Scarab beetle is one of the most severe agricultural pests in Japan, as it attacks a wide variety of crops both in the larval and adult stages. Consequently, elimination and growth control is of considerable commercial importance. As a result, a significant amount of research has been undertaken to identify the sex pheromones involved in the breeding cycle. The sex pheromones of this beetle were extracted and examined by Lea [4]. The active substance was found to be (R,Z)-5-(-) (oct-1-enyl)oxacyclopentane-2-one. The synthetic pheromones were separated on a Chiraldex G-TA column, 20 m long, 0.25 mm I.D. carrying a film of stationary phase 0.25 mm thick. Chiraldex G-TA is a γ-cyclodextrin derivatized with trifluoracetyl anhydride, giving a fairly polar, open-structured modified cyclodextrin. The separation was carried out isothermally at 120°C. This particular stationary phase gave a retention time difference between the enantiomers of 7 minutes at an absolute retention time of about 50 minutes.

From the examples given, it is clear that, as a result of their natural volatility, the analysis of essential oils has become one of the major application areas for chiral gas chromatography. In fact, the problems of separating the enantiomers of the essential oils was one of the driving forces that stimulated the development of *chiral* gas chromatography. The successful resolution of enantiomers has been due, firstly, to the very high efficiencies available from open tubular columns and secondly, to the remarkable chiral selectivity and thermal stability offered by the cyclodextrin based stationary phases.

Pharmaceutical Applications of Chiral Chromatography

Two factors have contributed to the emphasis on chiral GC in drug analysis. Firstly, the importance of the enantiomeric character of the drug has been well established and was discussed in the first chapter. As a result the Food and Drug Administration has required that the physiological effect of both or all enantiomers of any drug that can exist in chiral form must be determined. In addition, the chiral purity of any commercially available drug must also be monitored and controlled. Secondly, many modern drugs have relatively small molecular weights and consequently are volatile or can easily be made into volatile derivatives. Furthermore, GC capillary columns can easily provide the high efficiencies necessary to separate very similar compounds with relatively small separation ratios. It follows, that chiral chromatography is used in all laboratories involved with drug analysis.

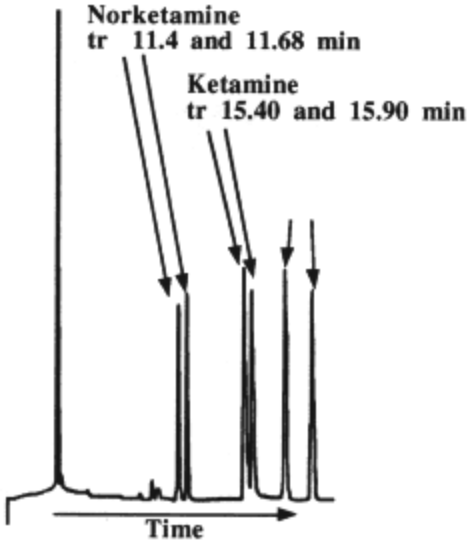


Figure 6.14
The Separation of the the Enantiomers of
Ketamine and its Metabolites Norketamine
and Dehydro-norketamine
Courtesy of ASTEC Inc.

Ketamine, originally developed as an anesthetic, was recently investigated as a potential drug that would reverse the problem of protein metabolism in AIDS patients. Analysis of the distribution of the drug in various body fluids was complicated by the presence of two chiral metabolites. A 30 m long, 250 µm I.D., Chiraldex G-TA column, operated isothermally at 160°C, with helium as the carrier gas, and 3 Kg/cm^2 head pressure, was shown to separate all 6 enantiomers as their trifluoryl acetyl derivatives as shown in figure 6.14. The high efficiencies and the general versatility of this stationary phase, that provides strong dispersive and polar interactions, makes it especially useful for the separation of substances with multiple chiral centers and in the presence of metabolites. The use of a 5m retention gap allowed the direct injection of 7 µl of plasma.

The anti-inflammatory drug, ibuprofen, exists in two enantiomeric forms which also can be separated on open tubular columns coated with derivatized β-cyclodextrin. An example of the separation of the isomers of ibuprofen is shown in figure 6.15.

Figure 6.15
The Separation of the
Enantiomers of Ibuprofen
Courtesy of ASTEC Inc.

The column used was 30 m long, 0.25 mm I.D., and carried a film of (2,3-di-O-methyl)-β-cyclodextrin, 0.25 µm thick. It was operated isothermally at 130°C and helium was used as the carrier gas at an inlet pressure of 30 psi. Assuming the dead time was short, compared with the retention time, the separation ratio of the two isomers was about 1.04. It is clear that the two solutes are only just resolved to base line and so the analysis time could not be reduced, at least, not with the particular stationary phase and at 130°C.

Fluoxetine, also known as Prozac, which is commonly used as an antidepressant, comprises two enantiomers and is sold as a racemic mixture. The separation of the Prozac enantiomers on a modified cyclodextrin column is shown in figure 6.16.

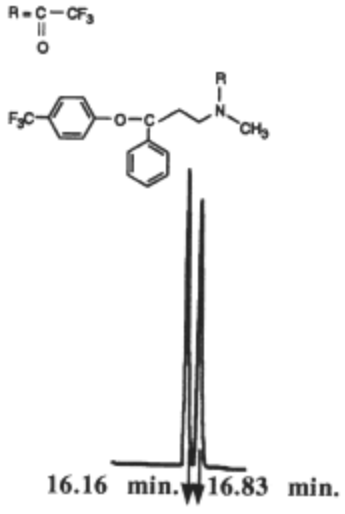


Figure 6.16
The Separation of the
Enantiomers of Prozac
Courtesy of ASTEC Inc.

The separation was carried out on a column 20 m long, 0.25 mm I.D., coated with a film of (2, 6-di-O-pentyl)-β-cyclodextrin, 0.25 µm thick. The column was operated isothermally at 190°C and hydrogen was used

as the carrier gas at an inlet pressure of 20 psi. It is seen that base line separation was just achieved and, again assuming the dead time is relatively short compared with the retention time, the selectivity, as given by the separation ratio of the two enantiomers, was about 1.04.

Amphetamine, methamphetamine and ephedrine all have similar structures and each has two enantiomers. Amphetamine is a central nervous system stimulant and an anorexic. Methamphetamine is also an anorexic and is frequently used for treating attention disorders with hyperactivity. Ephedrine, on the other hand, although of similar structure has quite different physiological effects. Ephedrine (1) is a commonly used as a bronchodilator whereas (d) ephedrine (pseudoephedrine) is used as a decongestant. The separation of the six isomers of amphetamine, methamphetamine and ephedrine is shown in figure 6.17.

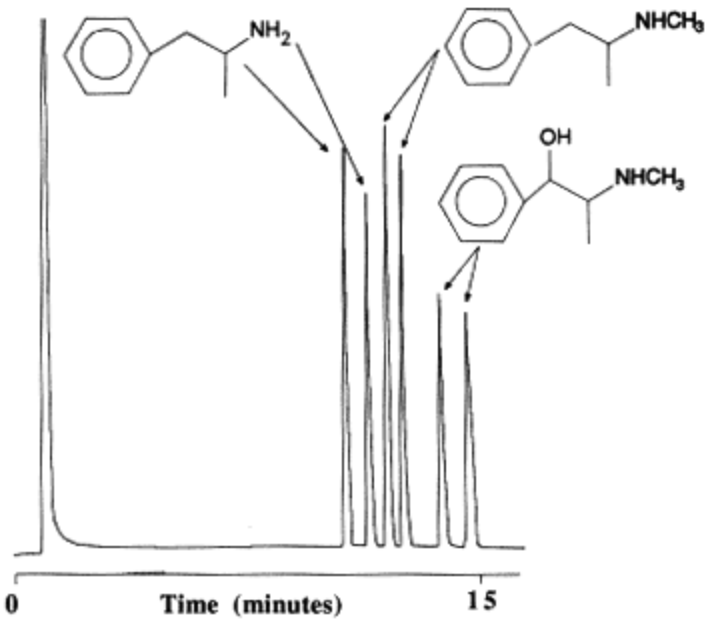


Figure 6.17
The Separation of the Enantiomers of Amphetamine,
Methamphetamine and Ephedrine
Courtesy of ASTEC Inc.

The chromatogram is of the trifluoro acetyl derivatives and it is seen that an excellent resolution of the enantiomers is achieved, and the analysis was complete in 15 minutes. The separation was carried out on a column 30 m long and 0.25 mm I.D., using (2,6-di-O-pentyl-3-propionyl)-γ-cyclodextrin as the stationary phase. Isothermal development at 130°C was used with helium as the carrier gas at an inlet pressure of 35 psi. This is a particularly elegant separation and demonstrates that the same phase system can be effective in resolving enantiomers from different substances, but which have basically similar structures.

General Applications of Chiral Chromatography

In environmental testing, the potential carcinogenity of the aromatic hydrocarbons make their separation and analysis important. However, they can pose some separation problems due to the closely similar chemical nature of, for example, the *m*- and *p*-xylenes.

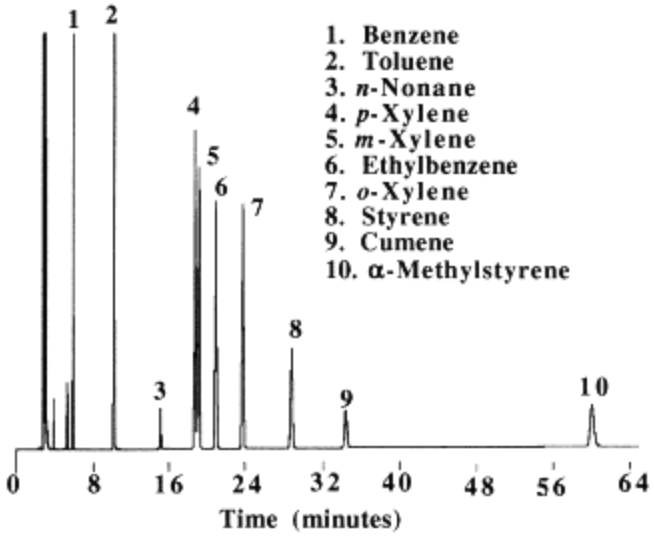


Figure 6.18
The Separation of Some Aromatic Hydrocarbons
Courtesy of Supelco Inc.

These isomers differ in structure, and, although not optically active, have similar spatial differences to pairs of enantiomers. Consequently,

stationary phases that separate enantiomers can also be used for separating spatial isomers that are not necessarily optically active. The separation ratios of such isomeric pairs, even on cyclodextrin stationary phases, is also very small, often in the 1.02–1.03 range. It follows, that the use of high efficiency capillary columns is imperative, if reasonable analysis times are to be realized. A separation of a mixture of hydrocarbons including some aromatics is shown in figure 6.18. A column 30 m long, 0.25 mm I.D., carrying a film of permethylated β-cyclodextrin 0.25 µm thick, was used for the separation. The column was operated isothermally at 50°C and helium was use as the carrier gas at a flow velocity of 20 cm/s. It is seen that baseline separation is achieved for the *m*- and *p*-xylenes and that separation ratio for the two isomers was about 1.03. Chiral stationary phases can be used very effectively for separating spatial isomers other than those that are optically active.

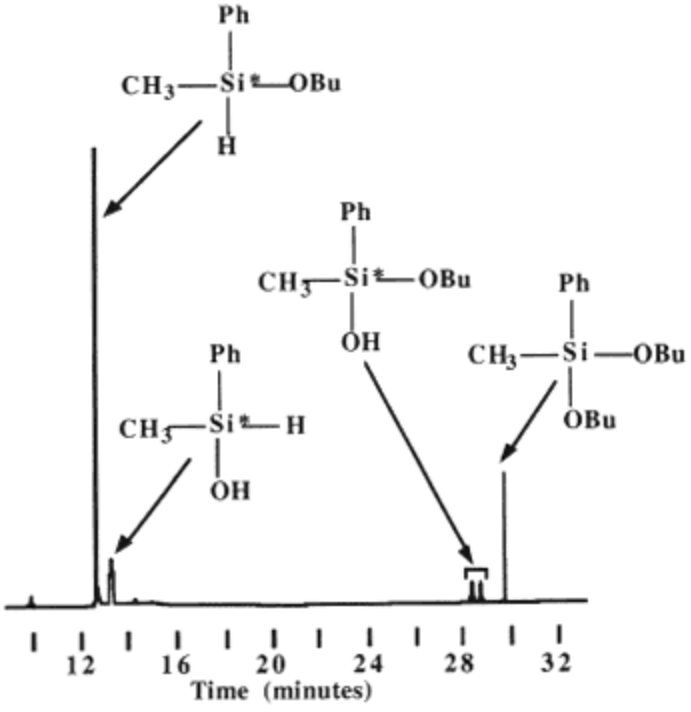


Figure 6.19
The Separation of Some Chiral Silicon Compounds
Courtesy of Supelco Inc.

Finally, an example would be appropriate of the use of chiral GC to separate the enantiomers of substances in which the chiral atom was not carbon. The separation of some compounds containing a chiral silicon atom is shown in figure 6.19. The sensitivity of the detector to silicon compounds apparently leaves a little to be desired, but nevertheless, adequate for monitoring the separation. It appears that the chiral selectivity depends largely on polar interactions. The enantiomers of the least polar solute remain unseparated, those of the solute having intermediate polarity are just beginning to be separated, whereas the enantiomers of the strongly polar solute are completely resolved. This separation was accomplished using a standard chiral stationary phase and capillary column. The column was 30 m long, 0.25 mm I.D., carrying a film of permethylated β-cyclodextrin, 0.25 μm thick. The operating temperature was programmed fro 100°C to 220°C at 2°C/min. Helium was used as the carrier gas a velocity of 20 cm/s.

GC analyses employing chiral stationary phases can be used to separate all kinds of spatial isomers containing any type of chiral atom. The only prerequisite is that the solutes are either intrinsically volatile or derivatives can be formed that are adequately volatile.

Synopsis

The choice of a phase system for a specific enantiomer separation is difficult and the selection has been confused by many diffuse explanations of solute selectivity given in the literature. Separations are achieved by entropic and enthalpic differences in the distribution system. The entropic contribution is provided by the unique spatial arrangement of the stationary phase structure. The enthalpic contribution is determined by the probability of interaction with the unique interactive site (the number of sites available) and the relative increase in interaction energy that results from the closer proximity of one enantiomer with the stationary phase site, relative to the other. The characteristics of a stationary phase can be evinced from the separation of test mixtures. The results will indicate the dispersive and polar character of the stationary

phase, and its selectivity towards spatial and chiral isomers. The chirality of the solutes and the stationary phase does not allow the precise prediction of elution order and similar types of stationary phase (*e.g.* the cyclodextrins) can frequently exhibit elution order reversal. In addition, in the case of the cyclodextrins, the chiral selectivity will vary with the type of cyclodextrin and the type of solute derivative that is used. Due to the structure of the cyclodextrins, retention can result from interactions arising from inclusion or exclusion of the solute from the natural cavities contained by the molecule. It general, the best phase system is usually determined by experiment and will not only be influenced by the stationary phase selected, but also the type of solute derivative that is employed. Chiral gas chromatography is one of the most frequently used techniques for the analysis of essential oils. It is used for the analysis of food and beverages, flavors, perfumes and medicinal oils. Due to the importance of chirality in the physiological response of many drugs, chiral gas chromatography is routinely used in drug analysis. The technique can be applied to the analysis of all types of spatial isomers, not merely those with chiral carbon atoms. Chiral gas chromatography also has a wide field of application in research, in environmental studies and environmental monitoring, in biochemistry and biotechnology, in forensic science and in all types of quality control.

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Chapter 7—
The Basic Liquid Chromatograph for Chiral Chromatography

The Basic LC Layout

The basic apparatus used in chiral LC (with the exception of the phase system) is sensibly the same as that used in general LC analysis. In chiral LC, occasionally a chiral detector is deemed to be appropriate, but for most analyses, the same type of detectors are also employed. A block diagram of an LC system suitable for chiral analysis is shown in figure 7.1.

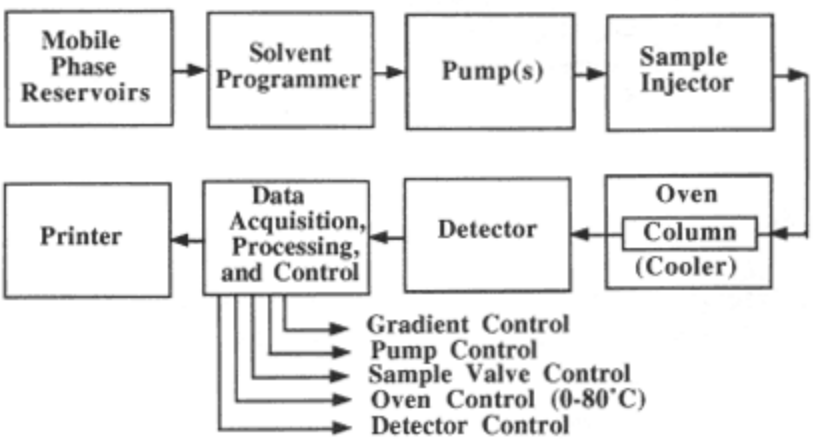


Figure 7.1
Block Diagram of Basic Liquid Chromatograph for Chiral Chromatography

For convenience, it can be considered that there are 8 essential units in a liquid chromatograph. However, the design and specifications of each unit can vary considerably, from one manufacturer to another. Consequently, appropriate specifications will be given for each unit that would make it suitable for most chiral analyses.

Mobile Phase Reservoirs

Reservoirs for three different solvents should be available for solvent programming. Four solvents are rarely (if ever) necessary and in the majority of LC separations, providing the basic principles of solvent selection are well understood, two solvents will usually be found adequate. Depending on local regulations, solvents may be required to be enclosed in a vapor proof container or a flame proof enclosure. It is generally advisable to provide each container air inlet with a tube filled with desiccant, to either keep the solvent dry, or maintain the water content constant. The reservoirs can be made of glass, stainless steel, Teflon or PEEK (polyether-etherketone) and if, for some reason, the reservoirs are required to contain biologically sensitive material, they may need to be constructed from titanium. Depending on the type of column that is used (normal columns or small bore columns) the capacity of the reservoirs can range from 100 ml to 1000 ml. The reservoir system should be designed so that the reservoirs can quickly and easily be changed or refilled. As LC detectors are highly sensitive, it is important to employ anhydrous solvents of high purity for the mobile phase

The Solvent Programmer

The solvent programmer is an apparatus that allows the composition of the mobile phase to be changed, in a defined manner, over a defined period of time, and is normally controlled by the chromatograph computer. There are basically two types of gradient programmer, one that mixes the solvents at high pressure, *prior to the column* and the other that involves solvent mixing that occurs at low pressures, *prior to the solvent pump*.

The High-Pressure Mixing Solvent Programmer

A diagram of a high-pressure solvent programmer is shown in figure 7.2. The system consists of three solvent reservoirs and it is seen that limiting the number of reservoirs to three is important as, with high-pressure mixing, each solvent reservoir requires its own solvent pump and high-pressure pumps can be very expensive. Each pump must be capable of operating at the maximum pressure required by the column, which will usually be about 3000–5000 p.s.i. The solvent from each reservoir passes to its own pump and each pump is controlled by the programmer. The output from each pump passes to a mixing chamber and thence to the sample valve of the chromatograph. The sample does not come in contact with any part of the gradient programmer and thus, unless labile substances are incorporated in the mobile phase, the materials of construction need not be bio-compatible.

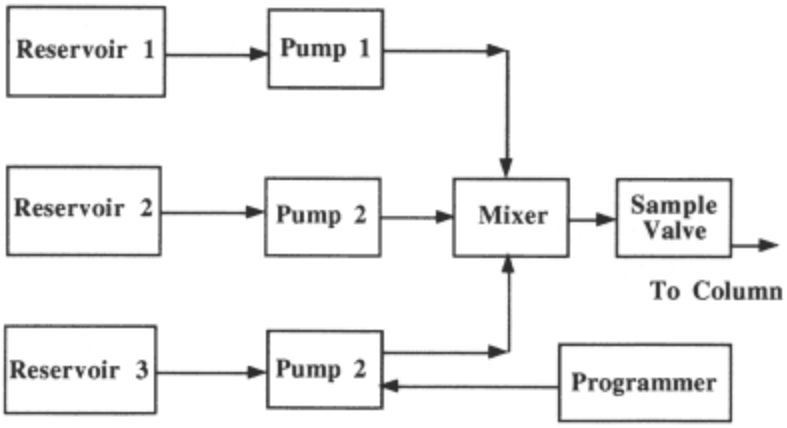


Figure 7.2
A Block Diagram of a High-Pressure Solvent Programmer

The programmer must allow the choice of solvent, solvent composition, shape of the gradient profile and the period of initial and final isocratic development. Modern programmers also allow the storage and recovery

of specific programs and facilities to allow the details of the program to be printed out with the analysis report.

The Low-Pressure Mixing Solvent Programmer

The low-pressure mixing solvent programmer mixes the different solvents prior to entering the pump. A diagram of one form of a low-pressure-mixing gradient system is shown in figure 7.3.

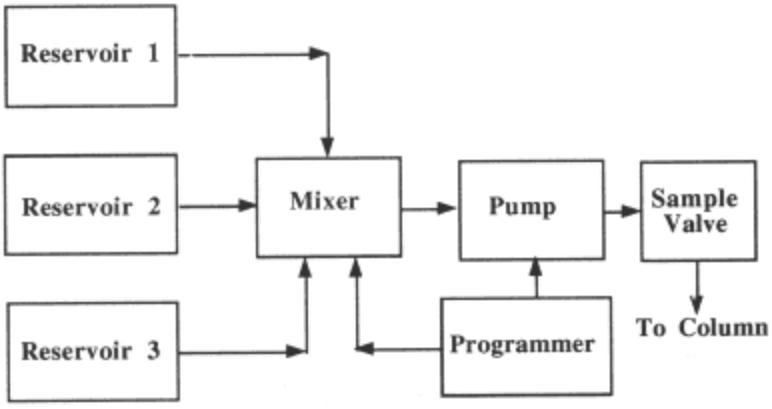


Figure 7.3
Diagram of a Low-Pressure Solvent Programmer

The solvent flows from the reservoirs, into the mixing valve which, in fact, consists of three time-controlled valves, one for each solvent. Each valve opens and closes, at a given frequency, and the individual flow from each valve is controlled by the portion of each cycle during which the valve is open. The output from the valve passes to a single pump and then to the sample valve. This system is less expensive as, although a special valve is necessary, only one pump is required.

The Mobile Phase Pump

The pump is usually a reciprocating type and has a maximum operating pressure of 3000–5000 p.s.i. However, in general practice, operating pressures are more often in the neighborhood of 2000 p.s.i. This is not usually a result of any limitation of the pump, but because sample valves,

although capable of operating well above 6000 p.s.i., will have a much longer working life when operated below 3000 p.s.i.. At lower pressures, there will be considerably less wear on the valve seats, particularly if they inadvertently come in contact with silica-based stationary phases. Nevertheless, some columns packed with very small particles and run at high flow rates may require to be operated at inlet pressures of 3000 p.s.i. or more. LC pumps are made of stainless steel, usually with non-return valves fitted with sapphire ball and seats to handle the high pressures, and sapphire pistons. A diagram of a single piston high pressure LC pump is shown in figure 7.4.

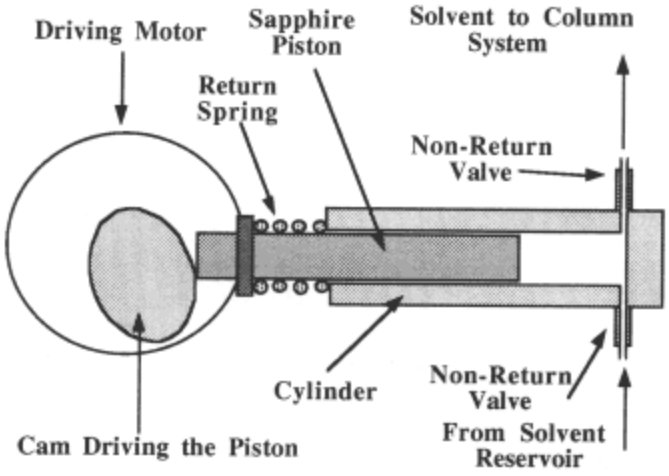


Figure 7.4
The Single Piston High-Pressure LC Pump

To reduce pulsation from a reciprocal pump, it may be fitted with twin cylinders or, alternatively, with some form of 'pulse damper'. Although pulses propagated through the column will theoretically cause a loss in column efficiency, the consequent loss in resolution is not usually very significant. The more serious problem associated with system pulses is the resulting detector noise which reduces the detector sensitivity. In order to accommodate a wide range of column diameters the pump should have a range of flow rates that extends from about 10 µl per minute to 10 ml per minute. Most modern pumps have two or sometimes three cylinders.

The Sample Valve

The sample valve can have either internal or external loops, the choice of which will largely depend on the volume of sample which, in turn, is determined by the column diameter. The internal loop valves have milled slots in the valve spigot that can have volumes ranging from about 0.1 µl to about 2 µl. External loops, on the other hand, consist of appropriate lengths of tubing that have sample volumes ranging from about 1.0 µl to 20 µl (or more) and are interchangeable. As the valve comes in direct contact with the sample it may need to be made from biocompatible materials such as PEEK or titanium. However, 'biocompatible' materials can be expensive, and it should be ascertained that the substances being separated are indeed labile in the presence of stainless steel, before resorting to the use of 'biocompatible' materials for construction. On the other hand, many compounds denature on the surface of stainless steel frits and render them impermeable and under such circumstances titanium frits can be essential..

The Internal Loop Valve

A diagram of the internal loop valve which utilizes only four ports is shown in figure 7.5.

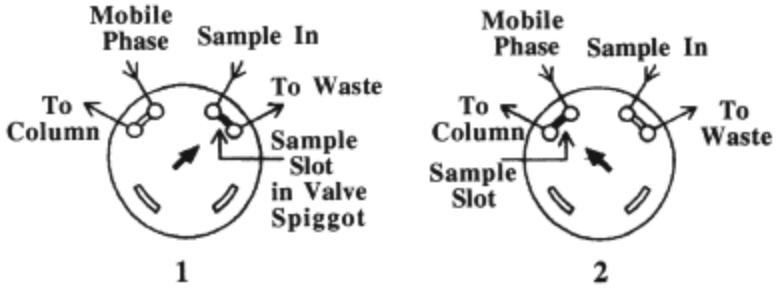


Figure 7.5
The Internal Loop Valve
Courtesy of Valco Instruments Inc.

The sample volume is contained in the connecting slot of the valve rotor and can deliver samples ranging from 0.1 µl to about 2.0 µl. The diagram on the left-hand side shows the sample being loaded into the valve by passing the sample from an appropriate syringe, through the rotor slot, to waste. During this period, the mobile phase supply passes through the valve and direct to the column. The valve is then rotated and the valve slot containing the sample is imposed between the mobile phase supply and the column. The sample is swept onto the column with the flow of solvent. A few seconds is allowed for the sample to be transferred to the column and the valve is then returned to the load position. This type of valve is used for short columns, perhaps 4.6 mm in diameter and 3 cm long packed with support particles only 3 µm in diameter. Alternatively, they might also be used, when employing somewhat longer columns (10 to 50 cm long) but only about 1 mm or so in diameter.

The External Loop Valve

The external loop sample system, which employs six ports, is depicted in figure 7.6.

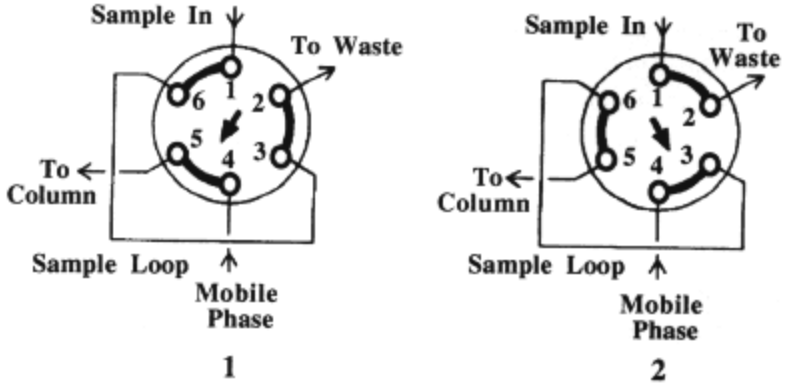


Figure 7.6
The External Loop Valve
Courtesy of Valco Instruments Inc.

The external loop sample valve has three slots cut in the rotor so that any adjacent pair of ports can be connected. In the loading position, shown on

the left, the mobile phase supply is connected by the rotor slot between port (4) and port (5) directly through to the column. In this position, the sample loop is connected across ports (3) and (6). Sample passes from the syringe into port (1) through the rotor slot to the sample loop at port (6) and the third slot in the rotor connects the exit of the sample loop to waste at port (2).

The sampling position is shown by the diagram on the right. On rotating the valve, the sample loop is interposed between the column and the mobile phase supply by connecting port (3) and (4) and ports (5) and (6) and the sample is swept onto the column. In the sampling position, the third rotor slot connects the syringe port to the waste port. After sampling, the rotor can be returned to the loading position, the system washed with solvent and the sample loop loaded in readiness for the next injection. For analytical applications, the sample loop can have a volume ranging from 1 to 20 µl, but for preparative work, loops with sample volumes of 1 ml or more can be placed on a preparative column.

Today, most liquid chromatographs that are used for routine analysis also include an automatic sampling device. This involves the use of some type of a transport mechanism that may take the form of a carousel or some form of belt conveyor system. The transporter carries a series of vials that are usually made to alternately contain sample and washing solvent. The sampling mechanism can be quite intricate, involving a complex sequence of operations that are automatically controlled by the device or, alternatively, by the chromatograph computer. The syringe plunger is usually operated pneumatically and the syringe is first washed with solvent, then it is rinsed with the sample, then reloaded with the sample and finally the contents are discharged into the column. After the analysis the next cycle commences with the next syringe washing procedure. In routine analytical laboratories, which often have very sophisticated LC assemblies, there may also be a sample-preparation robot which will automatically carry out such procedures as extraction, concentration, derivatization, etc. The robot is usually programmable, so that a variety of separation procedures can be carried in a sequence that

is unique for each sample. In laboratories that have a high throughput of samples, an automatic sampling device is often essential for the economic operation of the laboratory.

The Column and Column Oven

It is only over the last ten years that temperature has been used as a variable to enhance separations in liquid chromatography. In fact, even today, temperature is not generally used to control *retention* and *selectivity* because the change in free enthalpy of a given solute in one phase with temperature is generally very similar to that in the other phase. However, in chiral separations, where small changes in enthalpy can be essential, the use of an optimum temperature may be critical for the separation of a specific pair of enantiomers. Retention and selectivity are more frequently controlled by either changing the nature or porosity of the stationary phase, by changing the composition of the mobile phase or, if it is desired, to merely increase retention without altering the selectivity, by changing the column diameter or length. In chiral chromatography, however, both in GC and LC temperature has been found to be a critical parameter in controlling selectivity.

In addition, the effect of temperature on *column efficiency*, is now being frequently exploited, particularly in size exclusion chromatography (SEC) and in other separations where the standard free entropy dominates the separation (*i.e.* all chiral separations and the separation of all closely eluting isomers).

High efficiencies are essential in both SEC and chiral chromatography due to the very small separation ratios that occur and, to some extent, the limited peak capacity of the column. In practice, this means, if the peaks cannot be moved very far apart by suitable adjustment of the phase system, then the peaks must be kept very narrow to achieve resolution. Thus, high efficiency is used as an alternative to selectivity to accomplish the necessary separation.

The equation for the variance per unit length of a solute has already been discussed and is reiterated in equation (1):

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{f_1(k')d_p^2}{D_m}u + \frac{f_2(k')d_f^2}{D_S}u \qquad (1)$$

(λ) and (γ) are constants,

(D_m) and (D_s) are the diffusivities of the solute in the mobile and stationary phases respectively,

(dp) is the particle diameter of the packing,

(d_f) is the effective thickness of the film of stationary phase,

(k') is the capacity ratio of the solute,

and (u) is the linear velocity of the mobile phase.

It is seen from equation (1) that although in many instances the effect of temperature on the absolute value of (k') will be minimal in LC, there will be a very significant change in the diffusivity (D_m). It is therefore interesting to determine the effect of temperature on column efficiency (H) and retention time.

Now, by differentiating equation (1) and equating to zero an expression for the optimum velocity can be obtained:

$$\frac{dH}{du} = -\frac{B' D_m}{u^2} + \frac{(C_1 + \varepsilon C_2)}{D_m} \qquad (2)$$

where (B') is 2γ,

(C_1) is $f_1(k')d_p^2$

(C_2) is $f_2(k')d_f^2$

and (ε) is the ratio of D_m/D_s which will be sensibly constant over a limited range of temperature.

Thus, equating to zero and solving for (u_{opt}):

$$u_{opt.} = D_m \sqrt{\frac{B'}{(C_1 + \varepsilon C_2)}} \qquad (3)$$

Now substituting for (u) in equation (1) the minimum value of H, (H_{min}) that will provide the maximum column efficiency is given by:

$$H = A' + \frac{B' D_m}{D_m \sqrt{\frac{B'}{(C_1 + \varepsilon C_2)}}} + \frac{(C_1 + \varepsilon C_2)}{D_m} D_m \sqrt{\frac{B'}{(C_1 + C_2)}}$$

where (A') = 2λdp

Simplifying:

$$H_{min} = A' + \sqrt{B'(C_1 + \varepsilon C_2)} \qquad (4)$$

It is seen from equation (3) that the optimum velocity is directly related to the solute diffusivity and thus, to the temperature. It follows that increasing the temperature, increases the optimum velocity and thus provides the same efficiency but with shorter analysis time. However, the choice of operating temperature can be somewhat complicated. It will be seen later that there is a temperature at which the two enantiomers co-elute and, at the co-elution temperature, separation is impossible. As a consequence, the operating temperature must be chosen significantly above, or below, the coelution temperature to provide adequate selectivity. In addition, the operating temperature must also be *below* the *maximum permissible* for the particular stationary phase, as above this temperature the stationary phase degrades.

Now from equation (4), which is an expression for the minimum value of (H) (and consequently the maximum column efficiency) it is seen that the solute diffusivity has no effect on solute dispersion and consequently, the maximum column efficiency is more or less *independent of temperature*. This is because the effect of solute diffusion on dispersion

by longitudinal diffusion is inversely proportional to the effect of diffusion on dispersion by solute resistance to mass transfer. Thus, any change due to temperature effects on diffusion, will be canceled out by the two different dispersion processes. It follows, that where analysis time is critical then column temperature control can be very important. The available temperature range of LC ovens varies a little from instrument to instrument but an operational range from 0°C to 80°C would cover the majority of LC separations. One of the problems associated with the temperature control of ovens is the high thermal capacity of the column and the mobile phase. It is clear that the thermostatting medium also needs to have a high thermal capacity to facilitate a fast response to temperature changes and thus accurate temperature control. In general, due to the small heat capacity of gases, air-ovens are not very efficient. The use of a liquid thermostatting media, however, can have spillage problems, and, in addition, column replacement and the removal of end fitting tends to become a little messy. At this time, despite its theoretically poor technical performance, the air oven appears to be the most common due to it being cleaner to operate.

Another heating and cooling technique has been recently introduced to contemporary liquid chromatographs and that is the Peltier heat exchangers which are particularly efficient. Although a heat transfer medium to the column is still necessary, the heat supply and sink is simpler, cleaner and more controllable than the normal ohmic heating and refrigeration system. Basically, Peltier heating is the thermocouple effect in reverse. By passing a current through the junctions of a pair of dissimilar metals one junction generates heat and the other is cooled depending on the direction of current flow. Thus, one junction can be used to heat or cool the oven by adjustment of the magnitude of the current and its direction, providing a heat exchanger is available to remove heat from, or provide heat to the other junction. This type of heating has, so far, only been used for relatively small oven units but appears to be the temperature controlling device of the future.

Liquid Chromatography Detectors

Detector specifications have been discussed in chapter 5. They reveal the accuracy and precision attainable in quantitative analysis and also the lower concentration levels that are possible in trace analysis. As in GC, the five specifications of prime importance are detector response, detector noise level, detector sensitivity, or minimum detectable concentration, detector linearity and linear dynamic range [1]. The detector *response*, detector *noise level* and the detector *sensitivity* are relevant to trace analysis and the detector *linearity* and the *linear dynamic range* are pertinent in general quantitative analysis [2]. The linear dynamic range of LC detectors is usually much less than their GC counterparts which evinces more care in choosing the sample size in quantitative LC analyses. A GC detector may have a linear response that extends over a concentration range of five or six orders of magnitude. In contrast, an LC detector is more likely to have a linear dynamic range of only three orders of magnitude and some detectors may have considerably less than that. In addition, LC detectors have about two orders of magnitude less sensitivity than that of the GC, FID detector but this renders the restricted linear dynamic range less significant. The relatively low sensitivity of LC detectors usually evokes the use of some form of sample concentration when used for trace analysis.

There are a large number of LC detectors available but, of these, a relatively few are in common use. This is largely because some detectors are highly specific and others are very expensive. About 95% of all LC analyses are carried out using one of 5 different types of detectors, the UV adsorption detector, the fluorescence detector, the electrical conductivity detector, the light scattering detector and the refractive index detector. However, in addition to these basic detectors, for obvious reasons an example of a chiral detector will also be included.

The UV Detector

The UV detector is the most popular and generally useful LC detector and this is particularly true if multi-wavelength technology is included in the *genera* of UV detectors. The UV detector has the best combination of

sensitivity, versatility and reliability, despite the fact that it is insensitive to non-polar solutes and others that do not possess a UV chromaphore. Fortunately, the majority of compounds adsorb UV light somewhere in the range of 200–350 Å including those having one or more double bonds (π electrons) and all substances that have unshared (non-bonded) electrons (*e.g.* all olefins, aromatics and all substances containing -CO, -CS, -N=O and -N = N - groups).

The relationship between the intensity of UV light transmitted through the cell and the concentration of solute is given by 'Beers' Law:

$$I_T = I_o e^{-kLc}$$

where (I_O) is the intensity of the light entering the cell,

(I_T) is the intensity of the transmitted light,

(L) is the path length of the cell,

(c) is the concentration of the solute,

and (k) is the molar extinction coefficient of the solute for the specific wavelength of the UV light.

or:

$$\ln I_T = \ln I_o - kLc \qquad (5)$$

Changed to a slightly different form: $I_T = I_o 10^{-k'Lc}$

where (k') is the molar extinction coefficient of the solute.

Differentiating equation (5):

$$I_T = I_o 10^{-k'Lc}$$

From equation (6) it is seen that the detector sensitivity is determined by the magnitude of the extinction coefficient of the solute which will depend on the wavelength of the UV light that is used. Thus, the minimum detectable concentration can be changed by selecting a light

source of different wavelength. The detector sensitivity also depends on the path length of the cell. Unfortunately, the cell length cannot be increased indefinitely to provide higher sensitivity, as long cells will cause excessive peak dispersion, with consequent loss of resolution. The optimum cell length will be that which provides the minimum peak dispersion and minimum loss in resolution. There are two types of UV detector; the *fixed wavelength detector* and the *multi-wavelength detector*. Although the fixed wavelength detector is one of the least expensive detectors and can provide the highest sensitivity and have the minimum cell volume, it is not as popular as the multi wavelength detector. The probable reason is that the wide choice of wavelengths available with the multi-wavelength detector allow it to be used for a very wide range of substance types.

The Fixed Wavelength Detector

A diagram of a fixed wavelength UV detector is shown in figure 7.7.

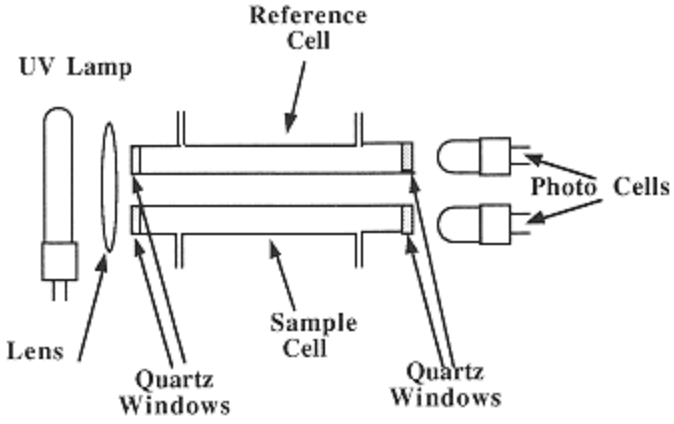


Figure 7.7
The Fixed Wavelength UV Detector

The detector consists of a small cylindrical cell (2.0 to 10.0 µl in volume) through which flows the eluent from the column. UV light passes through the cell and falls on a UV photoelectric sensor. The wavelength of the light will depend on the type of lamp that is used. There are a

number of lamps available that provide UV light having wavelengths ranging from about 210 nm to 280 nm. The most popular light source is the mercury vapor lamp. The mercury vapor lamp emits light at a wavelength that gives the detector the maximum versatility for detecting a wide range of solute types. The detector assembly has a sample and reference cell and the output from the sample cell is compared to that from the reference cell, thus eliminating any fluctuations in the light intensity emitted from the UV mercury vapor lamp.

The difference-signal is processed by a non-linear amplifier that converts the signal to one that is linearly related to concentration of solute in the sample cell. The fixed wavelength detector is inexpensive and, as the light is emitted at a specific wavelength(s), it has a high intensity and can provide a greater intrinsic sensitivity than the multi-wavelength UV detectors. However, the multi-wavelength detector can often compensate for this lack of sensitivity by operating at a wavelength where the absorption of the solute is a maximum and thus, provides the greatest response. The average specifications for a commercially available fixed wavelength UV detectors are as follows:

Sensitivity (toluene)	5×10^{-8} g/ml
Linear Dynamic Range	5×10^{-8} to 5×10^{-4} g/ml
Response Index	0.98 - 1.02

If designed with very small sensing cells, and used in conjunction with electronic systems with very small time constants, the fixed wavelength detector can give a very fast response at high sensitivity and very low peak dispersion. Consequently, it can be made suitable for monitoring very fast separations.

The Multi-Wavelength Detectors

There are basically two types of multi-wavelength detectors, the *dispersion detector*, that monitors the eluent at one wavelength only, and the *diode array detector*, that simultaneously monitors the eluted solute over a range of wavelengths. The former passes the light from a broad

emission light source through a monochromator, selects a specific wavelength, and allows it to pass through the detecting cell. The second also uses a broad emission light source, but all the light passes through the sensing cell, which is dispersed by means of a holographic grating and allowed to fall on an array of diodes. The dispersive instrument is no longer manufactured, so only the diode array detector will be described.

The Diode Array Detector

A diagram of a diode array detector is shown in figure 7.8. Light from the broad emission source such as a deuterium lamp is collimated by an achromatic lens system so that the total light passes through the detector cell onto a holographic grating. In this way, the sample is subjected to light of all wavelengths generated by the lamp. The dispersed light from the grating is allowed to fall on to a diode array. The array may contain many hundreds of diodes and the output from each diode is regularly sampled by a computer and stored on a hard disc.

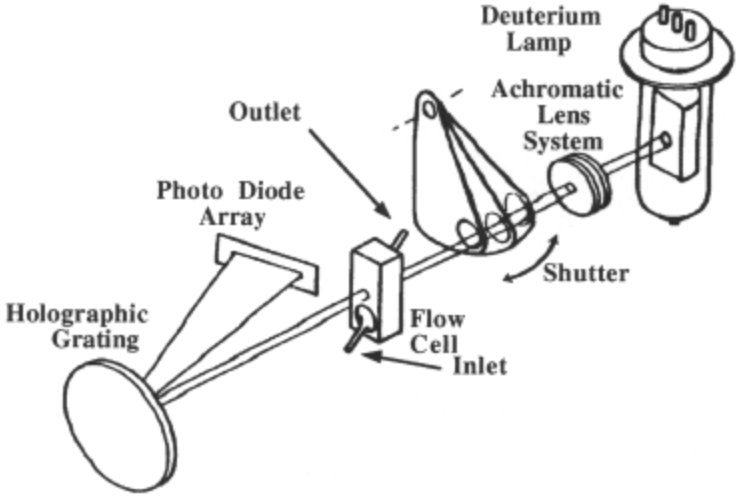


Figure 7.8
The Diode Array Detector
Courtesy of Hawlett-Packard Inc.

On completing the analysis, the output from any diode can be selected and a chromatogram produced displaying the adsorption of the light

having the wavelength that was falling on that particular diode. Most instruments will monitor the output of at least one diode in real time, so that the chromatogram can be followed as the separation develops. By noting the elution time of a particular peak, a UV adsorption spectrum of the solute can be obtained by recalling from memory the output of all the diodes at that particular time. This gives directly, the spectrum of the solute, *i.e.* a curve relating adsorption against wavelength. The diode array detector has some unique features, and the use of a diode array detector to verify the purity of a given solute is shown in figure 7.9. The chlorthalidone was isolated from a sample of tablets and separated by a reverse phase (C18) on a column, 4.6 mm I.D., 3.3 cm long, using the solvent mixture methanol/aqueous acetic acid solution (water containing 1% of acetic acid) : 35/65 v/v. The flow rate was 2 ml/min and the chromatogram produced, monitored at 274 nm, is shown in the lower part of figure 7.9.

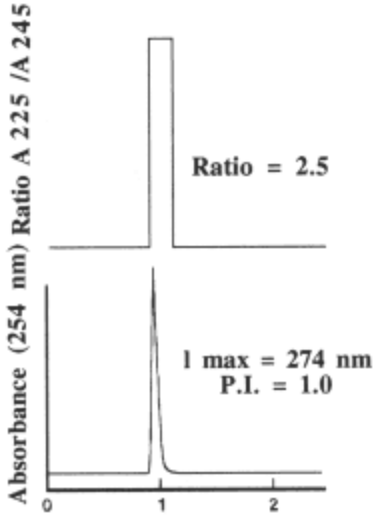


Figure 7.9
Dual Channel Plot from a Diode Array
Detector Confirming Peak Purity
Courtesy of the Perkin Elmer Corporation

As a diode array detector was employed, it was possible to ratio the output from the detector at different wavelengths and plot the ratio

simultaneously with the chromatogram monitored at 274 nm. Now, for a pure and homogeneous peak, the ratio of the adsorption at the two wavelengths (those selected being 225 and 245 nm) would remain constant throughout the elution of the entire peak. The upper diagram in figure 7.9 shows this ratio plotted on the same time scale and a clean rectangular peak is observed which confirms the purity of the chlorthalidone. Another example of the use of the diode array detector to confirm the integrity of an eluted peak is afforded by the separation of a mixture of aromatic hydrocarbons which is shown in figure 7.10. The separation was carried out on a column 3 cm long, 4.6 mm in diameter and packed with a C18 reversed phase on particles 3 μm in diameter. It is seen that the separation appears to be satisfactory, and all the peaks represent individual solutes.

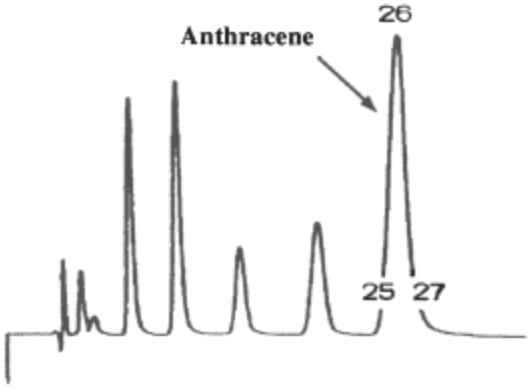


Figure 7.10
The Separation of Some Aromatic Hydrocarbons
Courtesy of the Perkin Elmer Corporation

However, by plotting the adsorption ratio, 250/255 nm for the anthracene peak it was clear that the peak tail contained an impurity. The absorption ratio peaks are shown in figure 7.11. The peak ratios shown clearly indicate that an impurity is present by the sloping top of the anthracene peak. Spectra taken at the leading and trailing edge of the anthracene peak are shown superimposed in figure 7.12. The presence of

the impurity is completely confirmed by the different spectra shown for the leading and trailing edges of the peak.

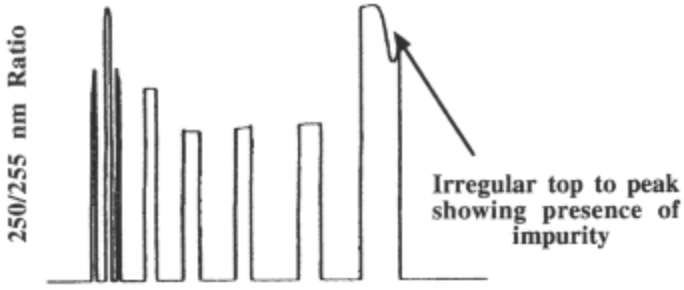


Figure 7.11
Curves for Adsorption Ratio, 250/255nm/Time
Courtesy of the Perkin Elmer Corporation

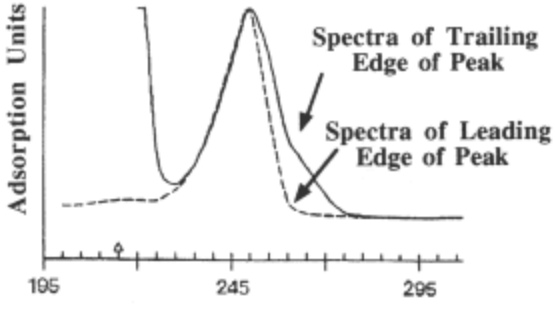


Figure 7.12
Superimposed Spectra Taken at the Leading and Trailing Edges of the Anthracene Peak
Courtesy of the Perkin Elmer Corporation

The impurity was eventually identified as about 5% t-butyl benzene. Typical values for their more important specifications of the diode array detector are as follows:

Sensitivity	1×10^{-7} g/ml
Linear Dynamic Range	5×10^{-7} to 5×10^{-4} g/ml
Response Index	0.97 – 1.03

The Electrical Conductivity Detector

The electrical conductivity detector is used almost exclusively in ion exchange chromatography and, as a consequence, is the second most commonly used detector in LC. It can only detect the presence of charged ions in the mobile phase and, accordingly only those substances that ionize. Consequently, the electrical conductivity detector is frequently used in the analysis of inorganic acids, bases and salts. The detection system is extremely simple and consists of only two electrodes situated in a suitable detector cell. A simple electrical conductivity detector sensing cell is shown in figure 7.13.

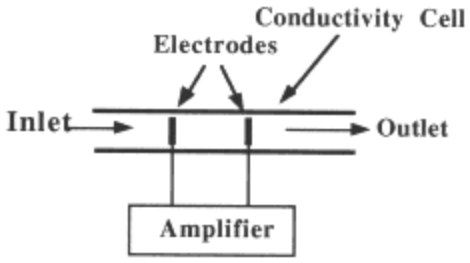


Figure 7.13
An Electrical Conductivity Detector Sensor

The electrodes are arranged to constitute one arm of a Wheatstone Bridge. When ions are present in the detector cell, the electrical resistance changes and the out-of-balance signal is passed to a suitable amplifier. The amplifier output is either digitized, and the binary number sent to a computer for storage, or is passed directly to a potentiometric recorder. The detector actually measures the electrical impedance between the electrodes which, by suitable non-linear amplification, provides an output that is linearly related to solute concentration. To avoid electrode polarization, an AC voltage (about 10 kHz) is applied across the electrodes to measure the cell impedance. Typical specifications for the conductivity detector are as follows:

Sensitivity	5×10^{-9} g/ml
Linear Dynamic Range	5×10^{-9} to 1×10^{-6} g/ml
Response Index	0.97 – 1.03

The chromatogram from the analysis of a mixture of alkali and alkaline earth cations at levels of a few parts per million is shown in figure 7.14. The cations lithium, sodium, ammonium, potassium, magnesium and calcium were present in the original mixture at concentrations of 1, 4, 10, 10, 5 and 10 ppm respectively. The separation obtained is shown in figure 7.14. A proprietary ion exchange column, IonPacCS12, was used and the mobile phase consisted of a 20 nM methanesulfonic acid solution in water. A flow rate of 1 ml/min was employed and the sample volume was 25 µl.

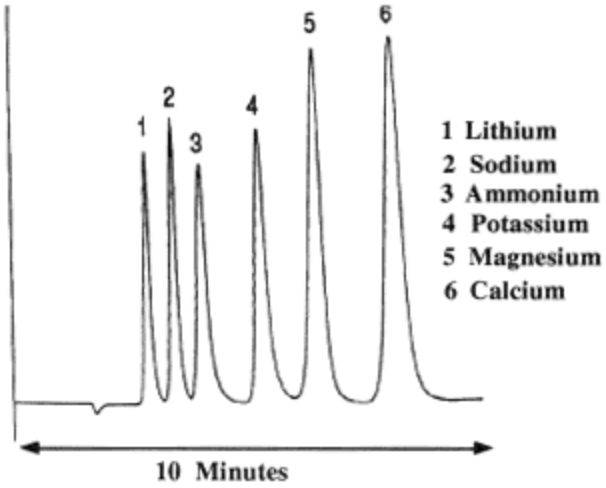


Figure 7.14
Determination of Alkali and Alkaline Earth Cations
Courtesy of Dionex Inc.

The separation is an example of the use of the *ion suppression* technique. The methanesulfonic acid solution has a high electrical conductivity and, if passed through the detector, would give a large detector output which would swamp the much smaller signal from the sample ions. Thus, after the mobile phase leaves the column (and after the methanesulfonic acid has achieved its purpose and helped produce the desired separation) the reagent must be removed. This ensures that the mobile phase entering the detector contains little or no ions, other than those being analyzed and

that there is minimal background conductivity. There are a number of ways of removing the methanesulfonic acid, but probably the simplest is to pass the mobile phase through a short reverse phase column situated between the column and the detector. The reverse phase column removes any organic material contained in the column eluent by strong dispersive interactions with the hydrocarbon chains of the reverse phase. Obviously, the ion suppression column will eventually saturate and need to be regenerated by desorbing the methanesulfonic acid with a strong dispersive solvent that is miscible with water, such as acetonitrile. The technique of ion suppression is frequently used in ion exchange chromatography when using the electrical conductivity detector. There are other ion suppression techniques available but a detailed discussion of them is outside the scope of this book. This detector is used frequently for the detection of amino acids, hydroxy acids and quaternary amines

The Fluorescence Detector

The fluorescence detector is probably the most sensitive LC detector and is often used for trace analysis. Molecules are excited by electromagnetic radiation to produce luminescence and this effect is called photoluminescence.

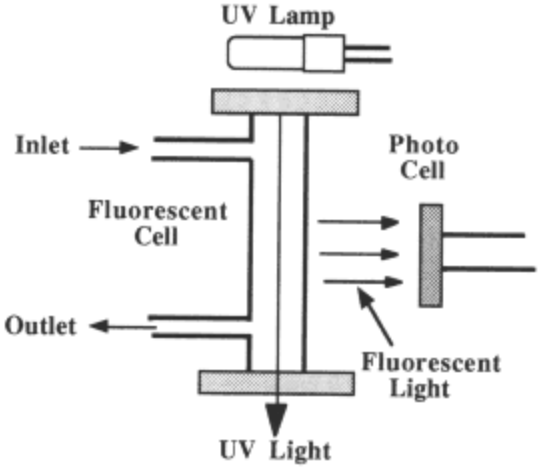


Figure 7.15
The Fluorescence Detector

If the release of electromagnetic energy is immediate, and stops upon the removal of the exciting radiation, the effect is called fluorescence, and it is this phenomenon that is utilized in the fluorescence detector. Although the detector is very sensitive, its response is only linear over a limited concentration range. In fact, for accurate work, the detector cannot be assumed to be linear over a concentration range of much more than two orders of magnitude. Unfortunately, the majority of substances do not naturally fluoresce and thus fluorescent derivatives of the substances may need to be synthesized to render them detectable. A diagram of the fluorescence detector is shown in figure 7.15. Light from a fixed wavelength UV lamp passes through a cell, carrying the column eluent, and the fluorescent light that is emitted by a solute is sensed by a photoelectric cell positioned normal to the direction of exciting UV light. The photo cell senses light of all wavelengths that is generated by fluorescence but the wavelength of the excitation light can only be changed by use of an alternative lamp. This simple type of fluorescence detector is relatively inexpensive, and can be extremely sensitive. Typical specifications for a fluorescence detector are as follows:

Sensitivity (Anthracene)	1×10^{-9} g/ml
Linear Dynamic Range	1×10^{-9} to 5×10^{-6} g/ml
Response Index	0.96 – 1.04

The ultimate fluorescence detector is one that uses a monochromator to select the excitation wavelength and a second monochromator to select the wavelength of the fluorescent light. This instrument gives the maximum versatility and sensitivity. However, this not a commonly used instrument, due to it being very complex and expensive, and will not be discussed here.

Fluorescent sensitivity is commonly enhanced by forming fluorescent derivatives with appropriate fluorescing reagents. This procedure can also be used in chiral chromatography providing the fluorescent derivatives are still enantiomeric and subject to the chiral selectivity of the stationary phase. There is, however, another method of using the fluorescence as a detection method and that is by employing a UV

photochemical reactor prior to the fluorescence detector in the manner depicted in figure 7.16.

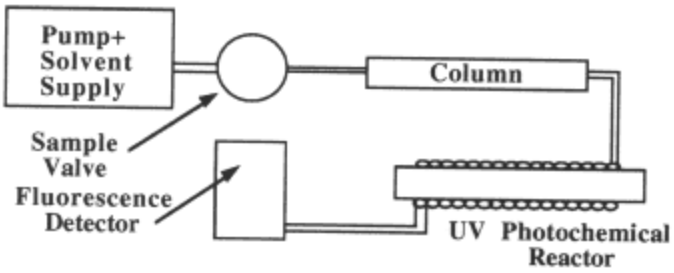


Figure 7.16
The Use of a Photochemical Reactor to Enhance Fluorescence Detection

The equipment is similar to a standard LC system except that a length of UV transparent tubing (usually coiled or woven and made from Teflon or quartz) connects the column outlet to the detector. The tubing passes round, by, or through, a strong UV beam of light (8 W) generated by a relatively high energy source. The strong light energy causes the solute to undergo a range of chemical changes and reactions (often quantitative) which usually result in the formation of highly fluorescent materials that are readily detected. This method has a wide range of applications beitmay, the actual mechanism of chemical change not always being well understood. As the photochemical reaction occurs *after* separation, the success of this system will not depend on the stability of the chiral centers and it does not matter if they are degenerated during the photochemical process. It will be important, however, that the reactor tube does not cause significant band dispersion during the passage of the solute to the detector, otherwise the separation achieved in the column will be lost as a result of peak merging in the reactor. A suitable low-dispersion reactor can be fabricated from 30 m of 0.3 mm I.D. Teflon tubing, crocheted in the form of a fabric cylinder, within which the light source is situated.

The Light Scattering Detectors

There are two types of light scattering detector the *evaporative light scattering detector* and the *liquid light-scattering detector*. The

evaporative light scattering detector incorporates a spray system that continuously atomizes the column eluent into small droplets. The droplets are allowed to evaporate, leaving the solutes as fine particulate matter suspended in the atomizing gas.

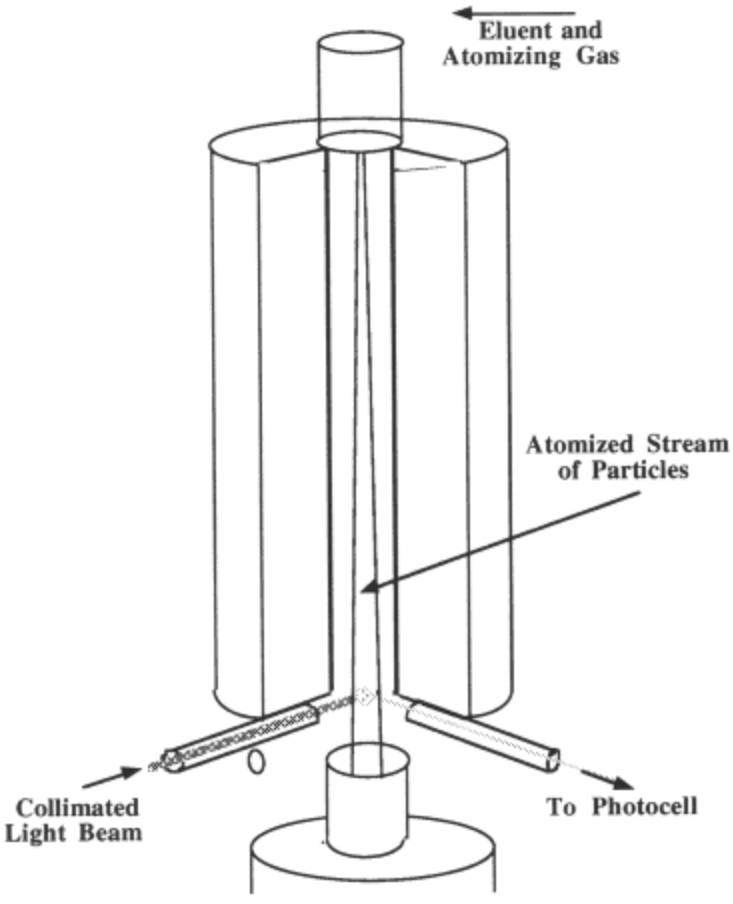


Figure 7.17
The Sensor of a Commercial Evaporative Light Scattering Detector
Courtesy of the Polymer Laboratories Inc

The atomizing gas may be air or an inert gas if so desired. The suspended particulate matter is arranged to pass through a light beam and the light scattered by the particles viewed at 45° by an appropriate sensor

system. The output of the sensor is electronically processed and then passed, either to a computer, or to a potentiometric recorder. The detector responds to all solutes that are not volatile and, as the light dispersion is largely Raleigh scattering, the response is proportional to the mass of solute present. To ensure linearity, the droplet size must be carefully controlled as it, in turn, controls the particle size of the dried solutes. The sensitivity of the detector is claimed to be between 10 and 20 ng of solute. However, in these terms it is difficult to compare it with other detectors. A diagram of the evaporative light scattering detector sensor, manufactured by Polymer Laboratories, that displays the basic principles involved, is shown in figure 7.17.

The eluent is atomized in a stream of nitrogen and the finely divided spray passes down a heated chamber to evaporate the solvent. The removal of the solvent converts the stream of droplets into a stream of particles which then pass through a collimated light beam. The light scattered by the particles, at an angle to the incident light, is focused onto a photomultiplier and the output is processed in an appropriate manner electronically. The device is fairly compact and relatively simple to operate.

Liquid light-scattering detectors respond to the light scattered by a polymer or large molecular weight substances in the column eluent as it passes through an appropriate sensor cell while illuminated by a high intensity beam of light. The light source is usually a laser that generates light at an appropriate wavelength for measurement. There are two basic forms of the detector: the *low angle laser light-scattering* (LALLS) detector and the *multiple angle laser light-scattering* (MALLS) detector. Both devices are in common use, but the multiple angle laser light-scattering detector is considered to have greater versatility, as it can also provide molecular dimensions as well as molecular weights. As the scattered light is measured at a very small angle to the incident light (virtually 0°), the low angle laser light-scattering detector signal can also be affected by scattering from contaminating particulate matter that is always present in the eluent.

The ratio of the intensity of the light scattered at an angle ϕ (I_ϕ) to the intensity of the incident light (I_o), for Rayleigh light scattering is given by:

$$\frac{I_\phi}{I_o} = \alpha \omega R_\phi \qquad (7)$$

where, (α) is the attenuation constant,

(ω) is a function of the refractive index,

and ($R\phi$) is Raleigh's constant.

Thus:

$$R\phi = \frac{I_\phi}{\alpha \omega I_o} \qquad (8)$$

Now, the molecular weight (M_w) of the solute is related to the Rayleigh factor by the following expression:

$$M_w = \frac{R\phi}{c(K - 2A_2 R\phi)} \qquad (9)$$

where (c) is the concentration of the solute,

(A_2) is a function of polymer-polymer interactions,

and (K) is the polymer optical constant.

Substituting for (R_ϕ) in (9) from (8):

$$M_w = \frac{\frac{I_\phi}{\alpha \omega I_o}}{c\left(K - 2B_2 \frac{I_\phi}{\alpha \omega I_o}\right)} = \frac{I_\phi}{c(\alpha \omega I_o K - 2B_2 I_\phi)} \qquad (10)$$

Where:

$$K = \frac{2\pi^2\eta^2}{\lambda^4 N\left(\frac{d\eta}{dc}\right)^2} \qquad (11)$$

where (η) is the solvent refractive index,

(l) is the wavelength of the light in vacuum,

and (N) is Avogadro's number.

Equation (9) gives the basic relationship between the molecular weight of the scattering material, the intensity of the scattered light and the physical properties of the materials and equipment being involved. Equation (10), however, contains constants, the magnitude of which are difficult to determine. Consequently, in practice a simple graphical procedure is used to determine the molecular weight of the solute without the need to determine all the pertinent constants. Rearranging equation (9):

$$\frac{1}{M_w} = \frac{c(K - 2A_2R\phi)}{R\phi} = \frac{cK}{R\phi} - 2cA_2$$

or:

$$\frac{cK}{R\phi} = 2cA_2 + \frac{1}{M_w} \qquad (12)$$

Now (c), (K), and (R_ϕ) are either known or can all be calculated from known data and light scattering measurements; thus, by plotting $\left(\frac{cK}{R\phi}\right)$ against (c) a straight line will be produced with the intercept being $\left(\frac{1}{M_w}\right)$.

The Multiple Angle Laser Light-Scattering (MALLS) Detector

In the multiple angle laser light-scattering detector, the scattering measurements are made at a number of different angles, none of which are close to the incident light. This reduces the problem associated with scattering from particulate contaminants in the sample. Data taken at a series of different angles to the incident light, allows the *root-mean-square (rms)* of the molecular radius $\langle r^2 \rangle^{1/2}$ to be calculated, in addition to the molecular weight of the substance. The relationship that is used is as follows:

$$\frac{cK}{R\phi} = a\langle r^2 \rangle^{1/2} \sin(\theta)^2 + bM_w \qquad (13)$$

In fact, theory can provide explicit functions for (a) and (b) but values for these constants are usually obtained from calibrating substances of

known molecular weights and molecular radii. Furthermore, each photocell will not have precisely the same response to low levels of light intensity and, consequently, calibration procedures are also necessary to take their different responses into account, to provide appropriate correction factors. The total number of different angles at which the scattered light is measured differs with different instruments. Commercial equipment that measures the intensity of the scattered light at 16 different angles is available. It is clear that the greater the number of data points taken at different angles, the more precise the results will be. A diagram of a (MALLS) detector system which measures the light scattered at *three* different angles is shown in figure 7.18.

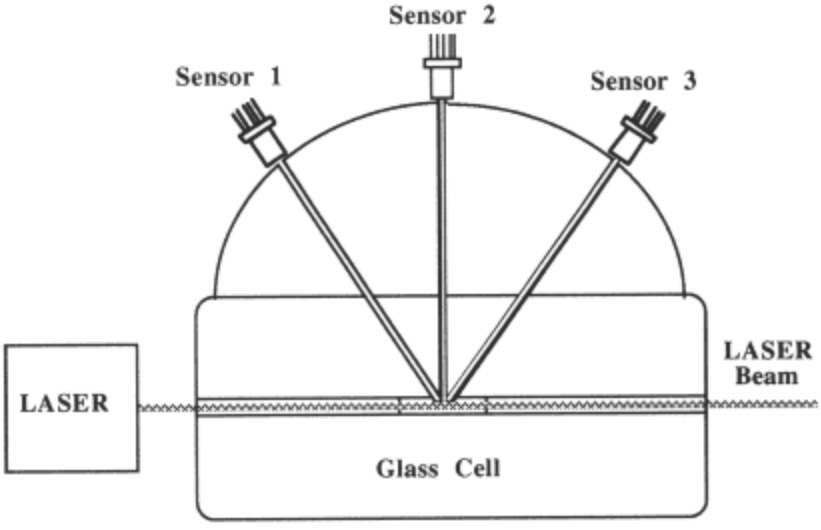


Figure 7.18
The Multiple Angle Laser Light-Scattering Detector (miniDawn®)
Courtesy of Wyatt Technology Corporation

This device, manufactured by Wyatt Technology Corporation, is called the miniDawn®. It contains no mirrors, prisms or moving parts and is designed such that the light paths are direct and not "folded". As seen in figure 7.18, light passes from the laser (wavelength 690 nm) directly through a sensor cell. Light scattered from the center of the cell passes

through three narrow channels to three different photocells, set at 45° and 90° and 135° to the incident light. Thus, scattered light is continuously sampled at three different angles during the passage of the solute through the cell. A continuous analog output is provided from the 90° sensor and all the sensors are sampled every 2 s. The molecular weight range extends from 10^3 to 10^6 Daltons and the *rms* radii from 10 to 50 nm. The total cell volume appears to be about 3 µl and the scattering volume is 0.02 µλ. The detector has a sensitivity, defined in terms of the minimum detectable excess Rayleigh ratio, of 5×10^{-8} cm^{-1} which is difficult to translate into normal concentration units but appears to be have a minimum detectable concentration of about 10^{-6} g/ml.

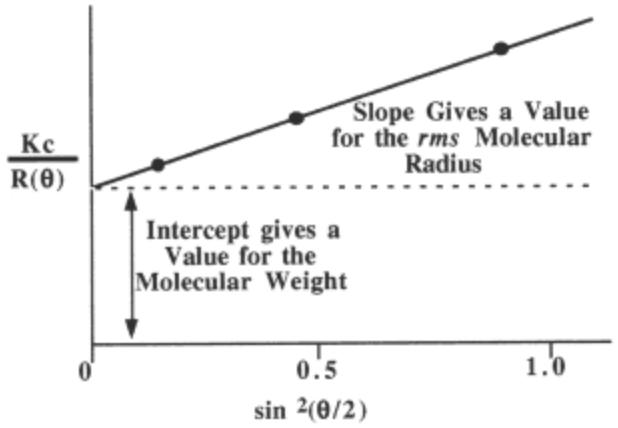


Figure 7.19
Calibration Curves

The relationship between the intensity of the scattered light, the scattering angle and the molecular properties are as follows:

$$\frac{cK}{R\phi} = 2cA_2 + \frac{1}{M_w P(\phi)}$$

where $P(\theta)$ describes the dependence of the scattered light on the angle of scatter and the other symbols have the meanings previously attributed to them.

In fact, the relationship between the angle of scattering, (θ), the molecular weight and the *rms* molecular radius of the solute is obtained using equation (13). Employing appropriate reference materials, graphs of the form shown in figure 7.19 can be constructed to evaluate constants (a) and (b) and thus permit the measurement of the molecular weight and molecular radius of unknown substances.

