BOOK 1

Chrom-Ed Book Series

Raymond P. W. Scott

PRINCIPLES AND PRACTICE OF CHROMATOGRAPHY

Chrom-Ed Book Series

Book 1 Principles and Practice of Chromatography
Book 2 Gas Chromatography
Book 3 Liquid Chromatography
Book 4 Gas Chromatography Detectors
Book 5 Liquid Chromatography Detectors
Book 6 The Plate Theory and Extensions for Chromatography Columns
Book 7 The Thermodynamics of Chromatography
Book 8 The Mechanism of Retention
Book 9 Dispersion in Chromatography Columns
Book 10 Extra Column Dispersion
Book 11 Capillary Chromatography
Book 12 Preparative Chromatography
Book 13 GC Tandem Systems
Book 14 LC Tandem Systems
Book 15 GC Quantitative Analysis
Book 16 LC Quantitative Analysis
Book 17 Silica Gel and Its Uses in Chromatography
Book 18 Thin Layer Chromatography
Book 19 Chiral Chromatography
Book 20 Sample Preparation

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Contents

Introduction

Chromatography, although primarily a separation technique, is mostly employed in chemical analysis. Nevertheless, to a limited extent, it is also used for preparative purposes, particularly for the isolation of relatively small amounts of materials that have comparatively high intrinsic value. Chromatography is probably the most powerful and versatile technique available to the modern analyst. In a single step process it can separate a mixture into its individual components and simultaneously provide an quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature and can range in complexity from a simple blend of two entantiomers to a multi component mixture containing widely differing chemical species. Furthermore, the analysis can be carried out, at one extreme, on a very costly and complex instrument, and at the other, on a simple, inexpensive thin layer plate.

The first scientist to recognize chromatography as an efficient method of separation was the Russian botanist Tswett (1), who used a simple form of liquid-solid chromatography to separate a number of plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography for this type of separation (color writing). Although color has little to do with modern chromatography, the name has persisted and, despite its irrelevance, is still used for all separation techniques that employs the essential requisites for a chromatographic separation,*viz.* a *mobile phase* and a *stationary phase*.

The technique, as described by Tswett was largely ignored for a along time and it was not until the late 1930s and early 1940s that Martin and Synge(2) introduced liquid-liquid chromatography by supporting the stationary phase, in this case water, on silica in a packed bed and used it to separate some acetyl amino acids. In their paper, they recommended replacing the liquid mobile phase by a suitable gas, as the transfer of sample between the two phases would be faster, and thus provide more efficient separations. In this manner, the concept of *gas chromatography* was created but again, little notice was taken of the suggestion and it was

left to Martin himself and A. T. James to bring the concept to practical reality nearly a decade later. In the same publication in 1941, the essential requirements for HPLC (High Performance Liquid Chromatography) were unambiguously defined,

"*Thus, the smallest HETP* (the highest efficiency) *should be obtainable by using very small particles and a high pressure difference across the column*".

Despite his recommendations, however, it was nearly four decades before this concept were taken seriously and the predicted high efficiency liquid chromatography columns became a reality. By the mid 1960s the development of all aspects of chromatography were virtually complete and since then, despite the plethora of publications that have appeared on the subject, the vast majority has dealt with applications of the technique and only a minority with fundamental aspects of the subject and novel instrumentation concepts.

Today, chromatography is an extremely versatile technique; it can separate gases, and volatile substances by GC, involatile chemicals and materials of extremely high molecular weight (including biopolymers) by LC and if necessary very inexpensively by TLC. All three techniques, (GC), (LC) and TLC have common features that classify them as chromatography systems.

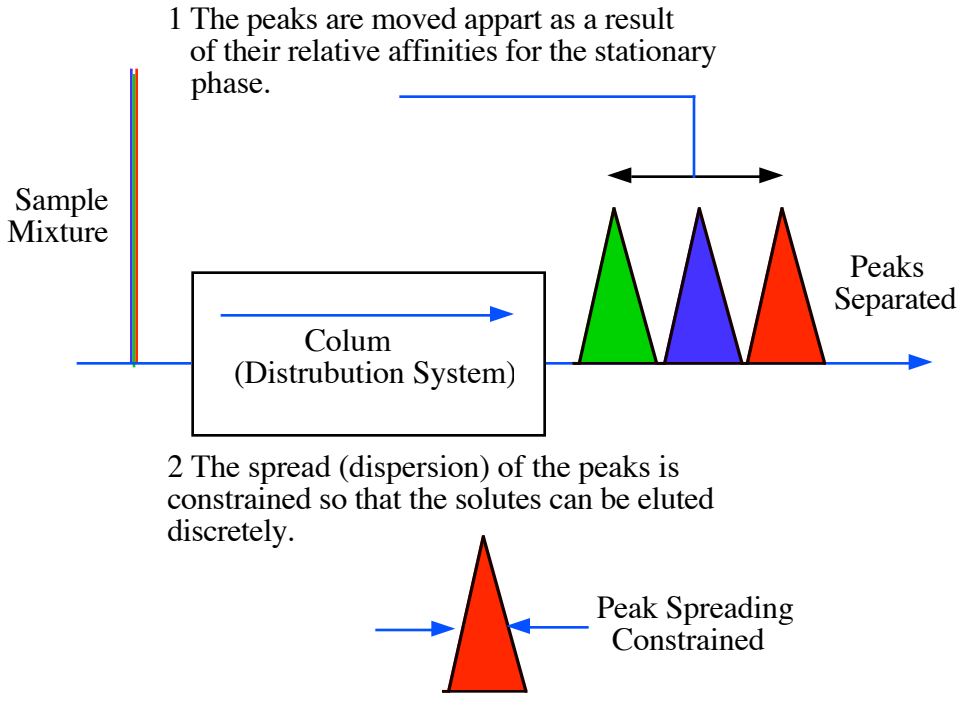
Chromatography has been defined as follows,

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase; ipso facto a separation is achieved.

In practice, the distribution system, (that part of the chromatographic apparatus where the solutes are distributed between the phases) can take

the form of a column such as a tube packed with particulate matter on which the stationary phase is bonded or coated. The mobile phase (which may be a gas or a liquid) passes under pressure through the column to elute the sample. The column form may also be a long, small-diameter open tube that has the stationary phase coated or bonded to the internal surface. Alternatively, the chromatographic system may take the form of a plate (usually glass) the surface of which is loaded with particulate matter to which the stationary phase is coated or bonded. The mobile phase (a liquid) is arranged to percolate up the plate (usually by surface tension forces) to elute the sample. The sample is injected into the mobile phase stream just before the front of the columns. The column is designed to allow two processes to take place that will produce the separation. Firstly, as a result of different forces between each molecular type and the stationary phase, each solute is retained to a different extent and, thus, the more weakly held will elute first and the more strongly held elute last. The process is diagramatically depicted below.

Two Processes Occur in the Column



The Function of the Column

Consequently, each solute will be sequentially eluted from the column in the reverse order of the magnitude of the interacting forces between each solute and the stationary phase. Secondly, the spreading of each solute band (that is its dispersion) must be constrained so that each solute is eluted discreetly. The first function of the column is achieved by choosing the appropriate phase system (the optimum stationary phase in GC and the optimum combination of mobile phase and stationary phase in LC) to separate the solutes. The second function is achieved by selecting the optimum physical properties of the column (column dimensions, particle diameter, mobile phase velocity etc.) to ensure that band dispersion is adequately constrained. As all chromatographic separations are carried out using a *mobile* and a *stationary* phase, the primary classification of chromatography is based on the physical nature of the *mobile* phase. The mobile phase can be a gas or a liquid which gives rise to the two basic forms of chromatography, namely, gas chromatography (GC) and liquid chromatography (LC).

Table 1 The Classification of Chromatography

MOBILE PHASE	STATIONARY PHASE
GAS Gas Chromatography **(GC)**	**LIQUID** Gas-Liquid Chromatography **(GLC)**
	SOLID Gas-Solid Chromatography **(GSC)**
LIQUID Liquid Chromatography **(LC)**	**LIQUID** Liquid-Liquid Chromatography **(LLC)**
	SOLID Liquid-Solid Chromatography **(LSC)**

The stationary phase can also take two forms, solid and liquid, which provides two subgroups of GC and LC, namely; gas–solid

chromatography (GSC) and gas–liquid chromatography (GLC), together with liquid solid chromatography (LSC) and liquid chromatography (LLC). The different forms of chromatography are summarized in Table 1. Most thin layer chromatography techniques are considered liquid-solid systems although the solute normally interacts with a liquid-like surface coating on the adsorbent or support or, in some cases an actual liquid coating.

The Development Process

A solute progresses through the chromatographic system, albeit through a column or along a plate, only while it is in the mobile phase. This process, whereby the substances are moved through the chromatographic system, is called chromatographic development. There are three types of chromatographic development, *elution development*, *displacement development* and *frontal analysis*. Elution development is now virtually the only development technique employed in both GC and LC although some displacement development is occasionally used in *preparative* LC.

In TLC, the development process is confused by the frontal analysis of the multicomponent solvent that occurs as the mobile phase moves through the system. In contrast, the solutes are transported across the plate by elution development. This apparent paradox will be explained in detail in due course.

Displacement Development

Displacement development is only effective with a solid stationary phase where the solutes are adsorbed on its surface. The sample mixture is placed on the front of the distribution system, and the individual solutes compete for the immediately available adsorption sites. Initially, all the nearby adsorbent sites will be saturated with the most strongly held component. As the sample band moves through the system the next available adsorption sites will become saturated with the next most strongly adsorbed component. Thus, the components array themselves along the distribution system in order of their decreasing adsorption

strength. The sample components are usually held on the stationary phase so strongly that they are eluted very slowly or even not at all. Consequently the solute must be displaced by a substance more strongly held than any of the solutes (called the displacer). The displacer, contained at a low concentration in the mobile phase, first displaces the most strongly held component. In turn this component will displace the one next to it. Thus, the displacer forces the adsorbed components progressively through the distribution system, each component displacing the one in front until they are all pass through the system. The solutes will be characterized by the order in which they elute and the amount of each solute present will be proportional to the length of each band, not the height. In displacement development the solutes are never actually *separated* from one another. The solutes leave the system sequentially and in contact, each somewhat mixed with its neighbor. This type of development is not used in analytical chromatography and only very rarely in preparative LC. However, displacement effects can occur in overloaded distribution systems and in the development of thin layer plates with multicomponent solvents.

Frontal Analysis

This type of chromatographic development is rarely used and probably is of academic interest only; it and can only be effectively employed in a *column* distribution system. The sample is fed continuously onto the column as a dilute solution in the mobile phase in contrast to displacement and elution development, where discrete samples are placed on the system and the separation subsequently processed. Frontal analysis can only separate part of the first compound in a relatively pure state, each subsequent component being mixed with those previously eluted. Consider a three component mixture, containing solutes (A), (B) and (C) as a dilute solution in the mobile phase that is fed continuously onto a column. The first component to elute, (A), will be that solute held *least* strongly in the stationary phase. Then the second solute, (B), will elute but it will be mixed with the first solute. Finally, the third solute (C), will elute in conjunction with (A) and (B). It is clear that only solute (A) is eluted in a pure form and, thus, frontal analysis would be quite

inappropriate for most practical analytical applications. This development technique has been completely superseded by elution development.

Elution Development

Elution development is best described as a series of absorption-extraction processes which are continuous from the time the sample is injected into the distribution system until the time the solutes exit from it. The elution process is depicted in Figure 1. The concentration profiles of the solute in both the mobile and stationary phases are depicted as Gaussian in form. Equilibrium occurs between the two phases when the probability of a solute molecule striking the boundary and entering one phase is the same as the probability of a solute molecule randomly acquiring sufficient kinetic energy to leave the stationary phase and enter the other phase. The distribution system is continuously thermodynamically driven toward equilibrium. However, the *moving* phase will continuously displace the concentration profile of the solute in the mobile phase forward, relative to that in the stationary phase which, in a grossly exaggerated form, is depicted in Figure 1.

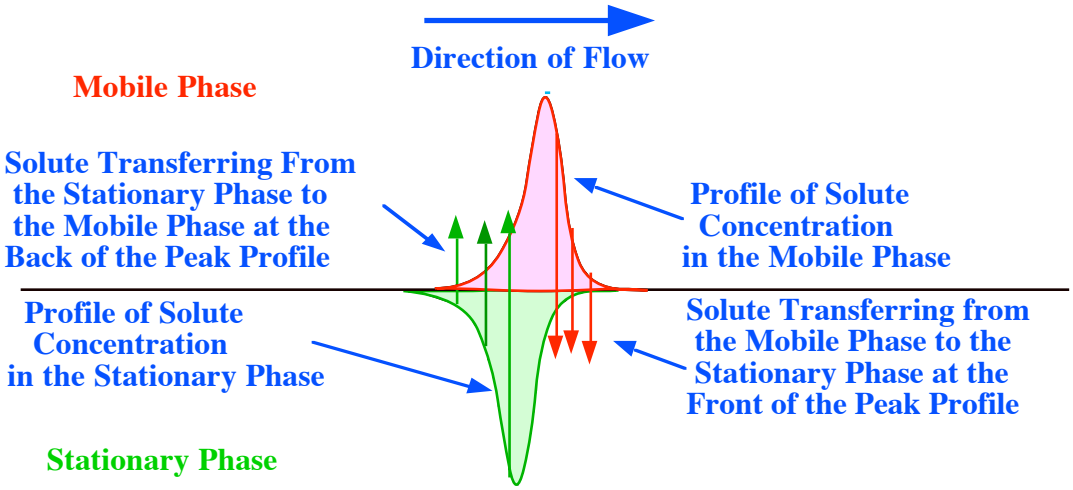


Figure 1. The Elution of a Solute Through a Chromatographic System

This displacement causes the concentration of solute in the mobile phase at the front of the peak to *exceed* the equilibrium concentration with respect to that in the stationary phase. As a consequence, a *net* quantity of solute in the front part of the peak is continually *entering* the

stationary phase from the mobile phase in an attempt to re-establish equilibrium. At the rear of the peak, the reverse occurs. As the concentration profile moves forward, the concentration of solute in the stationary phase at the *rear* of the peak is now in *excess* of the equilibrium concentration. A net amount of solute must now *leave* the stationary phase and enters the mobile phase to re-establish equilibrium. Thus, the solute moves through the chromatographic system as a result of solute entering the mobile phase at the rear of the peak and returning to the stationary phase at the front of the peak. However, that solute is always transferring between the two phases over the whole of the peak in an attempt to attain or maintain thermodynamic equilibrium. Nevertheless, the solute band progresses through the system as a result of a *net* transfer of solute from the mobile phase to the stationary phase in the *front half* of the peak. This *net* transfer of solute is compensated by solute passing from the stationary phase to the mobile phase at the *rear half* of the peak. Equilibrium processes between two phases is complicated, but a simplified explanation is as follows. The distribution of kinetic energy of the solute molecules contained in the stationary phase and mobile phase is depicted in Figure 2A and 2B. Solute molecules leave the stationary phase when their kinetic energy is equal to or greater than the potential energy of their interaction with the stationary phase. The distribution of kinetic energy between the molecules dissolved in the stationary phase at any specific temperature T, can be considered to take the form of a Gaussian curve as shown in Figure 2A. Other distribution functions might be more appropriate, but the specific nature of the function used will not affect the following explanation and so, for simplicity, the Gaussian function is assumed. The number of molecules at the boundary surface (N_1) that have a kinetic energy in excess of the potential energy associated with their molecular interactions with the stationary phase (E_A), (*i.e.,* those molecules represented by the red area of the distribution curve) will leave the stationary phase and enter the mobile phase. Those with an energy less than (E_A) will remain in the stationary phase. The distribution of energy of the solute molecules in the mobile phase is depicted in Figure 2B. The distribution is again taken as Gaussian in form and it is seen that the number of molecules (N_2) striking the surface that have an energy less than (E_A) (*i.e.,* the red area

in figure 2B) will remain in the stationary phase after entering the liquid, whereas the others having energies above (E_A) will collide with the surface and 'rebound'. 'Rebound' is, perhaps, a somewhat inappropriate term in this context.

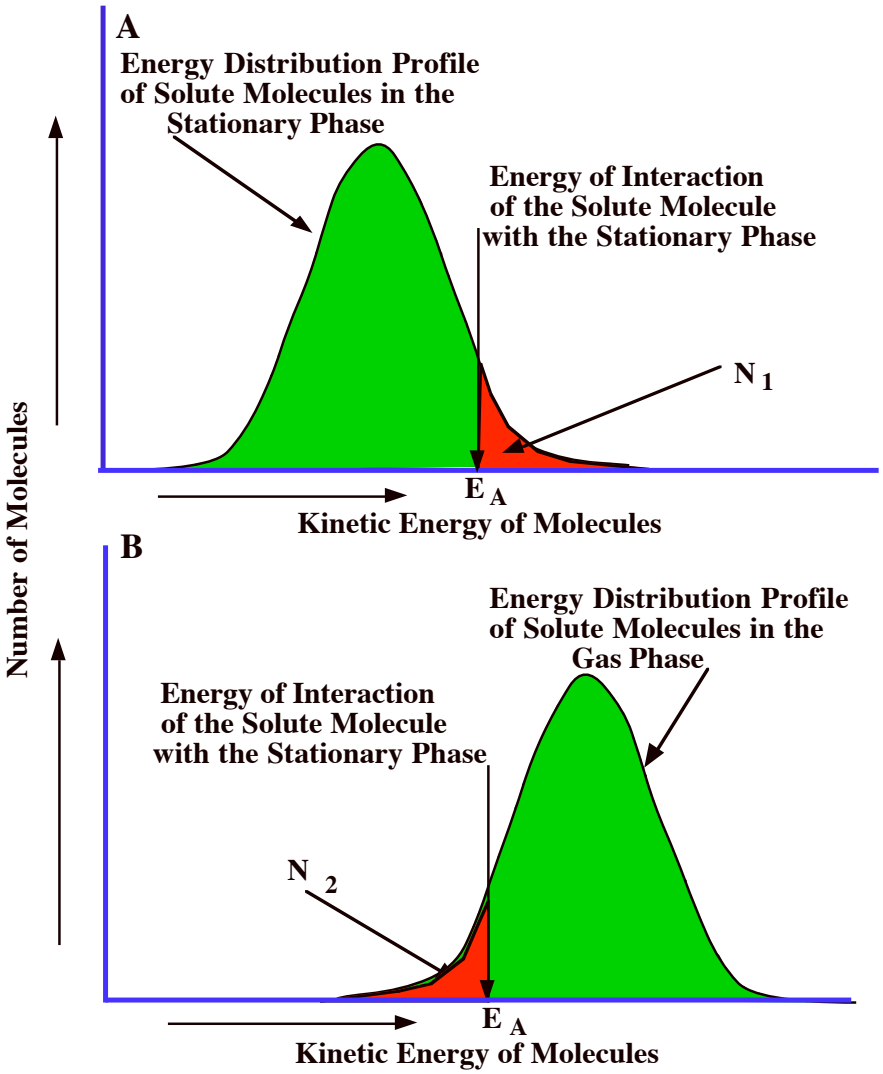


Figure 2. Energy Distribution of Solute Molecules in the Stationary and Mobile Phase

In fact, some may rebound, others may communicate their excess energy to a another solute molecule which will give it sufficient energy to enter the mobile phase.

In either case, the net effect is the same; there will be no net molecule transfer if its energy is too great.

Under equilibrium conditions,

$$N_1 = N_2$$

This description of the dynamics of solute equilibrium is oversimplified, but is sufficiently accurate for the reader to understand the basic principles of solute distribution between two phases.

Consider distribution between a gaseous mobile phase and a liquid stationary phase. As the temperature is raised the energy distribution curve in the gas moves to a higher range of energies. Thus, as the column temperature is increased, more solute molecules in the stationary phase will randomly acquire sufficient energy (E_A) to leave the stationary phase and enter the gas phase. Thus, the distribution coefficient of all solutes with respect to the stationary phase will be reduced as the temperature rises and it will be seen in due course that this will cause the band velocity of all the solutes to be increased.

Elution Development in Thin Layer Chromatography

The development processes that take place on a thin layer plate is complicated by the frontal analysis of the mobile phase itself. The mobile phases used to elute the solutes in TLC are usually multi-component, containing at least three individual solvents. If the plate is not pre-conditioned with solvent, there is an elaborate modification of the plate surface which is depicted, for a ternary solvent mixture, in Figure 3.

The edge of the plate is dipped into a tray of the solvent mixture which begins to migrate along the plate, driven by surface tension forces. The different solvents array themselves on the surface in the manner shown in Figure 3. The solvent that interacts most strongly with the stationary phase is extracted from the mixture and forms an adsorbed layer on the surface that corresponds to the area (X) in the diagram. The now binary mixture continues to migrate along the plate and the next solvent component that interacts most strongly with the stationary phase

(solvent B) is adsorbed as a layer on the surface corresponding to the area (Y).

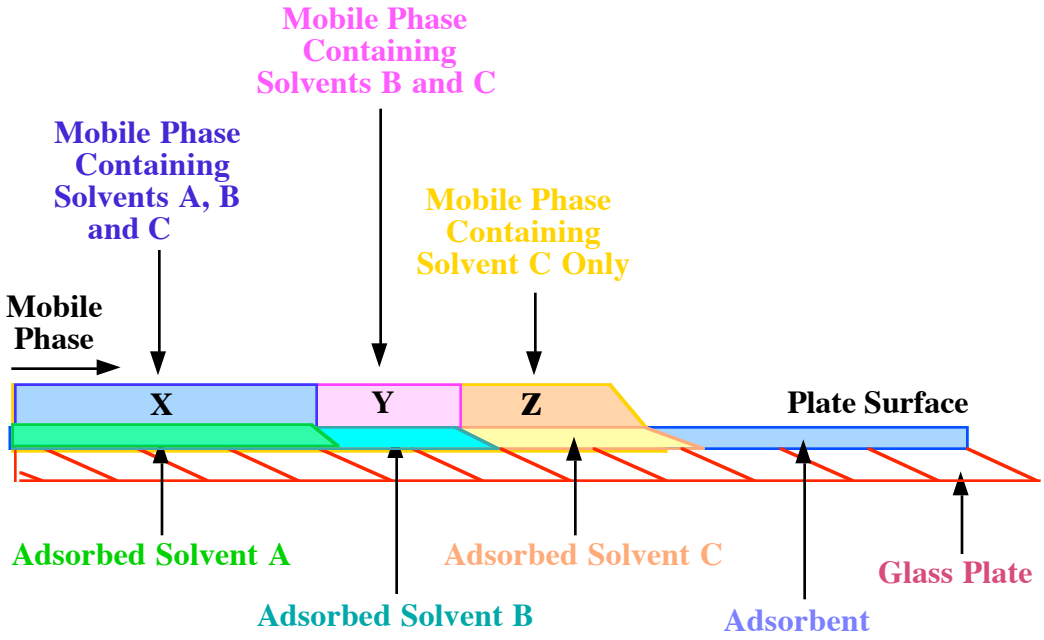


Figure 3. The Development of a Thin Layer Plate

Finally, the remaining solvent (C) with the weakest interactions with the stationary phase continues to migrate and cover the surface with a layer of solvent (C) in the area (Z). It is seen that the distribution system, which results from the frontal analysis of the three mobile phase components is now quite complex. The *solutes* will interact during the separation process. In the first section (X) solutes will be distributed between the ternary solvent mixture (A), (B) and (C) and the surface covered with solvent (A). In the next section (Y) the solutes will be distributed between a binary solvent mixture of (B) and (C) and a surface covered with solvent (B). Finally, distribution will take place in section (Z) between pure solvent (C) and a surface covered with solvent (C). Even this is an over-simplification, as the composition of the mobile phase in each section will not be constant but will decrease along the plate. Furthermore, as the separation progresses, the lengths of sections (X), (Y) and (Z) will continually increase. Such a system is extremely difficult to treat theoretically particularly as the boundaries are not as sharp as those depicted in Figure 3. In fact, the overall effect is as though

the separation was carried out sequentially on three separate sections of a plate, each section having a different stationary phase and mobile phase. In each section, the separation will then be achieved by elution development, but the overall effect will be a form of gradient elution.

The complexity of the system increases with the number of solvents used and, of course, their relative concentrations. The process can be simplified considerably by pre-conditioning the plate with solvent vapor from the mobile phase before the separation is started. Unfortunately, this only partly reduces the adsorption effect, as the equilibrium between the *solvent vapor* and the adsorbent surface will not be the same as that between the *liquid solvent* and the surface. It is clear that by forming a gradient by the frontal analysis of the mobile phase and carefully choosing the solvent mixture, very delicate *pseudo*-gradients can be created, which, in no small measure, accounts for the great versatility, popularity, and success of TLC.

Chromatography Nomenclature

Chromatography nomenclature has evolved over the years but it was not until the late 1950s that the various terms used for the characteristics of a chromatogram were rationalized. A summary of the nomenclature is shown diagramatically in figure 4. The various terms are defined as follows.

The *baseline* is any part of the chromatogram where only mobile phase is emerging from the column.

The *peak maximum* is the highest point of the peak.

The *injection point* is that point in time/position time when/where the sample is placed on the column.

The *dead point* is the position of the peak-maximum of an unretained solute.
The *dead time* (t_o) is the time elapsed between the *injection point* and the *dead point*.

