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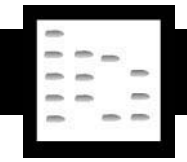
CHROMATOGRAPHY



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Introduction

Chromatography is the most widely used separation technique in chemical laboratories, where it is used in analysis, isolation and purification, and it is commonly used in the chemical process industry as a component of small and large-scale production. In terms of scale, at one extreme minute quantities of less than a nanogram are separated and identified during analysis, while at the other, hundreds of kilograms of material per hour are processed into refined products. It is the versatility of chromatography in its many variants that is behind its ubiquitous status in separation science, coupled with simplicity of approach and a reasonably well-developed framework in which the different chromatographic techniques operate.

Chromatography is essentially a physical method of separation in which the components of a mixture are separated by their distribution between two phases; one of these phases in the form of a porous bed, bulk liquid, layer or film is generally immobile (stationary phase), while the other is a fluid (mobile phase) that percolates through or over the stationary phase. A separation results from repeated sorption/desorption events during the movement of the sample components along the stationary phase in the general direction of mobile-phase migration. Useful separations require an adequate difference in the strength of the physical interactions for the sample components in the two phases, combined with a favourable contribution from system transport properties that control sample movement within and between phases. Several key factors are responsible, therefore, or act together, to produce an acceptable separation. Individual compounds are distinguished by their ability to participate in common intermolecular interactions in the two phases, which can generally be characterized by an equilibrium constant, and is thus a property predicted from chemical thermodynamics. Interactions are mainly physical in type or involve weak chemical bonds, for example dipole–dipole, hydrogen bond formation, charge transfer, etc., and reversible, since useful separations only result if the compound spends some time in both phases. During transport through or over the stationary phase, differential transport phenomena, such as diffusion and flow anisotropy (complex phenomena discussed later), result in dispersion of solute molecules around an average value, such that they occupy a finite distance along the stationary phase in the direction of migration. The extent of dispersion restricts the capacity of the chromatographic system to separate and, independent of favourable thermodynamic contributions to the separation, there is a finite number of dispersed zones that can be accommodated in the separation. Consequently, the optimization of a chromatographic separation depends on achieving favourable kinetic features if success is to be obtained.

The Family of Chromatographic Techniques

A convenient classification of the chromatographic techniques can be made in terms of the phases employed for the separation (**Figure 1**), with a further subdivision possible by the distribution process employed. In addition, for practical utility transport processes in at least one phase must be reasonably fast; for example, solid–solid chromatography, which may occur over geological time spans, is impractical in the laboratory because of the slow migration of

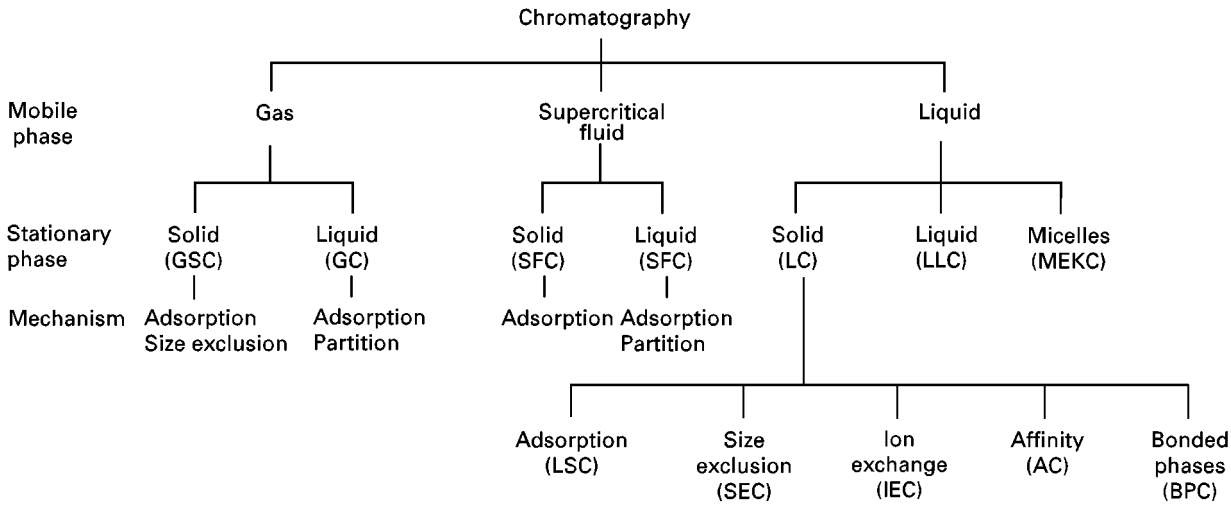

Figure 1 Family tree of chromatographic methods.

solutes through the crystal lattice. Two distinct phases are required to set up the distribution component of the separation mechanism, which explains why gas–gas chromatography does not exist and liquid–liquid separations are restricted to immiscible solvents. When the mobile phase is a gas the stationary phase can be a liquid or a solid and the separation techniques are called gas–liquid chromatography (GLC) and gas–solid chromatography (GSC). The simple term GC encompasses both techniques but, unless otherwise specified, it usually means GLC since this is the most common arrangement. Separations in GLC occur because of differences in gas–liquid partitioning and interfacial adsorption. In GSC the retention mechanism is governed by interfacial adsorption or size exclusion, if a solid of controlled pore size, such as a zeolite, is used as the stationary phase. When the mobile phase is a supercritical fluid (SFC) the stationary phase can be a liquid or a solid, and the distribution process may be interfacial adsorption or absorption.

When the mobile phase is a liquid the stationary phase can be a solid (liquid–solid chromatography, LSC) with interfacial adsorption as the dominant distribution process; a solid of controlled pore size (size exclusion chromatography, SEC), in which the distribution constant is characteristic of the ratio of the solute size to the dimensions of the stationary phase pore sizes; a solid with immobilized ionic groups accessible to solutes in the mobile phase with electrostatic interactions as the dominant distribution process (ion exchange chromatography or ion chromatography, IEC or IC); a solid with immobilized molecular recognition sites accessible to the analyte in the mobile phase (affinity chromatography, AC) in which the dominant distribution process is the three-dimensional specificity of the molecular interactions between the receptor and the analyte (a technique used in biotechnology); a porous solid coated with a film of immiscible liquid (liquid–liquid chromatography, LLC) in which the dominant distribution process is partitioning; or a solid with a surface containing organic groups attached to it by chemical bonds (bonded-phase chromatography, BPC) in which the dominant distribution processes are interfacial adsorption and partitioning.

Bonded phases in liquid chromatography are widely used to tailor solid phases for different applications, including LSC, SEC, IEC, IC and AC (**Figure 2**). Reversed-phase chromatography (RPC) is a particular form of bonded-phase chromatography in which the mobile phase is more polar than the stationary phase (for most practical applications the mobile phase is an aqueous solution). It is the most popular form of liquid chromatography because of its broad applicability to neutral compounds of wide polarity. In addition, by exploiting secondary chemical equilibria weak acids and bases can be separated by pH control (ion suppression chromatography, ISC); ionic compounds by using ion pairing with an additive of opposite charge (ion pair chromatography, IPC); and metal ions by the formation of neutral complexes (metal-complexation chromatography, MCC). By adding a surfactant to the mobile phase, micelles can be used to modify the overall distribution constant (micellar liquid chromatography, MLC), and a totally aqueous buffered mobile phase and a decreasing ionic strength gradient can be used to separate biopolymers with minimal disruption of conformational structure (hydrophobic interaction chromatography, HIC). Bonded-phase chemistry is also commonly employed to prepare stationary

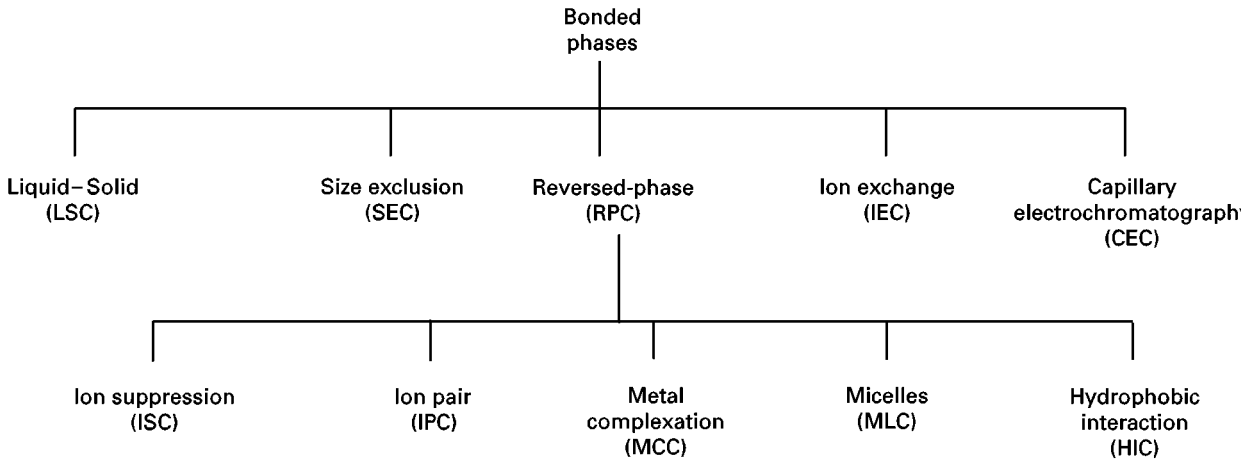

Figure 2 Applications of bonded phases in LC.

phases with immobilized enantiomer-selective groups for the resolution of racemates by chiral chromatography.

The mobile phase can be transported through or over the stationary phase by application of external pressure when the stationary phase is enclosed in a rigid container, or column. This is the ordinary mode of gas, supercritical fluid and liquid chromatography. If the stationary phase is distributed as a thin layer on a (usually) flat support, such as a sheet of glass or plastic, and the mobile phase is allowed to ascend through the layer by capillary forces, then this method is referred to as planar or thin-layer chromatography (TLC). The fundamental basis of the distribution mechanism between the mobile phase and the stationary phase is identical to that described for column liquid chromatography, only the separation format and transport mechanism for the mobile phase are different. TLC has largely superseded paper chromatography (PC) in contemporary practice. PC is mechanistically identical to TLC but, with a few exceptions, provides poorer separation characteristics. Bulk flow of liquid mobile phases containing an electrolyte can also be transported through a column by an electric field, through the process known as electroosmosis. When a column packed with a stationary phase is used this is called electrochromatography, or since columns of capillary dimensions are essential for this technique, capillary electrochromatography (CEC). The distribution process for neutral solutes is independent of the transport process, and separations occur by the mechanisms indicated for liquid chromatography. Ionic surfactants can form micelles as a continuous phase dispersed throughout a buffer. In an electric field these charged micelles move with a different velocity or direction to the flow of bulk electrolyte. Neutral solutes can be separated, if their distribution constant between the micelles and buffer are different, by micellar electrokinetic chromatography (MEKC). The stationary phase in this case is referred to as a pseudo-stationary phase, since it is not stationary, but moves with a different velocity to the mobile phase. Ionic solutes in CEC and MEKC are influenced by the presence of the electric field and are separated by a combination of chromatography and electrophoresis.