The Refractive Index Detector

The refractive index detector is the least sensitive of all the commercially available LC detectors. It is very sensitive to changes in ambient temperature, pressure changes, flow-rate changes and cannot be used for gradient elution. Nevertheless, despite these apparently overwhelming disadvantages, this detector has been found extremely useful for detecting those compounds that are non-ionic, do not adsorb in the UV and do not fluoresce. There are a number of optical systems used in refractive index detectors [2], but the simplest and most common in use, is the differential refractive index detector, the principle of which is shown diagramatically in figure 7.20.

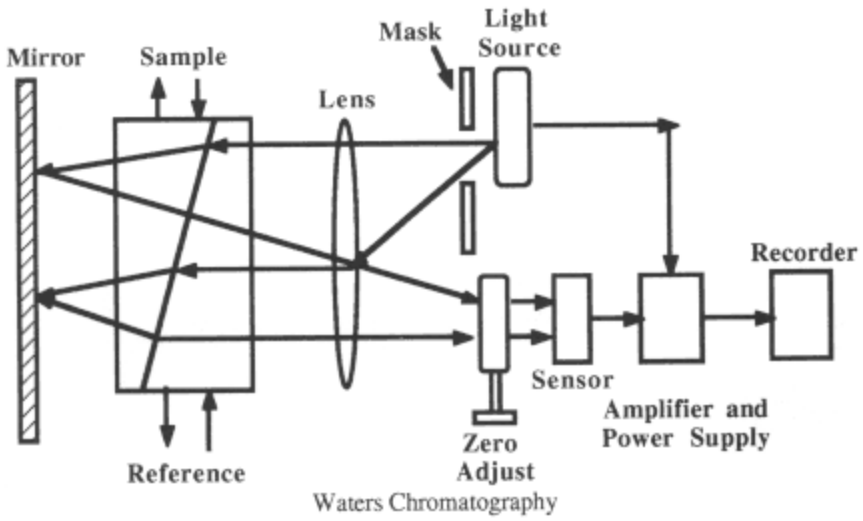


Figure 7.20
The Refractive Index Detector

The differential refractometer responds to the deflection of a light beam caused by the refractive index difference between the contents of the sample cell and that of the reference cell. A beam of light from an incandescent lamp passes through an optical mask that confines the beam to the region of the cell. A lens collimates the light beam which passes through both the sample and reference cells to a plane mirror. The mirror reflects the beam back through the sample and reference cells to a lens which focuses it onto a photo cell. The location of the beam, rather than its intensity, is determined by the angular deflection of the beam caused by the refractive index difference between the contents of the two cells. As the beam changes its position of focus on the photoelectric cell, the output changes and the resulting difference signal is electronically modified to provide a signal proportional to the concentration of solute in the sample cell.

The refractive index detector is used for those applications where, for one reason or another, other detectors are inappropriate or impractical. However, the detector has one particular area of application for which it is specially useful and that is in the separation and analysis of polymers. In general, for those polymers that contain more than six monomer units, the refractive index is directly proportional to the concentration of the polymer and is practically independent of the molecular weight. Thus, a quantitative analysis of a polymer mixture can be obtained by the simple normalization of the peak areas in the chromatogram, there being no need for the use of individual response factors.

Some typical specifications for the refractive index detector are as follows:

Sensitivity (benzene)	1×10^{-6} g/ml
Linear Dynamic Range	1×10^{-6} to 1×10^{-4} g/ml
Response Index	0.97 – 1.03

A typical application for the RI detector is afforded by the separation of the products of β-cyclodextrin hydrolysis, a chromatogram of which is

shown in figure 7.21. The separation was carried out on a TSKgel G-Oligo-PW column, 8 mm I.D., and 30 cm long, at 60°C and a flow rate of 1 ml/min. The TSKgel packing is a vinyl polymer based material suitable for separation by size exclusion using aqueous solvents. There are a number of grades of this material available that are suitable for separations, covering a wide range of molecular weights. It is seen that the products of the hydrolysis are well separated and almost all of the oligomers are resolved.

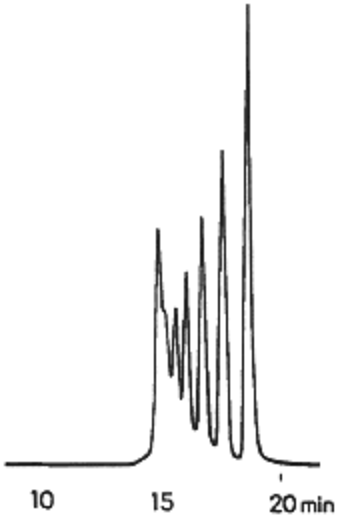


Figure 7.21
Separation of the Hydrolysis
Products of β-Cyclodextrin
Courtesy of TOYO SODA
Manufacturing Co. Lt

The refractive index detector is ideal for the detection of materials such as the cyclodextrins, as they contain only the elements hydrogen, carbon and oxygen. In addition, all the oxygen atoms exist as hydroxy groups or carbon-oxygen-carbon bonds and thus do not possess any UV chromaphores and do not induce fluorescence. Another example of the application of the refractive index detector to the analysis of cyclodextrins is given in figure 7.22 which depicts the separation of the three cyclodextrins. The separation was carried out on a β-naphthyl

ligand reverse phase column, 25 cm long, and 4.6 mm I.D., and represents the separation of 100 µg of the mixture. Pure degassed water was used as the mobile phase at a flow rate of 1 ml/min.

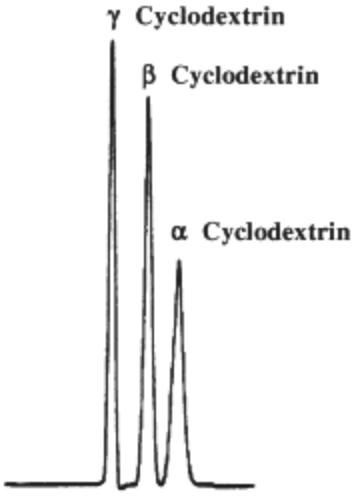


Figure 7.22
The Separation of the α-, β-, γ-Cyclodextrins Monitored with a Refractive Index Detector
Courtesy of ASTEC Inc

Chiral Detectors

The successful development of a chiral detector based on optical rotation measurement hinges to a large extent on the use of the Faraday effect. Consider the situation where a plane polarized beam of light passes through a medium that is subjected to a strong magnetic field. Under such circumstances the plane of the polarized beam will be rotated through a small angle (α), and it can be shown that the angle (α) is given by:

$$\alpha = VdH$$

where (V) is the Verdet Constant,

(d) is the path length

and (H) is the magnetic field strength.

This relationship is known as the Faraday effect. In practice, the magnetic field is generated in an air core coil inside of which is situated a rod, made from a glass having a high Verdet constant.

Now:

$$H = iN$$

where (i) is the current through the coil,

and (N) is the number of turns in the coil.

Thus:

$$\alpha = VdiN$$

Thus (α) can be controlled by varying (i). The rotational resolution ($\Delta\alpha$) may be as little as 10^{-5} degree but due to heat losses in the coil, a maximum practical value of ($\Delta\alpha$) will be about ± 2°. A diagram of the detector is shown in figure 7.23.

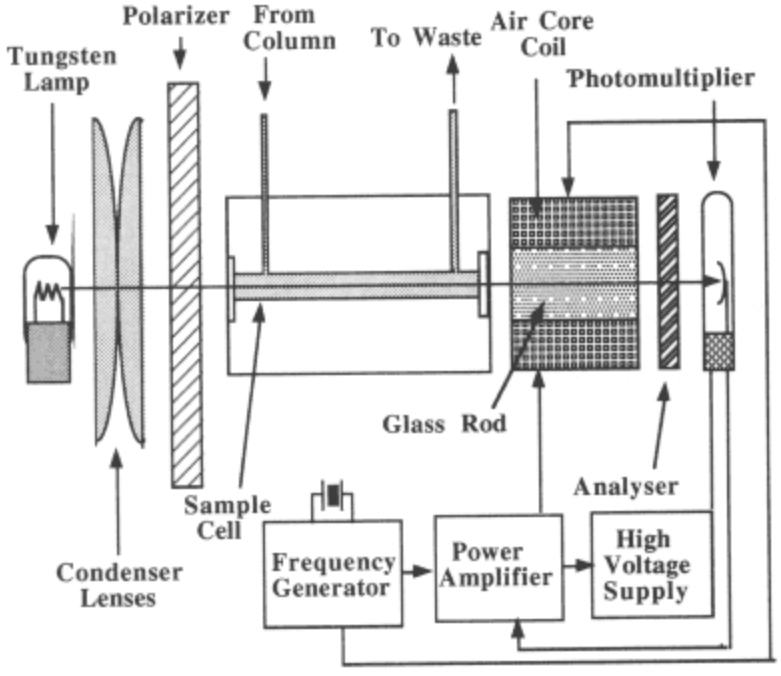
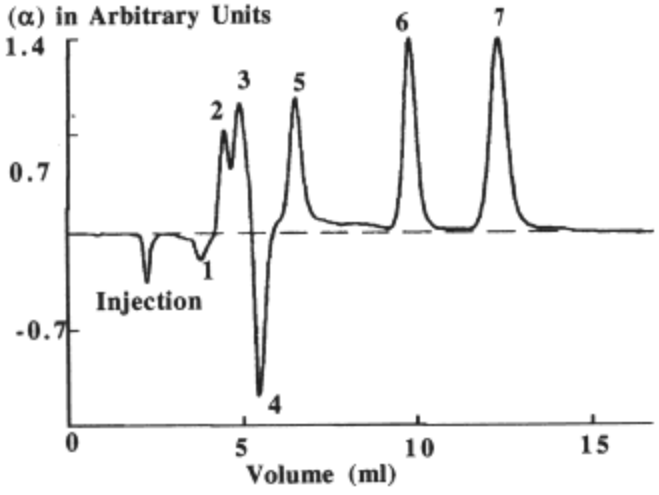


Figure 7.23
A Chiral Detector Monitoring Optical Rotation
Courtesy of JM Science Inc.

Light from a tungsten lamp passes through two condenser lenses to a polarizer. Plane polarized light from the polarizer then traverses through a temperature controlled cell to a Faraday modulator and thence, through an analyzer, onto a photomultiplier. The modulator is provided with a crystal controlled AC component. Consequently, if an optically active sample is present, the intensity of the light falling on the photo-multiplier will change. By the use of a phase sensitive amplifier in conjunction with electrical feed-back, the current though the modulator can be automatically adjusted, until the condition exists where no AC component appears on the photomultiplier output. Unfortunately, with present technology, the sensor must have a volume of about 40 µl, which is extremely large for use with the modern high efficiency LC columns employed in chiral chromatography. However, the urgent need for chiral detection is such that the large cell volume can often be accepted, and is partly accommodated by the use of very large diameter columns. The device must be rigidly supported and, if adequate stability is maintained, can measure a rotation (α) of 10^{-4} degree. The separation of some carbohydrates monitored by this type of detector is shown in figure 7.21. The separation was carried out on a column 12.5 cm long, 4.6 mm in diameter packed with Hypersil APS-1. The mobile phase was acetonitrile/water (8/2), at a flow rate of 0.5 ml/min. It is seen that the resolution of the mixture is maintained to a satisfactory level despite the very large cell volume. It is also seen that the direction of rotation is clearly and unambiguously indicated by the direction of the peak. The peaks represent between 20 and 40 µg of material, which also indicates reasonable sensitivity. From approximate calculations made from the chromatogram, the sensitivity for the peak appears to be 1.4×10^{-7} g/ml, which is only a relatively small factor less than that obtainable from the diode array UV detector.

However, it is extremely difficult to estimate the noise from the chromatogram shown in figure 7.24. A more accurate estimation of the detector sensitivity can be made from the chromatogram of some essential oil components shown in figure 7.25. The separation shown in figure 7.25 was carried out on a column 20 cm long, and 8 mm I.D.,

packed with Zorbax silica. The mobile phase was n-hexane/chloroform (4/1) and the flow rate was 0.5 ml/min. It is seen that the width of the second peak ((-)-α-terpinyl acetate) is about 0.16 mm and from the flow rate axis, 1.06 cm is equivalent to a volume of 5 ml. Thus the peak width at the base is about 0.75 ml.



(1) L-(–)Rhamnose	0.02 mg	(2) D-(+)-Xylose	0.04 mg
(3) L-(+)-Arabinose	0.04 mg	(4) D-(–)-Fructose	0.02 mg
(5) D-(+)-Glucose	0.02 mg	(6) Sucrose	0.02 mg
(7) D-(+)-Maltose	0.2 mg		

Figure 7.24
The Separation of Some Carbohydrates Monitored by a Chiral Detector
Courtesy of JM Science Inc.

The peak represented 3 µg of material and, taking the concentration at the peak maximum as twice the peak average concentration, the peak maximum concentration would be about 8 µm/ml. The peak height appears to be about three times the noise and so the sensitivity (that concentration that will give a signal equivalent to twice the noise) would

be about 5.3 µ/ml or, in more standard terms, 5.3×10^{-6} g/ml. As the chiral detector is a bulk property detector, a sensitivity of 5.3×10^{-6} g/ml seems far more realistic. Nevertheless, this sensitivity is a great improvement on many chiral detectors previously described.

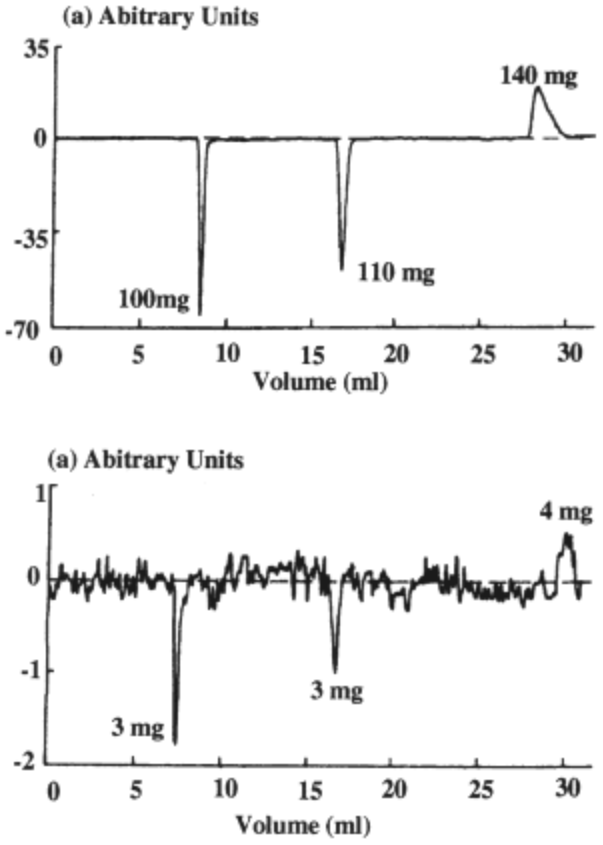


Figure 7.25
The Separation of Three Optically Active Fragrance Compounds Monitored by a Chiral Detector
Courtesy of JM Science Inc.

The value of a chiral detector in the analysis of physiologically active materials is clear, but the methods, so far, used have shown the detector to be somewhat insensitive. Nevertheless, development work on chiral

detectors is extremely active at this time, so hopefully, the sensitivity of the sensor will be significantly increased in the not too distant future.

Data Acquisition and Processing

It is not necessary for the analyst to know and understand the electronic circuitry associated with a data acquisition system, but to use the instrument effectively, some knowledge of the mathematical procedures that take place at each data processing stage will help reveal the limits of accuracy and the precision that can be expected from an LC analysis. A block diagram showing the stages involved in data acquisition and processing is shown in figure 7.26.

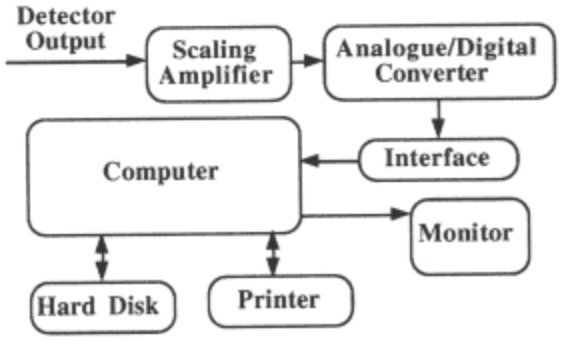


Figure 7.26
The Basic Stages in Data Acquisition and Processing

The detector output is usually in millivolts and thus is unacceptable for acquisition and digitizing by most analog to digital (A/D) converters. Consequently, the output of the detector is first processed by a linear amplifier, called the scaling amplifier, which converts the detector output in millivolts to a voltage range that is acceptable to the (A/D) converter, for example, 0–1 volt. The A/D converter then digitizes the output from the scaling amplifier to a binary number, which is temporarily stored in a register; this process is continuously repeated at a defined rate, called the 'sampling rate'. Each binary number, stored in the register is regularly read by the computer at the same frequency and stored on the

computer hard disk. When the analysis is complete, the computer accesses all the data from store, calculates the retention report, compares peak heights or peak areas to provide the quantitative analysis according to the processing program that is used, and finally prints out the results in tabulated form. The acquisition procedure, in a simple form, is shown diagramatically in figure 7.27.

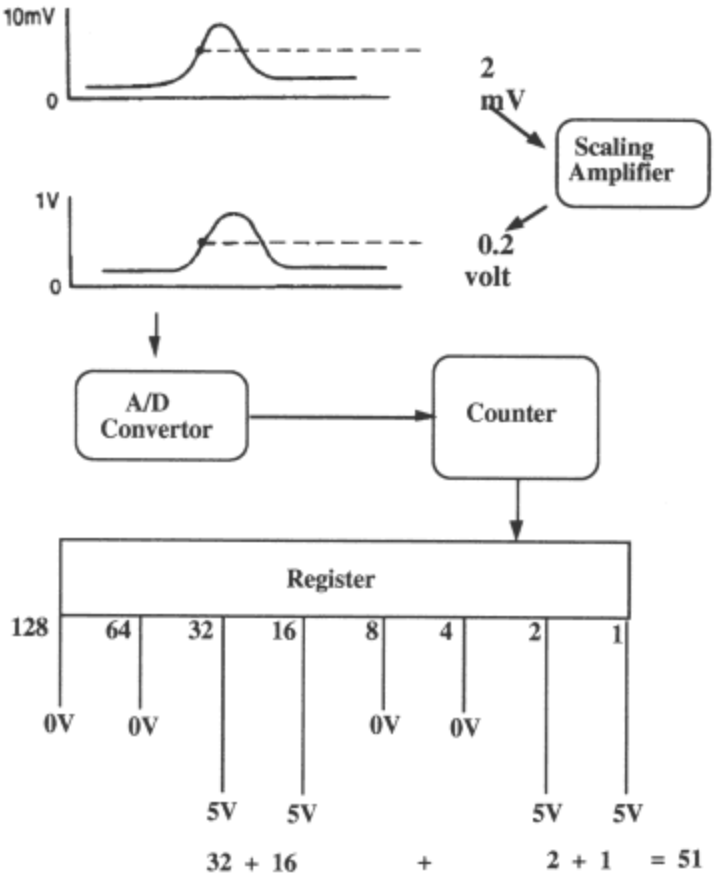


Figure 7.27
Stages of Data Acquisition

Most detectors provide an output that ranges from zero to 10 millivolts. As already stated the input voltage range required by most A/D converters is from zero to 1 volt. Consider the instantaneous output from

the detector is 0.2 mv. Now this must be scaled up by a factor of one hundred by the scaling amplifier to 0.2 volts.

Now the A/D converter must change the analog voltage of 0.2 volts to a digital number, the magnitude of which is determined by the number of "bits" that the computer employs in its processing. For example, if the processor uses eight bits then the largest decimal number will be 255 (in binary form 11111111). The digital data shown in figure 7.27 can be processed backward to demonstrate the A/D procedure. It is seen that the third and fourth most significant "bits" (which are counted from the far left) and the two least significant "bits" (which are counted from the far right) are at the five volt level, which as shown in figure 7.27 is equivalent to 51 in decimal notation (32+16+2+1, or in binary notation 00110011). It follows that the voltage that was converted must be $51/255 \times 1$ volt = 0.2 volt. It should also be noted that due to the limitation of 8 "bits", the minimum discrimination that can be made between any two numbers is $1/255 \times 100 \approx 0.4\%$. Thus the *number of bits* used for calculation *defines the minimum difference* between data values. Although 0.4% appears a little high, other factors in the analytical procedure (sample taking, sample preparation, sample injection, detector linearity etc.) can easily produce an accumulated error that is greater than the 0.4% resulting from the eight "bit" limitation. Many modern chromatography computer systems utilize more than eight "bits" (some as great as 12 bits or more) in their data processing but, in practice, eight "bits" is often quite adequate due to the magnitude of other instrumental limitations on precision.

Modern data processing software will identify and measure retention times and in some cases compare them with library values for solute identification. Peak heights and peak areas will be measured and, where necessary, corrected with appropriate response factors. The software included may also contain sub routines that can deconvolute the individual peaks from the composite envelope of unresolved peaks and

then calculate the area of the individual de-convoluted peaks. Such routines work well on peaks that are entrained in the tail of a major peak, but are not so accurate for composite envelopes, containing more than two unresolved peaks. The chromatographic system should, wherever possible, be optimized to obtain complete resolution of the mixture and it is not advisable to rely on mathematical techniques to aid in the analysis, as they will inevitably include some form of approximation. For further information on liquid chromatography apparatus and procedures the reader is referred to *Liquid Chromatography for the Analyst* [3] and the *Handbook of HPLC* [4].

Synopsis

The liquid chromatograph can be considered to contain *eight basic units* consisting of, solvent reservoirs, a solvent programmer, one or more pumps, a sample injector, a column oven/cooler, a detector, a computer and a printer. In most instruments, there are at least three *reservoirs* which can be made from glass, stainless steel or an appropriate plastic. They can have capacities ranging from 100 ml to 1000 ml. There are two types of solvent programmer. The high *pressure programmer* has a pump for each solvent and the composition of the solvent mixture is controlled by the flow rates from each pump which, in turn, are controlled by the computer. The output from each pump is blended in a mixer and then passed to the sample valve and column. The *low pressure programmer* employs timed valves, one for each solvent, and the solvent mixture is determined by the frequency and opening period of each valve which is also controlled by the computer. The output from the valves are blended in a mixing chamber and then passed to a high pressure pump. Most LC pumps are *dual-piston single-stoke* devices that consist of a stainless steel cylinder and a cam-driven sapphire piston. Sometimes dual pistons are used to reduce pumping 'noise'. The inlet and outlet ports of the pump are fitted with sapphire ball-and-seat non-return valves. There are two types of sample valve, the internal loop valve and the external loop valve. The sample volume of the *internal loop valve* can range from 0.1 to 1 µl and is contained in the spigot of the valve. The *external loop valve* used

in analytical LC has optional external loops with capacities ranging from 1 to 20 µl. For preparative work the loop volume may be increased to 100 milliliters. In most LC phase systems temperature has minimal effect on the magnitude of retention, or on selectivity, as the free enthalpy change is often similar in both phases. However, in chiral chromatography slight differences in retention can be very important to achieve resolution. Consequently, temperature is an essential operating variable in chiral chromatography. However, temperature also has a significant effect on solute diffusivity and thus the higher the temperature, the higher the optimum velocity, and the shorter the analysis time. Temperature has little, of no effect, on the minimum HETP of a column and thus little effect on the maximum column efficiency. The effect of temperature on analysis time and solute retention evokes the need for a precise control of the column temperature. The temperature range should be at least 0°C to 80°C and in some types of SEC separation, temperatures up to 200°C may be necessary. In modern liquid chromatographs, the use of ohmic heating and refrigeration has been replaced by Peltier heating and cooling. LC detectors have two to three orders of magnitude less sensitivity (minimum detectable concentration) than their GC counterparts and their linear dynamic range is also two to three orders less. Of the numerous different LC detectors available, the UV adsorption detector is the most commonly used which, in conjunction with the fluorescence detector, the electrical conductivity detector and the RI detector, comprise about 95% of all the detectors used in LC analyses. The *UV detector* measures the light adsorbed by the column eluent at wavelengths ranging from 200 Å to 350 Å employing a photoelectric sensor. The concentration of solute is not linearly related to the sensor output and thus must be electronically modified, in order to obtain accurate quantitative measurements. The most popular UV detector is the *diode array multi wavelength detector*. Light from a broad emission source passes through the sample and is then dispersed by a holographic grating across an array of photosensitive diodes. The output from each diode is continuously sampled at regular time periods and stored on the computer disk. Recalling the output from any *selected* diode and plotting it against time gives a chromatogram of

the separation. Recalling the output from *all* the diodes at a peak maximum, or any other specified time, and plotting the data against the wavelength associated with each respective diode, gives the UV spectrum of the solute or the spectrum of whatever is eluted at the selected time. Rationing the output for a pair of peaks, at two appropriate wavelengths, can confirm the purity of a pair of enatiomers as they should give identical responses. The *electrical conductivity detector* responds to any charged ions present in the column eluent and consists of two electrodes situated in the column eluent. A constant AC potential of about 1000 Hz is applied across the electrodes and the electrical impedance of the eluent situated between the electrodes is continuously monitored with an appropriate electronic circuit. The detector is used extensively in ion chromatography particularly for monitoring enantiomers of amines, acids, hydroxy acids and quaternary amines. The *fluorescence detector* is one of the most sensitive LC detectors. In its simplest form, light from a suitable excitation source passes through the sensor cell, and the fluorescent light emitted by the solute falls onto a photocell, positioned normal to the incident excitation light. The more sophisticated and expensive form of detector uses a broad wavelength light source, which is dispersed by a grating, so that light of a specific wavelength can be selected for excitation. In a similar manner, the fluorescent light can also be dispersed by another grating so that either light of a specific wavelength can be monitored, or a fluorescent spectrum obtained. With the exception of the mass spectrometer, this type of detector is probably the most selective available. Although the fluorescence detector is very sensitive, it only has a linear dynamic range of about two orders of magnitude. Solute sensitivity is often enhanced by the formation of fluorescent derivatives. An alternative procedure is to interpose a high energy UV photo-reactor between the column and the detector to produce (photochemically) fluorescent products. The reactor must be designed to produce minimum band dispersion. There are two types of *light scattering detectors*. The *evaporative light-scattering detector* which incorporates a spray system that continuously atomizes the column eluent into small droplets. The droplets are allowed to evaporate, leaving the solutes as fine particulate matter suspended in the atomizing gas. The

light scattered from a laser beam passing through the fog of fine particles, is sensed and electronically processed. The *liquid light-scattering detector* responds to the light scattered by a polymer or large molecular weight substances in the column eluent, as it passes through an appropriate sensor cell, while illuminated by a high intensity beam of laser light. By taking scattered light measurements at different scattering angles, the output can, as well as simply detecting the solute, also provide molecular dimensions and molecular weight information of the detected solute. The *refractive index detector* is probably the least sensitive of all LC detectors but is frequently used for detecting those substances that do not adsorb UV light, do not ionize and do not fluoresce. It is commonly used in the analysis of hydrocarbons, alcohols, carbohydrates and certain polymers. The RI detector is very sensitive to changes in temperature, pressure and flow rate and can not be used with gradient elution. The *chiral detector* senses those substances that are optically active, and obviously can provide a valuable service in chiral chromatography. The detector actually measures the rotation of polarized light that has passed through the column eluent. It has a sensitivity generally comparable to that of the refractive index detector, providing columns of adequate radius are employed. The device, however, is relatively expensive which limits its use, and it is more common to separate the chiral substances on a chirally selective column and detect the eluted enantiomers by more traditional methods of detection. The output from the detector is usually acquired by a computer and the analytical results calculated when the analysis is complete and printed out. There are four primary units involved in the data acquisition and processing system, a scaling amplifier, an A/D converter, a computer (including monitor) and a printer. The precision available from the system depends on the number of 'bits' involved in the A/D conversion. eight bits will give a resolution of 1/255 *ca* 0.4%. Although this resolution may appear inadequate, in most analyses accumulated errors involved in sampling, sample preparation, adsorption on chromatographic surfaces, detector linearity etc. are often significantly in excess of 0.4 %, and so 8 'bit' A/D conversion can often be quite adequate. Normally, a chromatogram of the separation is presented on the printer in real time, all data is stored

and processed using appropriate software and the analytical results presented in a standard format by the printer.

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3. R. P. W. Scott, *Liquid Chromatography for the Analyst*. Chromatography Science Series, vol. 67, Marcel Dekker, Inc., New York (1994).

4. E. Katz, R. Eksteen, P. Schoenmakers and N. Miller, *Handbook of HPLC*, Chromatography Science Series, vol. 78, Marcel Dekker, Inc., New York (1998).

Chapter 8—
Liquid Chromatography Chiral Stationary Phases

The fundamental difference between separations that are achieved by LC as opposed to GC is that in LC, there can be strong interactions between the solute and the mobile phase, in addition to those between the solute and the stationary phase. In GC, interactions between the solute molecules and those of the gas phase are weak, and relatively infrequent. However, interactions with the mobile phase in LC can be as strong, and even stronger than those with the stationary phase. This means that the retention and selectivity can be modified by solely changing the nature and/or composition of the mobile phase. In fact, by the use of gradient elution techniques, the interaction of the solute with the mobile phase can be continuously changed during development, with much the same results as those obtained by changing the temperature during development in GC. It follows that, the strong retention of a solute may result, on the one hand, from either strong interactions with the stationary phase or, on the other, from weak or reduced interactions with the mobile phase. However, it has been noted, that those chiral stationary phases that have *inclusion* possibilities as part of the retentive and selective mechanism, can exhibit chiral selectivity at both high and low concentration of the organic modifier (the dispersive component of the mobile phase). This makes the choice of the optimum gradient a daunting task. In most cases, under such conditions, the gradient must be shallow, and extend over a very limited concentration range.

The same type of molecular forces are involved as those in GC, except that, as the solutes no longer need to be volatile, ionic interactions can now be used to control retention, in addition to dispersive and polar interactions, as in GC. It will be seen that temperature can also be used to control retention in LC, in a somewhat similar manner to GC. The distribution coefficient of a solute between the two phases in LC will always result from both standard free entropy and enthalpy changes during distribution, as in GC. In addition, the separation of enantiomers will also depend primarily on a difference in the standard free entropy between the two isomers, that results from spatial variations and which are then augmented by standard free enthalpy differences.

A second difference, between gas and liquid chromatography, lies in the mode of solute dispersion. In the first instance, virtually all LC columns are packed (not open tubes) which introduces a dispersion process into the column that is not present in the GC capillary column. In a packed column the solute molecules will describe a tortuous path through the interstices between the particles and obviously some will travel shorter paths than the average, and some longer paths. Consequently, some molecules will move ahead of the average and some will lag behind, thus causing band dispersion. This type of dispersion is called *multipath dispersion* and is an additional contribution to longitudinal diffusion, and the two resistance to mass transfer contributions, to the overall peak variance.

A third difference, will result from the relatively small compressibility of the mobile phase. As opposed to a gas, which is highly compressible, when measuring retention volumes, there is no need for a mobile phase pressure correction in LC, unless extremely high pressures are used. Even then, due to the small compressibility of liquids, it will be a second order effect. The effect of pressure on solute interaction with the stationary phase, however, may well be more significant. This might occur, particularly, if the stationary phase matrix on the surface of the silica is polymeric in form, and thus somewhat compressible. Indeed, small changes in selectivity have be observed [1, 2, 3], in chiral separations as the result of increased column pressure. It should be

pointed, out that the change in retention due to pressure alone is not likely to be due to the compressibility of the mobile phase, but probably due to the distortion of the stationary phase.

The effect of pressure on diffusivity, and thus on band dispersion and column efficiency was critically examined by Katz and Scott [3] and Atwood and Goldstein [5]. Increase in pressure reduced the solute diffusivity and thus, increased the resistance to mass transfer term in the mobile phase. As a consequence, the peak dispersion increased and the efficiency fell as the pressure increased. If the column was not well thermostatted with a high capacity heat exchanging fluid (and appropriate liquid as opposed to a gas), Scott and Katz found there was a significant evolution of heat in the column resulting from the pressure, and a consequent change in column temperature. This, in turn, effected both the solute diffusivity and the magnitude of the capacity ratio, (k), of the solute. As a result of these interacting effects of temperature and pressure, the actual change in (k), solute diffusivity, and thus the column efficiency, tends to be irregular unless the column is very carefully thermostatted. This can be difficult, as the packing itself (silica gel) is a thermal insulator and, although the outside of the column can be maintained at a constant temperature, due to poor radial heat conduction, the center of the column can still be at an elevated temperature relative to the outer surface.

In chiral chromatography, very high pressures are not commonly employed and so the influence of pressure is normally a second order effect. However, for very precise work the column should be thermostatted with a medium having a high heat capacity. The column should be than 2 mm I.D. or less (to improve radial heat exchange across the column) and, if possible, the chromatographic conditions should be adjusted to obviate the need for very high pressures. The opposing effects of temperature and pressure on solute diffusivity make the effect of pressure on column efficiency very uncertain. It is difficult to predict the net effect of pressure change, as it will depend on whether the *reduction* in solute diffusivity by the effect of increased pressure will be

greater or less than the *increase* in diffusivity from the consequent rise in temperature and *vice versa*.

The most appropriate equation for predicting solute dispersion in a liquid chromatography column is the Van Deemter equation [6] which has been shown experimentally [7], to accurately describe peak dispersion in a packed column, particularly at mobile phase velocities around the *optimum velocity*. Furthermore, as all LC columns should be operated at, or close to, the optimum velocity for maximum efficiency, the Van Deemter equation is particularly important in column design and for predicting operating parameters.

The Van Deemter equation is given as follows:

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u}(1+\phi k) + \frac{f_1(k)\, d_p^2}{D_m} u + \frac{f_2(k)\, d_f^2}{D_S} u \qquad (1)$$

where (λ) is a packing constant (*ca.* 0.5),

(γ) is a packing constant (*ca.* 0.6),

(d_p) is the mean diameter of the particles,

(D_m) is the solute Diffusivity in the mobile phase,

(D_s) is the solute Diffusivity in the stationary phase,

(u) is the linear velocity of the mobile phase,

(ϕ) is a constant (usually close to zero),

(k) is the capacity factor of the solute,

(d_f) is the thickness of the film of stationary phase.

The original equation was introduced by Van Deemter for packed GC columns and consequently, the longitudinal diffusion term for the liquid phase was not included and $\frac{2\gamma D_m}{u}(1+\phi k)$ was replaced by $\frac{2\gamma D_m}{u}$. Furthermore, as the equation was developed for GC, where the diffusivity of the solute in the gas was four to five orders of magnitude greater than in a liquid, Van Deemter considered the resistance to mass transfer in the mobile phase to be negligible. As a result, the

function, $\frac{f_1(k)\, d_p^2}{D_m} u$, was also not included. The form taken by $f_2(k)$ was considered by Van Deemter to be: $\frac{8}{\pi^2}\frac{k}{(1+k)^2}$

and thus, the complete HETP equation for GC became:

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{8}{\pi^2}\frac{k}{(1+k)^2}\frac{d_f^2}{D_S} u \qquad (2)$$

However, in LC, the resistance to mass transfer in the mobile phase must be taken into account. Van Deemter *et al*. did not derive an expression for $f_1(k)$ for the mobile phase and it was left to Purnell [8] to suggest that the function of (k) employed by Golay [9], for the resistance to mass transfer in the mobile phase in his rate equation for capillary columns, would also be appropriate for a packed column in LC. Recalling that the form of $f_1(k)$ derived by Golay was:

$$\frac{1 + 6k + 11\, k^2}{24\,(1+k)^2}$$

Thus, Van Deemter's equation for LC becomes:

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{1+6k+11\, k^2}{24\,(1+k)^2}\frac{d_p^2}{D_m} u + \frac{8}{\pi^2}\frac{k}{(1+k)^2}\frac{d_f^2}{D_S} u \quad (3)$$

Equation (3) can put in a simplified form as follows:

$$H = A + \frac{B}{u} + C u \qquad (4)$$

where,

$$A = 2\lambda d_p,\ B = 2\gamma D_m,\ C = \left(\frac{1+6k+11k^2}{24(1+k)^2}\frac{d_p^2}{D_m} + \frac{8}{\pi^2}\frac{k}{(1+k)^2}\frac{d_f^2}{D_S}\right)$$

Equation (4) is the typical hyperbolic function expected for the variance per unit length of a column and indicates that there will be a minimum value of (H) for a particular value of (u). That is a maximum efficiency will be obtained at a particular linear mobile phase velocity as in the case of the capillary column.

The optimum mobile phase velocity can be obtained by differentiating equation (4) with respect to (u) and equating to zero, thus:

$$\frac{dH}{du} = -\frac{B}{u^2} + C$$

Equating to zero, *i.e.*dH/du = 0, and consequently: $-B/u^2 + C = 0$

$$u_{opt} = \sqrt{\frac{B}{C}} \qquad (5)$$

Substituting for (B) and (C):

$$u_{opt} = \left[\frac{2\gamma D_m}{\left(\frac{1 + 6k + 11k^2}{24(1+k)^2} \frac{d_p^2}{D_m} + \frac{8}{\pi^2} \frac{k}{(1+k)^2} \frac{d_f^2}{D_S} \right)} \right]^{0.5} \qquad (6)$$

and letting $D_s = \xi D_m$:

$$u_{opt} = D_m \left[\frac{48\gamma \pi^2 \xi (1+k)^2}{\left(\pi^2 \xi \left(1 + 6k + 11k^2\right) d_p^2 + 192 k d_f^2 \right)} \right]^{0.5} \qquad (7)$$

It is seen from equation (7) that the optimum velocity is directly proportional to the diffusivity of the solute in the mobile phase. To a lesser extent it also appears to be inversely dependent on the particle diameter of the packing (the particle size is an optional choice) and the film thickness of the stationary phase. The film thickness of the stationary phase is determined by the physical form of the packing, that is, in the

case of silica gel, the nature of the surface and in the case of a reverse phase, on the bonding chemistry.

Now, if it is assumed that $d_f << d_p$ (which in practice will normally be true) then:

$$u_{opt} = D_m \left[\frac{48\,\gamma\,\pi^2\xi(1+k)^2}{\left(\pi^2\xi\left(1 + 6\,k' + 11\,k^2\right)d_p^2\right)} \right]^{0.5} \qquad (8)$$

Furthermore, when k >> 1, that is, for well retained peaks, and bearing in mind that Giddings [10], predicted that for a well packed column (γ) should take a value approaching 0.6:

$$u_{opt} = D_m \left[\frac{4.36\,\gamma}{d_p^2} \right]^{0.5} \cong \frac{2.10 D_m}{d_p} \sqrt{\gamma} \equiv \frac{1.62\,D_m}{d_p} \qquad (9)$$

Under these circumstances, it is seen that the optimum velocity is directly proportional to the solute diffusivity in the mobile phase and inversely proportional to the particle diameter of the packing. Furthermore, as the diffusivity of the solute in both phases increases with the temperature, the optimum velocity will also increase with increase in temperature. However, the value of (k) will also change with temperature, as will the separation ratio of a given pair of solutes (α), and thus it is not clear at this stage whether the analysis time will increase or decrease with temperature. It is seen that the quality of the packing, also plays a part, in controlling the magnitude of the optimum velocity, but to a very less significant extent.

It is now possible to determine those factors that control the magnitude of H_{min}. Substituting the function for u_{opt} from equation (5) in equation (4) an expression for H_{min} is obtained:

$$H_{min} = A + \frac{B}{\sqrt{\frac{B}{C}}} + C\sqrt{\frac{B}{C}}$$

or:

$$H_{min} = A + 2\sqrt{BC}$$

Substituting for A, B, and C:

$$H_{min} = 2\,\lambda\,d_p + 2\sqrt{2\,\gamma\,D_m\left(\frac{1+6k+11\,k^2}{24\,(1+k)^2}\frac{d_p^2}{D_m} + \frac{8}{\pi^2}\frac{k'}{(1+k')^2}\frac{d_f^2}{D_S}\right)}$$

Noting that $D_s = \xi D_m$, and simplifying:

$$H_{min} = 2\,\lambda\,d_p + 2\sqrt{2\,\gamma\left(\frac{\left(1+6\,k+11\,k^2\right)d_p^2}{24(1+k)^2} + \frac{8}{\pi^2}\frac{k}{(1+k)^2}\frac{d_f^2}{\xi}\right)}$$

Again, assuming that, $d_f << d_p$, and $k' >> 1$, that is for well retained peaks, and that (γ) takes a value approaching 0.6:

$$H_{min} = 2\,\lambda\,d_p + 2\sqrt{\frac{2\,\gamma\,d_p^{\,2}}{24}}$$

$$= d_p\left(2\lambda + \sqrt{\frac{\gamma}{3}}\right)$$

It is seen that in packed LC columns the maximum efficiency possible is directly proportional to the particle diameter and the quality of the packing, and taking (γ) to be 0.5 as suggested by Giddings:

$$H_{min} = 1.45\,d_p$$

The efficiencies that can be expected at the optimum velocity from columns of different lengths, packed with particles of different size, are shown in table 8.1. As chiral separations often involve closely eluting peaks, the efficiency limitations disclosed in table 8.1 can be important in chiral chromatography.

Table 8.1 Efficiencies from Columns of Different Lengths Packed with Particles of Different Diameters

Column Length	Column Efficiency		
(cm)	3 µm	5 µm	8 µm
3	6900	4100	2600
5	11500	6900	4300
10	23000	13800	8600
25	—	34500	21500
40	—	—	30300

It is seen that within the limitations of the maximum pressure available from contemporary pumps, and the maximum operating pressure of sample valves, the smallest particles (3 µm in diameter) should be used. If efficiencies greater than 20,000 theoretical plates are needed then a longer column packed with larger diameter particles may be necessary as the larger particle diameter provide less flow impedance and allows the column to be operated at its optimum velocity.

Chiral Stationary Phases

There are a number of different materials employed as chiral stationary phases, but there are basically only five general types of stationary phase in common use in LC. The first type is the, now well established, protein based stationary phases. These stationary phases were the first to be developed and usually take the form of natural proteins bonded to a silica matrix. As they are proteins, they contain a large number of chiral centers of one configuration and are known to interact strongly with small analytes exhibiting strong chiral selectivity. There are specific interactive sites that provide chiral selectivity, but there are many other sites that contribute to general retention. These other sites can be significantly deactivated by mobile phase additives (*e.g.* octylamine) which reduces the overall retention and increases the chiral selectivity. The second type of chiral stationary phase consisted of relatively small molecular weight chiral substances bonded to silica and were pioneered by Pirkle [11]. Each bonded group has a limited number of chiral centers available but, due to their small size, there can be a large number of

groups bonded to the silica (as opposed to much larger complex chiral moieties), and thus a relatively high probability is maintained of the solute interacting with a chiral center. The particular advantage of the Pirkle chiral phases is that, as the overall interacting molecule is small, the extra chiral contributions to retention are also small, and thus the chiral selectivity becomes the dominant factor controlling retention. The third type is based on polymers of cellulose and amylose which were developed by Okamato [12]. These are derivatized to link appropriate interactive groups to the cellulose polymer which is then coated onto a silica support. The fourth type is based on the macrocyclic glycopeptides introduced by Armstrong [13]. These are materials that also contain a large number of chiral centers, together with molecular cavities in which solute molecules can enter and interact with neighboring groups. The spatial character of the solute will determine the degree of entry and consequently the proximity of interaction which, in turn, will determine the energy of interaction and the magnitude of the retention. Finally, the fifth group contains the cyclodextrin based materials that control retention in a similar manner to that previously described for GC. In LC, the cyclodextrin stationary phases are bonded to a support such as silica and are prepared using similar techniques to those for making reverse phases. The preparation of chiral stationary phases will be discussed in chapter 9.

Protein Based Stationary Phases

There are a number of commercially available protein based stationary phases which have been used to separate a wide range of chiral substances. The α_1-acid glycoprotein, sold under the name Chiral-AGP, is a very stable protein that has been bonded to 5 μm silica particles. This stationary phase is usually employed with simple binary solvent mixtures usually containing small amounts (1%–10%) of 2-propanol, ethanol or acetonitrile.

The concentration of solvent can critically control both the absolute retention of the enantiomers as well as the chiral selectivity. An example of the effect of solvent concentration on retention and selectivity is demonstrated in the chromatogram showing the separation of the Metroprolol isomers, depicted in figure 8.1. As the concentrations of solvent are relatively low, and the water content high, it is clear that the

major interactions involving retention are dispersive (hydrophobic) and not polar.

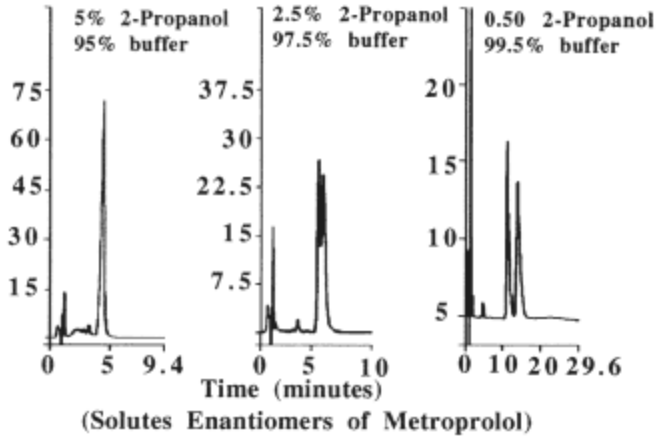


Figure 8.1
The Effect of Solvent Concentration on the Retention and
Resolution of Metroprolol Enantiomers on CHIRAL-AGP
Courtesy of ChromTech Sweden

It is also seen that the improved chiral selectivity resulting from the reduction in the 2-propanol content is mirrored by the increase in retention. This means that the enantiomer more closely interacting with the stationary phase is also selectively retained by dispersive interactions relative to its opposite isomer. The stationary phase also contains ionic groups that can interact ionically with the solute, providing the pH is adjusted to the pKa of the interacting ionic group so that it is ionizable and not in the form of a zwitter ion. The pI of AGP is 2.5 so it will carry an increasing negative charge up to a pH of 7.0.

An example of the effect of pH on the separation of the enantiomers of 2-phenoxypropionic acid is shown by the chromatograms in figure 8.2. It is seen that both the absolute retention and the chiral selectivity increases as the pH is reduced and that a pH change of only 2 units, results in a switch from the enantiomers being coincidentally eluted, to their being eluted with a very large separation factor. This indicates that the

enthalpic contribution to the separation is strongly (if not completely) dependent on ionic interactions and the separation is achieved while confining the analysis time to less than 5 minutes. In general, CHIRAL-AGP is commonly used for the separation of enantiomers carrying secondary and tertiary amines and for substances containing nitrogen in a ring.

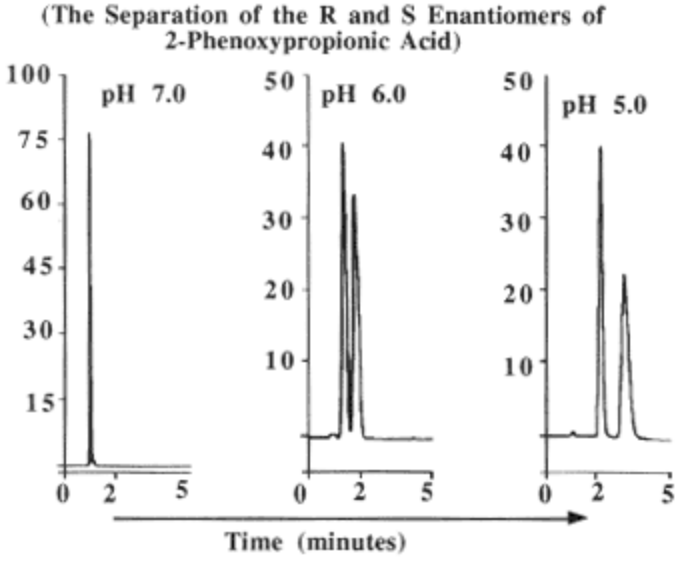


Figure 8.2
The Effect of pH on the Retention and Resolution of the
2-Phenoxypropionic Acid Enantiomers on CHIRAL-AGP
Courtesy of ChromTech Sweden

A protein with a similar behavior pattern is ovalbumin which is prepared from chicken egg whites. In general, the operating conditions are similar to AGP, albeit, that a reversal of the elution order of certain enantiomers has been reported.

Cellobiohydrolase has also been bonded to a silica gel matrix and used as an effective stationary phase. It is available commercially as CHIRAL-CBH. The bonded material also acts as a reversed phase column retaining substances largely by dispersive (hydrophobic) interactions. This

material is popular for separating the enantiomers of basic drugs, in particular, for the separation of enantiomers containing primary amines and drugs such as Normetanephrine and Octopamine. It is usually employed with a mixture of 2-propanol or acetonitrile with a phosphate buffer as the mobile phase. However, this is an enzyme that is easily destroyed by metals, so a chelating agent such as ethylenediaminetetra-acetate must be used to scavenge any contaminating metal ions in the system. In a similar manner to CHIRAL-AGP, the retention and chiral selectivity can be controlled by the pH of the mobile phase and the concentration of the organic solvent. Another protein based stationary phase is produced by bonding human serum albumin to a silica matrix. This material is also commercially available as CHIRAL-HSA.

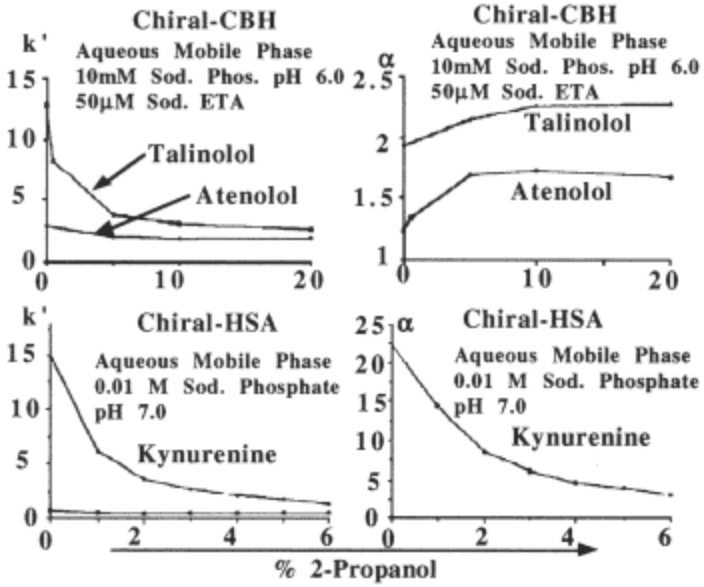


Figure 8.3
The Effect of 2-Propanol Concentration on Retention and Resolution of Enantiomers Separated on Two Different Protein Stationary Phases, CHIRAL-CBH and CHIRAL-HSA
Courtesy of ChromTech Sweden

The three protein stationary phases behave very similarly, in that retention and chiral selectivity is controlled by pH and the concentration of solvent in the mobile phase. However, the selectivity for a given enantiomer pair can be very different on each stationary phase despite the fact that they are all protein based. This is demonstrated in figure 8.3. The retention of the enantiomers of Talinolol and Atenolol decrease with increase in 2-propanol in the mobile phase which, if largely retained by dispersive interactions, would be expected. However, the *chiral selectivity* of the stationary phase to the two pairs of enantiomers of the stationary phase to the two pairs of enantiomers *increases* with the 2-propanol content of the mobile phase. This would indicate that the chiral selectivity was more likely to be due to polar interactions. However, it is interesting to note that although the retention of Kynurenine also falls with increase 2-propanol concentration, when separated on the CHIRAL-HSA phase, the chiral selectivity also *falls*.

In this case, it would appear the chiral selectivity was due to increased dispersive forces on the more retained isomer. This again indicates the difficulties met when trying to predict the degree of chiral selectivity that might be expected from a given chiral stationary phase, when used to separate a hitherto unknown specific chiral solute. It must again be stated, that although it is usually fairly easy to give a rational explanation as to why a specific separation has been achieved, its forecast, prior to achieving the separation, is more difficult. Considerably more research on chiral separations, particularly with respect to the physical chemistry involved, needs to be carried out before the optimum conditions that will separate a specific sample of chiral compounds can be accurately educed. However, certain chiral specific sites have been identified in some of these protein phases, *e.g.* human serum albumin, and where a clear stereogenic environment exists, retention and selectivity becomes more predictable.

An example of the practical use of a protein stationary phase for the separation of the enantiomers of methadone contained in a blood serum sample is shown in figure 8.4. The mobile phase employed was acetonitrile/0.01M phosphate buffer at a pH of 6.6 : 16/84 v/v. The

column was 10 cm long, 4 mm I.D., and operated at a flow rate of 0.7 ml/min. It is seen that an excellent separation of the enantiomers is obtained and the analysis was complete in about 12 min.

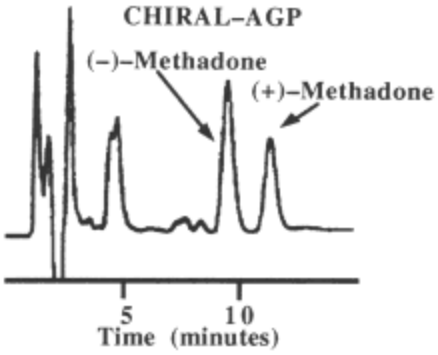
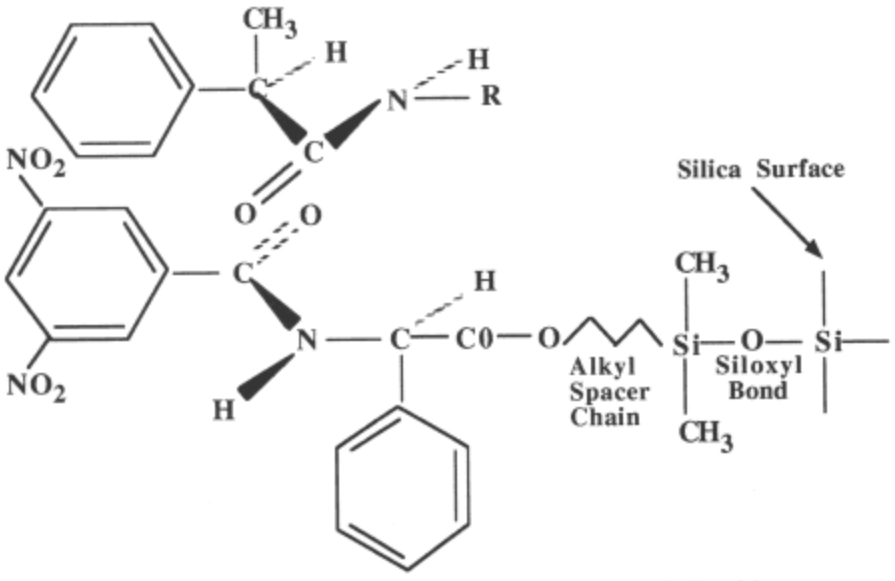


Figure 8.4
The Separation of the Enantiomers of Methadone in Blood Plasma on the Protein Stationary Phases, CHIRAL-AGP
Courtesy of ChromTech Inc.[6]

The Pirkle Type Stationary Phases

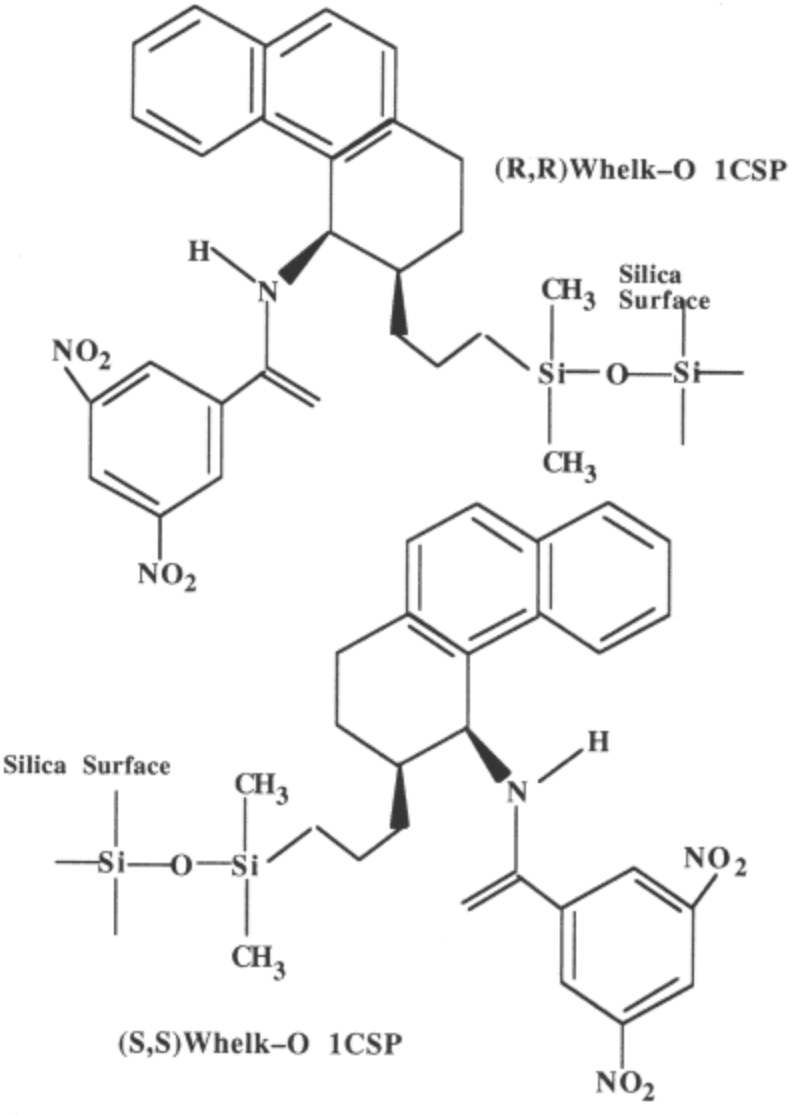
The small molecular weight chiral substances bonded to silica, commonly called the Pirkle phases, usually have a limited number of chiral centers but a large number of the groups bonded to the silica. There have been many types of these phases investigated and found effective for specific types of chiral separations. The system suffers from certain disadvantages which results from the spatial arrangement of the chiral center and other interacting moieties around it. The relative short bond between the chiral agent and the silica restricts the approach of some molecules, so that their chiral center can not interact with the chiral center of the stationary phase. However, for certain molecules, the spatial arrangement can be ideal. For example LC-(R)- and LC-(S)- dinitrobenzoylphenylglycine, the structure of which follows, is a typical example where the structure is appropriate to separate the enantiomers of 1-naphthymethylamide ibuprofen, which is thought to align itself in the manner shown below. The spatial arrangement around the stereogenic

center indicates one configuration will interact more strongly than its antipode.



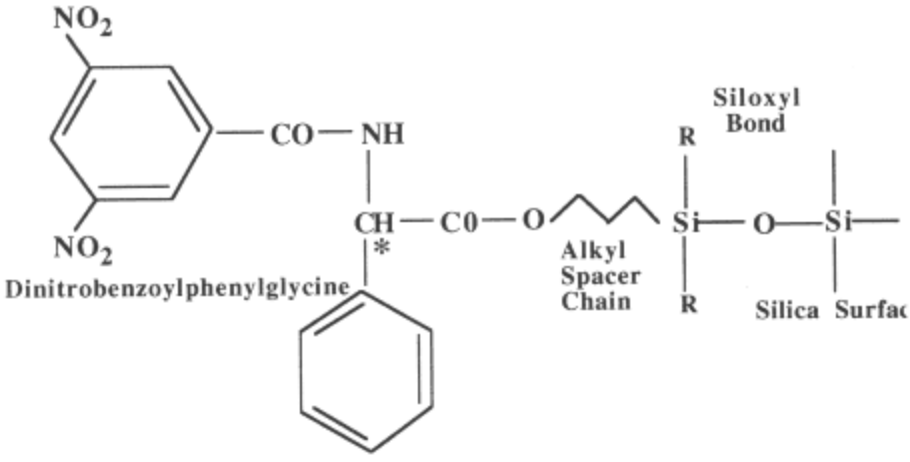
R= Naphthylmethylamide

In order to broaden the capabilities of the Pirkle concept, both polar and polarizable groups were introduced into the molecule. The most popular of this type of chiral stationary phase are the (R,R) Whelk-O1 and the (S,S)Whelk-O1 phases, the structures of which are shown below. These phases are more versatile and have a wider field of application than the phases previously described. The phases are covalently bonded to the silica and so they can be used with almost any type of solvent. However, they have been found to operate most effectively in the normal phase mode. It should be noted that the polarizable character of the aromatic ring is essential for the stationary phase to function well. As the Pirkle phases are generally available in both the (R) and (S) configurations, the reversal of the elution order of a pair of enantiomers is possible. This stationary phase was originally designed for the separation of the Naproxen enantiomers but has found a wide application to the separation of epoxides, alcohols, diols, amides, imides and carbamates.



Another, strongly poar, stationewry Pirkle phase synthesized in a similar manner is also shown below. this stationary phase is also very versatile, in that it can offer a variety of different interacting groups to the neighbouring groups of the chiral center of the solute. This stationary phase can provide strong polar interaction with its carbonyl group, its amide group and the nitrated aromatic nucleus. It can also interact

dispersively with the unsubstituted aromatic nucleus as well as providing induced dipole interaction with a strong polar group.



These types of columns can also be run under reversed phase or normal conditions of development.

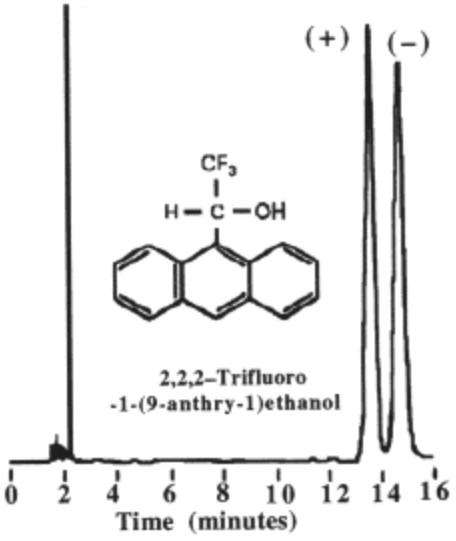


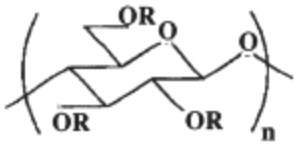
Figure 8.5
The Separation of the Isomers of
2,2,2-Trifluoro-1-(9-anthry-L) ethanol on a
SUPERCOSIL™ LC-(R)-Phenylurea
Stationary Phase under Normal Phase
Development Conditions
Courtesy of Supelco

Consequently, the mobile phase can consist of a hexane/ethanol mixture for normal development or a water/methanol for reverse phase development. In figure 8.5, the separation of the isomers of 2,2,2-trifluoro-1-(9-anthryl)ethanol on a LC-(R)-phenylurea stationary phase is shown developed under normal phase conditions.

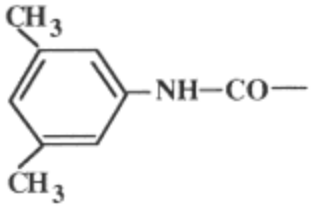
The column was 25 cm long, 4.6 mm I.D., and packed with silica particles 5 µm in diameter, carrying the bonded LC-(R)-phenylurea stationary phase. The mobile phase consisted of a hexane 2-propanol mixture 1000:15 and a flow rate of 1.0 ml/min. It is seen that an excellent separation was obtained that must have been achieved almost exclusively by dispersive interaction with the aromatic ring and the methyl group on the bonded moiety.

Coated Cellulose and Amylose Derivatives

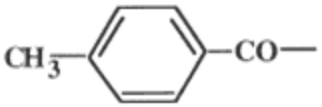
The cellulose types of chiral stationary phase currently available are coated on a wide pore silica support as opposed to being bonded to the silica surface. They take two basic forms, those derived from cellulose polymers and those derived from amylose polymers; they are reported to have molecular weights of up to 40,000 Daltons. The basic difference between the two polymers is that the cellulose adopts a linear structure, whereas the amylose forms a helical structure. Both cellulose adn amylose unit contains 5 chiral centers. As a result the polymers contain a large number of chirally active sites and thus a relatively high probability of chiral site interaction with the solute. The structure of the cellulose type of stationary takes the following form.



These type of phase were pioneered by Okamato [12] and there are two main derivatives that are used in LC as shown below.



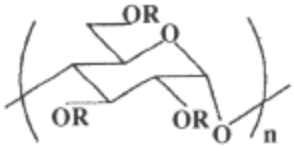
tris(3,5-Dimethylphenyl Carbamate



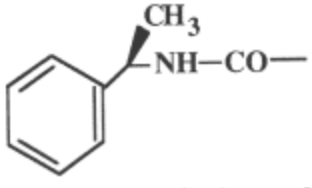
tris(4-Methyl Benzoate)

Both structures contain polar and dispersive interactive sites but, in addition, the aromatic nuclei can provide polarizability and thus will offer strong polar interactions with any strongly polar group appropriately situated on the solute molecule.

The amylose structure is somewhat different and is depicted below.



This material is also derivated with the tris(3,5-dimethylphenyl carbamate) but, in addition, has also been derivatized with tris[(S)-α-methylbenzyl carbamate]. The latter derivate, as well as providing polar, polarizable and dispersive sites, also contributes another chiral center to improve chiral selectivity. The structure of the tris (α methylphenyl carbamate) is as follows,



Tris[(S)-α-Methylbenzyl Carbamate]

Due to the fact that the stationary phase is coated on the silica and not chemically bonded to it, certain limitations are placed on the type of solvents, the linear velocity of the mobile phase and the operating

temperatures that can be employed. The solvents that are recommended for use as the mobile phase are heptane/alcohol mixtures which would indicate that the material is used largely in the polar phase mode (*i.e.* the dominant forces employed in the retention and selectivity are polar). To prevent peak tailing that may result from the presence of sites of extra high activity, small amounts of organic acids (TFA) or bases (TEA) may be used (typically 0.16%, maximum 1%) that may help to block any active sites and improve peak symmetry.

In practice, it has been found that certain derivatives (*e.g.* tris (3,5-dimethylphenyl carbamate) render the coating less culnerable to solvent dissolution. As a consequence this stationary phase can be used with buffered methanol/water or acetonitrile/water with certain care being taken. Therefore, the tris(3,5-dimethylphenyl carbamate) derivatives of both cellulose and amylose can, with caution, be used in the reversed phase mode. As example of a chiral separation using the different modes is shown in figure 8.6.

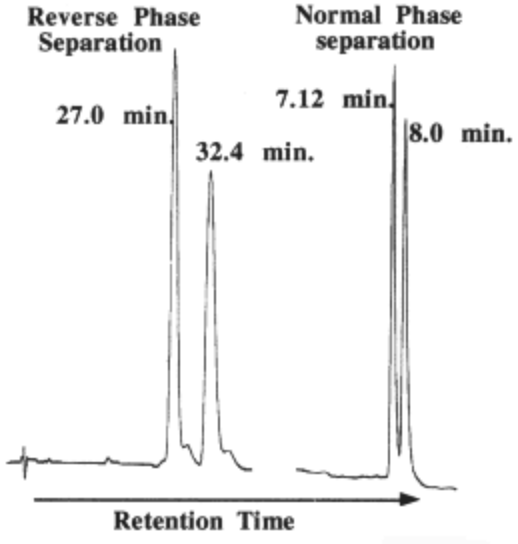


Figure 8.6
The Separation of a Pair of Enantiomers on a Carbamate Derivatized Cellulose-Type Stationary Phase Using Both the Reversed Phase and Normal Phase Mode

The solvent used in the reversed phase mode was methanol/aqueous-buffer: 65/35 v/v and in the normal phase hexane/ethanol: 95/5 v/v. As might be expected the separation ration realized from the reversed phase mode (α=1.20) is significantly greater than that obtained in the normal phase mode (α=1.13). This reflects the greater dispersive interactive capability of this type of derivative relative to its polar capability.

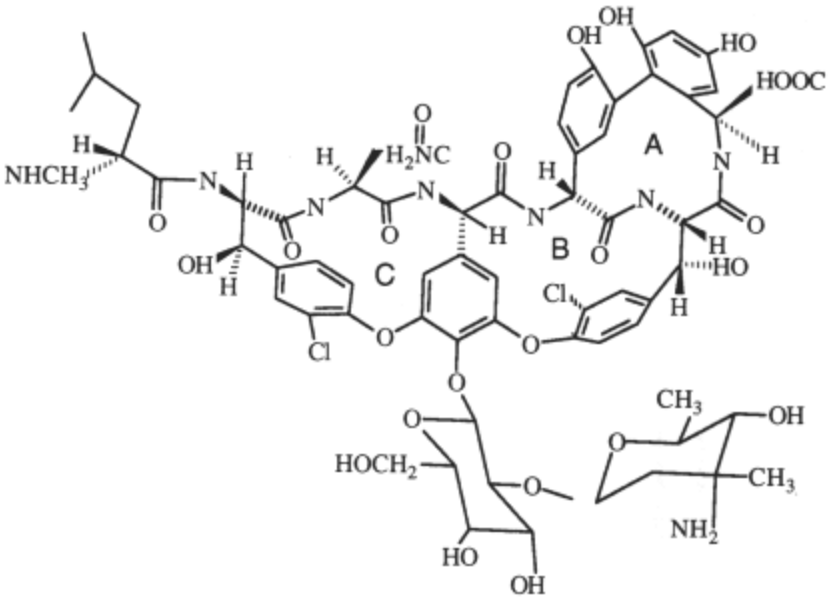
However, it must be pointed out that changing the development mode is often not reversible. For example, it is often possible to change from the polar mode of development to the reversed phase mode, but it is usually impossible to return to the polar mode after the change. This appears to be due to some morphological changes taking place in the stationary phase that destroys its chiral selectivity.

Marcrocyclic Glycopeptide Phases

The concept of using macrocyclic glycopeptides as chiral stationary phases was first introduced by Armstrong [13]. One methods of preparation is to covalently bond Vancomycin to the surface of silica gel particles. Vancomycin contains 18 chiral centers surrounding three 'pockets' or 'cavities' which are bridged by five aromatic rings. Strong polar groups are proximate to the ring structures to offer strong polar interactions with the solutes. This type of stationary phase is stable in mobile phases containing 0–100% organic solvent. The proposed structure of Vancomycin is shown in figure 8.7.