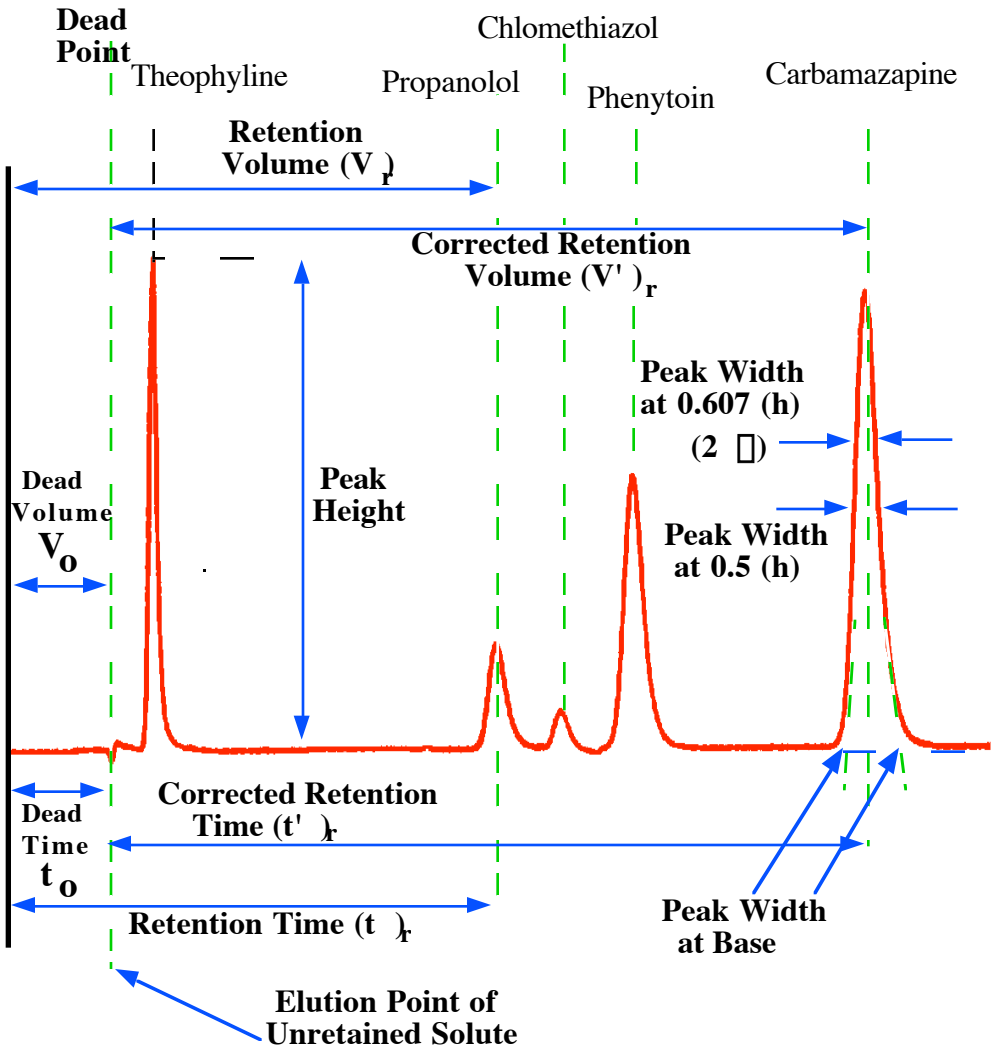


Figure 4 The Nomenclature of a Chromatogram.

The *dead volume* (V_O) is the volume of mobile phase passed through the column between the *injection point* and the *dead point*.

Thus, $V_O = Qt_O$ where Q is the flow rate in ml/min.

The *retention time* (t_r) is the time elapsed between the *injection point* and the *peak maximum*. Each solute has a characteristic retention time.

The *retention volume* (V_r) is the volume of mobile phase passed through the column between the *injection point* and the *peak maximum*.

Thus, $V_r = Qt_r$ where Q is the flow rate in ml/min.

Each solute will also have a characteristic retention volume.

The *corrected retention time* (t'_r) is the time elapsed between the *dead point* and the *peak maximum*.

The *corrected retention volume* (V'_r) is the volume of mobile phase passed through the column between the *dead point* and the *peak maximum*. It will also be the *retention volume* minus the *dead volume*.

Thus, $V'_r = V_r - V_o = Q(t_r - t_o)$ where Q is the flow rate in ml/min.

The *peak height* (h) is the distance between the *peak maximum* and the *base line* geometrically produced beneath the peak.

The *peak width* (w) is the distance between each side of a peak measure at 0.6065 of the peak height (*ca* 0.607h). The peak width measured at this height is equivalent to two standard deviations (2s) of the Gaussian curve and thus has significance when dealing with chromatography theory.

The *peak width at half height* ($w_{0.5}$) is the distance between each side of a peak measured at half the peak height. The peak width measured at half height has no significance with respect to chromatography theory.

The *peak width at the base* (w_B) is the distance between the intersections of the tangents drawn to the sides of the peak and the *peak base* geometrically produced. The peak width at the base is equivalent to four standard deviations (4s) of the Gaussian curve and thus also has significance when dealing with chromatography theory.

Factors Controlling Retention

The equation for the retention volume (V_r), as derived from the Plate theory (see Book 6 The Plate Theory and Extensions) is as follows,

$$V_r = V_m + KV_S$$

or $$V'_r = KV_S \qquad (1)$$

where (V_m) is the volume of mobile phase in the column
(V_S) is the volume of stationary phase in the column,
(K) is the distribution coefficient of the solute between the phases,
and (V'_r) is the corrected retention volume i.e., ($V_r - V_m$)

From equation (1) it is seen that the corrected retention volume is controlled by two parameters: firstly the *distribution coefficient* of the solute between the two phases and secondly, the *amount of stationary phase* that is available to the solute.

Consequently, the magnitude of (V'_r) is determined by (K) or (V_S) or both.

From equation (1) the conditions necessary to separate two solutes (A) and (B) can be deduced.

To separate solutes (A) and (B), $V'_{r(A)} <> V'_{r(B)}$,

which can be achieved by making either $K_{(A)} <> K_{(B)}$

or $V_{S(A)} <> V_{S(B)}$ or an appropriate combination of both.

Thus, to separate a mixture, either the values of (K) for all components, or the amount of stationary phase (V_S), available to each component, must be made to differ or, again, appropriate adjustments must be made to both.

Prior to discussing the parameters that determine the magnitude of (K) and (V_S) and how they can be changed, it is useful to develop the thermodynamic approach to the problem of solute retention in chromatographic separations.

The Thermodynamic Explanation of Retention

Classical thermodynamics provides an expression that describes the change in *free energy* of a solute when transferring from one phase to the other as a function of the equilibrium constant (distribution coefficient). 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.

In addition, classical thermodynamics provides an expression for (ΔG^o),

i.e., $$\Delta G^o = \Delta H^o - T\Delta S^o$$

where (ΔH^o) is the Standard Enthalpy Change,
and (ΔS^o) is the Standard Entropy Change.

Thus,

$$\ln K = -\left[\frac{\Delta H^o}{RT} - \frac{\Delta S^o}{R}\right] \qquad (2)$$

or,

$$K = e^{-\left[\frac{\Delta H^o}{RT} - \frac{\Delta S^o}{R}\right]} \qquad (3)$$

It is seen that if the *standard entropy change* and *standard enthalpy change* for the distribution could be calculated then the distribution coefficient (K) and, consequently, the retention volume could also be predicted. Unfortunately, these properties are difficult, if not impossible, to isolate and estimate and so the magnitude of the overall distribution coefficient can not be estimated in this way. Nevertheless, once the phase system has been identified, with sufficient experimental data being available, empirical equations can be developed to optimize a given distribution system for a specific separation. Computer programs, based on this rationale, are available for LC to carry out such optimization procedures for solvent mixtures having three or more components. Nevertheless, the appropriate stationary phase is still usually identified from the types of interactions that need to be exploited to effect the required separation. By measuring the retention volume of a solute over a range of temperatures equation (2) can also be used to identify the type of retention mechanism that is operative in a particular separation .

Rearranging equation (2)

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

Noting, $V' = KV_S$

$$\log V' = -\frac{\Delta H_o}{RT} + \frac{\Delta S_o}{R} - \log V_s$$

Thus, a curve relating log(V') to 1/T will give a straight line the slope of which will be proportional to the *standard enthalpy* and the intercept will be related to the *standard entropy* and, two, the dominant effects that control the distribution system can be identified from such curves. Such curves are called Vant Hoff curves and an example of two Vant Hoff for two different types of distribution systems are shown in figure 5.

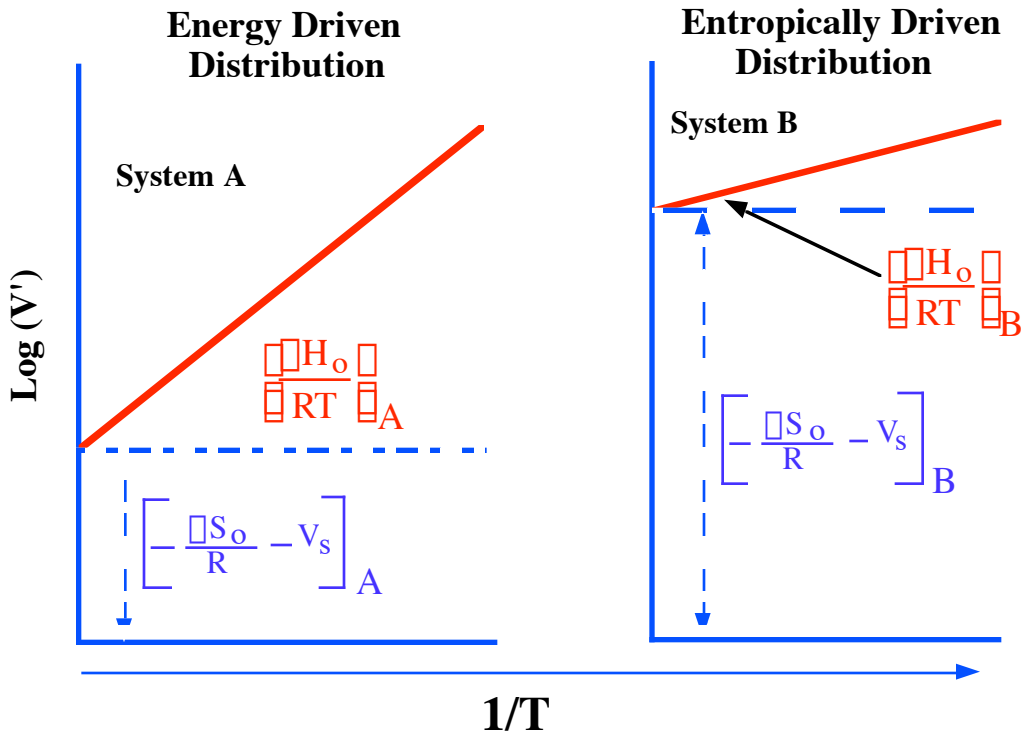


Figure 5 The Vant Hoff Curves for Two Different Distribution Systems.

It is seen that distribution system (A) has a large enthalpy value $\left[\frac{\Delta H_o}{R\,T}\right]_A$ and a low entropy contribution $\left[\frac{\Delta S_o}{R} - V_s\right]_A$. The large value of $\left[\frac{\Delta H_o}{R\,T}\right]_A$ means that the distribution is *predominantly controlled* by *molecular forces*. The solute is preferentially distributed in the stationary phase as a result of solute interactions with the stationary phase being much greater than those with the mobile phase. As 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, for distribution system (B) there is only a small enthalpy change $\left[\frac{\Delta H_o}{R\,T}\right]_B$, but a high entropy contribution $\left[\frac{\Delta S_o}{R} - V_s\right]_B$. Thus, the distribution is *not* predominantly controlled by molecular forces. The entropy is a measure of the degree of randomness that a solute molecule experiences in a particular phase. The more random and 'more free' the solute molecule is in a particular phase, the greater its entropy. A large negative entropy change means that the solute molecules are more restricted or less random in the stationary phase (B). and this loss of freedom is responsible for the greater solute retention. The change in entropy in system (B) is the major contribution to the change in free energy, so ,

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

Chiral separations and separations made by size exclusion are examples of entropically driven systems. Chromatographic separations are not exclusively "energetically driven" or "entropically driven". In most cases retention has both "energetic" and "entropic" components which, by careful adjustment, can be made to achieve very difficult and subtle separations.

Thermodynamics show that there are two processes controlling distribution but does not indicate how the distribution can be managed or controlled. To do this, it is necessary to identify how the magnitude of (K) and (V_S) are controlled. In general, (K) is usually determined by the nature and strength of the intermolecular forces between the solute and the two phases. The availability of the stationary phase (the magnitude of (V_S)) is largely determined by the geometry of the stationary phase.

Factors Affecting the Magnitude of the Distribution Coefficient (K)

The magnitude of (K) is determined by the relative affinity of the solute for the two phases. Those solutes interacting more strongly with the stationary phase will exhibit a larger distribution coefficient and will be retained longer in the chromatographic system. Molecular interaction results from intermolecular forces of which there are three basic types.

Molecular Forces

All intermolecular forces are electrical in nature. The three different types are termed *dispersion forces*, *polar forces* and *ionic forces*. All interactions between molecules are composites of these three forces.

Dispersion Forces

Dispersion forces were first described by London (3), and for this reason were originally called 'London's dispersion forces'. London's name has now 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.

Glasstone (4) suggested that dispersion forces could be best described as follows,

"*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 are typically those that occur between hydrocarbons and because of them, hexane is a liquid boiling at 68.7°C and not a gas. In the biotechnology field, dispersive interactions are often referred to as 'hydrophobic' or 'lyophobic' interactions, probably because dispersive substance (*e.g.*, aliphatic hydrocarbons) do not dissolve readily in water. For academic interest only, to a first approximation the interaction energy, (U_D), involved with dispersive forces has been calculated to be (5), $U_D = \frac{3h\nu_o\alpha^2}{4r^6}$

where (a) is the polarizability of the molecule,
(n_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 magnitude of 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,
(n) is the number of molecules per unit volume.

If (r) is the density of the medium and (M) is the molecular weight, then the number of molecules per unit volume is $\frac{N\rho}{M}$ 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 $\frac{M}{\rho}$, 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 4.

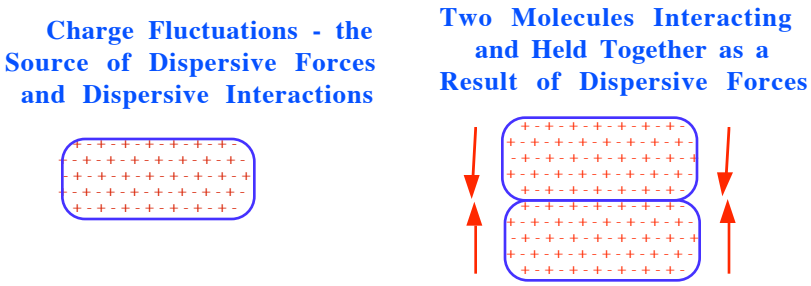


Figure 6 Dispersive Interactions

Dispersive interactions are not the result of a localized charge on any part of the molecule, but from 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.

Polar Forces

Polar interactions arise from electrical forces between localized charges resulting from permanent or induced dipoles. They cannot occur in isolation, but must be accompanied by dispersive interactions and under some circumstances may also be combined with ionic interactions. Polar interactions 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.

Dipole-Dipole Interactions

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

$$U_P = \frac{2\square\square^2}{r^6}$$

where ($\square$) is the polarizability of the molecule,
($\square$) 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, the magnitude of which can vary widely. Unfortunately, values of the dipole moment, taken from bulk measurements over a range of temperatures, does not always give a correct indication of the strength of any polar interactions that it might have with other molecules. For example, water, an extremely polar molecule, has a dipole moment of only 1.76 debyes. Similarly, the dipole moment of methanol, another extremely polar substance, is only 2.9 debyes. Unusually low values of dipole moments for strongly polar substances is often due to internal electric field compensation when more than one dipole is present in the molecule.(*e.g., water* associates strongly with itself by very strong polar forces or 'hydrogen bonding' . Methanol also associates strongly with itself in a similar manner. Examples of possible associates of water and methanol are shown in figure 5.

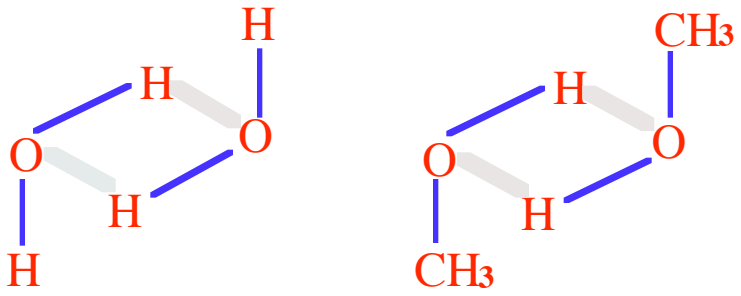


Figure 7 Possible Self Associates of Water and Methanol

Thus, with such associates (should they exist) the electric field from each dipole would oppose that from the other, resulting in a reduction in the *net* field as *measured externally*. It follows, *bulk* properties may not reflect the true value for the dipole moment of the individual dipoles. A molecule, however, approaching a water or methanol molecule would experience the uncompensated field of the single dipole and interact accordingly.

The *polarizability* of a substance containing no dipoles will give an indication of the *strength of any the dispersive* interactions that might take place with another molecule. In contrast, due to 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. A diagrammatic impression of a dipole-dipole interaction is shown in figure 8.

It is seen that the dipoles interact directly, but it is important to realize that with the dipole-dipole interaction is the dispersive interactions from the charge fluctuations on both molecules. The net interactive force 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.

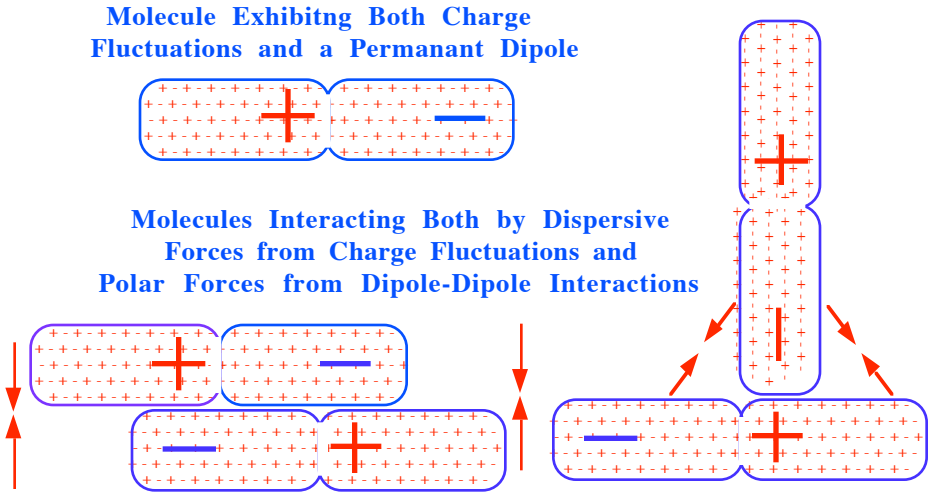


Figure 8 Polar Interactions: Dipole-Dipole Interactions

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, a diagrammatic impression of a dipole-induced dipole interaction is shown in figure 9.

Induced dipole interactions are always accompanied by dispersive interactions just as dipole interactions take place coincidentally with dispersive interactions. Thus, compounds such as aromatic hydrocarbons can be retained and separated purely on the basis of dispersive interactions, for example in GC using an hydrocarbon stationary phase. In addition, they can be retained and separated by combined induced-polar and dispersive interactions in LC using silica gel as a stationary phase and a dispersive mobile phase such as *n*-heptane. Molecules need not exhibit one type of interaction only.

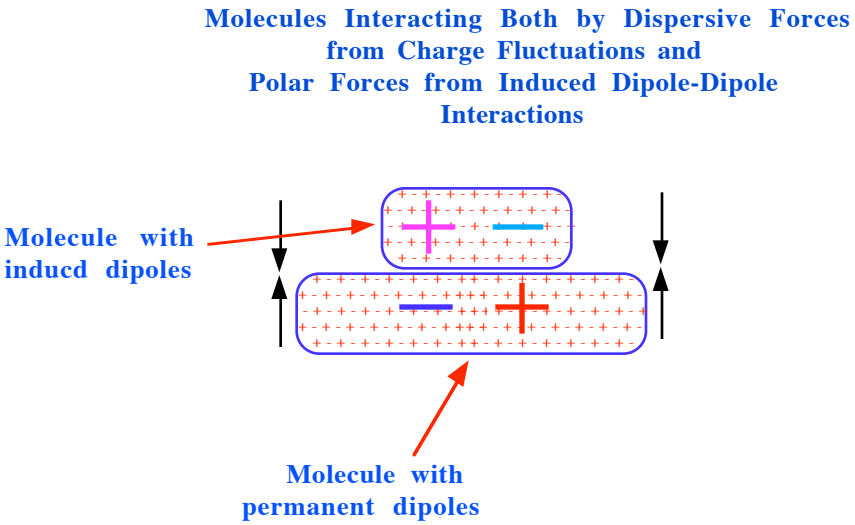


Figure 9 Polar Interactions: Dipole-Induced Dipole Interactions

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.

Ionic Forces

Polar compounds possessing dipoles, have no net charge. 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 10.

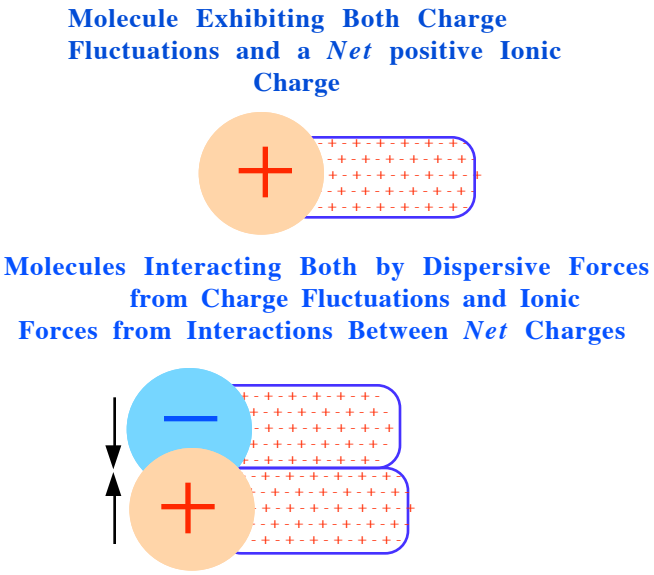


Figure 10 Ionic and Dispersive Interactions

A molecule can have many interactive sites comprised of the three basic types, dispersive, polar and ionic. Large molecules (for example biopolymers) may 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 biotechnologist call "hydrophilic" or lyophilic". These terms are not based on physical chemical argument but have evolved largely in the discipline of biology.

Hydrophobic and Hydrophilic Interactions

The term "hydrophobic interaction" 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 term may have been provoked by the immiscibility of a dispersive solvent such as *n*-heptane with a very polar solvent such as water.

n-heptane and water are immiscible, *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 is sufficiently strong to allow mutual solubility.

The term "hydrophilic force", literally meaning "love of water" force, appears to merely be 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. 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 remained in the lye and was consequently termed "lyophilic". Thus, glycerol mixes with water because of its many hydroxyl groups and is very polar and hence is a "hydrophilic" or "lyophilic" substance.

Hydrophobic and hydrophilic terms are extensively employed in biotechnology to describe the interactive character of the molecule as a whole. 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. Thus, 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 polar and dispersive interactions respectively.

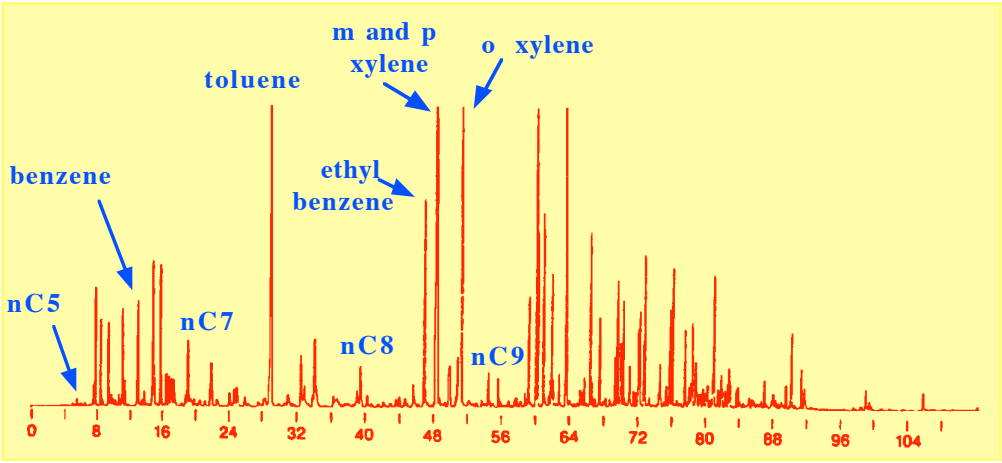
Molecular Forces and Chromatographic Selectivity

To chose a suitable stationary phase for a particular separation it is necessary to select a substance with which the solutes will interact relatively strongly. If the solutes to be separated are predominantly dispersive, then a hydrocarbon-like stationary phase would be appropriate, which, in GC, might be a high molecular weight hydrocarbon such as squalane. The operating temperature would be chosen so that the kinetic energy of the dissolved solutes molecules was sufficiently high to provide adequate partial vapor pressure for each and thus permit elution in a reasonable time.

Interactions in the mobile phase are extremely weak in GC, (5) and are not employed to influence selectivity. In LC, an appropriate dispersive stationary phase might be a bonded phase with a long aliphatic chain. To ensure that the selectivity resided predominantly in the stationary phase, a complementary polar and weakly dispersive mobile phase would be used. In LC, it is usual to allow one type of interaction to dominate in the stationary phase while a different type of interaction remains controlling in the mobile phase.

Separations Based on Dispersive Interactions

Separations based solely on dispersive interactions in GC must employ a nonpolar stationary phase such as a hydrocarbon or an alkyl silicone polymer. The separation of unleaded gasoline separated on a 50-m fused silica capillary column coated with 0.5 μm film of a methysilicone polymer is shown in figure 11.

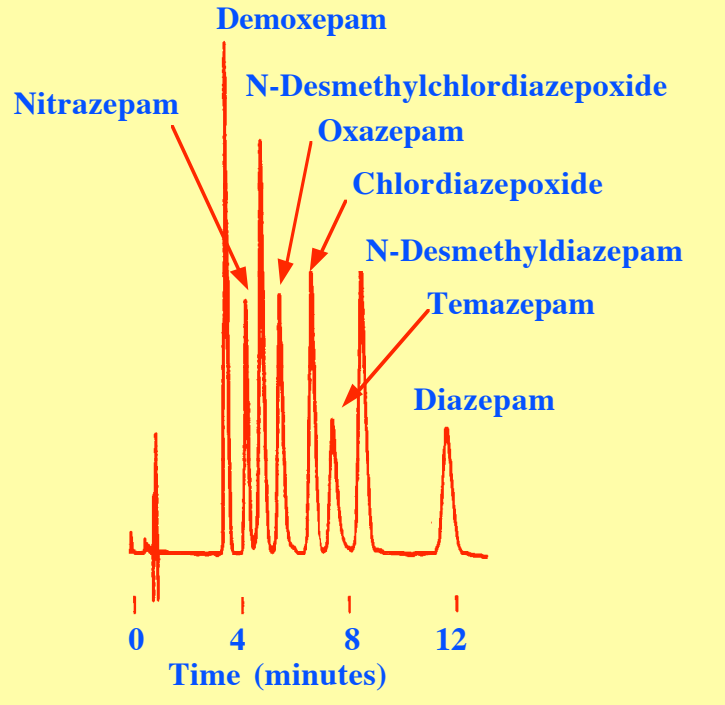


Courtesy of Supelco Inc.