Mode of Zone Displacement

In nearly all chromatographic systems, transport of solute zones occurs entirely in the mobile phase. Transport is an essential component of the chromatographic system since the most common arrangement for the experiment employs a sample inlet and a detector at opposite ends of the column, with sample introduction and detection occurring in the mobile phase (GC, SFC, LC, MEKC). In planar chromatographic systems (TLC, PC), sample introduction and detection is performed in the stationary phase, but the detection is of solute zones that have been transported different distances by the mobile phase. In GC the movement of solute molecules from the stationary to the mobile phase is controlled by the vapour pressure of the solutes in the column, and is usually manipulated by varying temperature. At an optimum temperature sample molecules will spend some of their time in the mobile phase, where they will be transported through the column, and some time in the stationary phase, where they are differentiated by their capacity for intermolecular interactions with the stationary phase. Displacement of solute zones can be achieved in three distinct ways: frontal analysis, elution and displacement (**Figure 3**).

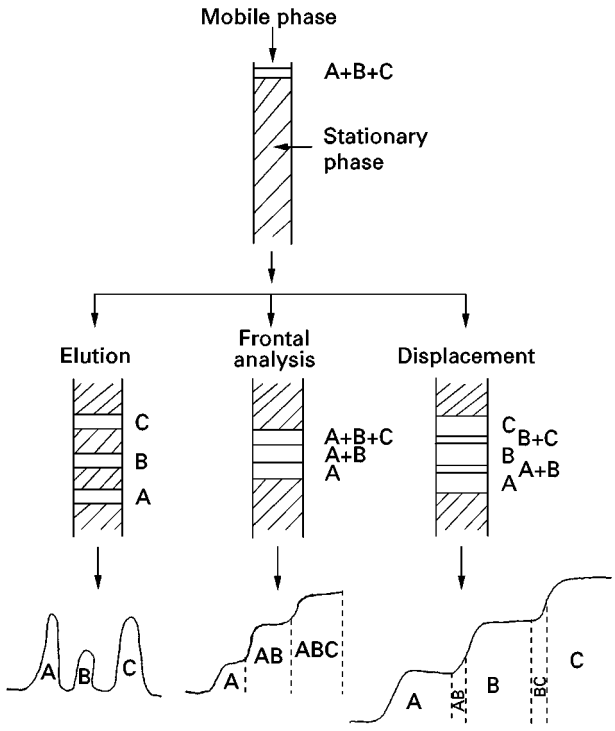

Figure 3 Mode of zone displacement in chromatography.

In frontal analysis, the mobile phase introduces the sample continuously onto the column (or the sample is the mobile phase) until eventually the column is saturated with the sample and the component with the lowest affinity for the stationary phase is displaced from the column by sample components of greater affinity. When the zone of pure component has completely exited the column it is followed by a mixture containing the next component, and so on. Frontal analysis can be used to obtain thermodynamic data from chromatographic measurements and to isolate a less strongly retained trace component from a major component. However, quantitation for each component in a mixture is difficult, and at the end of the experiment the column is contaminated by the sample so that reuse requires stripping the sample from the column.

In displacement chromatography the sample is applied to the column as a discrete band and a substance (or mobile-phase component) with a higher affinity for the stationary phase than any of the sample components is continuously passed through the column. The displacer pushes sample components down the column and, if the column is long enough, a steady state is reached. A succession of rectangular bands of pure components then exits the column. Each component displaces the component ahead of it, with the last and most strongly retained component being forced along by the displacer. At the end of the separation the displacer must be stripped from the column if the column is to be reused. Displacement chromatography is used mainly in preparative and process chromatography, where high throughputs of pure compounds can be obtained (note that the contact boundary between zones may not be discrete and the collection of pure compounds may be restricted to the central region of the displaced zones).

In elution chromatography the sample is applied to the column as a discrete band and sample components are successively eluted from the column diluted by mobile phase. The stationary and mobile phases are normally at equilibrium prior to sample introduction. The mobile phase must compete with the stationary phase for the sample components; separation will only occur if the distribution constants for the various components, resulting from the competition, are different. Elution chromatography is the most convenient method for analysis and is the most common method of separation in GC, SFC, LC and MEKC. Development, a modification of the elution mode, is used in planar chromatography. Samples are applied to the dry layer as compact spots or bands and the layer subsequently contacted by the mobile phase, which ascends and moves the sample components to positions higher up the layer in the direction of mobile-phase flow. The separation is (usually) stopped before the mobile phase reaches the opposite edge of the layer and neither the eluent nor the sample components exit the layer. The two processes can be compared; all components travel the same distance and are separated in time using the elution mode in column chromatography, whereas all components have the same separation time and are separated in space (migration position) in planar chromatography using the development mode.

Chromatogram

The information obtained from a chromatographic experiment is contained in the chromatogram. When the elution mode is used this consists of a plot of the concentration or mass profile of the sample components as a function of the flow of the mobile phase or as a function of time. Typically the y-axis will be detector response and the x-axis time or volume of mobile phase in column chromatography or migration distance in planar chromatography. The position of each peak in the chromatogram is characteristic of the identity of the compound and the area under the peak is a function of the concentration or amount of each compound. Peak widths in the chromatogram are controlled by solute-dependent kinetic factors, which in turn can be used to deduce values for

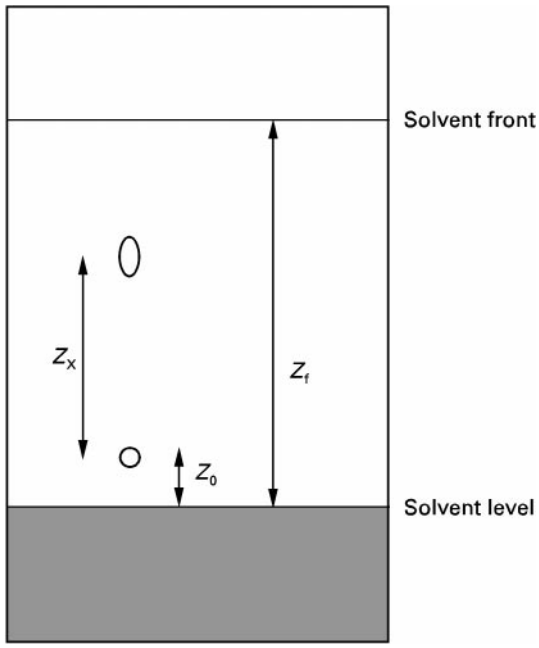

Figure 4 Calculation of the R_F value in planar chromatography. Z_X distance moved by the sample from the sample origin; Z_0, distance between the solvent entry position and the sample origin; Z_f, distance between the solvent entry position and the solvent front.

characteristic physical properties of either the solute or the mobile and stationary phases.

The position of a peak in the chromatogram is made up of two contributions: (1) the time (or volume of mobile phase) required by a compound that does not interact with the stationary phase to reach the detector from the sample inlet, called the column hold-up time or dead time; and (2) the time that individual compounds spend in the stationary phase (all compounds spend the same time in the mobile phase). The column hold-up time is a feature of the experimental system and is not fundamentally related to solute properties. Because of this, retention time is not a useful parameter for column comparisons. A more useful term is the retention factor (previously known as the capacity factor), k, defined as the ratio of the time the solute spends in the stationary phase to the time it spends in the mobile phase. The ratio of the retention factors for two solutes is called the separation factor, α, which by convention is always expressed with the larger retention factor in the numerator ($\alpha \geq 1$). The separation factor expresses the ease with which the chromatographic system can separate two compounds, and is directly related to the difference in free energy for the interactions of the two compounds in the chromatographic system. It is a major optimization parameter, as we shall see later. In planar chromatography retention is usually expressed as the retardation factor, R_F, equivalent to the ratio of the distance migrated by the solute zone, Z_X, to the distance moved by the solvent front, $Z_f - Z_0$, measured from the sample application position, $(1 \geq R_F \geq 0)$, as illustrated in **Figure 4**. The planar chromatographic retardation factor and the column retention factor are simply related by $k = (1 - R_F)/R_F$.

Peak Shape Models

For an ideal separation the peaks in the chromatogram are usually considered to be Gaussian. This is a convenient, if not always accurate, model and peak asymmetry can arise from a variety of instrumental and chromatographic sources. The most common types of peak distortion are skewness (the peak front is sharper than the rear) and tailing (the rear of the peak is elongated compared to the front). Although instrumental sources of peak asymmetry should, of course, be minimized, chromatographic sources cannot always be avoided. Curve fitting by computer offers the possibility of deconvoluting chromatographic peak profiles into their individual contributions. The exponentially modified Gaussian function, obtained by the combination of a Gaussian function with an exponential decay function (that provides for the asymmetry in the peak profile), is often an acceptable description of chromatographic peaks in analytical applications.

Chromatographic sources of peak asymmetry result from mechanical effects, for example the formation of voids in the stationary-phase bed and excessive extra-column volumes, and from isotherm characteristics. Most of the theory of analytical chromatographic separations is based on a linear isotherm model where the compositions in the stationary and mobile phases are proportional and characterized by a distribution constant that is independent of sample size and composition (**Figure 5**). The peaks resulting from a linear chromatography model are symmetrical and can be characterized by a normal distribution. The width of the chromatographic zone is proportional to retention and can be obtained directly from peak shape considerations. The extent to which the properties of the chromatographic system contribute to zone broadening (peak widths) is given by the number of theoretical plates, N. For a normal distribution this is equivalent to $(t_R/\sigma_t)^2$, where t_R is the retention time and σ_t is the peak standard deviation in time units. Simple algebraic manipulation of this formula permits calculation of N from the peak width at base or half-height, etc. For column comparison purposes the height equivalent to a theoretical plate, H, equivalent to the column length divided by N, is generally used.

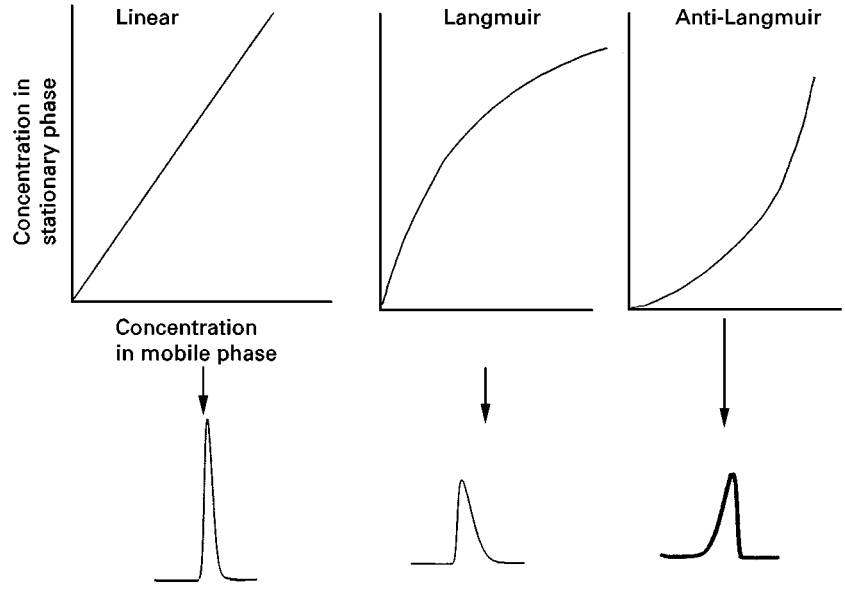

Figure 5 Influence of isotherm type on peak shapes.