Vancomycin is a very stable chiral stationary phase, has a relatively high sample capacity, and when covalently bonded to the silica gel surface. It can be used with mobile phases with a high water content, as a reversed phase, or with a high solvent content, as a largely polar stationary phase. for example, when used as a reversed phase strongly polar THF -water mixtures are very effective mobile phases. Conversely, when used as a polar stationary phase, *n*-hexane=ethanol mixtures are appropriate. Vancomycin has a number of ionizing groups and this can be used over a range of different pH values (pH 4.0 to 7.0) and exhibit a wide range of retention

characteristics and chiral selectivities. Ammonium nitrate, triethylammonium acetate and sodium citrate buffers have all been used satisfactorily with this stationary phase.



A, B and C are inclusion cavities. Molecular weight 1449. Chiral centers 18. pK's 2.9, 7.2, 8.6, 9.6, 10.4, 11.7. Isoelectric point 7.2

Figure 8.7
The Proposed Structure of Vancomycin
Courtesy of ASTEC Inc.

Other than controlling the pH, the effect of the chosen buffer has little or no effect on chiral selectivity. This is verified by the chromatograms shown in figure 8.8. It is seen that virtually the same selectivity is obtained from all three buffers irrespective of the actual chemical nature of the buffers themselves. It is interesting to note, however, that, although the difference is exceedingly small, the slightly greater separation ratio obtained from the buffer containing triethylamine might reflect the relatively strong dispersive character of the ethyl groups in the buffer molecule. The enthalpic contributions to retention can be strongly dispersive, and/or strongly polar, or result from induced dipole

interactivity. The aromatic rings will allow induced dipole interactions with the stationary phase and conversely, the strong polar groups on the stationary phase can induce dipole interaction with polarizable groups on the solute.

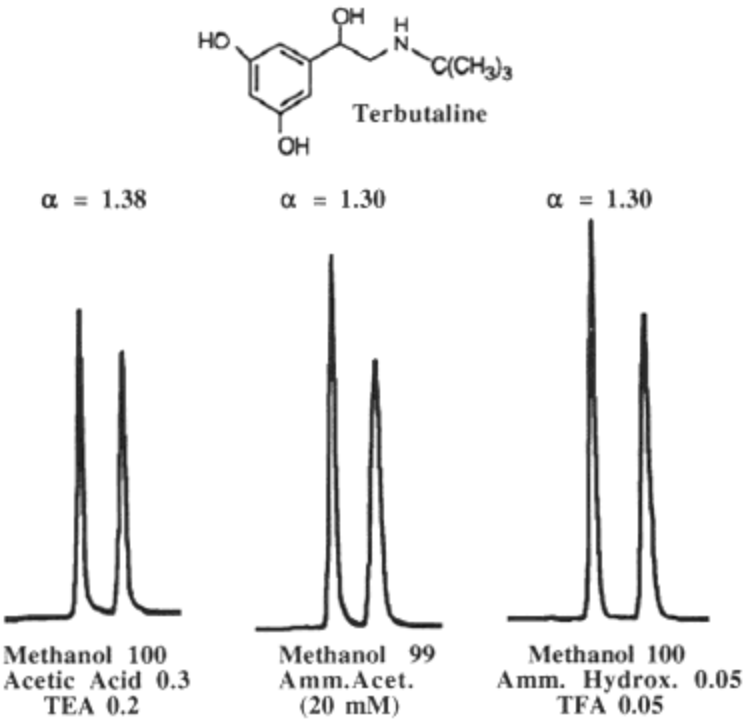


Figure 8.8
The Separations of the Isomers of Terbutaline Employing Different Buffer Solutions

The cavities are more shallow than those in the cyclodextrins and thus interactions are weaker however, this allows more rapid solute exchange between the phases, and thus higher column efficiencies. An example of the use of the stationary phase to separate the enantiomers of 3-methyl-5-phenylhydantoin is shown in figure 8.9. The separation is carried out under two conditions, the first used pure ethanol as the mobile phase, which is strongly dispersive, and in the second, a mobile phase that contains 90% of water. In the first case, the ethanol provides extremely

strong dispersive interactions in the mobile phase which will significantly exceed any dispersive interactions involved with the stationary phase.

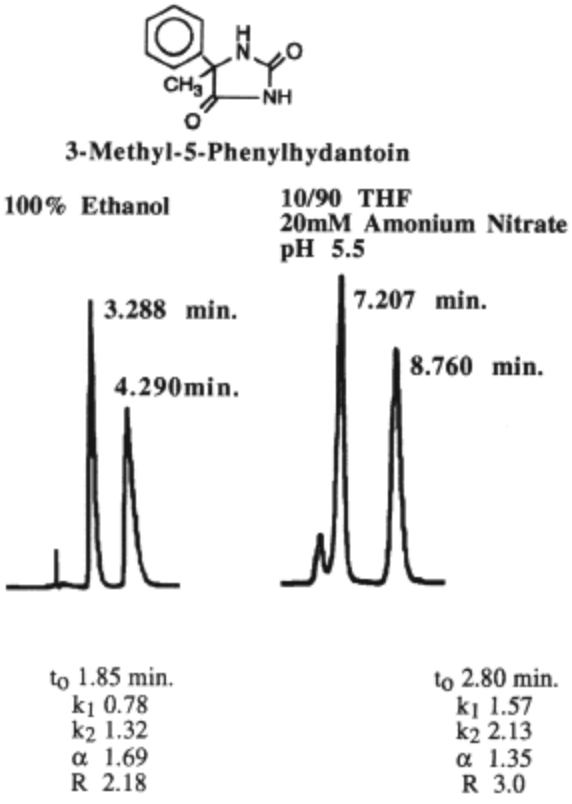
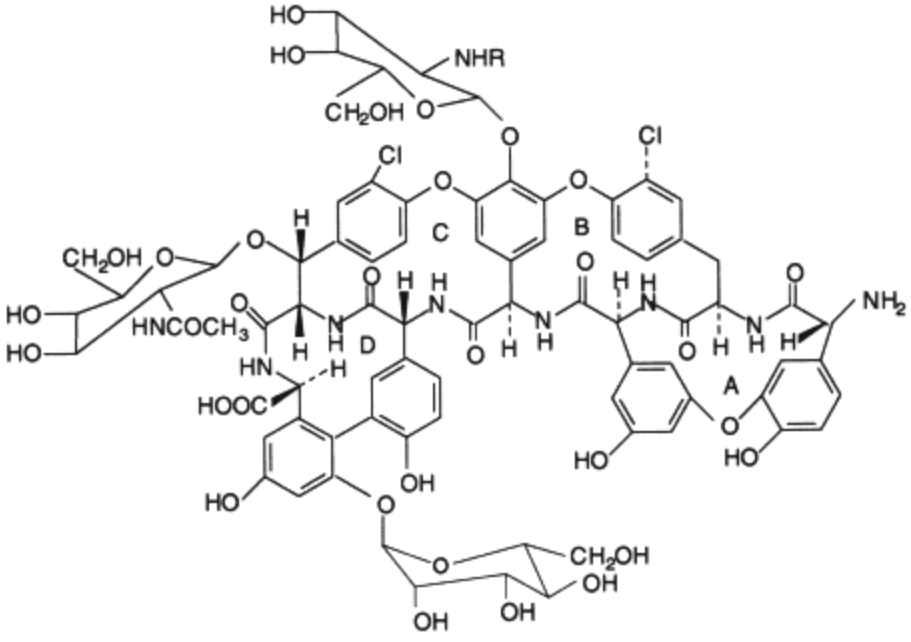


Figure 8.9
The Separation of the Enantiomers of
3-Methyl-5-Phenylhydantoin Using Polar and Dispersive Interactions
Courtesy of ASTEC Inc.

It follows that the remaining dominant retentive forces will be polar or ionic in nature. In the second case, the mobile phase is predominantly water and thus provides very strong polar interactions with the solute but very weak dispersive interactions. It also follows, that the retention forces of the stationary phase, in this case, will be dominantly dispersive

in nature. This demonstrates a very useful flexibility in the use of Vancomycin. By adjustment of the mobile phase composition, it a can be effective for separating solutes that depend largely on dispersive interactions (hydrophobic) to provide retention and selectivity or, alternatively, achieve the separation by exploiting ionic or polar interactions (hydrophilic).

Another macrolytic glycopeptide used in chiral chromatography is the amphoteric glycopeptide Teicoplanin which is commercially available under the trade name of CHIROBIOTIC T.



A, B, C and D are inclusion cavities. Molecular weight 1885. Chiral centers 20, Sugar moieties 3, and R is CH_3-decanoic acid

Figure 8.10
The Proposed Structure of Teicoplanin
Courtesy of ASTEC Inc.

This material is also bonded to 5 µm silica gel particles by multiple covalent linkages. Teicoplanin contains 20 chiral centers surrounding

four molecular 'pockets' or 'cavities'. Neighboring groups are strongly polar and aromatic rings provide ready polarizability. The proposed structure of Teicoplanin is shown in figure 8.10. This stationary phase is claimed to be complementary to the Vancomycin phase and can be used with the same types of mobile phase, one often providing chiral selectivity, when the other does not. Teicoplanin can be used in a reversed phase mode using strongly polar mixtures such as acetonitrile/aqueous buffer: 10/90 v/v, THF/aqueous buffer 10/90 : v/v, and ethanol/aqueous buffer: 20/80 v/v). It can also be used as a polar stationary phase using *n*-hexane/ethanol mixtures as the mobile phase. In some cases it is advisable to control the pH even when the solutes are not ionic, suitable buffers being ammonium nitrate and triethylamine acetate.

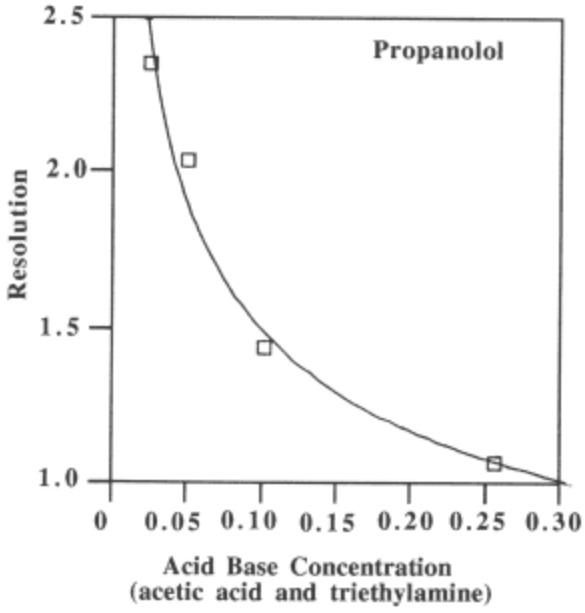


Figure 8.11
Graph of Resolution against Acid Base Concentration
(Acid/Base Ratio 1:1)

One of the most useful and efficient mobile phases for use with the macrocyclic stationary phases, and with Chirobiotic T in particular, consists of 100 part of methanol with the addition of acid and base

(acetic acid and triethylamine) in the range of 1.0 to 0.1 parts. This system has been termed the *polar/organic mode* of development. The solvent system has been used very successfully for enantiomers that contain two groups that are capable of interacting with the stationary phase. It has also been found that one of these groups must be on or (α) to the stereogenic center. The best concentration of the acid and base components appears to depend on the strength of the interaction between the active group and the stationary phase.

A graph relating the resolution of the enantiomers and the acid/base composition is shown in figure 8.11. It is seen from figure 8.11 that the acid/base concentration is quite critical. If this type of mobile phase is applicable, there are several advantages. The system offers simplicity and versatility and, in addition, it uses a relatively inexpensive, non-toxic, solvents. An example of the system in the separation of the Propranolol enantiomers is shown in figure 8.12.

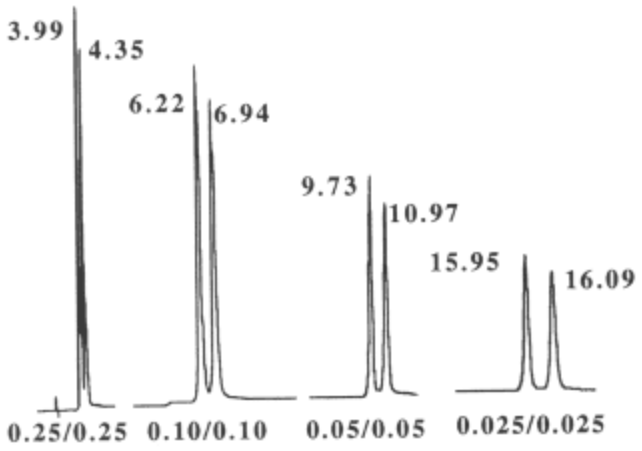


Figure 8.12
The Separation of the Enantiomers of Propranolol Employing
Different Acid/Base Ratios

The separations were carried out on a column 25 cm long, 4.6 mm I.D., packed with Chirobiotic T. The mobile phase was methanol containing acetic acid and triethylamine in the concentrations shown in figure 8.12.

The column was operated at room temperature and at a flow rate of 2 ml/min. Teicoplanin is stable over a pH range of 3.8 to 6.5 although it can be used for limited periods of time outside this range. In a similar manner to Vancomycin, by choice of the mobile phase, Teicoplanin can be used in the reversed phase mode, retention and selectivity depending largely on dispersive (hydrophobic) interactions between the solute and the stationary phase. An example of the use of Teicoplanin in the reversed phase mode is shown in figure 8.13.

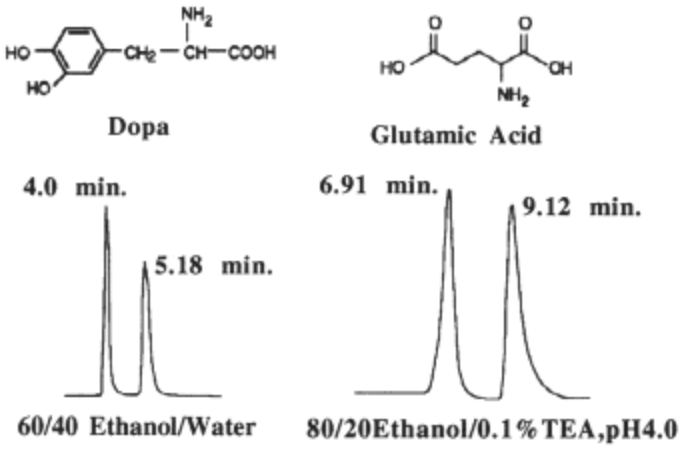


Figure 8.13
Chiral Separations on the Stationary Phase, Teicoplanin, Used
in the Reversed Phase Mode
Courtesy of ASTEC Inc.

It is seen that separation ratios of about 1.3 are easily realized indicating fairly strong chiral selectivity to the particular solutes. It should be appreciated that this stationary phase has a molecular weight of only 1185 and yet has 20 chiral centers. This provides a relatively high probability of the solute interacting with a chiral center. In addition, the dispersive groups that are proximate to the chiral centers provide strong enthalpic contributions to the differential standard free energy of distribution between the two enantiomers. The stationary phase can also be easily employed in the polar mode by using a strongly dispersive mobile phase such hexane/ethanol mixtures. The presence of the strongly

dispersive hydrocarbon completely swamps any possible competing dispersive interactions with the stationary phase and thus the solutes are retained almost solely by polar and/or (when applicable) by ionic interactions. Two examples of the use of teicoplanin operated in polar mode are shown in figure 8.14

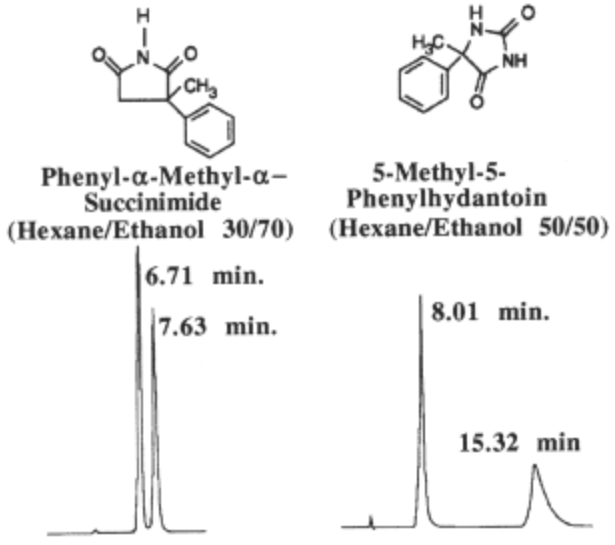


Figure 8.14
Chiral Separations of the Enantiomers of
Phenyl-α-Methyl-α-Succinimide and 5-Methyl-5-hydantoin
on the Stationary Phase Teicoplanin Used in the
Polar Phase Mode
Courtesy of ASTEC Inc.

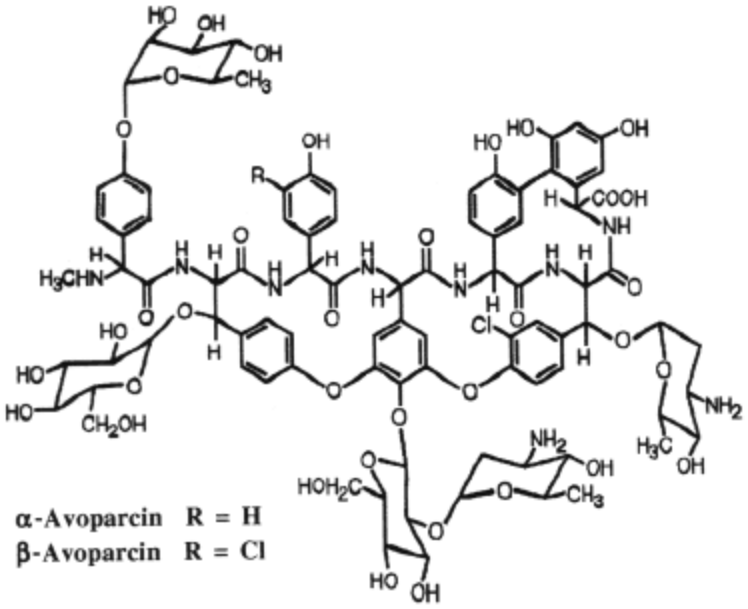
It is seen that the stationary phase is equally effective when polar interactions are exploited to achieve the separation. The separation ratio for the enantiomers of phenyl-α-methyl-α-succinimide is about 1.14, a moderately useful selectivity. However, the separation ratio for the enantiomers of 5-methyl-5-phenylhydantoin is 1.91, an extremely high selectivity which reflects both the highly polar nature of the solute and the possible inclusion of ionic interactions with the amino groups.

Berthod *et al.* [14] has demonstrated that the Teicoplanin chiral stationary phase can be used to separate a very wide range of underivatized amino

acids and imino acids. They reported the separation of 54 compounds including all the chiral protein amino acids. Aqueous solvents were used as the mobile phase and no buffers or salts were found necessary. This work on amino acids was augmented by that of Peter *et al*. [15], who separated more than 30 *unnatural* amino acids such as phenylalanine and tyrosine analogs and analogs containing 1,2,3,4-tetrahydroisoquinoline, tetraline, 1,2,3,4-tetrahydro-2-carboline, cyclo-pentane, cyclohexane, cyclohexene, bicyclo[2,2,1]heptane or heptane skeletons. In general, there is no better chiral stationary phase for the separation of amino acid isomers than Teicoplanin, even after considering the crown ethers and copper ligand stationary phases.

Another macrolytic glycopeptide which has been introduced relatively recently and has been used as a chiral stationary phase, is the glycopeptide, Avoparcin [16]. This stationary phase is commercially available under the trade name of CHIROBIOTIC A. Avoparcin is an antibiotic complex produced by *Streptomysces candidus* and is commonly used in animal feedstuffs. In particular, it is used to prevent necrotic enteritus in chickens. Commercially, it available as a product called Avotan that contains 10% of Avoparcin, and which is used generally in animal feeds other than chicken feed) to promote animal growth. Unfortunately, the large scale use of Avoparcin appears to give rise to a Vancomycin resistant enterococci in the feces of chickens and pigs. The structure of avoparcin is shown below. There are two forms of Avoparcin the unsubstituted α-Avoparcin structure and the chlorinated structure β-Avoparcin, the molecular weights being 1909 and 1944 respectively. The ratio of α-Avoparcin to β-Avoparcins is about 1:4. The mixture is a white, hygroscopic, amorphous solid with no clearly defined melting point, soluble in water, dimethylformamide and dimethylsulphoxide and slightly soluble in methanol. The aglycon portion of Avoparcin contains three connected semi rigid macrocyclic rings (one 12-membered, and two 16-membered) which form a pocket providing possible solute inclusion. The glycopeptide contains seven aromatic rings with four phenol moieties, four carbohydrate chains, 16 hydroxyl groups, one carboxylic acid, two primary amines, one secondary amine,

six amide linkages, two chlorine atoms for β-Avoparcin (only one for α-Avoparcin) and 32 stereogenic centers.



It is clear that, there is a wide diversity of interactive possibilities ranging from weak and strong dispersive interactions, to polar interactions that span from induced dipole interaction, through dipole-dipole interaction, to strong hydrogen bonding. In addition, at the right pK, basic and acidic ionic interactions can also be invoked. More importantly, with 32 stereogenic centers the probability of interaction between chiral centers of solute and stationary phase is relatively high. This particular macrocyclic antibiotic has not been commercially available for long and so its range of application and its particular advantages have not yet been established. It does however appear to have all the necessary properties to make an extremely useful chiral stationary phase. This situation may have changed by the time this book is published and more information may have become available

Cyclodextrin Based Stationary Phases

Although the cyclodextrin based GC stationary phases have already been discussed, it is now necessary to consider them in the light of their use as LC stationary phases. The three basic cyclodextrins, α, β and γ are also used in LC but they, or their derivatives are bonded to silica gel, particles, which are then packed into a column. The cyclodextrin structure is shown in figure 8.15.

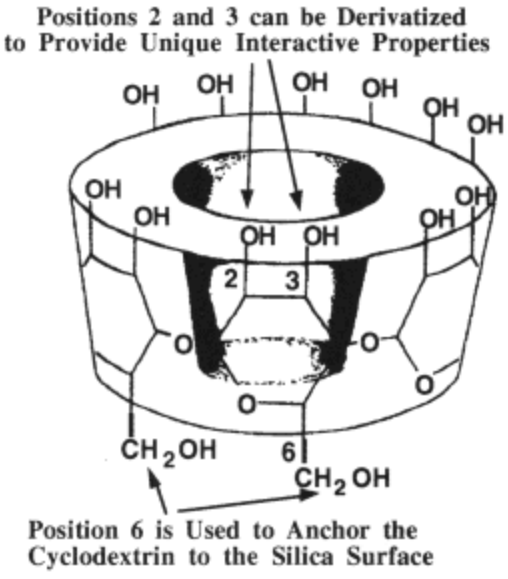


Figure 8.15
Bonding Sites on the Cyclodextrin Structure
Courtesy of Supelco Inc.

One or two of the primary hydroxyl groups (position 6) are used to link the cyclodextrin to the silica surface. The secondary hydroxyl groups (positions 2 and 3) can be derivatized selectively, usually first in position 2 and then subsequently in position 3. A number of different derivatives of the cyclodextrins have been synthesized to provide specific types of interaction to increase their chiral selectivity.

X-ray data has indicated that the β and γ structures are quite rigid whereas the α structure appears to exhibit some flexibility. Thus solute molecules, if spatially suitable, can be included and interact by dispersive, polar of ionic forces with any neighboring groups to which they are approriately close. The inclusion of a solute by the cyclodextrin structure is depicted in figure 8.16. Thus, because of the spatial differences between isomers, this can result in some interactive selectivity. This will take the form of another type of entropic contribution to the standard free energy of distribution which, as already discussed, will also induce an attending enthalpic contribution.

The concept, depicted in figure 8.16, is a grossly over simplified impression of the inclusion phenomena. It must be remembered that all molecules at the temperatures at which chromatographic separations are carried out are in continuous and violent motion as a result of their thermal energy.

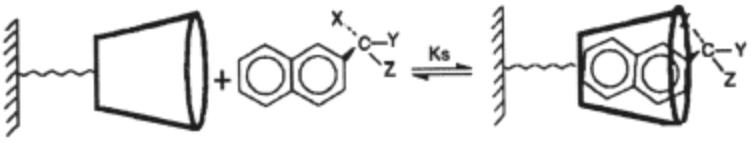
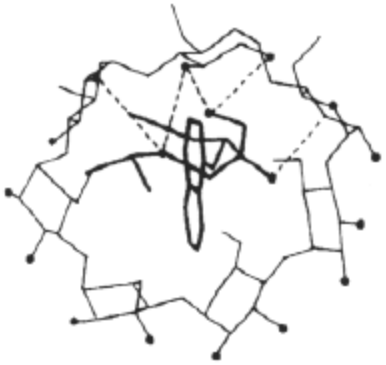


Figure 8.16
Diagram Depicting Solute Inclusion in the Cyclodextrin Structure
Courtesy of ASTEC Inc.

The diagram can only represent a statistical possibility and the situation as shown can only be transient to say the least. Nevertheless, during the passage of a solute through a column, a molecule may randomly and transitorilly assume a position something like that depicted. Furthermore, inclusion is considered by many an important factor contributing to retention.

Thus, there are basically two different interactive processes by which cyclodextrins can retain solutes and resolve enantiomers. One process involves molecular interaction after inclusion, in which case the proper

cavity size must be chosen to achieve the desired selectivity. The other process involves the usual interaction of the solute at the surface of the stationary phase. If the enantiomer molecules are relatively small, and inclusion takes place, then all three cyclodextrins must be considered in stationary phase selection. If the enantiomer molecules are large (*e.g.* 3 to 5 aromatic rings) and inclusion is not possible, then only the γ-cyclodextrin need be considered. In addition, inclusion can be inhibited by the types of interactive groups that are on the solute molecule itself. In general, if the solute molecule contains dispersive groups, including the halogens, sulfo, and phospho groups, inclusion can readily take place. In contrast, polar groups, amines, aldehydes, ketones, acids etc. do not readily enter the cyclodextrin cavities and interact preferentially with the surface. As an interesting corollary, if a stereogenic center contains a functional group that results in inclusion, then although the enantiomers may be well retained there will be little or no chiral selectivity. A computer model of R-Propanolol oriented in a β-cyclodextrin cavity is shown below.



There is considerable evidence supporting the concept of solute inclusion in the cyclodextrin cavity during chromatographic development including a number of NMR studies [17].

Derivatizing the cyclodextrins result in certain selective reactions and a general distribution of the derivatives about a mean. As already stated the 2-OH and 6-OH groups are the most reactive while the 3-OH group is significantly less reactive to derivatization. Armstrong *et al.* [18]

obtained a plasma desorption mass spectrum for a mixture of O-(S)-2-hydroxypropyl-derivatized β-cyclodextrin which is shown in figure 8.17

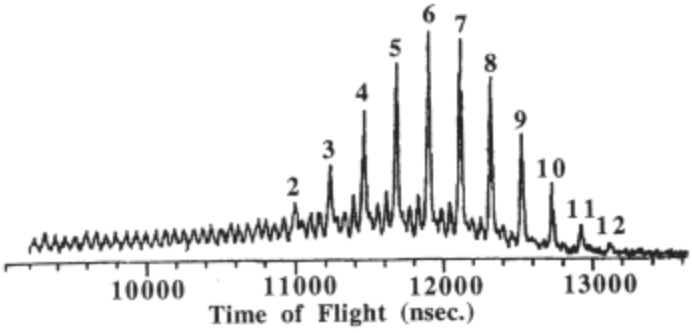


Figure 8.17
The Plasma Desorption Spectrum of a Mixture of
O-(S)-2-Hydroxypropyl-Derivatized
β-Cyclodextrin
Courtesy of Anal. Chem. [Ref. 10]

The number above the peaks denote the number of substituted hydroxy propyl groups per cyclodextrin moiety. It is seen that there is a (more or less) symmetrical distribution of substituents about a mean of 6 hydroxyl groups reacted per cyclodextrin structure. There also appears to be a minimum of about 2 and a maximum of 12 substituents per moiety. This distribution, that results from substitution reaction, shows that the substituted cyclodextrin phases are not necessarily homogeneous substances and that their net chromatographic properties, including their chiral selectivity, will be the average effect of a number of differently substituted hydroxyl groups.

The cyclodextrins can tolerate relatively high buffer concentrations and are stable from pH 3 to pH 14. However, the stability of the silica matrix restricts the pH range from 3.0 to about 7.0, as silica is significantly soluble at a pH of 8.0 and higher. Cyclodextrin type stationary phases may be operated in the polar or reversed phase mode. As with the other LC stationary phases, mobile phases with high water contents have little dispersive properties and thus the dispersive interactions with the stationary phase can be exploited. Conversely, if strongly dispersive

solvents are employed, the dispersive character of the stationary phase is swamped, and the retention and selectivity will result from polar and or ionic interactions. These polar interactions will take place with the many hydroxyl groups that are intrinsically present or with other polar or ionic centers that may have been introduced by derivatization. The sites of derivatization are shown in figure 8.18 together with an example of a naphthyl carbamate derivative attached to the 2 or 3 positions.

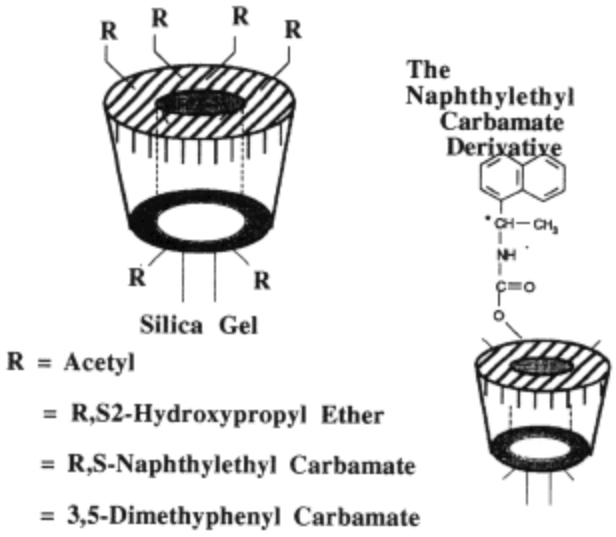


Figure 8.18
The Bonding and Derivative Positions on the Cyclodextrin Structure
Courtesy of ASTEC Inc.

There are a large number of possible derivatives that can be prepared and almost every one will probably possess some unique property that will enhance the separation of certain enantiomeric pairs. The examples given in figure 8.18 are those selected by the chiral stationary phase manufacturer ASTEC as those more broadly useful for chiral separations. Other stationary phase manufacturers may well select other types of derivatives for optimal use.

The cyclodextrin cavity readily accepts certain functional groups as well as the aromatic nucleus. For chiral selectivity one of the enantiomers must fit closer to the neighboring groups to allow stronger interaction with either the surface diol groups or the groups of a derivative. If significant solute penetration into the cavity is achieved, then it can interact with the more dispersive groups that are situated within the cavity. The separation of the enantiomers of a rather complex molecule is shown in figure 8.19.

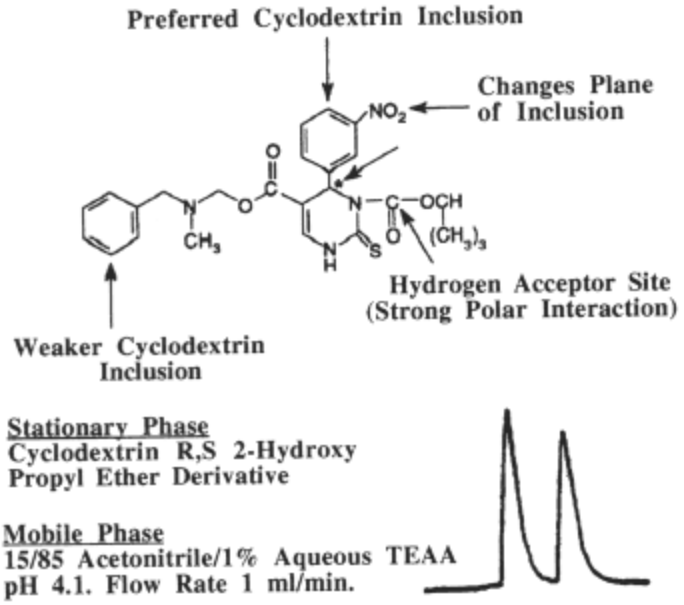


Figure 8.19
A Chiral Separation that is Interpreted to Result from Molecular Group Inclusion
Courtesy of ASTEC Inc.

The separation was achieved on a β-cyclodextrin that carried the hydroxy propyl ether group attached to the lip of the cavity in the 2 and 3-positions. A possible and rational explanation for the chiral selectivity that is achieved is suggested in the diagram. However, it is important to

appreciate that the suggested interactive mechanism has not been proved although the explanation is quite plausible.

As with other chiral stationary phases, dispersive interactions with the cyclodextrin structure are controlled with polar solvents, polar interaction controlled with dispersive solvents and, if ionic interactions are present, these will be controlled by both the pH and the type of buffer that is employed. Small changes in pH can be quite critical and a the effect of buffer type on chiral selectivity, under certain circumstances can be quite profound. an example of the effect of pH and buffer type is depicted in figure 8.20.

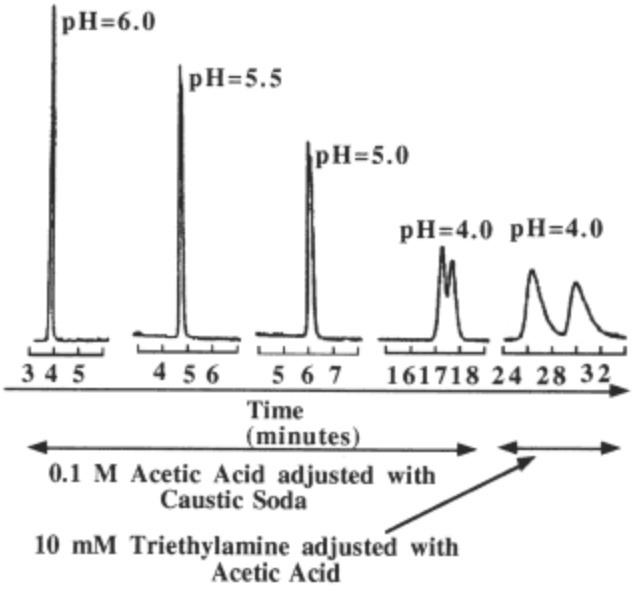


Figure 8.20
The Effect of pH and Buffer Type on Chiral Selectivity.
Courtesy of ASTEC Inc.

The enantiomeric pair that are separated was Dansyl D,L-phenylalanine separated on cyclodextrin column at a flow rate of 1.5 ml/min. It is seen that a change in pH from 5.0 to 4.0 has produced a remarkable increase in chiral selectivity. Even more significant is the effect of changing the

buffer from acetic acid adjusted with caustic soda, to triethylamine adjusted with acetic acid. This change has resulted in the complete resolution of the two enantiomers. However, it should also be noted that the retention time is increased from 18 min to about 32 min indicating that either interactions between the solute and the stationary phase are significantly stronger or the interactions between the solute and the mobile phase have become significantly weaker. Another example of the effect of different buffer concentrations on chiral selectivity is afforded by the separations shown in figure 8.21.

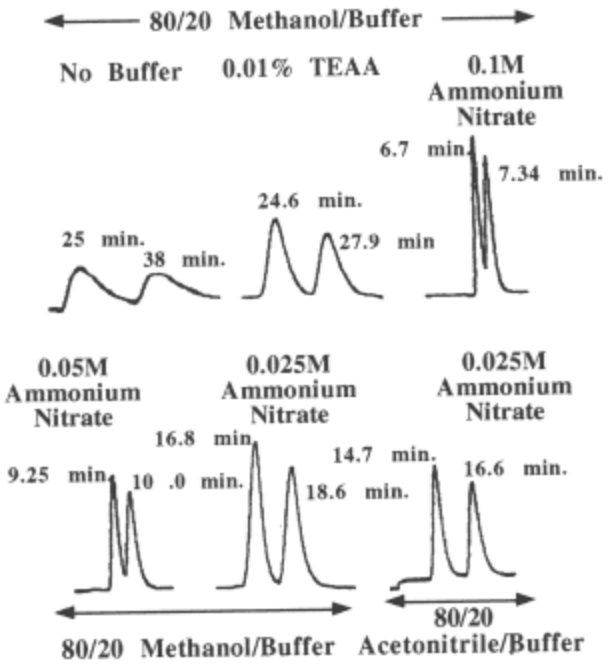


Figure 8.21
The Effect of Buffer Concentration on Chiral Selectivity
Courtesy of ASTEC Inc.

The same enantiomers are being separated as shown in figure 8.20, but the buffer used at different concentrations is ammonium nitrate. It is seen that although good selectivity is achieved in the presence of very low concentrations of ammonium nitrate and in the absence of the buffer, the

peaks are very broad and the analysis time is lengthy. At a relatively high concentration of ammonium nitrate (0.1 M) the separation is much more rapid (the distribution kinetics are much faster) but much of the chiral selectivity is lost. At the intermediate concentration of 0.025 M, the kinetics are still reasonably fast, the selectivity is maintained, and thus the enantiomers are well resolved. It is also interesting to note, that if the buffer concentration is kept constant, and the organic solvent changed to acetonitrile, the resolution is little effected.

It is seen that, as with the other types of LC stationary phases, some experimental work is essential if the optimum conditions to achieve the separation is to be identified. The best stationary phase, solvent, solvent concentration, buffer, buffer concentration and pH must be ascertained, and this can only be achieved by starting from experimentally confirmed conditions for the separation of similar types of solutes. If the sample to be separated is unique, then a rather laborious set of experiments need to be carried following the guidelines given by the supplier of the stationary phases that have been selected for use. It should be pointed out that the recommended procedure will vary between stationary phase types, and between suppliers.

Synopsis

In LC, as opposed to GC, there are strong interactions between the mobile phase and the solute thus providing a further variable to control the separation. These interactions can be increased during the separation by solvent programming, which greatly extends the polarity range of the solute mixture that can be separated. However, when inclusion modifies the normal type of interaction the relationship becomes more complex. The same interactions, dispersive, polar and ionic, are active in the retention process and the separation is controlled thermodynamically by changes in the standard free energy of distribution. LC separations are carried out almost exclusively, in packed columns, that exhibit a third type of solute dispersion called multipath dispersion. This arises from the tortuous path the solute takes when passing through the interstices between the packing. Some molecules will travel shorter paths than the

average, and some longer paths, and thus cause band dispersion. The best equation that described the dispersion in a packed column is derived by Van Deemter *et al*. The equation for the variance per unit length of the column takes the form of a hyperbolic function in (u) the linear velocity, from which, by differentiating and equating to zero, an expression for the optimum velocity and minimum variance per unit length can be obtained. There are five general types of LC chiral stationary phases. They are the protein based phases bonded to silica, the small molecular weight chiral compounds bonded to silica (the Pirkle phases), the cellulose and amylose polymers type phases that are coated onto silica gel, the macrocyclic glycopeptides that are bonded to silica and the cyclodextrin based materials that are also bonded to silica. There are several protein type stationary phases, α_1-acid glycoprotein, cellobiohydralase and human serum albumin being examples. These stationary phases are used with mobile phases with a high water content (mostly > 90%) and thus separations are largely by dispersive interactions. There are quite a large number of small chiral molecules that are used as Pirkle stationary phases, some of the more common being the substituted urea carbamates. These types of stationary phases can be operated either in the reversed phase mode or the normal mode. The cellulose or amylose polymers are derivatized in order to link groups such as carbamates or benzoates to the free hydroxyl groups. The material is coated onto the silica, not chemically bonded, and thus is vulnerable to attack by solvents. Heptane- alcohol mixtures are usually employed as the mobile phase, so separations largely depend on polar interactions. However, the tris(3,5-dimethyl-phenyl carbamate) derivative is claimed to be stable with high water content mobile phases and thus can be used in the reversed phase mode. The macrocyclic glycopeptides are chemically bonded to silica gel. Two popular materials are Vancomycin and Teicoplanin and a third Avoparcin has been recently introduced. Retention and selectivity is controlled by both the solvent composition, pH of the mobile phase and the type of buffer. They contain a large number of chiral centers that improves the probability of chiral selectivity. They also contain shallow inclusion centers that augment spatial selectivity. These stationary phases are stable and can be easily

used in either the reversed phase mode or the normal mode. Teicoplanin is particularly useful in the separation of underivatized and blocked amino acids. The cyclodextrin phases can be made form α, β or γ cyclodextrins and offer a large number of chiral centers and, as result of their unique structure, contain a number of substantially deep inclusion centers. These inclusion centers allow selective intrusion by appropriately sized molecules and thus can play an important part in chiral selectivity. The cyclodextrins can be derivatized and the natural chiral selectivity augmented with appropriate dispersive or polar groups situated close to the chiral centers and on the lip of the inclusion areas. The stationary phase is chemically bonded to a silica substrate and is stable to a wide range of solvents. It does not, however, readily tolerate mobile phases having a pH greater than 7.0. Many cyclodextrin based stationary phases can be used in both the reversed phase and normal phase mode of development.

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Chapter 9—
The Preparation of LC Chiral Stationary Phases and LC Chiral Columns

Virtually all LC chiral stationary phases are either chemically bonded to, or are coated on, the surface of silica gel. Silica gel is an amorphous, highly porous, partially hydrated form of *silica* which is a substance made from the two most abundant elements in the earth's crust, silicon and oxygen. The silica gel used in LC can take two forms, spherical and irregular. Although irregular silica that has been ground by air jet abrasion is well rounded, and very similar in physical form to spherical silica, spherical silica is seen as a 'state of the art' material and thus is the silica most commonly used. The process of making silica of either form is complex [1] and is not relevant to discuss here, but the properties of silica are indeed important.

The Supporting Matrix for Chiral Stationary Phases

The matrix of the silica gel particle consists of a core of silicon atoms joined together with oxygen atoms by siloxane bonds (silicon -oxygen-silicon bonds). However, on the surface of each primary particle some residual, uncondensed hydroxyl groups from the original polymeric silicic acid remain. It is these residual hydroxyl groups that confer upon silica gel its polar properties and it is with these hydroxyl groups that the silane reagents react to form the chiral bonded phases. The silica gel surface, however, does not simply consist of uncondensed hydroxyl

groups, but is quite complex and contains more than one type of hydroxyl group. It also contains strongly bound or 'chemically' adsorbed water and loosely bound or 'physically adsorbed' water depending on the history of the gel. Theoretically, any surface hydroxyl group can be one of three types. Firstly, it may be a single hydroxyl group that is attached to a silicon atom which has *three* siloxane bonds joining it to the gel matrix. Secondly, it can be one of two hydroxyl groups attached to the same silicon atom which, in turn, is joined to the matrix by only *two* siloxane bonds. These are called Geminal hydroxyl groups. Thirdly, it can be one of three hydroxyl groups attached to a silicon atom which is now only joined to the silica matrix by a *single* siloxane bond. An example of each type of hydroxyl bond is shown in Figure 9.1.

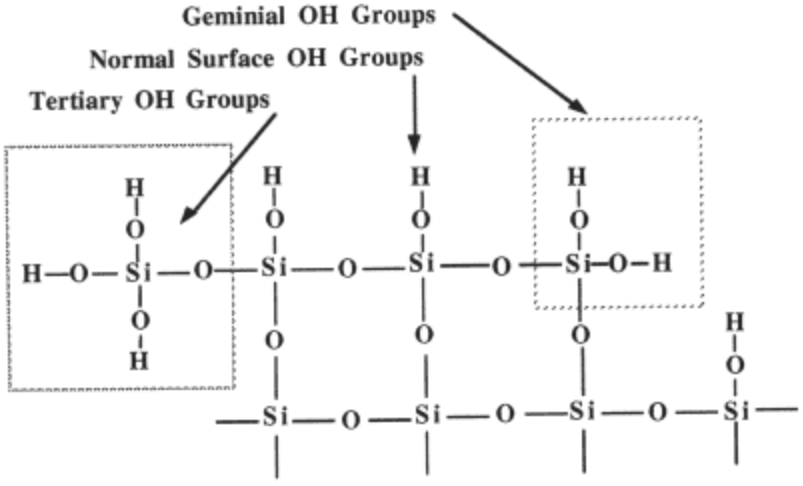


Figure 9.1
The Hydroxyl Groups on the Surface of Silica gel

The probability of any one type of hydroxyl group being present and consequently, its fraction of the total, has been estimated by Sindorf and Maciel employing NMR techniques [2,3], and it appears a reasonably safe assumption that the single hydroxyl group is likely to by far the most prolific, which is also the type to which the chiral groups are most likely to be attached. The next most common, appeared to be the geminal hydroxyl groups followed by the least common, tertiary hydroxyl group.

However, the silica surface is even more complex than the above diagrams would imply, as water can be hydrogen bonded to the hydroxyl groups and multi-layers of water physically adsorbed on top of these or on siloxane groups. Various types of hydrogen bonded water that might occur on the silica gel surface are depicted in figure 9.2. It must be emphasized that *none* of the above structures has been experimentally confirmed in an unambiguous manner but all are reasonably possible.

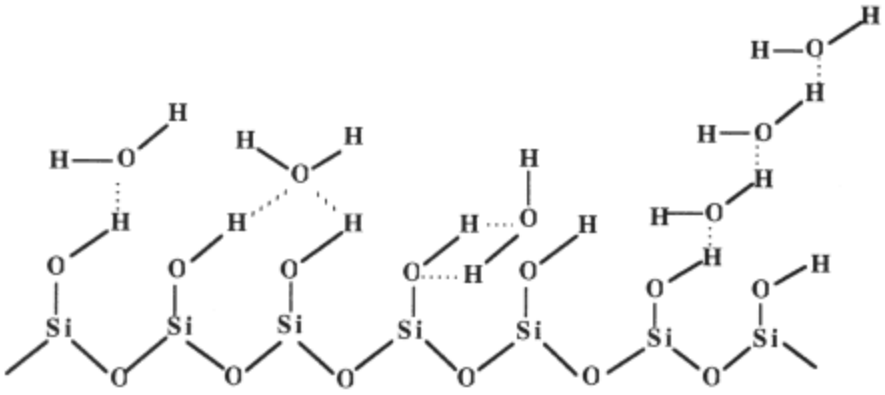


Figure 9.2
Adsorbed Water on the Surface of Silica Gel

Before bonding material to the surface, it is usual to heat the silica gel to at least 120°C and often to 200°C to remove all the adsorbed water. If the water is not removed, it will compete with the silanol groups for reaction with the derivatizing agent and may also cause a polymeric type of bonded phase to be formed.

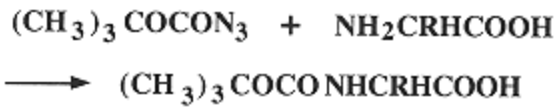
In chromatography, there are three physical properties of silica gel that are important. The *pore size*, the *surface area* and the *particle diameter*. The effect of the particle diameter has already been discussed. The efficiency of a column increases as the particle diameter of the packing is reduced. However, the column flow-impedance is also increased as the particle diameter is reduced and a higher pressure is necessary to achieve the optimum mobile phase velocity. The pore size and surface area of silica gel tend to vary inversely with one another. The smaller the pore size the higher the surface area. In addition, the larger the pore size, the

lower the mechanical strength of the particle. In chiral chromatography, both the stationary phase molecules, and often the solute molecules, may be relatively large, and so the pores can not be too small, or either the bonded moiety, or the solute may excluded. In practice, most chiral stationary phases are either bonded or coated to silica having a mean pore size ranging from 60 Å to 150 Å or more. The normal average pore size of non-chiral bonded phases used in LC are about 100 Å. As already stated, despite its similarity to contemporary air-jet ground, irregular silica, spherical silica is now the most common media (if not the only form of silica particles) that is used to produce modern chiral stationary phases. Spherical silica particles used for the production of chiral phases are 3 µm or 5 µm in diameter and have pore sizes that range between 100 Å and 200 Å.

The Preparation of Protein Stationary Phases

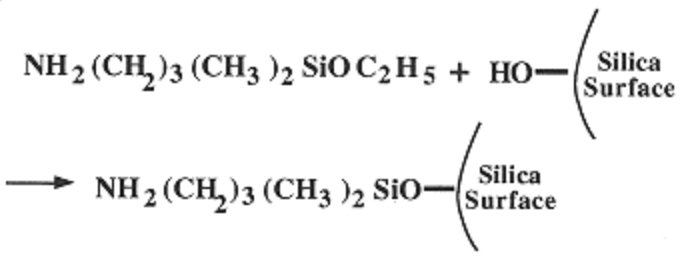
Polypeptide and protein phases must be immobilized onto the surface of the silica gel by reaction with the surface hydroxyl groups. There are a number of different synthetic procedures available to achieve this, some of which employ techniques used in the solid state synthesis of peptides. There are two or three stages to this process depending on the condition of the peptide. If the peptide has been synthesized, then the amino group will probably be protected and then it can be bonded to the silica surface by a two stage reaction. If the peptide is not protected then the free amino group must be protected before reaction. The first stage is to bond an appropriate organic moiety to the silica surface to which the peptide or protein can be attached. It is important to employ a silicon_oxygen-silicon bond to the surface hydroxyl group to ensure a chemically stable attachment. The carbon-oxygen-silicon bond is easily broken by hydrolysis and therefore should not be used. The protected peptide is then reacted with the attached group and bonded to the silica. The protection group can the be removed leaving the free amino group at the end of the chain. Depending on the groups available on the surface for reaction, a protein can be made to react directly with the bonded moiety on the silica surface.

The amino group of a free peptide can be protected in many ways, one using t-butyloxycarbonyl azide is an example.

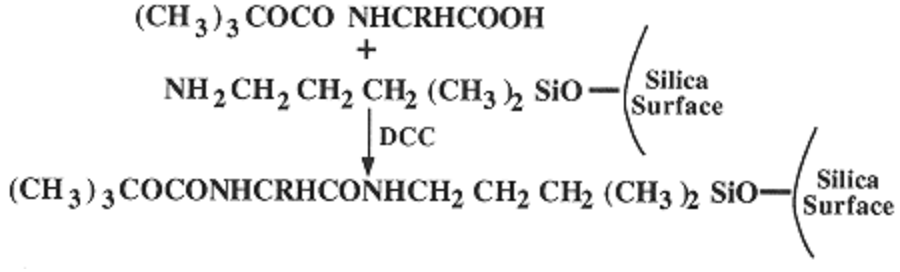


The reaction is carried out at a controlled alkaline pH in a pH-stat, or in the absence of a pH-stat, magnesium oxide, sodium bicarbonate or triethylamine have been used to promote the reaction.

The reacting group is then bonded to the silica. For example, the silica is reacted with 3-aminopropyldimethylethoxysilane. The reaction is carried out in refluxing toluene containing the silica, and the silyl reagent is added slowly over a period of about 30 minutes. The mixture is refluxed for about 2 hours, cooled filtered, washed with methanol and dried. The reaction proceeded as follows.

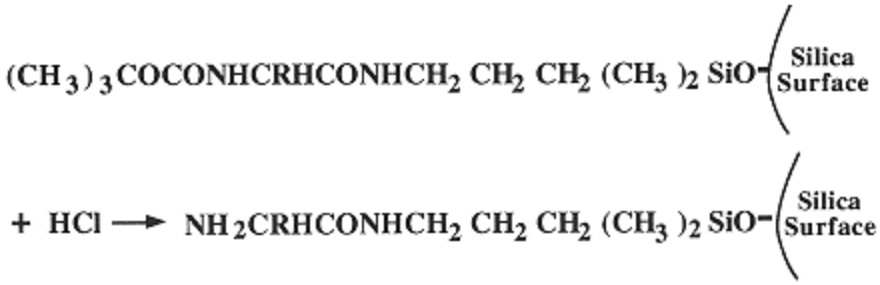


The protected peptide can then be coupled to the amino group on the surface of the silica in dichloromethane using dicyclohexyldiimide as the coupling agent as follows.



If the peptide is large and insoluble in dichloromethane then more polar solvents may be necessary.

Finally the peptide bonded to the silica gel can be deprotected with hydrogen chloride in a suitable solvent.

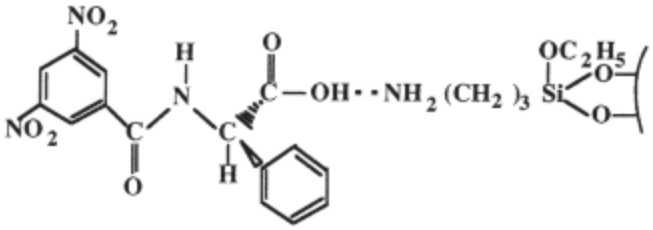


In this way a variety of relatively large peptides can be bonded to silica. There are many chemical pathways that can be used to bond peptides and proteins to silica surfaces and this is just one example. Another method will be given when the preparation of the macrocyclic peptides stationary phases are discussed. In principle, there are two groups on a protein that can be used to couple it to the silica and they are free amino groups and free carboxyl groups. In some cases, when there are a number of both groups available on the protein molecule, care must be taken not to couple the protein molecules together as opposed to the silica surface. These type of stationary phases have a limited range of pH over which they will remain stable. Despite the phase itself being stable, and the silicon-oxygen-silicon bond being chemically durable, very few of these type of phases bonded to silica can be used at pH values above 7.0 due to the siloxyl bonds being broken and the consequent disruption of the silica matrix itself.

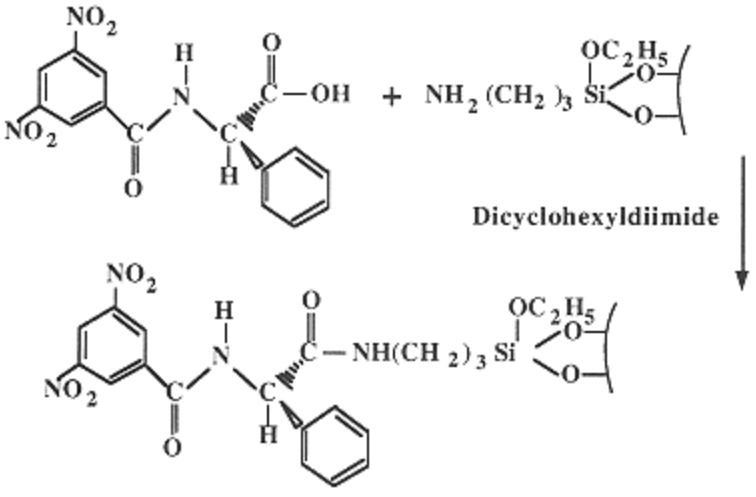
The Preparation of the Pirkle Stationary Phases

The Pirkle phases are small molecular weight materials with a limited number of chiral centers and are generally more simple to prepare than the other phases. One of the first methods of linking a chiral moiety to a silica surface was by ionic bonding. Phenylglycine was treated with benzoyl chloride to produce the benzoyl derivative, which was then reacted with the 3-amino propyl group that was linked to the silica via one or more siloxyl bonds. A solution of the (R)-1-(3,5-dinitrobenzoyl)glycine in THF solution is added to a suspension of the aminopropyl silica and the mixture well stirred simply producing the

ionically bonded stationary phase. The silanizing reagent again is 3-aminopropyldimethoxyethoxysilane. The structure of the bonded material is represented as follows.



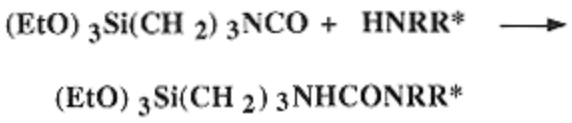
This type of material, obviously, can be somewhat labile in aqueous solvents and would only be usable under certain buffered conditions. However, when this stationary phase is used with normal phase solvents (dispersive or hydrophobic solvents) the system is stable. The obvious next stage was to couple the protected glycine directly to the amino propyl group using dicyclohexyldiimide as the coupling agent. This reaction proceeds as follows.



This type of bonding between the chiral moiety and the silica is obviously far more stable although it has been reported that the amide bond is vulnerable to extremes of pH and high salt concentrations.

One of the more common methods of synthesis is to use an isocyanate to react with a hydroxyl group to form a carbamate or to an amino group to form a substituted urea. There are a number of silyl reagents available that contain an amino propyl, a hydroxy propyl or an isocyanatopropyl group which can be bonded to silica.

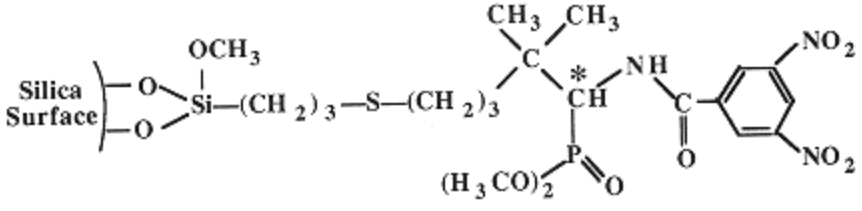
For example, starting with 3-isocyanatopropyltriethoxy silane this can be reacted with an appropriate chiral amine in the following manner.



where R* indicates an optically active compound

The urea derivative is then bonded to the silica by the ethoxy silyl reagents in the usual manner. For example, the silica is dispersed in toluene and the ethoxy silyl derivative added and the mixture shaken for 2 or 3 hours. Half the toluene is removed by distillation and more toluene added and again half the toluene removed by distillation. The derivatized silica is then filtered, washed with hot toluene and diethyl ether and dried in *vacuo*.

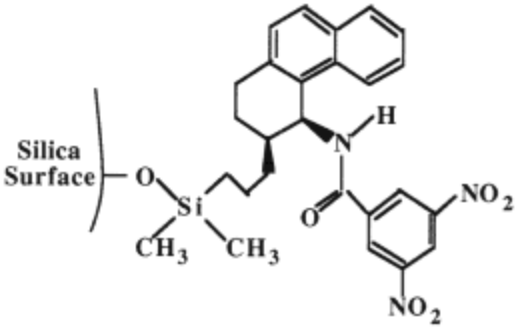
Two of the more common Pirkle type stationary phases in use today are the α-Burke 1 and the Whelk-01. The structure of α-Burke 1 is given below.



The material was synthesized from dimethyl N-3,5-dinitrobenzoyl-α-amino-2,2-dimethyl-4-pentenyl phosphonate which was covalently bound

to mercaptopropyl on 5 µm silica particles. This stationary phase has found fairly wide application but is particularly useful for separating β-'blockers' in their underivatized form.

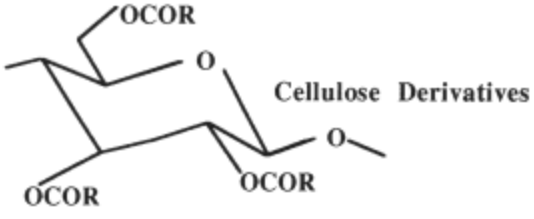
The Whelk-01 was designed specifically for the separation of underivatized non-steroidal anti-inflammatory drugs. The structure of the Whelk-01 stationary phase is given below.



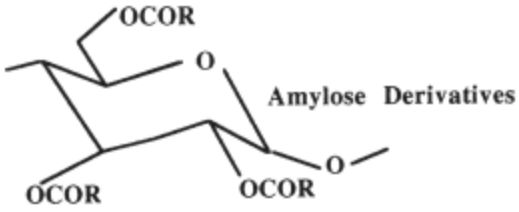
This material is synthesized from 4-(3,5-dinitrobenzamido)-tetrahydro-phenanthrene which is also covalently bonded to silica particles 5 µm in diameter. It is used to separate the underivatized isomers of drugs such as Ibuprofen and Naproxen. It is a fairly stable phase and is available in both enantiomeric forms allowing elution order reversal if so desired.

The Preparation of Cellulose and Amylose Stationary Phases

Both cellulose and amylose contain 5 chiral centers per unit and thus the polymeric material offers a large number of chirally interactive centers and a high probability of interaction. There are basically two common types of cellulose and amylose derivatives that are used as stationary phases. The first type are simple esters as shown below

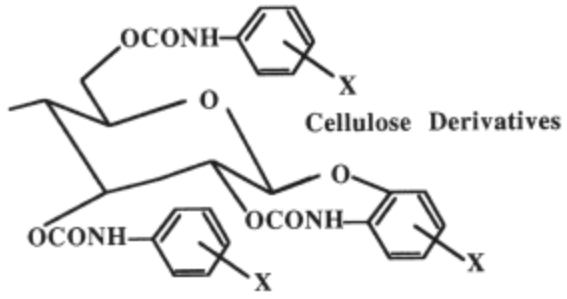


R may be aliphatic, aromatic or substituted aromatic. The second type, the amylose esters are similar in form.

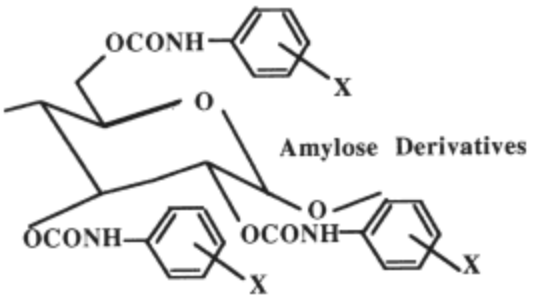


The ester derivatives are usually formed from the acid chlorides such as acetyl chloride or benzoyl chloride.

The more stable, and probably the more popular derivatives, are the carbamates which are synthesized from the appropriate isocyanate. They take the following forms.



The substituent groups can be 3,5-dimethyl, 3,5-dichloro, 3,5-difluoro, or 4-*tert*-C_4H_9.



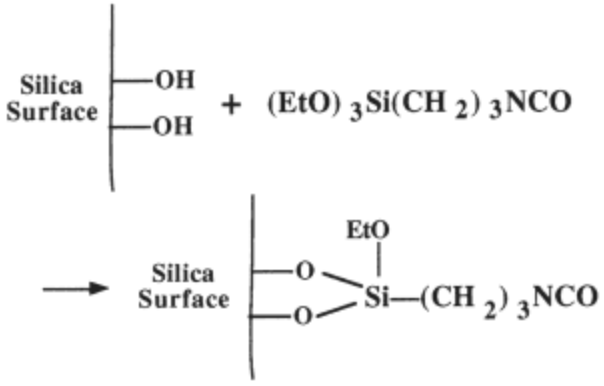
The substituent groups can be 3,5-dimethyl, 3,5-dichloro, or 3,4,5-trimethyl.

The derivatives are formed by reacting the polysaccharides with the substituted isocyanates in pyridine and the desired material is isolated as the methanol-insoluble fraction. Prior to coating, the silica particles are treated with 3-aminopropyltriethoxysilane using the standard procedure. The polysaccharides derivatives are then dissolved in tetrahydrofuran and adsorbed on the aminopropylsilica. The stationary phase loading should be about 25% w/w. The derivatized cellulose and amylose have been used extensively in the separation of chiral drugs of all types.

The Preparation of Macrocyclic Glycopeptides Phases

Although the macrocyclic peptides can be bonded to a number of different substrates, the material used most commonly in LC, is silica gel. The bonding reagents may be organosilanes that are terminated by carboxylic groups, amine groups, epoxy groups and isocyanate groups. Examples of such reagents are 2-(carbomethoxy)-ethyltrichlorosilane, 3-aminopropyldimethylethoxysilane,(3-glycidoxypropyl) trimethoxysilane and 3-isocyanatopropyltriethoxylsilane, respectively. Thus, the linkage attatching the peptides to the silica may be ether, thioether, amine, amide, carbamate or urea. An example of a reaction sequence that could be used to attach a peptide to silica employing 3-isocyanatopropyltriethoxylsilane as the bonding agent is as follows.

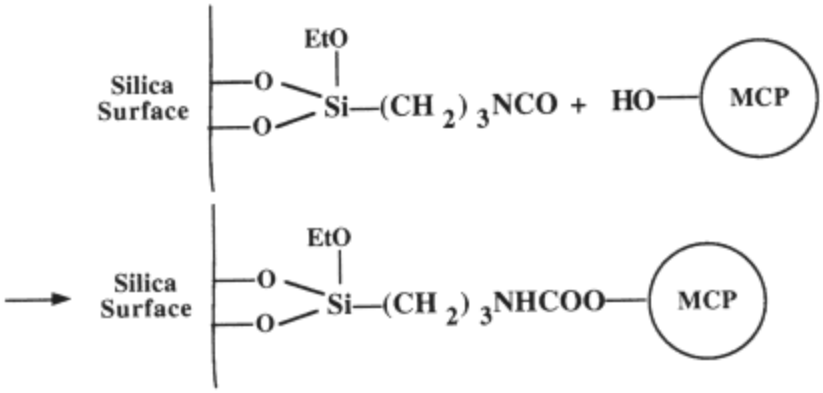
Initially the 3-isocyanatopropyltriethoxylsilane is reacted with the silica surface hydroxyl groups in the following manner



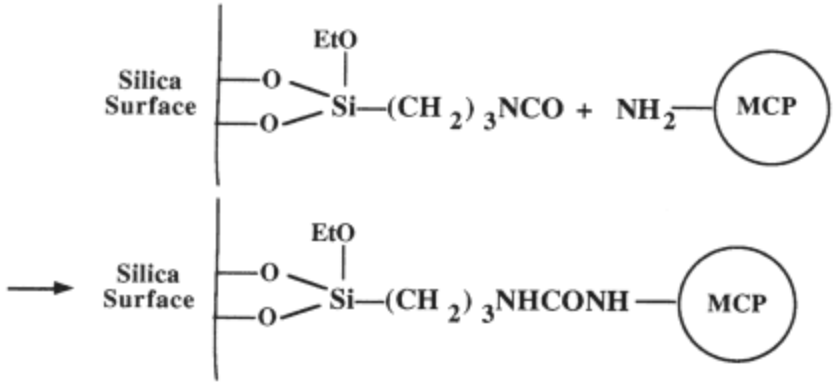
This is carried out by adding the silica (*e.g.* spherical silica having 5 µm particle diameter and a mean pore size of 100 Å) that has been dried *in vacuo* over phosphorus pentoxide to a solution of the silyl reagent contained in toluene. The mixture is refluxed and the ethanol produced in the reaction with the silanol groups periodically distilled off. The silica particles are then filtered, washed well with hot toluene and dried.

The next stage is to react the peptide with the isocyanate group on the silica. The particular bond used to attach the peptide to the carbamate group will depend largely on the nature of the cyclic peptide that is used. Prior to reaction, the cyclopeptide may be derivatized and such groups as 3,5 dimethylphenyl added to the macromolecule to improve, or change, the interactive nature of the material with regard to chromatographic retention and selectivity.

The reaction is carried out by adding the dry peptide (carefully dried in *vacuo* at 100°C over phosphorus pentoxide) dropwise, to a dispersion of silica particles, in anhydrous *N,N*-dimethyl formamide. The reaction is allowed to proceed under dry nitrogen at about 90°C to 95°C. If the isocyanate reacts with a hydroxyl group on the peptide then a carabamate bond is formed in the manner depicted below.



Alternatively, if the isocyanate reacts with an amino group present on the peptide structure then the peptide will be attached to the silica by a urea bond in the manner depicted below.

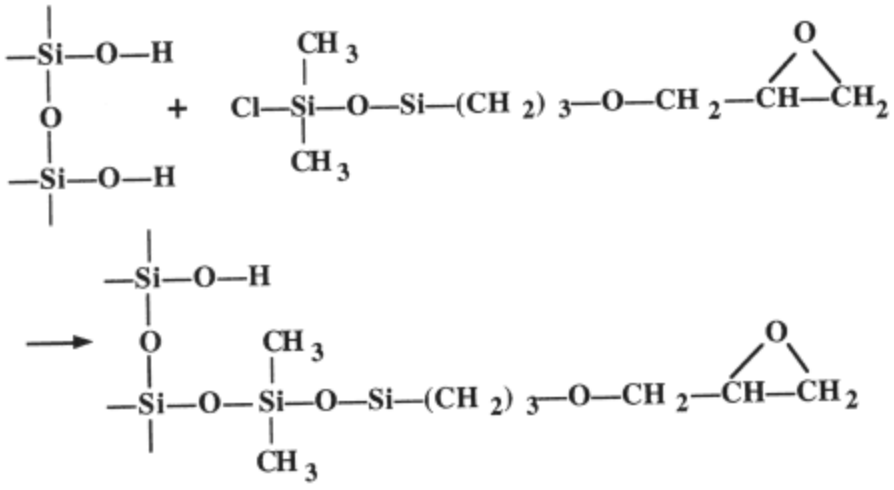


In general, the macrocyclic glycopeptides are more stable than the traditional protein phases and have a higher loading capacity. In comparison to the cellulose and amylose phases, the glycopeptides can tolerate a much wider range of solvents and consequently have greater versatility.

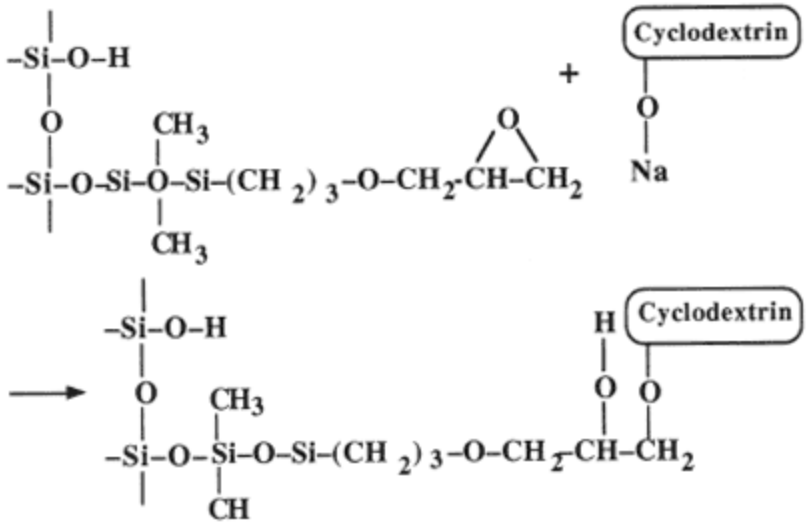
The Preparation of Cyclodextrin Based Stationary Phases

The three cyclodextrins, α, β and γ are all used as LC stationary phases bonded to a suitable support such as silica. The cyclodextrins may be appropriately derivatized as previously discussed. They can be bonded to the silica using a variety of silane reagents, but that which is most commonly employed is one of the three 3-glycidoxypropyl silanes such as, 3-glycidoxypropyl trimethoxy silane, 3-glycidoxypropyldimethyl-chloro-silane and 3-glycidoxypropyltriethoxy silane. The reagent is first attached to the silica by the following reaction.

Spherical silica particles, about 5 μm in diameter, are dried for about 12 hours at 170°C to remove all adsorbed water and then dispersed in 250 ml of dry toluene. The mixture is then refluxed and any remaining water azeotropically removed. The silane reagent is then added and the reaction allowed to proceed at 95°C for about 3 hours. The mixture is then cooled, filtered, washed several times with toluene and methanol and then dried at 60°C over phosphorus pentoxide *in vacuo*.