Figure 11 A Chromatogram of the Hydrocarbons Contained in Unleaded Gasoline Using a Dispersive (Nonpolar) Stationary Phase

Helium was employed as the carrier gas at a flowrate of 20 ml/min. The temperature of the column was programmed from 35°C to 200°C at 2°C per min. The solute retention increases with the molecular weight irrespective as to their being simple alkanes or aromatics with polarizable nuclei. This is because, interactions with the aromatics are purely

dispersive and related to their molar volume. As a consequence, they fall in the expected molecular weight sequence for the paraffins. An example of the use of dispersive interactions in LC is afforded by the separation of some benzodiazepines on a reverse-phase column shown in figure 12. The column, 15 cm long, 4.6 mm I.D., contained a bonded reversed phase (dispersive phase) having aliphatic chains 8 carbon atoms long. The mobile phase was a mixture of acetonitrile and an aqueous solution of potassium dihydrogen phosphate and triethylamine to act as a buffer (pH 3.15). The retentive interactions with the stationary phase are solely dispersive. In contrast, the interactive character of the mobile phase was largely polar but accompanied by some dispersive interactions with the acetonitrile.



Courtesy of Supelco Inc.

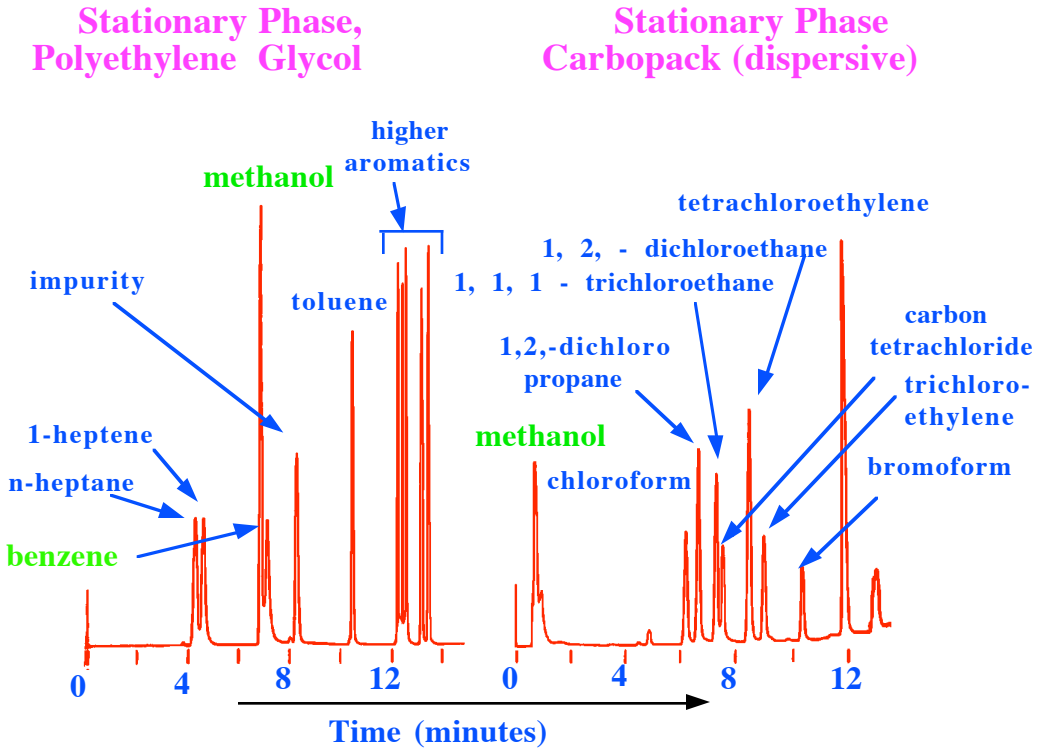
Figure 12 The Separation of Eight Benzodiazepines

It is seen that an excellent separation is obtained. To reduce the retention of the solutes the concentration of acetonitrile would need to be increased. This would increase the competitive dispersive interactions in the mobile phase and thus elute the solutes more rapidly. However, the

column efficiency would remain the same and thus some resolution would be lost.

Separations Based on Polar Interactions

Two separations by GC of some airborne contaminants shown in figure 13 illustrate the different selectivity that can be obtained by using dispersive or polar stationary phases. GC separations demonstrate the effects of phase selectivity very elegantly as there are no significant interactions with the mobile phase and, therefore, retention differences can be exclusively attributed to the nature of the stationary phase.



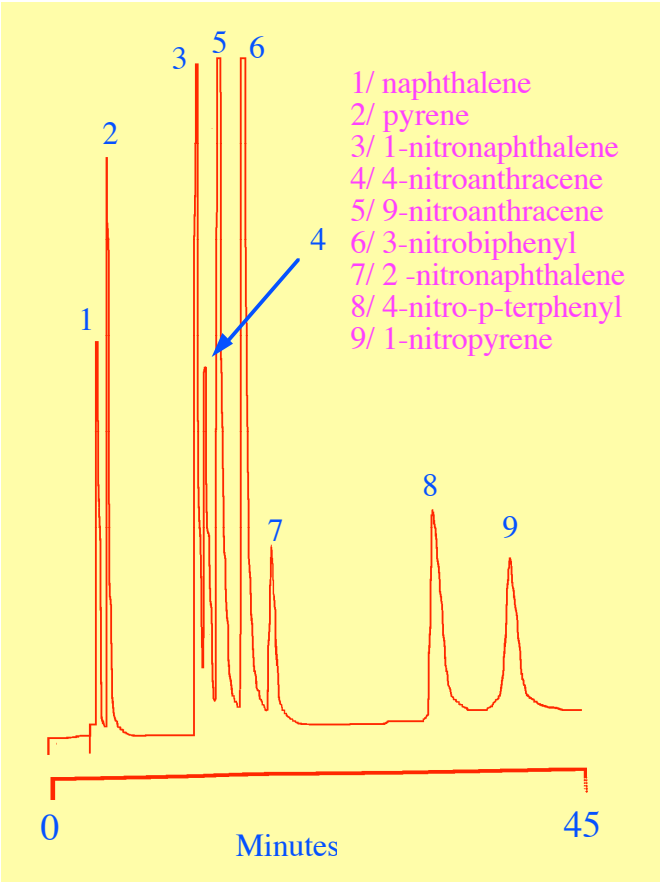
Courtesy of Supelco Inc.

Figure 13 The Separation of Some Airborne Contaminants by GC

The solutes that disclose the nature of the interactions that are taking place with the two stationary phase are *methanol* and *benzene*. On the polyethylene glycol stationary phase, methanol (B. Pt. 64.7°C, polar) and benzene (B. Pt. 80.1°C, polarizable) are eluted well after *n*-heptane (B. Pt. 98.4°C, dispersive) and 1-heptene (B. Pt. 93.6°C, dispersive and slightly polarizable). The strong polar interactions between the OH

groups of the polyethylene glycol and both the OH groups of the methanol and the polarized nucleus of the benzene are far greater than the dispersive interactions with heptane and heptene. This difference occurs despite the greater molecular weights and higher boiling points of heptane and heptene. On the stationary phase Carbopack, methanol, weakly dispersive, is eluted almost at the dead volume while the more dispersive solutes are extensively retained.

An example of the use of induced dipoles to separate polarizable substances is afforded by the analysis of some aromatic and nitroaromatic hydrocarbons by LC using silica gel as the stationary phase.

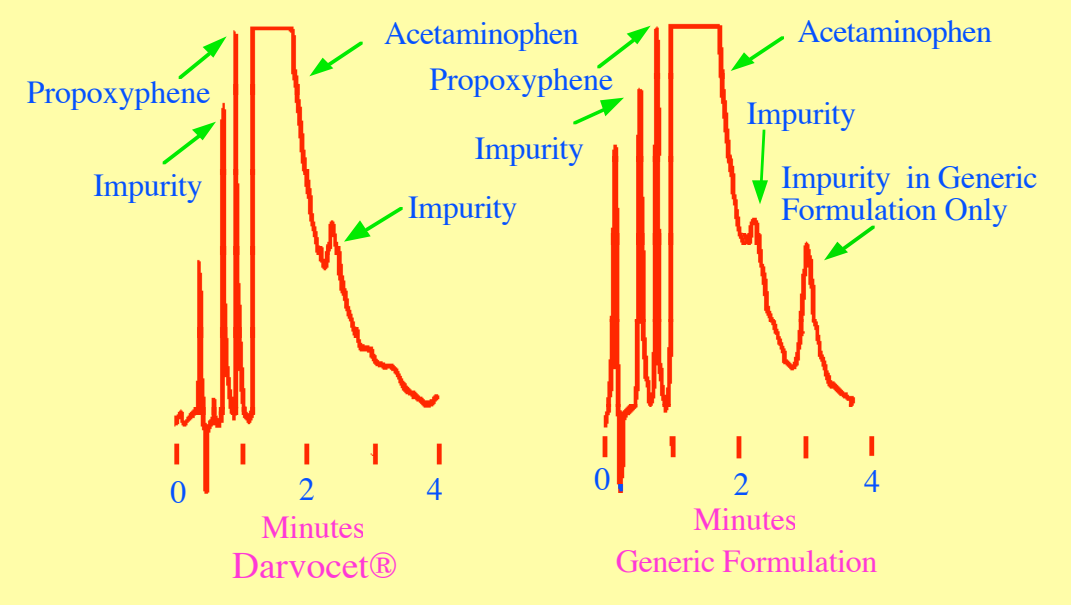


Courtesy of Supelco Inc.

Figure 14 The Separation Aromatic and Nitro-Aromatic Hydrocarbons

A small-bore column 25 cm long and 1 mm I.D. was employed, packed with silica gel having a particle diameter of 10 □. The mobile phase was *n*-hexane at a flow-rate 50 □l per min. The solutes of interest are

naphthalene and pyrene, the first two peaks. The two solutes are well separated and, as they have no permanent dipole, and as dispersive interactions with the silica gel are weak, they are selectively retained almost exclusively by induced dipole interactions. These interactions occur between the strong dipoles of the silanol groups on the silica gel surface and the induced dipoles on the aromatic nucleus resulting from their proximity to the silanol groups. To ensure that polar interactions dominate in the stationary phase the mobile phase consists of the dispersive solvent *n*-hexane. An interesting example of polar selectivity by hydroxyl groups on the surface of native silica is shown by the analysis of Darvocet® and its generic equivalent in figure 15.



Courtesy of Supelco Inc.

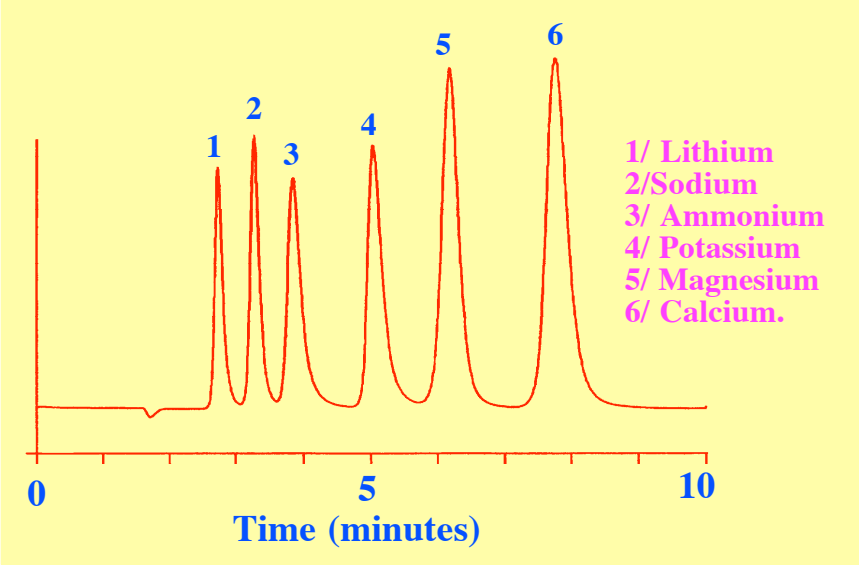
Figure 15 The Analysis of Acetaminophen Formulations

Darvocet® is an acetaminophen product in which it is the active ingredient. Other substances are present which also contain polar groups and thus, the sample lends itself to separation on the polar stationary phase, silica gel. The analysis was completed in less than 4 minutes using a short column 3.3 cm long and 4.6 mm in diameter packed with silica (particle size of 3 □). The column appears to be significantly overloaded, but the impurities are well still separated from the main component and a substance in the generic formulation that was not present in the

Darvocet® clearly indicated. The mobile phase was 98.5% dichloromethane with 1.5% v/v of methanol containing 3.3% ammonium hydroxide. Although, the ammoniacal methanol helped to decrease extreme polar activity from specially active adsorption sites on the silica surface, the overall interaction of the solutes with the stationary phase was predominately polar. In contrast solute interactions with the methylene dichloride in the mobile phase would be almost exclusively dispersive.

Separations Based on Ionic Interactions

Ionic materials are not volatile under the conditions normally employed in GC, so, ionic interactions cannot be exploited in GC stationary phases to control retention. However, they are very important in LC, and *ion exchange chromatography* (the name given to LC separations that employ ionic interactions to control retention) is widely used to analyze ion mixtures. The use of ionic interactions to separate some alkali and alkaline earth cations is shown in figure 16.



Courtesy of Whatman Inc.

Figure 16 The Separation of Cations by Ion-Exchange Chromatography

The column used was IonPacCS12 (a proprietary cation exchange column) and the mobile phase was a 20nM solution of methanesulfonic acid in water. The flow rate was 1 ml/min. and 25□l of sample was injected. The separation almost exclusively involved ionic interactions as any dispersive interactions between a metal ion and the stationary phase would be very small indeed.

The Control of Chromatographically Available Stationary Phase (V_S)

The volume of stationary phase that is made available to the solutes can be controlled in a number of ways. Firstly, the stationary phase loading on the column can be varied to adjust the retention as required. A *specific* stationary phase loading may be selected, to either improve the resolution, or to reduce the analysis time, or in some instances, to increase the *sample* load. Sometimes, the stationary phase loading is reduced so the column is more amenable to specific compounds (*e.g.* to prevent proteins from being denatured).

Secondly, the stationary phase can contain molecules of a special shape that can only make *close* contact with molecules having a complementary shape. Other molecules can not interact so closely with the stationary phase and consequently, the stationary phase available to them will be restricted. This approach is exploited in chiral chromatography where the stationary phase is made to consist largely of a specific enantiomer that confers chiral selectivity to the distribution system

Thirdly, the stationary phase can be attached to the surface of a porous support, and the pore size chosen to be commensurate with the size of the solute molecules to be separated. Under such circumstances the molecules that are smaller than the pores will enter the matrix of the material and have more stationary phase available to them. Conversely, the larger molecules will be excluded from the pores and, consequently, come in contact with much less of the stationary phase. Size selectivity, achieved by the use of porous solids, is utilized in size exclusion chromatography (SEC) where solutes are separated almost exclusively

on the basis of molecular size. The separation of chiral compounds can be successfully utilized in both GC and LC; size exclusion chromatography, however, is not greatly used in GC and is almost exclusively confined to LC.

The Effect of Stationary Phase Loading on the Performance of a Chromatographic System

The stationary phase content of a column can affect a separation in two ways. The more stationary phase there is in a column, the more the solutes will be retained, the further they will be apart and the greater the separation. Any change in stationary phase, however, will change the retention of all solutes proportionally and thus the separation will only increase, if the *peak widths* remain unchanged. Increasing the amount of stationary phase will usually increase the thickness of the stationary phase film, which, as is shown in Book 7 will increase peak dispersion. It follows that there will be a specific stationary phase loading that provides the best compromise between separation and band dispersion (6) and thus provides the maximum resolution. The loading can be quite critical for open tubular columns in GC. Thus, the stationary phase loading cannot be increased indefinitely to separate the peaks as, eventually, the peaks will start spreading to a greater extent than they are being separated.

Increasing the stationary phase load on a GC column (packed or open tube) will allow the sample placed on the column to be increased. A large sample is often necessary in trace analysis to provide sufficient material for detection. Under such circumstances the column may be overloaded giving a very broad asymmetric peak which may obscure the trace materials of interest. This asymmetric dispersion is due to solute-solute interaction in the mobile and stationary phases causing a nonlinear adsorption isotherm. The subject of adsorption isotherms will not be discussed here and it is sufficient to say that the asymmetric dispersion can be reduced by increasing the stationary phase in the column.. A larger amount of stationary phase, will, even with a larger charge, reduce the sample concentration in the stationary phase and thus the deleterious high sample concentrations are never reached.

The stationary phase loading of a an LC column is not modified the same way as a GC column. This is because, irrespective of the type of support material, the amount of stationary phase in an LC column is primarily determined by its surface area. In addition, the amount of available stationary phase on a bonded phase can be modified by adjusting the molecular size (chain length) of the bonded material.

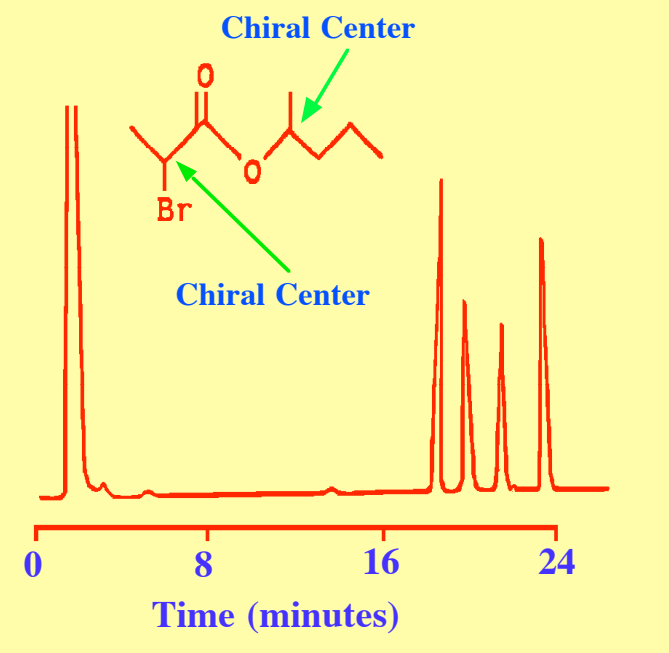
The chain length of the bonded material can be critical when separating proteins as dispersive interactions between the alkane chains and the dispersive (hydrophobic) groups of the protein can be strong enough to cause structural deconformation; (*i.e.*, the protein becomes denatured). Reducing the chain length of the bonded material, the dispersive forces can be reduced significantly and the deconformation diminished. In practice, carbon chains only two or four carbon atoms long are among those most commonly used for separating labile proteins.

Stationary Phase Limitation by Chiral Selectivity

The extent to which an enantiomer can interact with the stationary phase depends on how close it can approach the molecules of the stationary phase. If the stationary phase is also chiral in nature, it is likely that one enantiomer in the sample will fit closely to the stationary phase surface whereas the other will be stearically excluded and thus have less stationary phase with which to interact. The first chiral separations in GC were reported by Gil-Av *et al*. as in 1966 (7), but, surprisingly, the use of GC for the separation of enantiomers has only recently been investigated and developed into a practical system. The use of chiral stationary phases in GC has been dogged by entantiomeric instability arising from the racemization of both the chiral stationary phase and the chiral solutes at elevated temperatures. In addition, at the elevated temperatures necessary to elute the solutes in a reasonable time, the chiral *selectivity* of the stationary phase can also be impaired.

A thermally stable chiral stationary phase was produced by Frank, Nicholson and Bayer (8) in 1977 by the co-polymerization of

dimethylsiloxane with (2-carboxypropyl) methoxysilane and L-valine-t-butylamide.



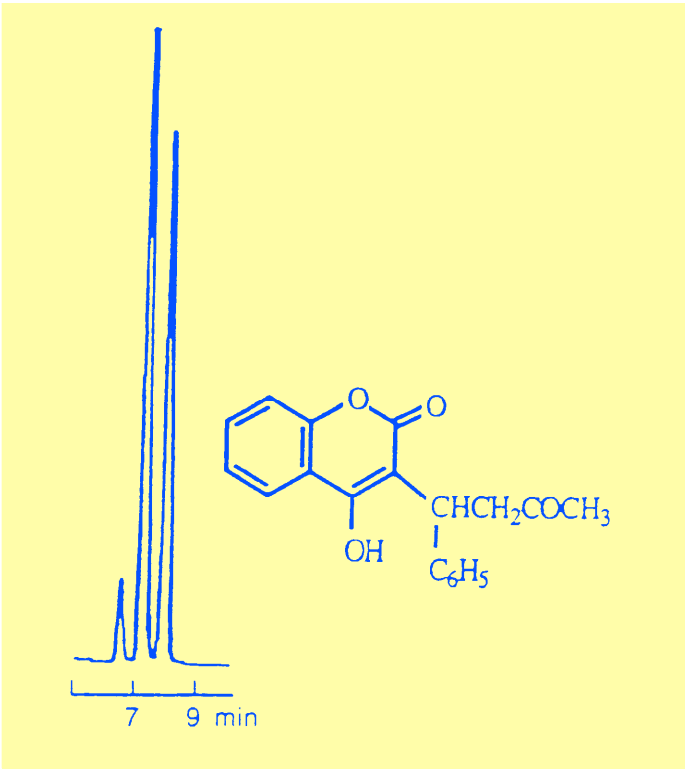
Courtesy of ASTEC Inc.

Figure 17 The Separation of the Enantiomers of □-Halocarboxylic Acid Esters on a □-Cyclodextrin-Based Stationary Phase

This material was relatively stable up to 220°C with little racemization but, was not made commercially available until 1989. Presently, there are a number of effective GC chiral stationary phases available, some of the more effective being based on cyclodextrin,. The separation of the enantiomers of an □-halocarboxylic acid ester on a fused silica open tubular column coated with a □-cyclodextrin product is shown in figure 17. The column was 10 m long and operated at 60°C using nitrogen as the carrier gas.

The use of LC for chiral separations is easier to carry out and generally more efficient. A number of racemic mixtures can be easily separated using a reverse-phase column and a mobile phase doped with a chiral reagent. In some cases, the reagent is adsorbed strongly on to the stationary phase, under which circumstances, the chiral selectivity resides in the stationary phase. Conversely, if the reagent remains predominantly

in the mobile phase, then the chiral selectivity will be in the mobile phase. Camphor sulphonic acid and quinine are examples of mobile phase additives. The most common method used to achieve chiral selectivity is to bond chirally selective compounds to silica in a similar manner to a reverse phase (*e.g.*, example of which is afforded by the cyclodextrins).



Courtesy of ASTEC Inc.

Figure 18 The Separation of Warfarin Isomers on a CYCLOBOND Column

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 chiral structures and the beta- cyclodextrin has 35 stereogenic centers. CYCLOBOND is a trade name used to describe a series of cyclodextrins chemically bonded to spherical silica gel. The separation of the isomers of Warfarin is shown in figure 18. The column was 25 cm long and 4.6 mm in diameter packed with 5 micron CYCLOBOND 1. The mobile phase

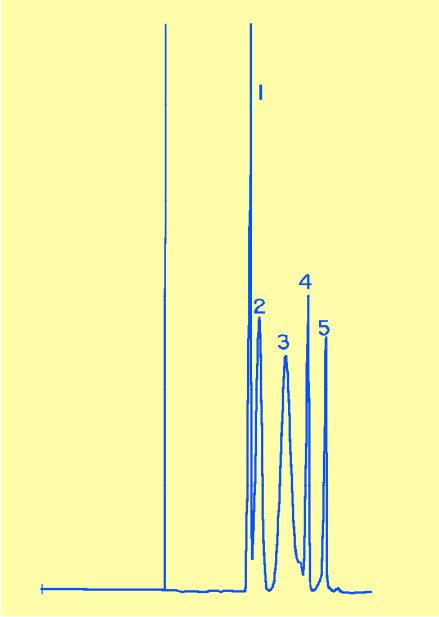
was approximately 90%v/v acetonitrile, 10%v/v methanol, 0.2 %v/v glacial acetic acid and 0.2%v/v triethylamine. It is seen that an excellent separation has been achieved with the two isomers completely resolved.

Stationary Phase Limitation by Exclusion

Size Exclusion Chromatography might imply that solute retention was determined solely by the size of the molecule. However, this can only be true if the magnitude of the interaction forces between the solute and each phase is the *same*. This situation can be closely approached by the appropriate choice of the mobile phase. Under such circumstances the larger molecules, being partially or wholly excluded, will elute first and the smaller molecules elute last.

It is important to understand that, even when the dominant retention mechanism is controlled by molecular forces, if the stationary phase or supporting material contains pores of size commensurate with those of the solute molecules, *exclusion will still partly control retention*. This is because the larger molecules will interact with less stationary phase and be eluted *relatively* faster than if they had interacted with the same amount of stationary phase as the smaller molecules. The two most common exclusion media used in LC are silica gel and macroporous polystyrene divinylbenzene resins. Figure 19 shows an exclusion chromatogram of a series of molecular weight standards obtained on silica gel.

The column length was 50 cm and the mobile phase tetrahydrofuran (THF). The THF would be strongly adsorbed on the silica surface and thus the solutes would be distributed between pure THF in the mobile phase and THF on the surface of the silica. As a consequence, the interactions are virtually identical in the two phases and the retention was determined almost exclusively by stationary phase availability. The molecular diameter of solute standards were (1) 11,000Å, (2) 240Å, (3) 49.5Å, (4) 27.1Å and (5) 7.4Å.



Courtesy of Supelco Inc.

Figure 19 The Separation of a Mixture by Exclusion Chromatography

Until relatively recently, silica has been the most commonly used exclusion media for the separation of high molecular weight hydrocarbons and polymers. However, it was not so successful in the separation of polymeric materials of biological origin. More recently the micro-reticular macroporous polystyrene gels have been introduced and found to be very useful for the separation of biopolymers by size exclusion. These materials have even replaced many of the traditional applications of silica gel.In summary, in all types of chromatography, solute retention is controlled by either the magnitude and probability of interaction and/or by the amount of stationary phase that is available to them. However, even if, by appropriate choice of the phase system, the solutes are separated, unless the peak dispersion is contained to allow the individual solutes to be eluted discretely, the mixture will not be resolved.