Nonlinear isotherms (nonlinear chromatography) result in the production of asymmetric peaks. Langmuir isotherms are frequently observed for adsorption interactions on surfaces with an energetically heterogeneous distribution of adsorption sites with incompatible association/dissociation rate constants. For sorbents with monolayer coverage, Langmuir-type isotherms result when solute–stationary phase interactions are strong compared with solute–solute interactions. Because the interactions between solutes are comparatively weak, the extent of sorption decreases following monolayer formation, even though the concentration in the mobile phase is increasing. In this case the concentration of the component in the stationary phase at equilibrium is no longer proportional to its concentration in the mobile phase and the peak shape and retention time will depend on the sample composition and amount. Anti-Langmuir type isotherms are more common in partition systems when solute–stationary phase interactions are relatively weak compared with solute–solute interactions, or where column overload results from the introduction of large sample amounts. Such conditions are common in preparative chromatography, where economic considerations dictate that separations are optimized for production rate and to minimize mobile phase consumption and operating costs.

Flow through Porous Media

For an understanding of zone dispersion in chromatography, an appreciation of the mobile-phase linear velocity through different porous media is important. Gases are highly compressible and an average linear velocity for the column is used. Liquids can be considered incompressible and the average and outlet velocity should be about the same. Supercritical fluids are often assumed to be incompressible for the purpose of calculation, more for convenience than reality, with local velocity changes reflecting changes in density along the column. For packed columns containing porous particles with fluid mobile phases, the flow of mobile phase occurs predominantly through the interstitial spaces between the packing particles and the mobile phase occupying the particle pore volume is largely stagnant. Slow solute diffusion through this stagnant volume of mobile phase is a significant cause of zone broadening for condensed phases. The mobile-phase velocity for a chromatographic system may be determined by dividing the column length by the retention time of an unretained and unexcluded solute from the pore volume (average velocity) or the retention time of an unretained and excluded solute (interstitial velocity).

The mobile-phase flow profile and changes in local velocity are products of the driving force used to induce bulk flow of mobile phase through the separation system. These driving forces can be identified as capillary, pneumatic or electroosmotic forces. Capillary forces are responsible for the transport of the mobile phase in planar chromatography (PC and TLC). These forces are generally weak and result in a mobile-phase velocity that decreases with migration distance from the solvent starting position (**Figure 6**). Capillary forces are incapable of providing a sufficiently high velocity

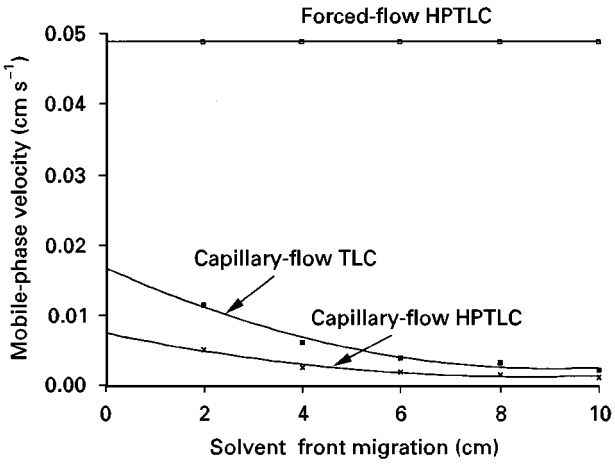

Figure 6 Relationship between mobile-phase velocity and migration distance for capillary-controlled and forced-flow development in planar chromatography. (Reproduced with permission from Poole CF and Wilson ID (1997) *Journal of Planar Chromatography* 10: 332, copyright © Research Institute for Medicinal Plants).

to minimize zone broadening. This has a number of consequences: zone brodening is largely dominated by diffusion; the useful development length for PC is set by the range of acceptable mobile-phase velocities; separation times are increased; and the separation potential of PC is less than that predicted for a constant and optimum mobile-phase velocity.

Pneumatic transport of the mobile phase is commonly employed in column chromatography. The mobile phase is pressurized externally to the column (a simple high pressure cylinder with regulator in the case of a gas, or a mechanical pump for liquids). The pressure gradient across the column provides the driving force to overcome the resistance to flow presented by the stationary phase and the rest of the system. In LC, Darcy's law relates the properties of the mobile phase, characteristic features of the column, and the external pressure required to obtain a useful mobile-phase velocity. This law can be stated as:

$$u = \Delta P K_0 d_P^2 / \eta L \qquad [1]$$

where u is the mobile phase velocity, ΔP is the pressure drop across the column, K_0 is the column permeability, d_P is the average particle diameter, η is the mobile phase viscosity, and L is the column length. Since a minimum value for u is required for acceptable column performance and separation times, and the available column pressure drop is constrained to an upper limit by material and safety considerations, then there is a finite limit to the range of permissible d_P^2/L values that can be used. Thus a compromise must be accepted between separation time and efficiency, which results in an upper limit to the number of theoretical plates that can be obtained for fast separations or the use of long separation times when very large numbers of theoretical plates are required for a separation.

Bulk liquid flow under electrophoretic conditions occurs by electroosmosis. At the column wall or particle surface (packed columns) an electrical double layer results from the adsorption of ions from the mobile phase or dissociation of surface functional groups. An excess of counterions is present in the double layer in comparison with the bulk liquid and in the presence of an electric field shearing of the solution occurs only within the very thin diffuse part of the double layer, transporting the mobile phase through the column with a nearly perfect plug profile (**Figure 7**). The velocity of the bulk liquid flow

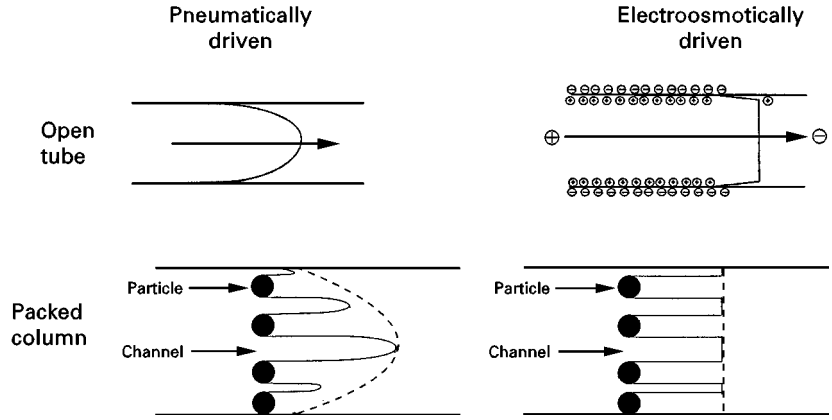

Figure 7 Flow profile for an open tube and a packed column using pneumatic and electroosmotic driving forces.

is given by:

$$u = \varepsilon\xi E/4\pi\eta \qquad [2]$$

where ε is the solution dielectric constant, ξ is the zeta potential (potential at the boundary between the charged surface and the start of the diffuse part of the double layer), and E is the electric field strength. Note that there is no explicit dependence on the particle size and column length, which limit the total efficiency of columns when the flow is pneumatically driven. The column length and column internal diameter, however, cannot be treated as independent variables in MEKC and CEC, but are related through Joule heating of the electrolyte and its effect on the mobile-phase flow profile. Heat is generated homogeneously throughout the electrolyte but the temperature variation across the column diameter is parabolic. Radial temperature gradients between the centre of the tube and the column wall cause zone broadening resulting from sample diffusion and solvent density and viscosity differences in the direction of flow.

Zone Broadening

Rate theory attempts to explain the kinetic contribution to zone broadening in column chromatography as the sum of three main contributions: flow anisotropy (eddy diffusion), axial diffusion (longitudinal diffusion), and resistance to mass transfer. Flow anisotropy is illustrated in **Figure 8**. When a sample band migrates through a packed bed, the individual flow paths must diverge to navigate around the particles such that individual flow streams are of unequal lengths. These variations in flow direction and rate lead to zone broadening that should depend only on the particle size and homogeneity of the column packing. Flow anisotropy can be minimized by using particles of small diameter with a narrow particle size distribution in columns with a high and homogeneous packing density. For open-tubular columns, flow anisotropy is not a contributing factor since the streamlines have no obstacles in their way to cause disruption of the sample profile.

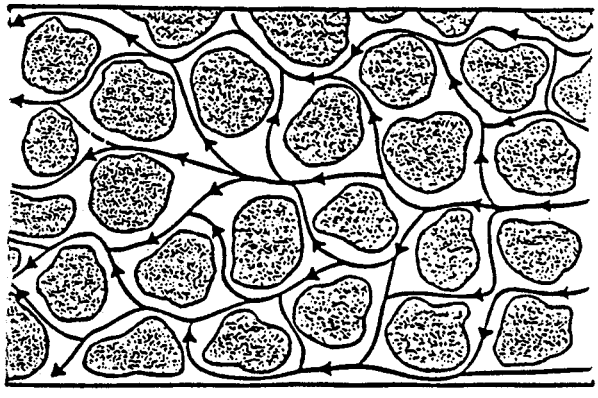

Figure 8 Representation of flow anisotropy in a packed column.

Axial diffusion is the natural tendency of solute molecules in the mobile phase to redistribute themselves by diffusion from a region of high concentration to one of lower concentration. Its contribution to zone broadening depends on the solute diffusion coefficient in the mobile phase and the column residence time. Diffusion of solute molecules occurs in all directions but only the components in the plane of mobile-phase migration contributes to the peak profile observed in the chromatogram.

Resistance to mass transfer in either the stationary or mobile phases is a consequence of the fact that mass transfer in the chromatographic system is not instantaneous and equilibrium may not be achieved under normal separation conditions. Consequently, the solute concentration profile in the stationary phase is always slightly behind the equilibrium position and the mobile-phase profile is similarly slightly in advance of the equilibrium position (**Figure 9**). The

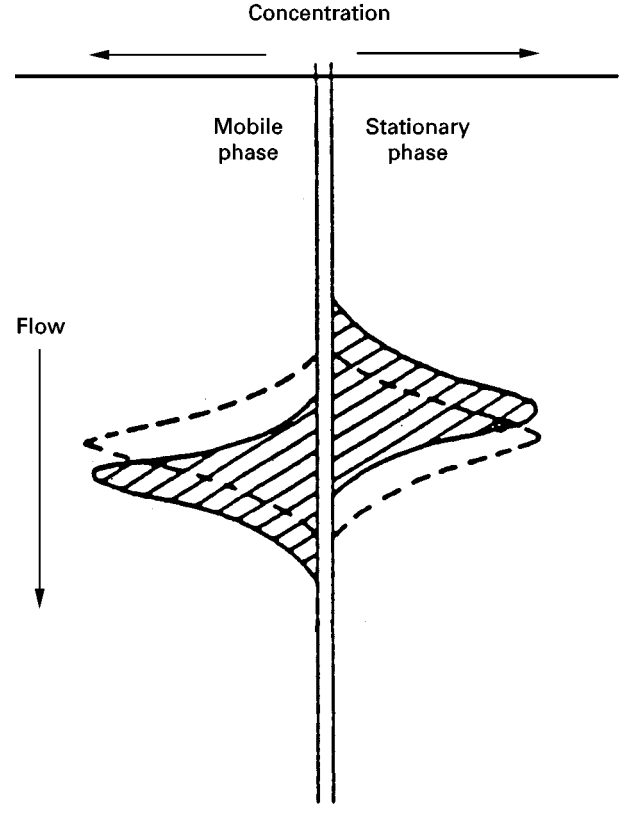

Figure 9 Representation of resistance to mass transfer in the mobile and stationary phases. The dashed line represents the equilibrium position and the solid line the actual position of the solute zones.