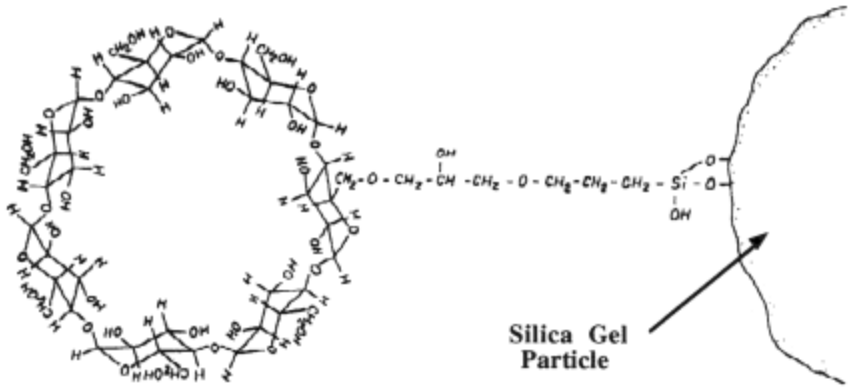


The synthetic procedure varies a little with the type of silane reagent that is used and this will depend on the type of linkage that is employed between the cyclodextrin and the active group of the silane reagent. In a similar manner, the method of linkage used will also determine the experimental conditions used to bond the cyclodextrin to the silica. An example of the bonding process is depicted below.



In practice, this bonding is carried out with the cyclodextrin in a solvent such as dimethyl formamide or pyridine. Theoretically, the linkage can occur through either the primary or secondary hydroxyl groups on the cyclodextrin, but more commonly, with the primary groups, as they are less sterically hindered.

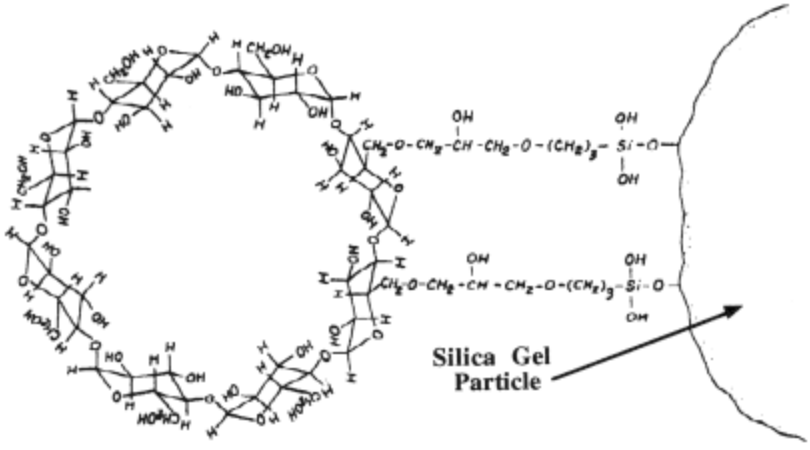
If the silane only contains a single functional group to interact with the silica surface, then a single bond will link the cyclodextrin to the surface of the silica. In cases where the di- or tri-chloro or methoxy groups are used to attach the silane to the silica, it is possible that more than one bonding link may occur between the silane reagent and the silica. This will depend on the relative positions of the hydroxyl groups on the silica surface and the orientation of the reacting chloro or methoxy groups. If reaction occurs between two of the chloro or methoxy groups and the silica surface a structure similar to that shown below could be educed.



This type of bonding would depend heavily on atomic distances between the reacting groups and the silanol groups on the silica surface matching almost exactly. The positions of the chloro or methoxy groups are certain and invariate, but, unfortunately, silica gel is not a crystalline substance and, as discussed earlier in the chapter, is amorphous and irregular in its molecular arrangement. It follows that the probability of the atomic distances matching is small, but not necessarily impossible.

Consequently, there may well be a small proportion of bonds of the form shown in the diagram that link the cyclodextrin to the silica gel surface.

There is yet another possible mode of attachment. In the reaction between the bonded silane and the cyclodextrin more than one hydroxyl group on the cyclodextrin macromolecule may react with the silica surface, locking the cyclodextrin to the surface with two silane chains. This type of linking has a much greater probability, as once the first bond has been made, the cyclodextrin group can rotate and a second hydroxyl group randomly react with another silane group. This type of bonding is depicted below.



This double bond attachment is substantiated by carbon/hydrogen analysis before and after bonding. Furthermore, the basic high stability of the cyclodextrin bonded phases support the idea that a significant number of these types of double bond attachments are, in fact, present. In addition, it is not theoretically impossible that one cyclodextrin molecule could be anchored to the silica by three or more chains, although such attachment bonds have not been experimentally confirmed or identified. These types of phases have been used very successfully in the resolution of the enantiomers of a number of β-'blockers' (*e.g.* Propranolol, Atenolol,

Timolol etc.) and a wide range of other types of drugs that occur as racemates.

Column Packing Techniques

Virtually all contemporary LC columns are slurry packed and the procedure can be expected to give column efficiencies close to the theoretical maximum from the material used. *i.e.* $1.5d_p$ <H> $2d_p$ (where (d_p) is the particle diameter). For obvious reasons, slurry packing techniques are held highly proprietary by many column suppliers and essential details are often not reported, though some general guidelines can be given. Nevertheless, is must be said that unless some very special columns are to be prepared, it is far easier and more economic to purchase the columns already packed from a reliable supplier. Columns are generally made from stainless steel 1, 2, or 4.6 mm I.D and common lengths are 3, 5, 10, 15 and 25 cm. Some substances of biological origin, such as certain proteins are thought to denature in contact with stainless steel, in which case the column can be made from titanium, PEEK or some other inert material of appropriate strength. It is particularly important, when analyzing plasma samples, to use titanium or teflon frits in the column rather than stainless steel to avoid denaturation of the protein, deposition of protein at the head of the column or racemization of certain the analytes such as the diazapams.

The packing, carrying the selected stationary phase should also have a very narrow particle distribution, as well as having the specified mean particle diameter. This is essential if a stable, closely packed, dense bed is to be obtained. It is also extremely important that steps are taken to ensure that each particle packs as an individual that impinges on the bed as it is formed and not as an aggregate of particles. For example, if particles 10 µm in diameter are being packed it is very easy to allow aggregates 40 µm or more in diameter to form in the packing solvent. These aggregates form conglomerate clumps in the column which initially results in poor column efficiency. Eventually, however, there is further deterioration and bed settling occurs, with the consequent formation of column voids at the beginning of the column, followed by a

further fall in column efficiency. This problem can be very significant when packing reverse phase materials and often soluble surfactants must be added and/or certain solvents must be included in the slurry mixture to prevent this particle aggregation. Many of the solvent mixtures appearing in the literature for use in slurry packing are not universally applicable to all silica or bonded phases. Some experimentation may be necessary before a satisfactory mixture is achieved and an appropriate "aggregation preventing" solvent or surfactant identified.

In addition to the removal of any aggregates that have been formed, the packing must be well dispersed by sonication with appropriate dispersing agents. The particles must be well disseminated in the slurry before packing so that they do not aggregate or settle out during the packing process. In principle, there are two types of solvent that could be appropriate for slurry packing. Firstly, the solvent could be made to have the same density as the packing material and thus, eliminate the possibility of settling completely. This type of solvent mixture is called a 'balanced density' solvent, but has the disadvantage of requiring components that are either expensive, toxic or difficult to handle. However, in general, balanced density solvent mixtures have a relatively low viscosity and thus, do not require such high pressures to pack the column successfully. Secondly, the dispersing solvent can be made to have a relatively high viscosity and thus, as Stokes Law predicts, will ensure a very slow settling rate for the particles. Solvent mixtures that are viscous have the advantage that the components used are relatively inexpensive but unfortunately the more viscous solvent requires the use of much higher packing pressures. In practice, solvent mixtures are often used that have a viscosity that is only large enough to ensure that if the packing procedure is performed as rapidly as possible, the packing is complete before significant settling can take place. This approach achieves an optimum compromise between solvent economy and high packing pressures. The apparatus used for slurry packing is shown in figure 9.3. Appropriate high pressure pumps that can be used for LC column packing are made by Haskel and operated by air pressure with

pneumatic amplification. A pump having a maximum pressure of 10,000 p.s.i. would be appropriate for packing most LC columns.

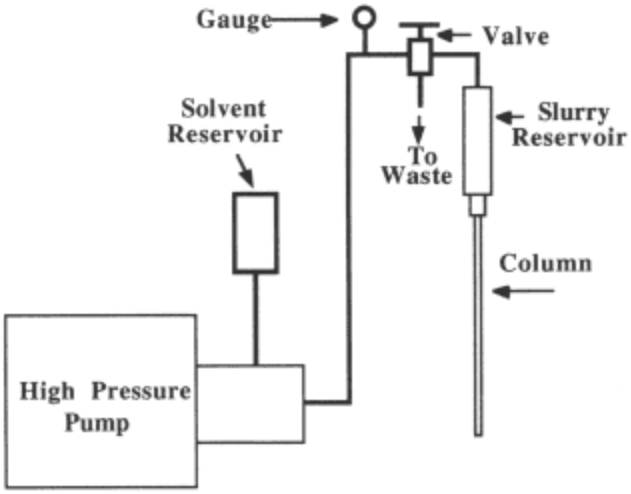


Figure 9.3
Slurry Packing Apparatus

The pump is supplied with solvent from a reservoir and is then connected to a pressure gauge which, in turn, is connected to another valve. The exit from the valve is connected to the slurry reservoir which in turn is connected to the column to be packed. All unions, fittings, valves etc. employed with the system must be capable of accommodating the full pressure available from the pump. For high pressure operation Sno-Trik fittings are recommended. A scheme for slurry packing an LC column is as follows.

1. The column should first be cleaned by the series of solvents recommended by Karger [4] *viz.* chloroform, acetone, water, 50% v/v phosphoric acid, 10%v/v nitric acid, water (until neutral), acetone and finally chloroform again. This treatment will remove all grease, extrusion lubricants, surface oxides and detergents that are present. The column is then connected to the slurry reservoir. Alternatively, the column can be washed with a residue free proprietary detergent such as

Liqui-Nox®. The column is filled with the detergent, sonicated, for a few minutes, washed well with water followed by acetone and then dried.

2. Unless the packing material is obtained from a commercial supplier it should be sieved through a 200 mesh screen to remove any large particles and in particular any *contaminating fiber* from filter paper, filter bed fibers etc. Another method of fines and light material removal is by sedimentation. The packing is mixed with excess solvent, well shaken and allowed to settle for a few minutes. The supernatant liquid is then decanted away. The process can be repeated until the upper layer of solvent, after sedimentation, is clear.

3. To determine the best solvent and surfactant concentration, a slurry mixture should be made up prior to packing, and dispersed by sonic vibration. The rate of sedimentation should be carefully checked over the anticipated time of packing to ensure that the solvent is sufficiently viscous and confirm that no significant settling takes place. When an appropriate solvent mixture is identified, the slurry should be prepared using 1–3%w/v mixture of the packing in the solvent and should then be well shaken and examined under a microscope.

4. The slurry must then be well dispersed by ultra sonic-vibration for at least 10 minutes. During the sonic process, a vacuum should be applied to the vessel to degas the slurry. The dispersion should then again be examined by a microscope to ensure no aggregation remains.

5. The column is filled with the slurry solvent and the slurry transferred to the packing reservoir. The following two steps are then carried out as rapidly as possible.

6. The tube from the valve to the reservoir is filled with solvent, the valve closed and the tube connected to the slurry reservoir. A shield should be placed in front of the packing system to protect the operator from any accident.

7. The pump is brought to the packing pressure and the valve rapidly opened.

8. The pump is allowed to force liquid through the reservoir and packed column until a constant flow-rate is attained (usually 5–10 minutes). The valve is then turned off, the pump stopped and the excess pressure in the system allowed to fall to gradually to zero. The column is now packed.

The column should then be disconnected from the slurry reservoir and the top of the column capped with a frit and end fitting. The column can then be connected to the chromatograph, washed free of packing solvent and then finally tested. A chromatogram from a high efficiency column packed in 1 m lengths, and subsequently joined, is shown in figure 9.4.

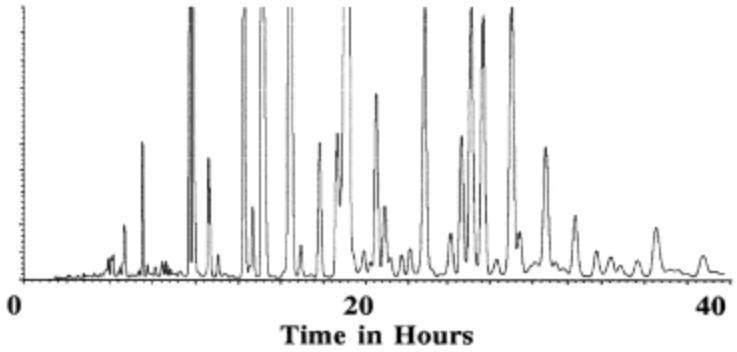


Figure 9.4
Chromatogram of an Aromatic Hydrocarbon Extract from Coal
Obtained from a High Efficiency Small Bore Column

The column was 2 m long, 1 mm I.D., packed with a reverse phase having a particle diameter of 10 µm. The inlet pressure was 6000 p.s.i., the mobile phase was an acetonitrile/water mixture and the chromatogram was developed isocratically. The column efficiency was about 250,000 theoretical plates but it should be noted that the retention time is over forty hours. Unfortunately, very high efficiencies can only be achieved by employing very high pressures or accepting long retention times [5]. In the particular example given, the maximum operating pressure of the sample valve was 6000 p.s.i.

Mechanical Packing Equipment

Slurry packing techniques carried out in the manner described above can produce excellent efficiencies for columns with internal diameters up to about 2 inches. For columns having diameters above 2 in however, it is found that the column performance deteriorates fairly rapidly and voids appear at the top of the column. This is a result of 'bridging' that occurs in the column which normally helps to support the packing but in larger diameter columns is unstable and tends to collapse and cause the packing to settle. The effect of 'bridging' in column packing is depicted diagramatically in figure 9.5.

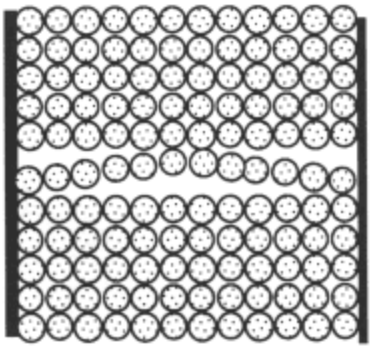


Figure 9.5
The 'Bridging' Effect in a Column Bed

Figure 9.5 shows a 'bridge' of packing particles extending across the column, supported by frictional forces between the terminal particles and the column wall. The formation of these 'bridges' occurs during packing and causes a void to appear on either side of the bridge. Even with the most efficient form of slurry packing, this 'bridging' seems to happen; nevertheless, providing the column is not too wide, the 'bridges' are fairly stable and do not either significantly impair the column performance or diminish the lifetime of the column. However, when the column diameter exceeds about 2 cm the 'bridges' become unstable and can collapse during use, causing the packing to settle and voids to appear at the top of the column. Sometimes the bed only partly settles and the void remains inside the column and there is no physical evidence for the

cause of column deterioration. This effect occasionally happens in analytical columns, but is very common in larger diameter columns that have been slurry packed. The process of 'bridging and mechanical methods of packing, has been discussed in detail by Colin *et al*. [6]. There are two packing procedures that have been developed to obviate the effect of 'bridging' and column deterioration in large bore columns and they are 'Packing by Radial Compression' and 'Packing by Axial Compression'.

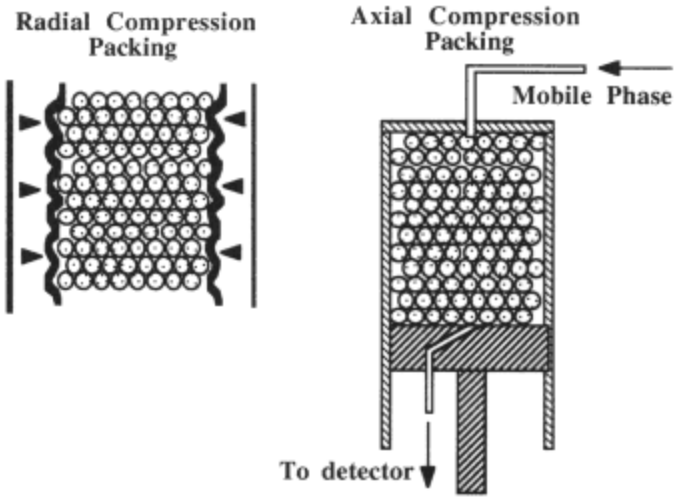


Figure 9.6
Radial and Axial Comprssion Packing Techniques

These two packing techniques can be used for column diameters up to about 10 cm; columns having diameters greater that 10 cm usually employ particles with diameters greater than 20 µm and thus, can be dry packed. The two mechanical packing methods are depicted in figure 9.6.

Radial Compression Packing

Radial compression is achieved by employing a column with a flexible wall situated inside an outer tube of stainless steel. The flexible-walled column must be made of an inert plastic such as PTFE and an hydraulic fluid fills the intervening space between the outside of the flexible

column wall and the inside of the outer case. The column is filled with a thick slurry of the selected packing material, and a pressure is applied to the hydraulic fluid, compressing the packing and expressing the packing fluid. This ensures that the whole of the bed is compressed, and not merely from one end, as it would be with normal slurry packing procedures. This procedure appears to eliminate the bridging that takes place and as a consequence eliminate any column voids and thus prevents column deterioration.

Axial Compression Packing

Axial compression packing employs a cylinder and piston as the chromatography column. The principle of the device is also shown in figure 9.6; the cylinder is filled with a slurry of the selected packing, a frit placed at either end and the top replaced. Frits having a wide diameter will need supporting discs to provide mechanical strength. The piston is then activated by a hydraulic jack, the slurry is compressed and the packing fluid expressed from the tube at the top of the column. This procedure minimizes any bridging that might occur and, as the hydraulic pressure is maintained on the packing during the use of the column, any bed movement that might occur during use is immediately accommodated by compression. As a result, no column voids are formed, either in the packing itself, or at the top of the column. It is of course, important to ensure that the column inlet pressure never exceeds the packing pressure otherwise the reverse would occur and the column would be unpacked. One other important advantage of the method is that the column can be very easily unpacked and the packing material recovered. This is achieved by removing the top of the column and extruding the packing by applying hydraulic pressure to the piston.

There are a number of techniques available to the chromatographer that can be used to pack a column. The best and most appropriate method will depend on the particle size of the packing, the scale of the separation and the character of the material to be separated. However, contrary to popular belief, there is no magic associated with column packing, but the procedure does need a little experimental skill, patience, and some

experience helps. Nevertheless, using the procedures outlined in this chapter it should be possible for an inexperienced chromatographer to pack an LC column and obtain close to the expected efficiency.

Synopsis

Virtually all chiral stationary phases are bonded or coated onto silica gel particles. Silica gel is an amorphous solid consisting of silicon atoms joined by oxygen atoms, on the surface of which are free hydroxyl groups to which other molecules can be chemically bonded. The surface contains various amounts of adsorbed free water that can be removed by heating to an appropriate temperature. The important chromatographic properties of silica gel are determined by its pore size, its surface area and the diameter of the silica gel particles. In most LC analyses, the mean pore size is about 100 Å. The most common particle diameter is 5 µm. In general, the stationary phase is attached to the silica by first bonding a silane compound containing an appropriate reaction group to the silica, and then bonding the molecules of the chiral stationary phase to the attached silane group. The silane group is usually attached to the silica by methoxy groups or chlorine atoms attached to the silane with the elimination of methanol or hydrochloric acid. There are a number of linkages that can be use to attach the stationary phase molecules, some of which are amide, carbamate and urea bonds. Protein phases are often linked to the silane group by peptide bonds and are probably the least stable of all the chiral stationary phases. The small molecular weight chiral molecules (the Pirkle phases) are attached to the silica with carbamate and urea bonds and are far more stable. Cellulose and amylose based stationary phases are usually derivatised with either acid chlorides to produce ester derivatives or isocyanates to produce carbamate derivatives. The derivatized cellulose or amylose is coated onto large pore (650 Å–1000 Å) silica particles and not bonded to them. Cellulose and amylose stationary phases are very effective but can only be used with a restricted range of solvents and at relatively low flow rates due to their mode of attachment. The macrolytic peptides are attached in a similar manner to the protein stationary phases but are far more stable

and can be used with a infinate variety of solvents. These materials are usually reacted directly with an appropriately derivatized silica gel. The cyclodextrins, after derivatization, are usually reacted with a silane reagent and joined *via* an ether bond by using a silane containing an epoxy group. The cyclodextrins, under certain conditions can be linked to the silica by more than one silane chain. These chiral stationary phases are also very stable, can be made by suitable derivatization to have a range of interactive characteristics, and have a wide field of application in chiral chromatography. Columns are slurry packed and usually have diameters of 1, 3, 4 or 4.6 mm and lengths of 3, 5, 10, 15 and 25 cm. For efficient columns, the particles should also have a very narrow range of size distribution. It is important to be certain there is no particle aggregation during packing and this is achieved by the correct choice of solvent or surfactant, which is added to the packing solvent before dispersing the particles. In preparative columns, bridges can occur during the packing process which results in unstable columns and poor efficiency. This effect can be reduced by using radial or axial compression techniques. Column packing is not difficult but needs some experience. Unless dictated by economy, it is more efficient to buy packed columns than to try to pack them in house.

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4. B. L. Karger, K. Conroe and H. Engelhardt, *J. Chromatogr.*, 8(1970)242

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Chapter 10—
Column Temperature and Mobile Phase Composition: Their Effects on Column Length and Analysis Time

The cost of any chromatographic separation is indemnified in two currencies, *pressure* and *time.* In general, the highest pressure that is usable (normally determined by the life expectancy of the sample valve or, under some circumstances, the pump) determines the smallest particle diameter that can be used. In fact, for a given maximum available (or usable) inlet pressure, there will be an optimum particle size that will achieve a given separation in the minimum time. A discussion of this aspect of column optimization is outside the scope of this book, but details are given elsewhere [1].

In any event, today, the particle size of the support is not really a practical variable (hopefully this may change sometime in the future) as it is largely controlled by the stationary phase or column supplier. As a consequence, the column inlet pressure is normally adjusted to that which provides the optimum mobile phase velocity. It should again be emphasized that, due to the dependence of peak dispersion on velocity, if excess theoretical plates are available, the retention time should not be reduced by *increasing* the mobile phase *velocity.* A more efficient method for reducing the analysis time is to reduce the column *length,* while maintaining the same optimum velocity. Increase in mobile phase velocity may be a short term option for unique sample, but for product monitoring or quality control analyses, where there is a large throughput of similar samples, reduction in column length will give shorter analysis

times and be more economically advantageous. At the time of writing this book, the most popular particle diameter used for chiral LC packings appears to be 5 µm. This diameter has been chosen, partly because such particles can be packed into columns having relatively long lengths, and partly because 5 µm particles are easier to pack reproducibly, particularly in columns more than 3 cm in length. It follows, that having arbitrarily defined the particle diameter, and consequently indirectly defined the column inlet pressure (that pressure which gives the optimum velocity), *time* is the only remaining currency that can be utilized for separation efficiency and cost-effectiveness.

The optimum conditions that will separate a given pair of isomers, in the minimum time, can be extremely difficult and tedious to determine in practical chiral LC, although the procedure is possible. This is because the separation ratio *and* the capacity factor of the enantiomer pair changes independently with *both* the solvent composition and the operating temperature. However, before discussing optimizing procedures, some problems that arise in measuring corrected retention times, capacity ratios and separation ratios of solute pairs that are eluted close together, need to be considered.

In order to optimize a separation and produce it in the minimum time, the capacity ratios and separation ratios must be measured for a given pair of enantiomers under known conditions of mobile phase composition and temperature (this will be discussed in detail later in this chapter). Unfortunately, when two peaks are eluted close together, which frequently occurs in chiral chromatography, the positions of the peak maxima are distorted due to the immediate presence of the other peak. An example of this problem is shown in figure 10.1, where the peaks are simulated and added, and the composite envelope plotted over the envelope of each individual peak. It is seen that the actual retention difference, if taken from the maxima of the envelope, will give a value of less than 60% of the true retention difference. Unfortunately, this type of error will probably not be taken into account by most data processing software. It follows, that if such data is used in an attempt to calculate the

necessary efficiency needed to achieve a baseline separation, the results will be grossly in error.

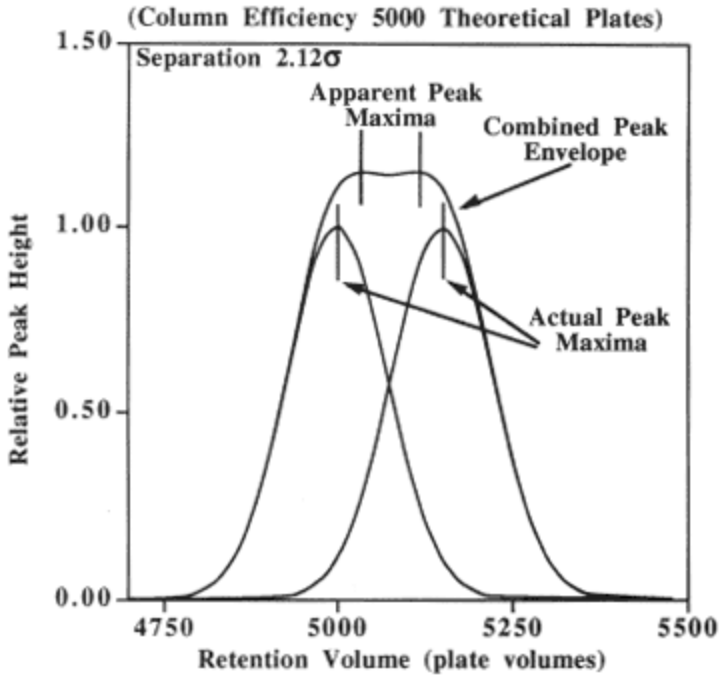


Figure 10.1
Position of the Peak Maxima of Unresolved Peaks

Another serious error can occur if it is known that the two peaks are eluted unresolved, and the retention time of the maximum of the envelope is taken as the mean retention time of the two isomers. This measurement can only be true if the peaks are *absolutely symmetrical* and the two isomers are present in the *same proportion.* The effect of different proportions of each isomer on the retention time of the composite envelope is shown in figure 10.2. It is seen that the position of the peak maximum of the composite envelope is significantly different from the mean of the retention times of the individual peaks. Furthermore, this is an ideal case where the peaks are considered to be truly Gaussian in shape and are completely symmetrical. Asymmetric peaks could distort the position or the peak maxima of the envelope even

more. In general, the retention time of a composite peak should never be assumed to have a specific relationship with the individual retention times of the unresolved pair.

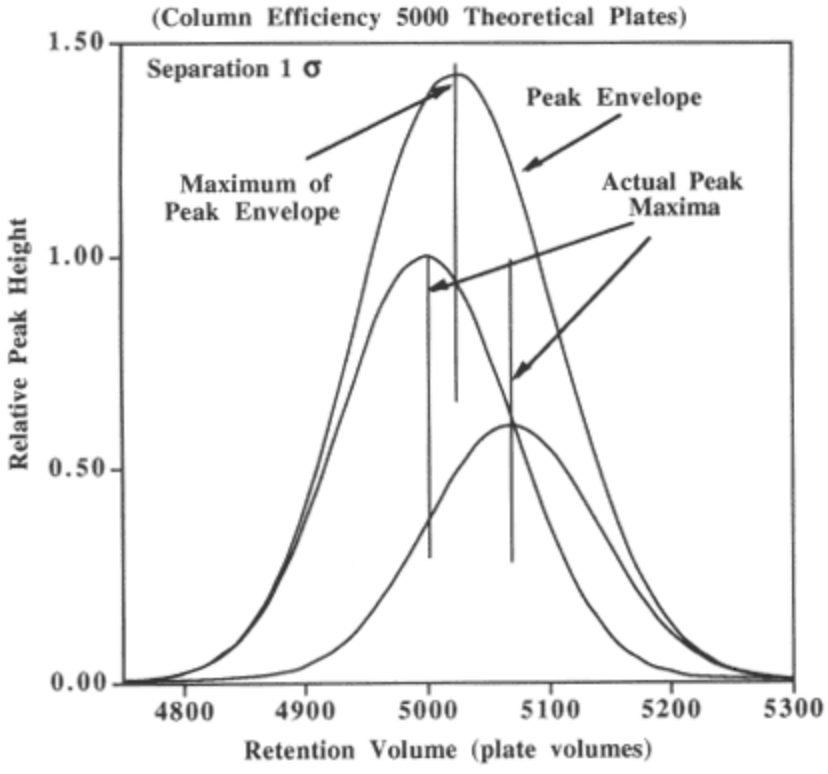


Figure 10.2
The Maximum of a Composite Peak Formed by Two Closely Eluting Peaks of Different Magnitude

Another error can arise when the two partially resolved peaks are asymmetrical (*e.g.* peaks that are described by the Poisson equation). An example of this situation is shown in figure 10.3. It is clear that there are two errors that occur. Firstly, the retention times, as measured from the peak envelope, will not be accurate for each isomer. Secondly, because the peaks are asymmetrical (and most LC peaks tend to be asymmetrical to the extent shown in the figure 10.3) the second peak appears higher

than the first, implying that the second eluted isomer is present in the mixture at a higher concentration.

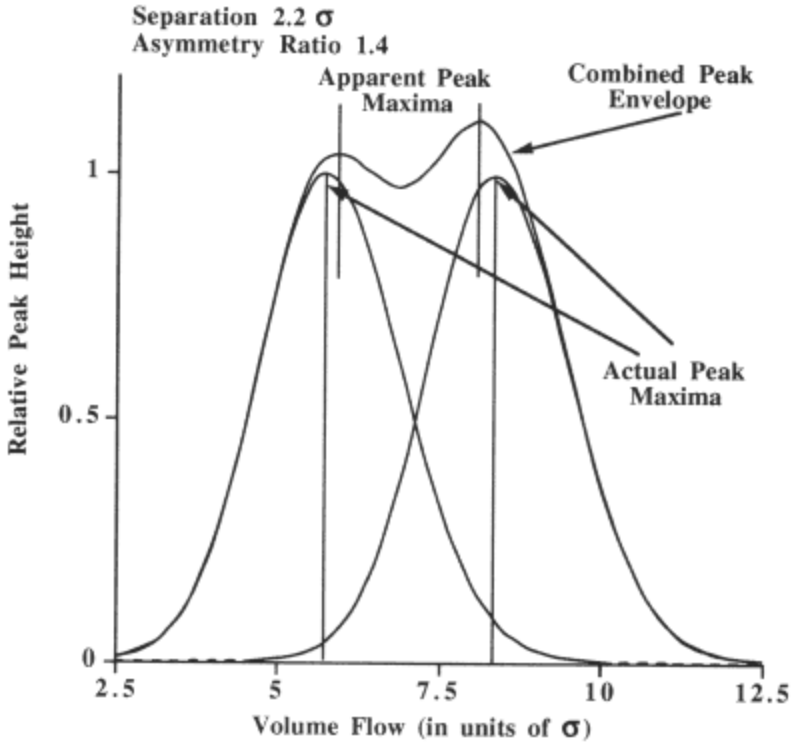


Figure 10.3
The Effect of Peak Asymmetry on The Apparent Composition of a Closely Eluting Pair of Solutes

It is clear that considerable care must be taken when assessing closely eluting peaks. *In fact, if the resolution is inadequate, the measurements must be taken on the individual isomers and chromatographed separately on the column.* It is important to know the value of the separation ratio above which, accurate measurements can be made on the peak maxima of the individual peaks in the envelope. In figure 10.4, the apparent peak separation ratio is shown, relative to the actual peak separation, in standard deviation units (σ) for columns of different efficiency. These have been theoretically calculated. It is seen that for a low efficiency

column (2500 theoretical plates) the separation ratio must be greater than about 1.055 before accurate measurements can be made on peak maxima in the composite curve. For a high efficiency column having 10,000 theoretical plates, the separation ratio need only be in excess of about 1.035, before accurate measurements can be made from peak maxima on the composite curve. These minimum values, however, assume that the measurements will be precise. Unfortunately, the measurements may have a random error standard deviation of 2–3%. As a consequence, the practical minimum separation ratio that can be used for accurate measurements will be proportionally greater than those calculated.

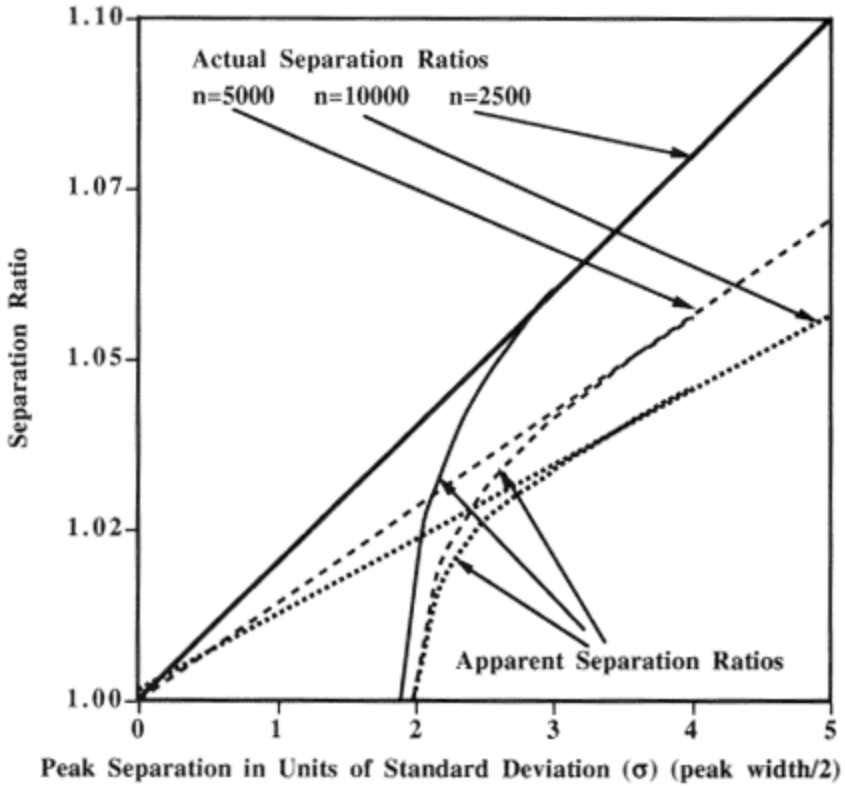


Figure 10.4
Graphs Showing Apparent Separation Ratio Relative to Actual Separation in Units of (σ) for a Pair of Closely Eluting Peaks

The calculation of optimum column conditions for a chiral separation depends on the accurate measurement of retention data of the individual peaks over a range of temperatures and solvent composition. It follows, that if resolution is poor, the data *must be* obtained from the elution of *single enantiomers.*

An Optimization Procedure for a Chiral Separation

The optimization procedure depends on identifying the function of solvent composition and temperature that will provide a precise value for the capacity ratios of the two solutes. It will then be possible, using a relatively simple computer program, to identify the various operating parameters of the chromatographic system that will provide the separation in the minimum time. In the example given, a separation system operating in the normal mode will be used.

A somewhat similar procedure can be described, using the reversed phase mode, but it is more complex, as aqueous mixtures almost always include association of water with the other solvent. This makes the binary mixture of solvent and water, a ternary mixture, consisting of the water, the solvent and solvent associated with water. Treatment of this model requires the use of the equilibrium constant for the water solvent associate and requires some physical chemical measurements to be made.

Consider the separation of two enantiomers (R) and (S). If the enantiomers (R) and (S) have corrected retention volumes of (V'_R) and (V'_S) respectively and a separation ratio of (α) when eluted by a binary mixture of two solvents (X) and (Y), the corrected retention volumes can be described by:

$$V'_{(R)} = f_R(T, X_\varphi) \qquad (1)$$

and:

$$V'_{(S)} = f_S(T, X_\varphi) \qquad (2)$$

where (T) is the column temperature

and ($X\varphi$) is the volume fraction of solvent (X)

and the capacity factors can be defined as:

$$(k_R) = f_R(T,X_\varphi)/V_o \quad \text{and} \quad (k_S) = f_S(T,X_\varphi)/V_o \qquad (3)$$

In addition:

$$\alpha = \frac{f_S(T,X\varphi)}{f_R(T,X\varphi)} \qquad (4)$$

Recalling the equation for the number of theoretical plates that are required to effect a given separation from chapter 5, [2]:

$$n = 16\frac{(1+k)^2}{k^2(\alpha-1)^2} \qquad (5)$$

This equation presupposes that the peak maxima are separated by (4σ) (four standard deviations of the Gaussian curve, and that both peaks have the same efficiency). It also assumes that this resolution is adequate for the analytical accuracy required. If a larger separation is necessary, then the factor 16 must be appropriately increased. Thus, substituting for (k) and (α):

$$n = 16\frac{(V_o + f_R(T,X\varphi,))^2}{f_R(TX\varphi)^2\left(\frac{f_S(T,X\varphi,)}{f_R(T,X\varphi,)}-1\right)^2}$$

$$n = 16\frac{(V_o + f_R(T,X\varphi,))^2}{(f_S(T,X\varphi,) - f_R(T,X\varphi,))^2} \qquad (6)$$

Now, the minimum value of (H) (the *variance per unit length* of the column or the *height of the theoretical plate*) has been shown to be given by the approximation:

$$H_{min} = 1.45\,d_p$$

which for a 5 µm particle, H_{min} (in cm) will be:

$$H_{min} = 1.45 \text{ x } 0.0005 = 0.00072\,cm$$

Thus, the column length (L) will be given by:

$$L = nH_{min} = 0.00072 \times 16 \frac{(V_o + f_R(T, X\varphi,))^2}{(f_S(T, X\varphi,) - f_R(T, X\varphi,))^2}$$

$$= 0.0115 \times \frac{(V_o + f_R(T, X\varphi,))^2}{(f_S(T, X\varphi,) - f_R(T, X\varphi,))^2} \qquad (7)$$

Now, the separation time (t) is given by:

$$t = \frac{L}{u_{opt}}(1+k_L) = \frac{nH_{min}}{u_{opt}}(1+k_L)$$

Now, it was shown on page 227, equation (9), that:

$$u_{opt} = \frac{1.62\,Dm}{d_p}$$

Thus:

$$= \frac{1.45\,n\,d_p}{\frac{1.62\,Dm}{d_p}}(1+k_L) = \frac{0.895\,n\,d_p^2}{Dm}(1+k_L)$$

where (k_L) is the capacity ratio of the last eluted peak and which may, or may not, be the second peak of the chiral pair.

Substituting for (n) and simplifying:

$$t = 14.3 \frac{(1+k)^2(1+k_L)}{D_m k^2 (\alpha - 1)^2} d_p^2$$

Now, as the mobile phase is a liquid, (D_m) will have a value within the range of 1.25×10^{-5} cm^2/s to about 2.75×10^{-5} cm^2/s thus, taking a mean value of 2.00×10^{-5} cm^2/s for (D_m), and (d_p) as 5 µm, an approximate value for (t) will be given by:

$$t = \frac{0.179(1+k)^2(1+k_L)}{k^2(\alpha - 1)^2}$$

Replacing (k) and (α) by their thermodynamic expressions,

$$t = \frac{0.179(V_o + f_R(T, X\varphi,))^2 (V_o + f_L(T, X\varphi,))}{(f_S(T, X\varphi,) - f_R(T, X\varphi,))^2} \qquad (8)$$

It must be emphasized that the minimum analysis time will be achieved only at those values of (T) and (X_φ) for which the expression for (t) is a minimum and which will need to be ascertained with the use of a computer.

Now, the algebraic forms of $f_R(T)$ and $f_S(T)$ in the original expressions are well known and can be precisely defined in thermodynamic terms.

$$V'_R = f_R(T) \quad \text{and} \quad V'_S = f_S(T)$$

In contrast, however, the algebraic forms of $f_R(X_\varphi)$ and $f_S(X_\varphi)$ in the expressions:

$$k_R = f_R(X_\varphi)/V_o \quad \text{and} \quad k_S = f_S(X_\varphi)/V_o$$

are *not known precisely* (although approximations have been suggested) and, even if they were, the relationships,

$$k_R = f_R(T) f_R(X_\varphi) / V_o$$

and

$$k_S = f_S(T) f_S(X_\varphi) / V_o$$

have certainly not been confirmed in chiral chromatography, and cannot at this stage be assumed.

If, however, (V'_R) and (V'_S) can be measured for practical ranges of (T) and (X_φ), then the separation ratios, the required number of theoretical plates and the column length that will provide the minimum analysis time, can also be calculated, and an approximate value for the minimum

analysis time also assessed. The thermodynamic function relating (V') and (l/T) can be used with confidence, but it must be emphasized that due to the small values of (α) that are usually encountered in chiral LC, an overall approximation for $f_R(X_\varphi)$ and $f_S(X_\varphi)$ *cannot* be used. Consequently, in order to carry out an optimization procedure with any degree of confidence, the nature of $f_R(X_\varphi)$ and $f_S(X_\varphi)$ for the particular enantiomer pair of interest must be determined (albeit by a curve fitting procedure to *accurately* measured retention data). This procedure can be lengthy and tedious and unless carried out with great accuracy will be of little value. Nevertheless, if the laboratory sample load is high enough, and the analysis time is important, then the procedure will be economically advantageous.

The Experimental Determination of $f_R(X_j)$, $f_S(X_j)$, $f_R(T,X_j)$ and $f_S(T,X_j)$

The chromatographic system chosen to examine was the chiral stationary phase Chirobiotic V (as already described, this is a macrocyclic glycopeptide called Vancomycin bonded to silica gel particles 5 µm in diameter) which was used in the normal phase mode (solute retention controlled largely by polar interactions), employing mixtures of hexane and ethanol as the mobile phase.

The solutes chosen were (S) and (R) 4-benzyl-2-oxazolidinone. The apparatus consisted of an LC pump, a 1 µ1 internal loop sample valve and the column was situated in a thermostatically controlled oven. The oven could be operated at temperatures between 5°C and 85°C with a precision of ±0.2°C. The mobile phase was passed through a preheater, consisting of 1 m length of coiled stainless steel tube, 0.010 in. I.D., situated in the thermostat, between the sample valve and the column. The column outlet was connected directly to a UV detector operating at 220 nm. The eluent from the detector was passed to a 50 ml Grade A burette and the retention volumes were measured directly in ml of mobile phase. The burette was read with an accuracy of 0.02 ml. The output of the detector was displayed on a digital meter and an electronic recorder/integrator.

The experimental procedure was as follows. The burette was read, the sample injected and the pump started. The detector output was observed by the digital meter and when a peak emerged the pump was shut off at the peak maximum. The system was allowed half a minute to reach equilibrium and the burette read again. The difference between the readings was taken as the retention volume being measured. The dead volume was taken as the elution volume of ethanol, the sample solvent.

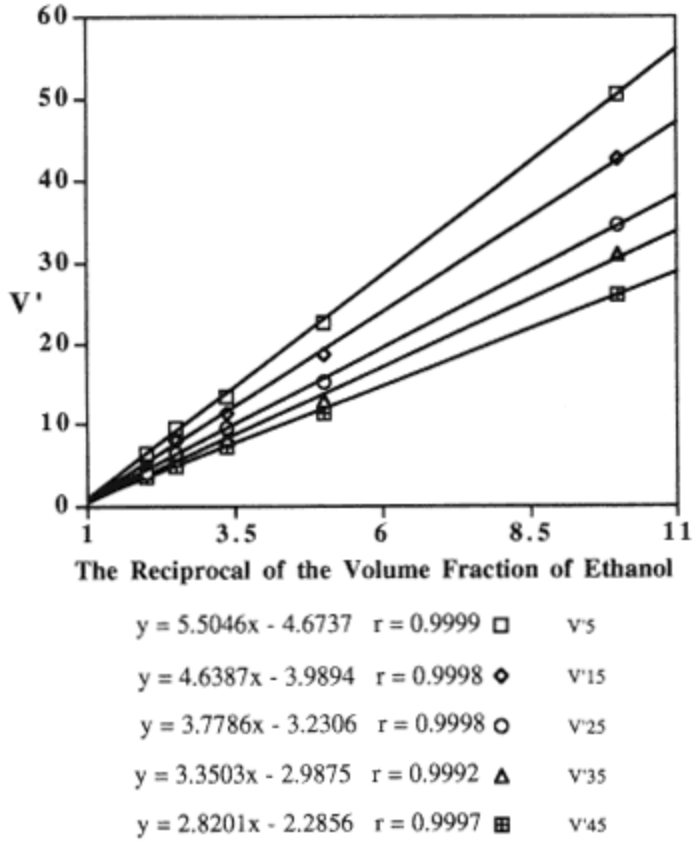
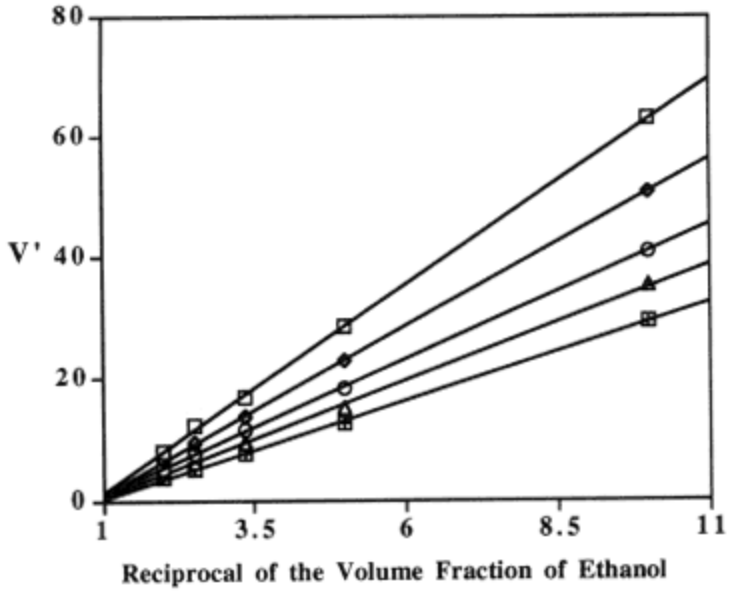


Figure 10.5
Graph of Corrected Retention Volume of the (R) Isomer/Reciprocal
of the Volume Fraction of Ethanol

The retention volume and the dead volume were measured in duplicate at 0.1, 0.2, 0.3, 0.4 and 0.5 volume fractions of ethanol in hexane and at

5°C, 15°C, 25°C, 35°C and 45°C at each mobile phase composition. If the duplicates differed by more than 3% then a third measurement was taken. The results obtained, expressed as corrected retention volume against the reciprocal of the volume fraction of ethanol, are shown in figures 10.5 and 10.6 for the (R) and (S) isomers respectively.



y = 6.7778x - 5.3171 r = 0.9998

y = 5.4855x - 4.3875 r = 0.9999

y = 4.4093x - 3.4047 r = 0.9998

y = 3.8055x - 3.2343 r = 0.9995

y = 3.1736x - 2.7196 r = 0.9997

Figure 10.6
Graph of the Corrected Retention Volume of the (S) Isomer/the
Reciprocal of the Volume Fraction of Ethanol

The relationship between the corrected retention volume and the reciprocal of the volume fraction of one component of the mobile phase is fairly well established but does not seem to be generally employed. This relationship stems from the pioneering work of Purnell and his

group[3], who showed that the retention of a solute on a mixed phase in GC was proportional to the volume fraction of one component in the *stationary phase* mixture. This was supported by the work of Scott [4], who showed that the retention of a solute in LC was inversely proportional to the volume fraction of one solvent in a *mobile phase* containing a binary mixture of dispersive solvents. Scott and Kucera [5], explained this on the basis that, in both cases, the volume fraction of a given solvent, set the probability of a solvent molecule interacting with a solute molecule, in much the same way as the partial pressure of a gas determined the probability of a molecular collision with another molecule of gas. These papers aroused considerable controversy [6] but the results were further substantiated by the work of Scott and Simpson [7], Laub and Purnell [8], Hurtubise *et al*. [9] and Robbins and McElroy [10]. As stated this simple relationship *only applies* if the components of the mobile phase *do not strongly interact* and *form an associate*. The published relationships between corrected retention volume, (k) and the distribution coefficient have been reviewed by Laub [11,12].

The results in figures 10.5 and 10.6 show the relationship between volume fraction of solvent (v) and the corrected retention to be as follows.

$$V' = \frac{A_R}{v} - B_R \quad (9A) \qquad V' = \frac{A_S}{v} - B_S \quad (9B)$$

The equations (9A) and (9B) are the functions $f_R(X_\varphi)$ and $f_S(X_\varphi)$, respectively. Now, if Log(A_R) and Log (A_S) for each temperature are plotted against 1/T then the van't Hoff isotherms shown in figure 10.7 are obtained. It is seen that excellent straight lines are obtained in accordance with that expected from thermodynamic theory. In a similar way the intercepts from figure 10.5 and 10.6 can be plotted against 1/T and the results obtained are shown in figure 10.8.

Although a linear relationship is obtained, the correlation coefficient is not high. However, as the contribution of the intercept to V' is relatively small, the equation for the intercept will be within the 3% limit of the experimental measurements.

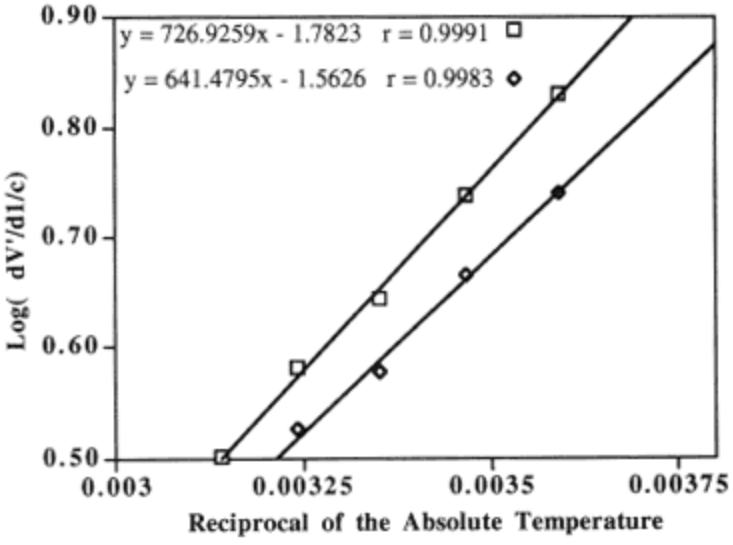


Figure 10.7
Graph of d(V')/d(1/Vol.Fraction) from the V'/1/ Volume Fraction of Ethanol Curves against the Reciprocal of the Absolute Temperature

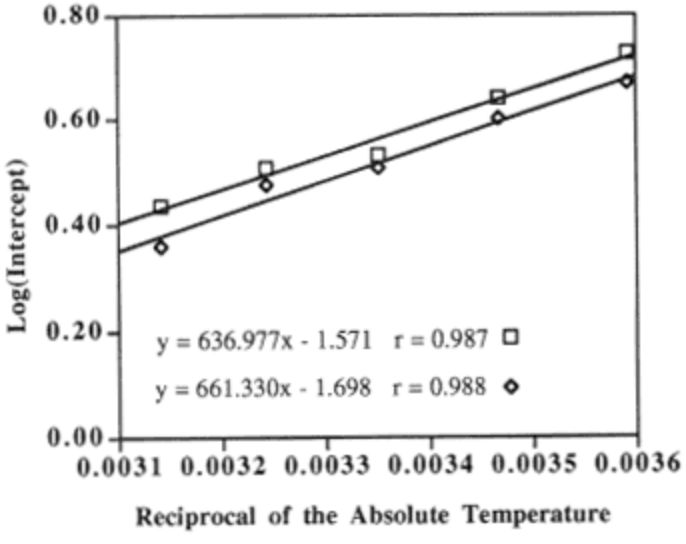


Figure 10.8
Graph of Log(Intercept) from the V'/1/ Volume Fraction of Ethanol Curves against 1/T

It is seen that the intercept can also be described by an exponential function of the reciprocal of the absolute temperature.

Thus:

$$B_R = e^{\frac{F_R}{T} - G_R} \quad \text{and} \quad B_S = e^{\frac{F_S}{T} - G_S}$$

It is now possible, from the experimental data, to define (V') for either solute at any temperature any solvent composition.

From equation (9):

$$V_R' = \frac{A_R}{v} - B_R \quad \text{and} \quad V_S' = \frac{A_S}{v} - B_S$$

It has already been shown that:

$$A_R = e^{\frac{C_R}{T} - D_R} \quad \text{and} \quad A_S = e^{\frac{C_S}{T} - D_S}$$

Thus:

$$V_R' = \frac{e^{\frac{C_R}{T} - D_R}}{v} - e^{\frac{F_R}{T} - G_R} \quad \text{and} \quad V_S' = \frac{e^{\frac{C_S}{T} - D_S}}{v} - e^{\frac{F_S}{T} - G_S}$$

and (k_R) and (k_S) will be

$$k_R = \frac{\frac{e^{\frac{C_R}{T} - D_R}}{v} - e^{\frac{F_R}{T} - G_R}}{V_o} \quad \text{and} \quad k_S = \frac{\frac{e^{\frac{C_S}{T} - D_S}}{v} - e^{\frac{F_S}{T} - G_S}}{V_o} \qquad (10)$$

and:

$$\alpha = \frac{\frac{e^{\left(\frac{C_S}{T} - D_S\right)}}{v} - e^{\left(\frac{F_S}{T} - G_S\right)}}{\frac{e^{\left(\frac{C_R}{T} - D_R\right)}}{v} - e^{\left(\frac{F_R}{T} - G_R\right)}} \qquad (11)$$

The data to be used in calculating the necessary operating conditions are obtained from figures 10.7 and 10.8 and are tabulated in Table 10.1.

Table 10.1 Basic Data for Optimization

Symbol	Data	Symbol	Data
C_S	726.93	C_R	641.48
D_S	-1.782	D_R	-1.571
F_S	636.98	F_R	661.33
G_S	-1.563	G_R	-1.698

Dead Volume V_o = 2.785 ml

Using the experimental data given in table 10.1, the expressions given in equations (10) and (11) can be put into explicit form:

$$k_S = \frac{e^{\frac{726.93}{T} - 1.782}/v - e^{\frac{636.98}{T} - 1.563}}{2.785} \qquad (12)$$

$$k_R = \frac{e^{\frac{641.48}{T} - 1.571}/v - e^{\frac{661.33}{T} - 1.698}}{2.785} \qquad (13)$$

$$\alpha_{S/R} = \frac{e^{\frac{726.93}{T} - 1.782}/v - e^{\frac{641.48}{T} - 1.563}}{e^{\frac{641.48}{T} - 1.571}/v - e^{\frac{661.33}{T} - 1.698}} \qquad (14)$$

Equations (12), (13) and (14) allow the values of (k_S), (k_R) and (αSR) to be calculated at any temperature and any solvent composition. These parameters will allow the efficiency necessary for resolution to be calculated and from that, the column length and the analysis time can also be determined. Using equations (12) and (13) the corrected retention volumes were calculated for the data points experimentally measured and the calculated values are shown plotted against the theoretical values in figure 10.9. It is seen that excellent agreement is obtained with a slope of 1.0008 and a small intercept of 0.042. This validates both the theoretical

argument and the equation, so they can be used with confidence in column design.

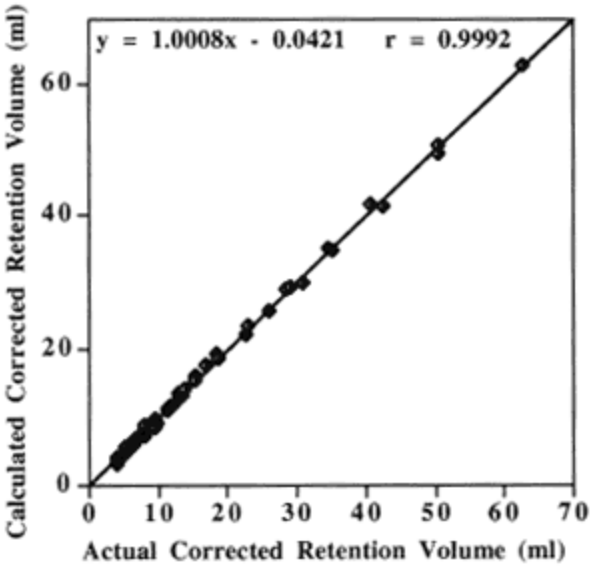


Figure 10.9
Graph of Calculated Corrected Retention Volume Values
against those Experimentally Measured

The change in the (k) with solvent composition and temperature can be easily examined and by using a simple program (in this case written in 'True Basic' language) and equations (12) and (13), the inversion of the elution order of the two isomers can be predicted. It is clear, that if the straight lines produced by plotting Log (V') against 1/T for the isomers have different slopes, then they must intersect, and, after intersection, the elution order will be reversed.

The curves relating the corrected retention volume of the two isomers to the reciprocal of the absolute temperature is shown in figure 10.10. It is seen that the reversal occurs at about 120°C. In many examples, the temperature at which the reversal of the elution order occurs, may be out of practical range. Unfortunately, this was true for this particular example due to the thermal instability of the stationary phase above *ca*

85°C. In addition, most bonded phases loose their chiral selectivity at about 86°C and even decomposed at more elevated temperatures

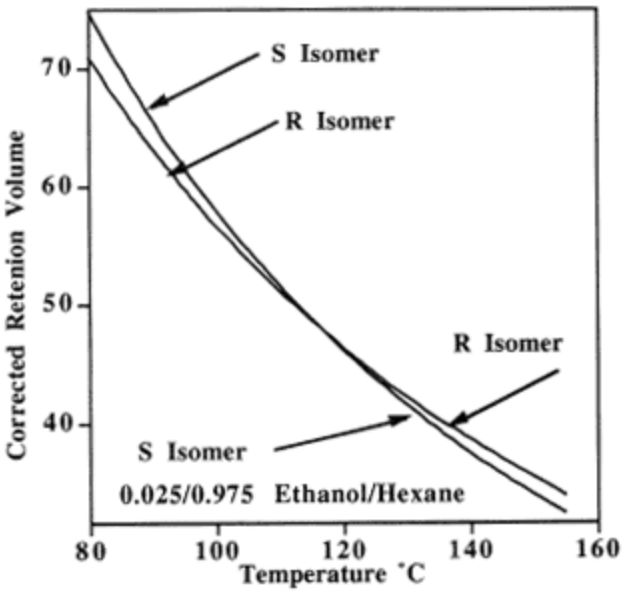


Figure 10.10
Curves Relating the Corrected Retention Volume of the Two Isomers to Temperature Showing Elution Order Reversal

Consequently, the reversal shown in figure 10.9 is difficult to experimentally confirm. In contrast, the GC stationary phases are much more stable, and the reversal of elution order is more easily demonstrated. However, at the time of writing this book, a paper was published by Karlsson and Aspergren [13], that displayed curves similar to those shown in figure 10.10 and which also predicted elution order reversal but, within the normal operating temperature range employed in LC. In fact, the authors showed that the order of elution of the two enantiomers of Mosapride (a new drug for gastrointestinal treatment) could be reversed by either changing the temperature or changing the mobile phase composition. The curves relating log(k') against the reciprocal of the absolute temperature for the Mosapride enantiomers are

shown in figure 10.11. The separation was carried out on a Chiral-AGP column with acetonitrile/phosphate buffer (pH 6.9) : 92/8 v/v. The inversion temperature is about 24°C and it is clear that, if the particular separation had been carried out solely at room temperature, and the effect of temperature on resolution had not been examined, it could have been concluded that the particular stationary phase showed no selectivity towards that particular pair of enantiomers.

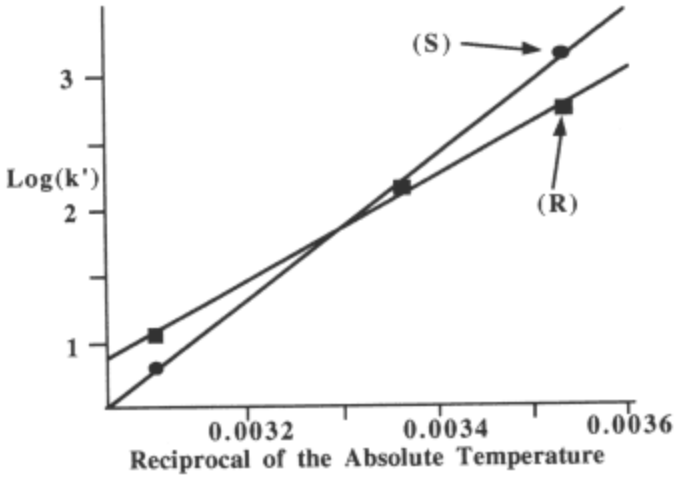


Figure 10.11
Graph Relating Log(k') against 1/T for the Enatiomers of Mosapride
Courtesy of Chromatographia [Ref. 11]

A simple computer program written in 'basic' can be used to identify the optimum column length and other operating conditions. A copy of the program used in this study is shown in figure 10.11. The program is written in an extended manner to simplify explanation. Initially, the retention volume of both isomers need to be measured at three widely dispersed mobile phase compositions, and each at three widely dispersed column temperatures. The dead volume is taken as the retention volume of the solvent peak (the sample being made up in one pure component of the mobile phase). All measurements must be very accurate and made in duplicate.

```
10 LET C2=726.93
20 LET D2=1.7823
30 LET F2=661.33
40 LET G2=1.698
50 LET C1=641.48
60 LET D1=1.5626
70 LET F1=636.98
80 LET G1=1.571
90 LET V=2.785
100 Let S=1000000000000000
110 For J=0.025 to 1.0 step 0.025
120 For I= 1 to 85 step 1
130 Let I1=I+273.4
140 let K2=((10^((C2/I1)-D2))/J)-10^((F2/I1)-G2)/V
150 let K1=((10^((C1/I1)-D1))/J)-10^((F1/I1)-G1)/V
160 Let A=K2/K1
170 Let N=16*((1+K1)^2)/((K1^2)*(A-1)^2)
180 Let L=N*0.00072
190 LET T=L/0.045
200 IF T>S THEN 290
210 Let S=T
220 let H1=K1
230 Let H2=K2
240 Let H3=A
250 Let H4=J
260 Let H5=I
270 Let H6=N
280 Let H7=L
290 Next I
300 Next J
310 Print H4,H5
320 Print H1,H2,H3
330 Print S,H6,H7
340 END
```

Figure 10.12
Program for Examining Chromatography Retention Data

From the data, graphs similar to figures 10.5, 10.6, 10.7 and 10.8 need to be constructed. The slopes and intercepts from the graphs equivalent to those given in figures 10.5 and 10.6 are used to construct graphs equivalent to those given in figures 10.7 and 10.9. The slopes and

intercepts from the graphs equivalent to figures 10.7 and 10.9, are noted and used in the program. C1 and C2 are the slopes D1 and D2 the intercepts from figure 10.7. F1 and F2 are the slopes and G1 and G2 the intercepts from figure 10.8. Enter the dead volume in line 90. Enter in line 110 the concentration range of solvent that is to be considered, in terms of volume fraction and the step between each concentration. Similarly, enter in line 120 the temperature limits and temperature step in °C. Line 130 changes the temperature to absolute values, lines 140 and 150 calculate the capacity factors of the two enantiomers and line 160 the separation ratio. Lines 170, 180, 190, calculate the necessary efficiency (to separate the enantiomers by 6σ), the column length, and the separation time (the second eluted enantiomer) respectively. Lines 200 to 280 contain a simple logic sequence that selects the conditions that provide the minimum analysis time. Lines 290 and 300 complete the search loops and lines 310 to 330 instruct the computer to printout, the solvent composition and temperature that will provide the separation in the minimum time. They also instruct the computer to print out the capacity factors of the two enantiomers, the separation ratio at the optimum conditions, together with the necessary column efficiency and the column length.

The separation chosen to examine is not difficult as, with the best combination of temperature and solvent composition, a fairly high separation ratio can be obtained, and furthermore the high separation ratios are obtainable at relatively large capacity ratios. The results, printed on the computer screen by the program, for the separation examined are shown in table 10.2.

Due to the simplicity of the separation in pure ethanol at 1°C, the number of theoretical plates necessary is very small and the column length, 0.25 cm, is so short, it would be virtually impossible to construct and operate. It follows that a practical column, (probably the smallest will be 3 cm long) would be used at a higher flow rate. However, reducing the analysis time in this way, is unlikely to shorten the minimum time as calculated, unless smaller particles were used which would then constitute an entirely different system. A 3 cm column, if used for the above

separation, would provide 4170 theoretical plates, which, under optimum temperature and solvent conditions, is widely in excess of that required.

Table 10.2 Chromatographic Specifications for the Separation of the Enantiomers of 4-Benzyl-2-Oxazolidinone

Optimum Solvent Composition (J)	Pure Ethanol
Optimum Temperature (I)	1°C
Capacity Factor of 1st Enantiomer (k_R)	3.938
Capacity Factor of 2nd Enantiomer (k_S)	5.510
Separation Ratio($\alpha_{S/R}$)	1.399
Analysis Time	5.68 s
Required Theoretical Plates	355
Column Length	0.256 cm

Operating at the optimum mobile phase velocity, a 3 cm column would require an analysis time of 66.5 s. This would still be a fairly fast analysis and, if desired, could be further reduced, without impairing the required resolution, by operating at higher velocities.

In many chiral separations, it is often a struggle to adequately resolve the enantiomers and achieve adequate separation ratios. In such cases, the above procedure may be essential to achieve the minimum analysis time. In addition, the time would be much extended, the column longer, and the efficiency that was needed, would be much greater. The same type of approach can be used for reversed phase systems employing water which, if in a binary mixture, will associate with the other solvent and produce a ternary mixture. Unfortunately, the procedure becomes extremely involved and its discussion is more appropriate to a text on chromatography theory and, consequently, is outside the scope of this book.

Synopsis

The currencies used to account for a chromatographic separation are pressure and time. The maximum pressure is limited by the tolerance of the sample valve and the capability of the pump. The analysis time can be

reduced by increasing the linear velocity (if excess column efficiency is available) or by reducing the column length, the latter being the more efficient. Optimizing a chiral separation can be tedious, due to the amount of the retention data that must be collected, and the precision necessary for its measurement. However, it is easier to optimize a separation that is developed in the normal phase mode, than it is in the reverse phase mode. This is due to the association of water with polar solvents producing an additional component in the solvent mixture. Taking retention data from closely eluting peaks should be avoided as the data can be significantly distorted by the immediate presence of the other peak. Depending on the column efficiency, data *should not* be collected from peaks with separation ratios of less than 1.05. In the normal phase mode, solute retention is inversely proportional to the volume fraction of the polar solvent in the mobile phase. In addition, the effect of temperature on retention is predicted by thermodynamic theory. Consequently, taking retention data over a range of solvent compositions and temperatures, permit an explicit equation to be developed, that will predict retention for any solvent mixture at any temperature. Relating experimental measurements to those calculated shows excellent correlation and very little divergence. As the slopes of the log(k) curves against the reciprocal of the absolute temperature differ for each enantiomer, they must intersect at a specific and unique temperature. It follows, that two enantiomers will be resolved at temperatures either side of that for coelution, but their elution order will be reversed. Unfortunately, higher temperatures are not often a practical option in LC, due to the inherent thermal instability of the stationary phase. A simple computer program can calculate the capacity ratios of the two isomers, their separation ratio, the optimum column length, operating temperature and solvent composition for minimum analysis time. The minimum data for this calculation must be obtained at three temperatures for three different solvent mixtures.

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Chapter 11—
Chiral Liquid Chromatography Applications

Each of the five main types of LC chiral stationary phase have fairly wide areas of application. As a result, there is much overlap in the use of the different stationary phases, and many specific chiral separations can be accomplished by any one of the five stationary phases, providing the optimum operating conditions for the particular stationary phase are identified. Probably the most important applications come from the pharmaceutical and biochemical industries and, to some extent, from the clinical laboratories. The other important application source for chiral chromatography is the essential oil industries, but, as most of the components of essential oils are, by definition, fairly volatile the applications from this source are usually separated by GC.

Another aspect of chiral separations must also be borne in mind when discussing applications, and that is the importance played by the contemporary high efficiencies that are now available from open tubular columns and columns packed with very small particles. Even with the present day technologies, separation ratios may still be relatively small and separationns are achieved largely by the high efficiencies available. It is interesting to note, that the first separation of *m*- and *p*-xylene was achieved by GC in 1958, on the stationary phase polyethylene glycol with no special spatial orientation. However, this was only possible because a packed column having 50,000 theoretical plates was available.

Chiral separations are a technical challenge because the enantiomers are chemically and physically very similar, and most chiral pairs show

relatively small separation ratios on all types of column, including those that are chirally selective. In practice, a chiral separation is achieved, firstly, by choosing a distribution system which gives the greatest possible separation ratio between the isomers (*i.e.* a stationary and mobile phase combination that exhibits maximum chiral selectivity) and, secondly, using the phase system in conjunction with a column having sufficient efficiency to attain resolution. Chiral chromatography has universal applicability, can be carried out with relatively simple and well established apparatus and, as a consequence, has become a 'global' analytical technique.

If efficiencies could be increased sufficiently, perhaps by using particles 1 μm in diameter, and techniques were developed to efficiently and reproducibly pack them, a single chiral stationary phase might be all that is necessary to separate the majority of enantiomeric mixtures. Such a system is, no doubt, part of the future. It must, however, be preempted by pumps and sample valves that can provide and tolerate the necessary high pressures. In addition, there must be detectors with sufficient sensitivity to furnish an adequate linear dynamic range of response to accommodate the inherent low loading capacity of such columns.

Due to the catholic performance of the different types of stationary phase, several examples of the use of each type will be given. Each type, however, will not be identified with a particular industry or application.

The Protein Stationary Phases

The protein stationary phases were the first types to be developed and are still used very effectively for the separation of chiral mixtures. An interesting example of the use of an α_1-acid glycoprotein column for the separation of the Verapamil enantiomers is afforded by the work of Rasymas *et al*. [1]. Verapamil is a calcium channel antagonist and is used extensively as an antianginal and antihypertensive agent. In addition, due to its negative dromotropic capacity, it is used as an antiarrythmic agent to control supraventricular tachhyarrythmias. It is used as a racemic

mixture, but it has been shown that the two enantiomers have different pharmacokinetic and pharmacodynamic characteristics. *In-vitro* and *in-vivo* studies have indicated that the (R)-(+) enantiomer has a greater effect on the atrioventricular conduction than does the (S)-(-) enantiomer. Further investigations in humans has suggested that the (S)-(-) enantiomer has greater plasma clearance and lower bioavailability than the (R)-(+) enantiomer.