Peak Dispersion in a Chromatographic Column

The first comprehensive approach to dispersion in chromatographic columns was taken by Van Deemter (8) who developed the dispersion

equation for a packed GC column. Van Deemter's development did not take into account the compressibility of the mobile phase which was dealt with later by Katz, Ogan and Scott (9). A simple form of this theory will be given that does not accommodate the compressibility of the mobile phase but a more detailed and comprehensive treatment is given in Books 6 and 7.

Van Deemter *et al*. assumed that there were four band spreading processes responsible for peak dispersion, namely, *multi-path dispersion*, *longitudinal diffusion*, *resistance to mass transfer in the mobile phase* and *resistance to mass transfer in the stationary phase*. Van Deemter derived an expression for the variance contribution of each process to the overall variance per unit length of the column. Furthermore, as the individual dispersion processes can be assumed to be random and *non-interacting*, the total variance per unit length of the column can be obtained from a sum of the individual variance contributions.

The Multi-Path Effect

The multi-path effect is diagramatically depicted in figure 20.

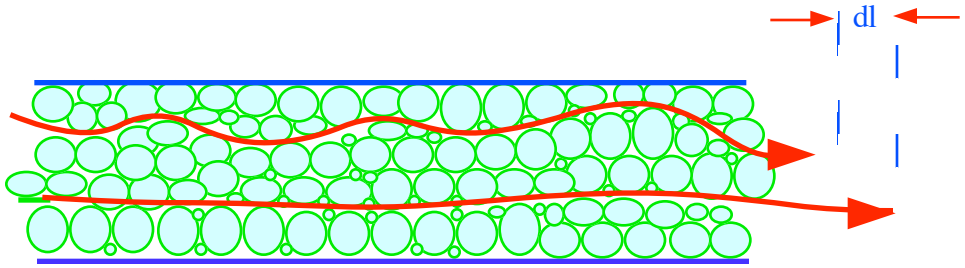


Figure 20 Multipath Dispersion

When flowing through a packed column, the individual solute molecules will describe a tortuous path through the interstices between the particles. As a result, some will randomly travel shorter routes than the average and some longer. It follows, that those molecules taking shorter paths will move ahead of the mean and those that take the longer paths will lag behind the mean. This will result in a differential distance traveled (dl) as shown in figure 3. This differential flow of the solute molecules results in band dispersion.

Van Deemter *et al*. derived the following function for the multi-path variance contribution (σ^2_M) to the overall variance per unit length of the column,

$$\sigma^2_M = 2 \lambda d_p \qquad (4)$$

where (d_p) is the particle diameter of the packing,
and (l) is a constant that depended on the quality of the packing.

Longitudinal Diffusion

Solutes when contained in a fluid naturally diffuse and spread driven by their concentration gradient. Thus, in a chromatographic column a discrete solute band will diffuse in the gas or liquid mobile phase. It also follows, that because the diffusion process is random in nature, a concentration curve that is Gaussian in form will be produced. This diffusion effect occurs in the mobile phase of both GC and LC columns. The diffusion process is depicted in figure 21.

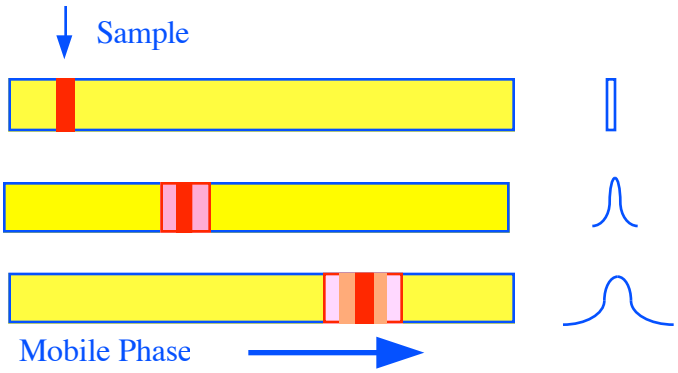


Figure 21 Peak Dispersion by Longitudinal Diffusion

The longer the solute band remains in the column, the greater will be the extent of diffusion. The time the solute remains in the column is inversely proportional to the mobile phase velocity, so, the dispersion will also be inversely proportional to the mobile phase velocity. Van Deemter *et al*. derived the following expression for the variance contribution by longitudinal diffusion, (σ^2_L), to the overall variance/unit length of the column.

$$\sigma_L^2 = \frac{2\gamma D_m}{u} \quad (5)$$

where (D_m) is the diffusivity of the solute in the mobile phase,
(u) is the linear velocity of the mobile phase,
and (γ) is a constant that depended on the quality of the packing.

The Resistance to Mass Transfer in the Mobile Phase

During passage through a chromatographic column, the solute molecules are constantly and reversibly transferring from the mobile phase to the stationary phase. This transfer is not instantaneous; time is required for the molecules to pass (by diffusion) through the mobile phase to reach the interface and enter the stationary phase. Those molecules close to the stationary phase enter it immediately, whereas those molecules some distance away will find their way to it some time later. Since the mobile phase is continually moving, during this time interval, those molecules that remain in the mobile phase will be swept along the column and dispersed away from those molecules that were close and entered the stationary phase immediately. This process is depicted in figure 22. The diagram shows 6 solute molecules in the mobile phase and the pair closest to the surface, (1 and 2), enter the stationary phase immediately. While molecules 3 and 4 diffuse through the mobile phase to the interface, the mobile phase moves on. As a consequence, when molecules 3 and 4 reach the interface, they enter the stationary phase some distance ahead of the first two. Finally, while molecules 5 and 6 diffuse to the interface, the mobile phase has moved even further down the column until molecules 5 and 6 enter the stationary phase ahead of molecules 3 and 4. Thus, the 6 molecules, originally relatively close together, are now spread out in the stationary phase. This explanation, although over-simplified, gives a correct description of the mechanism of mass transfer dispersion.

Van Deemter derived the following expression for the variance contribution by the resistance to mass transfer in the mobile phase, ($\sigma_{R\,M}^2$),

$$\square^2_{RM} = \frac{f_1(k')d_p^2}{D_m} u \qquad (6)$$

where (k') is the capacity ratio of the solute, and the other symbols have the meaning previously ascribed to them.

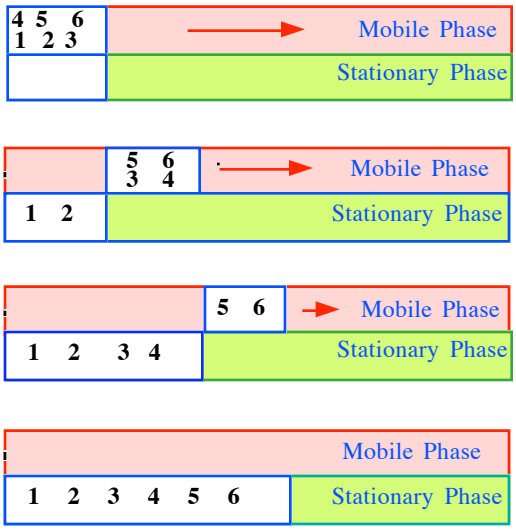


Figure 22 Resistance to Mass Transfer in the Mobile Phase

The Resistance to Mass Transfer in the Stationary Phase

Dispersion due to resistance to mass transfer in the stationary phase is exactly analogous to that in the mobile phase. Solute molecules close to the interface will leave the stationary phase and enter the mobile phase before those that have diffused further into the stationary phase and have a longer distance to diffuse back. Thus, as those molecules that were close to the surface will be swept along in the moving phase, they will be dispersed from those molecules still diffusing to the surface. The dispersion resulting from the resistance to mass transfer in the stationary phase is depicted in figure 23. Molecules 1 and 2 (the two closest to the surface) will enter the mobile phase and begin moving along the column. Their movement will continue while molecules 3 and 4 diffuse to the interface at which time they will enter the mobile phase and start following molecules 1 and 2 down the column.

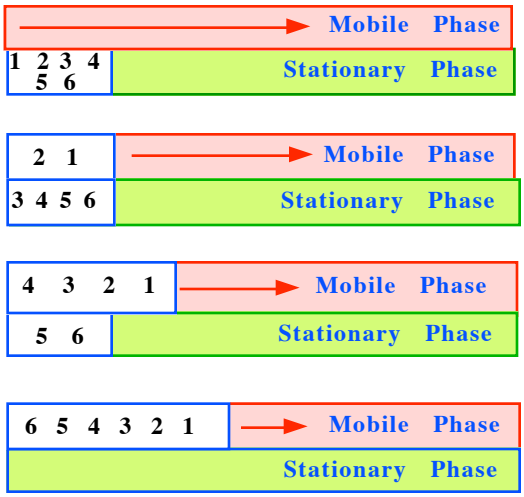


Figure 23 Resistance to Mass Transfer in the Stationary Phase

All four molecules will continue moving down he column, while molecules 5 and 6 diffuse to the interface. By the time molecules 5 and 6 enter the mobile phase, the other four molecules will have been smeared along the column and the original 6 molecules will be dispersed.

Van Deemter derived an expression for the variance from the resistance to mass transfer in the stationary phase, (σ^2_{RS}), which is as follows:

$$\sigma^2_{RS} = \frac{f_2(k')d_f^2}{D_S} u \qquad (7)$$

where (k') is the capacity ratio of the solute,
(d_f) is the effective film thickness of the stationary phase,
(D_S) is the diffusivity of the solute in the stationary phase,
and the other symbols have the meaning previously ascribed to them.
Now, applying the law of Summation of Variances,

$$\sigma^2_x = \sigma^2_M + \sigma^2_L + \sigma^2_{RM} + \sigma^2_{RS} \qquad (8)$$

where (σ^2_x) is the total variance/unit length of the column.

Thus substituting for (σ_M^2), (σ_L^2), ($\sigma_{R\,M}^2$) and ($\sigma_{R\,S}^2$) from equations (4), (5), (6) and (7), respectively,

$$\sigma_x^2 = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{f_1(k')d_p^2}{D_m}u + \frac{f_2(k')d_f^2}{D_S}u \qquad (9)$$

Equation (9) is the Van Deemter equation that describes the variance per unit length of a column in terms of the physical properties of the column contents, the distribution system and the linear velocity of the mobile phase. Alternatively the Van Deemter equation can be expressed in the form,

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{f_1(k')d_p^2}{D_m}u + \frac{f_2(k')d_f^2}{D_S}u \qquad (10)$$

where (H) is the Height of the Theoretical Plate. The relationship between (H) and (σ_x) is explained in Book 6.

Hence the term "HETP equation" for equation (10). This form of the Van Deemter equation is very nearly correct for LC but, due to the compressibility of the gaseous mobile phase in GC, neither the linear velocity nor the pressure is constant along the column. Furthermore, as the diffusivity, (D_m), is a function of pressure, the above form of the equation can only be approximate. However, equation (10) generally gives the correct form of the relationship between (H) and the linear velocity (u). It also predicts that there will be an optimum velocity that gives a minimum value for (H) and thus, a maximum efficiency. Pressure corrections for retention volume and the height of the theoretical plate are derived in Books 6 and 7.

The Golay Equation for Open Tubular Columns

The corresponding equation describing dispersion in an open tubular column was developed by Golay (10) for GC columns but is equally applicable to LC columns and to dispersion in connecting tubes. The

Golay equation differs from equation (10) in that, as there is no packing, there can be no multipath term. Consequently, the equation contains only three functions. One function describes dispersion from longitudinal diffusion and the other two describes dispersion from the resistance to mass transfer in the mobile and stationary phases, respectively. The Golay equation takes the following form:-

$$H = \frac{2 D_m}{u} + \frac{f_1(k') r^2}{D_m} u + \frac{f_2(k') r^2}{K^2 D_s} u \qquad (11)$$

where (r) is the column radius,
and other symbols have the meaning previously ascribed to them.

Open tubular columns behave in exactly the same way as packed columns with respect to pressure. As the column is geometrically simple the respective functions of (k') can also be explicitly developed.

The Efficiency of a TLC Plate

TLC plate efficiency is a measure of its capacity to restrain solute dispersion and maintain narrow spots as the solutes migrate along the plate. An explicit equation that describes the dispersion in TLC has not been rigorously developed, nevertheless, high efficiencies are realized in much the same way as they are in GC and LC. Primarily, the particle size of the silica layer must be made as small as possible and the layer must be spread in a thin, homogenous film on the supporting plate. TLC plate efficiency is measured in a similar manner to column efficiency but slightly modified. It is very difficult, if not impossible, to identify the positions of the points of inflexion on a TLC spot, but if the visible edges of the spot are assumed to occur at four standard deviations of the spot distribution, then it is still possible to assess the efficiency. In general it is considered that over 95% of the material in the spot is confined within 4 standard deviations of the spot dispersion.

If the diameter of the spot (d), corresponds to four standard deviations, then applying the same rationale as with the packed column,

$$\frac{\text{Ret. Distance}}{\text{Peak Width}} = \frac{Z_s}{d} = \frac{\sqrt{n}}{4}$$

where (Z_S) is the retention distance of the solute.

Thus, $$n = 16\left(\frac{Z_s}{d}\right)^2$$

It shout be pointed out, however, the method contains implicit assumptions that may not necessarily be valid. Besides assuming that the visible limits of the spot correspond to four standard deviations, the basic assumption that the value of (K) is constant throughout the development, is also tacitly made and this is certainly not so. In fact, this procedure would give similar errors to those that would arise from calculating the efficiency of an LC column under conditions of *gradient elution*. Nevertheless, the method does allow the relative performance of different plates to be accessed and in this way can be helpful.

The Basic Column Chromatograph

A chromatograph consists of five basic units and these units, although possibly designed differently for different systems, are essential for all types of chromatograph, including both gas and liquid chromatographs. The layout of all the five essential units is shown in figure 24. They consists of a mobile phase supply, a sampling system, a column and column oven including a temperature controller and temperature programming system. The temperature controller and programmer will probably have its own microprocessor which may also be under the control of the data acquisition and processing computer.

The column eluent will pass to an appropriate detector and its associated electronics. There may be more than one detector and they may be employed in parallel or in series and individually controlled and monitored. The final unit will be a computer and data acquisition system

and may include a simple potentiometric recorder to monitor the chromatogram in real time, if this is not provided by the computer.

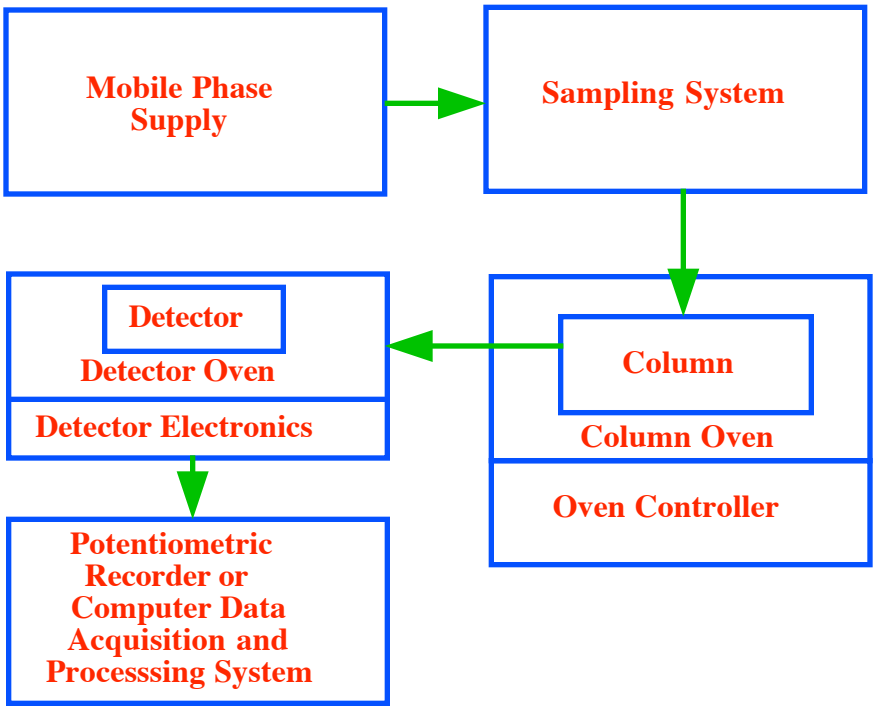


Figure 24 The Basic Chromatograph

The Mobile Phase Supply

The first unit, the mobile phase supply, can range in complexity from a simple gas cylinder connected to a flow controlling valve for a gas chromatograph, to a complex multi-piston pump supplied by four or five solvent reservoirs and fitted with both flow programming and gradient elution facilities, each with its own controlling micro-processor. For a gas chromatograph the gas supplies will vary depending on the type of detector employed and column that is used. If gases are being analyzed then a katharometer detector will probably be appropriate and thus helium will be used as the carrier gas to provide the best sensitivity. If a flame ionization detector (FID) is to be used then oxygen or air and hydrogen will be needed for the detector and helium or nitrogen must be available as the mobile phase. If an argon ionization detector is to be used then an argon supply will be necessary. Flow controllers are usually supplied to each gas supply, often under microprocessor control and

fitted with inline flow controllers. Such a system can provide flow programming facilities if so desired.

The mobile phase supply for a liquid chromatograph usually has a capacity for at least four solvents which are normally housed in a flame proof environment and solvent vapors are removed by a suitable vapor scavenging device. The solvents can be selected by a microprocessor or manually by suitable valves and these pass either directly to a dual piston pump for isocratic development or to a solvent programmer and thence to a pump for gadient elution. Care must be taken to minimize the volume existing between the solvent programmer exit and the sample valve otherwise the gradient will be distorted by the logarithmic decay function of the interstitial volume.

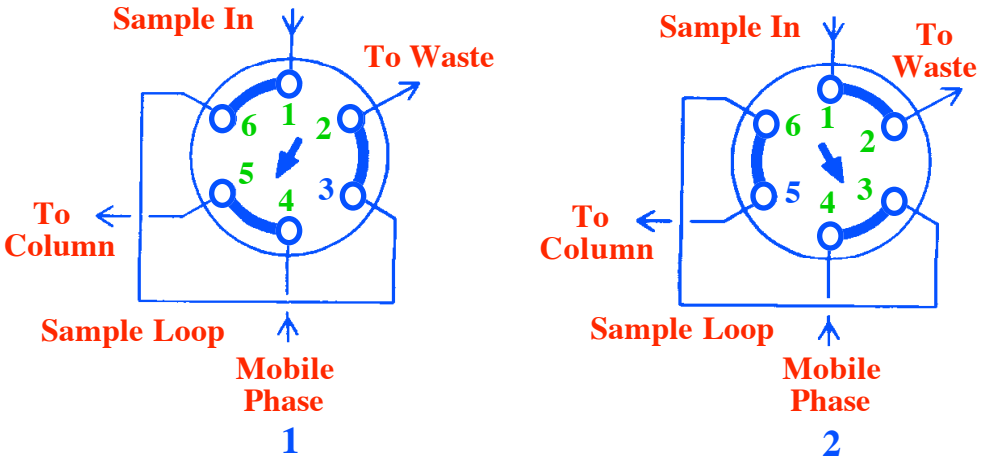
The Sampling System

Gas samples are generally placed on a GC column using an external loop sampling valve but liquid samples are usually injected onto the column by a syringe via a heated injector. Sample are placed on an LC column directly with either an internal or external loop sample valve the valve being connected directly to the column. The external loop sample system, employing six ports, is depicted in figure 25.

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 either from a syringe or other sample device 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.



Courtesy of Valco Instruments Inc.

Figure 25 The External Loop Valve

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 □l, but for preparative work, loops with sample volumes of 1 ml or more can be placed on a preparative column.

Modern liquid chromatographs that are used for routine analysis usually 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 alternately contain sample and washing solvent. The sampling can involve a complex sequence of operations that are controlled by a microprocessor. The syringe plunger is operated pneumatically and the syringe is first washed with solvent, then rinsed with the sample, reloaded with the sample and the contents discharged into the column. 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. which can be considered as part of the sampling

system. 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

GC ovens usually require an operating range from about 5˚C to about 400˚C although the majority of GC analyses are carried out between temperatures of 75˚C an 200˚C. In contrast, LC column ovens cover a more limited range of temperatures *viz*., 0˚C to 120˚C. Temperature programming is an essential feature of all GC column ovens and is necessary to handle a sufficiently wide molecular and polarity range of samples. Linear programming is the most common although other functions of time are often available. LC column ovens are rarely provided with temperature programming facilities as the technique appears to be far less effective compared with GC, gradient elution being a far more ffective alternative. The thermostatting medium used in GC ovens is almost exclusively 'forced air' as the heat capacity of the GC mobile phase (*i.e*., a gas) is relative small. Consequently, air has sufficient heat capacity to change the column temperature rapidly without significant cooling from the carrier gas. Air ovens are also employed in LC column ovens but are far less effective as the mobile phase, a liquid, has a much higher heat capacity and thus a stronger cooling effect. This problem is partly alleviated by using mobile phase preheaters but these introduce a significant volume between the solvent supply system and the column which will distort the profile of any solvent gradient that is employed. Nevertheless, a liquid thermostatting medium introduces difficulties when changing columns and with column detector connections and is thus, not commonly used. The temperature program can be controlled by a microprocessor incorporated in the programmer or can be controlled from a central computer that governs the operation of the whole instrument.

The GC column can be a packed or open tubular and thus the oven must be capable of taking both. The open tubular column is by far the

most popular partly because they are considered state of the art and not because they necessarily provide an improved performance. Open tubular columns will always provide the highest efficiencies but, if correct operating procedures are adopted, in general, analyses carried out on packed columns, are likely to provide greater accuracy and better precision and repeatability. Packed GC columns are usually made of stainless steel or glass and open tubular column almost exclusively fused quartz.

Almost all LC columns are packed, although they can vary widely in length and diameter depending of the nature of the sample and the resolution required. They are usually manufactured of stainless steel or titanium (reputed to provide greater stability for labile materials of biological origin) and the connection to the sample valve and detector should be as short as possible and have a very small diameter to reduce extra column dispersion.

Detector and Detector Electronics

There is a wide range of detectors available for both GC and LC each having their own particular areas of application. In general the more catholic the response, the less sensitive the detector and the most sensitive detectors are those that have a specific response. The performance of all detectors should be properly specified so that the user can determine which is most suitable for a specific application. Such specifications are also essential to compare the performance of different detectors supplied by alternative instrument manufactures. Detector specifications should be presented in a standard form and in standard units, so that detectors can be compared that function on widely different principles. The more important detector specifications are summarized in table 2.

The Detector Output

Most practical detectors must have a linear output, *e.g.*,

$$y = Ac$$

where (y) is the output of the detector in appropriate units,
(c) is the concentration of solute in the mobile phase,
and (A) is a constant.

Table 2 Detector Specifications

Specifications	Units
Dynamic Range	(D_R) g/ml (*e.g.* 3 x 10^{-9} to 6 x 10^{-5})
Response Index	(r) dimensionless
Linear Dynamic Range	(D_{LR}) g/ml (*e.g.* 1 x 10^{-8} to 2 x 10^{-5})
Detector Response	(R_C) Volts/g or (specific units of measurement/g)
Detector Noise Level	(N_D) usually in millivolts but may be in specific units (*e.g.* Refractive Index Units)
Sensitivity or minimum detectable concentration	(X_D) g/ml (*e.g.* 3 x 10^{-8}) but may be in specific units (*e.g.* Absorption Units)
Total Detection System Dispersion	(σ^2_D) (ml^2 often ml^2)
Cell Dimensions	(length (l), and radius (r)), (cm)
Cell Volume	(V_D), ml.
Overall Time Constant (sensor and electronics)	(T_D), seconds (sometimes milliseconds)
Pressure Sensitivity	(D_P) usually in the USA p.s.i, in Europe MPa
Flow Rate Sensitivity	(D_Q) usually in ml/min
Temperature Range	^oC

All are designed to provide a response that is as close as possible to linear for accurate quantitative analysis. However, the output from some detector sensors *may* not be linearly related to the solute concentration and appropriate signal modifying circuits must be introduced into the detector electronics to provide a linear output (*e.g.*, the output from a light adsorption sensor will be exponential and consequently it must be used with a logarithmic amplifier to produce an output that is linearly related to solute concentration).

The most important detector specification is *sensitivity* as it defines the minimum concentration of solute that can be detected. It is best defined a function of the detector response and the noise level The detector response (R_C) can be defined as the voltage output for unit change in solute concentration or as the voltage output that would result from unit change in the physical property that the detector measures, *e.g.* refractive index or carbon content. Detector noise is the term given to any perturbation on the detector output that is not related to an eluted solute. It is a fundamental property of the detecting system and determines the ultimate sensitivity or minimum detectable concentration that can be achieved. Detector noise has been arbitrarily divided into three types, '*short term noise*', '*long term noise*' and '*drift*' all three of which are depicted in figure 26.

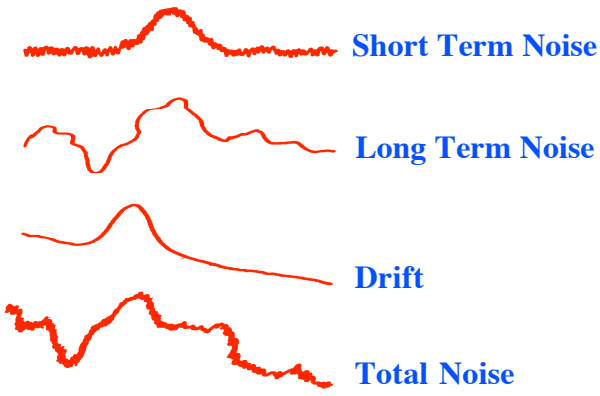


Figure 26 Different Types of Noise

Short term noise consists of baseline perturbations that have a frequency that is significantly higher than the eluted peak. Short term detector noise can be easily removed by appropriate noise filters without significantly affecting the profiles of the peaks. Its source is usually electronic, originating from either the detector sensor system or the amplifier. Long term noise consists of baseline perturbations that have a frequency that is similar to that of the eluted peak. This noise is the most significant as it is indiscernible from very small peaks in the chromatogram. Long term noise *cannot* be removed by electronic filtering without affecting the profiles of the eluted peaks. In figure 26, it

is clear 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 in the sensing cell. Drift are baseline perturbations that have a frequency that is large to that of an eluted peak. Drift is almost always due to either changes in ambient temperature, changes in mobile flow rate, or column bleed in GC; in LC drift can be due to pressure changes, flow rate changes or variations in solvent composition. A combination of all three sources of noise is shown by the trace at the bottom of figure 26.

The detector noise is defined as the maximum amplitude of the combined short– and long-term noise measured over a period of 15 minutes. The detector must be connected to a column and mobile phase passed through it during measurement. The detector noise (N_D) is obtained by constructing parallel lines embracing the maximum excursions of the recorder trace over the defined time period as shown in figure 27.