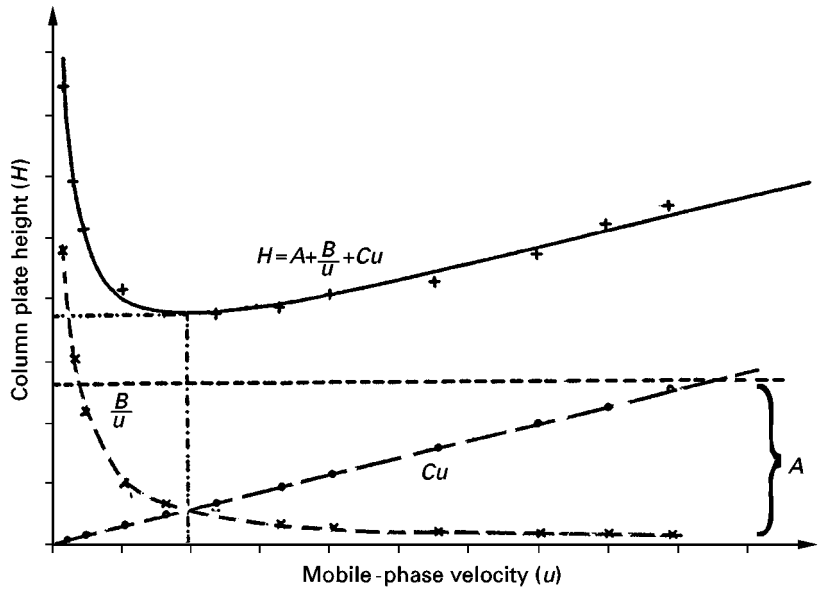

Figure 10 van Deemter plot of the column plate height as a function of the mobile-phase velocity. The solid line represents the experimental results and the broken lines the theoretical contribution from flow anisotropy (*A*), axial diffusion (*B/u*) and resistance to mass transfer (*Cu*).

resultant peak observed at the column exit is broadened about its zone centre, which is located where it would have been for instantaneous equilibrium, provided that the degree of nonequilibrium is small. Contributions from resistance to mass transfer are rather complicated but depend on the column residence time, mobile-phase velocity, stationary-phase film thickness, the particle size for packed columns, the solute diffusion coefficients in the mobile and stationary phases, and the column internal diameter.

The relationship between zone broadening (column plate height) and the mobile-phase velocity is given by the hyperbolic plot known as a van Deemter curve (**Figure 10**). The solid line represents the experimentally observed results and the dotted lines the contributions from flow anisotropy (*A* term), axial diffusion (B/u) and resistance to mass transfer (Cu). In this generic plot we see that there is an optimum velocity at which a particular chromatographic system provides maximum efficiency (a minimum column plate height). The position of this optimum velocity and the general curvature of the plot strongly depend on the characteristics of the chromatographic system, as shown by the values given in **Table 1**.

Gas Chromatography

Gases of low viscosity with favourable solute diffusivity, such as hydrogen and helium, are commonly used as mobile phases in GC. For these gases the minimum in the plate height occurs at a high optimum mobile-phase velocity, resulting in efficient and fast separations. At these high mobile-phase velocities the contribution from axial diffusion to the column plate height is minimized. For thin-film columns, resistance to mass transfer in the mobile phase is the main cause of zone broadening, while for thick-film columns resistance to mass transfer in the stationary phase is equally important. Since diffusion in gases is relatively favourable, the column internal diameters required to maintain an acceptable contribution from resistance to mass transfer in the mobile phase offer little difficulty in practice. For supercritical fluids, solute diffusivity is not as favourable as for gases and in the case of liquids must be considered unfavourable. The unfavourable slow optimum mobile-phase velocity in SFC (in practice open-tubular columns are operated at 10 or more times the optimum velocity to obtain an acceptable separation time) requires significantly smaller internal diameter capillary columns than those needed for GC to minimize resistance to mass transfer in the mobile phase. At mobile-phase velocities used in practice the contribution of axial diffusion to the column plate height is negligible compared with the contribution of resistance to mass transfer in the mobile and stationary phases. For fast, high efficiency separations, column internal diameters < 100 μm are required and much smaller diameters are preferred. As densities and solute diffusivity become more liquid-like, column dimensions for reasonable

Table 1 Characteristic values for column parameters related to zone broadening

Parameter	*Mobile phase*		
	Gas	*Supercritical fluid*	*Liquid*
Diffusion coefficient ($m^2\ s^{-1}$)	10^{-1}	10^{-4}–10^{-3}	10^{-5}
Density ($g\ cm^{-3}$)	10^{-3}	0.3–0.8	1
Viscosity (P)	10^{-4}	10^{-4}–10^{-3}	10^{-2}
Column length (m)			
Packed	1–5	0.1–1	0.05–1
Open-tubular	10–100	5–25	
Column internal diameter (mm)			
Packed	2–4	0.3–5	0.3–5
Open-tubular	0.1–0.7	0.02–0.1	< 0.01
Average particle diameter (μm)	100–200	3–20	3–10
Column inlet pressure (atm)	< 10	< 600	< 400
Optimum velocity ($cm\ s^{-1}$)			
Packed	5–15	0.4–0.8	0.1–0.3
Open-tubular	10–100	0.1–0.5	
Minimum plate height (mm)			
Packed	0.5–2	0.1–0.6	0.06–0.30
Open-tubular	0.03–0.8	0.01–0.05	> 0.02
Typical system efficiency (N)			
Packed	10^3–10^4	10^4–8×10^4	5×10^3–5×10^4
Open-tubular	10^4–10^6	10^4–10^5	
Phase ratio			
Packed	4–200		
Open-tubular	15–500		

performance start to approach values similar to those for LC and are not easily attained experimentally. Slow diffusion in liquids means that axial diffusion is generally insignificant but mass transfer in the mobile phase is also reduced, requiring columns of very small internal diameter, preferably < 10 μm, which are impractical for general laboratory use. Packed columns dominate the practice of LC while open-tubular columns are equally dominant in the practice of GC, with both column types used in SFC.

Packed columns in GC are prepared from comparatively coarse particles of a narrow size distribution and coated with a thin homogeneous film of liquid for high performance. The relatively large particle size and short column lengths are dictated by the limited pressure drop employed for column operation. For thin-film columns, resistance to mass transfer in the mobile and stationary phases is the main cause of zone broadening with a contribution from flow anisotropy. For thick-film columns, resistance to mass transfer in the stationary phase tends to dominate. The intrinsic efficiencies of open-tubular columns and packed columns of similar phase ratio are comparable, but because the two column types differ greatly in their relative permeability at a fixed column pressure drop, much longer open-tubular columns can be used. Thus, packed GC columns are seldom more than 5 m long while columns with lengths from 10 to 100 m are commonly used in open-tubular column GC, resulting in a 100-fold increase in the total number of theoretical plates available. In general, packed columns are used in GC for those applications that are not easily performed by open-tubular columns, for example separations that require a large amount of stationary phase for the analysis of very volatile mixtures, or where stationary phases are incompatible with column fabrication, preparative and process-scale GC, etc.

Liquid Chromatography

The intrinsic efficiency per unit length of packed columns in LC increases as the particle diameter is reduced. It can also be increased by using solvents of low viscosity, which result in smaller contributions to the column plate height from resistance to mass transfer and flow anisotropy. Operation at low mobile-phase velocities compared to GC further minimizes the contributions from resistance to mass transfer in the mobile phase at the expense of longer separation times. The pressure drop required to maintain a constant mobile-phase velocity is proportional to the ratio of the column length to the particle diameter squared. Since the available operating pressure is finite, the column length must be reduced as the

particle diameter is decreased. Consequently, most separations in LC are performed with a total of about 5000–20 000 theoretical plates that is largely independent of the particle size. However, since the retention time at a constant (optimum) mobile-phase velocity is proportional to the column length, this arbitrary fixed number of plates is made available in a shorter time for shorter columns packed with smaller diameter particles. Thus the principal virtue of using particles of a small diameter is that they permit a reduction in the separation time for those separations that do not require a large number of theoretical plates.

Conventional column diameters in analytical LC at 3–5 mm are comparatively large so as to minimize zone broadening from extracolumn effects in earlier instrument designs and have become the *de facto* standard dimensions, even though instrument capabilities have improved over time. Smaller diameter columns have been explored to reduce mobile-phase consumption (which is proportional to the square of the column radius) and to enhance mass detection through reduction in peak volumes, but offer no improvement in the intrinsic column efficiency, except perhaps for columns with a low column diameter-to-particle size ratio. Capillary columns of 0.1 to 0.5 mm internal diameter packed with 3–10 μm particles can be used in relatively long lengths for the separation of complex mixtures, where a large number of theoretical plates is required. Such columns probably minimize the contribution form flow anisotropy while at the same time providing a better mechanism for the dissipation of heat caused by the viscous drag of the mobile phase moving through the packed bed. The operation of these columns is still pressure-limited and separation times an order of magnitude greater than for GC have to be accepted as the price for high efficiency.

The enhancement of intraparticular mass transport is particularly important for the rapid separation of biopolymers, whose diffusion coefficients are perhaps 100-fold smaller than those of low molecular weight compounds in typical mobile phases used in LC. Also, the high surface area porous packings used for small molecules may be too retentive for biopolymers with a significant capacity for multisite interactions. For these compounds short columns packed with 1.5 and 2 μm pellicular or porous particles are used for fast separations. Longer columns containing perfusive particles of a large size with large diameter through-pores to promote convective transport can also be used for fast separations. Perfusive particles are also used for the preparative-scale separation of biopolymers.

Supercritical Fluid Chromatography

In SFC, mobile-phase modification of the stationary phase and its dependence on fluid density, together with the variation of fluid density along the length of the column, result in additional sources of zone broadening that cannot be treated in an exact way. Packed columns used in SFC are identical in type to those used in LC. When separations can be achieved with a modest number of theoretical plates (up to about 80 000), then packed columns provide much faster separations, perhaps up to an order of magnitude, than open-tubular columns, which are generally preferred when very large numbers of theoretical plates are required.

Systems with Electroosmotic Flow

Plug flow in CEC results in a smaller contribution to the plate height from flow anisotropy and transaxial diffusion compared with pressure-driven column liquid chromatography, while contributions to the plate height that are flow-profile-independent are the same. The absence of a pressure drop in electroosmotically driven systems provides the necessary conditions to achieve a larger total number of theoretical plates in CEC in a reasonable time through the use of smaller particles and longer columns (see **Table 2** and **Figure 11**). Under normal operating conditions CEC columns have the potential to provide column plate numbers 5–10 times higher than LC columns. Ultimately the performance in CEC is limited by Joule heating, which causes additional zone broadening and restricts applications of CEC to the use of microcolumns, since columns with a small internal diameter (<100 μm) are required for efficient heat dissipation. The dominant cause of zone broadening in MEKC is axial diffusion, with significant contributions from slow sorption–desorption kinetics between the analyte and micelles and electrophoretic dispersion arising from the polydispersity

Table 2 Achievable theoretical plate numbers in HPLC and CEC

Particle size (μm)	*HPLC*		*CEC*	
	Length (cm)	*Plates/ column*	*Length (cm)*	*Plates/ column*
5	5	55 000	50	115 000
3	25	45 000	50	170 000
1.5	10	30 000	50	250 000

Column pressure drop = 400 atm for HPLC and the field strength <30 kV in CEC for operation at the minimum point in the van Deemter plot.