The determination of the enantiomers in blood serum was carried out in two stages. The first stage is to isolate and collect the racemic mixture from the serum. The second stage, was to resolve the collected racemate and to quantitatively determine the enantiomers present. Serum (1 ml) was added to 50 µl of a standard containing 200 ng of Imipramine and 400 µl of 5 N caustic soda. The mixture was shaken and 5 ml of *t*-butyl ether added and the mixture shaken for a further 10 min. The mixture was then centrifuged for 10 min at 1200 *g* and the aqueous layer frozen in a bath of solid CO_2 and acetone. The ether layer was decanted from the container and evaporated to dryness at 45°C under a stream of filtered air.

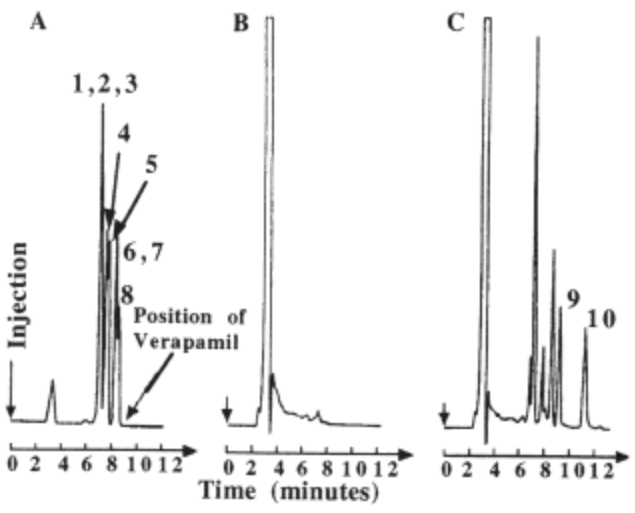


Figure 11.1
Separations Achieved in Stage 1 of the Analysis of Verapamil
Courtesy of J. Liquid Chromatogr. Ref. (1)

The residue was taken up in 50 µl of methanol, and the total volume injected onto the column. The results obtained are shown in figure 11.1. A Dynamax Microsorb CN column, 25 cm long, 4.6 mm I.D, packed with particles 5 µm in diameter, was used with a mobile phase containing tetrahydrofuran/acetonitrile/0.3 M sulfuric acid : 15/84.6/0.4 v/v/v at a flow rate of 1 ml/min. In figure 11.1, the first chromatogram (A) shows the separation of 8 metabolites of Verapamil and the position of the elution of Verapamil, if it had been present. There is obviously no interference. The second chromatogram (B) is of an extracted drug-free predose serum. The third chromatogram (C) is from a serum sample taken 4 hr after a 120 mg oral dose of Verapamil hydrochloride. Peaks (9) and (10) are for Verapamil and the internal standard Imipramine respectively. The eluate containing the Verapamil isomers was concentrated on a rotary evaporator at 37°C for 10 min. in a stream of filtered air. The tube was rinsed with 200 µl of acetonitrile, again concentrated and the residue taken up in 50 µl of double distilled water.

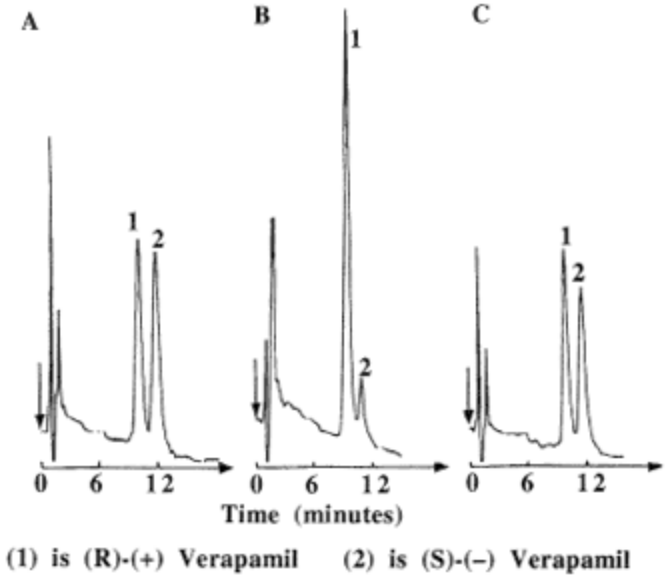
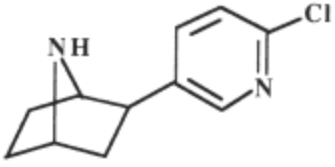


Figure 11.2
The Separation of the Enantiomers of Verapamil
Courtesy of J. Liquid Chromatogr. Ref. (1)

The whole sample was then injected onto the chiral column, 10 cm long, 4.0 mm I.D., packed with silica gel particles, 5 µm in diameter, bonded with α_1-acid glycoprotein (AGP). The results obtained are shown in figure 11.2. The separation ratio between the enantiomer pair is 1.23. Chromatogram (A) is the separation of a 50 ng sample of the racemic pair. Chromatogram (B) is from a blood serum sample taken 4 hours after the oral administration of 120 mg of racemic Verapamil. Chromatogram (C) is from a blood serum sample taken 15 min after the intravenous infusion of 15 mg of racemic Verapamil. It is clear that this type of analysis will allow an accurate pharmokinetic study of the relative biochemical degradation or excretion of the two enantiomers.

The Separation of the Enantiomers of Epibatidine

Epibatidine, *exo*-2-(6-chloro-3-pyridile-7-azabicyclo[2.2.1]heptane, the structure of which is shown below, is an alkaloid extracted from the skin of the Ecuadorian frog, *Epipedobates tricolor*. This alkaloid has been reported to possess potent, non-opioid, analgesic properties. In order to investigate the physiological properties in more detail Watt *et al*. [2], synthesized the material and also developed an analytical procedure for identifying and measuring the enantiomers present in the natural product.



The separation was carried out on a CHIRAL-AGP column, (a protein stationary phase, α_1-glycoprotein), 10 cm long, 4 mm I.D. The mobile phase consisted of 3% v/v acetonitrile/10 mM dipotassium hydrogen phosphate and 5 mM pentane sulfonic acid : 5/100 v/v, which was adjusted to a pH of 7.4 with *ortho*-phosphoric acid. The separation was carried out at ambient temperature, at a flow-rate of 1 ml/min. Samples were dissolved in ethanol and 2 µl samples were injected, containing 1 µg of each isomer. The separation obtained is shown in figure 11.3.

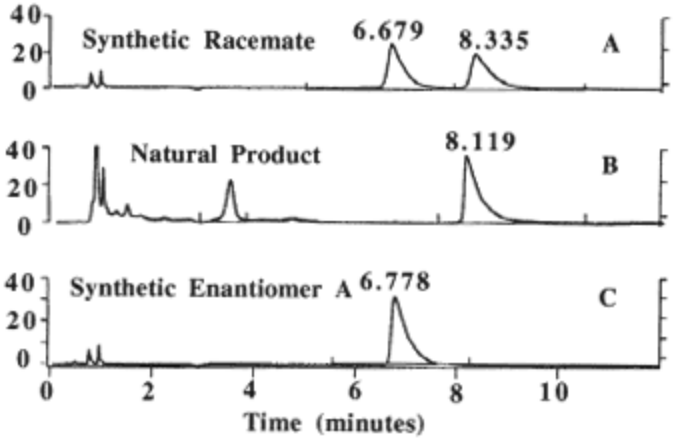


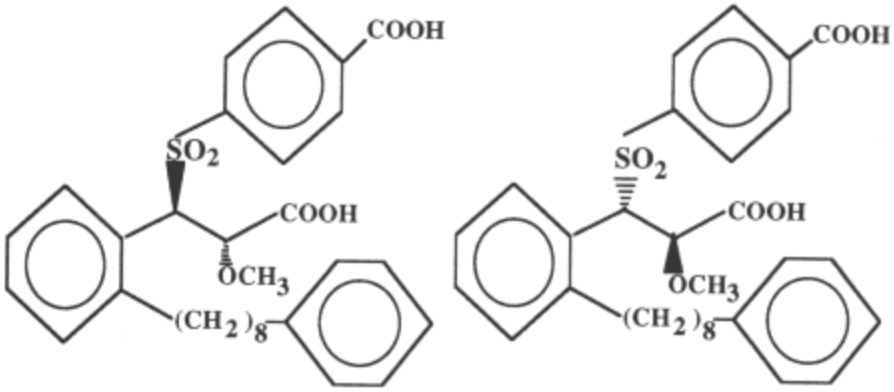
Figure 11.3
The Separation of the Enantiomers of Epibatidine
Courtesy of J. Liquid Chromatogr. Ref. (2)

Chromatogram (A) is a sample of the synthesized racemic material, (B) is a sample of the natural product and (C) is a sample of the opposite enantiomer of the natural product. Chiral liquid chromatography is widely used for this type of investigation providing a fast and accurate means of enantiomer identification.

Techniques for Improving the Detection Sensitivity of the Enantiomers of an Leukotriene Antagonist

Leukotriene D_4 (LTD_4) is formed from the metabolism of arachidonic acid by 5'-lypoxygenase and is considered to be an important receptor-mediator of human bronchial asthma. As a consequence, specific leucotriene LTD_4 receptor antagonists are potential therapeutics for the treatment of asthma. The compound (R,RS)-β(4(carboxyphenyl)-sulfonyl)-α-methoxy-2-(8-phenyloctyl)benzene propionic acid (SK&F 107310) is a potent and selective LTD_4 antagonist. The compound has two chiral centers and thus four possible optical isomers. Ho *et al.* [3] developed a method for separating the two enantiomers, the structure of which is shown below and also demonstrated that by the simple use of an

appropriate additive to the mobile phase, the sensitivity of the system could be greatly enhanced.



The separation was also carried out on a CHIRAL-AGP column, (stationary phase, α_1-glycoprotein), 10 cm long, 4 mm I.D. The mobile phase was a mixture of ethanol/20 mM potassium dihydrogen phosphate buffer (pH 4.6) 40/60 v/v.

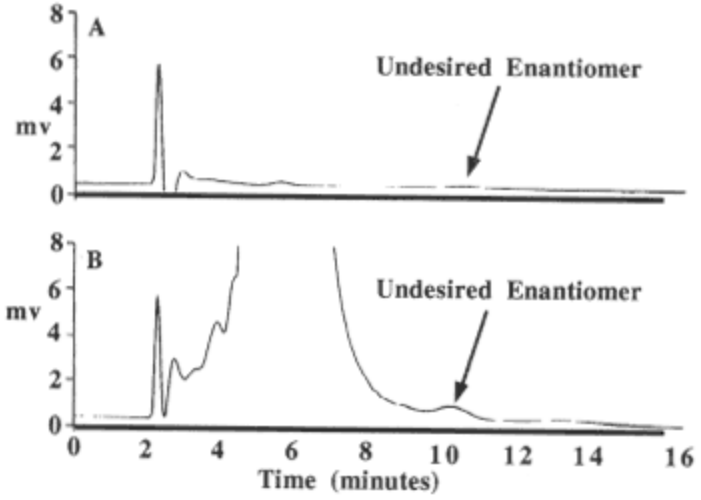


Figure 11.4
The Separation of the Isomers of (R,RS)-β (4(carboxyphenyl)-sulfonyl)-α-methoxy-2-(8-phenyloctyl)benzene Propionic Acid
Courtesy of J. Liquid Chromatogr. Ref. (3)

The separation obtained on the untreated column is shown in figure 11.4. The upper chromatogram is obtained on a new, untreated column and the scarcely discernible peak represents 0.002 mg/ml of the undesirable isomer. The lower chromatogram represents the same mass of enantiomer mixed with 0.53 mg/ml of the other isomer. The column was then pretreated with a mobile phase composed of ethanol/20 mM potassium dihydrogen phosphate buffer (pH 4.6)/triethylamine : 40/60/0.1 v/v/v, at a flow rate of 0.5 ml/min for about 40 min. and then equilibrated with the original mobile phase. The results obtained from the pretreated column are shown in figure 11.5.

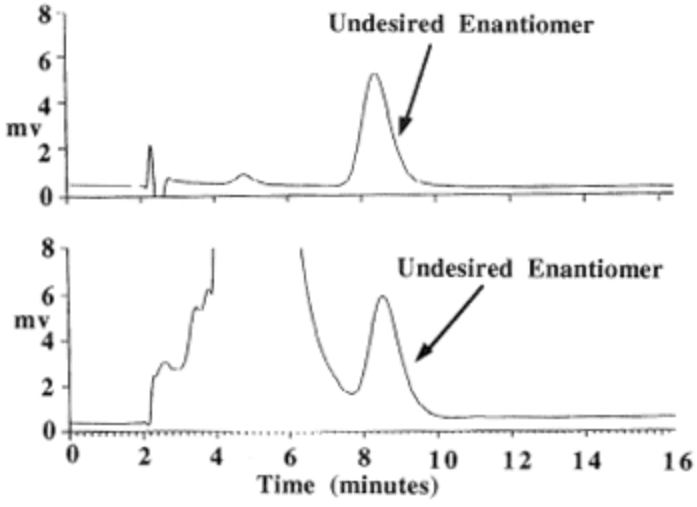


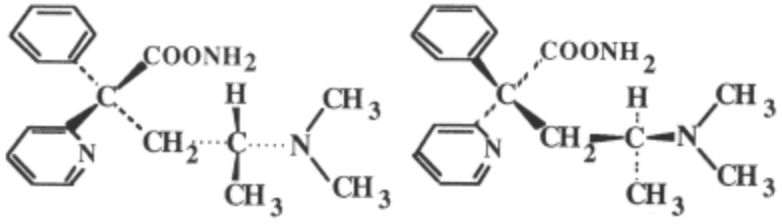
Figure 11.5
The Separation of the Isomers of (R,RS)-β
(4(carboxyphenyl)-sulfonyl)-α-methoxy-2-(8-phenyloctyl)benzene
Propionic Acid
Courtesy of J. Liquid Chromatogr. Ref. (3)

It is seen that the triethylamine has deactivated some high energy interactive sites and allowed the second isomer to be eluted and monitored. The authors, also found that by using dioctylamine, the deactivation was further improved, perhaps indicating that the high energy sites were dispersive in character. This would also indicate that

the overall retention and the chiral selectivity was, in all probability, dominantly controlled by polar interactions.

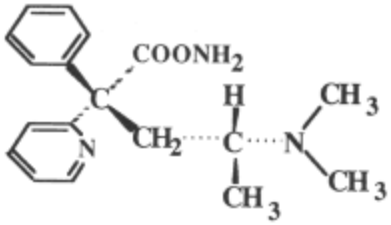
The Separation of the Enantiomers of Vamicamide Contained in Blood Serum and Urine

Vamicamide is a relatively recent and potent anticholinergic agent and is used as a racemic mixture of 4-dimethylamino-2-phenyl-2-(2-pyridyl)-valeramide. Preliminary studies on guinea pig ileum appeared to indicate that the R,R-enantiomer was 80 times greater in anticholinergic activity than the S,S-isomer. The structure of the two isomers, together with the S,R-isomer which, was used as an internal standard, are shown below.



R,R–Vamicanide

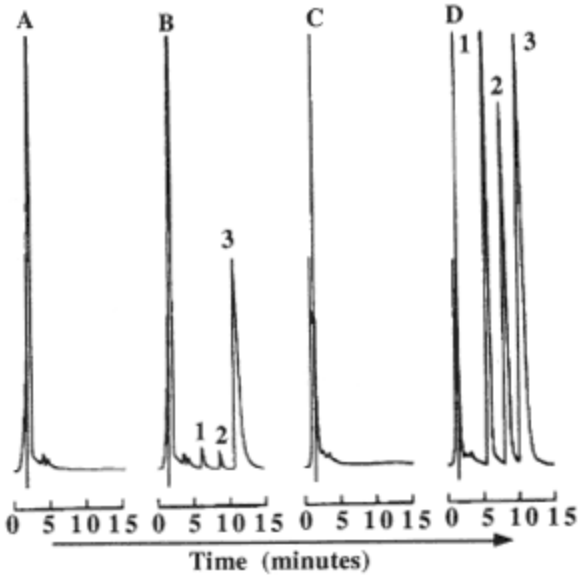
S,S–Vamicanide



S,R–Vamicanide (Internal Standard)

It follows, that a technique was required for the separation and estimation of both isomers present in blood serum and urine and a suitable procedure was developed by Susuki *et al.* [4]. The drug enantiomers were extracted in the following manner. A sample of serum or urine (1 ml) was treated with 100 µl of the standard solution (S,R-Vamicanide, containing 5 µg per ml for serum, 10 µg per ml for urine), 100 µl of distilled water and 1 ml of 0.1 M caustic soda. The mixture was then extracted with 5 ml of diethyl ether for 10 min with mechanical

shaking. The mixture was centrifuged, the organic phase removed and treated with 200 µl of 0.1% phosphoric acid and shaken. The mixture was again centrifuged and 150 µl of the organic layer neutralized with 1 M caustic soda (3.5 µl for serum and 4.0 µl for urine) and 100 µl was injected onto the column. The recovery of the drug by this procedure was shown to be about 80%. The Chiral-AGP column (α_1-acid glycoprotein) was 10 cm long, 4 mm I.D., equipped with a 1 cm long, 3 mm I.D. guard column. The mobile phase was acetonitrile/0.02 M phosphate buffer (pH 6.3) : 1/20 v/v, and used at a flow rate of 0.9 ml/min. The separation obtained is shown in figure 11.6.



1-S,S-Vamicamide, 2 = R,R-Vamicamide and 3 = the Internal Standard

Figure 11.6
The Separation of the R,R and S,S Isomers of
4-Dimethylamino-2-Phenyl-2-(2-Pyridyl)-Valeramide
Courtesy of J. Liquid Chromatogr. Ref. (4)

It is seen that the isomers are well separated; chromatograms (A) and (C) are the blank serum and urine samples respectively. Chromatograms (B) and (D) are from samples taken 2 hours after the oral ingestion of 18 mg

of racemic Vamicamide, from serum and urine samples, respectively. It is clear that the protein phase separates the enantiomers very efficiently and the overall technique highly practical.

In general, the bonded protein phases, especially α_1-acid glycoprotein, have been extensively used for the analysis of chiral drugs very successfully. In some circumstances analysis times may be a little longer than those obtained with alternative chiral stationary phases.

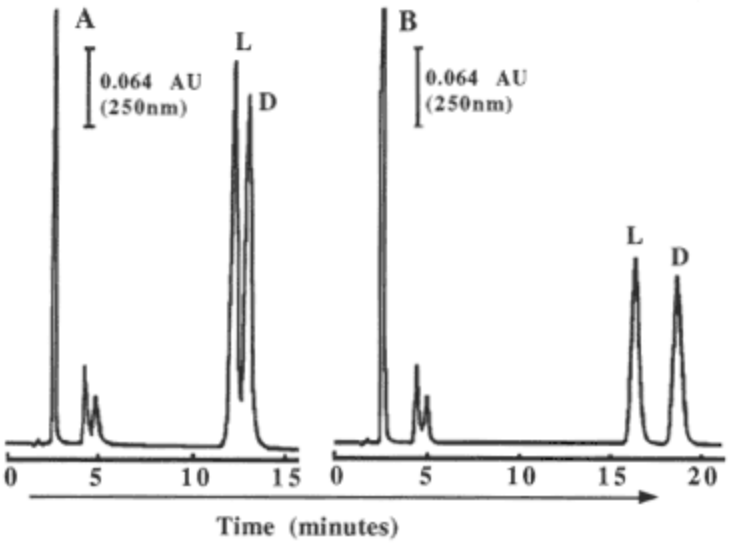
The Pirkle Stationary Phases

The Pirkle type chiral stationary phases are relatively straightforward to manufacture, are quite stable, and exhibit good chiral selectivity to a wide range of solute types. These classes of chiral stationary phase are also popular for the separation of many drug enantiomers and for amino acid analysis. The majority of the Pirkle phases are bonded to 5 µm spherical silica particles and packed into columns having lengths that range from 5 to 25 cm, using the slurry packing technique that has already been described. The common covalent linkage to the silica is achieved using aminopropyl silyl reagents and after linking to the silica, the amino group is reacted with an appropriate carbamate to form a urea bond. A range of different groups can be bonded in this way, the most common contemporary material being Whelk-01, synthesized from 4-(3,5-dinitrobenzamido)-tetrahydro-phenanthrene. However, over recent years a number of different Pirkle type phases have been introduced, such as N-3,5-dinitro-benzoyl-α-amino-2,2-dimethyl-4-pentenylphosphonate (known as the α-Burke 2 chiral stationary phase) and are still available and in general use.

The Separation of the Enantiomers of Some Amino Acids

An example of the separation of two derivatized amino acids on a naphthyl urea Pirkle phase is shown in figure 11.7. The phase was multiple-bonded to the silica and was contained in a column 10 cm long, 6 mm I.D. The mobile phase was acetonitrile/0.15 M sodium acetate (pH

5.0) 70/30 v/v and the flow rate 1 ml/min. Each peak represents 250 ng of the original underivatized enantiomer.

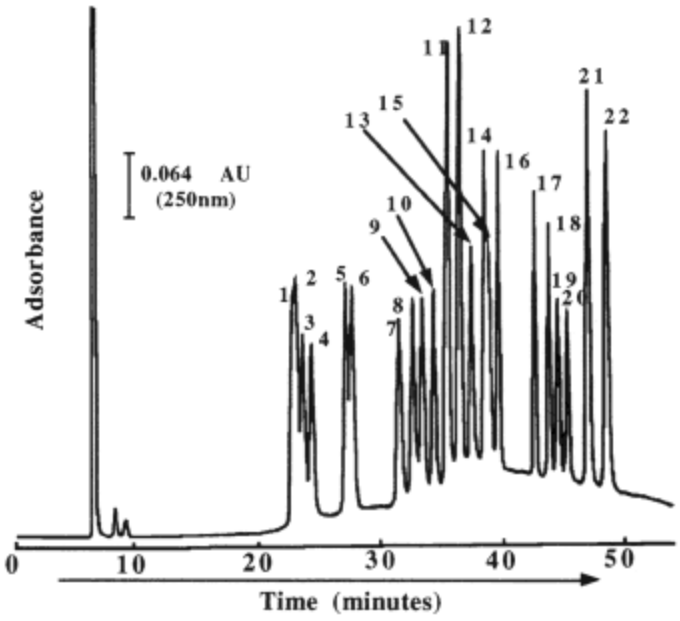


(A) *p*-Bromophenylcarbamyl Threonine (B) *p*-Bromophenylcarbamyl Phenylalanine

Figure 11.7
The Separation of Derivatized Threonine and Phenylalanine
Courtesy of J. Liquid Chromatogr. Ref. [5]

The separation of the *p*-bromophenyl derivatives of the enantiomers of a number of amino acids on the same stationary phase is shown in figure 11.8. One mobile phase component (A) was 50 mM phosphate buffer (pH 6.0) and the second component (B) pure acetonitrile. The gradient used was isocratic for 12 min 30% (B), then programmed for 12 to 29 min from 30% (B) to 47% (B), then from 29 to 49 min 47 to 67% (B) and finally, from 49 to 57 min, 67 to 93% (B). The flow rate was 1 ml/minute. It is seen that the chiral selectivity allowed all the enantiomers to be separated and the analysis time was less than 50 min. The solutes were eluted largely by increasing the dispersive character of the mobile phase and thus the chiral selectivity was probably dominated by polar interactions. The basic character of the mobile phase, *i.e.* dispersive,

polar or ionic usually accounts for the major contribution to the chiral selectivity, but subtle differences can play an important part in achieving the actual separation as evinced by the next example.



1. L-Serine
2. D-Serine
3. L-Threonine
4. D-Threonine
5. L-Alanine
6. D-Alanine
7. L-Valine
8. D-Valine
9. L-Methionine
10. D-Methionine
11. L-Leucine and Isoleucine
12. D-Leucine and Isoleucine
13. L-Tyrosine
14. L-Phenylalanine
15. D-Tyrosine
16. D-Phenylalanine
17. L-Tryptophan
18. D-Tryptophan
19. L-Lysine
20. D-Lysosine
21. L-Cystine
22. D-Cystine

Figure 11.8
The Separation of a Series of Amino Acid Derivatives
Courtesy of J. Liquid Chromatogr. Ref. [5]

The Effect of Chain Length of the Mobile Phase Dispersive Solvent Component on Chiral Resolution

The use of predominantly polar interactions, to control retention and chiral selectivity, invokes the use of dispersive mobile phases having

either high solvent content or strongly dispersive solvents. Fell and Dyas [6], examined the effect of chain length of the alcohol used in the mobile phase on the chiral resolution of the enantiomers of Propanolol. The stationary phase was 3,5-dinitrobenzoyl-α-phenylglycine covalently bonded to aminopropyl silica particles, 5 µm in diameter, contained in a column 10 cm long, 4.6 mm I.D. The separation of the phenylurea derivatives of the enantiomers of Propanolol using *n*-dodecane and *n*-pentane as the dispersive mobile phase solvents is shown in figure 11.9.

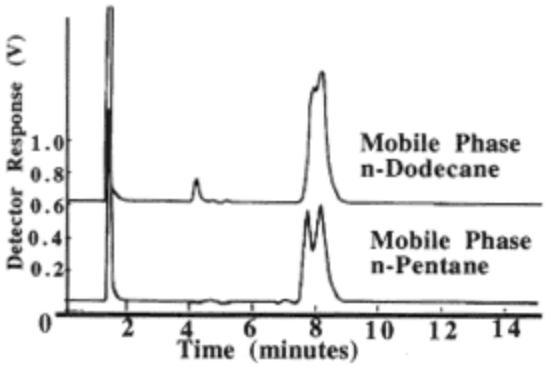


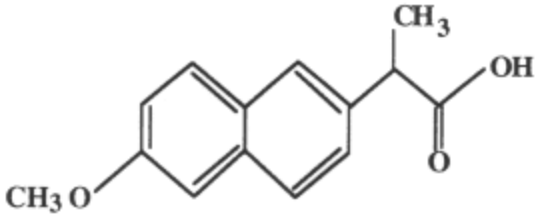
Figure 11.9
The Effect of Chain Length of Alcohol on the
Resolution of the Propanolol Enantiomers
Courtesy of Royal Society of Chemistry, Ref. [6]

It is seen that the use of the lower molecular weight dispersive solvent, *n*-pentane gave a better resolution than that obtained employing *n*-dodecane. The authors claimed that both the selectivity (as determined by the magnitude of the separation ratio) was improved as well as the column efficiency as a result of using the lower molecular weight solvent *n*-pentane. However, bearing in mind the apparent reduction in the separation ratio that occurs when peaks are eluted very close to one another (as discussed in the early part of chapter 10), it would appear from the results shown in figure 11.9, that if there was a change in separation ratio for the two enantiomers, then the change was very small indeed. It seems more likely, that the major part of the improvement in

resolution arose from the higher column efficiency, which would be a consequence of the higher solute diffusivity expected from the lower molecular weight hydrocarbon. It should again be emphasized that, when trying to determine the separation ratio for two closely eluting peaks, each enantiomer should be chromatographed separately and the separation ratio calculated from the two (k) values. To reduce run-to-run errors, a standard should be added to the samples containing the individual enantiomers and any change that may occur due to variable operating conditions can be corrected by using the data for the standard.

Stationary Phase Modification to Improve the Separation of Naproxen Enantiomer

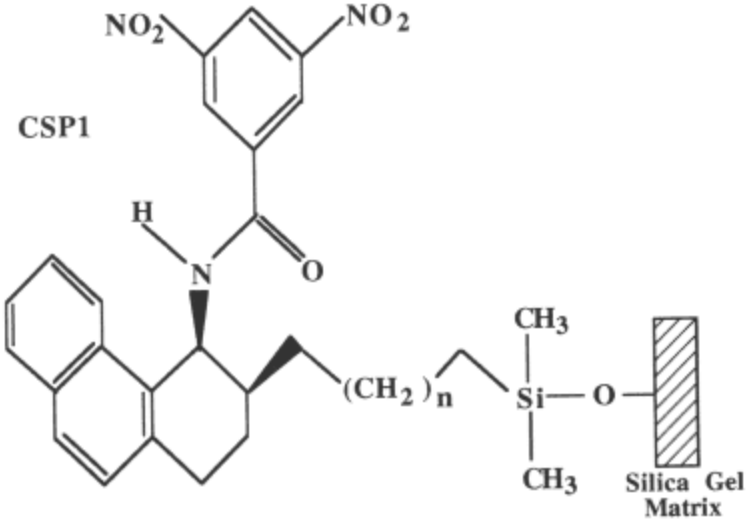
Naproxen, (S)-6-Methoxy-α-methyl-2-naphthaleneacetic acid, is a popular anti-inflammatory, analgesic and antipyretic drug which is also used in veterinary medicine largely for its anti-inflammatory properties. Its structure is as follows:



Naproxen

As it is enantiomeric, the resolution of its isomers is an important analysis for quality control purposes. Originally, the stationary phase CSP-1, the structure of which is shown below, was synthesized by Pirkle *et al*. [7]. It is seen that this material contained a long aliphatic chain connecting the structure with the chiral center to the silica gel surface. An alternative stationary phase, CSP-2 which eliminated the long aliphatic chain and replaced it with a single methylene group was synthesized by Pirkle and Welch [9]. This material was found to have significantly greater chiral selectivity than the long chain material. The rational behind the synthesis was that the shorter chain would favor a different spatial interaction between the solute and the chiral center. However, it must also be pointed out that removal of the C9 chain would

more than double the probability of the solute interacting with that part of the structure containing the chiral center, which would, without doubt, also increase chiral selectivity.



In addition, the elimination of the long aliphatic chain would also greatly reduce the dispersive interactions of the solute with the stationary phase and thus, relatively augment the polar interactions.

Table 11.1 Relative Chiral Selectivity of the Stationary Phases

	CSP 1			CSP 2		
Compound	**k1**	**k2**	**a**	**k1**	**k2**	**a**
Naproxen	3.96	8.95	2.26	1.71	5.01	2.93
Ibuprofen	0.94	1.05	1.12	0.19	0.28	1.47
Ketoprofen	4.53	5.03	1.11	1.39	1.79	1.29
Flurbiprofen	1.63	1.94	1.19	0.37	0.59	1.59
Pirprofen	2.53	3.49	1.38	0.85	1.54	1.81
Fenoprofen	1.48	1.81	1.22	0.38	0.61	1.61
Cicloprofen	3.03	5.18	1.71	1.16	2.50	2.15
Tioprofenic Acid	6.15	6.70	1.09	2.02	2.48	1.23

The results obtained for a series of underivatized profens are shown in table 11.1. It is clear that the significantly reduced retention of Naproxen on the CSP 2 results from the removal of the strong dispersive interactions that would take place between the solute and the 9 methylene groups. These dispersive interactions would dilute the differential chiral interactions of the solute with the structure containing the chiral center and thus, decrease the chiral selectivity. The true explanation probably involves, to some extent, all these effects. The separation of the enantiomers of Naproxen is shown in figure 11.10.

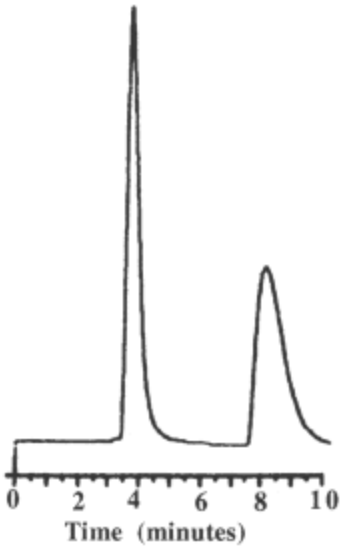


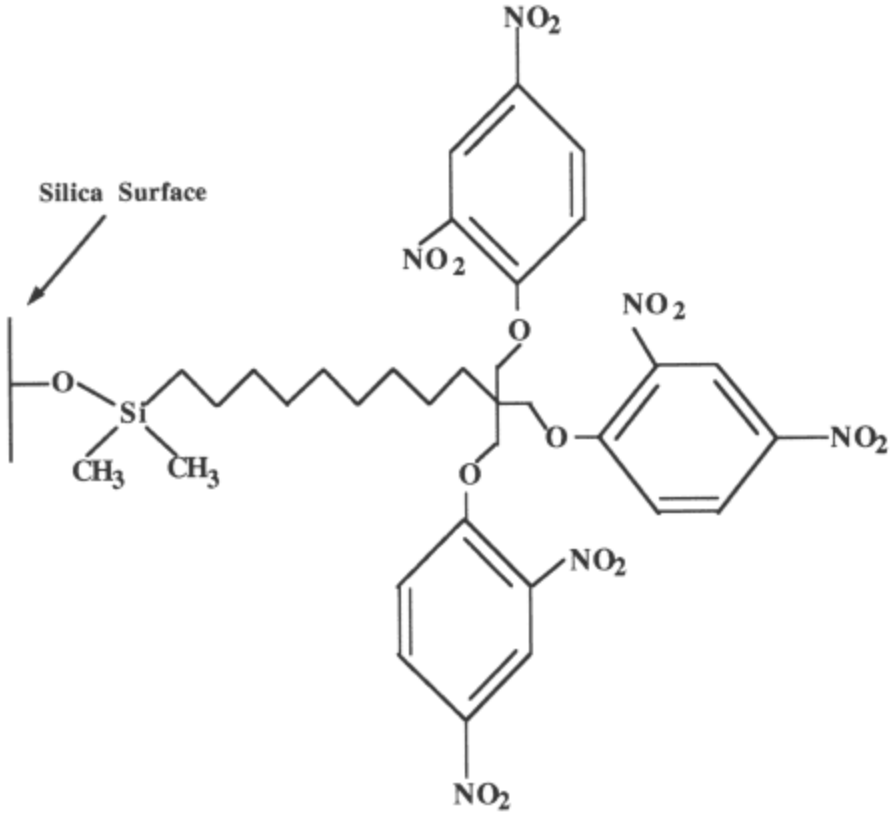
Figure 11.10
The Separation of the Enantiomers
of Naproxen on Stationary Phase
CSP 2
Courtesy of the Journal of Liquid
Chromatography (Ref. 8)

The separation was carried out using the CSP-2 stationary phase bonded to silica gel having a particle size of 5 µm. The stationary phase was packed into a column 25 cm long, 4.6 mm I.D. and operated at ambient temperature. The mobile phase consisted of in *n*-hexane/2-propanol

containing 1 g/l of ammonium acetate : 80/20 v/v, and was used at a flow rate of 2 ml/minute.

The Separation of Fullerenes

In the fullerene synthesis, a number of different fullerenes are simultaneously produced. The poor solubility of the fullerenes, together with their weak interaction with conventional stationary phases, evoked the development of a specific stationary phase for their resolution. The following structure was synthesized to facilitate the separation of the fullerenes by liquid chromatography.



The principal of the interaction depended on the strongly polar groups of the 2,4-dinitro aromatic rings inducing dipoles in the fullerene structure, thus allowing strong polar interactions between the solute and the

stationary phase. The interaction was augmented by the tripodal arrangement of the nitro aromatic groups that could provide multipoint interaction with the spherical highly polarizable fullerene structure. An example of the separation of some fullernes on the tripodal stationary phase is shown in figure 11.11.

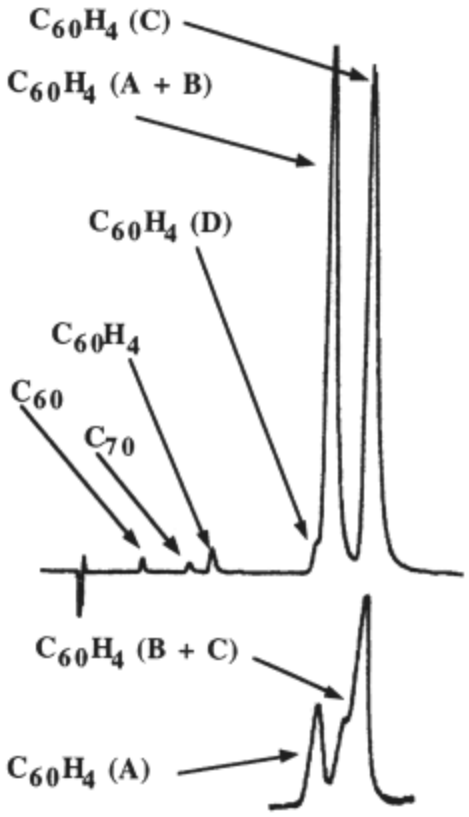


Figure 11.11
The Separation of Some Fullerenes
Courtesy of Regis Inc. [Ref.9]

Although this is not an enantiomeric separation, the problem is similar as the separation ratios between the solutes are normally very small due to their chemical similarity. The separation shown in figure 11.11 also demonstrates some interesting effects of the mobile phase. The column

used was 25 cm long, 1 cm I.D., packed with silica particles 5 µm in diameter carrying the stationary phase. The upper separation was developed using a mobile phase comprising toluene/hexane : 40/60 v/v, at a flow rate of 4.5 ml/min. The lower chromatogram was developed using a mobile phase consisting of a methylene chloride/*n*-hexane : 18/82, v/v mixture, at a flow rate of 9 ml/min. It is clear that the mobile phase containing the polarizable solvent eluted the solutes much faster than the mobile phase containing dispersive solvents only, indicating that polar interactions are dominant in the elution process. It is also clear that the $C_{60}H_4$ B component was eluted more rapidly by the toluene component, than the methylene chloride component, and thus was the isomer with the greater polarity.

The Cellulose and Amylose Stationary Phases

The most effective cellulose and amylose based chiral stationary phases are those derivatized with the various substituted tris(3,5-dimethylphenyl carbamates). Okamoto *et al.* [10], carried out an extensive study on the effect of the different substituent groups on the chiral selectivity of the stationary phase to a range of protected amino acid derivatives. An example of one of their separations is given in figure 11.12.

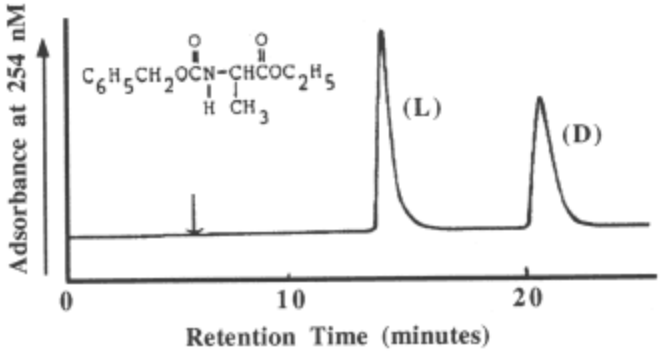


Figure 11.12
The Separation of N-Benzyloxycarbonyl Alanine Ethyl Ester
on Cellulose Tris(3,5-Dimethylphenylcarbamate)
Courtesy of J. Chromatogr. Ref. (10)

The column was 25 cm long, 4.6 mm I.D., and the eluent was hexane/2-propanol : 90/10 v/v. The stationary phase was thus operated in the normal mode, so retention and selectivity would be largely controlled by differential polar interactions. Okamoto *et al*. reports a large amount of data for a number of different amino acids, protected and derivatized in a variety of ways. Some selected examples of the results obtained for alanine derivatives on both a range of cellulose and amylose columns are shown in Tables 11.2 and 11.3. The data is selected, more from the point of view of discussing the different properties of the stationary phases, than to provide specific reference retention and selectivity data.

Table 11.2 Capacity Ratios and Separation Ratios of Some Protected Alanine Derivatives Separated on Cellulose Columns

	Ac-Ala-OEt			**Ac-Ala-OBzl**			**Bz-Ala-OEt**		
Column	**k**		**a**	**k**		**a**	**k**		**a**
3,5-Dimethyl	1.09	+	1.12	5.52	-	1.04	1.51	D	1.34
4-Methyl	1.05	-	1.25	1.65	-	1.32	1.26	L	1.12
4-Ethyl	0.68	-	1.49	1.18	-	1.58	0.66	L	1.29
H	4.74	-	1.14	7.69	-	1.22	2.01		1.00
4-F	2.08	-	1.36	3.11	-	1.51	2.62	L	1.49
4-Br	1.61	-	1.63	2.58	-	1.65	2.74	L	1.22

Firstly, in assessing data, such as that shown in tables 1.1 and 11.2, it is important *not* to try to derive a rational explanation for the figures in too great a detail. It is almost certain that a second set of data will completely confound the initial speculations, and invoke an entirely different *scenario* to account for retention and selectivity. There are, however, some important generalizations that can be educed from the data. Firstly, it is clear that the benzyl ester of the acetyl alanine is retained on all stationary phases to a greater extent than either the acetylated or benzoylated alanine ethyl esters. It would appear that a larger and polarizable ester group, produces a greater overall stationary phase-solute interaction, than a large polarizable blocking group on the amine fragment. However, it is also seen that this increased retention is

not mirrored by strong chiral selectivity, as indicated by the separation ratios. This would indicate that the ester group does not contribute to the same extent to the *relative* interactions of the two enantiomers. It is also seen that the underivatized carbamate results in the greatest retention and thus the strongest solute-stationary phase interactions. However, the separation ratios for the underivatized carbamate, with one exception, are the smallest and thus exhibits the poorest *chiral* selectivity. Hence, the *raison d'être* for the substituted carbamate derivatives. It is also seen that, in general, the 4-ethyl, 4-fluoro and 4-bromo substituted carbamates give the greatest chiral selectivity. Thus, although, due to the nature of the mobile phase, polar interactions must dominate, with respect to the alanine derivatives examined, the chiral selectivity appears to arise from significant excess dispersive interactive forces.

Table 11.3 Capacity Ratios and Separation Ratios of Some Protected Alanine Derivatives Separated on Amylose Columns

	Ac-Ala-OEt			**Ac-Ala-OBzl**			**Bz-Ala-OEt**		
Column	**k**		**a**	**k**		**a**	**k**		**a**
3,5-Dimethyl	0.73	+	1.53	2.33	+	1.37	1.64	D	1.43
4-Methyl	2.93	-	1.19	3.73	-	1.24	3.26		1
H	3.19	-	*ca.*1	4.12	-	*ca.*1	3.58	L	*ca.*1
4-Cl	4.11	-	*ca.*1	5.53	-	*ca.*1	4.18	L	*ca.*1
3,5-Dichloro	2.27	-	1.09	4.57	+	1.14	3.56	D	*ca.*1

The data in table 11.3 indicates that the benzyl ester of the acetyl alanine is also retained to the greatest extent on amylose as well as cellulose. However, on derivatized amylose, the chiral selectivity is also the greatest for this alanine derivative. This is in direct contrast to the selectivity exhibited from the derivatized celluloses. The unsubstituted carbamate and the 4-Cl substituted carbamate give the greatest retention, indicating strong general polar interactivity, but virtually no chiral selectivity at all. The 3,5-dimethyl and 4-methyl substituted derivatives of amylose show the best chiral selectivity for the alanine derivatives

examined, but in general poorer selectivity than that obtainable from the derivatized celluloses. The relative merits of the two types of phases for chiral selectivity will vary from one type of solute to another, and for some solutes the derivatized amyloses have provided better chiral selectivity than the derivatized celluloses. Unfortunately, the best type of stationary phase for an unknown enantiomeric separation can only be estimated from the results obtained for similar solute types. The relevance of the chosen stationary phase must then be confirmed by experiment.

The Separation of Some Chiral Drugs on Different Cellulose Derivatives

Okamato *et al*. [11] also examined the relative performance of cellulose and amylose derivatives for the separation of a group of racemic drugs. At the same time, they demonstrated generally the versatility of the cellulose based chiral stationary phases for the separation of a range of physiologically active compounds. Examples of such separations are shown in figure 11.13 (A–F).

The enantiomers of Mephenesin are shown separated in figure 11.13 (A) on a stationary phase consisting of coated cellulose derivatized with tris(3,5-dimethylphenylcarbamate). A mixture of *n*-hexane/2-propanol/diethyl-amine : 80/20/0.11 v/v/v was used as the mobile phase. In figure 11.13 (B), which shows the separation of the enantiomers of Tolperisone, the stationary phase was cellulose derivatized with tris(3,5-difluoro-phenylcarbamate) and a mixture of *n*-hexane/2-propanol : 90/10 v/v was used as the mobile phase.

In both examples, the mobile phase flow rate was 0.5 ml/min. As both mobile phase mixtures were strongly dispersive in character, the retention and selectivity was dominated largely by polar interactions. The separation of the Tolperisone was quite adequate, but it would appear that the very strong polar character of the Mephenesin resulted in some strong adsorption on some active sites on the stationary phase.

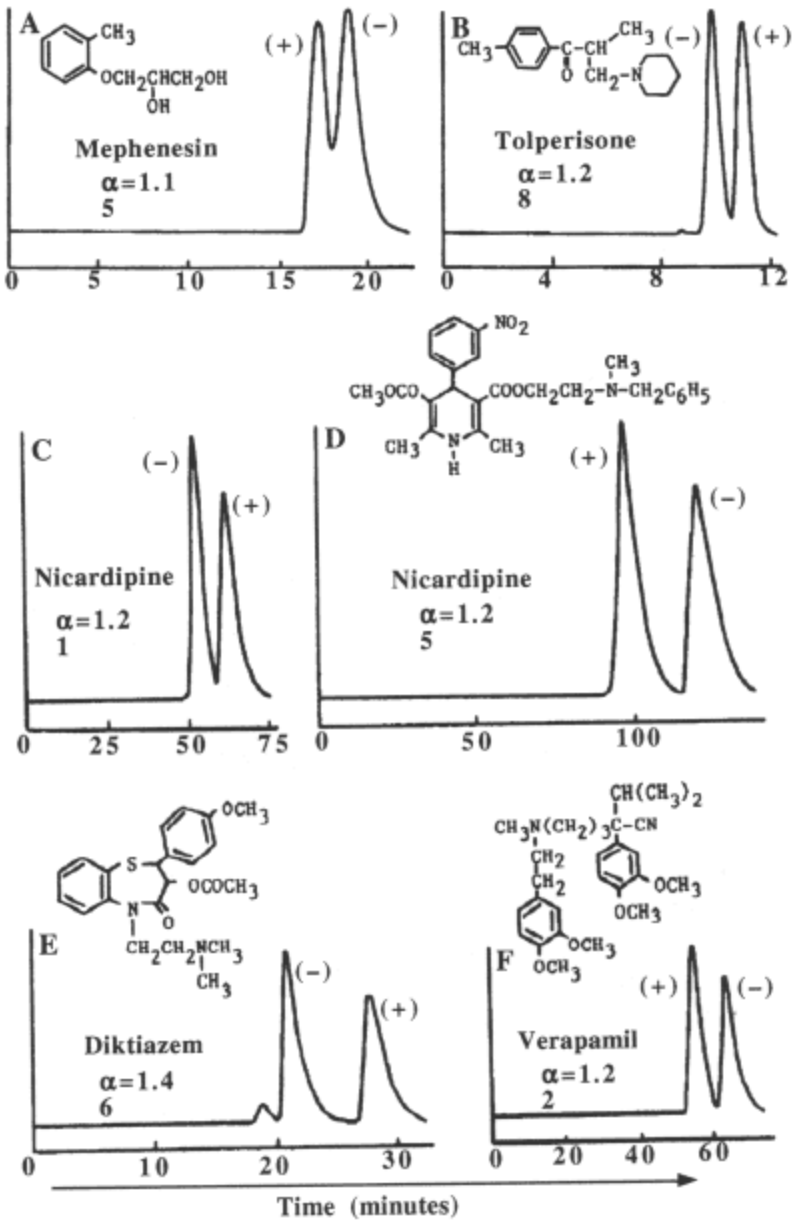


Figure 11.13
The Separation of Different Drug Enantiomers
Courtesy of J. Liq. Chromatogr. (Ref. 11)

This appears to have produced some peak asymmetry with accompanying reduced efficiency and resolution. The separation of the isomers of Nicardipine on cellulose tris(4-tert-butylphenylcarbamate is shown in figure 11.13 (C). The mobile phase was *n*-hexane/2-propanol : 90/10 v/v. It is seen that the chiral selectivity is very good, but the retention time was excessive, at 75 minutes. Again, the strong dispersive nature of the mobile phase indicates that, both retention, and chiral selectivity, was determined largely by polar interactions between the solutes and the stationary phase.

The same solute, Nicardipine, is shown separated under somewhat different conditions in figure 11.13 (D). The stationary phase was bis(3,5-dichlorophenylcarbamate and the same mobile phase was employed as that in the separation in figure 11.13 (C). It is seen that the chiral selectivity is better, but the retention is also greater extending the separation time to 100 minutes. It is also seen, that the peaks are significantly asymmetric indicating interaction with high energy sites that are probably on exposed silica gel.

The separation of the enantiomers of Diktiazem is shown in figure 11.13 (E). The stationary phase was tris(3,5-dimethylphenylcarbamate and the mobile phase was the same as that used in figure 11.13 (C) and (D). It is seen that the system exhibits a high selectivity for the two enantiomers, but there is significant peak asymmetry, due probably to interaction with high activity polar sites on exposed silica. The selectivity and retention will be largely due to polar interactions between the solute and the stationary phase, the solutes being eluted largely by dispersive interactions with the mobile phase.

Finally, figure 11.13 (F) shows the separation of the isomers of Verapamil on cellulose tris(3,5-difluorophenyl carbamate). In this separation, the mobile phase was *n*-hexane/2-propanol/diethylamine : 80/20/0.1 v/v/v. It is seen that adequate separation is achieved and that the peaks are a little more symmetrical, probably because the high activity sites on the exposed silica are blocked by the trace of

diethylamine present in the mobile phase. The basic diethylamine will strongly interact with the acidic hydroxyl groups of the silica significantly reducing their availability for interaction with the solute. The use of diethylamine in this way is a common and popular method of reducing solute interaction with exposed silanol groups, when using cellulose and amylose based stationary phases *coated* on silica.

It is clear that the cellulose and amylose derivatives can be very useful for the separation of drug enantiomers, and due to the high density of chirally active sites, is particularly useful for preparative work.

The Separation of the Enantiomers of Propranolol, Metroprolol and Atenalol As Fluorescent Enhanced Derivatives on Cellulose Tris(3,5-dimethylphenylcarbamate) Coated on Silica Gel

Yang *et al*. [12] developed a method for separating a number of β-blockers as their fluorescent derivatives, which were formed by reacting them with an electrophilic fluorogenic reagent, 4-(*N*-chloroformethyl-*N*-methylamino-7-*N*-*N*-dimethylaminosulfonyl-2, 1, 2-benzoxydiazole (DBD-COCl).

These derivatives show intense fluorescence at long excitation wavelengths (450_{Ex} and 560_{Em} nm) and thus significantly reduce the level of detection. It was found that Propranolol, Metoprolol and Atenolol reacted directly with reagent forming 1:1 adducts by reaction with the secondary amino group of the β-blockers. The DBD-COCl reagent reacted readily with the drugs under mild conditions, with no catalyst, and the reaction was complete in about 5 min. The initial separation was carried out on a TSKgel ODS-80T column, 15 cm long, 4.6 mm I.D., packed with 5 μm particles. A different gradient was employed for each derivative. A gradient was used to develop the separation that commenced at 50% aqueous solution of acetonitrile and increased to 80% acetonitrile over 40 min. for Propanolol, from 40% to 48% over 15 min and the increasing to 100% at 30 min for Metroprolol and from 33% to 42% in 10 min and then to 100% at 60 min. for

Atenolol. The separations obtained are shown in figure 11.15. Peak 2 represents the DBD derivative of each drug, peak 1 DBD-COOH and peak x was ascribed to the anhydride produced by the reaction of DBD-COCl with DBD-COOH. The drug derivatives were collected and then subjected to separation on a chiral column.

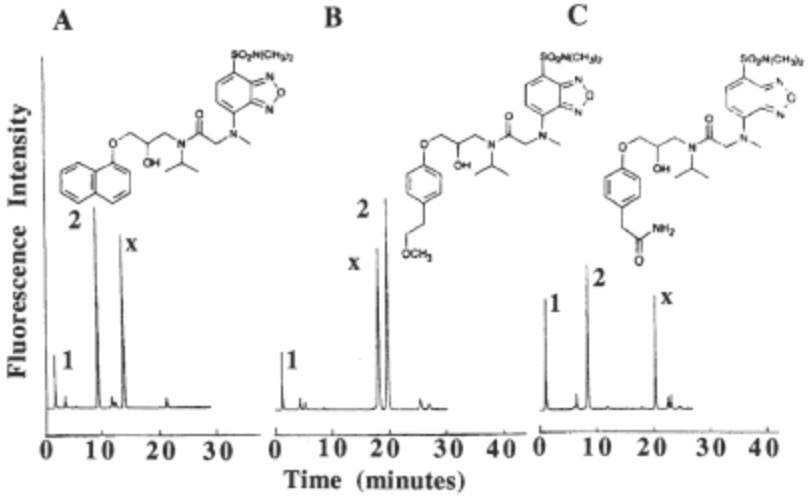


Figure 11.14
The Separation of Derivatized Propranolol (Pro) Metroprolol (Met) and Atenolol (Ate)
Courtesy of the Royal Society of Chemistry, Ref. [12]

The use of CHIRACEL OD-R (a cellulose carbamate) and CHIRACEL OJ-R (a cellulose ester) were used to separate the individual enantiomers. It was found that the enantiomers of the DBD-Pro were well separated on the CHIRACEL OD-R column. However, the DBD-Met enantiomers could not be separated on the CHIRACEL OD-R column but were well separated on the CHIRACEL OJ-R column as were the DBD-Ate isomers. The separation of the DBD-Met and DBD-Ate isomers are shown in figure 11.15 (chromatogram A, DBD-Met and chromatogram B, DBD-Ate). Each enantiomeric pair represents 50 pmol of the original drug. The separation was carried out on the CHIRACEL OJ-R column (15 cm long, 4.6 mm I.D., packed with particles 5 μm in diameter coated

with the cellulose ester. The mobile phase used for the separation of DBD-Met was methanol/acetonitrile : 90/10 v/v, at a flow rate of 0.5 ml/min, the separation ratio was 1.33. The mobile phase used for the separation of DBD-Ate was methanol, also at a flow rate of 0.5 ml/min, the separation ratio being 1.53.

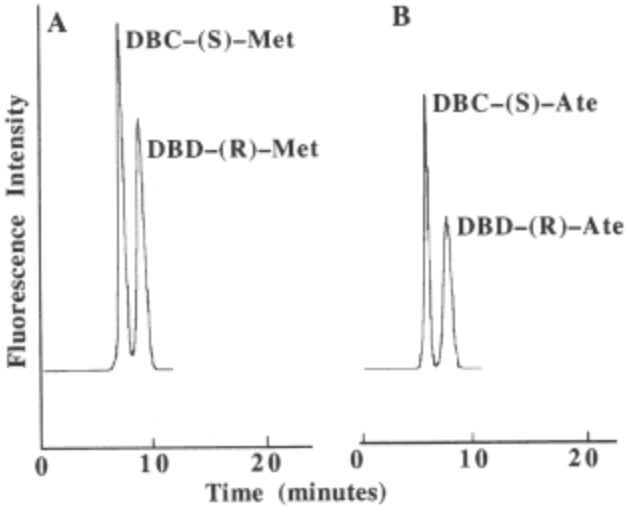


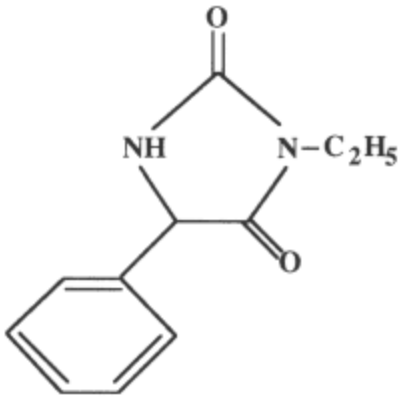
Figure 11.15
The Separation of Derivatized Metoprolol and Atenolol at High Sensitivity
Courtesy of the Royal society of Chemistry, Ref. [12]

The excitation wavelength was 450 nm and the emission wavelength was 560 nm. The fluorescent derivatives were found to be stable at 4°C for over 1 week. The detection limits at a signal-to-noise ratio of 3 were 50 fmol for both (S)- and (R)-Propanolol, 12 and 17 fmol for (S)- and (R)- Metroprolol respectively and 15 and 20 fm for (S)- and (R)-Atenolol respectively.

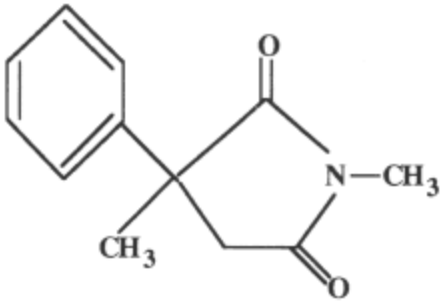
The Separation of the Enantiomers of Two Anticonvulsants

Ethotoin, (3-ethyl-5-phenyl-2,4-imidazolidinedione) is an anticonvulsant that is freely soluble in alcohol, ether, benzene and in dilute solutions of

alkali hydroxides but only sparingly soluble in cold water, although slightly more soluble in hot water. It is commonly used therapeutically as an anticonvulsant. The structure of Ethotoin is as follows.



Another anticonvulsant of somewhat similar structure is Methsuximide, 1,3-dimethyl-3-phenyl-2,5-pyrrol-indinedione, a substance freely soluble in alcohol, the enantiomers of which have also been separated on a cellulose based chiral stationary phase. The structure of Methsuximide is as follows.



The enantiomers of both anticonvulsants were separated on a CHIRAL OJ column which contained a cellulose ester coated on silica gel particles 5 µm in diameter. The separation was carried out at ambient temperature at a flow rate of 1 ml/min. The separations obtained are shown in figure 11.16. The ethotoin enantiomers were separated using a *n*-hexane/2-

propanol mixture 9/1 v/v, as the mobile phase. The methsuximide enantiomers were separated using ethanol as the mobile phase.

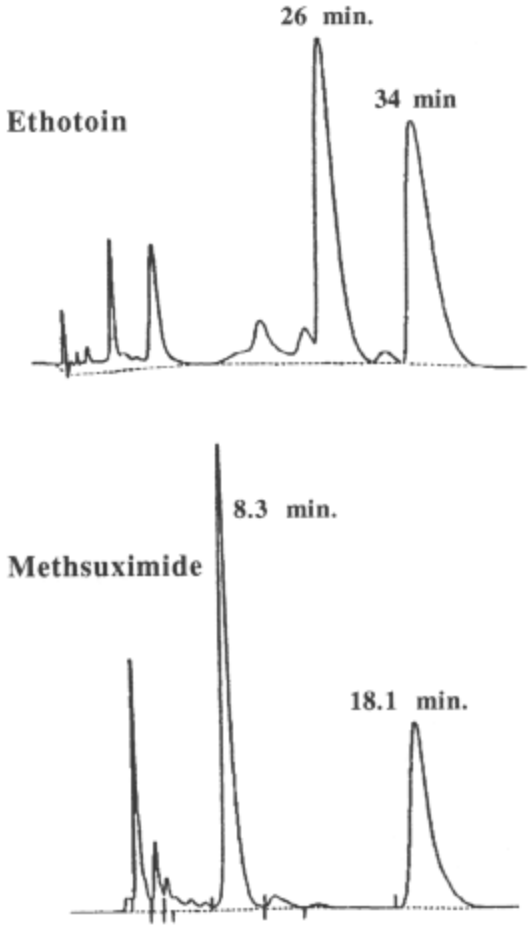


Figure 11.16
The Separation of the Enantiomers of the
Anticonvulsants Ethotoin and Methsuximide
Courtesy of CHIRAL Technologies Inc.

It is seen that the enantiomer pairs were well separated and as the solvents were largely dispersive in character, it would appear that the

chiral selectivity arose largely from differential polar interactions between the solutes and the stationary phase.

The Macrocyclic Glycopeptides Stationary Phases

There are two popular macrocyclic glycopeptides in common use, the first utilizes Vancomycin bonded to silica and the second Teicoplanin. A third, Avoparcin is likely to become available in the near future. Vancomycin has 18 chiral centers, and three shallow molecular cavities, and so some inclusion can also take place, augmenting the entropy contribution to retention and selectivity. There are many dipoles on the molecule covering a wide range of polar intensities, providing a span of polar interactions. There are also polarizable groups that can provide induced dipole interactions and a range of dispersive groups to provide dispersive (hydrophobic) interactions. It has pK values of 2.9, 7.2, 8.6, 9.6, 10.4 and 11.7. Teicoplanin has 20 chiral centers and 4 fused rings and, like Vancomycin, has a complex structure that provides a range of polar, dispersive and potential ion interactive sites. These types of stationary phase are very stable, can be used in the polar or reversed phase mode, and have been shown to have a wide field of application.

The Separation of the Enantiomers of Three Racemic Substituted Pyridones on Different Macrocyclic Glycopeptide Stationary Phases

This example, described by Chen *et al*. [13] demonstrates the complementary use of the two macrocyclic chiral stationary phases to separate a range of difficult chiral separations. It will be seen that where one type of chiral stationary phase fails to achieve a separation, the other can often succeed. Vancomycin and Teicoplanin were prepared in the manner described by Armstrong [14], and bonded to silica gel particles 5 μm in diameter and packed into columns 25 cm long and 4.6 mm I.D. The results that were obtained are shown in figure 11.17. All three racemates were developed under identical conditions. The mobile phase was methanol/1% triethylammonium acetate buffer, pH 4.1 : 1/90 v/v, used at a flow rate of 1 ml/minute.

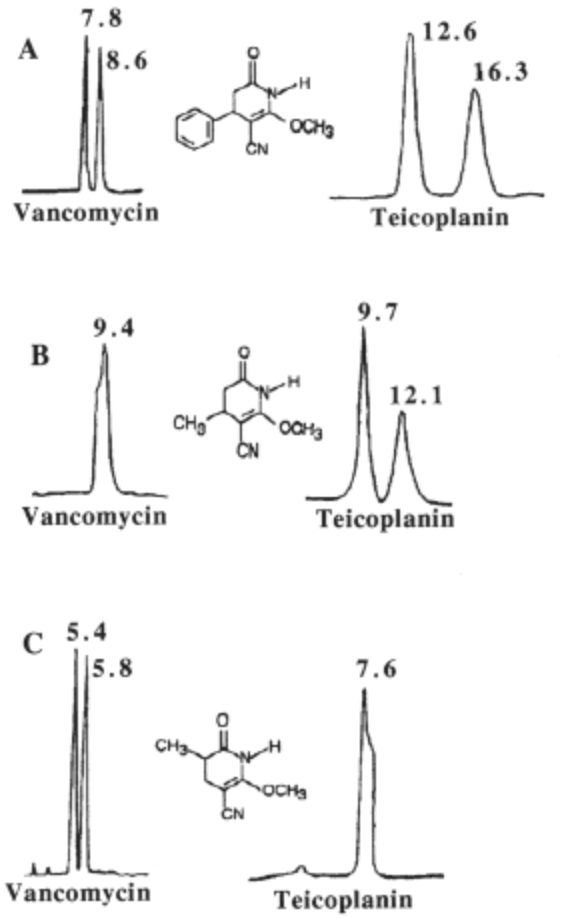


Figure 11.17
The Separation of Three Racemic Substituted Pyridones on Two Macrocyclic Chiral Stationary Phases
Courtesy of J. Liq. Chromatography Ref. [13]

It is seen that all three of the enantiomeric pairs could be separated either on the Vancomycin or the Teicoplanin. However, the retention times of the different solutes varied widely from about 5 min to over 16 min. It is clear that although one single macrocyclic antibiotic will not separate

all optical isomers, two or more of these stationary phases will significantly increase the probability of success.

Examples of the Use of Vancomycin in Both the Normal Phase and Reversed Phase Modes for the Separation of the Enantiomers of Five Biologically Active Substances

The dual development technique was demonstrated by Armstrong *et al*. [14], on a column which was 25 cm long, 4.6 mm I.D., and packed with 5 μm silica gel particles on which the Vancomycin was attached. In the reversed phase mode, the mobile phase consisted of acetonitrile/1% triethylammonium acetate, pH 7.0 : 10 /90 v/v. In the normal phase mode, the mobile phase consisted of a 2-propanol/*n*-hexane : 50/50 v/v. In both modes, the separation was carried out at room temperature (22°C), and at a flow rate of 1 ml/minute.

Two racemic mixtures were used; one mixture contained Bromacil, Devrinol and Coumachlor, which were separated by reversed phase development and the other, *N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine and 3-[2-(2-bromoacetamido)-acetamido]-proxyl, which was separated by normal phase development. Bromacil, 5-bromo-6-methyl-3-(1-methyl-propyl)uracil and Devrinol, 2-(α-naphthoxy)-*N*,*N*-diethylpropionamide are herbicides. Coumachlor is a rodenticide, similar to Warfarin, which produces delayed action on the prothrombin level and blood clotting, which results in death by hemorrhage. Its chemical name is 3-[1-(4-chlorophenyl)-3-oxybutyl]-4-hydroxy-2H-1-benzopyran-2-one and is used extensively in pest control. The structures of Bromacil, Devrinol and Coumachlor are included in figure 11.18A. Similarly, structures of *N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine and 3-[2-(2-bromoacetamido)-acetomido]-proxyl are included in figure 11.18B. The separations obtained are shown in figure 11.19. Figure 11.18A shows the separation developed under reversed phase conditions, and figure 11.18B shows the separation developed under normal phase conditions. It should be recalled that under reversed phase development, the solutes are retained largely by dispersive interactions between the solute and the stationary

phase. In normal phase development, solutes are retained largely by polar forces between the solutes and the stationary phase.

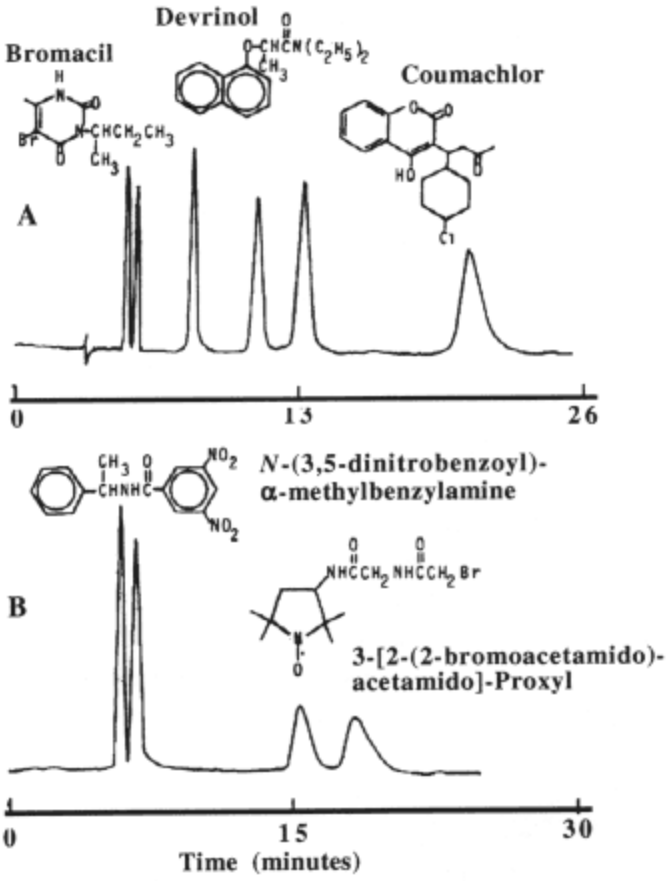


Figure 11.18
The Separation of Some Physiologically Active Substances on Vancomycin using Normal Phase and Reversed Phase Mode of Development
Courtesy of Anal. Chem [Ref. 14]

It is seen that the Vancomycin can be used equally effectively in either development mode, the separation in both modes are clean, and the peaks well shaped allowing easy, quantitative assessment.

The macrocyclic glycopeptide Teicoplanin has been used very successfully by Irv Wainer and his group at Georgetown University, to monitor the enantiomers of Albuterol in blood plasma. Albuterol (supplied under the names of Proventil and Ventolin) is used to dilate breathing passages and make breathing easier. It is often used to prevent bronchospasm in certain cases of lung diseases.

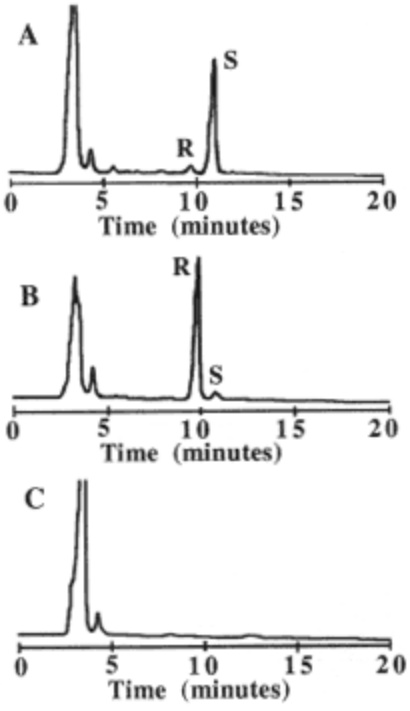


Figure 11.19
The Separation of the Enantiomers of Albuterol in Extract of Spiked Blood Plasma
Courtesy of Karen Fried Georgetown University

Albuterol is inhaled into the lungs (2 to 30 mg) employing a nebulizer, typically, several times a day. The Albuterol was extracted from the plasma and then separated on a Chirobotic T column. The column was 25 cm long, 4.6 mm I.D., and packed with 5 mm particles carrying the stationary phase. The mobile phase consisted of a mixture of

methanol/acetonitrile/glacial acetic acid/diethylamine, 50/50/0.3/0.2 : v/v/v. The separation was carried out at room temperature at a flow rate of 1 ml/min. The separation obtained is shown in figure 11.19, (A) and (B) display chromatograms of the two enantiomers, the plasma containing 25 ng/ml of Albuterol and (B) is plasma blank.