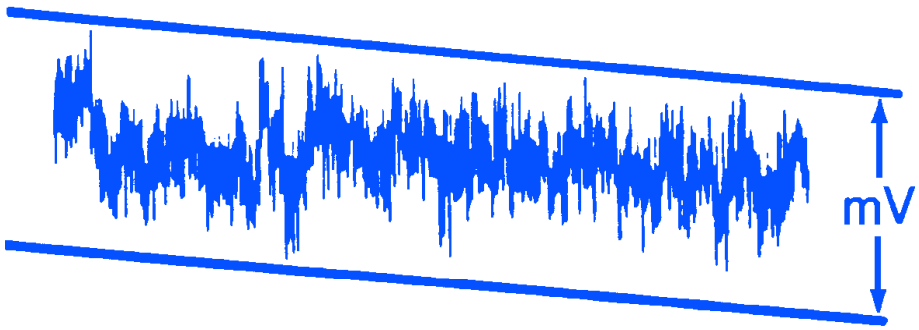


Figure 27 Measurement of Detector Noise

Detector sensitivity or minimum detectable concentration (MDC) is defined as the minimum concentration of solute passing through the detector that can be unambiguously discriminated from noise, conventionally taken when the signal to noise ratio is *two* and this criteria has been adopted for defining detector sensitivity.

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

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

The two important ranges that are specified for a detector are the *dynamic* range and the *linear dynamic* range. The dynamic range (D_R) extends from the minimum detectable concentration (*i.e.* the sensitivity) to that concentration at which the detector no longer responds to any increase. The dynamic range is not usually pertinent to general analytical work but is important in preparative chromatography. The linear dynamic range or detector linearity is as important as sensitivity for any detector that is to be used for quantitative analysis. It is defined as the concentration range over which the detector response is linearly related to the concentration of solute passing through it.

That is, $V = A C_m$

where (A) is a constant,
(C_m) is the concentration of solute,
and (V) is the output of the detector.

Because of the imperfections in mechanical and electrical devices practical detectors can only approach this ideal response. A measure of linearity that is specified in numerical terms so that comparisons can be made between detectors can be obtained as follows. It is assumed that for a closely linear detector the response could be described by the following power function

$$V = A C_m^r \qquad (1)$$

where (r) is defined as the Response Index

It follows that for a truly linear detector, r = 1, and the proximity of (r) to unity will indicate the extent to which the response of the detector deviates from true linearity. In addition if (r) is not unity but is known then appropriate corrections can be made to the response and improved accuracy can be achieved.

The three remaining important specifications are *pressure sensitivity* which is particularly crucial if multidimensional chromatography is

envisaged,*flow rate sensitivity* which is important if flow programming is contemplated and, of course, the maximum operating temperature to ensure the detector is not damaged by overheating. A GC detector must be situated in a separate oven and maintained at a temperature at least 15˚C above that of the column oven to ensure that no solute condenses in the detector and causes detector fowling. In GC any connecting tube between the column and the detector must also be heated to a temperature above that of the oven to prevent condensation.

Data Acquisition and Processing System

A diagram of a chromatographic data acquisition and processing system is shown in figure 28.

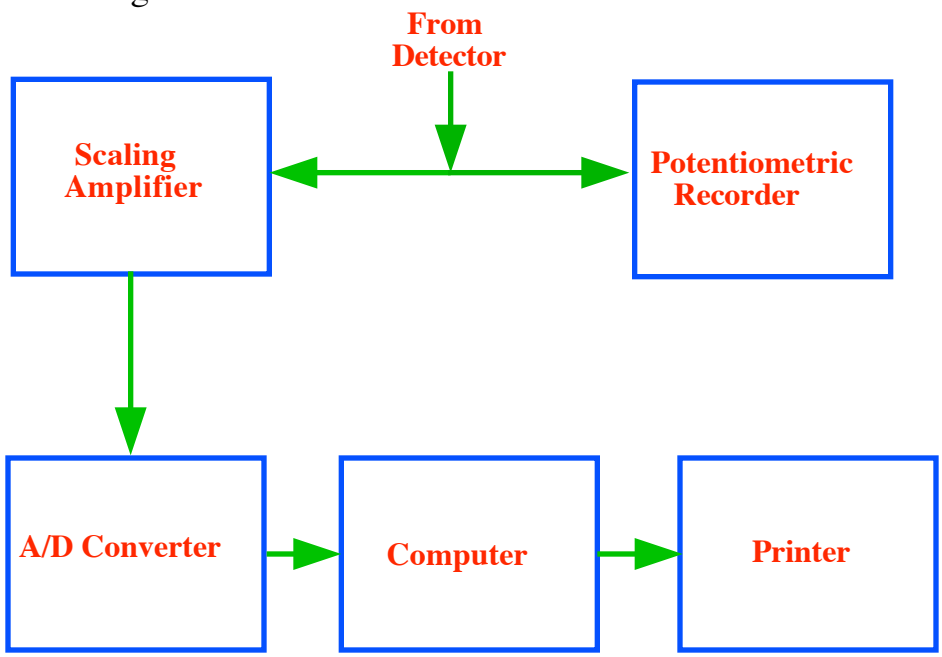


Figure 28 The Data Acquisition and Processing System

The actual format of the data system can vary widely from manufacturer to manufacturer. Most systems have a means for real time monitoring the detector output either by using an ancillary recorder or by computer software, the chromatogram being drawn by the computer on the printer. The signal from the detector is first scaled, usually to a range from 0 to 5 volt by a suitable amplifier and the scaled signal is then digitized by means of an A/D converter. The data is then regularly read

by the computer and stored on disk. The data may be partially processed 'on the fly' or processed at the completion of the analysis.

Thin Layer Chromatography Apparatus

Thin layer chromatography appears to have been first developed and utilized by Schraiber in 1939 (11). Schraiber working with Izmailov at the Khar'kov Chemistry and Pharmacy Research Institute employed the techniques for the analysis of pharmaceuticals. In her own words,

" *It occurred to us that a thin layer of the sorbent could be used in lieu of a strip of paper; also we felt that the flat bed could be considered as a cut-out of the adsorbent column. We believed that in carrying out the separation process in such a layer, the process would be accelerated significantly. In our work, we deposited a drop of the solution being investigated on the flat adsorbent layer and observed the separation into concentric circular zones which could become visible because of their fluorescence in the light of a UV lamp.*"

Schraiber not only invented thin layer chromatography in 1939 but also was the first to use fluorescence as the separation indicator or detection system. Unfortunately, Schraiber's work does not seem to have been heeded and the technique appears to have been rediscovered by Kirchner in 1951 (12).

Although thin layer chromatography (TLC) phase systems are basically the same as those used in LC, the equipment required is far simpler and very much less expensive. Furthermore, as many separations can be carried out simultaneously by multiple spotting, analysis times are much shorter and there can be as many as 60 samples per plate which, in effect means that each analysis will only take about 5 seconds to complete. Resolution obtained from TLC is far less than that obtainable by LC but, as a result of the cost advantage, the technique is very widely used. In fact, despite the many advances that have taken place in LC techniques over the past years, the use of TLC for routine analyses continues to grow. However, samples containing multiple components cannot be separated by TLC due to restricted plate capacity. In TLC all the solutes

must be contained by the plate whereas in LC, as the solutes are eluted from the column, the component capacity is much greater.

Thin Layer Chromatography Chambers

A diagram of two simple thin layer chromatography development chambers is shown in figure 29.

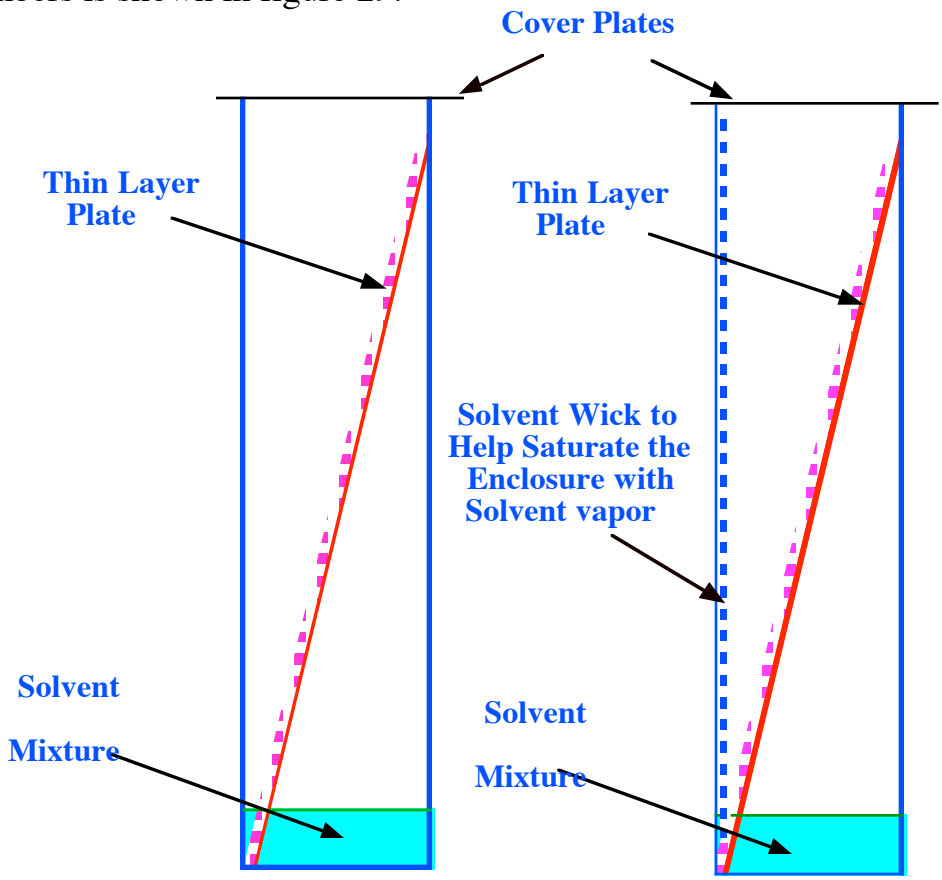


Figure 29 The Normal Method of Thin Layer Plate Development

Solvent must be prevented from evaporating from the plate surface during development as this would cause the composition of the developing solvent to change. In the simplest case, the developing chamber can consist of a round or square glass jar fitted with a glass cap. Sufficient solvent mixture is placed in the chamber to raise the level about a centimeter from the base. The plate is then placed in the chamber with the end where the samples have been placed dipping into the solvent and the cap replaced. The sample spots must not be at, or below, the surface of the solvent mixture or they will be washed from

the plate. To improve the air space saturation with solvent, the walls, or part of the walls, of the chamber are sometimes covered with filter paper to act as a wick that soaks up the solvent and provides a greater surface area for evaporation. The use of a paper wick is depicted on the right-hand side of figure 29.

The saturated solvent vapor in the chamber not only prevents solvent evaporating from the plate surface but partly controls the retention mechanism by surface deactivation. The solvents are selectively adsorbed on the surface of the stationary phase causing the solutes to interact, not with the native silica surface, but with the silica surface covered with the most strongly interacting solvent. It should be emphasized, however, that the equilibrium between the *solvent vapor* and the plate will not be the same as the equilibrium between the *solvent* and the plate. For example, for a binary mixture of solvents having concentrations of solvent in the gas phase of c_1 and c_2 respectively,

$$c_1 \neq x_1 \text{ and } c_2 \neq x_2$$

where (x_1) is the molar fraction of solvent (1)
and (x_2) is the molar fraction of solvent (2)

In fact, *Raoult's Law* applies and

$$\frac{c_1}{c_2} = \frac{x_1 P_1}{(1 - x_1) P_2} \text{ where } x_2 = (1 - x_1)$$

and (P_1) and (P_2) are vapor pressures of pure solvents (1) and (2) respectively. One form of apparatus that can be used for the pre-equilibrium of a thin layer plate is shown in figure 30.

The apparatus is very similar to that used for normal development but a separate reservoir contains the developing solvent. The plate is placed in the enclosure and allowed to come into equilibrium with the solvent vapor for a few minutes. The plate is then lifted and placed so that the end now dips into the developing solvent and the separation processed in the usual way. There are two main effects resulting from the pre-saturation of a TLC plate. These affects are depicted in figure 31. Firstly the velocity of the solvent front is increased relative to that of the unsaturated plate. Secondly, as a significant amount of the solvent at the

front is adsorbed onto the silica surface of the unsaturated plate, the separated components are much closer to the solvent front.

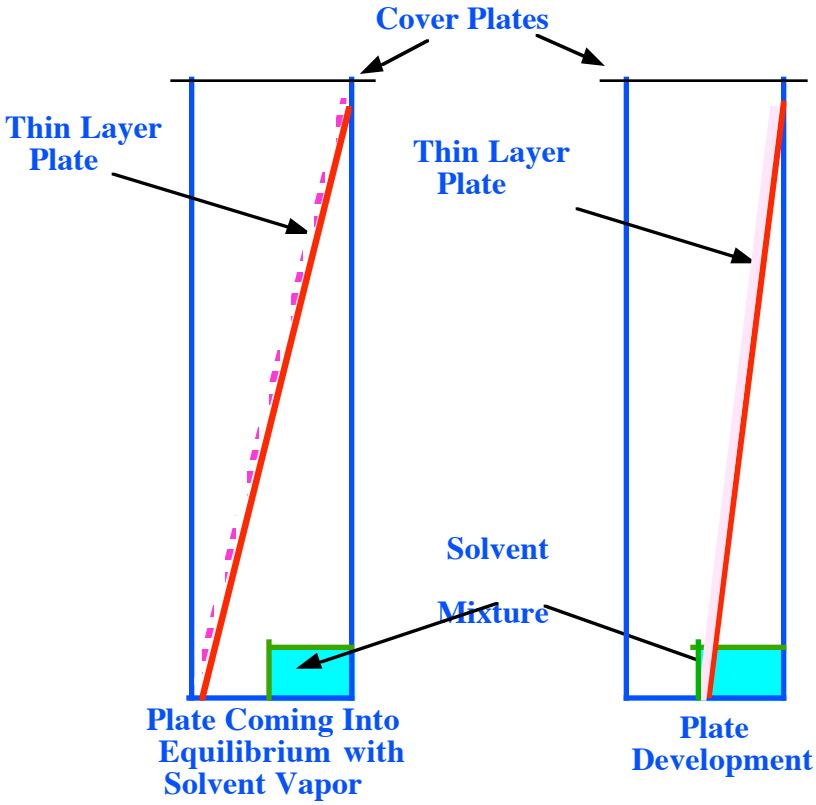


Figure 30 Apparatus for Establishing the Pre-Equilibrium of a TLC Plate

Consequently, the apparent advantage resulting from the faster moving front of the pre-saturated plate on the analysis time is offset by the closer proximity of the spots to the solvent front in the unsaturated plate. In most cases, the effect of pre-saturation on the actual separation is small but can be important for special mixtures where subtle changes in retention can make the necessary difference.

Continuous Plate Development

The normal development of a thin layer plate is limited by its physical dimensions but a continuous development procedure has been used employing special equipment. An apparatus used for the continuous development of a thin layer plate is shown in figure 32. The plate is held

horizontal and inverted so that the stationary phase layer faces downwards and rests on a second glass cover plate.

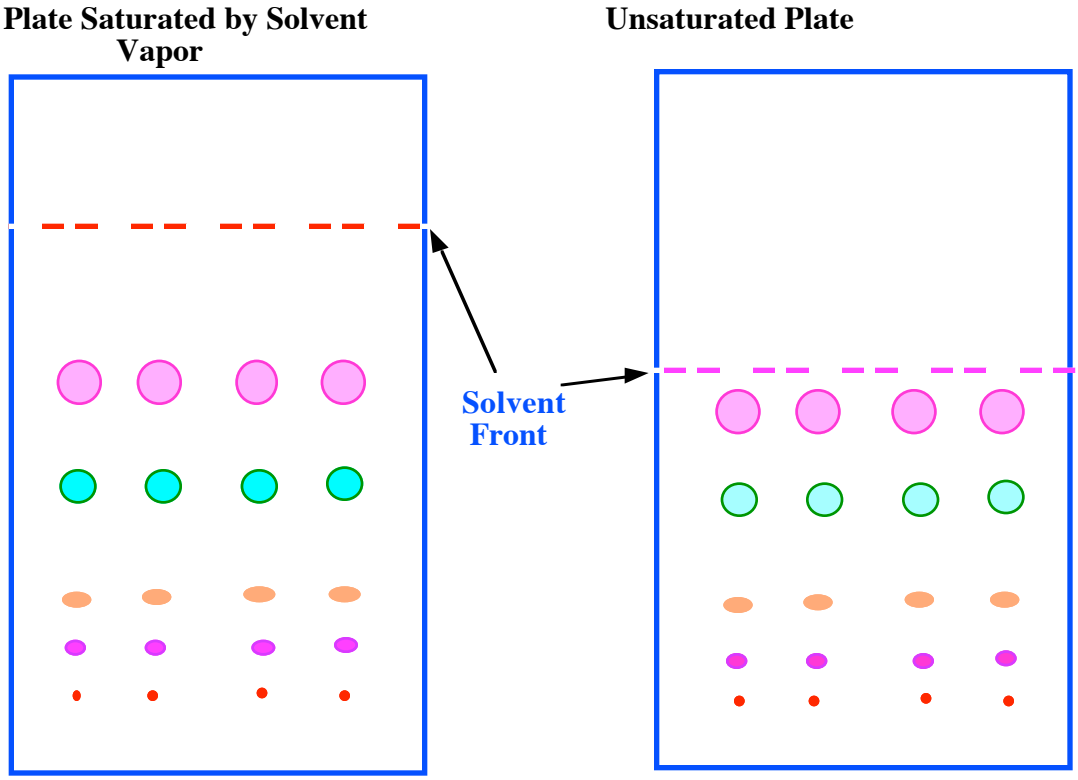


Figure 31 Effect of Plate Saturation on Plate Development

A wick transfers the solvent from the reservoir to the stationary phase coating which is sandwiched between the two glass plates.

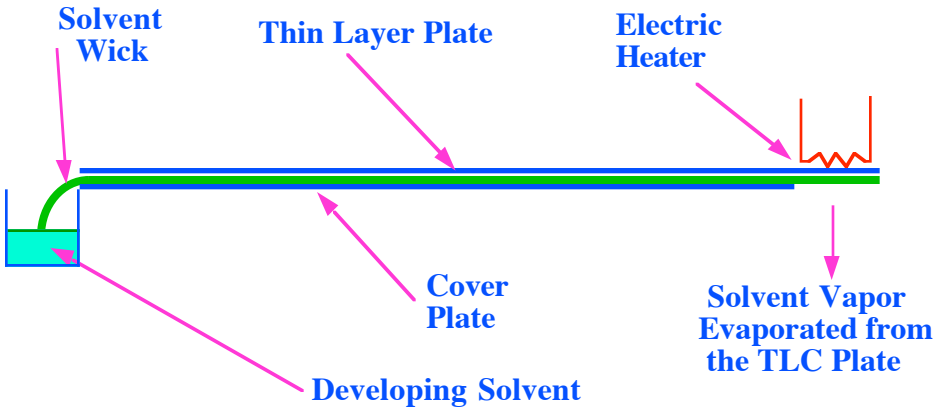


Figure 32 Apparatus for the Continuous Development of a Thin Layer Plate

The whole system is situated in a suitable chamber to prevent solvent evaporation from the reservoir. The solvent passes along the plate by surface tension forces in the usual way until it reaches the end of the plate. A small area at the end of the plate is exposed and heated electrically to evaporate the solvent as it arrives. In this way development can be continued and the system now resembles an LC column of lamina shape. The value of this technique is a little questionable as its intent is to simulate an LC column, in which case it would be preferable to use an LC column in the first place.

Sample Application

Samples should be placed on a TLC plate as a spot that is as small as possible. Sample volumes of 1 to 2 μl are common but when using the so called high performance TLC plates (HPTLC plates) coated with particles 5-7 μm in diameter a maximum loading of about 100-200 nl can only be tolerated. Ideally the sample area should be circular and not greater than 1 mm in diameter, on any HPTLC plate. As this requires the use of samples a few nanoliters in volume, most will need to be concentrated. Manually, the sample can be applied with a micro-pipette and the solvent is then allowed to evaporate, This procedure is repeated until sufficient sample is placed on the plate. Micro-syringes can also be employed to place a sample on to the plate. With care, and a little local heating, sample concentration can also be accomplished. The contents of the syringe are slowly but continuously discharged onto the plate and at the same time the solvent is progressively evaporated. This procedure can be automated, using computer controlled syringes and, in this way, samples can be focused onto the plate and constrained to a very small area.

There are various devices that are commercially available that will apply samples to a TLC plate either individually or as multiple groups. An example of a device for automatically concentrating a sample and then placing it on a plate is shown in figure 33.

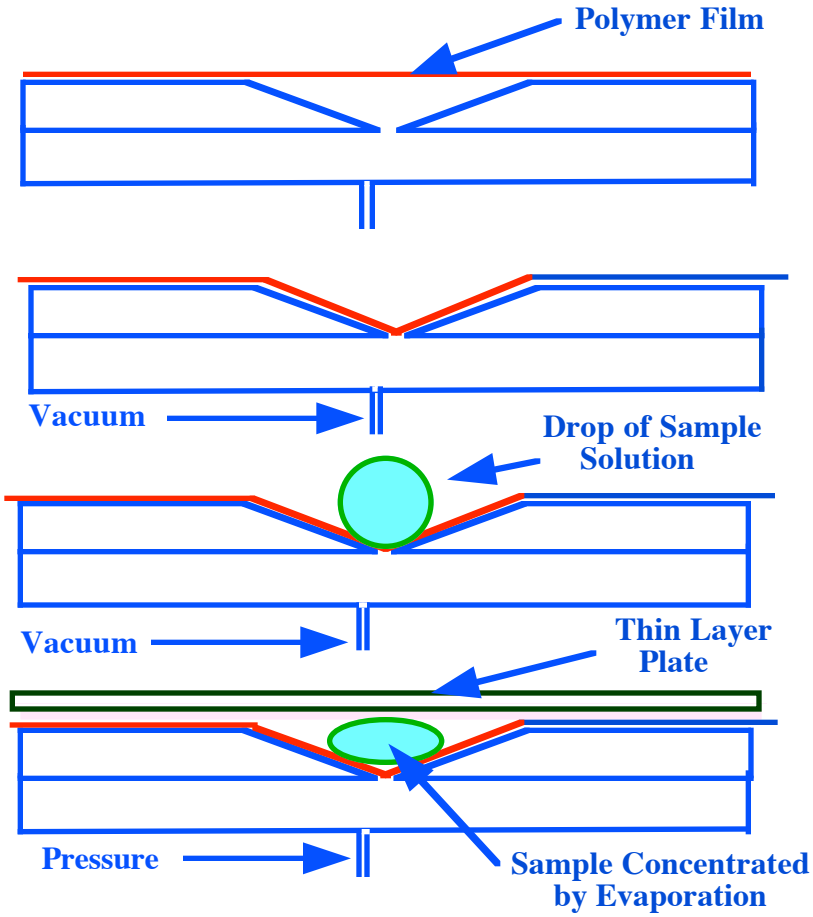


Figure 33 A TLC Sampling Device Incorporating Sample Concentration

The device consists of a plate with a small indentation, at the center of which is an aperture that can be connected either to a vacuum or to a source of gas pressure. A polymer film is placed over the plate surface and a vacuum applied to suck the film into the indentation. A small quantity of sample solution is placed in the indentation and the solvent evaporated. This procedure is repeated until sufficient sample is present on the film for a satisfactory TLC separation. It is important that the sample is *not* evaporated to *dryness* as the transfer of solid materials to the thin layer plate can be *very inefficient*. When sufficient sample has been accumulated and the droplet is still liquid, the thin layer plate is placed over the film and a positive pressure now applied to the aperture at the center of the indentation. The film extends to the surface of the TLC plate and the sample is transferred by contact, as a result of surface

tension forces and the adsorptivity of the TLC plate surface. The plate is then conveyed to the solvent chamber and the separation developed in the usual way.

If larger samples are required for semi preparative work, sample *bands* can be applied to the plate as opposed to sample *spots*. Sample bands can be applied either by using TLC plates with *concentrating zones* or alternatively by using *band applicators*. A diagram of a TLC plate that includes a concentrating zone is shown in figure 34.

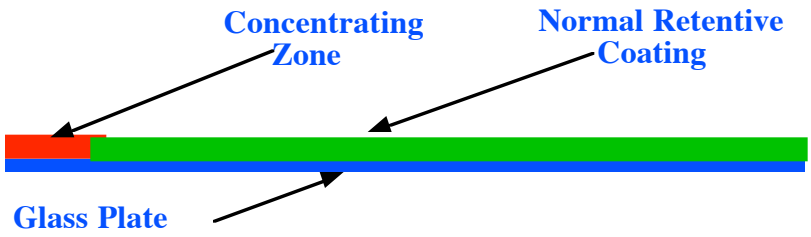


Figure 34 TLC Plate with Sample Concentrating Zone

The concentrating zone is about 2 cm wide and consists of a coating made from relatively large particles of silica with a relatively low surface area and consequently low retentive capacity. The concentrating band is coated closely adjacent to the normal retentive coating which, consists of the usual particles 5-7 mm in diameter but with a much higher surface area and, thus, much greater retentive capacity. Several samples of a few microliters or more can be placed sequentially on to the concentrating zone and the solvent allowed to evaporate until there is an adequate quantity of sample on the plate. The sample is now spread along the concentration zone in a fairly broad band. When the plate is developed the solutes move rapidly through the concentration zone due to its low retentive character to the interface between the layers. At the interface the solutes are slowed down by the more retentive layer and are thus focused as a sharp band at the front of the plate. As development proceeds, the solutes separate in the normal high retentive layer in the usual manner. This procedure has other advantages. If the sample is contaminated with salts or biological polymers, these will be trapped in the concentration zone and, thus, will not pass onto the separation region of the plate and effect the quality of the separation. Band applicators

operate differently and are usually fully automated. The sample is atomized in a stream of air or nitrogen depending on the nature of the sample and its tendency to oxidation. A diagram of the type of atomizer used in band application is shown in figure 35.