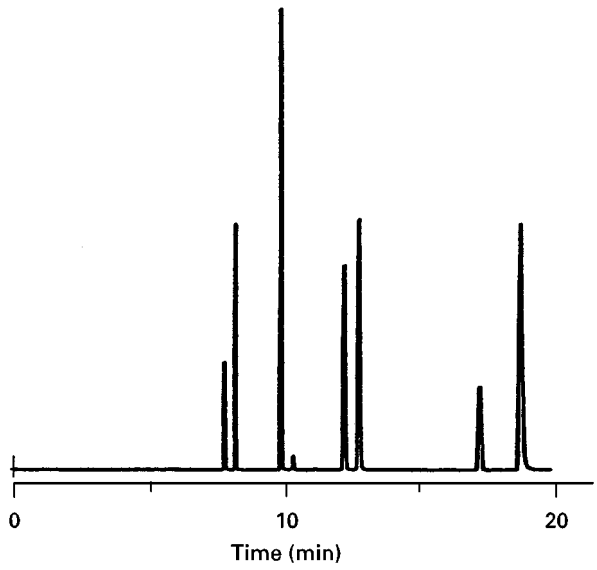

Figure 11 Separation of aromatic compounds by CEC on a 50 cm × 50 μm i.d. fused silica capillary column packed with 1.5 μm spherical octadecylsiloxane-bonded silica gel with 70% (v/v) acetonitrile buffer as mobile phase, temperature 25°C, and field strength 30 kV.

of micelle sizes. Resistance to mass transfer in the mobile phase is minimized by the capillary dimensions of the column and the small size and homogeneous distribution of the micelles throughout the mobile phase combined with the near-perfect plug flow of the mobile phase. Thermal dispersion, as described for CEC, is an additional potential source of zone broadening resulting from radial temperature gradients. Separations in MEKC are typically carried out with between 100 000 and 500 000 theoretical plates. Adsorption of solutes on the column wall can greatly reduce the potential column efficiency and experimental conditions should be optimized to minimize these contributions whenever possible.

Planar Chromatography

The consequence of the suboptimal mobile-phase velocity in planar chromatography obtained by capillary-controlled flow is that zone broadening is dominated by diffusion. Since the mobile-phase velocity varies approximately quadratically with migration distance, solutes are forced to migrate through regions of different local efficiency and the plate height for the layer must be expressed by an average value (**Figure 12**). Each solute in the chromatogram experiences only those theoretical plates over which it migrates, with solutes close to the sample application point experiencing very few theoretical plates and those close to the solvent front experiencing up to an upper limit of about 5000. High performance layers, with a nominal average particle size of about 5 μm, provide more compact zones than coarser particles, provided that the solvent front migration distance does not exceed about 5–6 cm; beyond this point zone broadening exceeds the rate of zone centre separation. When the development length is optimized the separation performance of conventional layers (average particle size about 10 μm) is not very

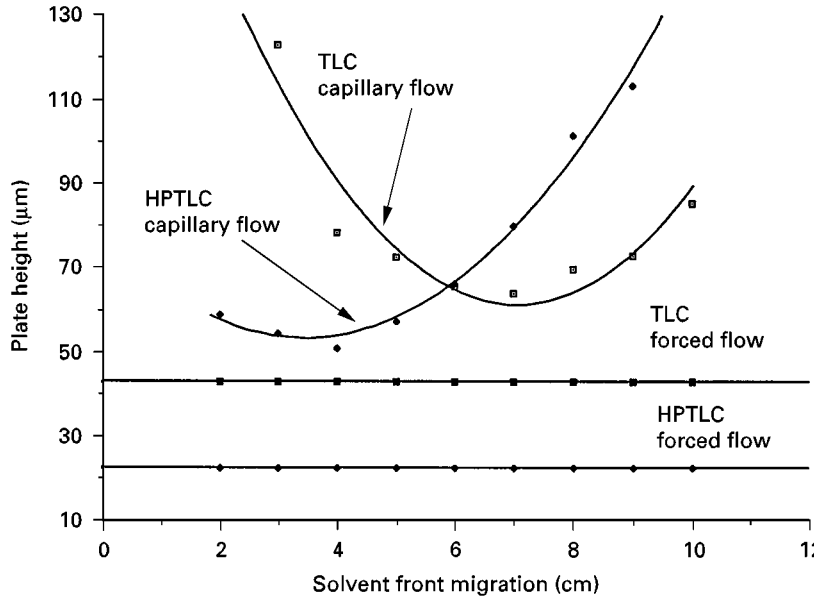

Figure 12 Variation of the average plate height as a function of the solvent front migration distance for conventional and high performance silica gel layers with capillary-controlled and forced-flow development. (Reproduced with permission from Poole CF and Poole SK (1997) *Journal of Chromatography A* 703: 573, copyright © Elsevier Science B.V.)

different from that of the high performance layers; the primary virtue of the latter is that a shorter migration distance is required to achieve a given efficiency, resulting in faster separations and more compact zones that are easier to detect by scanning densitometry. The minimum in the average plate height under capillary-controlled conditions is always greater than the minimum observed for forced-flow development, indicating that under capillary-controlled flow conditions the optimum potential performance is currently never realized in full. Under forced-flow conditions the minimum in the plate height is both higher and moved to a lower velocity compared with values anticipated for a column in LC, (**Figure 13**). Also, at increasing values of the mobile-phase velocity, the plate height for the layer increases more rapidly than is observed for a column. At the higher mobile-phase velocities obtainable by forced-flow development, resistance to mass transfer is an order of magnitude more significant for layers than for columns. The large value for resistance to mass transfer for the layers may be due to restricted diffusion within the porous particles or is a product of heterogeneous kinetic sorption on the sorbent and the binder added to layers to stabilize their structure. The consequences for forced-flow TLC are that separations will be slower than for columns and fast separations at high flow rates will be much less efficient than for columns, although in terms of total efficiency and separation speed the possibilities for forced-flow development are significantly better than those of capillary-controlled separations (**Figure 14**).

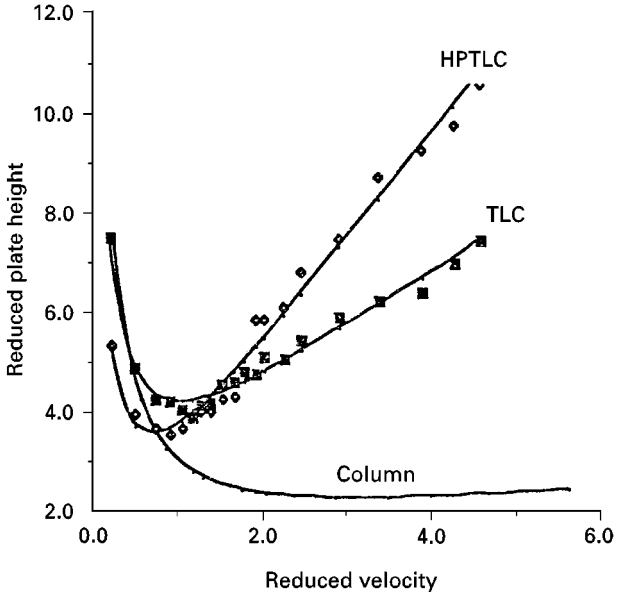

Figure 13 Plot of the reduced plate height (H/d_P) against the reduced mobile-phase velocity (ud_P/D_M) for a high performance and a conventional TLC layer using forced-flow development superimposed on a curve for an ideal LC column. (Reproduced with permission from Fernando WPN and Poole CF (1991) *Journal of Planar Chromatography* 4: 278, copyright © Research Institute for Medicinal Plants.)

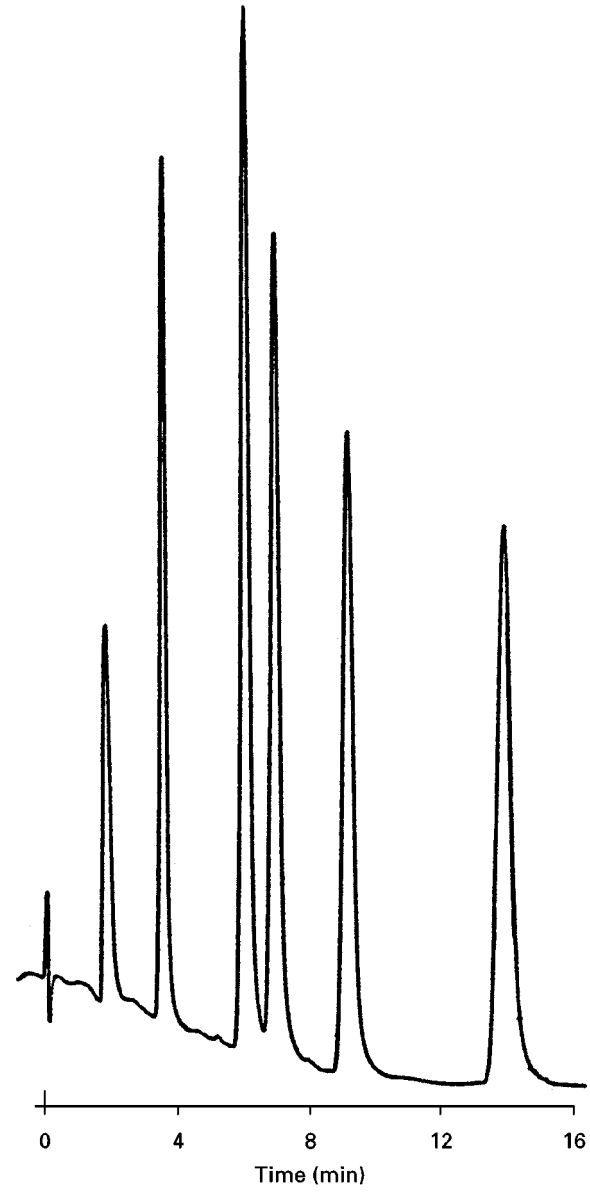

Figure 14 Separation of polycyclic aromatic hydrocarbons by forced-flow TLC with online detection (elution mode). A silica gel high performance layer, migration distance 18 cm, with hexane as the mobile phase (0.07 cm s^{-1}) was used for the separation. (Reproduced with permission from Poole CF and Poole SK (1994) *Analytical Chemistry* 66: 27A, copyright © American Chemical Society).

Separation Quality

The general object of a chromatographic separation is to obtain an acceptable separation (resolution)

between all components of interest in a mixture within the shortest possible time. The resolution between two peaks in a chromatogram depends on how well the peak maxima are separated and how wide the two peaks are. This can be expressed numerically by the ratio of the separation of the two peak maxima divided by the average peak widths at their base. Baseline separation of the peaks is achieved at a resolution of about 1.5 but a value of 1.0, representing about 94% peak separation, is taken as an adequate goal for components that are difficult to separate. Resolution is also simply related to the properties of the chromatographic system. For this purpose it is convenient to consider a simple model of a three-component mixture in which the optimum column length is dictated by the number of theoretical plates required to separate the two components that are most difficult to separate, and the total separation time is dictated by the time required for the last peak to elute from the column. The resolution of the two peaks that are most difficult to separate is then related to the column variables by:

$$R_S = (\sqrt{N}/2) \times [(\alpha - 1)/(\alpha + 1)] \times k_{AV}/(1 + k_{AV}) \quad [3]$$

where k_{AV} is the average value of the retention factor for the two peaks, or in an approximate form by:

$$R_S = (\sqrt{N}/4) \times [(\alpha - 1)/\alpha] \times k_2/(1 + k_2) \quad [4]$$

for peaks with approximately equal base widths in which the elution order of the peaks is $k_2 > k_1$.