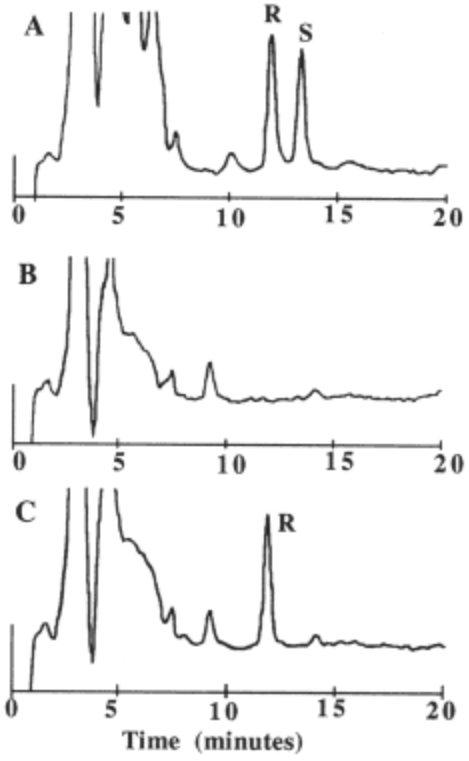


Figure 11.20
The Separation of the Enantiomers of
Albuterol in Human Blood Plasma Extract
after Treatment
Courtesy of Karen Fried Georgetown
University

The analysis of human blood plasma after treatment with the drug, is shown in figure 11.20. Chromatogram (A) is from an extracted human plasma, spiked with 4.0 ng/ml of Albuterol. Chromatogram (B) is from the predose plasma sample, and chromatogram (C) from a plasma sample taken 45 min after receiving 2.5 mg of racemic Albuterol by a nebulizer.

The presence of the two enantiomers is clearly detected and can be quantitatively assayed. The analytical procedure was also used for the assay of the drug in dog plasma and the results obtained are shown in figure 11.21.

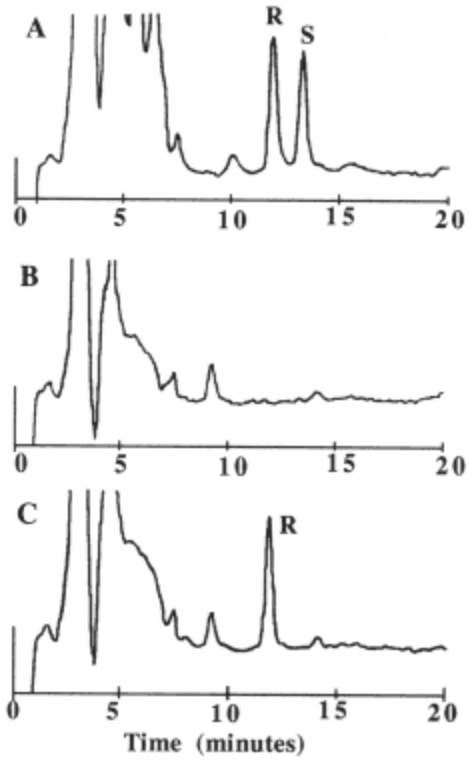


Figure 11.21
The Separation of the Enantiomers of
Albuterol in Canine Blood Plasma Extract
after Treatment
Courtesy of Karen Fried Georgetown
University

Chromatogram (A) is from 1 ml of canine plasma, spiked with 2.5 ng/ml of racemic Albuterol. Chromatogram (B) is from a predose plasma sample from the dog, and chromatogram (C) is from 0.5 ml of dog plasma taken 5 min after the administration of 0.02 mg/kg (R)-(-) Albuterol. It is seen that an excellent recovery is achieved and the

individual enantiomer unambiguously identified with more than adequate sensitivity.

The Separation of the Enantiomers of Ibuprofen on Vancomycin

Ibuprofen, (α-methyl-4-(2-methylpropyl)benzene-acetic acid), is a well known proprietary non-steroidal anti-inflammatory drug used to reduce pain, fever and inflammation, particularly in arthritis. The drug exists as two enantiomers and has been successfully separated by liquid chromatography using Chirobiotic V (Vancomycin).

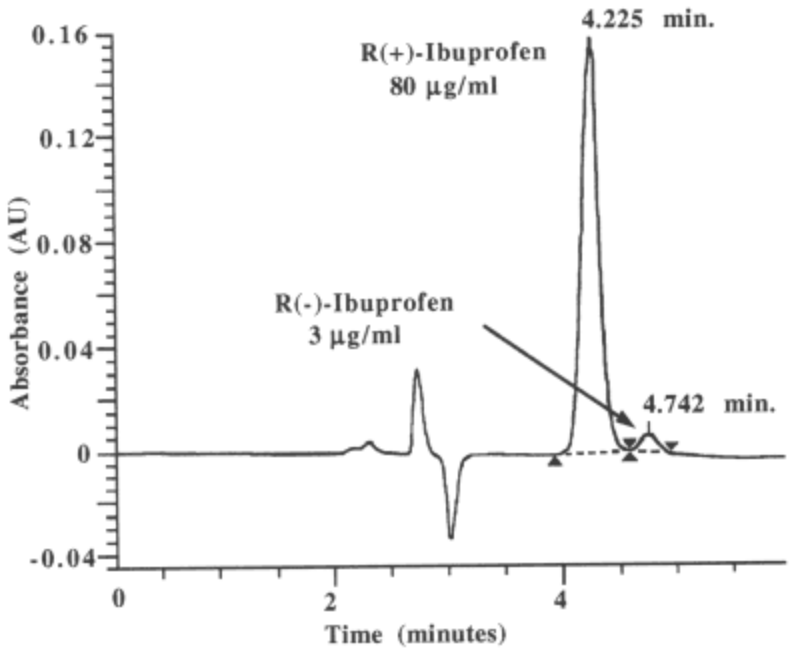


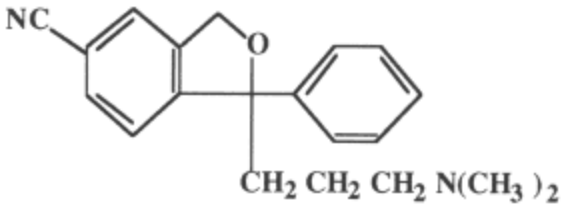
Figure 11.22
The Separation of the Enantiomers of Ibuprofen on Vancomycin
Courtesy of Dr. Reinhard Kupferschmidt, Gebro Broschek GmbH Pharmezeutische

The separation is shown in figure 11.22. The column was 15 cm long, 4.6 mm I.D., and operated at room temperature at a flow rate of 0.75 ml/minute. The mobile phase consisted of tetrahydrofuran/50 mM

ammonium phosphate (adjusted to a pH 7.0 with 25% ammonium hydroxide which had been filtered through 0.45 μm filter) : 10/90 v/v. The resolution and sensitivity of the procedure emphasizes the value of this type of stationary phase for drug analysis and also possibly for forensic purposes.

The Separation of the Isomers of Citalopram

Citalopram, also known as Nitalapram, is 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile and exists in enantiomeric form. The structure of Citalopram is as follows.



The drug acts as a serotonin uptake inhibitor and is therapeutically categorized as an effective antidepressant. Citalopram is often used in cases of acute depression. It is usually employed as the hydrobromide which is crystallized from isopropanol.

It is usually monitored in blood or urine samples, using a chiral LC technique. It is necessary to resolve, and determine the enantiomers of the free Citalopram (CIT), its dimethylated metabolites (DCIT) and its didimethylmetabolites (DDCIT). A Chirobiotic V column (Vancomycin bonded to silica) was used for this purpose which was 25 cm long, 4.6 mm I.D., and packed with 5 μm particles.

The results obtained are shown in figure 11.23. Chromatogram (A) is from a 1 ml sample of mobile phase spiked with 100 ng racemic (CIT), 50 ng of racemic (DCIT) and 40 ng of racemic (DDCIT). It is seen that all the enantiomers are separated. Chromatogram (B) is from a 1 ml sample of serum taken from a patient routinely treated with 50 mg/day of (CCIT).

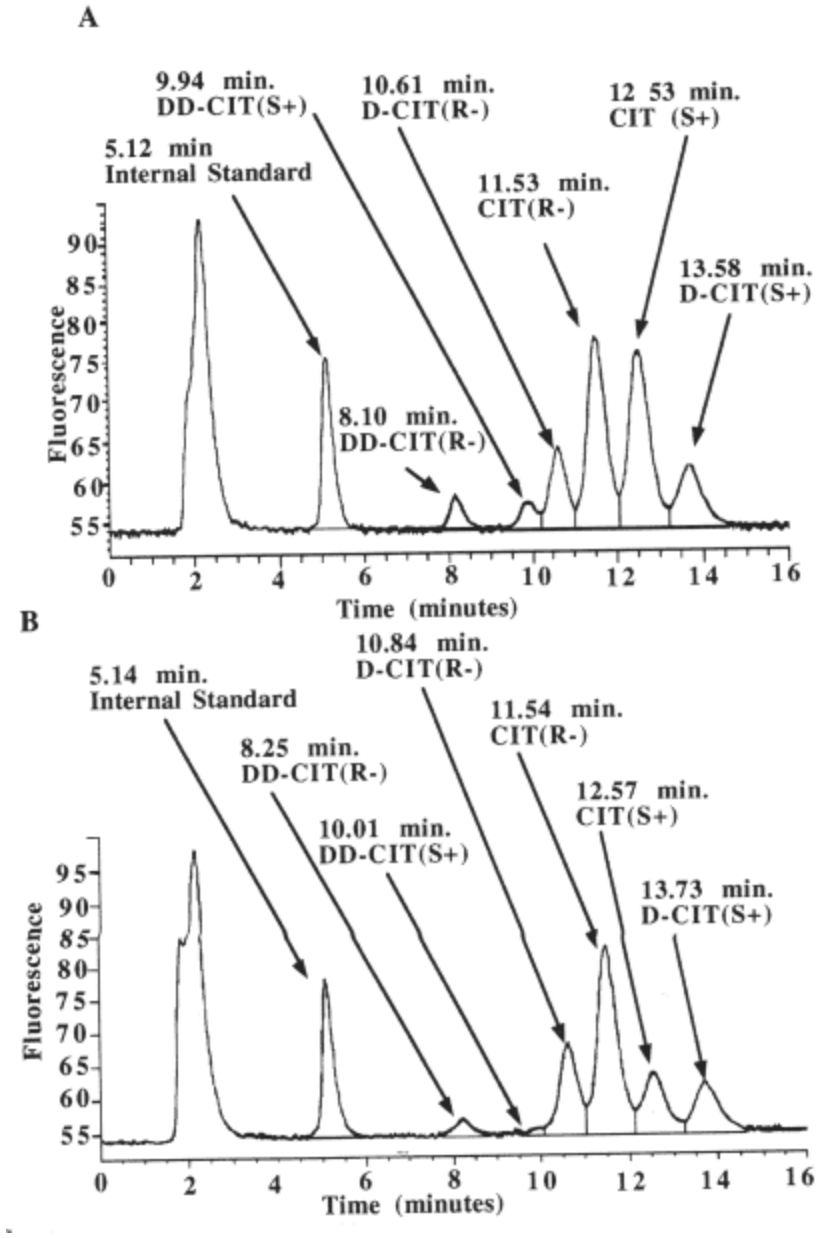


Figure 11.23
The Separation of the Enantiomers of Citalopram on a Vancomycin Stationary Phase
Courtesy of Dr. Markus Kosel and Dr. Pierre Baumann, University Hospital, Site de Cary, Prilly-Lausanne

The measured concentrations were 51.6 ng/ml R(-)-CIT, 17.5 ng/ml S(+)-CIT, 26.9 ng/ml R(-)-DCIT, 18.9 ng/ml S(+)-DCIT, 9.5 ng/ml R(-)-DDCIT and S(+)-DDCIT was below the limit for quantitative analysis. The chromatograms displayed in figure 11.23 are quite remarkable in that they display the separation of 6 enantiomers in a single analysis.

The Separation of the Isomers of 2- and 3-Bromo Phenylalanine

The isomeric bromophenylalanines were separated as their FMOC derivatives by reacting them with 9-fluorinylmethyl-chloroformate.

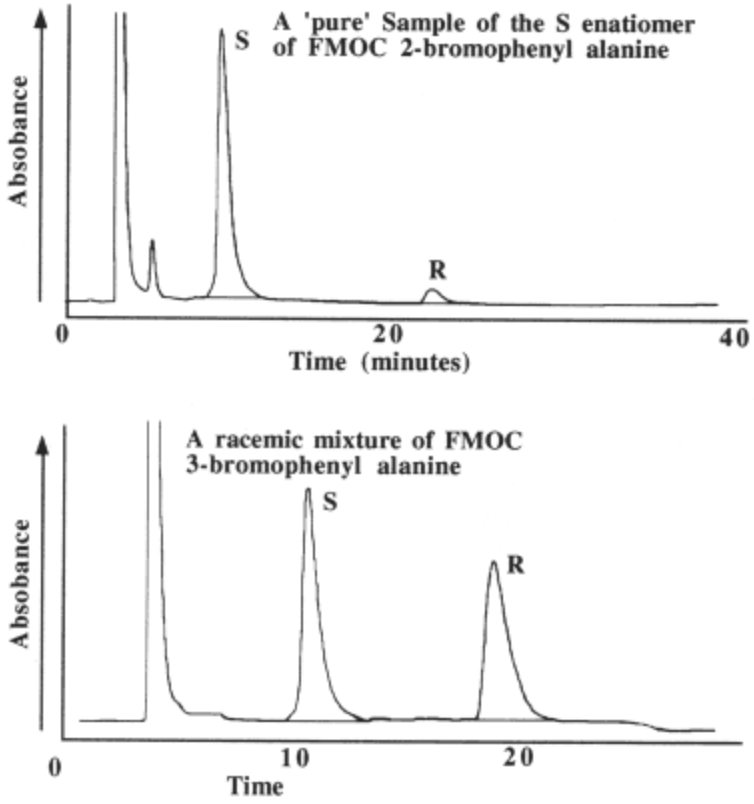


Figure 11.24
The Separation of the Enantiomers of 2 -Bromophenylalanine and 3-Bromophenylalanine
Courtesy of Chirotech Technology Ltd.

The separation was carried out on a CHIROBOTIC T column, 25 cm long, 4.6 mm I.D., packed with 5 µm particles. The mobile phase was programmed from methanol/1% triethylamine acetate (pH 4.5) : 40/60 v/v to methanol/1% triethylamine acetate (pH 4.5) : 60/40 v/v over 20 min. The flow rate was 1.0 ml/min and the sample solvent acetone. It is seen that the selectivity of the stationary phase was extremely high, the two enantiomers being very well separated. It is also interesting to note, that the 'pure' (S) enantiomer did, indeed, contain a significant amount of the (R) enantiomer. This type of high selectivity is often achieved with these type of stationary phases.

The Cyclodextrin Based Stationary Phases

The cyclodextrin based chiral stationary phases are some of the more popular materials used for contemporary chiral separations. One of their distinct advantages lies in their unrestricted and successful use with all types of solvent. In particular, they can be used very effectively in the reversed phase mode (a method of development that is not possible with some other chiral stationary phases) and, as well as being usable in a normal phase. They can also be used in the so-called *polar organic mode*, where the polar constituents of the mobile phase can be anhydrous diethylamine or glacial acetic acid, but in the complete absence of water. The cyclodextrins and their derivatives are widely used for all types of chiral separations, they have a good sample capacity, and can often be used for preparative separations. Cyclodextrin-based phases are readily available, usually covalently bonded to spherical silica gel particles 5 µm in diameter. There are numerous examples of the use of cyclodextrins in chiral separations and the following are some applications that illustrate their general use.

The Use of a Cyclodextrin Based Chiral Stationary Phase to Separate Blocking Agents in Conjunction with Optically Active Fluorescent Edman-Type Reagents to Enhance Detection

Toyo'oka and Liu [15] used the optically active Edman type fluorescent reagents, 4-(3-isothiocyanatepyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiaz-

ole [(r)-(-) and (S)-(+)-NBD-PyNCS] and 4-(3-(3-isothiocyanatepyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxydiazole [(R)-(-)- and (S)-(+)-DBD-PyNCS] to produce diastereomers that could be separated on a simple reversed phase column and, at the same time, provide enhanced sensitivity. The reagents reacted quantitatively with primary and secondary amino functional groups, under mild conditions (55°C for 10 min.), in the presence of triethylamine, to produce the corresponding fluorescent thiourea derivatives. The purity of the reagents were ascertained by separation on a cyclodextrin column and the results are shown in figure 11.25.

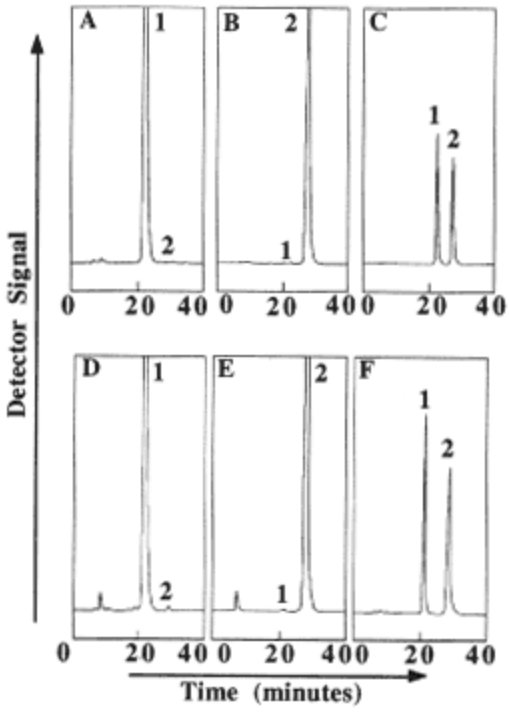


Figure 11.25
The Separation of Enantiomeric Pairs of
NBD-PyNCS and DBD-PyNCS on Utron
ES-PhCD, a Cyclodextrin Based Stationary Phase
Courtesy of Royal Society of Chemistry, [Ref. 15]

The separation was carried out on a derivatized cyclodextrin column (ES-PhCD) 15 cm long and 6 mm I.D., packed with 5 µm particles. Chromatogram A shows the elution of the (R)-(-)-NBD-PyNCS isomer, B, the elution of the (R)-(+)-NBD-PyNCS isomer and C, the separation of the racemic mixture. The mobile phase consisted of a mixture of acetonitrile/methanol/water : 3/3/4 v/v/v. Chromatogram D shows the elution of the (R)-(-)-DBD-PyNCS isomer, E, the elution of the (R)-(+)-DBD-PyNCS isomer and F, the separation of the racemic mixture. In this case, the mobile phase consisted of a mixture of acetonitrile/water : 8/2 v/v. It is seen that the cyclodextrin based stationary phase elegantly resolves both pairs of enantiomers. The separation of the diastereomers formed by reacting the reagents with some chiral drugs is shown in figure 11.26.

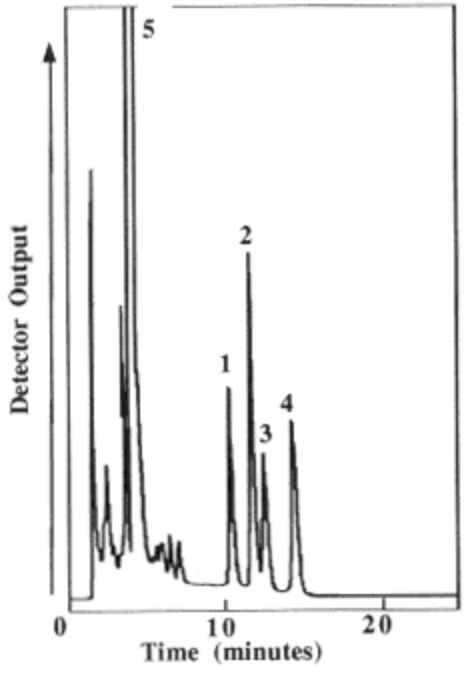


Figure 11.26
The Separation of the Diastereomers of Propanolol and Alprenolol on a Reversed Phase Column
Courtesy of Royal Society of Chemistry, [Ref. 15]

The column was the Intersil ODS 80A, 15 cm long, 4.6 mm I.D., packed with 5 µm particles. The largest peak in the chromatogram was the reagent itself. Peak 1 was (R)-(+)-Propanolol, peak 2 was (S)-(-)-Propanolol, peak 3 (R)-(+)-Alprenolol and peak 4 (S)-(-)-Alprenolol. Each peak, except that of the reagent, represents 25 ng of the original drug.

Determination of Enantiomers in Blood Serum by Direct Injection

In order to carry out *in vivo* pharmacological profiling of enantiomeric drugs, the direct analysis of biological fluids is highly desirable to reduce sample preparation time, and the chance of enantiomeric change. Unfortunately, many chiral stationary phases, such as the Pirkle types phases and the derivatized cellulose phases, demand the use of mobile phases that are incompatible with the biological fluids. Haginaka and Wakai [16] suggested that the silica should first be reacted with (3-glycidoxypropyl)trimethoxysilane, to cover a significant part of the silica surface with 'spacers', and then the remaining silanol groups reacted with cyclodextrin-carbamoylated triethoxysilane to attach the chiral agent.

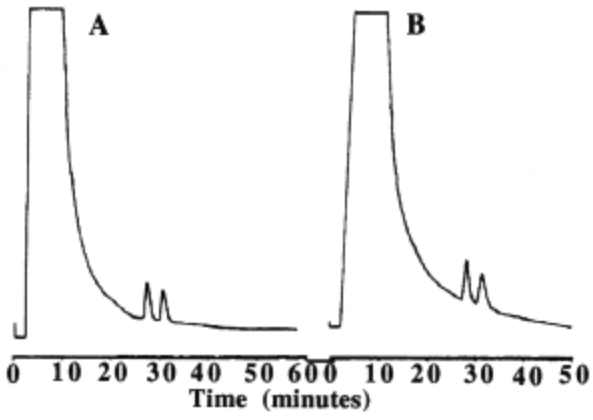


Figure 11.27
The Separation of the Hexobarbital Enantiomers Contained in Blood Serum by Direct Injection on a Cyclobond Phase
Courtesy of J. Liq. Chromatogr. [Ref. 17]

Subsequent treatment, would convert the epoxy group to diols that would insulate the analytes from the silica surface. Stalcup and Williams [17] analyzed a series of these type of materials and found that there was about 10 times as much spacer on the surface (*ca.* 2 μmol/m^2) as there was derivatized cyclodextrin (*ca.* 0.2 μmol/m^2).

This type of chiral stationary, however, proved to be very effective. The separation of the enantiomers of hexabarbital on this stationary phase by the direct injection of blood serum is shown in figure 11.27. Chromatogram A was obtained after 20 injections of serum and chromatogram B after 60 consecutive injections of blood serum. It is seen that here is very little column deterioration and that, although the tail of the major peak has become a little extended after 60 injections, the column could still be used very effectively for the analysis (it should be noted, that it is essential to employ titanium frits in the column, to prevent protein precipitation, which could occur on stainless steel frits).

The Use of 2-Quinoxal Chloride for Precolumn Derivatization in the Separation of Hydroxy Carboxylic Acids Enantiomers

A number of hydroxy carboxylic acids have been shown to exhibit significant biological activity and thus, they are frequently assayed in biological fluid for diagnostic purposes. The majority of the hydroxy carboxylic acids that have diagnostic use, exist in enantiomeric form, and it has been found that their enantiomeric ratio can provide some detailed information regarding the biological processes involved. Unfortunately, the hydroxy acids are somewhat difficult to detect in sufficiently low concentrations for general diagnostic use. Consequently, Brightwell *et al.* [18], used the fluorescing reagent, 2-quinoxal chloride to enhance detector sensitivity, and a derivatized β-cyclodextrin to achieve the separation of the isomers.

A sample of the acid was reacted at room temperature for 30 min with the same weight of the 2-quinoxal chloride in a 2 ml flask containing 1 ml of acetonitrile and 1 drop of triethylamine. In the case of acid-insolubility, small quantities of water were added until solubility was

achieved. The reaction mixture was diluted appropriately and 5 µ1 injected onto the column. The stationary phase was CYCLOBOND 1 2000SN, a β-Cyclodextrin derivatized with naphthylethylcarbamate. The column was 25 cm long, 4.6 mm I.D., and operated at room temperature. The mobile phase used for development consisted of a mixture of acetonitrile/triethylamine/acetic acid : 1000/5/2.5 v/v/v at a flow rate of 1 ml/min.

The separation of the enantiomers of lactic acid, caprilic acid, palmitic acid and hydroxyhexacosanoic acid are shown in figure 11.28.

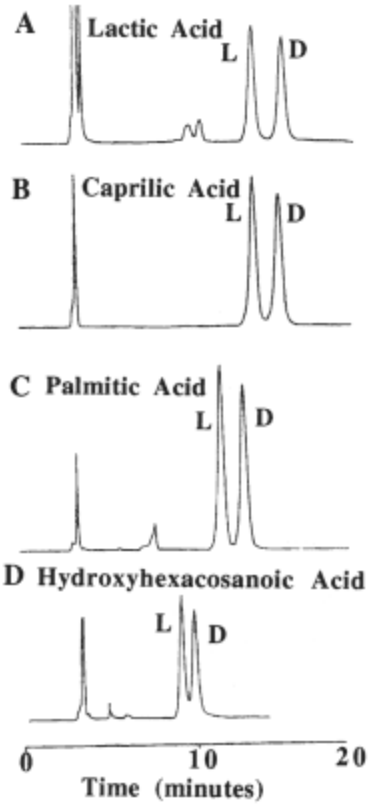


Figure 11.28
The Separation of Hydroxy Carboxylic Acids Using 2-Quinoxal Chloride for Precolumn Derivatization
Courtesy of the J. Liq. Chromatogr.
[Ref. 18]

It is seen that the retention and the chiral selectivity decreases as the solute becomes less polar and more dispersive in interactive character. This indicates that both the retention and chiral selectivity is largely dependent on polar interactions between solute and stationary phase. This technique was applied to the evaluation of some samples of (-)- and (+)-hexahydromandelic acid, are the results are shown in figure 11.29.

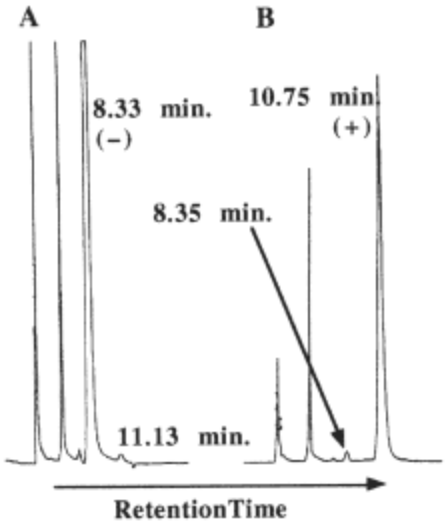


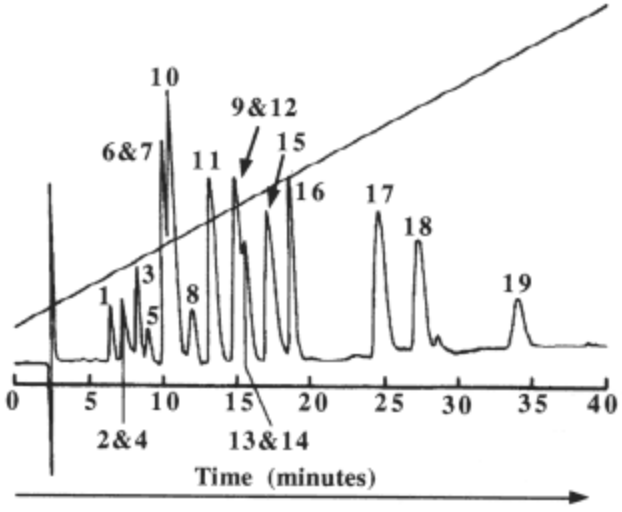
Figure 11.29
The Analysis of Some Commercial Samples
of Hexahydromandelic Acid
Courtesy of the J. Liq. Chromatogr. [Ref. 18]

It is seen that the peaks are symmetrical, there is excellent chiral selectivity, and there is more than adequate sensitivity for the accurate quantitative assay of the enantiomeric purity. In the analysis of lactic acid, the D-enantiomer could be detected at a level of 0.1 % w/w.

The Separation of the Chlorophenols on a b-Cyclodextrin Bonded Phase

Chlorophenols and their salts are manufactured throughout the world for use as fungicides, wood preservatives, antiseptics, bactericides and disinfectants. As a consequence of their extensive use, considerable amounts of chlorophenols are discharged in industrial effluents, into the

water system. Chlorophenols are not easily degradable and tend to persist in the environment, and as they are highly toxic, they can induce considerable health problems if not carefully controlled. In view of the importance of the chlorophenol assay, Paleologou *et al.* [19] developed an liquid chromatography procedure employing a β-cyclodextrin bonded phase to selectively retain the chlorophenols for their quantitative analysis. Although this analysis does not involve the separation of enantiomers, it does involve the separation of molecules of similar composition, but with different spatial structure (positional isomers). It was thought that the inclusion properties of the cyclodextrins would provide the necessary structural selectivity.



No.	Substance
1	2-Chlorophenol
2	3-Chlorophenol
3	4-Chlorophenol
4	2,6-Dichlorophenol
5	3,5-Dichlorophenol
6	2,4-Dichlorophenol
7	2,5-Dichlorophenol
8	2,3-Dichlorophenol
9	3,4-Dichlorophenol
10	2,4,6-Trichlorophenol

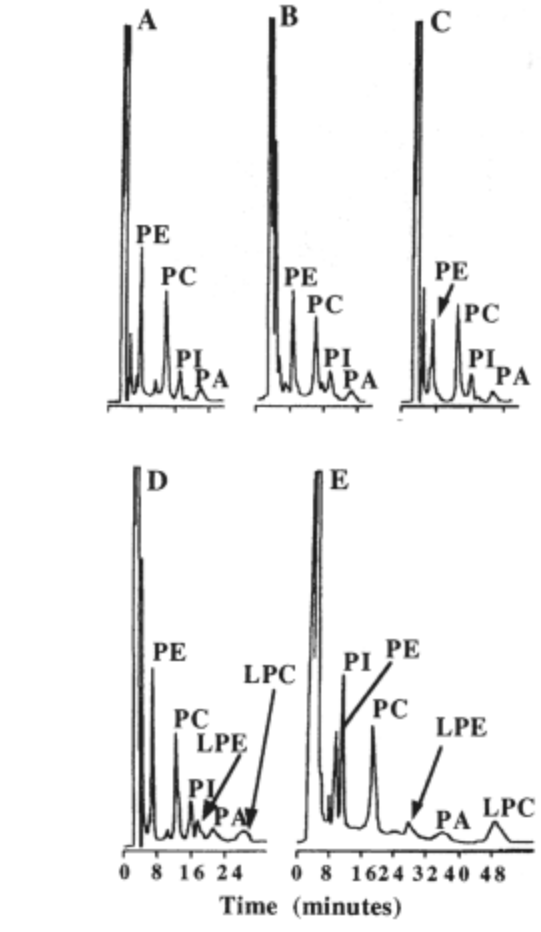
No.	Substance
11	2,3,6-Trichlorophenol
12	2,3,4-Trichlorophenol
13	2,3,5-Trichlorophenol
14	3,4,5-Trichlorophenol
15	2,4,5-Trichlorophenol
16	2,3,4,5-Tetrachlorophenol
17	2,3,5,6-Tetrachlorophenol
18	2,3,4,6-Tetrachlorophenol
19	Pentachlorophenol

Figure 11.30
The Separation of Monoaromatic Chlorophenols
Courtesy of the J. Chrom Sci. [Ref. 19]

In addition, as a result of the dimensions of the cyclodextrin cavity, it would also exclude substances that might possibly interfere with the analysis, and cause the chlorinated compounds to be eluted early in the chromatogram. The column was a CYCLOBOND 1, 25 cm long, 4.6 mm I.D., packed with 5 µm particles of derivatized silica gel. It was found that gradient elution was necessary to separate the maximum number of the chlorophenols in a reasonable time. The separation obtained is shown in figure 11.30. The separation was carried out at 50°C. and the gradient used was linear commencing with a solvent mixture comprising methanol/water : 27/73 v/v, the water containing 0.01 M triethylammonium acetate, buffered at pH 4.0. The methanol concentration was increased at 1% per min. until all the solutes were eluted. Under optimum conditions, however, it was found that only 15 of the 19 chlorophenols could be resolved and quantitatively assayed.

The Separation of Major Soybean Phospholipids on b-Cyclodextrin Bonded Silica

In plants that are used to provide oils, genetic engineering has yielded a number of interesting modifications that has improved both the quality and quantity of the oil produced. Phospholipids are important substances that occur in animal and plant cell membranes and are carried though into the oil. An accurate assay for such materials, and the distribution of the different species in modified oils, can indicate the quality and stability of the oil. There have been numerous analytical procedures developed for the analysis of phospholipids. However, with the advent of the derivatized cyclodextrin columns, Abidi *et al*. [20] developed a new method for assaying phospholipids in soya bean oil, using a normal phase development technique and a derivatized β-cyclodextrin as the stationary phase. It was found necessary to carry out a preliminary separation on silica gel, using a 10 min linear gradient from chloroform/t-butyl methyl ether : 75/15 v/v to methanol/chloroform/ammonium hydroxide : 92/1/7 v/v/v. The phospholipids were collected as a group for subsequent separation on the cyclodextrin column. A flow rate of 0.5 ml/min was employed.



PA	Phosphatidic Acid (egg)
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PC	Phosphatidylcholine
PL	(Standard)

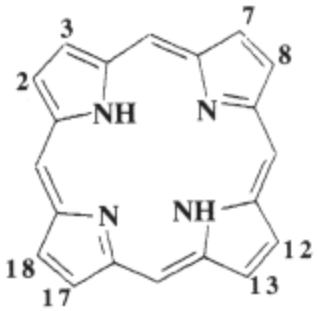
Figure 11.31
The Separation of the Phospholipids from
Genetically Modified Soya Bean Oils
Courtesy of the J. Liq. Chromatogr. (Ref. 20)

The collected sample was then separated on a derivatized β-cyclodextrin column 25 cm long, 4.6 mm I.D., and packed with particles 5 µm in diameter. The separation that was obtained is shown in figure 11.31. The sample 0.5 to 1.0 µl in volume and containing about 3 mg/ml, were injected onto the column. The mobile phase used was *n*-hexane/isopropanol/ethanol/water 5 mM tetramethylammonium phosphate, pH 6.3) : 35/32/726.8/5.5 v/v/v/v, which was replaced gradually with methanol/chloroform/ammonium hydroxide 92/1/7 v/v/v, over a period of 10 min. Chromatograms A, B, C and D are from different genetically modified soya bean oils, and Chromatogram E is from an unmodified soya bean oil. This example shows the use of the unique character of the cyclodextrin phase for separating materials that are neither chiral, or of such a size that inclusion plays a significant part in the retention mechanism.

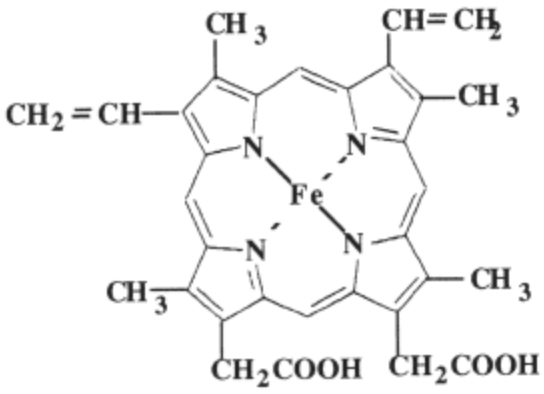
The Separation of Porphyrins on an g-Cyclodextrin Stationary Phase

In the biosynthesis of heme, the polycarboxylated porphyrins are essential intermediate metabolites.

The Basic Porphyrin Structure



The Heme Structure



Under certain pathological conditions, heme occurs free in tissues and it also occurs in normal tissues as the prosthetic group of a number of hemoproteins, such as hemoglobin, myoglobins, peroxidases, catalases and cytochrome c. It is that part of hemoglobin that gives it its color.

The excessive production of porphyrins and their excretion in biological fluids indicates that the heme synthetic pathway is disturbed. The basic porphyrin structure and the structure of heme is given above. A disturbance in the heme biosynthetic pathway can be of genetic origin or result from exposure to toxic chemicals. The relative levels of porphyrins in urine, stool or blood is necessary to diagnose, among other health disturbances, lead poisoning and iron deficiency anemia. There are five porphyrins found in normal urine that may contain four to eight carboxylic acid groups, *e.g*. copro-, pentacarboxyl-, hexacarboxyl-, heptacarboxyl- and uroporphyrin. The structure of these porphyrins are given in the following table.

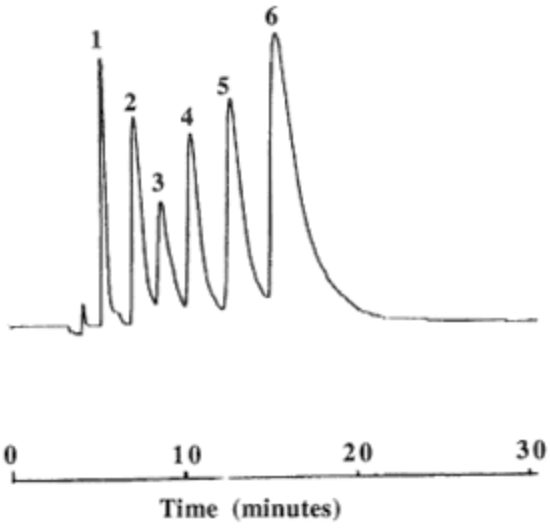
Table 11.4 Porphyrin Structures

				Side Chain Substitution				
Porphyrins	**2**	**3**	**7**	**8**	**12**	**13**	**17**	**18**
Unoporphyrin	A	P	A	P	A	P	A	P
Heptacarboxylporphyrin 1	M	P	A	P	A	P	A	P
Hexacarboxylporphyrin 1 (*cis*)	M	P	M	P	A	P	A	P
Hexacarboxylporphyrin 1(*trans*)	M	P	A	P	M	P	A	P
Pentacarboxyporphyrin 1	M	P	M	P	M	P	A	P
Coproporphyrin	M	P	M	P	M	P	M	P

where A = $-CH_2COOH$, P = $-CH_2CH_2COOH$, M = $-CH_3$.

Although native and derivatized cyclodextrins are normally used for separating enantiomers, they are also extremely effective for separating positional or structural isomers and other types of complex molecular configurations. In fact, many separations of spatial isomers are virtually impossible to resolve on conventional LC columns. Numerous separations involve inclusion in the cyclodextrin cavity, but this is not possible if the solute molecules are too large. However, recent studies suggest that the larger molecules may sit on the top of the cavity like a lid, without forming conventional inclusion complexes [22]. As a consequence, Wu and Stalcup [21], explored the use of the γ-cyclodextrins with the larger cavity for the separation of porphyrins by liquid chromatography with a view to developing a diagnostic test.

The column used was a CYCLOBOND 11 (γ-cyclodextrin), 25 cm long, 4.6 mm I.D., and packed with particles 5 µm in diameter. The separation was carried out at 18°C using a mobile phase consisting of a mixture of acetonitrile/buffer (140 mM phosphate, pH 6.9) : 25/75 v/v at a flow rate of 0.8 ml/min. An example of the separation obtained is shown in figure 11.32.



1	Uroporphyrin
2	Heptacarboxylporphyrin
3	Hexacarboxylporphyrin(1?)
4	Hexacarboxylporphyrin(2?)
5	Pentacarboxylporphyrin
6	Coproporphyrin

Figure 11.32
The Separation of Some Polycarboxylated Porphyrins
Courtesy of J. Liq. Chromatogr. (Ref. 22)

For diagnostic purposes, the determination of the porphyrin must be carried out on biological samples, such as blood serum and urine. Furthermore, ideally the actual fluid itself needs to be injected, to avoid the labor and time involved in extraction and sample preparation. As a result of the high water content of the mobile phase used with the γ-cyclodextrin in the separation of the porphyrins, it would be compatible

with aqueous biological samples. An example of the separation of some porphyrins, spiked into a sample of blood serum, and then separated on the γ-cyclodextrin column, using the conditions described for figure 11.32 is shown in figure 11.33.

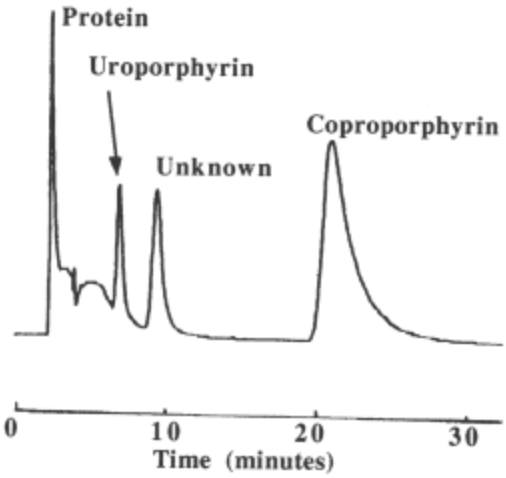


Figure 11.33
Chromatogram of a Serum Sample Spiked with
Uroporphyrin and Coproporphyrin
Courtesy of J. Liq. Chromatogr. (Ref. 22)

It is seen that a good separation is obtained and the method could easily be used quantitatively for diagnostic purposes.

Synopsis

All five major chiral stationary phases have a very wide range of applications. Nevertheless, the selectivity that they achieve is sometimes limited and the separation ratios of the enantiomers small. As a consequence, relatively high efficiencies are usually necessary to achieve the desired resolution. Examples of the use of the popular protein stationary phases are the separation of the Verapamil isomers, and the epibatidine and vamicamide enantiomers. These chiral stationary phases have also been used to separate optical isomers of a leukotriene antagonist. The Pirkle chiral stationary phases are also very well-liked as

they are easy to manufacture, are stable, and exhibit good chiral selectivity. Examples of their use that are discussed include the separation of the isomers of the derivatized amino acids and Naproxen and other Profen enantiomers. The effect of the chain length of the solvents used in the mobile phase are also demonstrated and it is shown that these stationary phases also separate other types of spatial isomers such as the fullerenes. Examples of the use of the cellulose and amylose stationary phases include the protected amino acids, several chiral drugs including Nicardipine, Diktiazem, Verapamil, Propanolol, Metroprolol and Atenolol and some anticonvulsants. Examples of the use of the more recent chiral stationary phases, the macrocyclic glycopeptides, are the three racemic substituted pyridones, a number of biologically active substances and Albuterol in blood plasma. The cyclodextrin based chiral stationary phases have the great advantage of being amenable to a wide range of solvents and thus can be easily used in the reverse phase or normal phase mode. Example of their use include some adrenergic blocking agents Propanolol, Alprenolol and the hexabarbital enantiomers. 2-Quinoxal chloride has been used as a fluorescence reagent for precolumn derivatization in the separation of hydroxy carboxylic acids enantiomers to improve the sensitivity. A number of spatial isomers have also been found to be well separated on the cyclodextrin bonded phases such as the chlorophenols and the porphorins and some soya bean phospholipids.

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Chapter 12—
Preparative Chiral Chromatography

Preparative chromatography is a complex subject and its comprehensive treatment would warrant a book to itself. However, preparative separations by chiral chromatography are becoming increasingly important. Consequently, a book on chiral chromatography must address the subject in a manner that will at least help the reader understand the basic problems associated with preparative separations, and to give some guidance as to how to isolate significant quantities of enantiomeric material.

The term preparative chromatography has a variety of meanings that depend somewhat on the complexity of the separation problem, and the type of application. To the biochemist, preparative chromatography may mean the isolation of a few milligrams of a substance for subsequent structure elucidation. To the organic chemist, preparative chromatography will often mean the isolation of 5–50 g of a pure intermediate for subsequent synthetic work. In preparative *chiral* chromatography, there are some special difficulties, or combination of difficulties and, in addition, some interesting advantages. Firstly, the substances to be separated are often very similar and have very small separation ratios. This means the stationary phase must be carefully chosen and the necessary efficiency obtained from the column. Secondly, the loading capacity of many chiral stationary phases, if not small, is certainly significantly smaller than those used in general LC. This means there will a limited choice of stationary phase Thirdly, in all probability, as a result

of the limited choice, the stationary phases that have appropriate selectivity, may not be ideal for preparative columns.

However, there are also some simplifying factors associated with chiral LC. For example, the sample for separation often contains only the two enantiomers of interest and thus constitutes a simple binary mixture. Under such circumstances there are no (or very little) contaminating substances present to eliminate, so that, in principle, the separation should be relatively straightforward. In addition, a simple binary mixture rarely requires gradient elution which is difficult and expensive on a preparative scale. A simple binary mixture also simplifies the operating process, and allows the chromatograph to be automatically managed and, under certain circumstances, permits significantly increased productivity.

Nevertheless, the operating procedure and the problems associated with preparative chiral chromatography are very similar to those met in general preparative work. Consequently, the general operating conditions and procedures in general preparative LC will be discussed, but particular emphasis will be given to those aspects that are important in chiral preparative work.

The Loading Capacity of a Column

The most common column system used in preparative chromatography is the axial flow cylindrical column and consequently this type of column will be considered first and in some detail. Radial flow columns, and simulated moving bed columns are also used very effectively in preparative chromatography, but these systems constitute very special and expensive apparatus. In addition, initial work with traditional columns will need to be carried out to evaluate and justify the necessary expense that is involved with these more sophisticated type of preparative separation systems.

It has been shown [1], that the maximum sample feed volume (V_i) or sample mass (M_i) that can be placed on a cylindrical column is directly proportional to the plate volume of the column:

$$M_i = A_m\sqrt{n}(v_m + Kv_s)$$

or:

$$V_i = A_v\sqrt{n}(v_m + Kv_s)$$

where K is the distribution coefficient of the solute,

(v_s) is the volume of stationary phase per plate,

(v_m) is the volume of mobile phase per plate,

(n) is the column efficiency,

(A_v) is a constant for sample volume,

and (A_m) is a constant for sample mass.

Now:

$$V_i = A_v\sqrt{n}(v_m + Kv_s)$$
$$= A_v\sqrt{n}\, v_m(1+k)$$

From the Plate Theory [1], $V_m = nv_m$, where (V_m) is the total volume of mobile phase in the column or the column dead volume.

Thus:

$$V_i = A_v\frac{V_m}{\sqrt{n}}(1+k)$$

Now:

$$V_m = \varepsilon\pi r^2 L$$

where (r) is the radius of the column,

(L) is the length of the column,

(ε) is the fraction of the column containing solvent.

Thus:

$$V_i = A_v \frac{\varepsilon \pi r^2 L}{\sqrt{n}}(1+k)$$

And as (e) is approximately 0.6:

$$V_i = A_v \frac{1.9 r^2 L}{\sqrt{n}}(1+k)$$

and:

$$M_i = A_m \frac{1.9 r^2 L}{\sqrt{n}}(1+k) \qquad (1)$$

The magnitude of (A_v) will be determined by the separation that is required, and (A_m) will be defined by both the loading capacity of the stationary phase and the shape of the adsorption isotherm.

Although, apparently simple, equation (1) has some very significant implications on preparative column design. It is clear from equation (1) that increasing radius and length of the column increases both the maximum sample volume and the maximum sample mass. However, increasing the column length will also increase the columns efficiency (unless the particle diameter is also increased).

This is all fairly obvious, but it is also apparent that increasing the column efficiency will have the opposite effect, that is, it *reduces* the maximum sample load. It follows that, having attained the necessary efficiency to achieve the required separation, then if the column is lengthened to increase the loading capacity for optimum performance, either the flow rate will need to be increased to reduce the efficiency and thus maintain the maximum loading, or the particle size will need to be increased to reduce the efficiency to its required value. Now, increasing the flow rate will also reduce separation time and thus increase productivity. Nevertheless, the alternative use of larger particles provides a more permeable column and thus can operate at a lower pressure and be constructed of lighter materials. In addition, as the sample is usually a simple pair of enantiomers, using automatic operation and injection on a long permeable column will permit multiple sampling, so that there can be a number of enantiomer pairs in the column being separated and eluted at any one time. This arrangement will also increase productivity.

To continue with the basic examination of factors that effect the efficient operation of preparative columns, the maximum sample volume will now be considered. The maximum sample volume is that which will limit the

loss of resolution to an acceptable and predefined level. An equation needs to be derived that will allow the maximum sample volume to be calculated.

The Maximum Sample Volume

Any finite volume of sample placed on to an LC column will have a band variance, and this variance will contribute directly to the final peak variance that results from the dispersion processes that take place in the column. It follows that the maximum volume of sample that can be placed on the column must be limited, or the column efficiency will be seriously reduced.

Consider a volume (Vi), injected onto a column, which will form a rectangular distribution on the front of the column. The variance of the peak eluted from the column will be the sum of the variances of the injected sample plus the normal variance of the eluted peak.

Thus:

$$\sigma^2 = \sigma_i^2 + \sigma_c^2$$

where σ^2 is the variance of the eluted peak,

σ_i^2 is the variance of the eluted sample,

and σ_c^2 is the variance due to column dispersion.

Now the maximum increase in band width that can be tolerated due to any extraneous dispersion process is obviously a matter of choice, and may be defined by the nature of the separation, and the purity of the required product. However, Klinkenberg [2] suggested a 5% increase in standard deviation (or a 10% increase in peak variance) was the maximum extra-column dispersion that could be tolerated without serious loss in resolution. This criteria is generally accepted in analytical LC.

Now the variance of the rectangular distribution of sample volume at the beginning of the column is $\left(\frac{V_i^2}{12}\right)$ and assuming the peak width is increased by 5% due to the sample volume:

$$\frac{V_i^2}{12} + \left(\sqrt{n}(v_m + Kv_s)\right)^2 = \left(1.05\sqrt{n}(v_m + Kv_s)\right)^2$$

Thus:

$$\frac{V_i^2}{12} = n\,(v_m + Kv_s)^2\left(1.05^2 - 1\right)$$

$$= n\,(v_m + Kv_s)^2 0.102$$

Consequently:

$$V_i^2 = n(v_m + Kv_s)^2 1.23$$

$$V_i = \sqrt{n}\,(v_m + Kv_s)\,1.1$$

Bearing in mind that:

$$V_r = n(v_m + Kv_s)$$

Then:

$$V_i = \frac{1.1\,V_r}{\sqrt{n}}$$

It is seen that the maximum sample volume that can be tolerated can be calculated from the retention volume of the solute concerned and the efficiency of the column. It is also seen, that the sample volume increases with the retention volume of the solute, and inversely as the square root of the efficiency.

Thus, the sample volume can be increased by adjusting the mobile phase so that the solute pair is eluted later. Seemingly, this would also increase the separation time, but as will be seen later, due the somewhat unique nature of most chiral separations, this may often be used to a production advantage. The calculation of maximum sample volume can be important, if the separation ratio of the enantiomer pair is small, and adequate resolution is only just attained. If, however, the stationary phase provides good chiral selectivity, with base line between the peak, then column overload could be justified.

Sample Volume Overload

Consider the separation depicted in figure 12.1 the retention parameters are labeled according to the plate theory as discussed in chapter 1. It is clear that the column could be heavily overloaded, to allow the peaks to disperse until they touched at the base, before resolution would be lost. Under these conditions the summation of variances cannot be used, as when the sample volume becomes excessive, the dispersion of the peak becomes, to the first approximation, the volume of the sample itself. Presented in a different way, the sample volume acts as a part of the mobile phase and contributes to the elution process in the same manner.

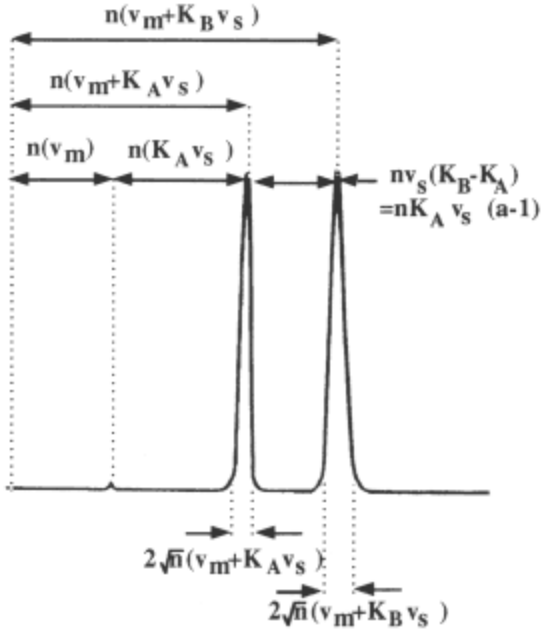

Figure 12.1
Theory of Volume Column Overload

Referring to figure 12.1, the peak separation in milliliters of mobile phase will be equivalent to the sample volume plus the sum of half the base widths of the respective peaks. Bearing in mind that half the peak width is equivalent to two standard deviations, then:

$$(\alpha-1)\,n\,K_A v_s = V_L + 2\sqrt{n}(v_m + K_A v_s) + 2\sqrt{n}(v_m + K_A v_s)$$

(K_A) and (K_B) are the distribution coefficients of solutes (A) and (B) and (α) is the separation ratio of solutes B and A respectively.

Rearranging:

$$V_L = (\alpha-1)\,n\,K_A v_s + 2\sqrt{n}\left((v_m + K_A v_s) + (v_m + K_A v_s)\right)$$

Noting that:

$$nK_A v_s = V'_A,\ nK_B v_s = V'_B,\ \frac{V'_A}{V_o} = k_A,\ \frac{V'_B}{V_o} = k_B,\ k'_B = \alpha k'_A$$

and $V_o = nv_m$

$$V_L = V_o\left[(\alpha-1)k'_A - \frac{2}{\sqrt{n}}(2 + k'_A + \alpha k'_A)\right] \qquad (2)$$

Taking a practical example of a column 40 cm long, 2 cm I.D., having a dead volume of 75.4 ml ($\varepsilon\,\pi\,r^2 1$), the maximum sample volume can be calculated for a range of separation ratio values (α) and capacity ratio values (k) for the first eluted peak. The results are shown in figure 12.2

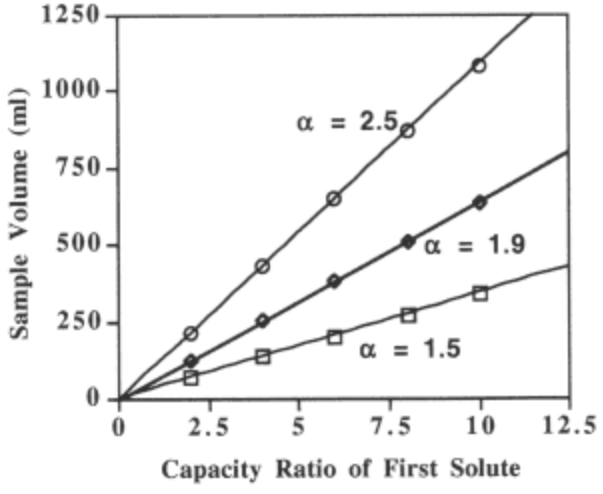

Figure 12.2
Graph of Maximum Sample Volume against (k')
for the First Solute

It is seen that, for the defined column packed with 20 µm particles, and operated at its optimum velocity to give 12,500 theoretical plates, if the separation ratio is as small as 1.5, and the first solute is eluted at a (k') value of 2.0, a volume as large as 66 ml can be still placed on the column. At the other extreme if the first solute is eluted at a (k') value of 10 and the separation ratio is 2.5 then the sample volume can be over 1 liter. It should be pointed out however, that the mathematical argument inherently presupposes that sample is injected onto the column as a *solution, in the mobile phase*. That is, the sample solvent does not change the elution conditions in any way. Also, inherent in the argument, is that the solute concentration in the sample solution is not strong enough to produce significant *solute/solute interaction* in the mobile phase and thus effect the conditions of elution.

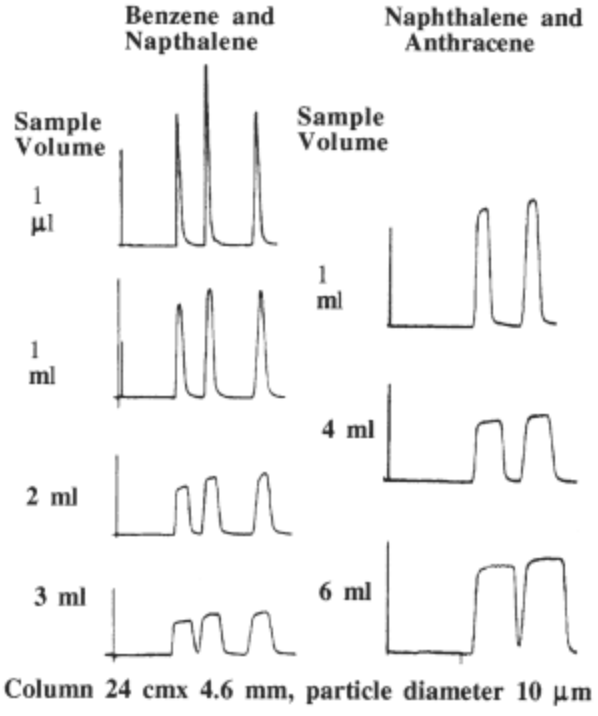

Figure 12.3
An Experimental Example of Volume Overload
Courtesy J. Chromatogr., Ref. [3]

An experimental example of column overload was demonstrated by Scott and Kucera [3] is shown in figure 12.2. The column dead volume was *ca* 2.5 ml. The chromatographic properties of the three solutes chromatographed on the column at 1 ml/min are shown in table 1.

Table 12.1 Chromatographic Properties of the Three Solutes Separated on the Column Used for Overload Experiments

	Benzene	**Naphthalene**	**Anthracene**
Capacity Ratio **k**	1.18	2.33	4.31
Efficiency **n**	1850	4480	5470
Retention Ratio **a**	—	1.97	1.85
Sample Volume $\mathbf{V_L}$	3.1 ml	6.1 ml	—

It is clear, that the column was operated well above its optimum flow rate so the theoretical efficiencies were not realized. In addition, dispersion in the sample loop also appears to reduce the efficiency of the two first peaks and it is not until the (k') of the solute reached 4, or more, were reasonable efficiencies realized. Nevertheless, the results clearly demonstrate the advantages of column overload. Referring to the chromatograms on the left hand side of figure 12.3, 10 µ1, 1 µ

l, 2 ml, and 3 ml of sample was placed on the column each containing 176 µg of benzene, 9 µg of naphthalene and 0.3 µg of anthracene. So all the samples contained the same mass of solute. It is seen that a sample volume of 3 ml (calculated from equation (2), *viz*. 3.1 ml) just permits the separation of benzene and naphthalene.

In a similar manner, 2, 4 and 6 ml of a solution each containing 9.0 mg of naphthalene was injected onto the column and the chromatograms obtained are shown on the right hand side of figure 12.3. It is seen that the experimental sample volume of 6 ml (6.1 ml calculated from equation 2) just permits the separation of naphthalene and anthracene. Destefano and Beachel [4], investigated the effect of volume and mass overload on resolution and concluded that it is better to overload a column with a large volume of a dilute solution of sample, than to

employ a small volume containing a high concentration of sample. The validity of this conclusion, however, appeared to depend on the capacity ratios of the eluting solutes.

Sample Mass Overload

The effect of excessive sample mass on the chromatographic process is extremely complex. The theory of mass overload is also intricate [5–7] and requires a considerable amount of basic physical chemical data, such as the adsorption isotherms of each solute measured over a wide range of concentration, before it can be applied to a practical problem. Unless the production size, and the production economy, will support the necessary basic data gathering, the problem of mass overload is more conveniently and thriftily approached from a simple experimental stance.

When a large mass is placed on an LC column, a number of effects can take place, depending on the ultimate concentration of solute at the point of injection. Initially, as the concentration of solute in the stationary phase increases, solute-solute interaction takes place, and the adsorption isotherm becomes non linear. A non linear isotherm will cause peak dispersion as well as peak distortion (peak asymmetry). As the sample mass is increased further, the concentration of solute in the mobile phase will also increase, although, assuming the solutes are reasonably retained, not to the same extent as that in the stationary phase. This is because in LC, the distribution coefficient is strongly in favor of the stationary phase. Nevertheless, the high concentration of solute in the mobile phase, modifies the mobile phase in much the same way as *another solvent* and thus increases the migration rate of the other solute(s) in the mixture. On, yet further increase in sample mass, solute-solute interaction can start to take place in the mobile phase causing an increase in elution rate for all the solutes.

These complex effects can be best illustrated by experiment. The same column was used for mass overload experiments as that employed in the volume overload experiment. In this case, the sample volume was kept

constant at 200 µ1, and a mixture of benzene, toluene and anthracene was placed on the column, the mass of benzene being increased progressively from 180 µg to 16.9 9 mg. An example of three of the chromatograms obtained are shown in figure 12.4.

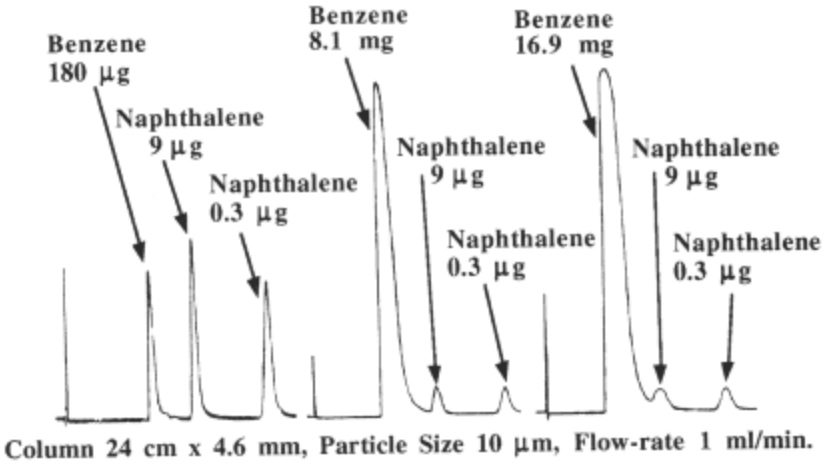

Figure 12.4
An Experimental Example of Mass Overload.
Courtesy J. Chromatogr., Ref. [3]

The mass of naphthalene and anthracene was kept at 9.0 µg and 0.3 µg respectively for all samples. Visual inspection of the chromatograms indicate that the benzene peak has broadened, and become asymmetrical, a direct result of the nonlinear adsorption isotherm. It is also seen that up to 16 mg of benzene could be injected onto the column before the peak merged into that of naphthalene. In fact, by sacrificing a small amount of benzene significantly more benzene (probably at least 30 mg) could have been injected before the major portion of the peak was contaminated with naphthalene. This compromise, which involves sacrificing a small quantity of the selected component to obtain a larger load, is common in preparative chromatography, and arises from the tailing that again results from the formation of a-non linear adsorption isotherm. In samples where the two components are present in the mixture at similar levels and are less well resolved, as in the separation of a pair of enantiomers, this compromise must be taken with caution. As both peaks

will be overloaded, and asymmetrical, the tail of one peak will merge with a high concentration at the sharp front of the following peak, and thus significant contamination of the second peak will occur. The results of the overload experiment are better examined quantitatively. Curves relating the retention distance of the front and back of each peak to the sample load are shown in figure 12.5.

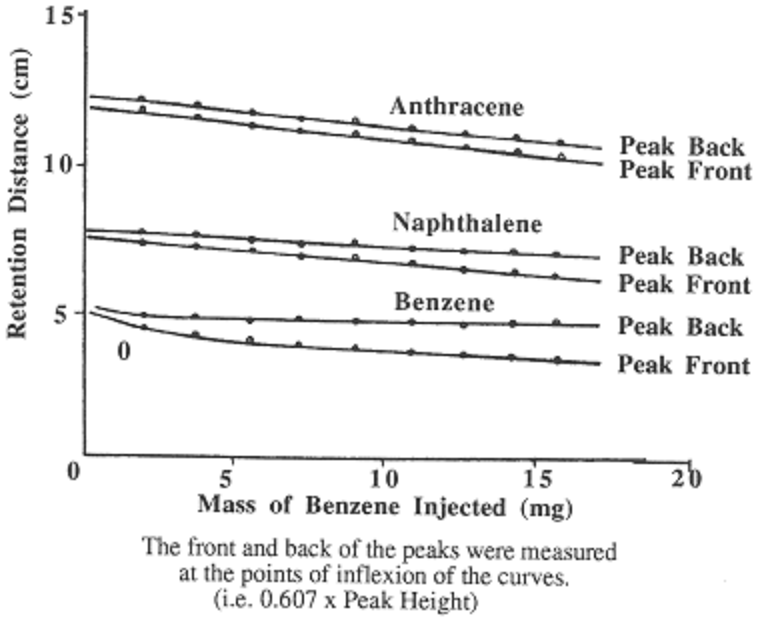

Figure 12.5
The Effect of Mass Overload on Retention
Courtesy J. Chromatogr., Ref. [3]

The different effects of mass overload are now clearly revealed. Firstly, it must be emphasized that the curves in figure 12.5 represent the movement of the peak extremes and are in no way related to the peak shape. It is seen that the retention of the rear of the major peak, benzene, hardly changes with sample mass as this represents low concentrations of benzene and thus is eluted in the normal manner. However, the retention of the peak front is reduced progressively as the sample mass is increased. This results from both the formation of a non-linear adsorption isotherm and the increased elution strength of the mobile

phase in contact with the benzene. In fact, as a result of solute-solute interaction the benzene is, in effect, *partially eluting itself*.

The effect of the mass overload on the other solutes is also clearly demonstrated, the presence of the high concentration of benzene in the mobile phase increases the elution rate of both the naphthalene and the anthracene. Its also seen, however, that the effect of the high concentration of the benzene on the closer eluting peak naphthalene is to produce band dispersion, whereas the anthracene band does not suffer significant dispersion and the retention of both the front and the rear of the anthracene peak are linearly reduced with sample mass. The chromatograms shown in figure 12.4 also show that the anthracene peak maintains its symmetry throughout all sample sizes. The impact of the high concentration of benzene on the elution of the other solutes only occurs while the solutes are still in contact with one another at the beginning of the column. Once the separation has started to develop, and the three solutes are no longer in contact, then the naphthalene and anthracene will be eluted in the normal manner. This also explains the significant band dispersion of the naphthalene peak, as, being closer to the benzene peak, experiences the effect of the high concentration of benzene. The increased dispersion due to mass overload on closely eluting peaks is of particular importance in the separation of enantiomers and may become the major load limiting factor. It follows, that the phase system that provides the highest separation ratio should be used, irrespective of any increased and, apparently unacceptable, retention time, as this can be compensated by multi-sample development.

Preparative Chromatography Apparatus

The conventional preparative chromatograph, although certainly more massive, is generally less complex than the analytical chromatograph. Although gradient elution has been used in preparative chromatography, it is avoided, if possible, due to the cost of solvents and solvent recovery.

As a consequence, gradient facilities are not usually fitted to preparative chromatographs. In addition, complex data acquisition systems are not required although programmable fraction collectors are usually necessary. The layout of a preparative chromatograph is depicted in figure 12.6.

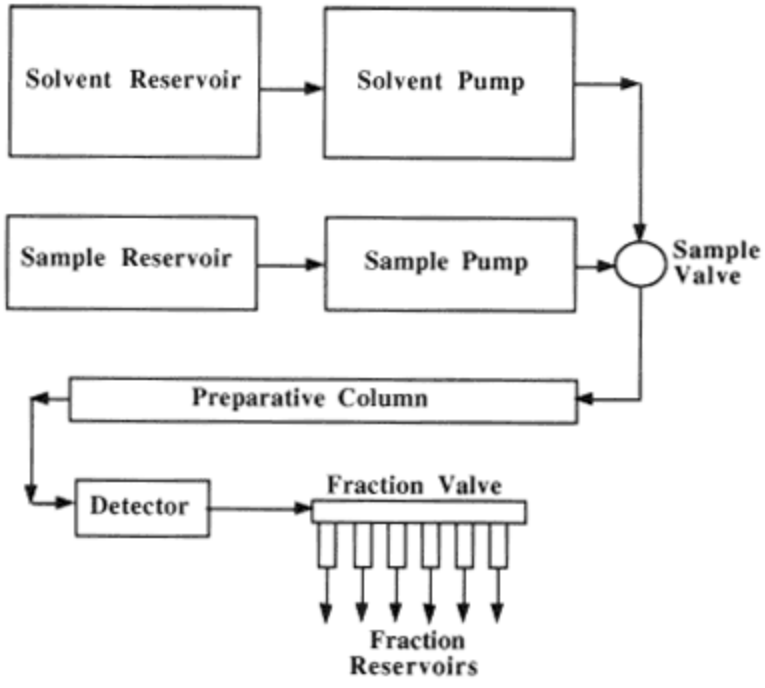

Figure 12.6
The Basic Preparative Liquid Chromatograph

Solvent Reservoirs

The solvent reservoir is usually made of glass or stainless steel and should have an appropriate capacity, normally several gallons. In general, most of the parts of a preparative chromatograph are made from stainless steel, however for biologically sensitive, or labile substances, biocompatible materials may be necessary for certain parts of the apparatus. To fabricate the bulky preparative components (*e.g.* the column) from titanium that have sufficient strength can become

inordinately expensive and plated base metal (*e.g*. using gold or some other suitable biocompatible metal) may be a more economic alternative.

Pumps

The pump must have adequate capacity, and for columns up to 1 in. diameter, pumping rates of at least 150 ml/min should be available. For larger columns, pumping rates of 500 ml to 1 liter per min may be necessary. Owing to the limited strength of large diameter columns pressures above 6000 p.s.i. are rarely required. Any glands involved in the pump should be made from appropriately inert material (*e.g*. Teflon). In preparative chromatography, the sample is usually placed on the column with a separate pump having its own sample reservoir, usually 1–2 *l* capacity. The required volume of sample is placed on the column by pumping the sample solution at a known rate, for a defined time. In addition, to the column and subsequent apparatus, the sample pump and reservoir also needs to be constructed of biocompatible material, if biosensitive materials are being separated.

Sample Valves

Sample valves, with appropriate loops, can also be used for smaller preparative separations, and sample loop capacities of 20 to 30 ml are practical. Above these volumes, however, a sample pump is recommended. It should also be pointed out that there can be considerable dispersion in open tubes, and the sample loop should not be left in-line during development. The valve should be returned to the 'flush' position immediately after injection, so that the 'tail' of the sample left in the loop does not cause dispersion and tailing on the column.

Preparative Columns

Preparative columns are usually made of stainless steel and must be designed to accommodate the inlet pressure necessary to obtain the required flow rate through the packed bed. This will be determined by the size of the particles selected for the packing. The larger the column diameter, the stronger must be the column and the thicker the walls. The

construction of wide columns (3 in. O.D. and greater), irrespective of the packing, can be extremely expensive and the cost must be included in the design considerations. Column having diameters greater than 0.5 in. need to have the frit supported on a suitable grid, as the frit material has limited strength. The porosity of the frit will be determined by the particle size of the packing. In order to minimize the pressure drop across the frit at high flow rates, the frit porosity should not be made unnecessarily small.

Preparative Detectors

Detectors that are used for preparative work must be able to function linearly at very high solute concentrations. The refractive index detector is probably the most useful of the analytical LC detectors for preparative work, but even at its lowest sensitivity setting, it may still be too sensitive. The multi wavelength UV detector can sometimes be used providing its conduits can tolerate the high flow rates. A very short path length cell can be installed, or a variable path length cell can be used. Another option is to set the wavelength at that which the solutes have very small extinction coefficients. If possible, a detector specifically designed for preparative work should be used, but there are a limited number of these available.