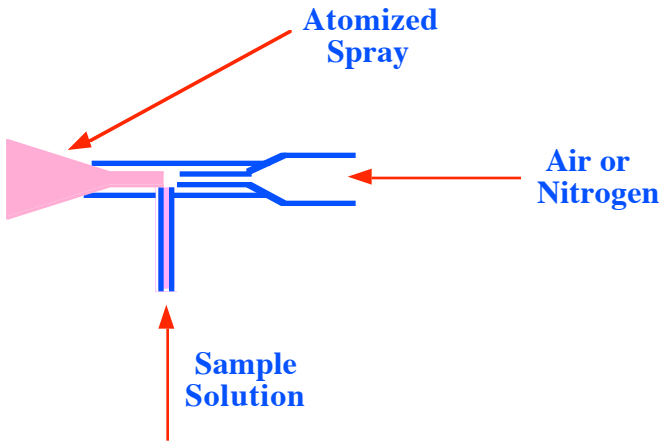


Figure 34 TLC Plate with Sample Concentrating Zone

The atomizer is mounted on a mechanical arm that can sweep from side to side while directing the atomized sample on to the surface of a TLC plate. The range and the number of sweeps, are usually under computer control and the speed of movement is adjusted such that the solvent is able to evaporate from a given area of sample before it receives the subsequent dose. After dosing, the plate is developed in the normal way. Some exceedingly novel and clever devices have been developed for TLC. These devices indeed improve the performance of the TLC analysis but are also expensive and in many cases tend to make the TLC system more like a liquid chromatograph. *The great advantage of TLC is its low cost and its relatively high separating capability*. If the required performance required is at the limit or beyond the capability of the technique, there is no point in trying to stretch it. The rational solution for the chemist or analyst would be to change to an alternative procedure such as liquid chromatography or to some other technique if more appropriate.

Chromatography Applications

Gas chromatography has an entirely different field of applications to that of liquid chromatography. In general, gas chromatography is used for

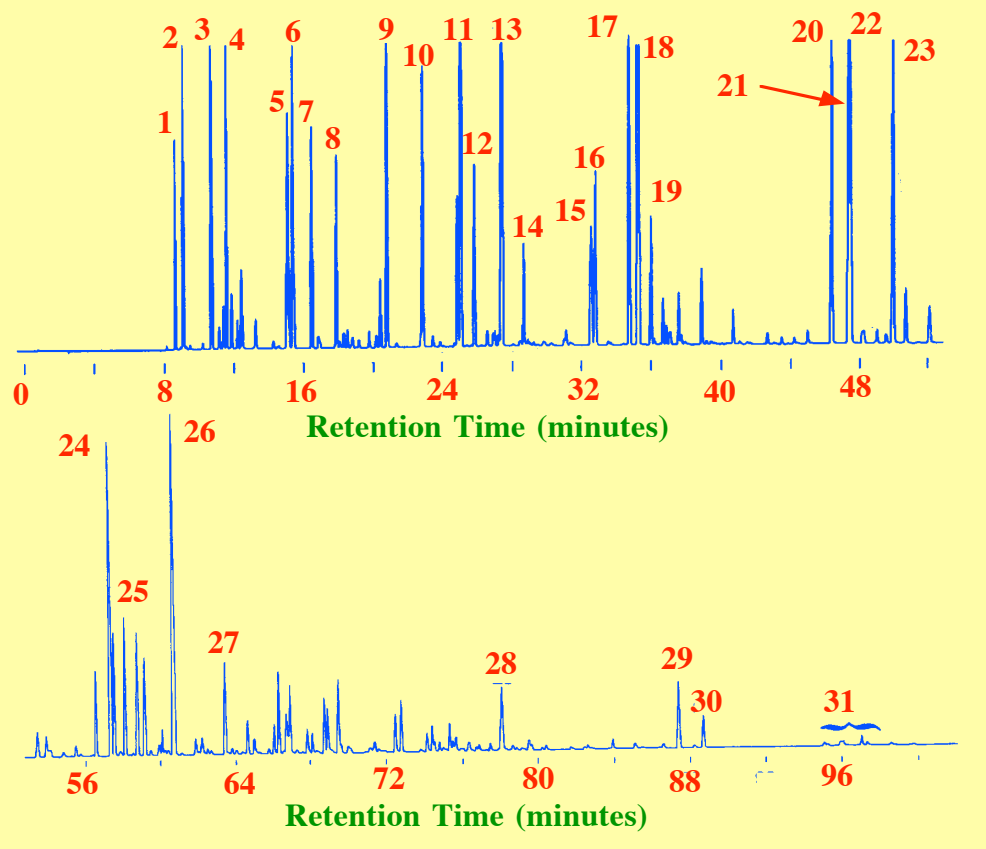
the separation of volatile materials and liquid chromatography for the separation of involatile liquids and solids. There are certain compounds, however, that can be separated with either techniques, and more importantly, many involatile substances such as amino acids, steroids and high molecular eight fatty acids can be derivatized to form volatile substances that can be separated by GC. The derivatization must be highly reproducible and usually proceed to completion in order to maintain adequate accuracy. The capillary columns in GC can have much higher efficiencies than their LC counterpart and thus GC can more easily handle multicomponent mixtures such as essential oils. On the other hand, only LC can separate the peptides, polypeptides, proteins and other large biopolymers that are important in biotechnology.

Gas Chromatography Applications

The most common hydrocarbon analysis carried out by GC is probably that of gasoline. The analysis of gasoline is typical of the type of sample for which GC is the ideal technique. It is this type of multicomponent mixtures containing very similar compounds that need the high efficiencies available from GC for a successful analysis. The separation of a sample of gasoline carried out on a long open tubular column is shown in figure 35. It is clear that the column had a very high efficiency which was claimed to be in excess of 400,000 theoretical plates. The column was 100 m long and only 250 μm I.D., carrying a film of the stationary phase, Petrocol DH, 0.5 μm thick. Petrocol DH is specially designed stationary phase for the separation of hydrocarbons and consists of bonded dimethylsiloxane, a very dispersive type of stationary phase, retaining the solutes approximately in the order of their increasing boiling points.

Nonpolar or dispersive stationary phases are employed for the separation of hydrocarbons (*e.g.* OV101, which is also a polyalkyl-siloxane, is widely used in packed columns). The flow velocity of 20 cm/sec., appears to have been taken from the ratio of the column length to the dead time. Thus, due to the pressure correction the actual effective linear velocity would be much less than that (see Book 7 Peak

Dispersion in Chromatographic Systems). Helium was used as the carrier gas which was necessary to realize the high efficiencies with reasonable analysis times.



1. Isobutane	12. 3–Methylhexane	23. *o*–Xylene
2. *n*–Butane	13. 2,3,4–Trimethylpentane	24. 1–Methyl–3–ethylbenzene
3. Isopentane	14. *n*–Heptane	25. 1,3,6–Trimethylbenzene
4. *n*–Pentane	15. 2,5–Dimethylhexane	26. 1,2,4–trimethylbenzene
5. 2,3–Dimethylbutane	16. 2,4–Dimethylhexane	27. 1,2,3–Trimethylbenzene
6. 2–Methylpentane	17. 2,3,4–Trimethylpentane	28 Naphthalene
7. 3–Methylpentane	18. Toluene	29. 2–Methylnaphthalene
8. *n*–Hexane	19. 2,3–Dimethylhexane	30. 1–Methylnaphthalene
9. 2,4–Dimethylpentane	20. Ethylbenzene	31. Dimethylnaphthalene
10. Benzene	21. *m*–Xylene	
11. 2–Methylhexane	22. *p*–Xylene	

Courtesy of Supelco Inc.

Figure 35 The Separation of Gasoline

The FID detector provided the necessary to wide quantitative dynamic range. The column temperature was held at 35°C for 15 min. to effect the separation of the low boiling, low molecular weight hydrocarbons,

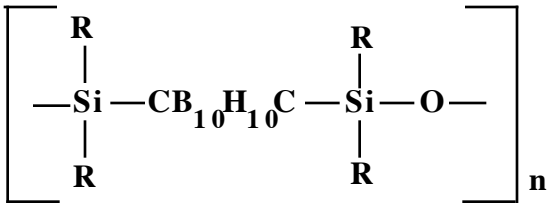
the temperature was then increased to 200˚C at 2˚C/min. and finally held at 200˚C for 5 min. to ensure the complete elution of the higher boiling components.

An excellent separation is obtained giving clearly separated peaks for the marker compounds which are of importance in fuel evaluation. Nevertheless, due to the complexity of the sample, exceedingly high efficiencies were necessary and so, the analysis time was about 100 min. Long analysis times are directly related to the use of long columns The complete analysis was carried out using only 0.1 μl of gasoline with a split of 100:1 at 250˚C (*ca* 1 μg) confirming the remarkable sensitivity of the FID for general analysis.

High Temperature GC Stationary Phases

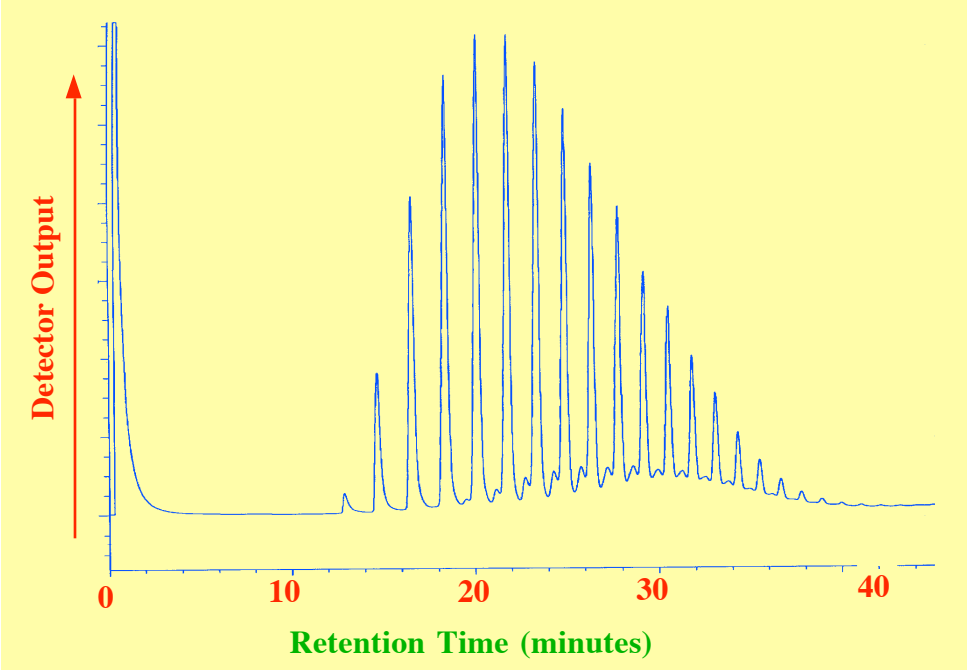
The major limitation of gas chromatography is the stability of the stationary phase at high temperatures. The higher the polarity and the higher the molecular weight of the solutes, the higher the temperature necessary to provide adequate solute partial vapor pressure to allow a gas chromatographic separation to be realized. Similarly, the stability of the solutes at high temperature can also become a problem. The solute must be thermally stable so that the partial pressure is sufficiently high to allow elution in a reasonable time. Nothing can be done with respect to the solute stability as this is determined by the nature of the sample.

There are certain materials hat can be used as stationary phases at remarkably high temperatures. These materials are based on the polymerization of carborane substituted siloxanes. An example of the empirical formula of a carborane silicone polymer is as follows,



where $CB_{10}H_{10}C$ represents the meta–carborane nucleus.

There are three commonly used carborane stationary phases, The first a dispersive phase, Dexsil 300 where the carboranes are linked with a methylsilicone polymer and can be used up to a temperature of 450˚C (an exceedingly high temperature for chromatographic separations). Some induced polarizability has been introduced into the carborane polymer by employing a methyl *phenyl* silicone and this resulted in the second carborane having intermediate polarity that can be operated up to a temperature of 400˚C. The introduction of a phenyl group makes it slightly less thermally stable. The third and most polar carborane is Dexsil 410 that contains methyl, □–cyanoethyl silicone (the polarity being contributed by the cyano group) which can be used up to 375˚C.



Courtesy of Mr. Andrew Lynn of the Dexsil Corporation

Figure 36 The Separation of a High Molecular Weight Hydrocarbon Wax on a High Temperature Stationary Phase

As would be expected the more polar the stationary phase the lower the temperature stability. An example of the use of Dexsil 400 to separate some very high boiling waxes is shown in figure 36. The column was programmed from 50˚C to 380˚C at 4˚C /min. and held at 380˚C for 6.5 min. The carrier gas flow rate was 30 ml/min. The wax components are

well resolved and the baseline appears very stable even a 380˚C. The stable base line, with no drift, indicates there is little or no decomposition of the solutes or the stationary phase, even at 380˚C. Stationary phases based on the carborane structure, can extend the temperature range of gas chromatography very significantly, However, having thermally stable stationary phases solves only half the problem, the solutes themselves must be equally stable.

Hydrocarbon Analysis

Due to the perceived toxicity and carcinogenic character of the aromatic hydrocarbons, the presence of these materials is carefully monitored in all areas where they might enter the human food chain. The analysis of water for aromatic hydrocarbons, particularly surface water in those areas where contamination might take place, is a common assay made by the public analyst. It is essential to be able to measure concentrations in the ppb levels, and thus GC method employing a high sensitive detector is essential. Nevertheless, even if a high sensitivity detector is employed, some sample concentration will be necessary to measure contaminants at such low levels.

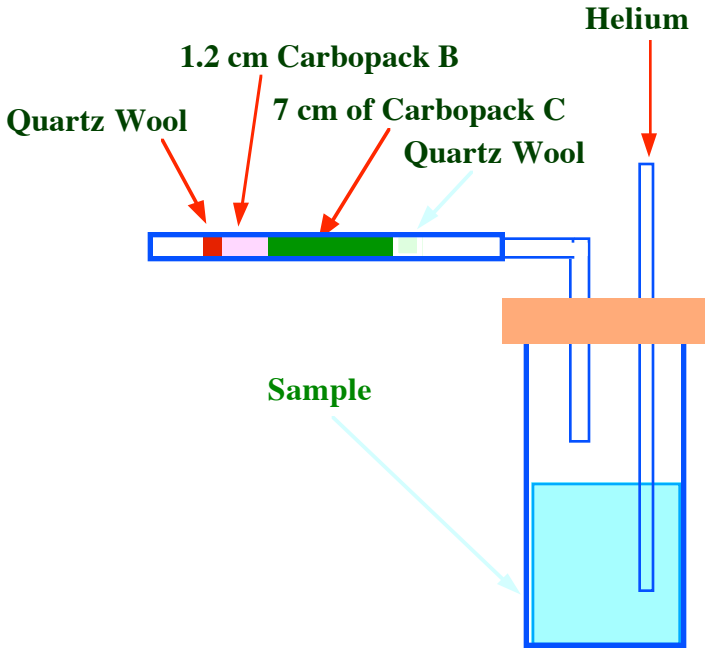


Figure 37. Purge and Trap System for Aromatic Hydrocarbons in Water

One method is the purge and trap procedure, using a solid adsorbent to remove the hydrocarbon vapors. A diagram of the purge and trap system is shown in figure 37. A 5 ml sample of spiked water was placed in a small vial through which a stream of helium was passed at a flow rate of 40 ml/min. The purge was carried out at room temperature for 11 min.

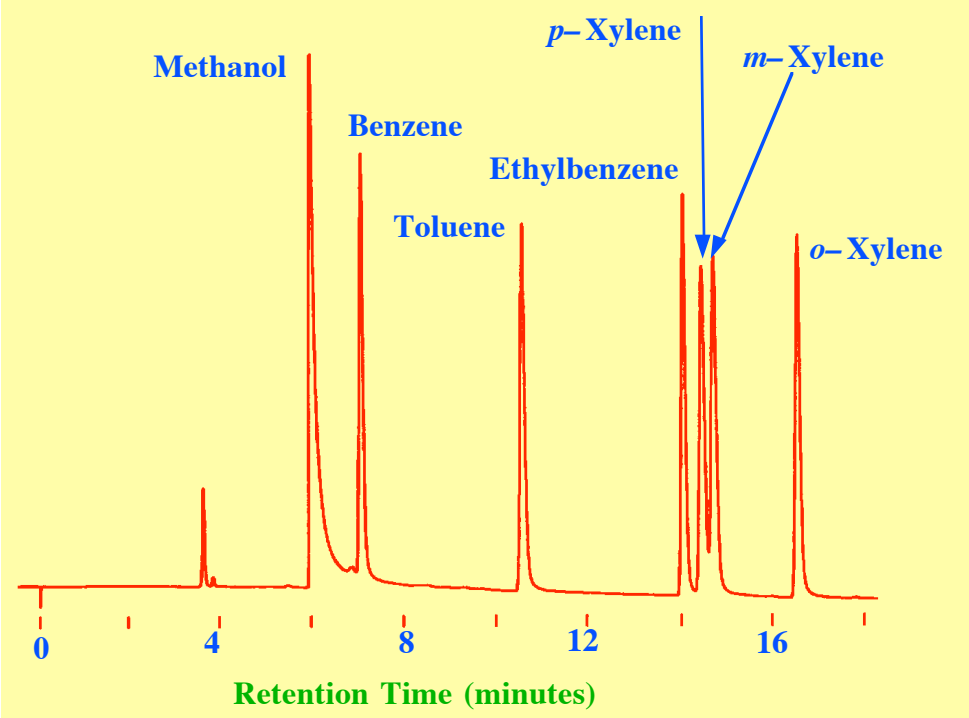
After bubbling through the sample, the helium passed through a stainless steel adsorption tube, 1/8 in. diameter having a 7 cm length packed with Carbopack C and a 1.2 cm length packed with Carbopack B. Carbopack C is 20/40 mesh, graphitized carbon, having a surface area of 10 m^2/g and the short length of Carbopack B packing had a surface area of about 100 m^2/g.

The short length of packing with the higher surface area (thus high adsorptive capacity) was to used to ensure that none of the sample material was eluted through the adsorption bed and lost. The sample vial was then removed and the trap purged dry with 40 ml/min. of dry helium for 5 min. The trap was then transferred to a small heating oven and the contents desorbed onto the column at 250˚C with a 4 min. bake at 260˚C. A chromatogram of the separation that was obtained is shown in figure 38.

The open tubular column used was 60 m long, 0.75 mm I.D. carried a 1 μm film of the stationary phase Supelcowax 10. This stationary phase is strongly polar and corresponds to a bonded polyethylene glycol. The strong fields from the hydroxyl groups polarize the aromatic nuclei of the aromatic hydrocarbons and thus retention was effected largely by polar interactions between the permanent and induced dipoles of the stationary phase and solute molecules respectively.

The flow rate was 10 ml/min. in conjunction with the FID detector. The column was held at 50˚C for 8 min. and then programmed to 100˚C at 4˚C per min. More than adequate separation is achieved and even the *m* and *p* xylenes are well resolved. This might indicate that a significantly shorter analysis was possible. The aromatic hydrocarbons were present

in the original aqueous solution at 10 ppb and so the 5 ml of water contained about 50 pg. of each aromatic hydrocarbon.



Courtesy of Supelco Inc.

Figure 38 The Separation of 10 ppb Quantities of Aromatic Hydrocarbons from Water

Essential Oils

Without the use of gas chromatography the analysis of essential oils would be extremely difficult. Prior to the technique being developed, only the major components of the oils could be separated, achieved by distillation with high efficiency columns. Even so, such columns rarely contained more than 100 theoretical plates (distillation plates), were very slow in operation, and took many days to complete an analysis. Due to the limited separation efficiency of the distillation column, even the major components were contaminated with traces of materials, many of which had strong olfactory intensity and thus confused the olfactory character of the major component. The gas chromatograph had a startling impact on the essential oil industry. Not only was the complex nature of the raw materials disclosed for the first time, but the character of each pure

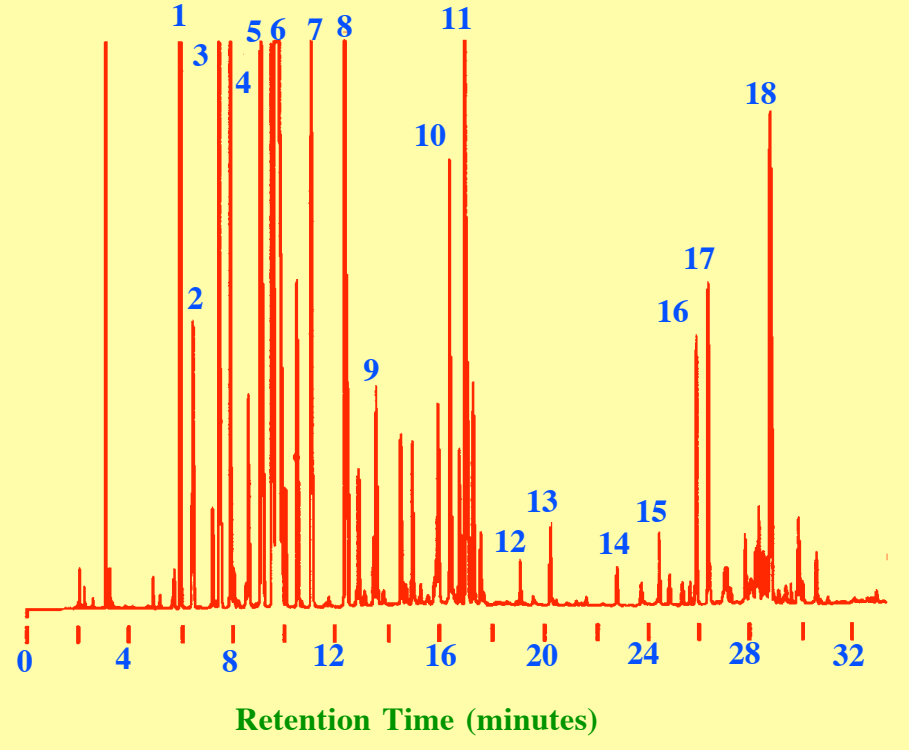
individual components could be accurately ascertained by olfactory assessment of the eluted peaks (using a non destructive detector such as the katherometer, and smelling them).

The first separations of essential oils were carried out on packed columns that provided limited efficiency but, nevertheless represented a tremendous advance on distillation. The introduction of the technique of temperature programming improved the separation even more. However, it was not until the capillary column, with its many thousands of theoretical plates, became commercially available that the true complex nature of many of the essential oils was revealed. The chemical structure of the individual components of many of the oils, elucidated by the GC/MS tandem systems, provided the knowledge necessary to synthesize a number of commercially important synthetic flavors. For example, the synthetic flavors that closely imitate those of the peach, melon and other fruits that are presently available to the contemporary food chemist are a direct result of the separating capabilities of gas chromatography.

An example of the separation of lime oil employing modern GC techniques is shown in figure 39. The separation was carried out on a SB–5 column, that contained poly(5%diphenyl-95%–dimethylsiloxane) as the stationary phase. Although the diphenyl group will contribute some induced polarizability capability to interact with polar solutes, it is largely a dispersive stationary phase, and thus substances are eluted roughly in order of their boiling points (excepting very polar solutes). The introduction of the diphenyl groups contributes more to phase temperature stability than it does to solute selectivity. The column was 30 m long, 250 μm I.D. carrying a film 0.25 μm thick of stationary phase. Helium was used as the carrier gas at a linear velocity of 25 cm/sec(set at 155˚C).

The column was held isothermally for 8 min. at 75˚C and then programmed up to 200˚C at 4˚c/min. and finally held at 200˚C for 4 min. The sample volume was 0.5 μl which was split at 100:1 ratio allowing about 5 μg to be placed on the column. It is seen from figure 5 that a very good separation is obtained that convincingly confirms the

complex nature of the essential oil. In practice, however, the net flavor or odor impact can often be achieved by a relatively simple mixture of synthetic compounds.



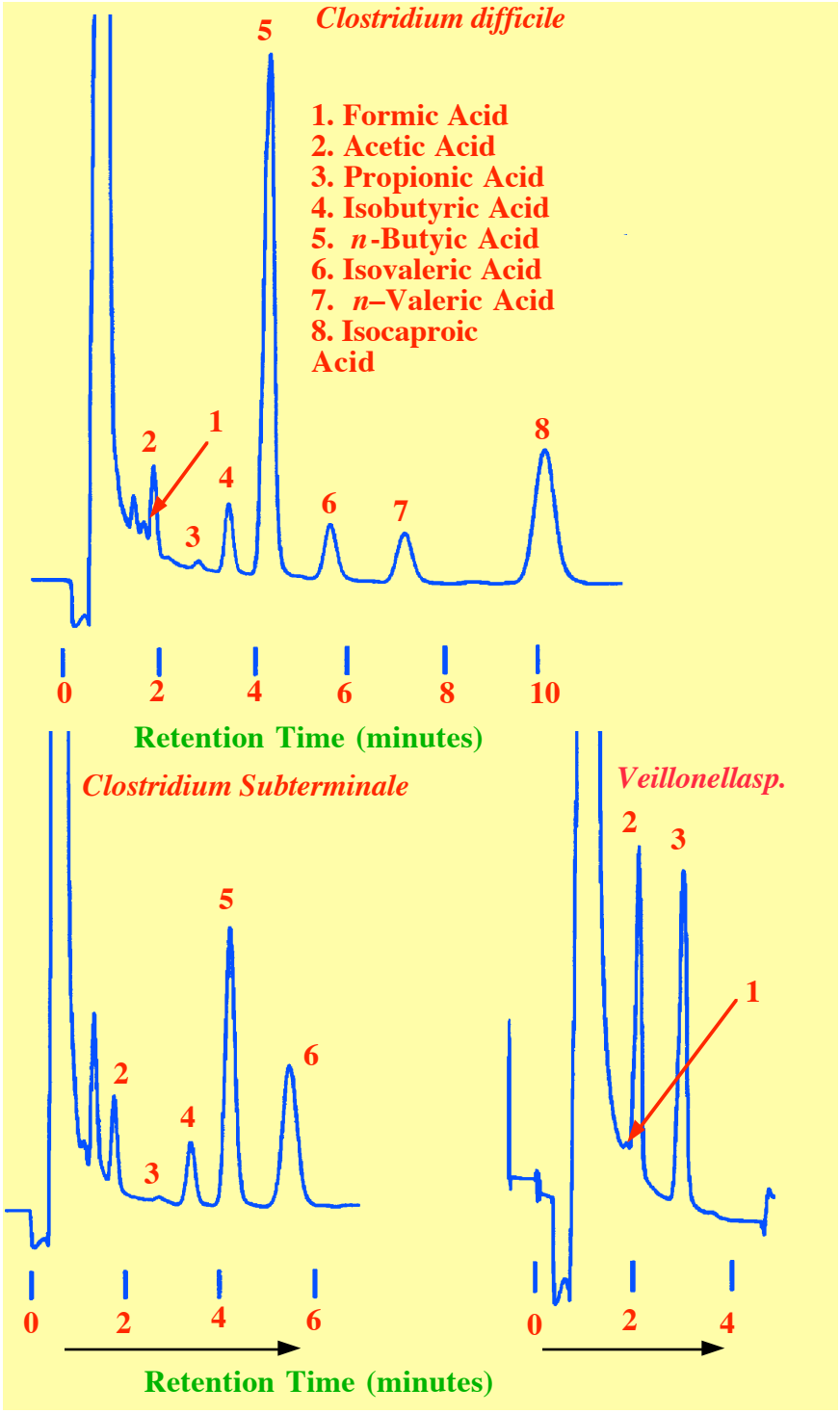
1. *a*–Pinene	7. □–Terpinene	13. Geraniol
2. Camphene	8. Terpinolene	14. Neryl Acetate
3. □–Pinene	9. Linalool	15. Geranyl Acetate
4. Myrcene	10. Terpinene–4–ol	16. Caryophyllene
5. *p*–Cymene	11. □–Terpineol	17.*trans*–□–Bergamotene
6. Limonene	12. Neral	18. b–Bisabolen

Courtesy of Supelco Inc.