Column Chromatography

To a reasonable approximation, the three contributions to resolution (efficiency, selectivity and time) can be treated independently and optimized separately. Resolution increases only as the square root of N, so although the influence of efficiency is the most predictable parameter in the resolution equation, it is also the most limited. In practice all separations have to be made in the range $N = 10^3$–10^6 (Table 1). For GC this full range is available, so that increasing the column length or, better, reducing the column internal diameter of an open-tubular column at a constant length (separation time is proportional to column length), is often an effective strategy. For LC only a modest number of theoretical plates can be obtained in a reasonable time. In this case the general approach is to use the maximum available value for N and optimize resolution by changing the other variables. SFC is an intermediate case in which the general strategy depends on whether the fluid is more gas-like or liquid-like.

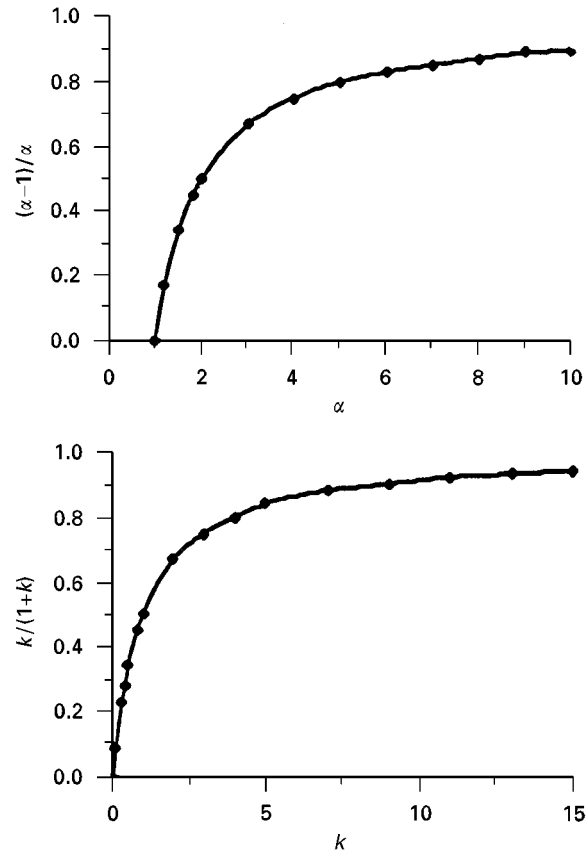

Figure 15 Influence of the separation factor (α) and the retention factor (k) on the resolution of two closely eluting peaks in column chromatography. (Reproduced with permission from Poole CF and Poole SK (1991) *Chromatography Today*, p. 31, copyright © Elsevier Science B.V.)

The separation factor determines the ability of the chromatographic system to differentiate between the two components based on the difference in their thermodynamic interactions with the mobile and stationary phases. When $\alpha = 1$ a separation is impossible but, as can be seen from **Figure 15**, only a small increase in α above unity is required to improve resolution considerably. At comparatively large values of α, resolution is little influenced by further changes; indeed, separations in which $\alpha > 2$ are easy to achieve. Selectivity optimization is the general approach to improve resolution in LC, where a wide range of mobile and stationary phases are available to choose from and a wide range of different retention mechanisms can be employed. Empirical or statistically based experimental approaches to selectivity optimization are often used because of a lack of formal knowledge of exact retention mechanisms for computer-aided calculations. Although powerful, selectivity optimization in LC can be a time-consuming process. The ease of achieving a separation by selectivity optimization can be

Table 3 Factors affecting resolution in column chromatorgraphy

Value of N needed for $R_S = 1$ at $k = 3$ for different values of α		*Value of N needed for $R_S = 1$ at different k values for $\alpha = 1.05$ and 1.10*		
α	*N*	*k*	*$\alpha = 1.05$*	*$\alpha = 1.10$*
1.005	1 150 000	0.1	853 780	234 260
1.01	290 000	0.2	254 020	69 700
1.02	74 000	0.5	63 500	17 420
1.05	12 500	1.0	28 220	7 740
1.10	3 400	2.0	15 880	4 360
1.20	1 020	5.0	10 160	2 790
1.50	260	10.0	8 540	2 340
2.00	110	20.0	7 780	2 130

illustrated by the data in **Table 3**, which indicate the number of theoretical plates required for a separation. These data can be compared to the data in Table 1, which indicates the number of theoretical plates available for different chromatographic systems. This is a clear indication of the need for selectivity optimization in LC and SFC, and the more relaxed constraints for GC.

Resolution will initially increase rapidly with retention, starting at $k = 0$, as shown in Figure 15. By the time k reaches a value around 5, further increases in retention result in only small changes in resolution. The optimum resolution range for most separations occurs for k between 2 and 10. Higher values of k result in long separation times with little concomitant improvement in resolution, but they may be necessary to provide sufficient separation space to contain all the peaks in the chromatogram.

The separation time is given by:

$$t_R = (H/u) \times 16R_S^2 \times [\alpha/(\alpha - 1)^2] \times (k_2 + 1)^3/k_2^2) \qquad [5]$$

If the separation time (t_R) is to be minimized, then the acceptable resolution should not be set too high ($R_S = 1$); the separation factor should be maximized for the most difficult pair to separate; the retention factor should be minimized ($k = 1$–5) for the most difficult pair to separate; and the column should be operated at the minimum value for the plate height corresponding to the optimum mobile-phase velocity.

Micellar Electrokinetic Chromatography

The resolution equation for MEKC is identical to eqns [3] and [4] but contains an additional term, $(t_M/t_{MC})/[1 + (t_M/t_{MC})k_1]$, to account for the limited elution range (all solutes must elute between the retention time of an unretained solute, t_M, and a solute totally retained by the micelles, t_{MC}; see **Figure 16**). The intrinsic efficiency of MEKC is much higher than column liquid chromatography, and optimization of the separation factor depends on a different set of parameters (changing surfactant type, use of additives, etc). Large values of the retention factor are unfavourable for obtaining high resolution since the additional term added to the resolution equation tends to zero at high k values. The optimum value of k for maximum resolution is around 0.8–5, corresponding to $(t_M/t_{MC})^{1/2}$. The retention factor is usually optimized by changing the surfactant concentration.

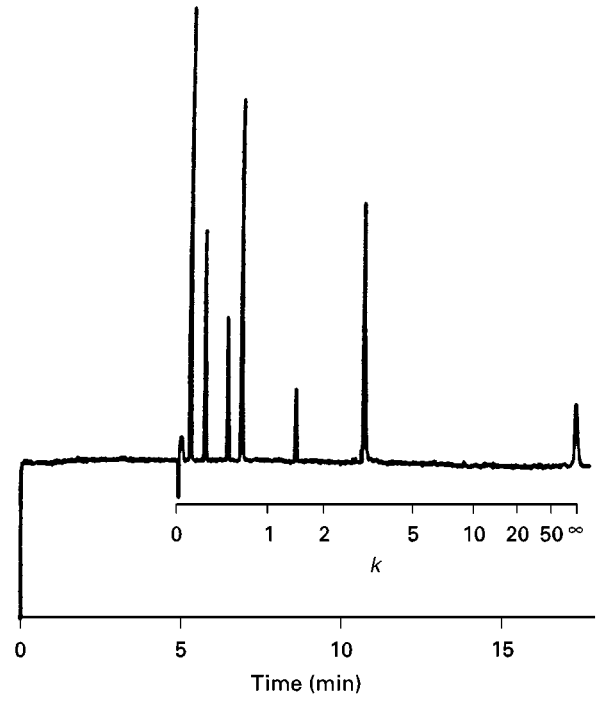

Figure 16 Separation of aromatic compounds by MEKC using a 65 cm (effective length 50 cm) × 50 μm i.d. fused silica capillary and a mobile phase containing 30 mmol L^{-1} sodium dodecyl sulfate and 50 mmol L^{-1} sodium phosphate/100 mmol L^{-1} sodium borate buffer (pH = 7) at a field strength of 15 kV. (Reproduced with permission from Terabe S (1989) *Trends in Analytical Chemistry* 8: 129, copyright © Elsevier Science B.V.)

Planar Chromatography

For a single development under capillary-controlled flow conditions the TLC analogue of the general resolution equation for column chromatography can be expressed in approximate form as:

$$R_S = [(N_1R_{F2})^{1/2}/4] \times [(k_1/k_2) - 1)] \times (1 - R_{F2}) \quad [6]$$

where N_1 is the maximum number of theoretical plates available corresponding to the solvent front position. The use of N_1R_{F2} is only a rough

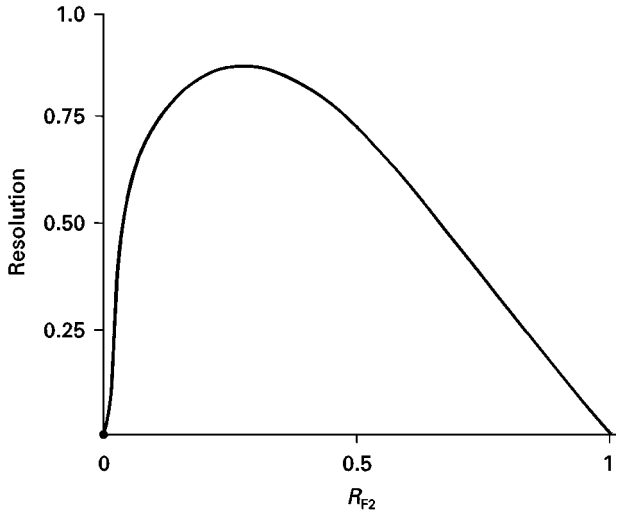

Figure 17 Variation of the resolution of two closely migrating zones as a function of the R_F value for the faster moving zone. (Reproduced with permission from Poole CF and Poole SK (1991) *Chromatography Today*, p. 669, copyright © Elsevier Science B.V.)

approximation for the number of theoretical plates that a particular zone has migrated across. Relatively small changes in selectivity have enormous impact on the ease of obtaining a given separation in TLC, since the total number of theoretical plates available for a separation is never very large. Separations in TLC are fairly easy when $R_{F2} - R_{F1} > 0.1$ and very difficult or impossible for $R_{F2} - R_{F1} \leq 0.05$ in the region of the optimum R_F value for the separation. Maximum resolution is obtained at an R_F value of about 0.3 and does not change much in the R_F range of 0.2 to 0.5, as can be seen in **Figure 17**. Resolution is zero for compounds that are retained at the origin or migrate with the solvent front.