Fraction Collectors

Fraction collection in preparative chromatography is usually achieved using a multiport valve and a number of collection vessels. The selector valve should be programmable on the basis of time, or be actuated by the detector output signal (preset at the appropriate signal level or signal derrivative) or, preferably, both. The valve should have at least six ports, or preferably ten. If the system is to be used solely to separate enantiomer pairs, then a six port valve would usually be adequate. Alternate ports of the valve should be connected to waste to reduce the volume of solvent that must be evaporated during product recovery. In addition, this simplifies solvent recovery for future separations. Product recovery when using normal phase solvents is best carried out by bulking

the fractions and removing the solvent in a rotary evaporator under reduced pressure. For reverse phase solvents that have a high water content, recovery can be best achieved by passing the fraction through a reverse phase, C18, column of high capacity. The solute and solvent is adsorbed, and the solute and solvent content of the fraction can be recovered by displacement with another solvent, and the solute recovered by evaporation.

Solvent Hazard

Unless solvent recycling can be employed, which is not always possible, the operation of large diameter columns inevitably involves the use of large quantities of solvent. It follows that there is a possibility of both fire and toxicity hazards. The solvent should be selected with care and if possible the entire chromatograph, including the solvent supply, electrically grounded, and the apparatus including fraction reservoirs should be located in a walk-in fume hood.

Packing Preparative Columns

There are a number of techniques available to the chromatographer that can be used to pack a preparative column. The best and most appropriate method will depend on the particle size of the packing, the scale of the separation and the nature of the material to be separated. Contrary to popular belief, there is no magic associated with column packing, but the procedure does need some experimental skill, patience, and experience helps. It also can require some expensive apparatus, if slurry packing is to be used. Consequently, before embarking on a preparative column packing project, the relative cost of purchasing commercially packed columns should be examined. The basic phase system to be used with a preparative chiral columns should be established using analytical scale columns. If the particle size is 20 μm or less, the column must be slurry packed, using similar techniques to those employed for analytical columns. Care must be taken not to operate at pressures in excess of the bursting strength of the tube used for the column. As the column diameter increases, the maximum permissible pressure rapidly falls

unless extremely thick walled tubing is used. The safe maximum pressure for any tube can usually be obtained from the tube suppliers.

If particle sizes in excess of 20 m are used, then the column can be dry packed, with appropriate longitudinal and radial sonic vibration. The variance per unit length obtainable from a preparative column should be less than 2 particle diameters (determined using analytical scale samples). It is worth remembering that, when designing preparative columns, it is better to obtain the necessary efficiency using a longer column packed with larger particles, than the converse. The long column will permit much larger charges (as already discussed) and will also allow multiple sample development techniques. In addition, the larger particles will provide greater column permeability, and thus lower pressures can be used. Lower pressures will, in turn, allow lighter and less expensive materials to be used in the construction of the preparative system.

The denigrating effect of 'bridging' that can occur when packing wide diameter columns has already been discussed in detail, together with mechanical methods of packing. A review of these methods has been given by Colin *et al*. [8].

Recycling Development

A method for improving the resolution of a pair of closely eluting solutes (such as enantiomers), on a preparative column of limited length, is to employ the technique of recycling. After the sample has been placed on the column, the eluent from the column is switched to the pump inlet and thus the mobile phase is continuously circulated round the column. This means that the column is used many times, and each time the sample passes through the column, the resolution is improved. Unfortunately, the resolution is not necessarily proportional to the number of cycles, as significant peak dispersion can occur each time it passes through the pump. Nevertheless, there is a substantial net gain in resolution on each cycle. This procedure can be very time consuming if long retention times are involved, but has the advantage of considerable solvent economy. The recycling procedure, in effect, artificially increases the column length and actually trades in *time* for *solvent economy.*

An example of the recycling process is demonstrated in the separation of the Warfarin enantiomers as shown in figure 12.7. The chromatogram shows the effect of three cycles. In the first cycle there is little or no visible separation. On the second cycle, the two enantiomers are beginning to separate. In the third cycle, the separation is improved further, and is sufficient to allow the collection of significant quantities of the individual isomers at a high purity. It is also seen that the process is fairly rapid as three cycles are completed in less than 12 minutes.

The separation was carried out on a CYCLOBOND 1 2000 column, 25 cm long, 1 in, I.D., using a mobile phase consisting of methanol/acetic acid/triethylamine : 100/0.3/0.2 v/v/v, at a flow rate of 12 ml/min.

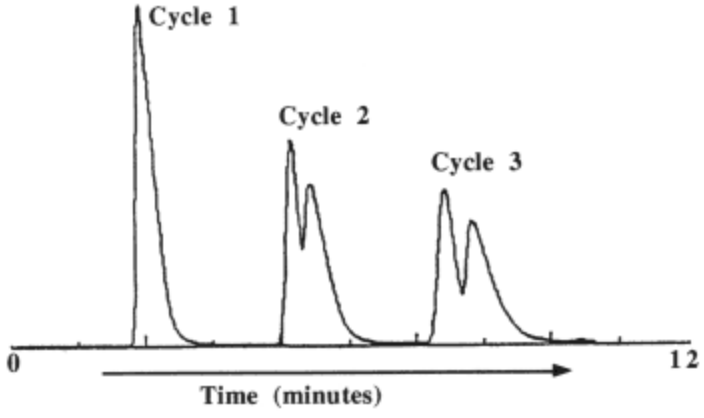

Figure 12.7
The Separation of the Warfarin Enantiomers by Recycling
Courtesy of ASTEC Inc.

An additional example of the technique of recycling, which includes a procedure called *peak shaving*, is the preparative separation of the enantiomers of 5-methyl-5-phenylhydantoin. The recycling and peak shaving procedure is depicted in figure 12.8. The column was 30 cm long, 2 in. I.D., packed with 10 m particles carrying Vancomycin as the chiral stationary phase. The mobile phase was ethanol and the flow rate 100 ml/minute. The sample load was 400 mg dissolved in 5 ml of ethanol.

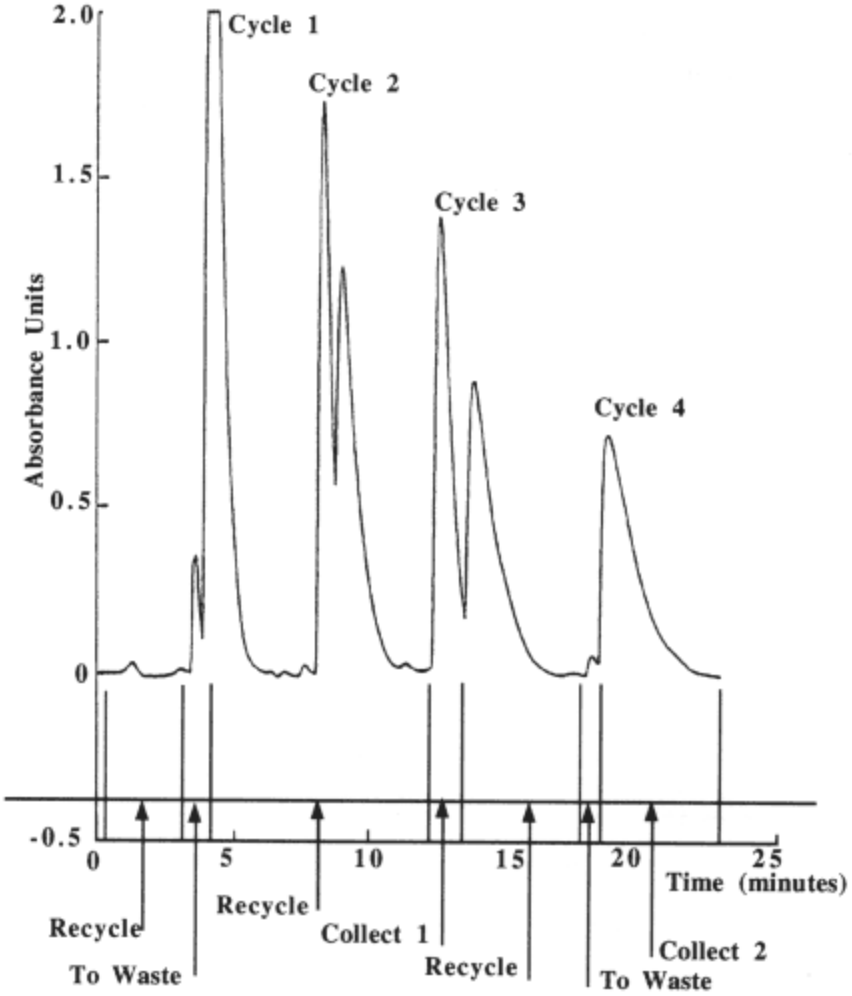

Figure 12.8
The Preparative Separation of the Enatiomers of 3-Methyl -5-Phenylhdantoin
Courtesy of ASTEC Inc.

It is seen in figure 12.8 that after the first cycle, there is very little resolution of the enantiomers, but an impurity is separated on the front of the composite peak. This peak is diverted to waste (shaved from the main peak). After the second cycle, the separation of the enantiomers has

begun, although insufficiently resolved for peak collection to be initiated. After the third cycle, the first major peak is shaved from the composite peak and, as the overloaded peak tails, the first peak will be collected virtually pure. The second peak will remain contaminated with a small amount of the first peak. After the third cycle is complete, the trace of the first peak is shaved from the major peak and passed to waste, and the remainder of the peak collected. It is seen that the four cycles are completed in less than 25 min and thus the output of the system would be nearly 1200 mg per hour. The recovery of each enantiomer was 98% and had an enantiomeric excess of 98%.

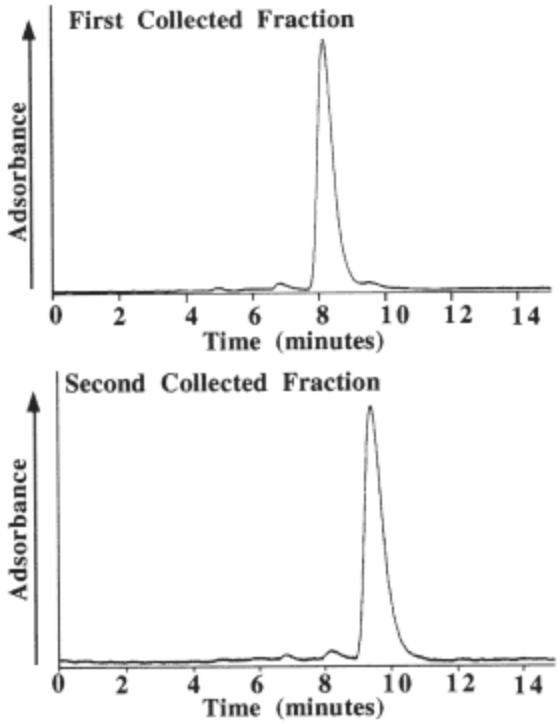

Figure 12.9
Chromatograms of the 5-Methyl-5-Phenylhydantoin Enantiomer Fractions
Courtesy of ASTEC Inc.

In addition, this procedure can be fully automated, providing the recycling and collection program is controlled by the detector signal or at least, if a time program is used is calibrated after each cycle by the detector signal. The purity of the products is confirmed by the chromatograms of each major fraction which are shown in figure 12.9. The chromatograms show that each fraction is very pure and this purity was obtained, using a 400 mg charge, in less than 25 minutes. It is clear that the recycling procedure, coupled with the peak shaving technique, can handle significant column loads and provides excellent solvent economy.

Alternative Preparative Techniques

Although there may well be an economic limit to the scale of preparative chromatography, there does not appear to be any practical constraint to column size. Columns of over a meter in diameter are reported to have been successfully operated, but there is very limited data available due to the highly proprietary nature of their use. Large diameter columns, however, must have an appropriate sample distribution system at the column inlet, to ensure the sample is dispersed evenly over the whole cross section of the column, in order to maintain high column efficiencies. Although the actual column may need to be custom made, solvent pumps, with high volume delivery at moderate pressures, are readily available. Most of the pipe fittings, valves, etc. can be obtained from manufacturers that supply equipment for general chemical plant operation. Large columns, handling large sample loads, will be effected by the heat generated in the column during solute absorption in the stationary phase. Consequently, appropriate heat exchange must be arranged to prevent the separation being denigrated in the larger columns due to localized temperature changes.

A number of alternative LC distributions systems (distribution systems other than simple columns) have been developed for preparative work which need to be discussed. Some of these have found limited use but, nevertheless, have been shown to be very effective for large scale chromatographic purification for certain types of application.

Unfortunately, the successful applications are largely proprietary and so operating details are often not available.

The Moving Bed Continuous Chromatography System

The concept of the moving bed extraction process was originally introduced for hydrocarbon gas adsorption by Freund *et al*. [9] and was first applied to gas liquid chromatography by Scott [10]. A diagram of the moving bed system suitable for GC was proposed by Scott and is shown in figure 12.10.

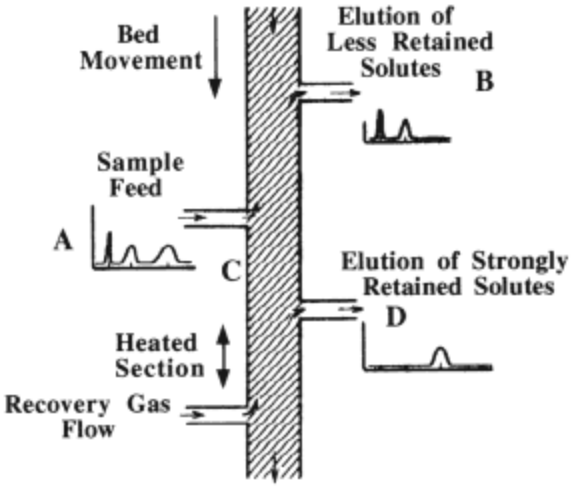

Figure 12.10
The Moving Bed Continuous Chromatography System
Courtesy of Butterworths Scientific Publications Ltd.
[Ref. 10]

Although the feasibility of this device was originally established for a gas chromatographic system, subsequently, its viability was also confirmed for liquid chromatography. The original GC system will be used here to explain the principal of the separating process. The moving bed system takes a *continuous* sample feed and operates in the following way. The stationary phase, coated on a suitable support, is allowed to fall down a column against and upward stream of carrier gas. In the original device

of Scott, the packing (dinonyl phthalate coated on brick dust) was contained in a hopper at the top of the column and was taken off from the bottom the column by a rotating disc feed table. As the packing was removed, it was returned to the hopper by a simple air-lift device. By suitable adjustment of the relative rates of upward carrier gas flow and downward stationary phase flow (contained on the falling support) some components were arranged to move upward with the carrier gas, and others move downwards with the stationary phase. Referring to figure 12.10, if the ordinary chromatogram of the mixture is that depicted at (A), the relative speed of the carrier gas and the stationary phase define an imaginary line on the chromatogram. Those components to the left of the line, move up with the carrier gas (B) and those components to the right of the line, move down with the stationary phase (C). The components that move down in the stationary phase are stripped out by arranging a portion of the column to be heated and a second stream of gas elutes them through a second port (D). This system, as described can not be used for liquid chromatography but a modified form, which will be discussed later, can be used very successfully using a liquid/solid distribution systems. Scott and Maggs [11] developed a theory that would predict the necessary operating parameters for the effective separation of a specific component from a multi component mixture. The equations they derived are as follows,

$$V_L = V_g\left(\frac{K^A_{\theta_1} K^B_{\theta_2}}{K^B_{\theta_1} - K^A_{\theta_1}}\right) \text{ and } V'_g = \frac{V_g K^A_{\theta_1}}{K^B_{\theta_1} - K^A_{\theta_1}}$$

$$V''_g = \frac{V_g}{K^B_{\theta_1} - K^A_{\theta_1}}\left(\frac{K^B_{\theta_2}}{e^{\mu\left(\frac{\theta_1-\theta_2}{\theta_1\theta_2}\right)}}\right)$$

$$\frac{l_1}{l_2} = \frac{\ln K^A_{\theta_1} + \frac{\mu(\theta_1-\theta_2)}{\theta_1\theta_2} - \left(K^A_{\theta_1}\exp\left(\frac{\mu(\theta_1-\theta_2)}{\theta_1\theta_2} + f\right)\right)}{\ln K^A_{\theta_1} - \left(\ln K^A_{\theta_1} - f\right)}$$

$$\frac{l_1}{l_3}=\frac{\ln K_{\theta_1}^{B}-\left(\ln K_{\theta_1}^{B}+f\right)}{\ln K_{\theta_1}^{A}-\left(\ln K_{\theta_1}^{B}+f\right)}$$

where (V_L) is the flow rate of stationary phase down the column,

(V_g) is the flow of gas at extraction section inlet,

(V''_g) is he flow of gas at the stripping section outlet,

($V_g+V'_g$) is the flow of gas at the extraction section outlet,

($V''_g-V'_g$) is the flow of gas at the stripping section outlet,

(V'_g) is flow rate of gas phase up the column,

(θ_1) is the temperature of the extraction section,

(θ_2) is the temperature of the stripping section,

(μ) is $dK/d\theta$ for component (A),

($K_{\theta_1}^{A}$) is the distribution coefficient of component (A) at (θ_1),

($K_{\theta_1}^{B}$) is the distribution coefficient of component (B) at (θ_1),

(l_1) is the length of the extraction section,

(l_2) is the length of the stripping section,

(l_3) is the length of the intermediate section,

(f) is the $\frac{\text{Volume of Stationary Phase per unit length}}{\text{Volume of Gas Phase per unit length}}$

Using this theory, Scott and Maggs designed a three stage moving bed system to extract pure benzene from coal gas. Coal gas contains a range of saturated aliphatic hydrocarbons, alkenes, naphthenes and aromatics (benzene, toluene and xylenes). They found that the theory only gave approximate values for the operating parameters, but values that were close enough to allow optimization by an iterative

experimental procedure. The separations they obtained are shown in figure 12.11.

It is seen that the material stripped form the top section contained the alkanes, alkenes and naphthenes and very little benzene. The material stripped from the center section consisted of almost pure benzene. The residue striped from the lower section contained the toluene, the xylenes and even the thiophene which elutes closely to the benzene. To eliminate the thiophene, however, it was necessary to loose some benzene to the

lower stripping section. Nevertheless the separation clearly demonstrates the effective use of the moving bed extraction technique.

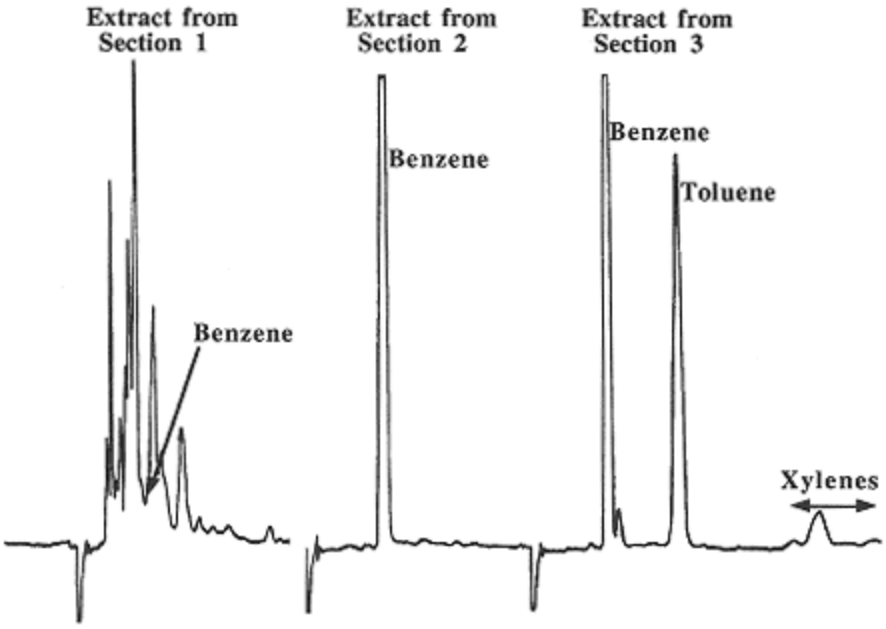

Figure 12.11
The Extraction of Pure Benzene from Coal Gas by continuous Extraction Using a Moving Bed Technique

The Simulated Moving Bed Preparative Chromatography System

Barker [12] and Barker and Deeble [13] used a novel modification of this principle involving a column in circular form. A diagram representing the wheel concept of Barker and Deeble is shown in figure 12.12. The ports to the column are fixed in positions at the periphery of a large wheel and the wheel is rotated. The wheel had a series of apertures situated around its periphery that were normally closed except when they coincided with the ports during rotation.

In this way, the bed moved continuously in one direction relative to the ports and the mobile phase moved in the opposite direction. As with the

moving bed system, some solutes (those that eluted at a rate slower than the wheel velocity) moved in the direction of the wheel movement while others (those that migrated faster than the wheel velocity) moved in the opposite direction with the movement of the mobile phase.

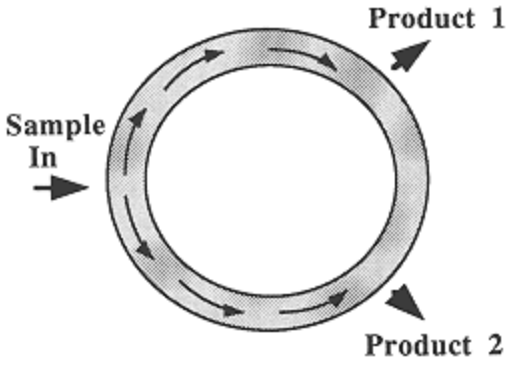

Figure 12.12
The Circular Simulated Moving Bed Column

The two fractions were collected from take off ports and the mobile phase returned for recirculation. This was the first form of the so called *simulated moving bed system*, and was used by Barker for continuous LC separations with moderate success. Leaks at the port seals on the wheel periphery were a source of difficulty, limiting the pressure that could be applied to the system to produce the mobile phase flow. The wheel was about 5 ft in diameter and thus was a somewhat heavy and rather cumbersome device.

The first practical simulated moving bed system was described by Hurrel in the late 1960s [14]. The system he developed was an ingenious extension of the large circular column devised by Barker. The *simulated* moving bed has two main advantages over the *actual* moving bed system. In addition to the technical simplicity, there is considerable adsorbent conservation and, as the mobile phase is recirculated, there is substantial solvent economy. The system used by Hurrel is shown in figure 12.13. The circular column is divided up into a number of sections, each section taking the form of a relatively short preparative column. The columns

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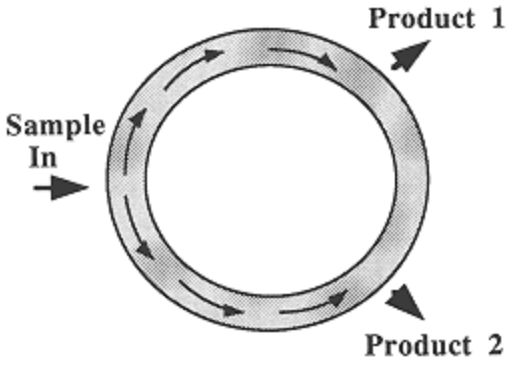

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12.15 and can be oriented in a number of different ways, although they are all basically similar to the Hurrel system.

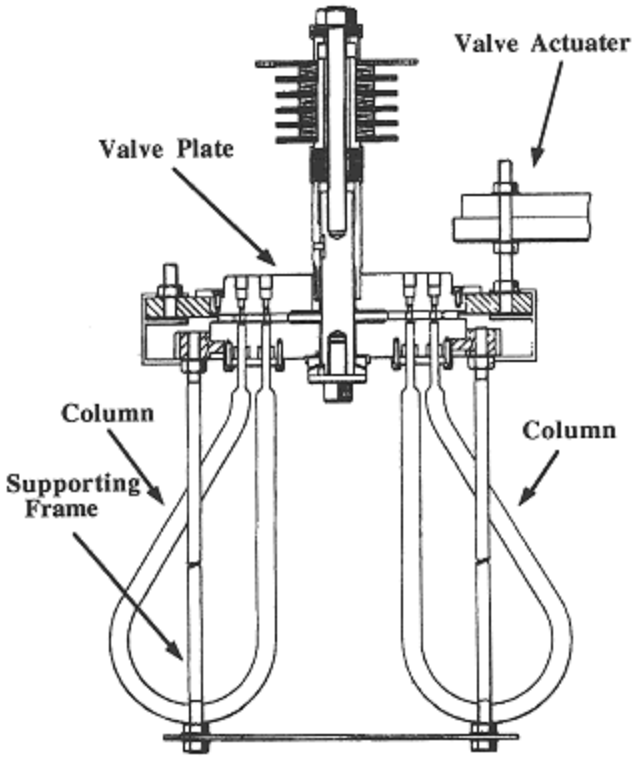

Figure 12.14
A Cross section of the Hurrel Disc Valve System and Columns.

The mobile phase passes through several stationary columns which contain the stationary phase. There are, in a similar manner, a number of different ports, one for the mobile phase and one for the return of the mobile phase. There is a central feed port and two take-off ports. These ports can, by appropriate valve programming (in modern systems these are individual valves and not necessarily disc valves), be connected sequentially to the link between each column. The apparent counter current movement of the stationary phase, relative to the mobile phase, is

achieved by valve switching which simulates the rotation of the ports between each column.

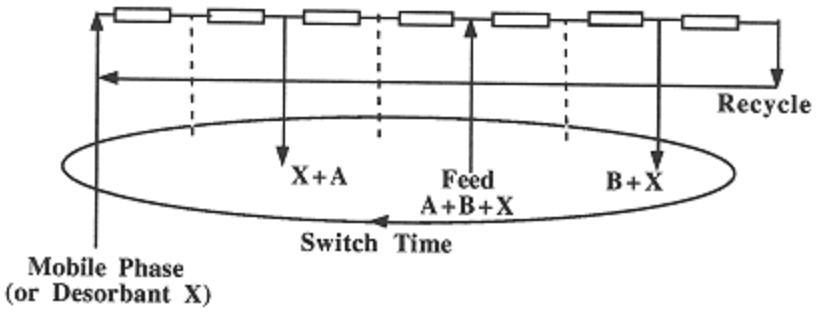

Figure 12.15
The Simulated Moving Bed Chromatography System

Exactly the same process takes place as that in the Hurrel system but, in effect, the valving makes the columns appear to move instead of the packing. Part of the feed moves with the mobile phase and is collected by a small take-off flow in front of the feed port (B + solvent). The other, more retained portion of the sample, accumulates in a column on the other side of the feed port and is collected by a another small take-off flow behind the feed port (A + solvent). This particular system ideally, produces two products and thus lends itself specifically to the separation of enantiomeric pairs. However, for effective separation with high purity yields, the stationary phase capacity for the two enantiomers must be fairly large and thus the phase system must be carefully selected. The technique has been successfully used to isolate single enantiomer drugs [15–17].

Radial Flow Chromatography

Another alternative chromatographic procedure for preparative separations is *radial flow chromatography*. The radial flow chromatography columns consist of two concentric porous cylindrical

frits between which the stationary phase is packed. A diagram of a radial chromatography column is shown in figure 12.16.

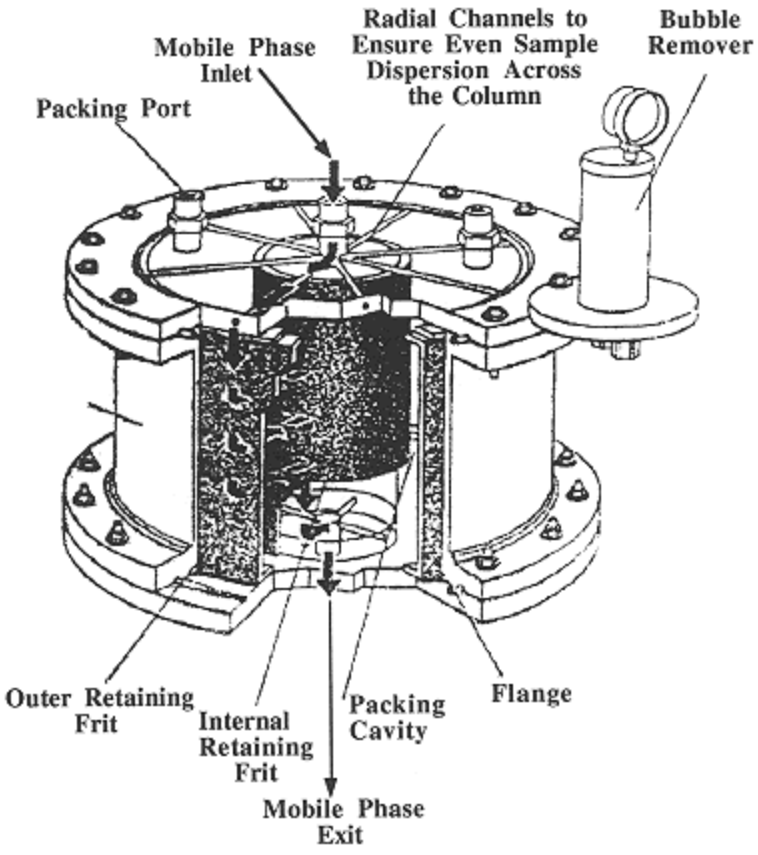

Figure 12.16
The Radial Flow Column

The mobile phase flows from the outer cylinder, across the radius of the column to the inner cylinder. The radial gap between the frits is the effective bed 'height.' The solid core houses the inner frit, through which the eluent percolates and exits at the base of the column. The outer frit is the column inlet, and consequently the sample initially has a large area of stationary phase with which to interact. The cross-sectional area of

packing decreases progressively as the solute moves to the center, but as the solute bands progressively decrease in concentration due to dispersion, this does not normally result in column overloading at the center. The sample is injected on the top of the column, where it is radially dispersed by radial channels (see figure 12.16) to ensure an even sample loading around the periphery of the column.

Although this type of column has a high capacity, it is also relatively short and thus not suitable for complex mixtures that need to be developed isocratically. They can, however, be used very effectively with gradient elution.

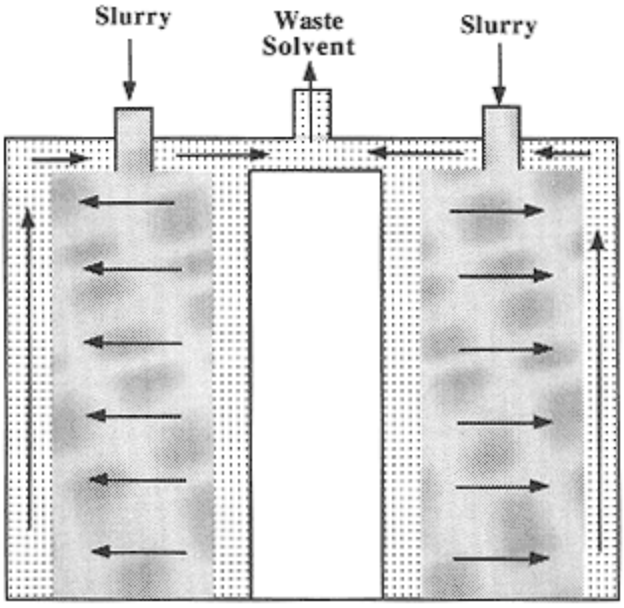

Figure 12.17
Packing Radial Columns

In practice, the gradient is made to 'lift' the solutes from the packing by the progressive increase in either the dispersive solvent component of the mobile phase, the polar component, salt concentration or the pH depending on the type of phase system selected. This means it is generally restricted to the separation of substances that have relatively large

separation ratios and differ significantly in the strength of their interaction with the stationary phase. In general this type of column would not be the first choice for chiral separations. The method used for packing radial columns is depicted in figure 12.17. Due to the crossectional area decreasing as the solute progresses through the bed, the linear velocity of the mobile phase also changes and so the optimum velocity can only be a compromise for the total bed length.

The packing in the form of a slurry passes directly into the column between the two frits. The column exit is closed, and the slurry solvent passes through the outer frit and exits via the normal mobile phase inlet port. The columns is easily unpacked by adopting the reverse procedure. An example of the use of the radial flow column to separate some large biomolecules is shown in figure 12.18.

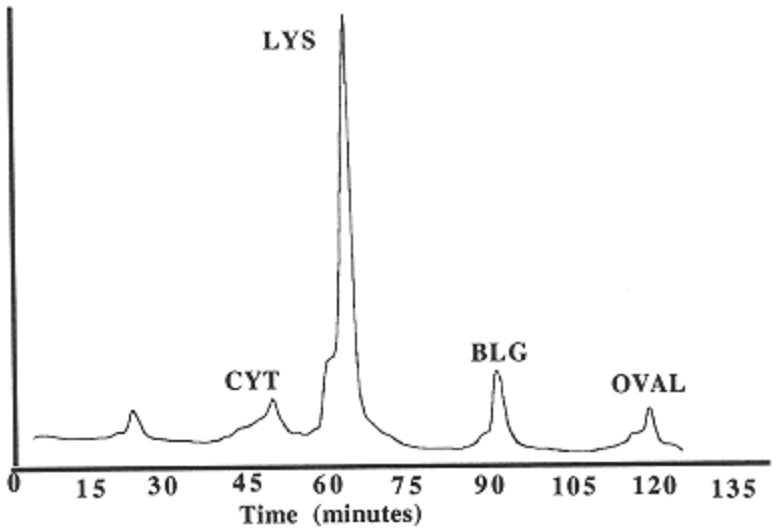

Figure 12.18
The Separation of Some Large Biomolecules Using Radial -Flow Reverse Phase Chromatography

The total column volume was 500 ml, and it was filled with a proprietary packing, IMPAQ^{R} RG2020-C18 which was basically a octadecane bonded phase. It was used in the reversed phase mode, with a linear

gradient from acetonitrile/0.1% TFA : 20/80 v/v, to acetonitrile/0.1% TFA : 50/50 v/v in 120 minutes at a flow rate of 46 ml per min.

The Preparative Separation of the Enantiomers of Chlorokynurenine

Details of this application were obtained by private communication from Dr. Lester Dolak and Dr. Eric Seest of the Upjohn Pharmacia Company, Kalamazoo. The separation was carried out on a 50 cm × 5 cm Chirobiotic T column (ASTEC) at 40°C. The feed solvent was difficult to make up due to the relative insolubility of the solute. It was eventually made up at a concentratiojn of 2 mg per ml by heating and stirring the chlorokynurenine in a 95% aqueous ethanol/water : 50/50 v/v, at 40°C. The filtered solution and the column were maintained at 40°C throughout the work; 400 ml of the solution (800 mg) was pumped onto the column at 50 ml/min for 8 minutes, and then eluted at 50 ml/min for 20 minutes.

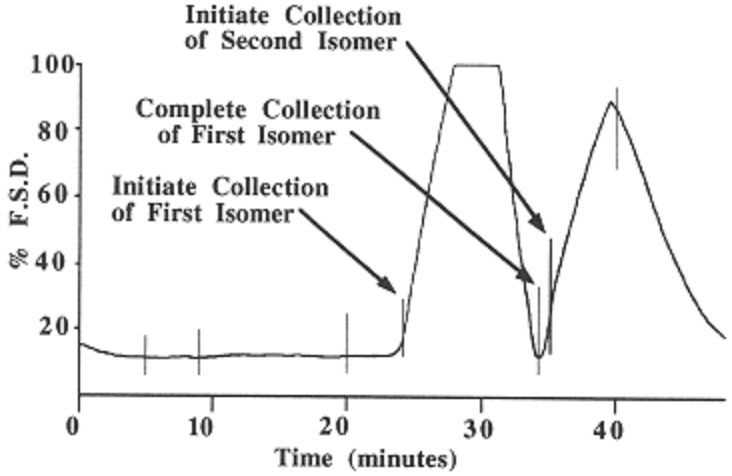

Figure 12.19
The Preparative Separation of the Enantiomers of Chlorokynurenine
Courtesy of Dr. Lester Dolak and Dr Eric Seest of Pharmacia Upjohn Inc.

The flow rate was increasd to 60 ml/min as soon as the second enantiomer began to emerge. The actual separation is shown in figure

12.19. It is seen that a very good separation was obtained and the results from an analysis carried out on an analytical Chirobiotic T column indicated that the first enantiomer was >99% pure, and the second enantiomer was 98% pure. The mid fraction, that was collected between the two main peaks, was recycled. The total cycle took 49 minutes and it is seen that the system operated very effectively.

The use of preparative chromatography for the separation of physiologically active enantiomers is now quite well established but, even so, it is only the beginning. The technique of LC is probably the only practical technique for the large scale resolution many racemic mixtures.

Synopsis

Preparative chromatography can include a wide range of sample sizes extending from a few milligrams to hundreds of grams. Preparative *chiral* chromatography is somewhat unique, as it mostly involves the separation of two very similar solutes, eluted relatively close together, and thus makes special demands on the separation process. The maximum sample load must often be placed on the column and, where possible, the column may need to be overloaded to achieve the necessary sample through-put. Increasing the column radius and columns length, increases both the maximum sample volume and the maximum sample mass. However, increased column length will also increase the column efficiency (unless the particle diameter is changed). Both the maximum sample volume to restrict dispersion to 10%, and the maximum permissible sample overload volume to maintain adequate enantiomeric purity, can be calculated from the basic chromatographic properties of the system. Mass overload results in the adsorption isotherm becoming non linear and causes peak broadening and peak distortion. The high concentration of the solute in the mobile phase also modifies its interactive properties and causes the retention of later eluting peaks to be reduced. Preparative chromatography apparatus is not complex and consists of a high capacity reservoir(s), high volume delivery mobile phase pump, a sample reservoir and pump, a preparative column, a low sensitivity detector and an appropriate fraction collector. Columns

packed with particles 20 µm and less in diameter need to be slurry packed, particles with diameters in excess of 20 µm can be dry packed. Preparative columns can also be mechanically packed using either radial compression or axial compression. Such techniques also allow the column to be easily unpacked, which permits efficient packing recovery. The column can be used repeatably for the same sample by recycling the mobile phase. In this process, the sample is recycled until the desired separation is obtained and is very effective if used with a peak shaving technique. This procedure is very economic with regard to both packing and solvent. Other preparative techniques include the moving bed continuous chromatography and the simulated moving bed chromatography. The latter uses a series of columns connected in series by a complicated valve system. The mobile phase flow rate is adjusted so that part of the sample moves in one direction in the column (in the direction of the movement of the mobile phase) while the remaining sample moves in the opposite direction with the apparent movement of the stationary phase. This can be very effective for a binary mixture such as a pair of enantiomers but the stationary phase capacity must be relatively large for it to be successful. The radial column is also an alternative for preparative chromatography. The separation is developed from the outside of a cylindrical bed to the inside. This process does not work well for solute pairs with small separation ratios and is thus not ideal for chiral separations. Liquid chromatography is probably the best separation technique available for the preparative scale resolution of many enantiomer drugs, and those substances that are used in relatively small quantities, but have a high intrinsic commercial value.

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Chapter 13—
Chiral Separations by Capillary Electrophoresis and Capillary Electrochromatography

Capillary Electrophoresis

Capillary electrophoresis, although carried out in a capillary tube, is not fundamentally a chromatographic technique. It evolved somewhat tenuously from the traditional gel electrophoresis and, as the separation procedure differs considerably from chromatography, the basic principles involved in an electrophoretic separation will be briefly described.

Electrically charged compounds can be transported in a gel or liquid under the influence of an electric field and this process has been termed electrophoresis. There are basically three different electrophoretic methods of separation and they are, zone electrophoresis, isotachophoresis and isoelectric focusing.

Zone electrophoresis is carried out in an electrolyte, across which an electric field is applied. The sample to be separated is placed in the center of the electrolyte, which could be a electrophoretic plate or capillary tube depending on the system being used. Those components carrying a negative charge migrate to the anode, while those carrying a positive charge migrate to the cathode. Eventually, the substances separate into

individual bands, their relative positions depending on their individual mobilities. A zone electrophoretic separation is depicted, diagramatically, in figure 13.1.

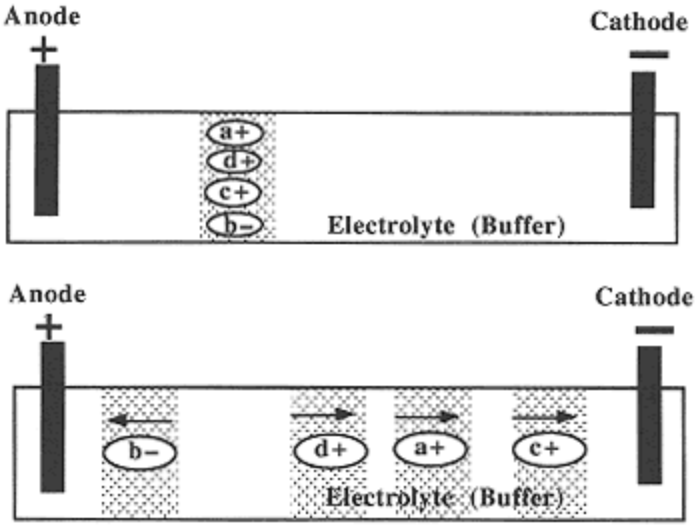


Figure 13.1
Zone Electrophoresis

Isotachophoresis

Although based on the same principle of electrophoretic migration, isotachophoresis is carried out in quite a different manner. The sample is placed at the junction between a leading and terminating electrolyte contained in a capillary tube. The leading electrolyte must have a higher mobility than any of the sample components, and the terminating electrolyte must have a mobility that is less than any of the sample components. In addition, the leading electrolyte should have a buffering capability at the pH at which the samples are to be separated. A separation by isotachophoresis is shown in figure 13.2. On application of the electric field, the compound with the highest mobility, (3), will migrate faster, leaving those moving at a slower rate, (1) and (2), behind. This results initially in a mixed zone being formed before the leading electrolyte and after the terminating electrolyte. The sample

components can never enter the leading electrolyte, because their mobility is less than that of the electrolyte. In a similar manner, the terminating electrolyte can never enter the sample mixture, as its mobility is less than those of the sample components.

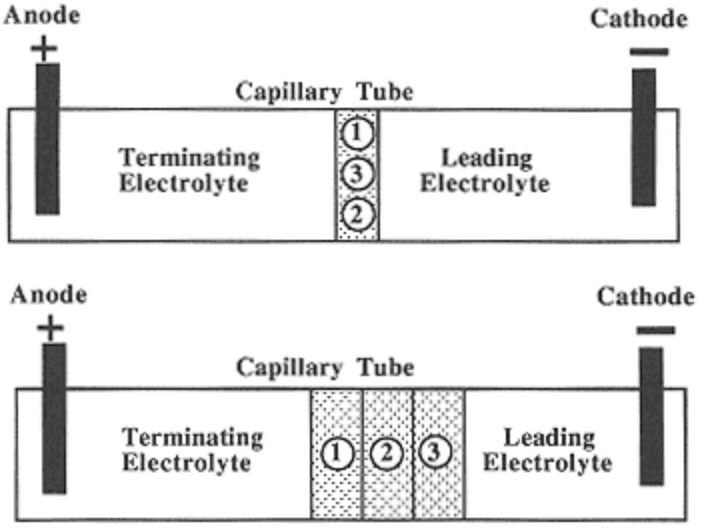


Figure 13.2
An Isotachophoresis Separation

Eventually each sample component is separated from its neighbor in order of their increasing mobilities, the one with the least mobility being situated next to the terminating electrolyte and that with the greatest mobility next to the leading electrolyte. The separation is depicted in figure 13.2. The bands in isotachophoresis are not dispersed by diffusion in the normal way, as any dispersion that does occur must result in the solute entering its neighboring bands. However, the solute will immediately be driven back as a result of their differential mobilities and thus the solute bands are *self-sharpening*.

Isoelectric Focusing

Isoelectric focusing is used to separate amphoteric substances and, as a consequence, the separation is based not on their differential mobilities but on their different isoelectric points (pI). The isoelectric point of an

ampholyte is that (pH) at which it has no net charge. At the (pI) the ampholyte will not migrate in an electric field as it will be existing probably as a zwitterion (an internal salt) with no charge. A separation due to isoelectric focusing is depicted in Figure 13.3.

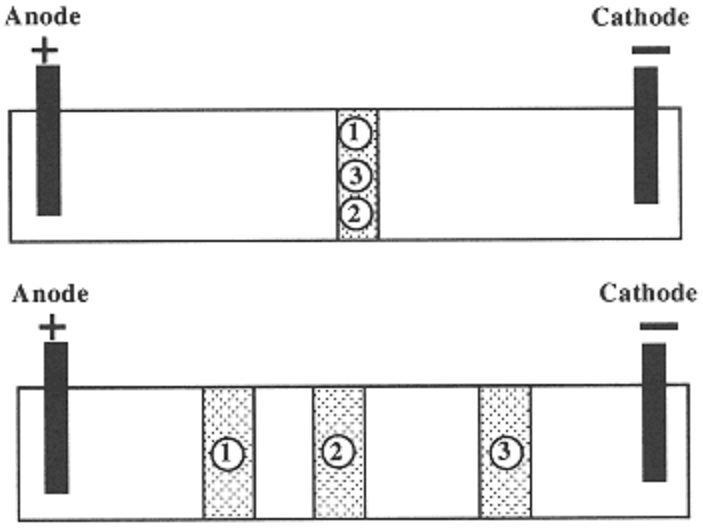


Figure 13.3
A Separation by Isoelectric Focusing

Using a mixture of ampholytes (usually polyamino polycarboxylic acids) contained in the capillary tube, the anode vessel containing an acid solution, and the cathode vessel filled with an alkaline solution, a (pH) gradient will be formed along the capillary tube on the application of an electric field. When this gradient is stabilized, the sample is introduced into the center of the electrically arranged ampholytes. Each substance will migrate under the applied field until it reaches the position where the (pH) is equal to its (pI) and, as at that point it will no longer be charged, it will come to a halt. If any solute tends to move out of the isoelectric point, it will immediately ionize and become charged again and under the electric field forced back into its isoelectric position; *i.e*. it is *focused*. As a result, a series of bands of solutes are formed in the capillary tube in the order of their increasing (pI) values. It is clear that the resolution of such a system can be extremely high and separation will

depend on all the substances having sufficiently different isoelectric points.

The fourth type of electrophoresis is a hybrid of the previous three, and can be used under certain conditions to separate enantiomeric materials. However, before discussing the technique itself, some mention must be made of electro-osmotic flow.

Electro-Osmotic Flow (Electro-Endosmosis)

The movement of a liquid, when in contact with a charged surface, situated in a strong electric field is called electro-endosmosis. The flow of liquid through a silica tube under electro-endosmosis is of 'plug' form, and does not exhibit the parabolic velocity profile that normally occurs in Newtonian flow. As a result of this, there is little, or no, resistance to mass transfer similar to that in open tubular columns. It follows, that there is very little band dispersion when the flow is electrosmotically driven and consequently extremely high efficiencies can be attained.

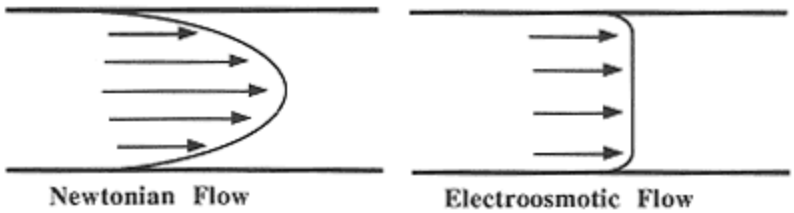


Figure 13.4
Newtonian Flow and Electro-endosmosis

The different types of flow are illustrated in figure 13.4. In electrophoretic separations, electro-osmotic flow can often affect the separation adversely, but it can also be used to advantage in placing samples onto the capillary electrophoretic system.

A diagram of the basic instrument used for capillary electrophoresis is shown in figure 13.5. It consists of two reservoirs, one carrying the anode and anode electrolyte, and the other the cathode and cathode

electrolyte. Each end of a fused quartz capillary dips into two reservoirs, thus joining them electrically. At one end of the tube, there is a 'T' join connecting to another reservoir containing the sample solution and, at the other end, a detector.

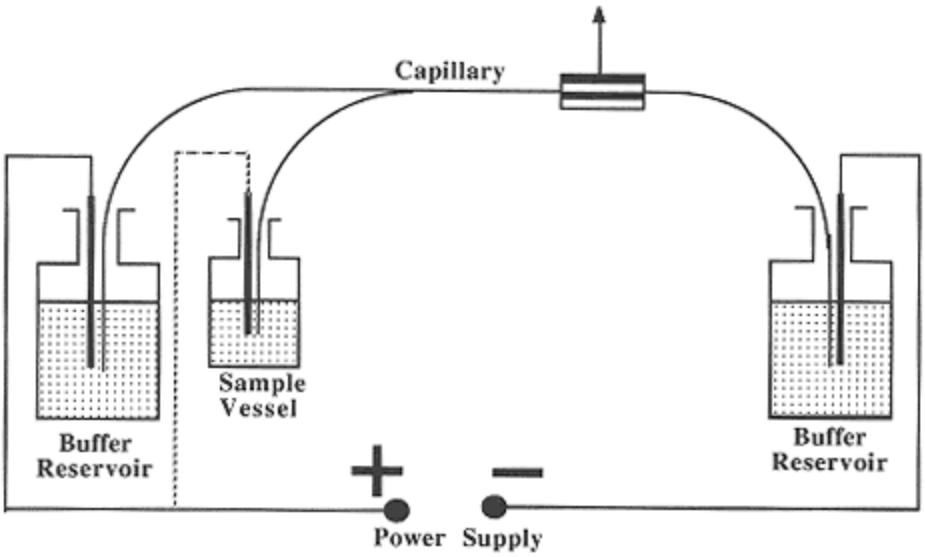


Figure 13.5
Capillary Electrophoresis Apparatus

The capillary is usually a polymer-coated fused silica tube and the detector consists of a small section of a similar tube, from which the polymer coating has been removed. Detection is achieved by a variety of procedures, the most common being the adsorption of UV light by the solutes as they pass the aperture in the polymer coating, usually employing a fiber optical system. Alternatively, the section of tube is irradiated by an appropriate laser, and the intensity of the fluorescent light monitored with an appropriate light-sensing cell. The sample is introduced into the system using electro-osmotic flow, by connecting a high potential to the electrode in the sample reservoir. When sufficient sample has passed into the tube, the high potential is again connected to the anode, in the anode reservoir, and the separation developed.

Unfortunately, most chiral substances are not ionic and so are not appropriate for electrophoretic separation. However, it has been shown that neutral, uncharged substances can be separated by partitioning the solutes between an ionic phase and miscelles containing long aliphatic chains. In effect, this is replacing an electrophoretic technique by a chromatographic system, where the mobile phase is driven by electro endosmosis.

The micelles form the second phase that can offer dispersive (hydrophobic), polar (hydrophilic) and ionic interactions with the solutes in contrast to largely polar interactions with the aqueous electrolyte. It is clear that this type of system could be also used with some slight modification for the separation of enantiomers. This type of separation has been termed miceller electrokinetic chromatography [1]. Watarai [2] carried the concept further and used a micro emulsion to the same purpose and termed the technique micro emulsion electrokinetic chromatography. In chiral, capillary electrophoresis, the chiral selector can also be an additive to the electrolyte, or take the form of an emulsion or be immobilized on the capillary tube surface as a traditional type of stationary phase.

The most common chiral additives used in chiral capillary electrophoresis with micellular solutions (mostly micelles of sodium dodecylsulphate) are derivatives of the three basic cyclodextrins. This system might be considered more of a chromatographic process than one that is electrophoretic, as the solutes are distributed between the aqueous electrolyte phase and the cyclodextrin/micelle phase. The derivatized cyclodextrin additive will also be distributed between the electrolyte and the micelles, the extent of which will depend on the type of derivatized cyclodextrin and its capacity for dispersive or polar interactions with the micelles. As the cyclodextrin additive itself partitions between the electrolyte and the micelle (albeit the distribution under certain circumstances may be small) some of the chiral additive will be distributed on the micelle surface and will act as a chiral stationary phase.

The movement of the solute, relative to the phase system, can be understood by considering the distribution system. If the micelle electrolyte system is treated as a chromatography distribution system with the micelles stationary, and the mobile phase moving at an electrosmotic velocity of (u), then from chromatography theory, the velocity of the solute relative to the micelles (v) will be given by:

$$v = \left(\frac{1}{1+k'}\right)u$$

where (k') is the effective capacity ratio coefficient of the solute between the micelles and the electrolyte

and (u) is the electrosmotic flow-velocity

In fact:

$$k' = \frac{Kv_M}{v_E}$$

where (v_M) is the effective volume of micelles present,

(v_E) is the effective volume of electrolyte present,

and K is the distribution coefficient of the solute between the micelles and the electrolyte

Now, in practice, the micelles will usually be traveling at a velocity (w) in the opposite direction to the electrolyte and so the actual velocity of the solute, (V), relative to the stationary column will be given by:

$$V = \left(\frac{1}{1+k'}\right)u - w$$

$$V = \left(\frac{v_E}{v_E + Kv_m}\right)u - w$$

It is seen that the solute can move either way depending on whether:

$$\left(\frac{v_E}{v_E + Kv_m}\right) < w \quad \text{or} \quad \left(\frac{v_E}{v_E + Kv_m}\right) > w$$

It is also seen that the solute velocity (V), and thus its retention time, will not only depend on the distribution coefficient of the enantiomer, but also depend on the relative electrosmotic velocities of the electrolyte and the micelles, together with the relative volumes of the electrolyte and micelles in the system. There is a further complication, the cyclodextrin additive must also behave as a solute and, although it is often assumed that it resides solely in the electrolyte and is not distributed in the micelles, this will depend on the character of the derivatized cyclodextrin and whether or not it can interact with the micelle. This will obviously differ from one derivative to another.

Thus, the chiral agent will also move in the system, which will be governed by a similar equation to that of the solute:

$$v_c = \left(\frac{1}{1 + k'_c}\right)u$$

where (k'_c) is the effective capacity ratio coefficient of the solute between the miscelles and the electrolyte,

and (v_c) is the velocity of the cyclodextrin derivative.

or:

$$V_c = \left(\frac{v_E}{v_E + K_c v_m}\right)u - w$$

where (K_c) is the distribution coefficient of the cyclodextrin derivative between the micelles and the electrolyte,

and (V_c) is the absolute velocity of the cyclodextrin derivative.

It is seen that the cyclodextrin derivative also moves through the system and the enantiomeric selectivity can either arise from specific interactions between the solute and chiral agent in the electrolyte, or as a result of specific interactions between the solute and the chiral agent adsorbed on the surface of the micelles. This is analogous to the different selectivity's that can be employed in LC, where the chiral agent can

either reside in the stationary phase, or in the mobile phase. Micellular electrokinetic chromatography, however, is more complicated in that the effective stationary phase is also moving and thus, it is more akin to counter current chromatography, with the flow rates electro-osmotically driven.

The mechanism of retention, however, is still basically simple. The chiral selectivity is explained in exactly the same way as in GC and LC. The entropic term that results from one enantiomer approaching the other more closely is still the primary agent. As in LC, this entropic contribution to retention is augmented by the ensuing enthalpic effect that results from relatively greater polar, dispersive or ionic interactions between the closer associating enantiomer and the stationary phase. Irrespective of the distribution system, beitmay, a gas and a liquid, a liquid and a liquid, a liquid and a solid or an electro osmotically driven liquid-liquid system, the separation will depend primarily on the spatial fitting of the two enantiomers with a third. Only as a secondary effect, will the magnitude of the differential retention be determined by the strength and nature of the molecular forces that can occur during interaction and, consequently, the structure of the enantiomers and that of the chiral agent. In electrokinetic chromatography, however, besides the nature of the phases and the relative volumes of them present in the system, the electrolyte velocity, and the second phase velocity will effect both resolution and analysis time. This in turn will depend on the salt concentrations and the nature of the electrolyte and that of the micelle material. Consequently, optimum conditions are usually determined by experiment or by comparison with other known separations of a similar nature.

In certain instances, enantioselectivity can be influenced by the addition of a second chiral additive such as alkylhydroxyalkyl cellulose [3,4], hexadecyltrimethyl ammonium bromide or cetylpyridinium chloride [3]. Extra additives, will not only introduce a *different* enantiomeric selectivity, but it will also increase the probability of the solute interacting with a chiral center and thus *increase* the selectivity. This

will take the form of apparent chiral synergism which has in fact been reported by Okafo *et al*. [5]. Guttman and co-workers [6] immobilized the cyclodextrin derivative by inclusion into a polyacrylamide gel. Cross linking [7], produced an allylcarbamoylated cyclodextrin-acrylamide copolymer gel. The chiral selectivity of the liquid gels was found not to be as great as the solid gels, but the columns were more efficient and thus the resolving power of the two systems were somewhat similar. Mayer and Schurig, [8,9], used columns coated with immobilized dimethylpolysiloxane containing permethylated β- or γ- cyclodextrin that was chemically bonded with an octamethylene spacer for the separation of drug enantiomers. Armstrong [10] also described the immobilization of permethylated β-cyclodextrin. by coupling the allyl or pentanyl substituted TM-β-cyclodextrin to a organohydroxysilane polymer.

Crown ethers have also been used in capillary zone electrophoresis and have been established as suitable for separating optically active primary amines by LC [11,12]. In addition, they have been used in conjunction with the cyclodextrins [13,14] to improved chiral separations. As already discussed, the introduction of a second chiral agent would be expected to improve the enantiomeric resolution, as, in addition to introducing a second type of chiral selectivity, it would also increase the probability of the solute interacting with a chiral center.

The common use of miceller electrokinetic chromatography evokes some discussion on the production of micelles. Surfactants can organize themselves into micelles, at or above the critical micelle concentration, and at temperatures in excess of the Krafft point. After the critical micelle concentration has been reached, the concentration of the surfactant monomer remains constant, and any excess of surfactant aggregates to form micelles. The diameter of the micelles is only 3–6 nm and the solution exhibits properties similar to homogenous solutions that have relatively high electrical conductivity. When employing miceller electrokinetic chromatography to separate enantiomers, chiral surfactants can be employed or, alternatively, chiral additives in conjunction with nonchiral detergents can be used. The separation is carried out in very

small diameter tubes (50–100 μm I.D.) to prevent excessive ohmic heating (Joule heating). The smaller the cross sectional area of the tube, the smaller the current for a given applied voltage and thus, less energy is generated as heat along the capillary tube.

There are two classes of chiral surfactants that have been used, optically active amino acids derivatives, and natural surfactants such as bile salts, digitonin and the saponins. Bile salts possess both dispersive (hydrophobic) and polar (hydrophilic) interactive sites and form chiral, helically shaped aggregates with a reversed micelle conformation. The bile acids have quite different chiral selectivity from the long chain surfactants. Their dispersive (hydrophobic) character appears to dominate in their interactive capabilities. An example of the alternative class of amino acid derivatives is *N*-dodecanoyl-L-valinate. These chiral agents must be used under neutral or alkaline conditions unless sodium dodecyl sulfate is also added to produce co-micellation, in which case the system can be used under acid conditions. Columns used with these agents can exhibit reduced efficiency, but this can be improved by adding urea, or small amounts of methanol, and by reducing the electro-osmotic flow, by adding glycerol.

It must be emphasized that a mixture containing micelles will not necessarily produce a simple, single component stationary (or non aqueous) phase. Not only will the solute be distributed between the micelles and the aqueous phase, but also, to a lesser or greater extent, will any other component in the electrophoretic solution. The presence of any other organic component distributed between the micelles and the aqueous phase will modify the solute interactions with *both* phases. In general, increased interaction of the solute with the micelles will slow the migration rate of the solute. Increased interaction of the solute with the aqueous phase will increase the migration rate of the solute.

Although peptides, proteins, polysaccharides and certain polymers have been used successfully in LC to separate chiral substances, they have found only limited use in miceller electrokinetic chromatography. Due to the large number of different chiral, dispersive and polar sites available

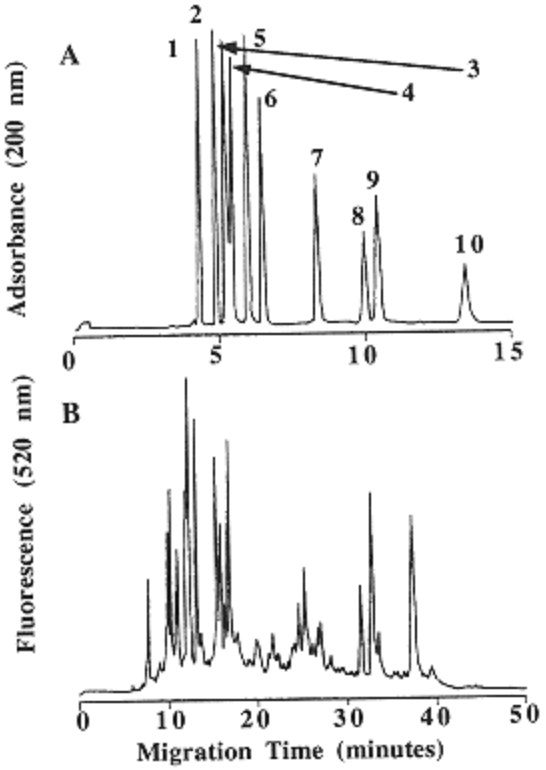
on each macromolecule, the probability of unique selectivity for a particular enantiomer may be very high. Unfortunately, due to the limited number of these special sites that will be available in the molecule, the *probability* of the solute interacting with the appropriate site will, in contrast, be very small. As a result, the selectivity necessary to achieve the separation is often not realized. Nevertheless, bovine serum albumin has been used as a buffer to separate the racemates of leukovorin, ibuprofen and DNS-amino acids [13,14] and bovine serum albumin cross linked with gluteraldehyde has been used to separate the isomers of tryptophan [15]. In addition, human serum albumin has also been used for the separation of dibenzoyl tartaric acid, amino acids and indole lactic acid [16].

Application Examples

The Separation of Peptides

The reproducible analysis of peptides was one of the first successes of capillary electrophoresis. Acidic peptides, that have proved difficult to separate by LC, are readily separated in low pH buffer systems, such as 100 mM sodium phosphate at pH 2.5. In figure 13.6A, the separation of 10 peptides has been achieved with baseline separation in 15 minutes. Each peptide was injected at a concentration 50 ng/ml in 5 mM phosphate, pH 2.5. The sample was introduced by an 8 s injection, at 8 kV, into a 27 cm × 50 μm capillary, equilibrated with 50 mM phosphate buffer and separated with a constant voltage of 10 kV at 28°C. The solutes were detected by UV adsorption at 200 nm.

In figure 13.6B is shown the separation of peptides labeled with CBQA (3-(*p*-carboxybenzoyl)-quinoline 2-carboxy aldehyde) following a trypsin digestion of cytochrome *c*. The cytochrome *c* (100 μg) was digested overnight at 37°C with 3 μg of sequencing grade trypsin. The sample was injected by a 10 sec pressure injection into a 57 cm × 75 μm capillary that had been equilibrated with 100 mM borate at pH 8.3 and separated at 25 kV constant voltage at 28°C. The solutes were detected by fluorescence excitation by a laser at 488 nm and sensed at 520 nm. The total on column mass was 4 fmol.



1	Bradykinin
2	Angiotensin
3	32-60 Mouse acetylcholinesterase
4	α-Melanin stimulating hormone
5	Thyrotropin-releasing hormone
6	Leutinizing-hormone releasing hormone
7	Leucine enkephalin
8	Bombesin
9	Methionine enkephalin
10	Oxytosin

Figure 13.6
The Separation of Some Peptides by Capillary Electrophoresis
Courtesy of Biotechniques (ref. 17)

The Separation of the Stereoisomers of a Mixture of Pseudoephedrine and Ephedrine

The separation of the pseudoephedrine and ephedrine isomers was carried out using β-cyclodextrins in the mobile phase. The separation, shown in figure 13.7, was carried out on a capillary 72 cm × 50 μm I.D. and the solutes were detected by UV adsorption at 214 nm. The mobile phase chiral additive was the sulfobutylether derivative of β-cyclodextrin.

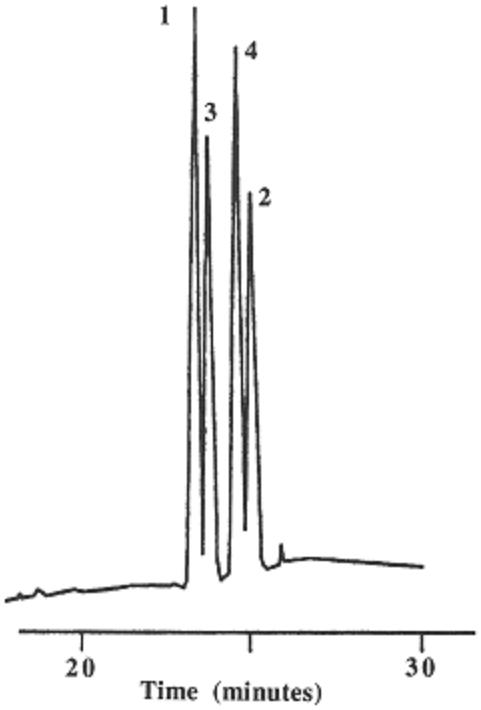


Figure 13.7
The Separation of the Stereoisomers of a
Mixture of Pseudoephedrine and Ephedrine
Courtesy of Journal of Chromatographic Science
(ref. 18)

The separation was carried out using 1.5 mM of the cyclodextrin derivative in 20 mM tris-phosphate buffer. A volume of 5 nl of the sample was hydrostatically introduced into the column for 10 s. and the separation developed under a potential of 30 kV. The cyclodextrin derivative was made up in a 50 mM phosphate solution pH 4.0, containing 5 mM sodium chloride. It is seen an excellent separation of each enantiomer is achieved.

The Effect of pH on the Separation of Duloxetine Enantiomers Using Hydoxypropyl-b-Cyclodextrin Additives

The separating system consisted of 75 cm × 50 μm capillary which was operated at 30 °C with an applied voltage of 23 kV. The enantiomers were detected by UV adsorption at 214 nm. The separation obtained is shown in figure 13.8

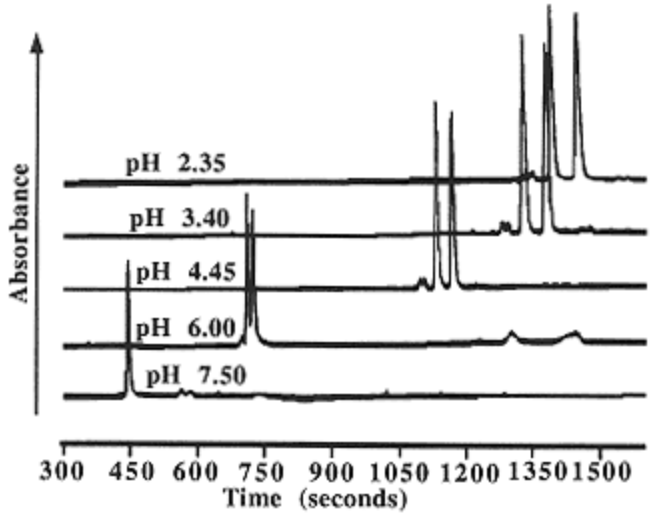


Figure 13.8
The Effect of pH on the Separation of the Enantiomers of Duloxetine Employing Hydroxypropyl-β Cyclodextrin
Courtesy of Eli Lilly and Co.

It is seen that good chiral selectivity is obtained at pH 4.5 or lower. The (R) enantiomer eluted first on hydroxypropyl-β cyclodextrin. It is clear

that pH plays an important role in electrophoretic separation even when chiral additives are employed.

The Separation of the Enantiomers of Some Amino Acids with the Chiral Additive Vancomycin

Vancomycin can be used very effectively as a chiral additive in capillary electrophoresis, but is in some ways inconvenient. as it adsorbs strongly in the UV. However, as low levels of the agent are employed, adequate sensitivity is often realized. Nevertheless, solutes are sometimes detected by fluorescence using an appropriate fluorescing derivative. The separation of the AQC derivative of phenylalanine, methionine and serine is shown in figure 13.9.

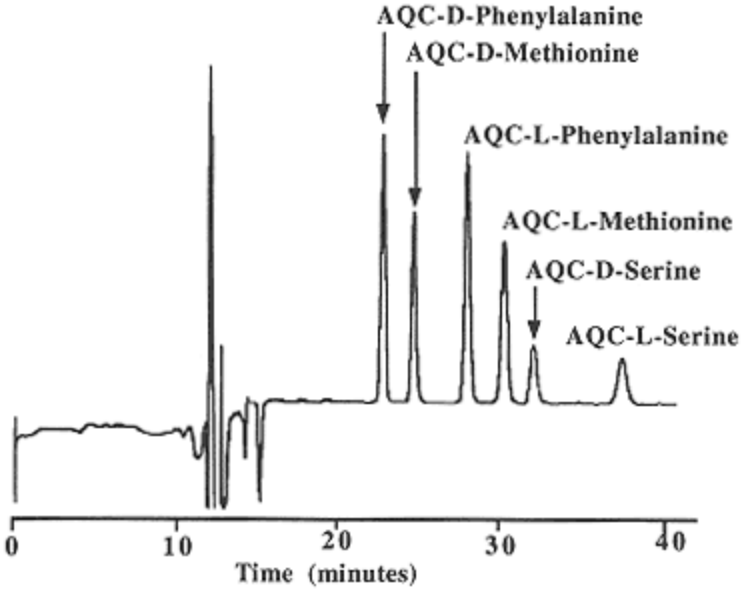


Figure 13.9
The Separation of the Enantiomers of the AQC Fluorescent Derivatives
Phenylalanine, Methionine and Serine
Courtesy of LC/GC. (Ref. 18)

The separation was carried out on a 30.5 cm silica tube, 50 μm I.D., containing 0.1 M phosphate buffer and 5 mM Vancomycin. The pH of the buffer was 7.0 and the electrophoretic voltage 5 kV.

The same system was used to separate the Dansyl derivatives of valine except that the pH was adjusted to 4.9. The separation that was obtained is shown in figure 13.10.