Figure 39 A Chromatogram of Lime Oil

The Identification of Bacteria by Their Volatile Fatty Acid Profiles.

The bacteria *genus clostridium* are rod shaped and found largely in soil.



Courtesy of Supelco Inc.

Chromatogram provided to Supelco by K.J. Hauser, Department of Pathology, Mount Sinai Medical Center, Milwaukee, WI.

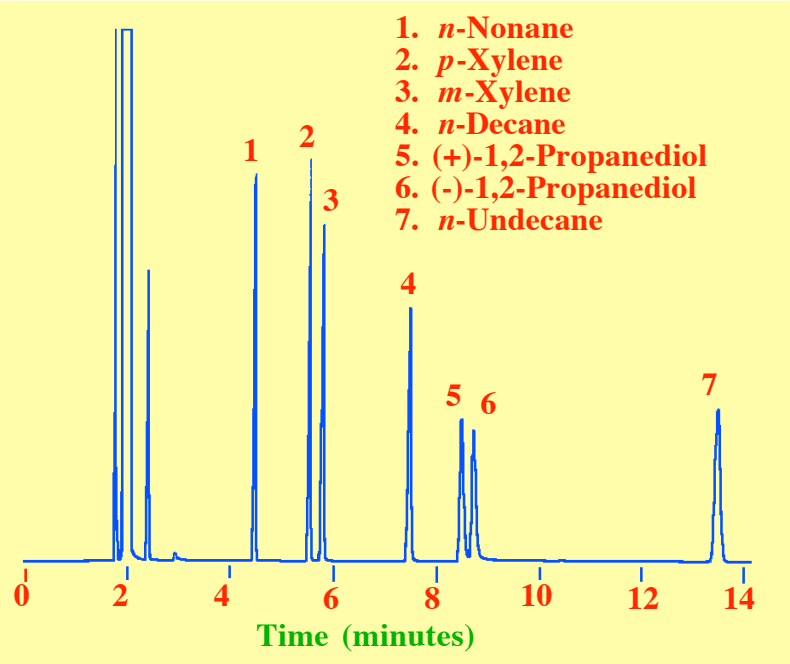
Figure 40 Volatile Fatty Acid Profiles from Different Bacteria.

Among these germs are those that can cause some of the most deadly diseases in man, such as tetanus (*clostridium tetani*) botulism, (*clostridium botulinum*) and gas gangrene, the deadly killer of wounded soldiers in the trench warfare of the first world war. The clostridium bacteria are particularly dangerous as they are very resistant to heat and thus all materials with which they may come in contact must be carefully sterilized. Samples are prepared using the following procedure. 2 ml of the acidified culture is placed in a conical centrifuge tube and 1 ml of diethyl ether added. The tube is sealed and well mixed and then centrifuged to break up the emulsion. The ether layer is pipetted from the tube and anhydrous sodium sulfate added. The mixture then is allowed to stand for about 10 min. to remove all traces of water. An appropriate volume of the dry ether extract is injected onto the column. An example of the fatty acid profiles for three different types of bacteria are shown in figure 40, two of which are clostridium. A packed column was employed carrying a 15 % loading of a proprietary stationary phase and 1% of phosphoric acid supported on 100/120 Chromosorb W AW, a processed diatomite. The column was made of glass, 6 ft long, 4 mm I.D. and operated isothermally at 145°C. Helium was used as the carrier gas at a flow rate if 60 ml/min. and 15 µl of the ether extract was injected onto the column. The three different types of bacteria gave quite different volatile fatty acid profiles. Furthermore it would appear that the acid profile; could be used as a means of identification.

Chiral Separations

An example of a chromatogram of a test mixture used by Supelco to demonstrate the chromatographic characteristics of their β-DEX column is shown in figure 41. 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 β-cyclodextrin gives it a rigid character and unique chiral selectivities. 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.



Courtesy of Supelco

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

Figure 41 Chromatogram of a Test Mixture for a Permethylated □-Cyclodextrin Stationary Phase

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 □-cyclodextrin.

Liquid Chromatography Applications

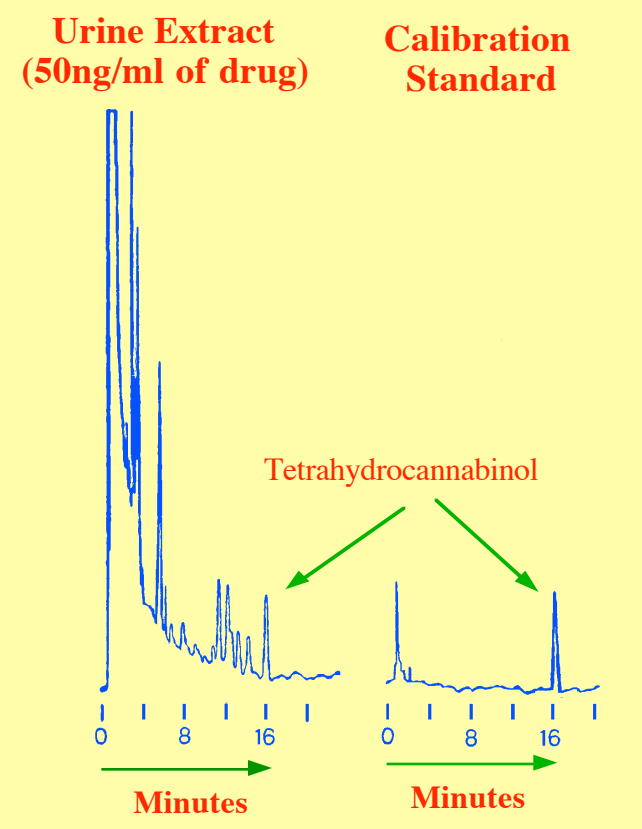
Liquid chromatography lends itself particularly to the separation of highly polar and large molecular weight materials that have very low

volatility and, thus, can not be separated by gas chromatography. In addition, however, the technique is also used in trace analysis (e.g., drugs and drug metabolites in blood) where a concentration procedure is necessary and the sample is eventually regenerated in a small volume of liquid that can be injected directly onto an LC column. In general, the sample is passed through a short length of tube packed with an adsorbent which selectively removes the material of interest. The adsorbent is then washed and the adsorbed material extract with a small amount of suitable solvent and the solvent then inject onto the column.

An example of the use of this technique is in the determination of tetrahydrocannabinol carboxylic acid in urine. This substance appears in the urine of those subjects that have recently smoked marihuana. The tetrahydrocannabinol carboxylic acid can be extracted from the urine by means of a solid state extraction cartridge packed with a C18 reverse phase (a strongly dispersive packing containing bonded octyldecyldimethyl chains). The urine sample can be used direct, without pretreatment and the materials of interest are irreversibly adsorbed on the reverse phase solely by dispersive interactions. The actual procedure for extracting the tetrahydrocannabinol carboxylic acid according to Supelco is as a follows (1).

5 ml of urine was diluted with 5 ml of water and 0.5 ml of 10M potassium hydroxide and placed in a silanized glass tube. Note the tube with a non-polar wall was used to ensure that none of the acid was adsorbed on the hydroxyl groups of the glass. The tube was heated to 60^{o}C for fifteen minutes and then cooled to room temperature. The sample was then adjusted to a pH of 4.5 with acetic acid. The C18 reversed-phase in the extraction tube was pre-conditioned with a mixture of 2 ml of methanol and 1 ml of 1% acetic acid. The hydrolyzed sample was allowed to percolate slowly through the tube by dropwise addition. The tube packing was then washed twice with 1 ml of a 20% aqueous solution of acetone, once with 1.5 ml aqueous 0.01M KH_2PO_4 , once with 0.5 ml of aqueous 0.01M Na_2HPO_4 and finally 0.5 ml of 1% aqueous acetic acid. The sample was then slowly desorbed from the reversed-phase with 1 ml of pure methanol and collected in a silanized

glass tube. The sample was reduced in volume by evaporation in a stream of nitrogen and finally made up to a volume of 250µl with methanol.



Courtesy of Supelco Inc.

Figure 42. Chromatograms of Tetrahydrocannabinol Car-boxylic Acid from Urine

The chromatograms obtained from a urine sample and a reference standard by this procedure is shown in figure 42.

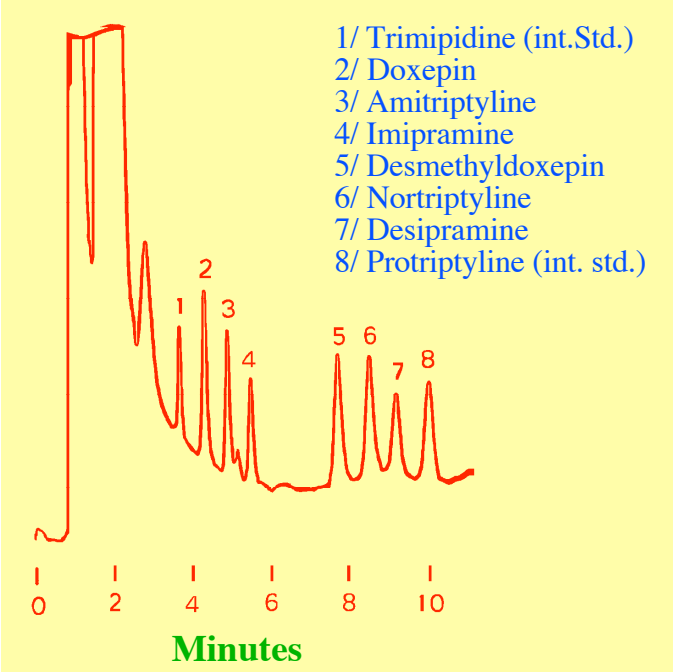
Column	LC 18
Column Length	25 cm
Column Diameter	4.6 mm
Column Packing	C18 Reverse Phase, (octadecyldimethyl chain)
Mobile Phase	55% acetonitrile 45% aqueous 1% acetic acid
Flow-Rate	2.5 ml/min.
Detector	UV adsorption at 280 nm
Sample Volume	100µl containing 500 ng tetrahydrocannabinol

This is a typical application for liquid chromatography using solid phase extraction cartridges. It is seen that the tetrahydrocannabinol carboxylic acid is clearly and unambiguously separated from the contaminating materials with an extraction efficiency of over 90%.

Another example employing a similar technique is the determination of the tricyclic antidepressant drugs in blood serum (13). The solid phase adsorbent used in this extraction is a weak ion exchanger and the material is preconditioned with a wash of 0.5 ml of 0.5M phosphoric acid followed by 1 ml of deionized water. A volume of 0.5 ml of the serum containing the tricyclic antidepressant drug standards is mixed with 0.5 ml of deionized water and allowed to percolate slowly through the packing. As in the previous example the drugs are held on the ion exchanger whereas the sample matrix materials pass through. The packing is then washed with 0.5 ml of 1.0M aqueous ammonium hydroxide and then two, 1 ml aliquots of 5% methanol in water. The sample is then desorbed by two separate aliquots of 1 ml of 0.22M ammonium hydroxide in pure methanol. Finally, the sample is collected in a silanized glass tube and the solvent removed by evaporation under stream of dry nitrogen. The volume of the sample is then made up to 250 □l and 100 □l placed on the column. The separation obtained is shown in figure 43. The separation was carried out using an LC-PCN column packed with a bonded phase carrying cyanopropylmethyl moieties on the surface. Thus, in contrast to the extraction process, which appears to be based on ionic interactions with the weak ion exchange material, the LC separation appears to be based on a mixture of interactions. There will be dispersive interactions of the drugs with the hydrocarbon chains of the bonded moiety and also weakly polar interactions with the cyano group. It is seen that the extraction procedures are very efficient and all the tricyclic antidepressant drugs are eluted discretely.

An example of the use of native silica is in the analysis of Darvocet® and its generic equivalent formulation. The separation obtained is shown in figure 44. Darvocet® is an acetaminophen product of which acetaminophen itself is the active ingredient together with other weakly

polar substances present. Consequently, the mixture lends itself to separation on silica gel. The analysis was completed in less than 4 minutes using a short column 3.3 cm long and 4.6 mm in diameter.



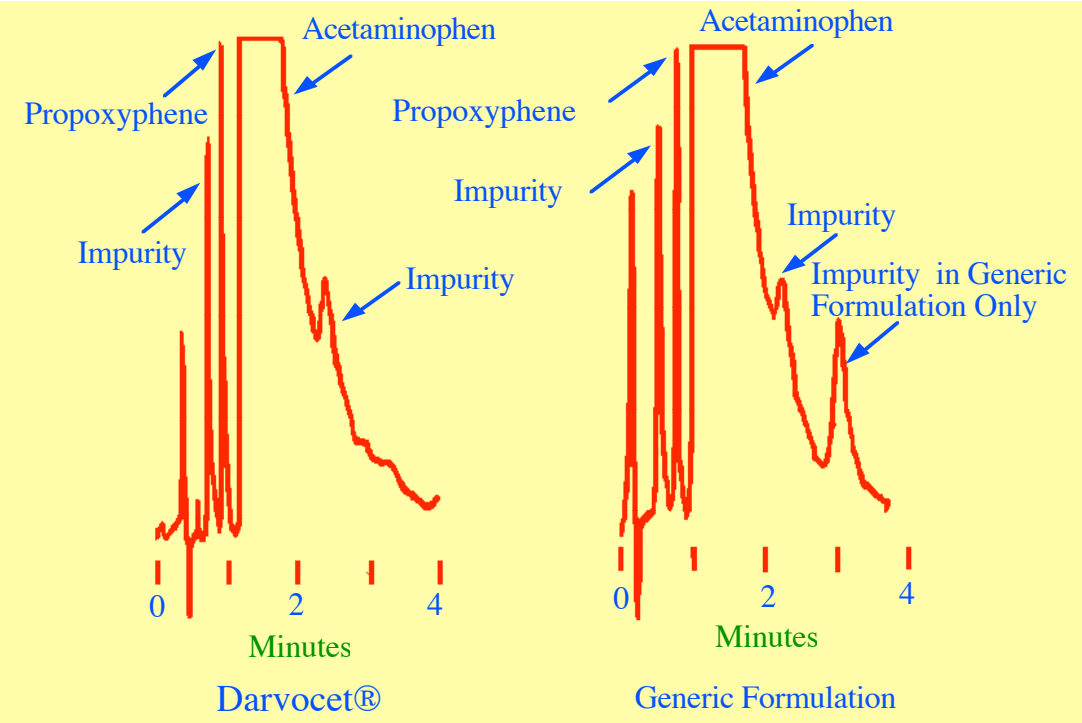
Courtesy of Supelco Inc.

Figure 43 Chromatogram of the Tricyclic Antidepressant Drugs from Blood Serum

The chromatographic conditions were as follows.

Column	LC-PCN
Column Length	25 cm
Column Diameter	4.6 mm
Column Packing	C18 Reverse Phase, (cyanopropile chain) Column Temperature 30°C
Mobile Phase	25%0.01M potassium phosphate (adjusted to pH 7 with 85% phosphoric acid)60% acetonitrile and 15% methanol
Flow-Rate	2 ml/min.
Detector	UV adsorption at 215 nm
Sample Volume	100□l

The silica packing had a particle size of 3 □ providing a maximum efficiency of about 5,500 theoretical plates. The column appears to be significantly overloaded in order to identify the impurities, Nevertheless, the impurities were well separated from the main component and the presence of a substance was demonstrated in the generic formulation that was not present in the Darvocet®.

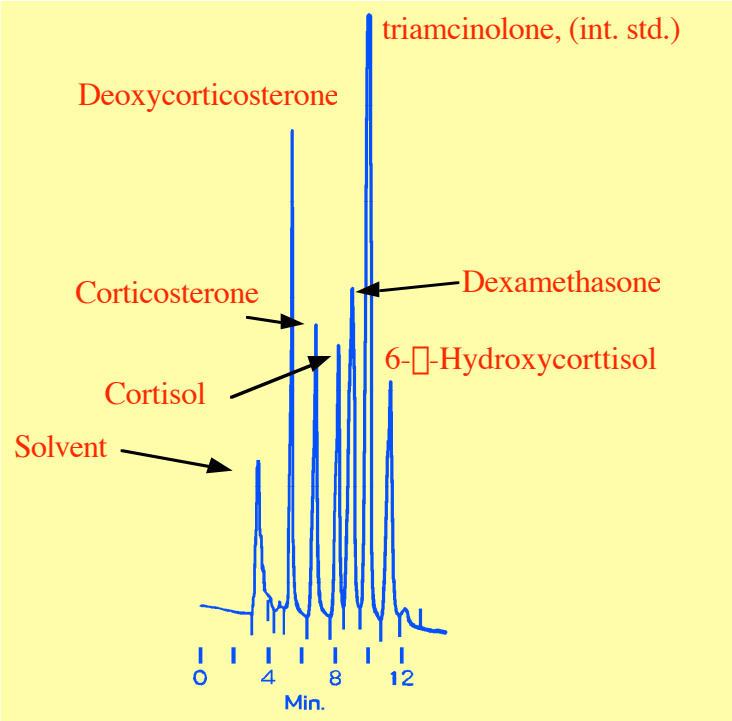


Courtesy of Supelco Inc.

Figure 44 The Analysis of Acetaminophen Formulations

The mobile phase consisted of a mixture of 98.5% dichloromethane with 1.5% v/v of methanol containing 3.3% ammonium hydroxide. The ammoniacal methanol deactivated the silica gel but the interaction of the solutes with the stationary phase would still be polar in nature. In contrast solute interactions with the methylene dichloride would be exclusively dispersive. The separation of some steroid hormones is another example of the use of silica gel as an interactive stationary phase the separation obtained is shown in figure 45. The column was 25 cm long, 4.6 mm in diameter, and packed with silica gel particle (particle diameter 5 □m) which gave a maximum efficiency at the optimum

velocity of 25,000 theoretical plates. The mobile phase consisted of 76% v/v n-hexane and 24% v/v 2-propyl alcohol at a flow-rate of 1.0 ml/min.



Courtesy of Supelco Inc.
(Supplied to Supelco by Dr. S. N. Rao and Prof. M. Okamato, Cornell University Medical College, New York)

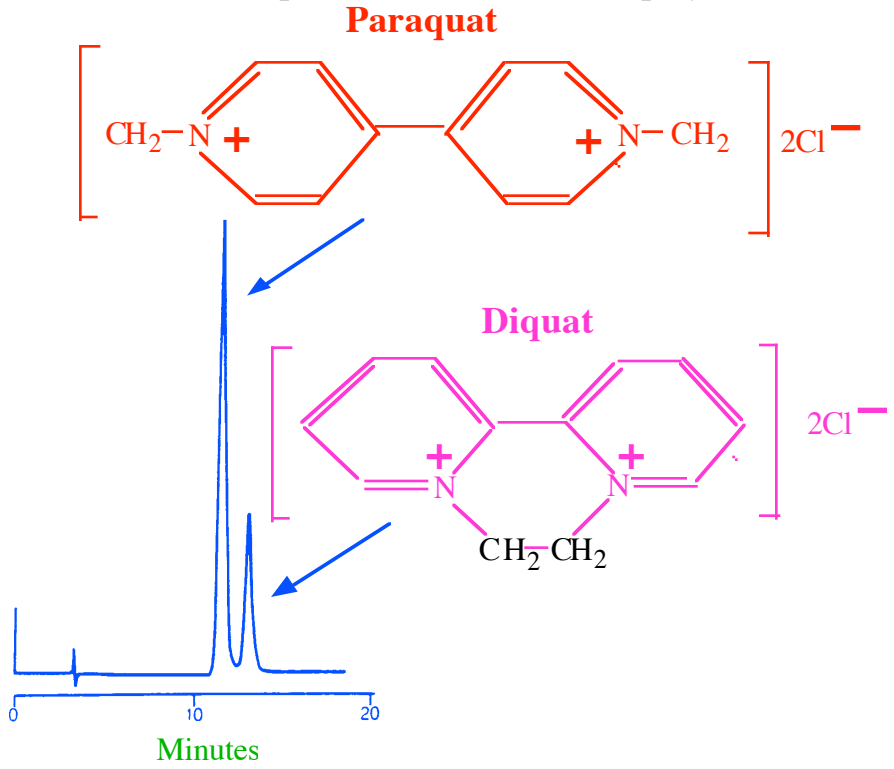
Figure 45 The Separation of Steroid Hormones

The steroid hormones are mostly weakly polar, consequently, the separation on silica gel, will be based primarily on polarity. The silica, however, was heavily deactivated by a relatively high concentration of the moderator 2-propyl alcohol and thus the silica gel surface would be covered with isopropanol and thus the interacting surface would be virtually pure 2-propyl alcohol. Whether the interaction is by sorption or displacement is difficult to determine. It is likely that the early peaks interacted by sorption and the late peaks possibly by displacement.

Ionic Interaction Chromatography

Ionic interaction chromatography, or ion chromatography as it is usually called, is typically carried out employing ion exchange resins as the stationary phase. There are some silica based ion exchange materials

available, but the bonded silicas tend to be unstable in the presence of high salt concentrations and at extremes of pH. As a consequence, they have very limited areas of application. Alternatively, the polystyrene divinyl benzene cross-linked polymers, are extremely stable to a wide range of salt concentrations and can function well within the pH range of 2.0 to 12.0. An obvious application area for ion exchange chromatography is in the separation of all types of anions and cations. Metal cations and inorganic anions are all separated predominantly by ionic interactions with an ion exchange resin. Organic acids and bases, however, would be retained by mixed interactions, as dispersive and polar interactions will take place between the solute molecules and the aromatic nuclei and the aliphatic side chains of the polymer base.



Courtesy of Asahi Chemical Industry Co. Ltd.

Figure 46 The Separation of the Herbicides Diquat and Paraquat

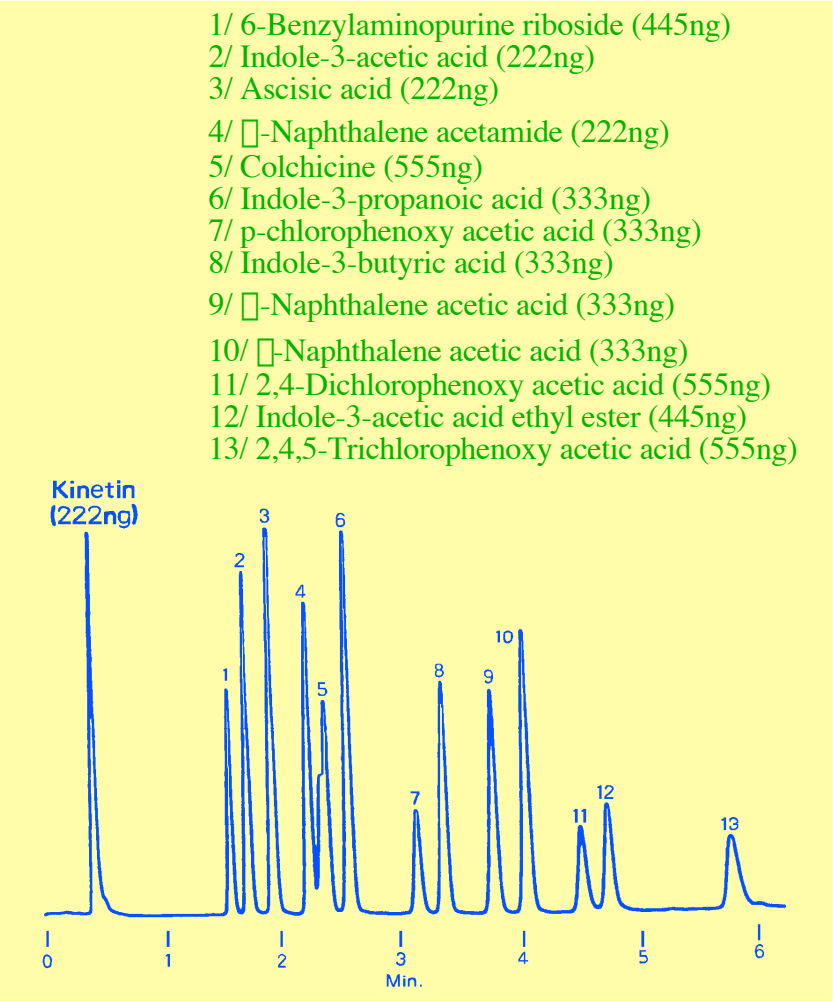
The separation of simple acids and bases require that the mobile phase is buffered appropriately according to the pK_a of the salts so that dissociation occurs and the ions are free to interact with the stationary phase. By employing mobile phase additives and using novel operating

conditions, ionic interactions can be used to separate a far wider range of materials than simple organic and inorganic anions and cations. An example of the use of ion interactions to separate the non-selective contact herbicides Diquat and Paraquat is given in figure 46. The column was fairly large (10 cm long and 7.6 mm in diameter) and was packed with cross-linked polystyrene beads, 9 μm in diameter, carrying -COOH groups as the interacting ion moieties. At the optimum flow rate the column would give about 5,500 theoretical plates but at the flow-rate used which was 1 ml/min. the efficiency would be considerably below that. Nevertheless, it is clear that a very good separation was obtained. An example of the use of a C18 reverse phase column that separates substances purely on the basis of dispersive interactions is shown in figure 47. The packing is incorporated in a short column 3.3 cm long, 4.6 mm in diameter and packed with particles 3 μm in diameter. The example of its use is in the separation of mixture of growth regulators

The efficiency expected from such a column when operated at the optimum velocity would be about 5,500 theoretical plates. This is not a particularly high efficiency and, as a consequence, the separation relied heavily on the phases selected and the gradient employed. The separation was achieved by a complex mixture of competitive interactions, ionic and dispersive interactions between the solutes and the stationary phase and ionic, polar and dispersive between the solutes and the mobile phase. The gradient started with an initial solvent mixture of 1% acetic acid and 1 mM tetrabutyl ammonium phosphate buffered to a pH of 2.8. Initially the tetrabutyl ammonium salt would be adsorbed strongly on the reverse phase and thus acted as an adsorbed ion exchanger. During the program, acetonitrile was added to the solvent and initially this increased the dispersive interactions between the solute and the mobile phase.