General Elution Problem

Constant separation conditions, for example isothermal operation in GC and isocratic elution in LC, are unsuitable for separating samples containing components with a wide retention range. Employing average separation conditions will result in a poor separation of early-eluting peaks, poor detectability of late-eluting peaks, and excessively long separation times. In GC there is an approximately exponential relationship between retention time and solute boiling point under isothermal conditions. For mixtures with a boiling point range $> c.$ 100°C it is impossible to identify a compromise temperature that will provide an acceptable separation. The solution in this case is to use temperature programming, flow programming, or both. Temperature programming is the most common and usually involves a continuous linear increase in temperature with time, although other programme profiles are possible, including segmented programmes incorporating isothermal periods. The reduction in separation time, increase in peak capacity, and nearly constant peak widths obtained are illustrated by the separation in **Figure 18**. The general elution problem in LC is solved using solvent-strength gradients. Here, the composition of the mobile phase is changed as a function of time. Binary or ternary solvent mixtures are commonly used as the mobile phase in which the relative composition of the strong solvent (that solvent with the capability of reducing retention the most) is increased over time. In SFC it is usual to programme the density, mobile-phase composition or temperature as a single factor, but it is also possible for some combination of parameters to be changed simultaneously. The goal remains the same, as indicated by the

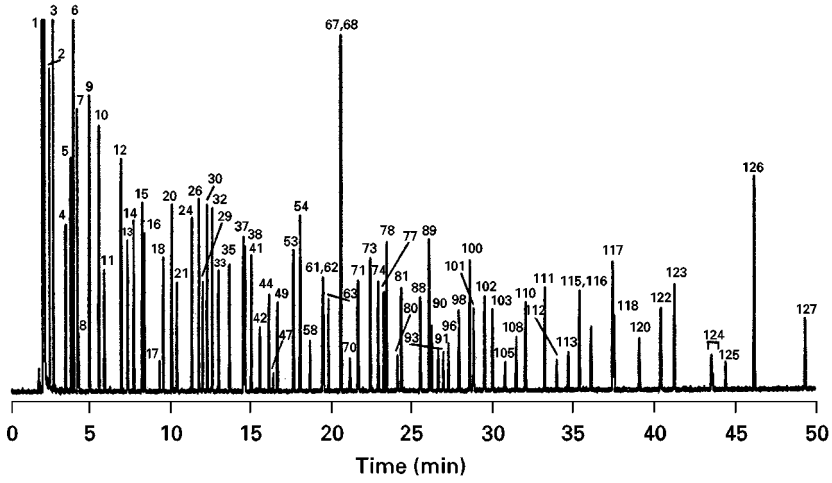

Figure 18 Temperature programmed separation of fragrance compounds by GC on a 30 m × 0.25 mm i.d. fused silica open-tubular column coated with DB-1, film thickness 0.25 μm, helium carrier gas 25 cm s^{-1} and temperature program 40°C (1 min isothermal) then 40–290°C at 5°C min^{-1}. (Reproduced with permission from J&W, copyright © J&W Scientific Inc.)

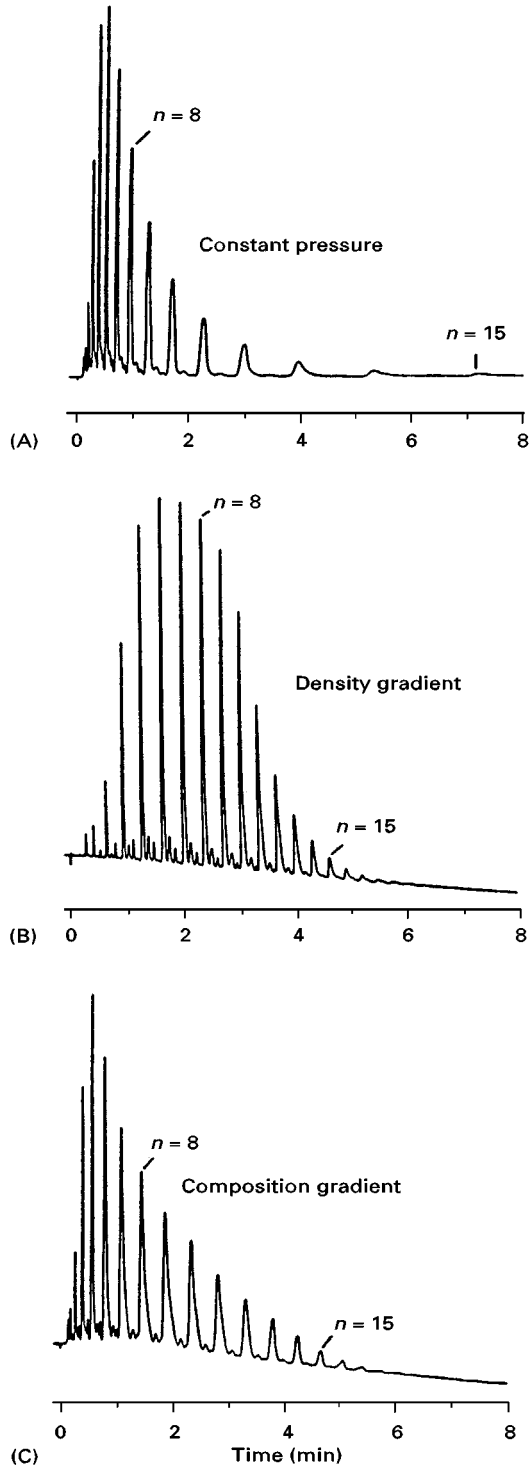

Figure 19 Separation of Triton X-114 by SFC using programmed elution on a 10 cm × 2 mm i.d. column packed with 3 μm octadecylsiloxane-bonded silica gel at 170°C with UV detection. (A) Carbon dioxide/methanol $(2 + 0.125)$ mL min^{-1} at 210 bar; (B) as for (A) with pressure programmed form 130 to 375 bar over 8 min; and (C) using a mobile-phase composition gradient from 0.025 to 0.4 mL min^{-1} methanol over 8 min at 210 bar. (Reproduced with permission from Giorgetti A, Pericles N, Widmer HM, Anton K and Datwyler P (1989) *Journal of Chromatographic Science* 27: 318, copyright © Preston Publications, Inc.)

density- and composition-programmed separation of oligomers in **Figure 19**.

Solvent-strength gradients in TLC are usually discontinuous and achieved through the use of unidimensional multiple development. This is accompanied by zone refocusing resulting in a larger zone capacity and easier-to-detect separated zones. All unidimensional multiple development techniques employ successive repeated development of the layer in the same direction with removal of the mobile phase between developments. Each time the solvent front traverses the sample zone it compresses the zone in the direction of development because the mobile phase contacts the bottom edge of the sample zone first where the sample molecules then start to move forward before those molecules ahead of the solvent front. Once the solvent front has reached beyond the zone, the refocused zone migrates and is broadened by diffusion in the usual way. When optimized, it is possible to migrate a zone a considerable distance without significant zone broadening beyond that observed for the first development. If the solvent composition is varied for all, or some, of the development steps during multiple development, then solvent strength gradients of different shapes can be produced. With increasing solvent-strength gradients it is usually necessary to scan the separation at a number of intermediate development steps corresponding to the development at which different components of interest are separated, since in later developments these zones may be merged again because of the limited zone capacity in TLC. Alternatively,

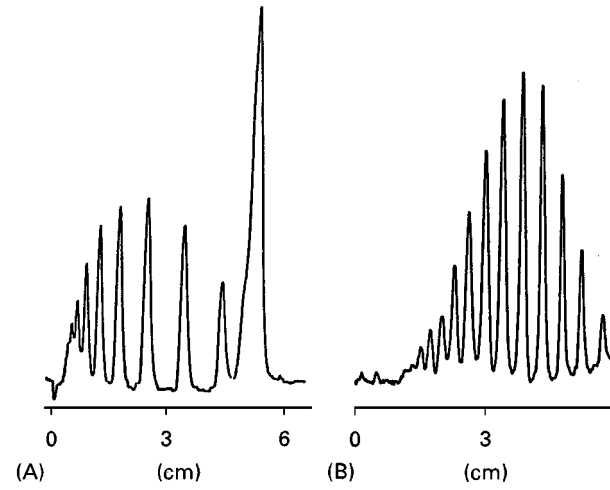

Figure 20 Separation of the 3,5-dinitrobenzoyl esters of poly(ethylene glycol) 400 by (A) a single conventional development and (B) by incremental multiple development with a stepwise gradient of methanol, acetonitrile and dichloromethane over 15 developments (Reproduced with permission from Poole CF, Poole SK and Belay MT (1993) *Journal of Planar Chromatography* 6: 438, copyright © Research Institute for Medicinal Plants.)

incremental multiple development can be used with a decreasing solvent-strength gradient. In this case, the first development distance is the shortest and employs the strongest solvent composition, while subsequent developments are longer and employ mobile-phase compositions of decreasing solvent strength. The final development step is the longest and usually corresponds to the maximum useful development length for the layer and employs the weakest mobile phase. In this way sample components migrate in each development until the strength of the mobile phase declines to a level at which some of the sample zones are immobile, while less retained zones continue to be separated in subsequent development steps, affording the separation of the mixture as a single chromatogram (**Figure 20**). Incremental multiple development with a decreasing solvent-strength gradient is easily automated.

Multidimensional and Multimodal Chromatography

The analysis of complex mixtures requires a very large peak capacity since the probability of peak overlap increases with the number of compounds requiring separation. Multidimensional and multimodal chromatographic systems provide a better route to achieving high peak capacities than is possible with single-column systems. The necessary characteristic of these systems is that the dominant retention mechanism should be different for each dimension. Other uses of multidimensional and multimodal chromatography include trace enrichment, matrix simplification, increased sample throughput, and as an alternative to gradient elution in LC.

Multidimensional column chromatography involves the separation of a sample by using two or more columns in series where the individual columns differ in their capacity and/or selectivity. Multimodal separations involve two or more chromatographic methods in series, for example, the online coupling of LC and GC (LC-GC) or SFC and GC (SFC-GC). Both methods involve the transfer of the whole or part of the eluent from the first column to another via some suitable interface. The function of the interface is to ensure compatibility in terms of flow, solvent strength and column capacity. The design requirements and ease of coupling differ significantly for the different chromatographic modes. Coupling GC-GC, SFC-GC, SFC-SFC, LC-LC, LC-GC and LC-TLC are routine and other combinations such as SFC-TLC, SFC-LC and GC-TLC have been described in the literature. Trace enrichment and sample clean-up on short pre-columns is finding increasing use in the automated determinations of drugs in biological fluids and crop protection agents in water by LC-LC. **Figure 21** illustrates the separation of a mixture of deoxyribonucleosides and their 5′-monophosphate esters using LC-LC with an anion exchange column and a reversed-phase column connected in series by a microvolume valve interface. The neutral deoxyribonucleosides are switched as a single peak for separation on the reversed-phase column while the phosphate esters are resolved by the anion exchange column. The separation time remains acceptable since both separations are performed almost simultaneously. TLC-TLC is commonly called two-dimensional TLC and is a widely used qualitative method of analysis. It is very easily performed by placing a sample at the corner of the layer and developing the plate in the normal way, evaporating the solvent, turning the plate through a right angle and developing the plate a second time at 90° to the first development. If adequately optimized this is a very

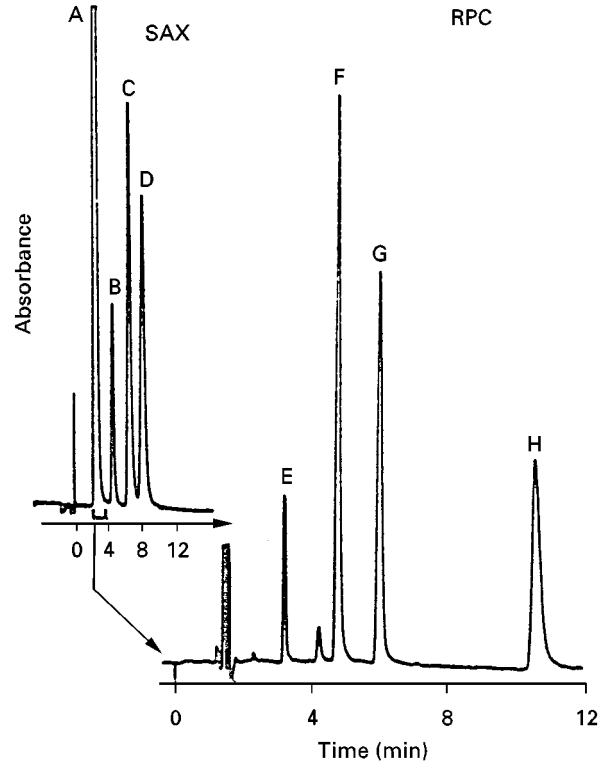

Figure 21 Separation of the major deoxyribonucleosides and their 5′-monophosphate esters by multidimensional LC-LC. The first column is a strong anion exchange column and the second a reversed-phase column. The unseparated nucleosides, A, are switched to the second column after which the 5′-monophosphate esters, B to D are separated on the IEC column and the parent deoxyribonucleosides, E to H, are separated on the RPC column. (Reproduced with permission from Sagliano N, Hsu SH, Floyd TR, Raglione TV and Hartwick RA (1985) *Journal of Chromatographic Science* 23: 238, copyright © Preston Publication, Inc.)

powerful separation method, but more frequently than not, solvents of different composition are used for the two developments employing retention mechanisms that differ in intensity rather than kind, and the zones are only dispersed around the diagonal between the two development directions and not uniformly over the whole layer.