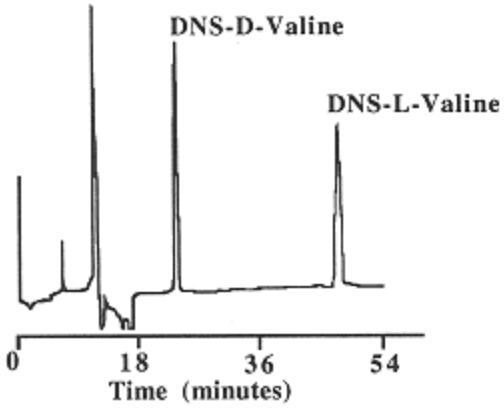


Figure 13.10
The Separation of the Enantiomers of the Dansyl Derivatives of Valine
Courtesy of J. Chromatogr. (Ref.19)

Separations can also be achieved very effectively in capillary electrophoresis using crown ethers as the chiral additive and example of this we given by Bereufer [18]. In figure 13.11, the separation of erythro-2-amino 3-phenylbutyric acid is shown as the first pair of solutes in chromatogram (A) and threo-2-amino-3-phenybutyric acid the second pair of solutes in chromatogram (A). The racemic pair in chromatogram (B) are those of threonine. In chromatogram (A) the buffer was 10 mmol of Tris adjusted with citric acid to a pH of 2.2 and 10 mmol C6-tetracarboxcylic acid. In chromatogram (B) the buffer was 10 mmol of Tris and 11 mmol of benzyltrimethylammonium chloride adjusted with citric acid to a pH of 2.2 and 15 mmol C6-tetracarboxylic acid [19]. The chiral stationary phase was a crown ether.

It is clear that many of the chiral agents used in LC can be equally effective in capillary electrophoresis.

This technique was used by Reilly and Risley [21] to separate some Dansyl glutamic acid enantiomers. The separation was carried out on a 27 cm neutral capillary 50 μm I.D. The buffer was 100 mM phosphate at a pH of 6.0. The results are shown in figure 13.12.

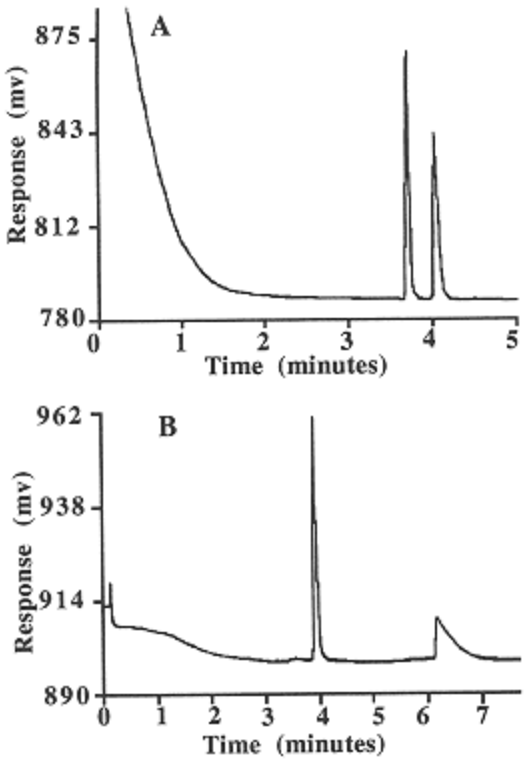


Figure 13.12
The Separation of the Enantiomers of Glutamic Acid
by Counter Current Capillary Electrophoresis
Courtesy of LC-GC. (Ref. 21)

The buffer used for chromatogram (A) contained 2 mM Vancomycin and chromatogram (B) 2 mM of the antibiotic A82846B. It is seen that there is no problem with detection, and the Vancomycin produces an excellent separation, the peaks of both enantiomers being very symmetrical. The

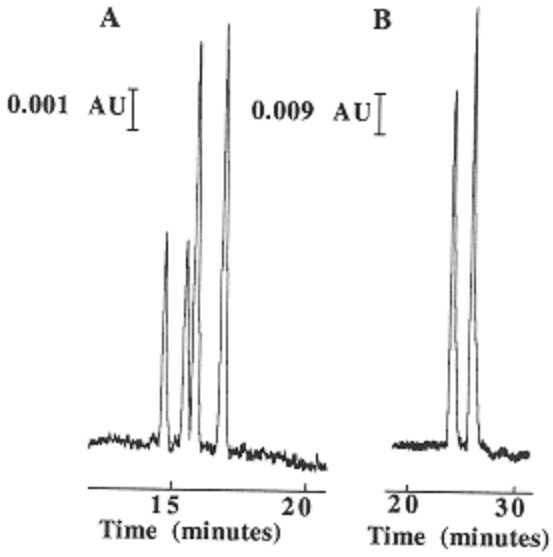


Figure 13.11
The Separation of (A) Racemic Erythro- and Threo-2-amino-3-phenylbutyric Acid and (B) Racemic Threonine
Courtesy of LC-GC. (Ref. 20)

The Separation of Enantiomers by Counter Current Capillary Electrophoresis

Although the macrocyclic antibiotics are very effective for separating chiral substances by capillary electrophoresis, as already stated they have the disadvantage of adsorbing strongly in the UV, and thus can make detection less sensitive. Park *et al*. [20] used a novel counter current capillary electrophoresis process, that incorporates the macrocyclic antibiotics ability to separate racemates, but also overcomes the problem of UV interference. A coated capillary is employed to suppress electro osmotic flow, which prevented the antibiotic from passing the detector window. The column is filled with buffer containing the Vancomycin and the sample is loaded onto the tube. When a voltage is applied, the chiral selector moves in the opposite direction to the solute and away from the detector window. As a consequence, there is no background adsorbance from the antibiotic when the solutes are sensed.

other antibiotic producing a good separation but the second enantiomer is a very asymmetric peak.

Synopsis

There are three electrophoretic methods of separation: zone electrophoresis, where the components are separated on the basis of relative mobilities; isotachophoresis, where the separation is again based on relative mobilities, but the solutes are sandwiched between leading and terminating electrolytes; and finally, isoelectric focusing, where the solutes are separated according to their isoelectric points. In all electrophoretic systems, movement of charged species is always accompanied, to a greater or lesser extent, by electro-osmotic flow. Electro-osmotic flow is the movement of a liquid, when in contact with a charged surface, under the influence of an electric field. The flow is 'plug' form and is not accompanied by the parabolic velocity profile associated with Newtonian flow. In 'plug' flow there is virtually no band dispersion resulting from resistance to mass transfer effects and thus very high efficiencies are attainable. The capillary electrophoretic apparatus consists of two reservoirs, each containing electrolyte, and fitted with electrodes. Each reservoir is inter-connected by fused quartz capillary tubing dipping into each reservoir. A third reservoir, containing another electrode, is connected by a 'T' to one end of the capillary, through which the sample is applied. At the other end of the tube is the detector. The most common detector is a UV absorption system measuring the intensity of the light passing through a cleaned section of the tube using fiber optics. Using the same physical system, detection by fluorescence measurement has also been found satisfactory. Chiral selectivity can be introduced into the buffer by employing standard LC stationary phases such as the crown ethers, the cyclodextrins or the macrocyclic antibiotics. Unfortunately, most chiral substances are not ionic, but these can be separated by introducing micelles (usually formed with sodium dodecylsulfate) or emulsions into the buffer. The chiral solutes partition between the long aliphatic chains of the micelles and the electrolyte as do the chiral additives themselves. In general, under an electric field the micelles move in the opposite direction to the

solute, and thus the actual movement of the solute relative to the capillary will be the difference between the micelle velocity and the solute velocity. The chiral selectivity arises from spatial interaction between the enantiomers and the chiral additive in exactly the same way as in LC and GC. Surfactants organize themselves into micelles at or above the critical micelle concentration and at temperatures in excess of the Krafft point. After the critical micelle concentration has been reached, the concentration of the surfactant monomer remains constant, and any excess of surfactant aggregates to form micelles. The diameter of the micelles is only 3–6 nm, and the solution exhibits properties similar to homogenous solutions that have relatively high electrical conductivity. In some cases chiral surfactants can be used, such as the saponins and bile salts. The distribution system with micelles is complex in that the chiral agents themselves, and the solutes, distribute between the micelles and the electrolyte. The capillary electrophoresis technique has been successfully employed to separate many different chiral substances including peptide enantiomers and a range of chiral drugs. A technique has been developed to avoid the strongly UV adsorbing macrocyclic antibiotics from interfering with UV detection. An internally coated capillary is used which eliminates electro osmotic flow and the electric field is arranged so that the chiral agent migrates in the opposite direction to the solute and, thus, never passes the detector window.

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Chapter 14—
An Experimental Approach to Chiral Chromatography

Introduction

Manufacturers of chiral phases usually provide an experimental protocol for their particular products and, in many instances, recommendations from these protocols will be included here. The rational behind the phase selection procedure, suggested by each manufacturer, differs considerably, and is sometimes a little obscure. However, in this section, the phase selection process will be discussed using the already established concepts of spatial orientation, entropic and enthalpic contributions to retention, and the different molecular forces that control retention.

There are a number of general considerations that need to be presented before detailed discussion of the individual phase systems commences. In any chiral separation the following conditions should be met,

1. The phase system must provide the necessary chiral selectivity to resolve the enantiomers of interest.

2. The phase system, in addition to providing the necessary chiral selectivity, must provide reasonably low capacity ratios, to ensure acceptable analysis times.

3. The phase system, and in particular the stationary phase, must be stable to the conditions under which it is to be used. (*e.g.* pH, solvent type, temperature, etc.)

4. If the analysis is to be a routine quality control assay, then, in LC, the solvents used should be non-toxic, easily recoverable, inexpensive and easily, and safely, disposable.

5. The phases (stationary phase and mobile phase) need to be economically priced, and readily available in a reproducible form.

The above conditions should be adhered to, irrespective of the technique that is used, the sample type, or the phase system selected.

The choice of the techniques is basically between GC or LC, as only under special circumstances would electrophoretic procedures be considered. If an electrophoretic system is deemed appropriate, then information should be sought from original references. In general, GC can provide much higher efficiencies and a much wider range of operating temperatures than LC. The GC procedure is simple and no subtle choice of mobile phase is necessary. After the stationary phase has been selected, the only important variable left to the analyst, is the operating temperature, or the temperature program. In addition, there is a more restricted range of stationary phases from which to select in GC, making the choice of stationary phase simpler.

In contrast, as has been discussed throughout the book, there are at least five different classes of LC stationary phase available and, within each class, there can be a number of alternative materials, each having different physical properties and unique chiral selectivities. The choice of phase system in LC is further complicated by the wide choice of solvents, and solvent mixtures that can be used with them. In addition, in some cases, a number of solvents must be excluded from choice, due to stationary phase instability. Thus in LC, after choosing the stationary phase from a wide range of possibilities, the optimum mobile phase components must be selected, together with the optimum mobile phase composition and then the optimum operating temperature. It is hardly surprising that, in LC the optimization process for a particular separation can be very confusing and, at the every least, tedious. To the newcomer,

it may appear almost impossibly difficult (*ipso facto* the contemporary popularity of practical training courses in chiral chromatography).

In this section, on experimental procedures, the subject will only be briefly surveyed. If the reader selects a particular commercial chiral stationary phase or column for use, then it is advisable to obtain the most recent product literature available from the supplier, as references and protocols are being continuously revised and updated.

Chiral Gas Chromatography

Chiral gas chromatography should be selected as the separation technique if the materials are volatile and stable at elevated temperatures. In addition, if the solutes can be derivatized to form a sufficiently volatile product without racemizing the enantiomers, or changing their racemic proportion, then GC may be the choice. GC offers much higher efficiencies, much higher peak capacities and significantly higher sensitivities than LC. It follows, that GC can easily contend with multicomponent mixtures, such as the essential oils and other complex mixtures from biological sources. In addition, the columns have short equilibrium times, trace impurities are easily assayed, and the analyses are shorter providing much faster sample throughput.

Derivatization

There are two ways of using derivatization in chiral GC. The first is to derivatize with a chiral reagent to produce diastereoisomers which can be separated on conventional GC columns. The second, is to derivatize with an achiral reagent to increase volatility and separate the derivatives on a column with a chiral stationary phase.

The first approach has met with limited success. There are certain disadvantages. The reagent must be enantiomerically pure, the resulting diastereoisomer must be adequately volatile and the derivatizing procedure must not produce racemization, either of the reagent or, the sample.

Derivatization with achiral reagents are more advantageous. The more volatile derivative usually exhibits greater column efficiency, the separations are rapid, and, in some cases, interactions between the derivative group and the stationary phase will enhance the chiral selectivity.

Table 14.1 Achiral Derivatization Methods

Compound Class	Derivatives
Alcohols and Carbohydrates	Acylation—esters Trimethylsilation—silyl esters Isocyanates—urethanes
Hydroxy Acids and Carboxylic Acids	Alkylation* Sylation Isocyanates—urethanes, amides
Amines	Acylation - trimethylsilylation
Amino Acids	Alkylation* Acylation
Amino-alcohols	Acylation * trimethylsilylation

* First Choice

Table 14.2 Derivatization Procedures

The Preparation of the Methyl Ester of a Carboxylic Acid

1. To approximately 1 mg of the sample, contained in a screw top vial, add 1 ml of 2 M methanolic hydrochloric acid. Place in boiling water for 30 min with the screw top tightly closed. Use a silicone rubber seal.

2. After 30 min, remove the cap, and allow the solution to evaporate to dryness. The removal of the last traces of water can be aided by the addition of methanol. Care must be taken not to loose any of the sample by evaporation.

(table continued on next page)

(table continued from previous page)

3. Dissolve residue in an appropriate solvent (diethyl ether, methylene chloride, methanol, ethanol, dimethoxypropane etc.) and inject appropriate sample onto the GC column.

The Preparation of the Trimethylsilyl Ester of a Carboxylic Acid

1. To approximately 5 mg of the sample, contained in a screw top vial, add 2 ml of bis-(trimethylsilyl)-trifluoroacetamide containing 10 % v/v of trimethylchlorosilane.

2. Add 0.1 ml of pyridine, seal vial and mix well.

3. Heat for 5 min at 45°C, cool to room temperature and inject sample onto column.

The Acetylation of Alcohols and Amines

1. To approximately 1 mg of the sample, contained in a screw top vial, add 0.2 ml of methylene chloride.

2. Add 0.2 ml of trifluoroacetic anhydride (or other appropriate anhydride), cap the vial and heat to 60°C for several minutes.

3. Remove cap from vial (using the appropriate precautions).

4. Place capsules back on hot-plate and evaporate the solution to near dryness. The final drying must be carried out with care, so that none of the sample is volatilized and lost.*

5. Dissolve residue in methylene chloride and inject onto the column.

*If there is concern over evaporation losses, the solution can be extracted twice with 5% sodium bicarbonate solution which will remove the free acid (the solution may foam and care must be taken in venting the vial). The methylene chloride can then be dried by passing it through a small bed of anhydrous sodium sulfate in a Pasteur pipette.

The Polysiloxane Based Stationary Phases

The Amino Acid-Peptide Polysiloxane Stationary Phases

There are two commonly used chiral GC stationary phases from which the analyst can choose. Firstly, there are the polysiloxane based stationary phases that contain amino acids or peptides included in the polymer or reacted with the polymer matrix, secondly, there are those that contain one of the cyclodextrins, or derivatized cyclodextrins

included in the polymer. The broad basis of choice for the peptide polysiloxane phases are as follows.

Peptide-Dimethylpolysiloxane Chiral Stationary Phases

The dimethylsiloxanes are dispersive in character (retention largely depending on dispersive (hydrophobic) interactions), and have relatively lower temperature stability. They are not in common use today, and would be appropriate for separations at temperatures (conservatively) up to about 150°C. Such materials would separate *dispersive solutes of small molecular weight* (solutes with alkyl chains, naphthene rings and aromatic rings).

Peptide-Phenylpolysiloxane Chiral Stationary Phases

Incorporating the chirally selective material into a phenylpolysiloxane polymer raises the operating temperature significantly and, in addition, the polarizability of the phenyl group allows induced dipole interactions (π interactions) with polar solutes. Materials based on the phenylsiloxane polymers can be used up to temperatures (conservatively) of about 180°C. These materials are suitable for the separation of *enantiomers that are essentially polar* but, insufficiently polar to render them involatile. Derivatives of polar substances would also separate well on phenylpolysiloxane polymer stationary phases, but the derivatizing procedure must not effect the enantiomeric ratio.

Cyanopolysiloxane Peptide Chiral Stationary Phases

The cyanyopolysiloxane chiral stationary phases can be used in two ways. The polymer can include the chiral agent, usually a peptide or dipeptide and thus provide an overall polar interaction with the solutes, which would be more suitable for the separation of *polar solutes*. The temperature stability of the cyano polymer phases is, however, less than that of the phenyl polymer phases and can only be used up to temperatures (conservatively) of about 160°C.

Alternatively, the cyano group can be hydrolyzed to the acid and coupled to an acid-protected amino acid or peptide. This stationary phase is

probably one of the more popular polysiloxane chiral stationary phases an is claimed to be stable up to 280°C. This type of stationary phase is largely polar, and thus the overall retention will be controlled by polar interactions. Such a material would be effective for the *separation of enantiomers where the chiral center was located relatively closely to polar groups* such as amino acids.

In practice, the sample should be injected onto the column (probably the bonded hydrolyzed cyano polysiloxane would be a good first choice.) and the temperature programmed from about 50°C to 200°C (depending on the upper *published* thermal limit of the stationary phase). The best chiral selectivity is usually obtained by operating isothermally. Thus, from the temperature program, the best isothermal temperature can be estimated and, from subsequent results at that temperature, the optimum operating temperature that will achieve the required separation in the minimum time, can be assessed by iteration. In general, the column length should be adjusted to provide just the separation that is necessary and in this way reduce the analysis time. Accelerating the separation by increasing the mobile phase velocity, although effective, is not the best way to reduce analysis time.

The Polysiloxane-Cyclodextrin Based Stationary Phases

The polysiloxane-cyclodextrin based stationary phases are probably the most popular as they have both good chiral selectivity, and high temperature stability. The cyclodextrins that are commercially available can have three different ring structures and can be derivatized with different interactive groups. In addition, they can also be included in diverse polysiloxanes, so there is a number of different stationary phases of this type available. Introducing alkyl or alkyl-acyl substituents into the cyclodextrin by derivatizing the hydroxyl groups produces materials that have lower melting points, are still thermally stable, and, more important, are soluble in the solvents used for coating capillary columns. The range of cyclodextrins available from Advanced Separation Techniques Inc. as stationary phases for GC including their maximum

allowable operating temperature (MAOT) are typical of those shown in table 14.3.

Table 14.3 Derivatized Cyclodextrin GC Chiral Stationary Phases

Stationary Phase	MAOT	Code
1. 2,6-di-O-pentyl-α-cyclodextrin	180°C	A-DA
2. 2,6-di-O-pentyl-β-cyclodextrin	180°C	B-DA
3. 2,6-di-O-pentyl-γ-cyclodextrin	180°C	G-DA
4. permethyl-(S)-2-hydroxypropyl-α-cyclodextrin	180°C	A-PH
5. permethyl-(S)-2-hydroxypropyl-β-cyclodextrin	180°C	B-PH
6. permethyl-(S)-2-hydroxypropyl-γ-cyclodextrin	180°C	G-PH
7. 2,6-di-O-pentyl-3-O-trifluouroacetyl-α-cyclodextrin	180°C	A-TA
8. 2,6-di-O-pentyl-3-O-trifluouroacetyl-β-cyclodextrin	180°C	B-TA
9. 2,6-di-O-pentyl-3-O-trifluouroacetyl-γ-cyclodextrin	180°C	G-TA
10.2,6-di-O-pentyl-3-O-propionyl-γ-cyclodextrin	180°C	G-PN
11.2,6-di-O-pentyl-3-O-butyryl-γ-cyclodextrin	180°C	G-BP
12. permethyl-β-cyclodextrin (in polysiloxane)	230°C	B-PM
13. 2,3-dimethyl-6-silyl-β-cyclodextrin (in polysiloxane)	250°C	B-DM

The cavity size (the α, β, and γ, cyclodextrins) can be chosen on the basis of the size of the solute molecule. If the molecule can fit into the cavity of a particular cyclodextrin, then, that cyclodextrin could permit inclusion interactions and thus might provide greater chiral selectivity. If the solute molecule is large compared with all the cavities of all three cyclodextrin, then inclusion interactions are not possible, and the γ-cyclodextrins are likely to be the most appropriate. Although the stationary phase B-PM has proved to be the most popular, in the majority of applications the performance of B-DM has been shown to be superior.

In choosing the stationary phase it can be generally said, that if nitrogen (amine) oxygen (ester alcohol) or a halogen is a functional group or atom attached to the stereogenic center, the G-TA stationary phase would be a first choice. If the molecule has few or no functional groups

attached to the chiral center then the B-PH would also be another frequently successful alternative. A list, showing the applicability of the different phases to different types of chiral separation, is shown in table 14.4. This list has been developed from published applications and represents contemporary knowledge. The stationary phases may well have wider areas of application than those indicated by the table.

Table 14.4 The Cyclodextrin Application Table

Stationary Phase	Application Class
Chiraldex A-DA	Single ring molecules, small cyclic amines, alcohols and epoxides
Chiraldex B-DA	Nitrogen heterocyclics, heterocyclics, some lactones, aromatic amines, sugars, amino acid derivatives, bicyclic compounds and epoxides.
Chiraldex G-DA	Aromatic amines containing 2 or more rings, large cyclic diols, Some heterocyclics, multi-ring compounds and those with bulky substituents.
Chiraldex A-PH	Small linear saturated amines, alcohols, carboxylic acids and epoxides.
Chiraldex B-PH	Structural isomers, unsaturated no aromatic compounds, Linear and cyclic amines and alcohols, acids, lactones, amino alcohols, sugars, bicyclic compounds and epoxides.
Chiraldex G-PH	Cyclic and bicyclic diols, steroids and carbohydrates.
Chiraldex A-TA	Small alcohols, amines, diols, propylene oxide.
Chiraldex B-TA	Broad range, alkyl alcohols, halogen acid esters, amino alkanes, amino acids derivatives, halogenated cycloalkanes, certain lactones, diols, alkyl halides, furan and pyran derivatives.
Chiraldex G-TA	Alcohols, polyols, amines, amino alcohols, lactones, halogenated carboxylic acid esters, furans, pyrans, epoxides, amino acids and glicidyl analogs.
Chiraldex G-PN	Epoxides, higher alcohols (>C4), lactones, aromatic amines (amphetamines).
Chiraldex G-BP	Amino acids, certain primary amines, oxides and furans.
Chiraldex B-PM	Carboxylic acids, alcohols, barbital's, diols, lactones, terpines and tertiaryamines.

(The bold type in the previous table indicates the more broadly applicable GC phases and has been used successfully in a high proportion of applications).

Cyclodextrin stationary phases are applicable to as wide variety of compounds, often without derivatization. Chiral separations by GC are becoming more and more important, and for very difficult separations, it is easy to increase the length of the capillary column to produce the necessary number of theoretical plates.

Chiral Liquid Chromatography

As a result of the development of thermally stable chiral stationary phases with good selectivity, many samples that were, hitherto, separated by LC are now separated on GC. They are separated, either in their natural state, or as volatile derivatives. LC is, therefore, now largely used for separating those substances that are involatile and cannot be made volatile by derivatization. Such substances include those that are highly polar or ionic, or those substances that have relatively high molecular weights. The general use of LC for chiral separations has been developed over many years, and so there are, now, a number of different types of chiral stationary phases available for LC. There are basically five different types of LC stationary phase which have been discribed at some length earlier in this book. The experimental techniques and the protocol for choosing the most applicable chromatography system will be discussed for each type of phase.

The Protein Bases Stationary Phases

There are three commonly used protein based LC chiral stationary phases, the α_1-acid glycoprotein phase (CHIRAL-AGP), cellobio-hydrolase (CHIRAL-CBH) and human serum albumin (CHIRAL-HSA). The properties of these stationary phases are a follows.

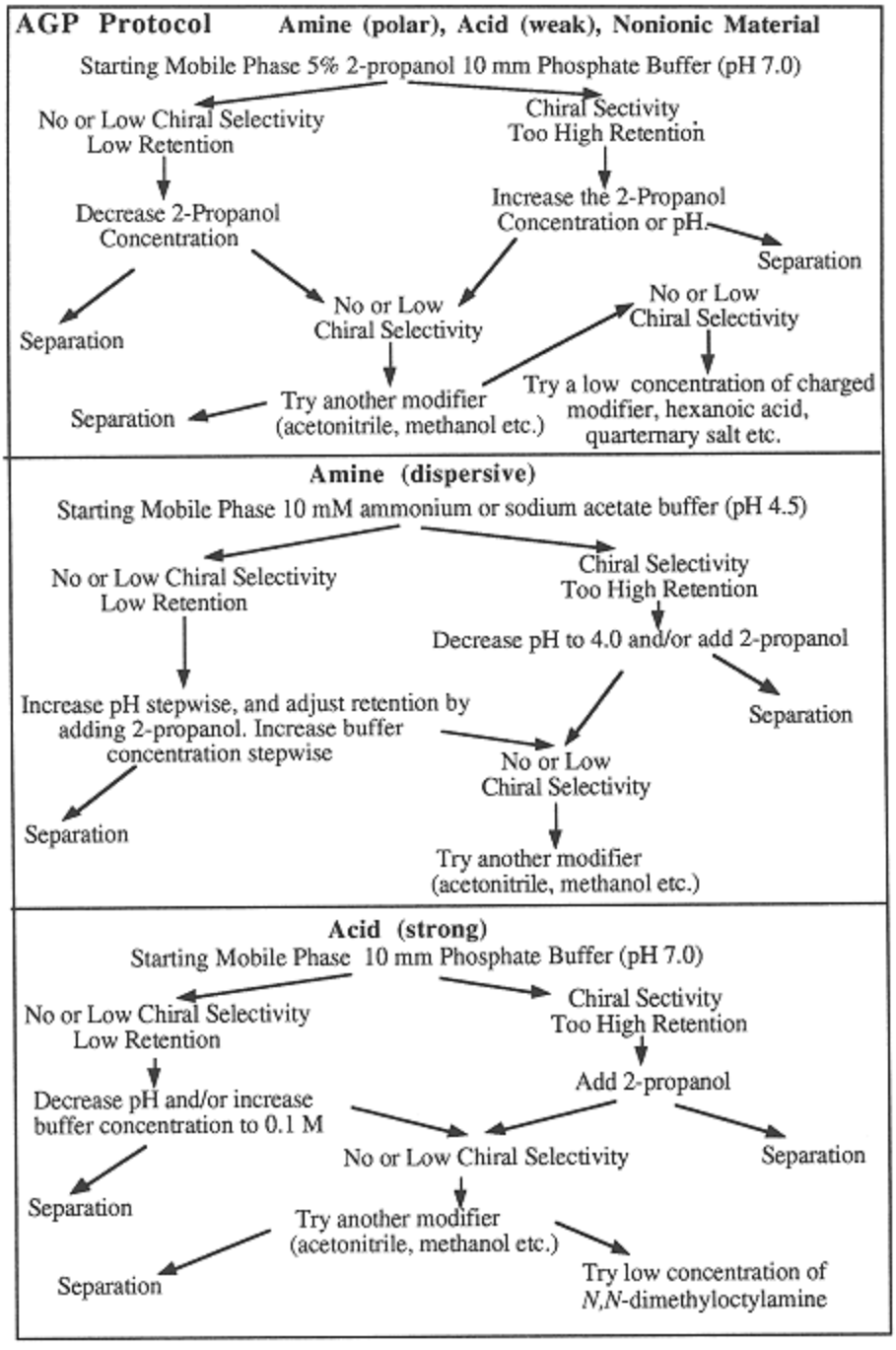
Chiral-AGP

The α_1-acid glycoprotein phase has extremely broad applicability and can separate the enantiomers of a wide range of drug compounds. It has

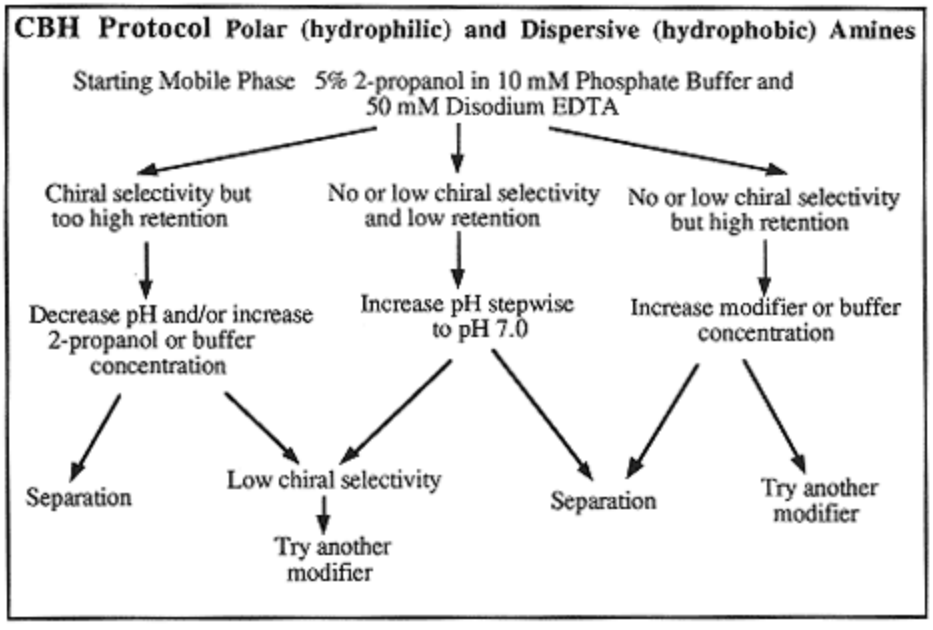
been used successfully for the separation of amines (primary, secondary, tertiary and quaternary), acids (strong and weak) and amides, esters, alcohols, etc. This stationary phase can be used with very simple mobile phase mixtures in the reversed phase mode, mostly with an aqueous buffer containing and organic modifier such as 2-propanol or acetonitrile. Retention and selectivity can be adjusted by changes in the buffer concentration, the pH or the concentration of organic modifier.

The most frequently used organic modifiers are 2-propanol, acetonitrile. methanol, ethanol, 1-propanol and tetrahydrofuran (THF). Normally increasing the organic modifier will reduce both retention and chiral selectivity. However, there are certain exceptions, where increasing the modifier content will decrease the retention and *increase* enantio-selectivity. Examples of these exceptions are the resolution of the enantiomers of Tiaprofen and Warfarin. This is probably due to the overall retention being controlled largely by dispersive interactions whereas the chiral selectivity results from polar interactions with polar groups proximate to the chiral centers. Chromtech provides the chart given below to help in the use AGP. The chart is largely self explanatory: when the organic modifier is increased, the dispersive interactions in the mobile phase increase, reducing the *relative* strength of the dispersive interactions with the stationary phase. Similarly increase in buffer concentration increases ionic interactions in the mobile phase.

Cellobio-hydrolase is a stable enzyme and is also used in the reversed phase mode. This stationary phase has a more narrow area of application than the AGP material, and is used preferentially to separate compounds containing one or more basic nitrogen groups, together with more polar groups (alcohol, phenol, carbonyl, amide, ether, sulfoxide, ester etc.) It has found particular use in the separation of basic drugs. The common mobile phase is a mixture of phosphate or acetate buffers and an organic solvent such as 2-propanol. As in the case of the AGP phases, retention and selectivity can be adjusted by changes in the buffer concentration, the pH or the concentration of organic modifier.


AGP Protocol
Amine (polar), Acid (weak), Nonionic Material
Starting Mobile Phase 5% 2-propanol 10 mm Phosphate Buffer (pH 7.0)
No or Low Chiral Selectivity
Low Retention
Chiral Sectivity
Too High Retention
Decrease 2-Propanol
Concentration
Increase the 2-Propanol
Concentration or pH.
Separation
Separation
No or Low
Chiral Selectivity
No or Low
Chiral Selectivity
Separation
Try another modifier
(acetonitrile, methanol etc.)
Try a low concentration of charged
modifier, hexanoic acid,
quarternary salt etc.
Amine (dispersive)
Starting Mobile Phase 10 mM ammonium or sodium acetate buffer (pH 4.5)
No or Low Chiral Selectivity
Low Retention
Chiral Selectivity
Too High Retention
Decrease pH to 4.0 and/or add 2-propanol
Increase pH stepwise, and adjust retention by
adding 2-propanol. Increase buffer
concentration stepwise
Separation
No or Low
Chiral Selectivity
Separation
Try another modifier
(acetonitrile, methanol etc.)
Acid (strong)
Starting Mobile Phase 10 mm Phosphate Buffer (pH 7.0)
No or Low Chiral Selectivity
Low Retention
Chiral Sectivity
Too High Retention
Add 2-propanol
Decrease pH and/or increase
buffer concentration to 0.1 M
No or Low Chiral Selectivity
Separation
Separation
Try another modifier
(acetonitrile, methanol etc.)
Separation
Try low concentration of
N,N-dimethyloctylamine

The chart giving an experimental protocol for CHIRAL-CBH is given below.

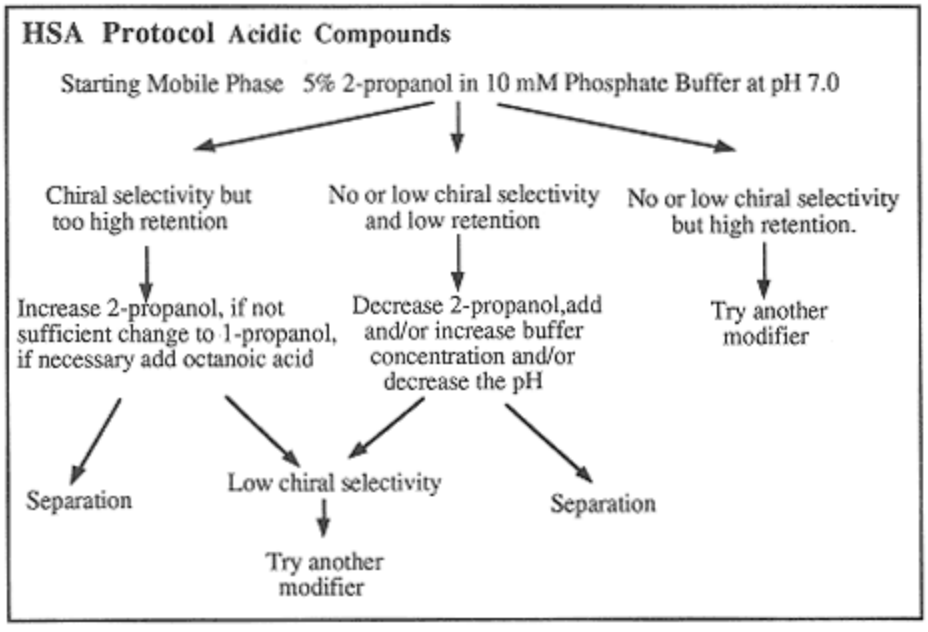


Again, the chart is relatively self explanatory and the recommended procedure is very similar to that given for CHIRAL-AGP It would appear that the manufactures of these phases recommend, in the first instance, the use of 2-propanol as the organic modifier (the agent that introduces dispersive interactions into the mobile phase). If necessary the alternative acetonitrile is then tried and after that there appears to be no specific choice. Changing to methanol will, provide a less dispersive, but more polar solvent, changing to THF will provide a more dispersive solvent.

Chiral-HSA

Bonded human blood serum has an even more restricted application range than CBH. It is also used in the reversed phase mode with mixtures of phosphate buffers and organic modifiers such as 2-propanol as the mobile phase. Retention and selectivity can be adjusted in the same manner as AGP and CBH. It's preferred use is to separate strong acids

and zwitterionic substances and certain non-ionic materials. The protocol for this stationary phase provided by Chromtech is given below. In general, over 95% of the separations that have been investigated, using these types of stationary phase, have required nothing more than a buffer and an organic modifier as the mobile phase.



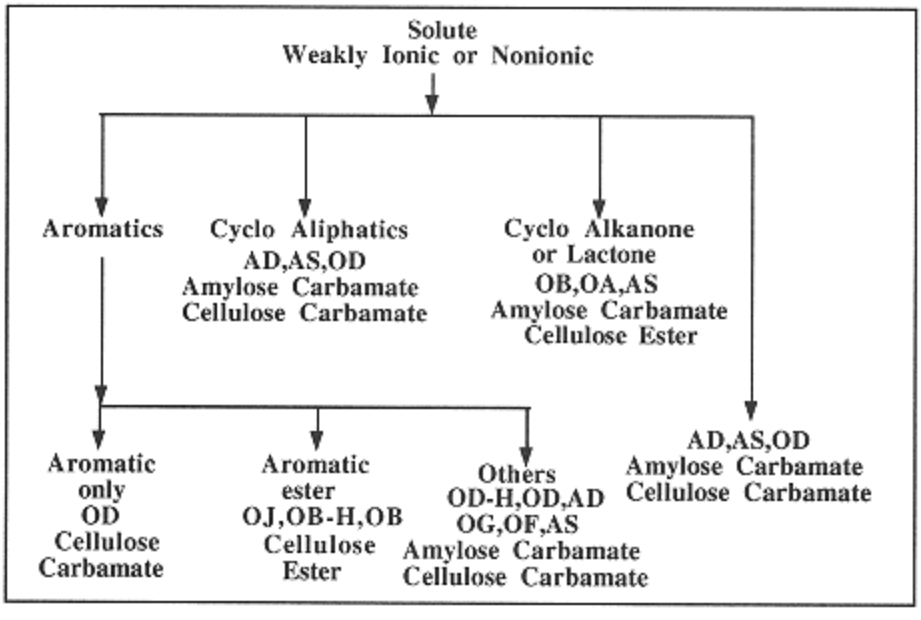
However, in certain cases, charged modifiers are needed, such as octanoic acid, quaternary compounds etc., to induce adequate enantioselectivity. Such additives, have been shown to be necessary when separating arylpropionic acids and profens, *i.e.* such substances as Ibuprofen and Naproxen.

The Cellulose Based Stationary Phases

The cellulose and amylose based stationary phases are more often effectively used in normal phase mode. The carbamates, however, are claimed to function in high concentrations of water, and have been used successfully with a mobile phase of pure buffer. There are three basic

types, the amylose carbamates, the cellulose carbamates and the cellulose esters. The range of stationary phases provided by Chiral Technologies Inc. together with their selection chart are shown below. In addition, the solvent mixtures recommended for use with each phase are also included.

Stationary Phase Type	Trade Name	Solvent
Amylose Carbamate	CHIRALPAK ® AD	Hexane/IPA
Amylose Carbamate	CHIRALPAK ® AS	Hexane/Ethanol
Cellulose Carbamate	CHIRALPAK ® OC	
Cellulose Carbamate	CHIRALPAK ® OD	Hexane/IPA
Cellulose Carbamate	CHIRALPAK ® OD-H	Hexane/Ethanol
Cellulose Carbamate	CHIRALPAK ® OD-R	
Cellulose Carbamate	CHIRALPAK ® OF	Hexane/IPA
Cellulose Carbamate	CHIRALPAK ® OG	Hexane/IPA
Cellulose Ester	CHIRALPAK ® CA-l	
Cellulose Ester	CHIRALPAK ® OA	
Cellulose Ester	CHIRALPAK ® OB	Hexane/IPA
Cellulose Ester	CHIRALPAK ® OB-H	Hexane/Ethanol
Cellulose Ester	CHIRALPAK ® OJ	
Cellulose Ester	CHIRALPAK ® OK	



Chiral Technologies Inc. provide a solvent protocol for use with their columns. If a normal phase development is to be used, a simple mixture of hexane/IPA : 90/10 v/v or heptane/IPA : 90/10 v/v is recommended as a good average stating mobile phase. Samples that are nonionic or weakly ionic are suitable for normal phase development. In general, the goal of sample optimization is to achieve a good separation of the enantiomers in 30 min or less. The following approximate rules can be helpful.

If chiral selectivity is observed in:	Chance of a satisfactory separation in 30 min. or less
R=0.8 in 10 min	probable
R=0.8 in 20 min.	possible
R<0.8 in 20	improbabl-change columns

The following approximation can also be useful.

Element content of sample	Initial mobile phase
Oxygen	hexane/IPA : 90/10 v/v
	(0.15% TFA if an acid is present)
Nitrogen	hexane/IPA : 80/20 v/v
	(0.15% DEA if a base is present)
Nitrogen and Sulfur	hexane/IPA : 70/30 v/v

Recommended Initial Conditions

1. Temperature 25°C (room temperature)

2. Flow rate 1 ml/min (compatible with available pressure)

3. Sample dissolved in mobile phase 2–5 mg/ml

Increasing the IPA content would decrease retention and probably selectivity. If the retention is still high, a change to a more polar solvent such as ethanol is recommended. Conversely, increasing the hexane content would increase retention and in most cases, also increase chiral selectivity. In general, lowering the temperature will increase retention raising the temperature will reduce retention. However, as already

discussed this will depend on the crossover temperature where coelution takes place.

In reverse phase separations, retention can be strongly pH and buffer dependent. An example of some starting conditions are as follows.

Sample Type	**Initial Mobile Phase**
Neutral	acetonitrile/water : 40/60 v/v
Basic	acetonitrile/0.1 M salt : 40/60 v/v/
Acidic	acetonitrile/ aqueous buffer pH 2.0 : 40/60 v/v/

Recommended Initial Conditions

1. Temperature 25°C (room temperature)

2. Flow rate 1 ml/min (compatible with available pressure)

3. Sample dissolved in mobile phase 2–5 mg/ml

To adjust the retention and selectivity, the solvent ratio can be changed in much the same way as the normal phase. However, now, as dispersive forces are controlling retention, increasing the buffer content will increase retention and probably chiral selectivity, whereas increasing the acetonitrile content will have the opposite effect. Temperature changes will also control retention but, again, the effect of change in temperature will depend on the cross-over temperature. Some cellulose based chiral stationary phases are not very thermally stable and so the operating temperature should be kept well within the recommended range. It follows, that temperature may not be a very effective variable for controlling retention and chiral selectivity as it is with other phases.

The cellulose and amylose stationary phases have been used successfully for the separation of a wide range of sample types. They have competently resolved a large number of different drug enantiomers, amino acids, protected amino acids, peptides etc. These types of stationary phase have a high concentration of chirally active centers, and therefore exhibit a high chromatographic capacity for many chiral

substances. As a result, the cellulose based stationary phases have been very effective in preparative chiral chromatography.

The Pirkle Stationary Phases

The Pirkle phases have evolved over the years and there are basically, two groups of phases, as shown in the table below.

Pirkle Chiral Phases

	Strong Polar Phases (p-Electron Acceptors)	**Polarizable Phases (p-Electron Donors)**
Packings	D-Phenylglycine	D-N-2-N-Naphthylalanine
	L-Phenylglycine	L-N-2-N-Naphthylalanine
	DL-Phenylglycine	DL-N-2-N-Naphthylalanine
	D-Leucine	(S)-N-1-N-Naphthylleucine
	L-Leucine	
	(R,R)-β-GEM 1	
	(S,S)-β-GEM 1	
	(R)-α-Burke-1	
	(S)-α-Burke-1	
Resolvable Compounds	Polarizable and Polar Compounds (π-Electron Donors) Aromatics	Strong Polar Compounds (π-Electron Acceptors) DNB or DNA Derivatives
Compound Types	Aromatic Alcohols	Alcohols
	Aromatic Amino Acids	Amino Acids
	Aromatic Amino Alcohols	Amino Alcohols
	Aromatic Thiols	Thiols
	Aromatic Amines	Amines
	Aromatic Sulfoxides	Diols
	Mandelic Acid Analogs	
	Aryl-Substitute Phthalides	
	Aryl-Substituted Lactams	
	Aryl-Substituted Succinimides	
	Aryl-Substituted Hydantoins	
	α-Hydroxy-α-Aryl Phosphonates	
	Aryl Propionic Acids	
	Phenoxybenzylamines	
	(β-Blockers)	

Examples	2,2,2-Trifluoro-1-(9-anthryl)-ethanol	1,2-Cyclohexane Diol
	Hexabarbital	2-Amino-1-butanol
	Propanolol	alanine
		Phenylethyl Alcohol

The first type are strongly polar (designated in the table as π-electron acceptors) and can therefore interact with a solute in two ways. If the

solute has one or more aromatic rings, then dipoles will be induced into the ring and dipole-induced dipole interaction can take place.

If, on the other hand the solute has a strong dipole, then dipole-dipole interaction can take place directly between the two dipoles. The second group contain polarizable structures(designated in the table as π-electron donors) such as aromatic rings, and, in this case, the opposite can occur. A strong polar solute can induce dipoles in the aromatic ring of the stationary phase and again dipole-induced dipole interaction will take place. It is interesting to note that phenylethyl alcohol is given as a suitable type of solute to be separated on the second class of stationary phase. This solute molecule has a permanent dipole from the alcohol group, a polarizable aromatic ring that can provide strong induced dipole interactions and finally the aromatic ring and the two methylene groups will provide strong dispersive interactions. This type of molecule will interact strongly with almost any other molecule by at least one of the three available interactive mechanisms.

By far the most popular, and the most extensively used Pirkle stationary phase today, is, without doubt, the Whelk-01 chiral stationary phase. This phase has already been described, but it should be recalled that it contains strong polar groups for dipole-dipole interactions, three aromatic nuclei to provide strong induced dipole interactions and finally, a naphthene ring together with sundry methylene and methyl groups to provide strong dispersive interactions. Like the phenethyl alcohol, just considered, it is capable of interacting strongly with almost any other type of molecule, whatever its interactive character. As such, it is not surprising, it is so versatile and popular stationary phase.

Most of the Pirkle phases, and in particular the Whelk-01 phase, are stable to all types of solvent and can be used either in the reversed phase mode, or the normal mode, which again, adds to its universal applicability. In the normal phase mode of development, hexane/IPA would be a good mobile phase from which to start. A mixture, hexane/IPA : 80/20 v/v, would be a practical scouting composition to assess the possible level of retention and chiral selectivity. Again,

increasing the dispersive interactions in the mobile phase, by increasing the hexane content, will increase the retention and probably the chiral selectivity. Conversely, increasing the polar solvent, will increase the mobile phase polar interactions, thus reducing retention time and chiral selectivity.

If a solvent composition cannot be found that will provide an appropriate combination of retention and chiral selectivity, then a different polar solvent, such as ethanol or acetonitrile, should be tried. Using a different polar solvent will change the polar/dispersive ratio of the solvent mixture, and thus provide an alternative balance between retention and chiral selectivity.

In the reversed phase mode, a good scouting solvent mixture might be acetonitrile/buffer : 20/80 v/v, which should give reasonably high retention and chiral selectivity, if the stationary phase and the dispersive solvent is appropriate. In this case, changes in water content has the reverse effect. Increasing the water content will reduce the dispersive interaction of the mobile phase with the solute and thus, increase the retention and probably selectivity. Conversely, reducing the water content will, by the same argument, increase retention and selectivity. If a mixture cannot be found that will provide adequate chiral selectivity and moderate retention, then the solvent will need to be switched to change the potential dispersive/polar interaction ratio of the overall solvent mixture.

The Whelk-01 stationary phase is relatively temperature stable and thus the operating temperature can be changed to give a final, and more subtle, opportunity to fine-tune the separation. However, the operating temperature must be kept with the published limits, for the safe operation of the stationary phase, as thermal degradation of chiral stationary phases is almost always irreversible. Having found a suitable solvent mixture that will provide satisfactory retention and chiral selectivity, it is always worth while to explore the effect of temperature on the separation as long as the temperature range does not exceed that recommended by the suppliers. Again, it must be recalled, that there will be a crossover

temperature where both enantiomers coelute. Thus, to increase chiral selectivity the temperature must adjusted further away from the coelution temperature.

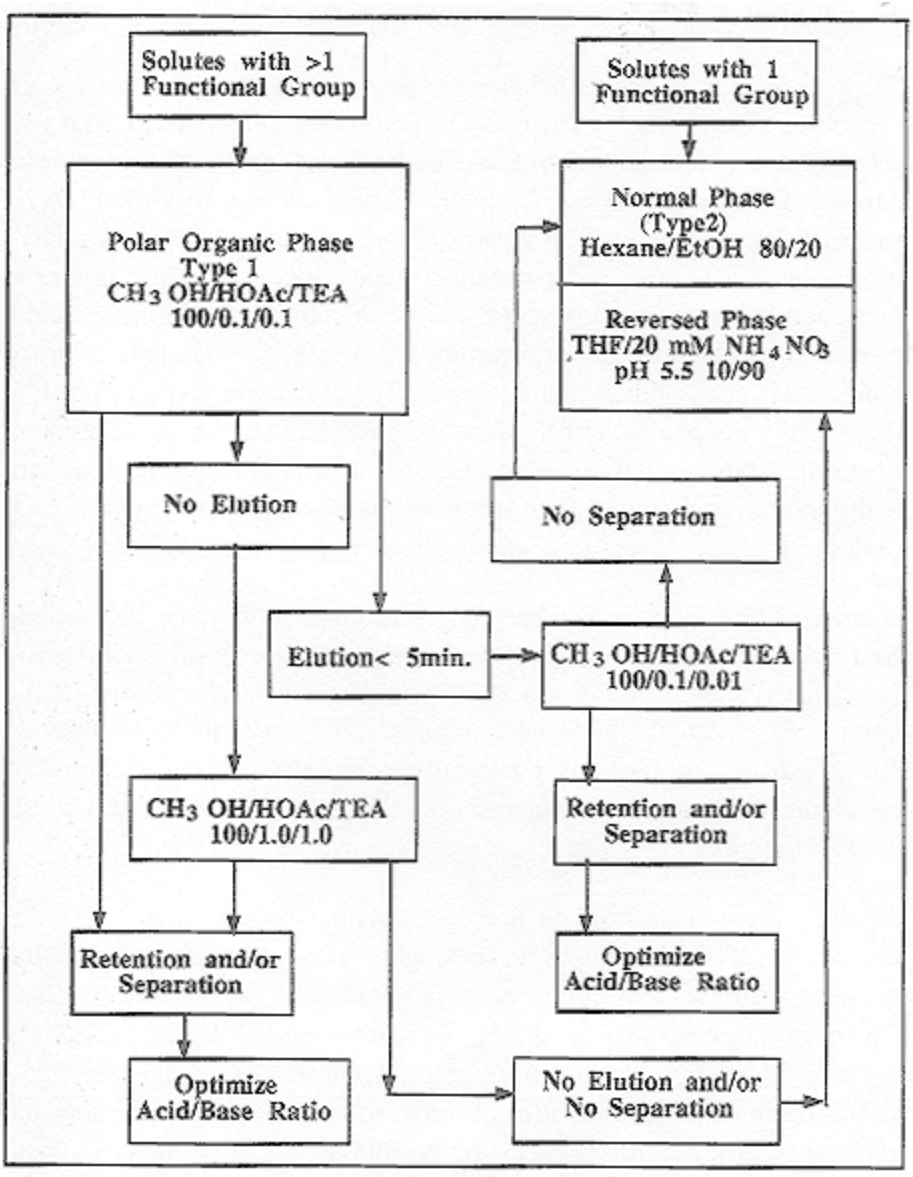
The Macrocyclic Glycopeptide Bonded Phases

The two most established macrocyclic glycopetide stationary phases are Chirobiotic V (Vancomycin) and Chirobiotic T (Teicoplanin). The two phases have unique chiral selectivities and are complementary to one another. In most instances, if separation fails on one, it is likely to be successful on the other. The macrocyclic glycopetide stationary phases have been successful in separating a wide range of sample types, in particular the phases show good chiral selectivity for amides, acids, esters, cyclic amines and are particularly useful for the separation of amino acids and N-blocked amino acids. The broad selectivity evinced by these stationary phase can be realized in all three modes of development, reversed phase, normal phase and the so-called polar organic phase, but is different in each mode. The stationary phase is uneffected by switching rapidly from one solvent system to another, during method development.

A method development scheme for Chirobiotic V is given below. The first consideration is the number of functional groups the enantiomers have, particularly those associated with the chiral center. If the compound has more than one functional group, *i.e.* the possibility of strong chiral selectivity is potentially good, then the first choice of development mode, as recommended by the manufacturers, would be the 'polar organic'.

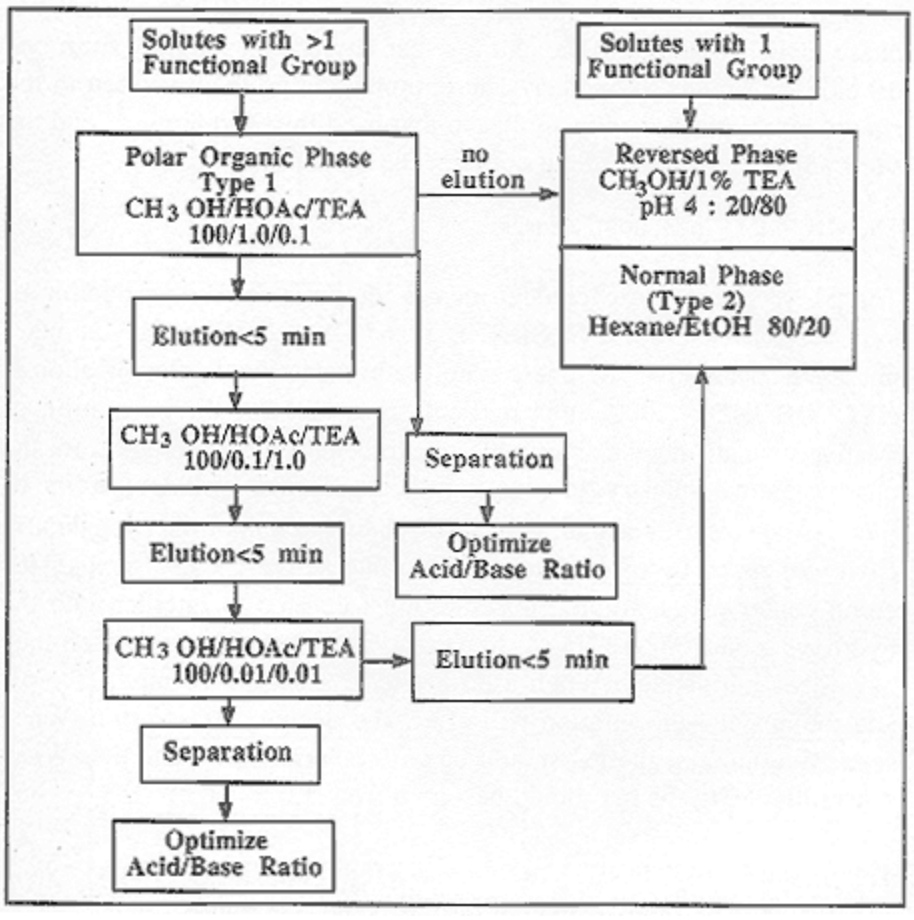
The key factor to achieving the best possible separation in the polar organic mode is to identify the best ratio of acid to base. The actual concentration of acid plus base only effects the retention. Each Chirobiotic phase has a different recommended initial acid/base concentration. If the solutes are eluting too fast, then the acid/base concentration should obviously be reduced. Conversely, increasing the acid/base concentration reduces the retention. The proximate practical range for the acid/base concentration is between 1.0% and 0.001%. If

increasing the acid/base concentration to 1% is still inadequate, then a reversed phase system is probably more appropriate.



A Method Development Protocol for CHIROBIOTOC V

If the acid/base concentration is reduced to 0.001% with inadequate results, then a normal phase development is probably the most suitable. Solutes that only have one functional group associated with the chiral center are more likely to be resolved using the conventional normal phase or reversed phase modes. Optimization of the reverse phase separation is accomplished in the usual way, by adjusting the organic modifier concentration and the nature of the modifier.



A Method Development Protocol for CHIROBIOTOC T

Acetonitrile, methanol, ethanol, IPA and tetrahydrofuran have all been shown to provide good selectivities for different solutes. In general,

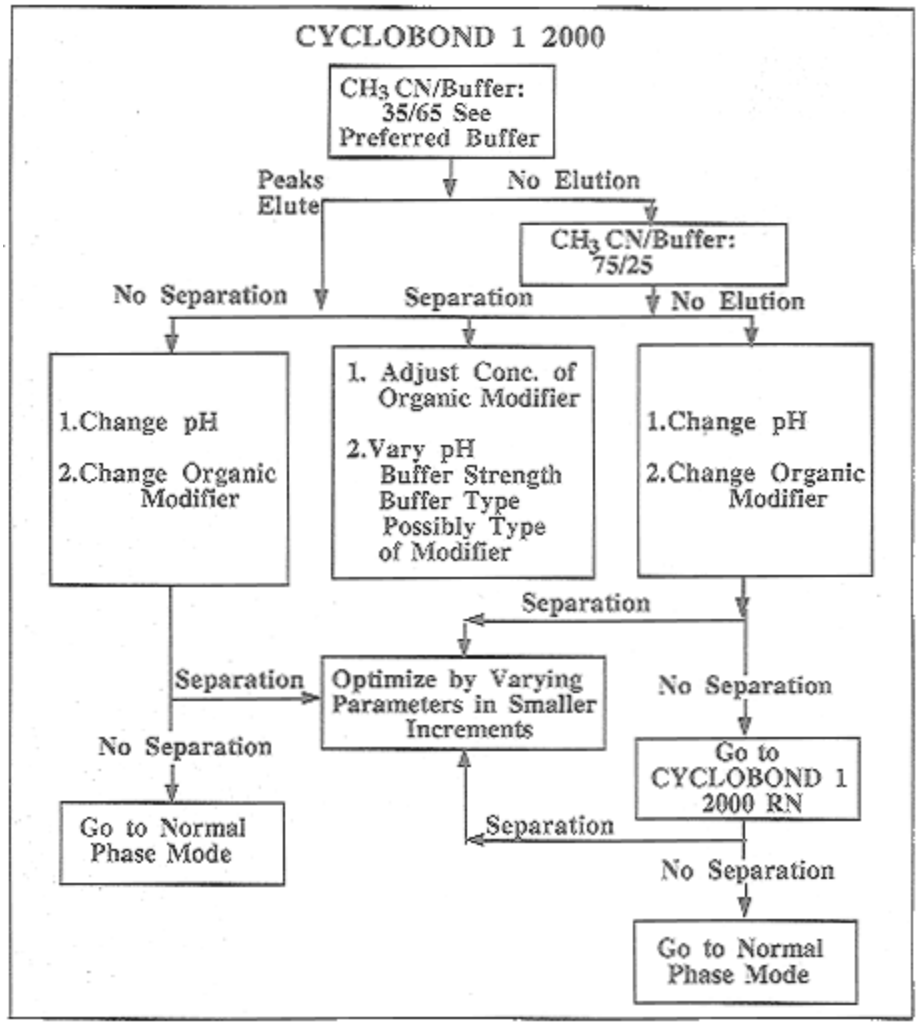
tetrahydrofuran appears to be the most useful for Chirobiotic V and methanol for Choribiotioc T. The protocol for Chirobiotic T is shown above.

Chirobiotic T exhibits a unique chiral selectivity for a number of classes of solutes. It is particularly useful for resolving the enantiomers of underivatized amino acids, N-derivatized amino acids and small peptides but is widely applicable to many classes of compounds. Chirobiotic T can also be used with a very wide range of solvents, and can be used with the polar organic system, in the reversed phase mode and in the normal phase mode. In addition, the chiral selectivity changes greatly from one development mode to another. The optimization protocol as seen in the chart Chirobiotic T is very similar to that used for Chirobiotic V and the same adjustments are made to optimize the separation.

The Cyclodextrin Bonded Phases

The β-, γ-, and the α-cyclodextrins are the basis for the production of the CYCLOBOND I, CYCLOBOND II and CYCLOBOND III stationary phases, respectively. The phase with the broadest field of application is CYCLOBOND I 2000. It is particularly useful for the separation of relatively small molecular weight enantiomers, typically drugs from the pharmaceutical industry. In choosing the appropriate cyclodextrin (*i.e.* α, β or γ), the size of the solute molecule relative to that of the cyclodextrin cavity needs to be taken into account. Interactive groups, next to the chiral center of the solute molecule, must be able to interact with the hydroxyl groups on the lip of the cavity, or any group with which they have been derivatized. When used in the reversed phase mode, the pH and the buffer concentration will effect the degree of resolution. When separating amines, the pH should be varied between 3.0 and 5.0. When separating acids, the pH should be varied from 5.0 to 7.0.

Temperature also has a pronounced effect on chiral selectivity, in general, the lower the temperature the better the chiral selectivity. In method development, it is necessary to determine both the retention ratio and the separation ratio at least at temperatures 5°C, 15°C and 25°C.

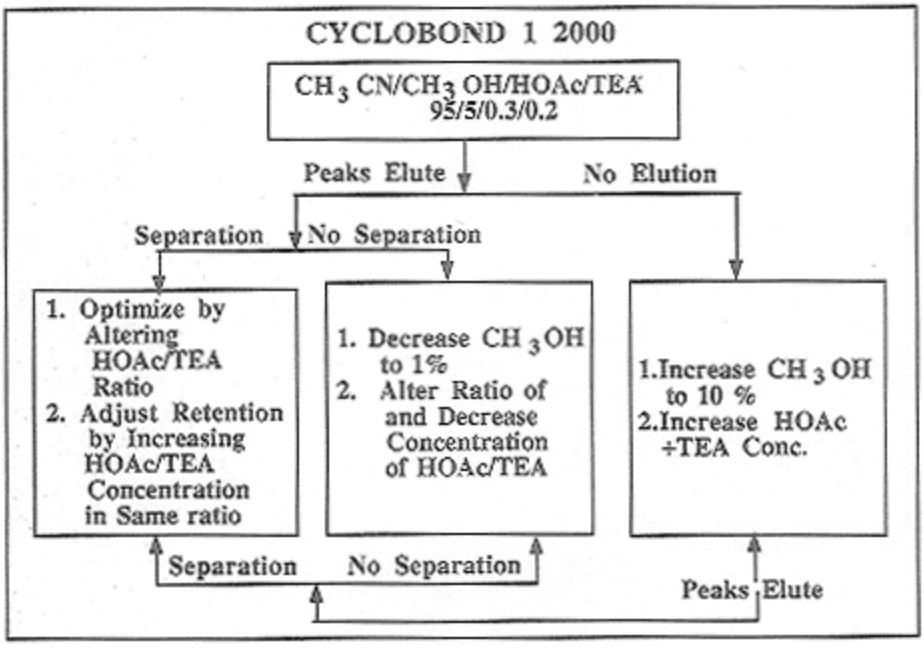


A Reverse Phase Method Development Protocol for CYCLOBOND 1 2000

A graph of retention ratio against the reciprocal of the absolute temperature can also be helpful in the manner previously discussed. The previous chart gives a method development protocol for CYCLOBOND 1 2000 operated in the reverse phase mode. The chart is self explanatory, the first solvent being acetonitrile/buffer 35/65 v/v. A method development protocol for CYCLOBOND 1 2000 operated in the polar

organic mode is as follows. This is for molecules that have at least 2 functional groups capable of interaction with the cyclodextrin surface. One functional group must be on the stereogenic center.

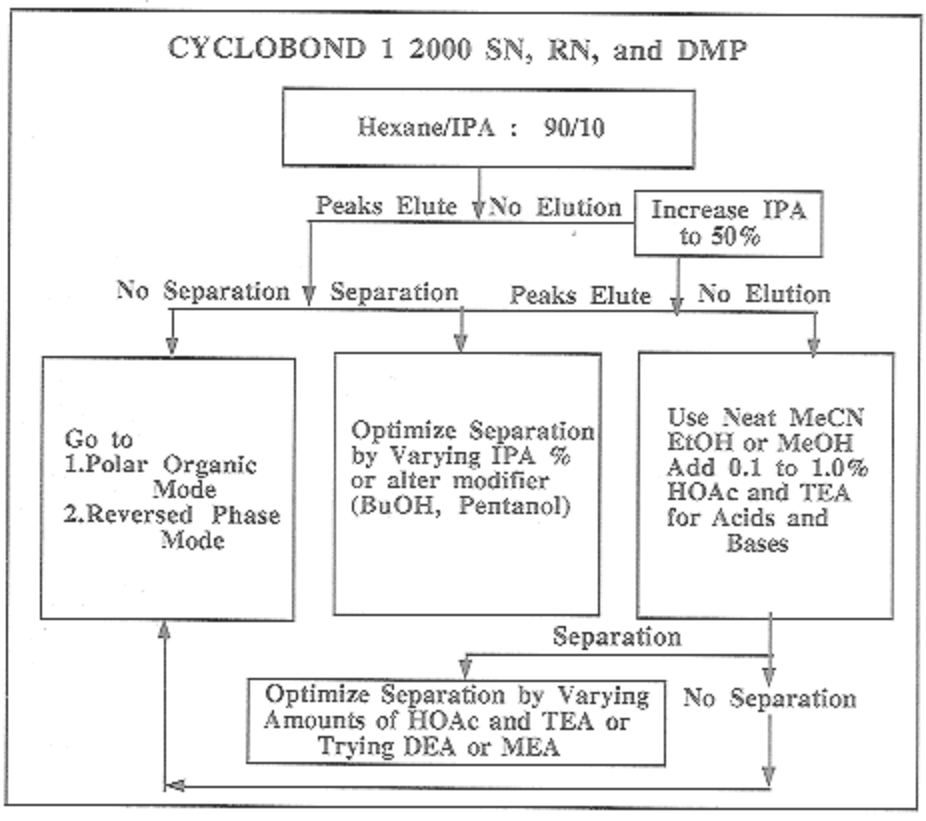
Preferred Buffers	Concentration Range	Preferred pH
TEAA/$TEAPO_4$	0.01%–2%	4.0 (alcohols, amines)
Ammonium Nitrate	10–500 mM	7.0 (acids)
Citrate	10–200 mM	
Ammonium Acertate	10–200 mM	
Acetic acid,TEA,DEA	10–25 µl/250 ml	



A Polar Organic Phase Method Development Protocol for CYCLOBOND 1 2000

Acetonitrile is the first choice for the organic moderator as it has a low viscosity and thus gives somewhat higher efficiencies. Unless the unique interacting properties of methanol are required to obtain the required chiral selectivity, this solvent should be avoided as it has a high viscosity and consequently lower efficiencies are often obtained. A list of preferred buffers is also given above. The polar organic solvents used

with the cyclodextrins are the same as those described previously for other stationary phases. They consist predominately of an organic solvent (*e.g.* acetonitrile) mixed with small quantities of dry glacial acetic acid and anhydrous triethylamine.



A Normal Phase Method Development Protocol for CYCLOBOND 1 2000

The chart is again largely self explanatory. This type of solvent system for chiral separations has a number of advantages. The column is more stable in this form of mobile phase, and the sample capacity is higher. Retention times are shorter and chiral selectivity appears to be improved. Finally, the solvent is easy to remove and thus, is appropriate for preparative separations. The following, is the protocol for normal phase development. If the solute is acidic and polarizable (or can be made

polarizable by introducing an aromatic ring using the derivatizing reagent 3,5-dinitrobenzoylchloride) then such substances are often easily resoved by the simple manipulation of the hexane/IPA ratio using CYCLOBOND 2000 SN as the chiral stationary phase. The preferred solvents and solvent concentrations are as follows.

Solvent	**Solvent Range**
Hexane/IPA	98–50% Hexane/2–50%IPA
Acetonitrile	100% 0.1 to 0.2 % Acetic Acid or TEA
Methanol or Ethanol	100% 0.1 to 0.2 % Acetic Acid or TEA
Acetonitril/Methanol	90–99% Acetonitrile/10–1% Methanol

Increasing the dispersive solvent hexane will increase the retention and probably the chiral selectivity. Increasing the polar solvent, will increase the polar interactions in the mobile phase, which will compete for the solute against the polar interactions between solute and stationary phase. The net effect will be a reduction in retention.

The cyclodextrins have become very popular due to their stability, reproducibility and high chiral selectivity. A list of examples of their use is given at the end of this chapter.

The procedure for finding the optimum conditions for the separation of a particular pair of enantiomers is very similar, irrespective of the stationary phase that is chosen. Certain phases may give a higher chiral selectivity than others, but the optimizing procedure will be very comparable. In general, raising the proportion of one solvent will increase the retention. Conversely, increasing the proportion of the other solvent will reduce retention. The same principle will hold with salts, buffers and the pH of the mobile phase. It is important to remember that there are only *three* basic types of interaction that can be exploited in the control of retention and selectivity, and they are dispersive, polar and ionic interactions. They may vary widely in relative strength, but they are the only basic interactions that are operating in the retention process.

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Appendix

The Solution of the Differential Equation that Describes the Rate of Change of Concentration in a Theoretical Plate with the Volume of Mobile Phase that Passes Through it.

$$\frac{dX_{m(p)}}{dv} = X_{m(p-1)} - X_{m(p)}$$

First consider the conditions of the above equation when an initial charge of concentration $X_{o(m)}$ has been placed on the first plate of the column, but chromatographic development has not commenced; that is v=0.

Then; $X_{m(p)} = X_{o(m)}$ when p=0

(*i.e.* for the first plate)

and: $X_{m(p)} = 0$ when p>0

(*i.e.* for any other plate in the column)

The first condition merely states that before the chromatographic development commences, the concentration in plate (0) is ($X_{o(m)}$) and is that resulting from the injection of the sample on the column. The second condition states that the remainder of the column is free of solute.

Thus, for plate p=0, and as there is no plate (p-1);

$$\frac{dX_{m(0)}}{dv} = X_{m(0)}$$

$$\frac{dX_{m(0)}}{X_{m(0)}} = dv$$

Intergrating:

$$\ln X_{o(m)} = -v + \text{constant}$$

When v = 0, $X_{o(m)} = X_{o(m)}$. Consequently, the constant = $\log_e X_o$.

Thus:

$$\log_e X_{o(m)} = -v + \log_e X_o$$

or:

$$X_{o(m)} = X_o e^{-v} \qquad (1)$$

For Plate 1:

$$\frac{dX_{m(1)}}{dv} = X_{m(0)} - X_{m(1)} \qquad (2)$$

Substituting for $X_{m(0)}$ from (1) in (2):

$$\frac{dX_{m(1)}}{dv} = X_o e^{-v} - X_{m(1)}$$

$$\frac{dX_{m(1)}}{dv} + X_{m(1)} = X_o e^{-v}$$

Multiplying throughout by e^v:

$$\frac{dX_{m(1)}}{dv} e^v + X_{m(1)} e^v = X_o$$

Now, this equation can be recognized as the differential of a product.

Hence:

$$\frac{d(X_{m(1)} e^v)}{dv} = X_o$$

Intergrating:

$$X_{m(1)} e^v = X_o v + k$$

Now, when v = 0, $X_{m(1)} = 0$, then, k = 0. Thus, $X_{m(1)} = X_o e^{-v} v$

In a similar way, it can be shown that,

for Plate (2):

$$X_{m(2)} = X_o \frac{e^{-v} v^2}{1.2}$$

for Plate (3):

$$X_{m(3)} = X_o \frac{e^{-v} v^3}{1.2.3}$$

Thus, for the nth plate:

$$X_{m(n)} = X_o \frac{e^{-v} v^n}{n!}$$

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