As the acetonitrile concentration increased, however, the concentration of adsorbed tetrabutyl ammonium salt would also be reduced and it would be eluted from the reverse phase with a resulting reduction in the ionic interactions of the solutes with the stationary phase. At higher concentrations of acetonitrile, the tetrabutyl ammonium salt would be

completely removed and the interactions of the solutes with the stationary phase would become almost exclusively dispersive.



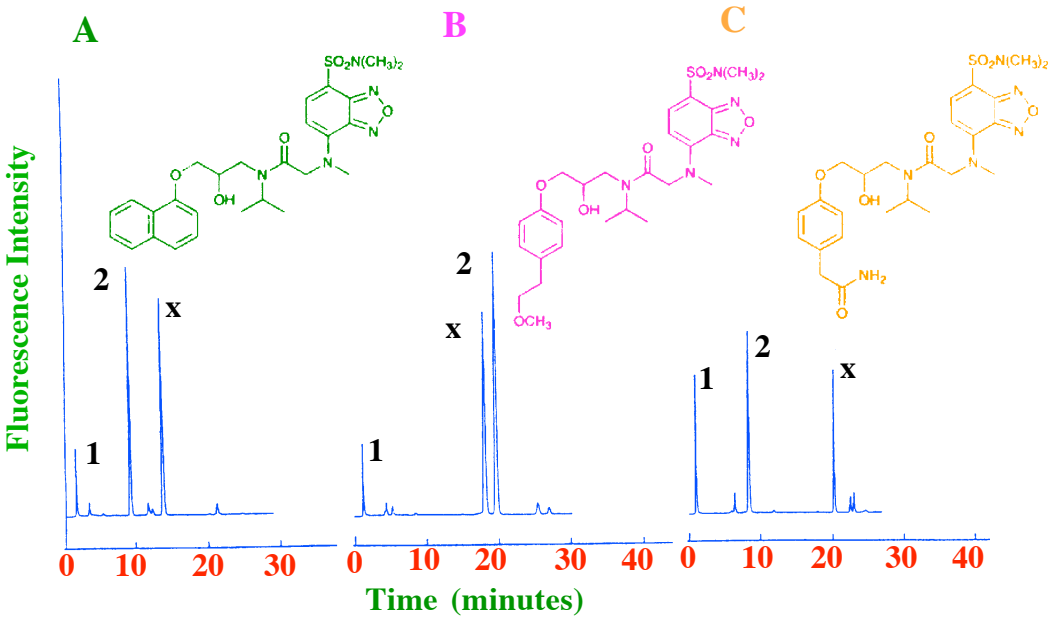
Courtesy of Supelco Inc.

Figure 47 The Separation of a Mixture of Growth Regulators on a C18 Reverse Phase C18 Column

This is an example where the phase system is complex and is required to be so, because limited column efficiency demanded clever phase selection to obtain the necessary differential retention.

Another liquid chromatography application that involves some novel derivatization is the separation of some '□ blockers', the enantiomers of propranolol, metroprolol and atenalol as fluorescent enhanced derivatives on cellulose tris(3,5–dimethylphenylcarbamate) coated on silica gel. This

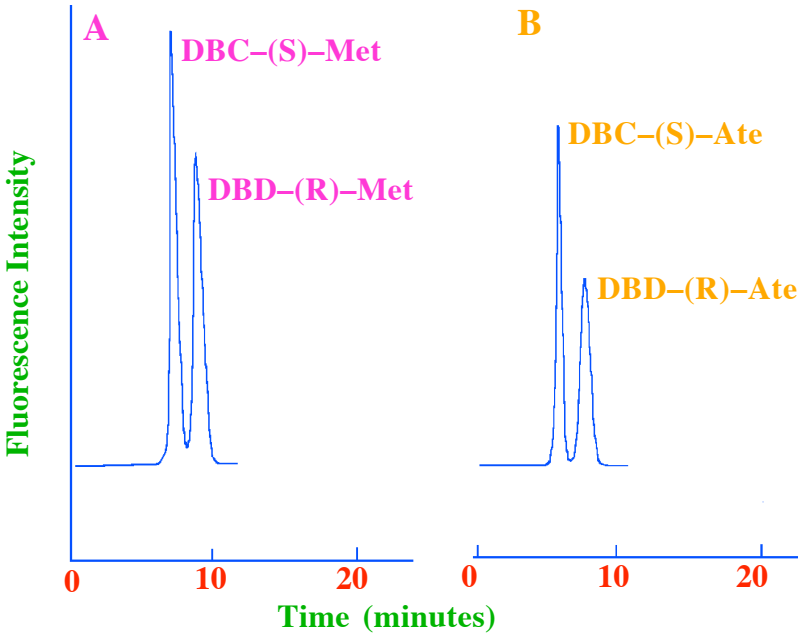
technique was developed by Yang *et al.* [14] and involved the synthesis of their fluorescent derivatives, which were formed by reacting them with an electrophilic fluorogenic reagent, 4-(*N*-chloroformethyl-*N*-methylamino-7-*N*-*N*–dimethylaminosulfonyl-2, 1, 2–benzoxydiazole (DBD-COCl). These derivatives 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, three very commonly used □ blockers, reacted directly with reagent forming 1:1 adducts by reaction with the secondary amino group of the □-blockers. The DBD-COCl reagent reacted readily with the drugs under mild conditions, with no catalyst, and the reaction was relatively fast and 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 □m particles. The solvent gradient used to develop the separation commenced with 50% aqueous solution of acetonitrile which was increased to 80% acetonitrile over a period of 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.



Courtesy of the Royal Society of Chemistry, Ref. [14]

Figure 48 The Separation of Derivatized Propranolol (Pro) Metroprolol (Met) and Atenolol (Ate)

The separations obtained are shown in figure 48. 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. CHIRACEL OD-R (a cellulose carbamate) and CHIRACEL OJ-R (a cellulose ester) were used as stationary phases to separate the individual enantiomers. 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 49 (chromatogram A, DBD–Met and chromatogram B, DBD–Ate).



Courtesy of the Royal society of Chemistry, Ref. [12]

Figure 49 The Separation of Derivatized Metoprolol and Atenolol at High Sensitivity

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 □m in diameter coated with

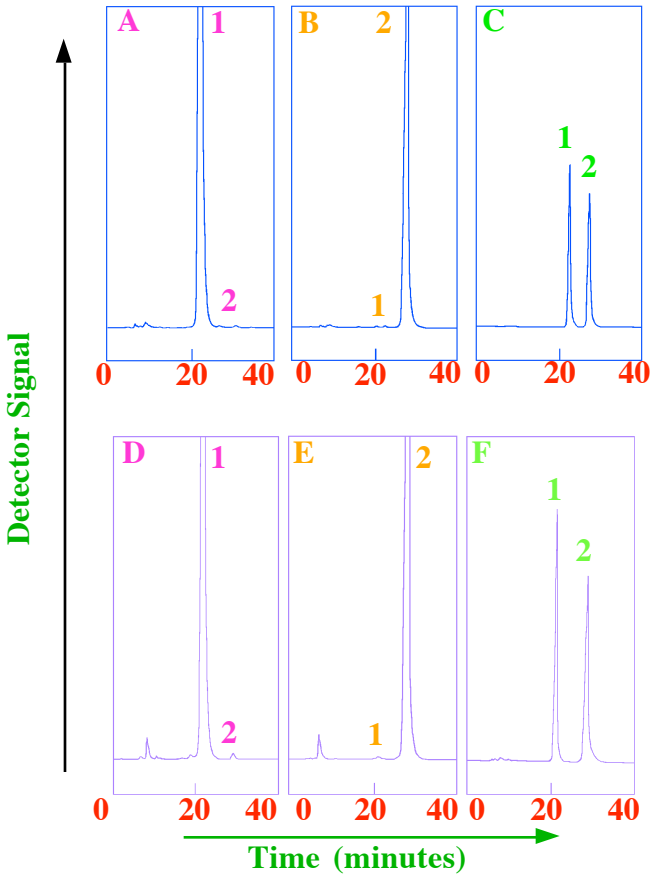
the cellulose ester. The mobile phase used for the separation of DBD–Met was methanol/acetonitrile : 90/10 v/v, at a flow rate of 0.5 ml/min., the separation ratio was 1.33. The mobile phase used for the separation of DBD–Ate was methanol, also at a flow rate of 0.5 ml/min., the separation ratio being 1.53. 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 cyclodextrin based stationary phases are some of the more popular and effective chiral stationary phases presently available. 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) as well as being very effective in a normal phase conditions. 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.

Cyclodextrin based chiral stationary phases have also been used to separate β blocking agents in conjunction with an optically active fluorescent Edman-type reagent 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-isothiocyanatepyrrol-idin-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 50.

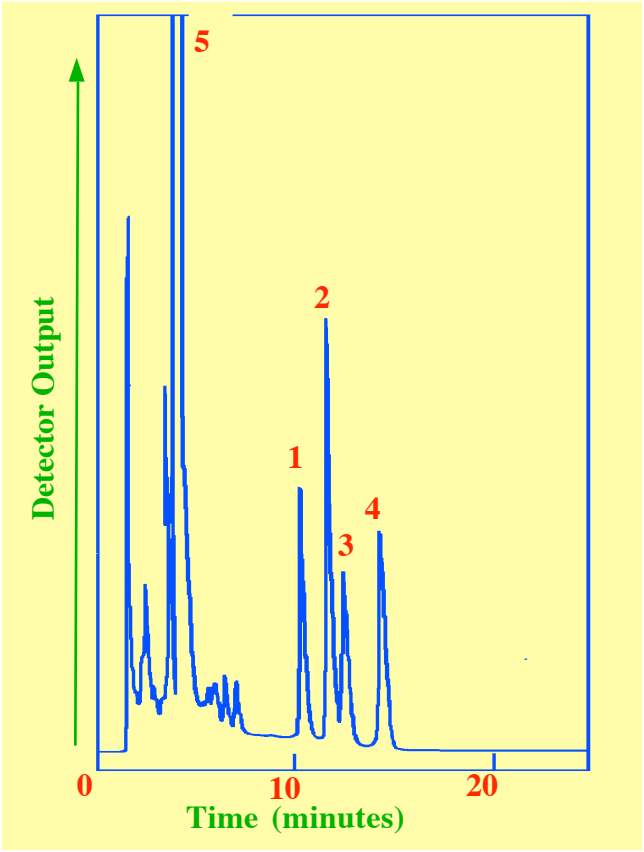


Courtesy of the Royal Society of Chemistry, [Ref. 15]

Figure 50 The Separation of Enantiomeric Pairs of NBD-PyNCS and DBD-PyNCS on Utron ES-PhCD, a Cyclodextrin Based Stationary Phase

The separation was carried out on a derivatized cyclodextrin column (ES-PhCD) 15 cm long and 6 mm I.D., packed with 5 □m 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 was 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 51.



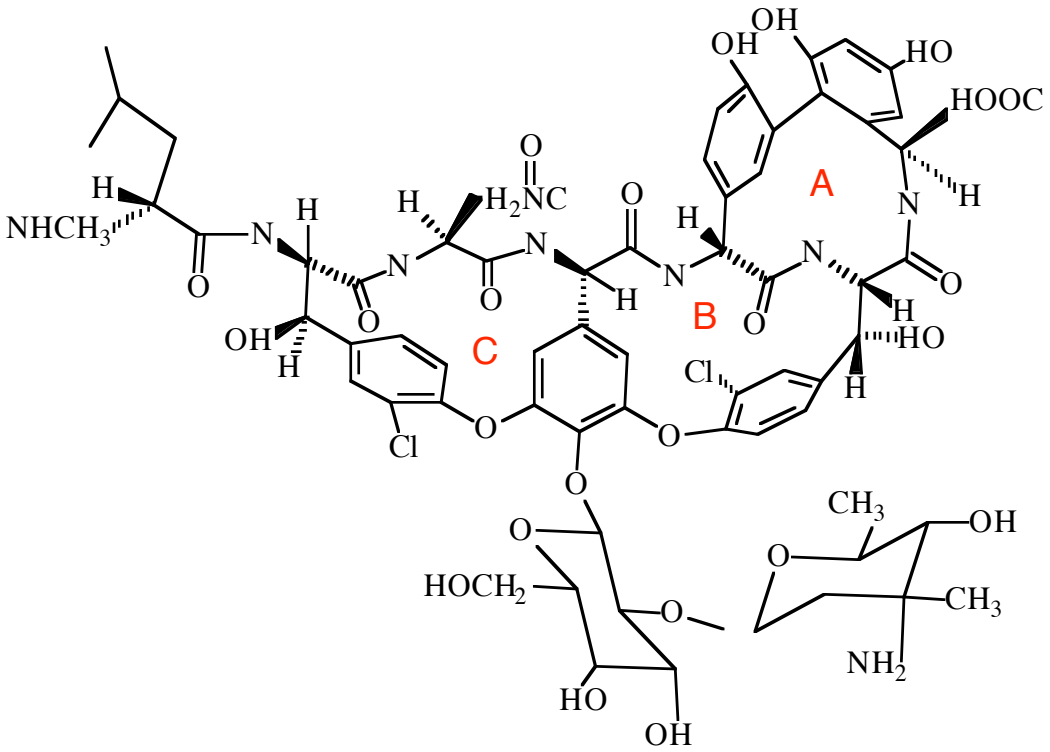
Courtesy of the Royal Society of Chemistry, [Ref. 15]

Figure 51 The Separation of the Diastereomers of Propanolol and Alprenolol on a Reversed Phase Column

The column was the Intersil ODS 80A, 15 cm long, 4.6 mm I.D., packed with 5 □m 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.

Another group of chiral stationary phase are the macrocyclic glycopeptide phases. The macrocyclic glycopeptides were first introduced by Armstrong [16]. One way of preparing the glycopeptide phases 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 52.



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

Courtesy of ASTEC Inc.

Figure 52 The Proposed Structure of Vancomycin

Vancomycin is a very stable chiral stationary phase, has a relatively high sample capacity, and when covalently bonded to the silica gel, has multiple linkages to the silica gel surface. It can either 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 the strongly polar THF–water mixtures would be very effective mobile phases. Conversely, when used as a *polar* stationary phase, *n*-hexane–ethanol mixtures would be more appropriate. Vancomycin has a number of ionizing groups and thus 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.

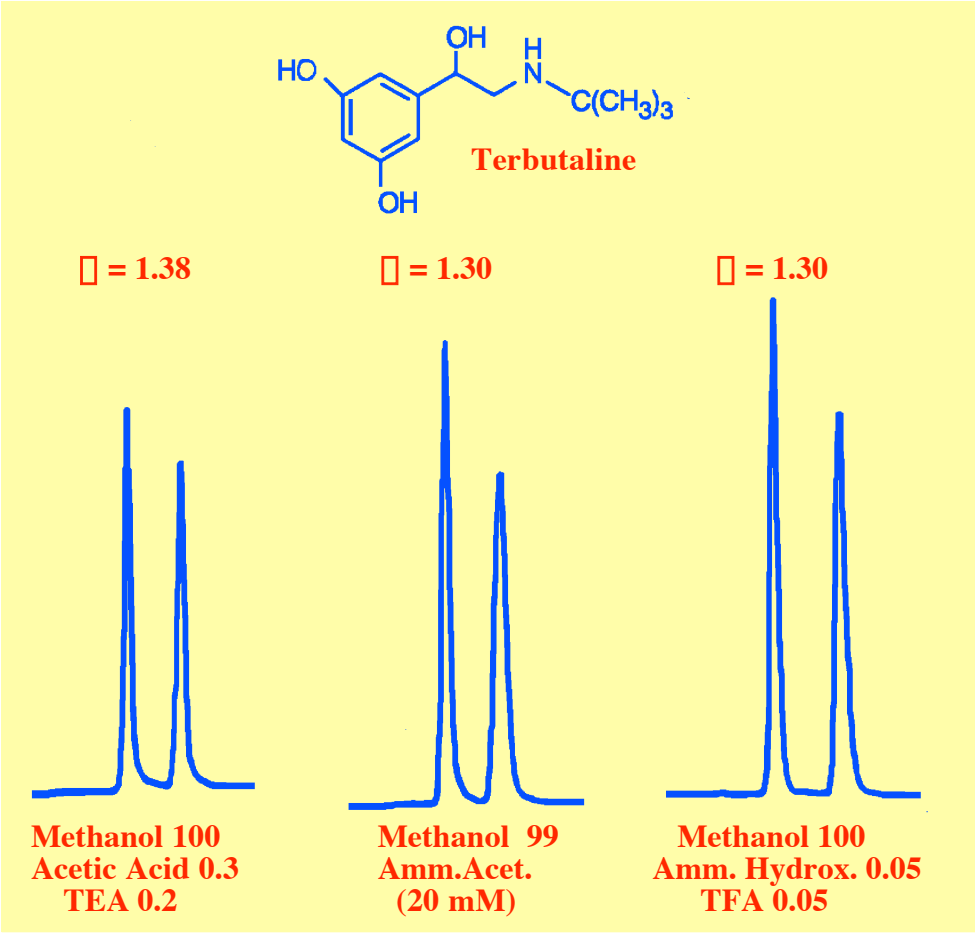
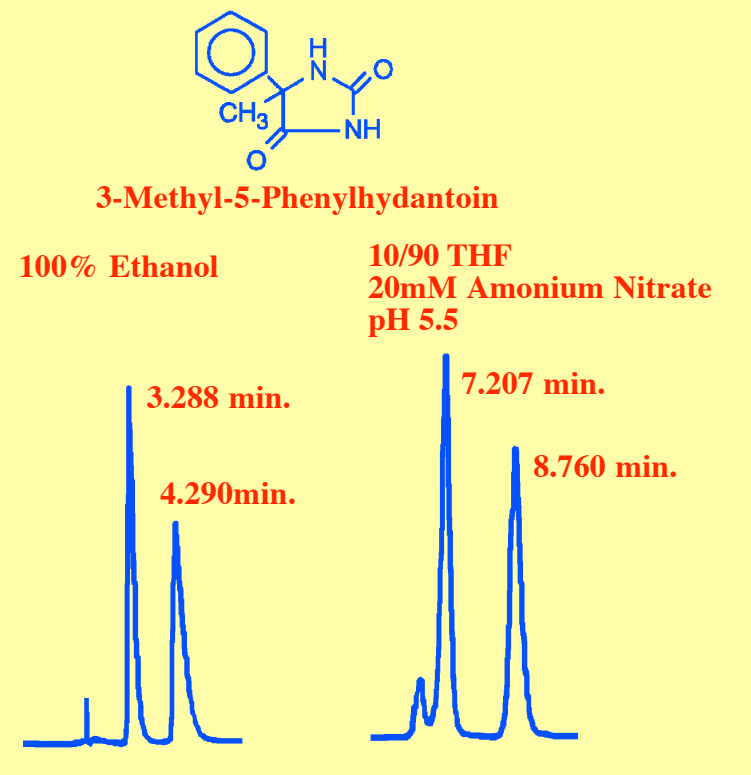


Figure 53 The Separations of the Isomers of Terbutaline Employing Different Buffer Solutions

Ammonium nitrate, triethylammonium acetate and sodium citrate buffers have all been used satisfactorily with this stationary phase. 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 53. It is seen that virtually the same selectivity is obtained from all three buffers irrespective of the actual chemical nature of the buffers themselves. However, 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.



t_o 1.85 min.	t_o 2.80 min.
k_1 0.78	k_1 1.57
k_2 1.32	k_2 2.13
□ 1.69	□ 1.35
R 2.18	R 3.0

Courtesy of ASTEC Inc.

Figure 54 The Separation of the Enantiomers of 3-Methyl-5-Phenylhydantoin Using Polar and Dispersive Interactions

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. 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 54.

The separation is carried out under two conditions, the first used pure ethanol as the mobile phase, which is relatively dispersive, and in the second, a mobile phase that contains 90% of water which is strongly polar. Pure ethanol provides extremely strong dispersive interactions in the mobile phase relative to that of the aqueous solvent which will be significantly more dispersive than any interactions involved with the stationary phase.

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 the very useful selective flexibility of Vancomycin. By adjusting the mobile phase composition, selectivity can be made to depend largely on dispersive interactions (hydrophobic) or, alternatively, to depend largely on polar interactions (hydrophilic).

Hosoya *et al*. and Kobayashi *et al*. (17), developed a method for packing uniformly sized polymer based LC packing materials that provided practically adequate column efficiency using a copoly-merization technique.

The uniformly sized, polymer-based packing was prepared from mixtures of alkyl methracrylate and glycerol dimethacrylate by copolymerization techniques using a multistep swelling and polymerization method. The resulting materials gave good efficiencies

and worked well with microbore columns. An example of their use in the separation of some polyaromatic hydrocarbons is given in figure 55. The column was 15 cm long, 4.6 mm I.D. and the mobile phase 80%(v/v) aqueous acetonitrile employed at a flow rate of 1 ml/min.

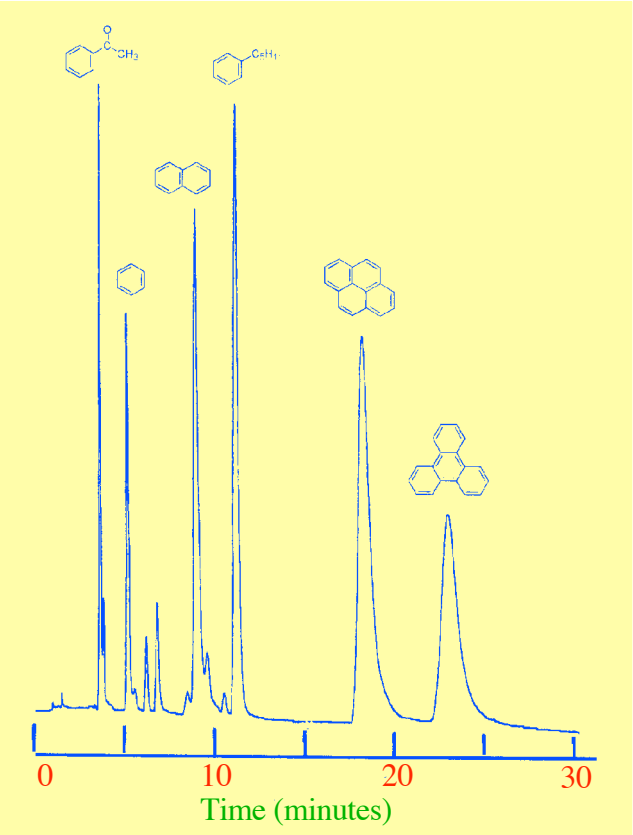
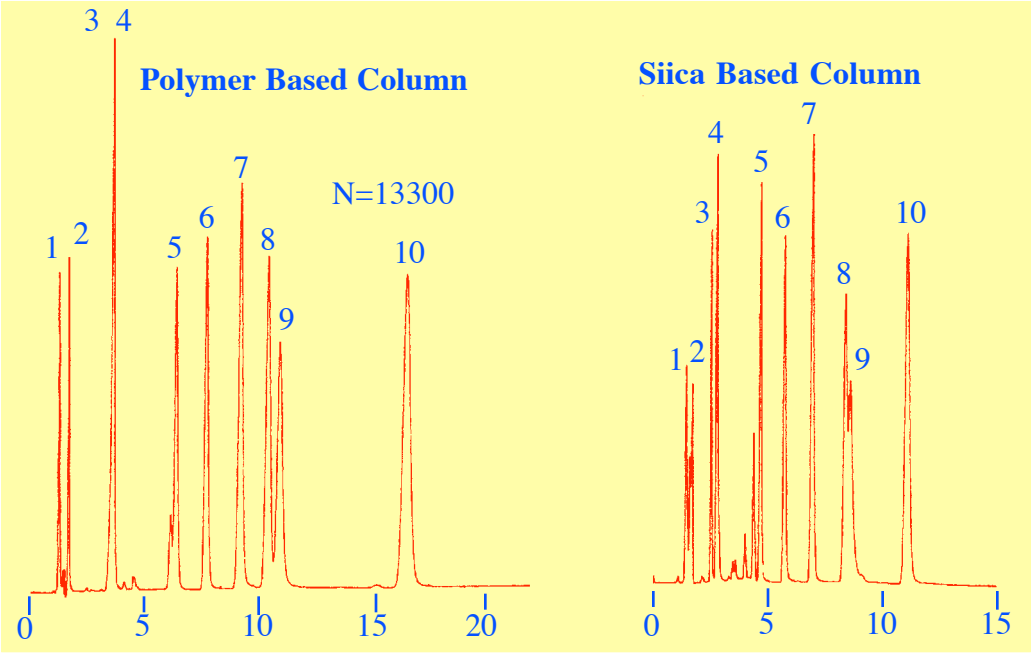


Figure 55 Chromatogram of Some Polyaromatic Hydrocarbons Separated on the Polymer Based Pickings

The temperature was 30˚C and a UV detector operating at a wavelength 254 nm was used to monitor the separation. The performance of the polymer based packings was compared with that of a commercial silica based packing using a synthetic mixture.

The chromatogram obtained for the mixture on the two columns is shown in figure 56. It is seen that although the polymer columns exhibits a large number of theoretical plates and uracil and caffeine, and toluene and phenylacetyl acetone are better separated, 2-ethyl pyridine and

phenol coelute are also reasonably well resolved on the silica based column.



1. Uracil
2. Caffeine
3. 2-ethylpyridine
4. phenol
5. butyl benzoate
6. benzene
7. N,N-diethylaniline
8. toluene
9. phenylacetyl acetone
10. naphthalene

Column length 15 cm, diameter 2 mm, mobile phase 50% (v/v) aqueous acetonitrile, flow rate 0.19 ml/min., temperature 40°C, detector UV at 254 nm.

Figure 56 The Relative Performance of a Column Packed with Polymer Based Material and that from a Column Packed with Silica Based Material

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