Mode Selection

Chromatography provides many different approaches for the separation of mixtures. There are many instances where the same mixture can be adequately separated by more than one approach. In this section we will take a mechanistic look at how solutes are separated by the common chromatographic techniques to provide some guidelines for method suitability.

If the only consideration were efficiency and speed, then GC would be the preferred technique. In practice, GC is restricted to thermally stable compounds with a significant vapour pressure at the temperature required for their separation. The upper temperature limit for common GC stationary phases is 200–400°C. Few compounds with a molecular weight greater than 1000 Da have sufficient vapour pressure to be separated in this temperature range, and many low molecular weight compounds are known to be labile at temperatures required for their vaporization. Derivatization techniques extend the scope of GC to otherwise labile compounds by forming thermally stable derivatives, often with increased volatility, and by tagging compounds with specific groups that simplify trace analysis using one of the selective and sensitive group or element-selective detectors available for GC.

Under typical conditions the mobile phase in GC behaves essentially as an ideal gas and does not contribute to selectivity. To vary selectivity either the temperature is changed or a new stationary phase (column) is employed for the separation. Temperature and separation time are closely connected in GC. The range over which temperature can be varied is usually short and will likely provide only a small change in selectivity, but because of the large number of theoretical plates available for a separation in GC, this may be sufficient to provide adequate resolution. Provided that stationary phases that differ in their relative capacity for intermolecular interactions are selected, then larger changes in selectivity can be anticipated by stationary-phase optimization. In modern column technology the most versatile group of stationary phases are the poly(siloxanes), which can be represented by the basic structure $-(R_2SiO)_n-$, in which the type and relative amount of individual substituents can be varied to create the desired variation in selectivity (R = methyl, phenyl, 3,3,3-trifluoropropyl, cyanoethyl, fluorine-containing alcohol, etc.) Special phases in which R contains a chiral centre or a liquid-crystalline unit are used to separate enantiomers and geometric isomers. Other common stationary phase include hydrocarbons, poly(phenyl ethers), poly(esters) and poly(ethylene glycols), although many of these phases are restricted to packed column applications because of difficulties in either coating or immobilizing them on the walls of fused-silica capillaries, favoured for the manufacture of open-tubular columns. The solvation parameter model provides a reliable systematized approach for selectivity optimization and the prediction of retention in GLC. For GSC the stationary phase is usually silica, alumina, graphitized carbon, organic polymer or zeolite porous particles (packed columns); or a thin layer dispersed over the inner surface of a capillary column with an open passageway down the centre (porous layer open-tubular column, or PLOT column). These materials are used to separate inorganic gases, volatile halocarbon compounds, low molecular weight hydrocarbons and, in particular, geometric and isotopic isomers.

LC and GC should be considered as complementary techniques. Since the only sample requirement for LC is that the sample has reasonable solubility in some solvent suitable for the separation, and since separations by LC are commonly carried out close to room temperature, thermal stability is not generally an issue. The large number of separation mechanisms easily exploited in the liquid phase provides a high level of flexibility for selectivity optimization. In general, many applications of LC can be categorized as those for which GC is unsuited and includes applications to high molecular weight synthetic polymers, biopolymers, ionic compounds and many thermally labile compounds of chemical interest.

Mode selection within LC is quite complicated because of the number of possible separation mechanisms that can be exploited, as illustrated in **Figure 22**. Preliminary information on the molecular weight range of the sample, relative solubility in organic solvents and water, and whether or not the sample is ionic, can be used as a starting point to arrive at a suitable retention mechanism for a separation. The molecular weight cutoff at 2000 indicated in Figure 22 is quite arbitrary and reflects the fact that size exclusion packings are readily available for the separation of higher molecular weight solutes, although size exclusion is not used exclusively to separate high molecular weight compounds because of its limited peak capacity. Wide-pore packing materials allow polymers with a molecular weight

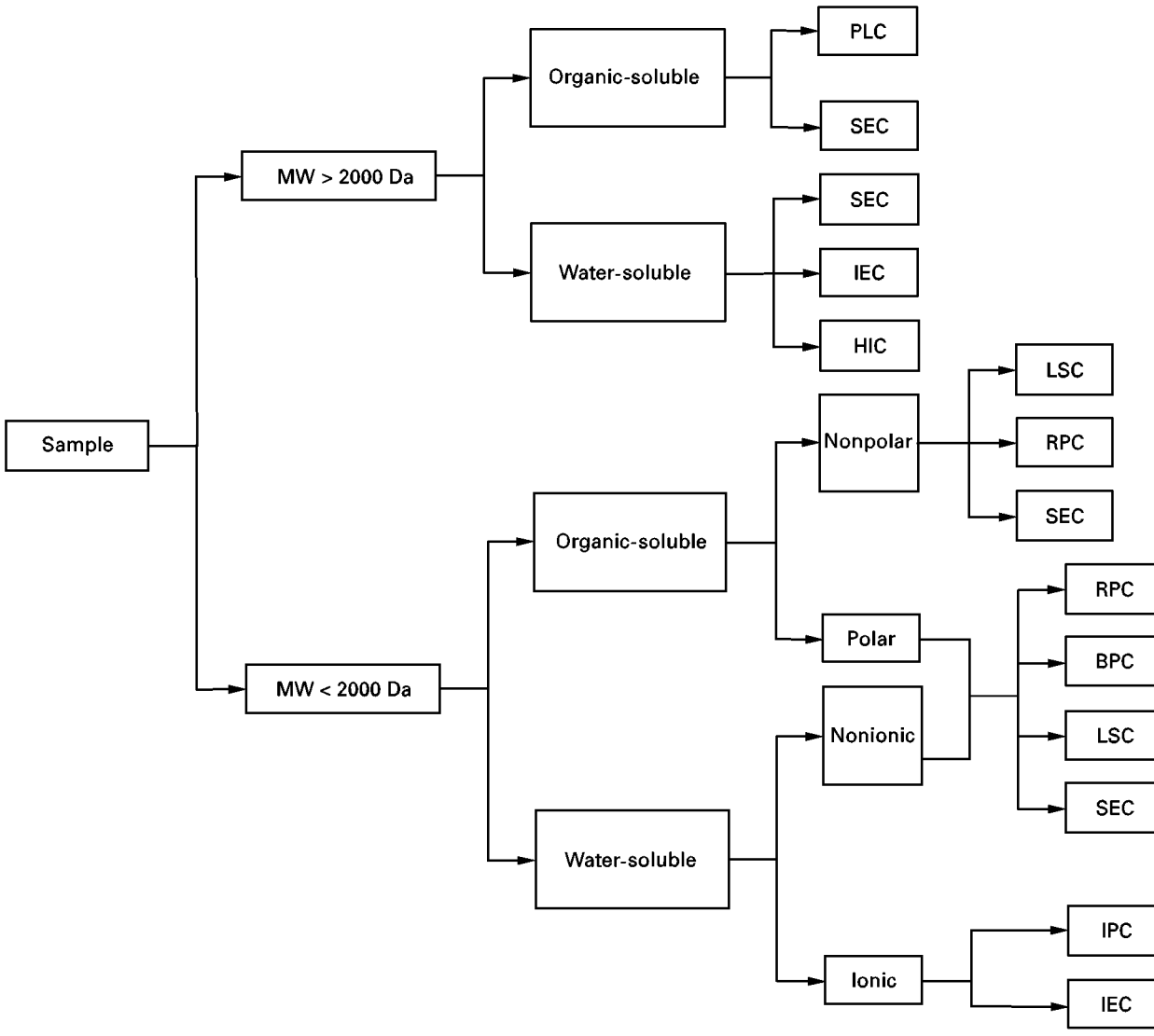

Figure 22 Selection of the separation mechanism in LC based on the criteria of sample molecular weight, solubility and conductivity. (Reproduced with permission from Poole CF and Poole SK (1991) *Chromatography Today*, p. 455, copyright © Elsevier Science B.V.)

exceeding 2000 to be separated by conventional sorption and ion exchange mechanisms.

Liquid–solid chromatography (LSC) is characterized by the use of an inorganic oxide or chemically bonded stationary phase with polar functional groups and a nonaqueous mobile phase consisting of one or more polar organic solvents diluted to the desired solvent strength with a weak solvent, such as hexane. A characteristic of these systems is the formation of an adsorbed layer of mobile-phase molecules at the surface of the stationary phase with a composition that is related to the mobile-phase composition but generally not identical to it. Retention is essentially determined by the balance of interactions the solute experiences in the mobile phase and its competition with mobile-phase molecules for adsorption sites at the surface of the stationary phase. The position and type of polar functional groups and their availability for interaction with discrete immobile adsorption sites is responsible for selectivity differences when silica or alumina are used as stationary phases. The ability of LSC to separate geometric isomers has been attributed to the lock–key type steric fitting of solute molecules with the discrete adsorption sites on the silica surface.

Reversed-phase liquid chromatography (RPC) is characterized by the use of a stationary phase that is less polar than the mobile phase. A chemically bonded sorbent or a porous polymer could be used as this stationary phase, while for most practical applications the mobile phase contains water as one of its major components. RPC is ideally suited to the

separation of polar molecules that are either insoluble in organic solvents or bind too strongly to inorganic oxide adsorbents for normal elution. RPC employing acidic, low ionic strength eluents is a widely established technique for the purification and characterization of biopolymers. Other favourable attributes include the possibility of simultaneous separation of neutral and ionic solutes; rapid equilibrium between phases facilitating the use of gradient elution; and the manipulation of secondary chemical equilibria in the mobile phase (e.g. ion suppression, ion pair formation, metal complexation and micelle formation) to optimize separation selectivity in addition to variation in solvent type and composition of the mobile phase. A large number of chemically bonded stationary phases of different chain length, polarity and bonding density are available to complement mobile-phase optimization strategies. About 70% of all separations performed in modern LC are by RPC, which gives an indication of its flexibility, applicability and ease of use. The main driving force for retention in RPC is solute size because of the high cohesive energy of the mobile phase compared to the stationary phase, with solute polar interactions, particularly solute hydrogen bond basicity, reducing retention. These findings strongly reflect the properties of water, which is the most cohesive of the solvents normally used in LC, as well as a strong hydrogen bond acid.

Ion exchange chromatography (IEC) is used for the separation of ions or substances easily ionized by manipulation of pH. Stationary phases are characterized as weak or strong ion exchangers based on the extent of ionization of the immobile ionic centres, and as anion or cation exchangers based on the charge type associated with the ionic centres. Thus, sulfonic acid groups are strong, and carboxylic acid groups are weak, cation exchangers. Most of the metal cations in the Periodic Table have been separated by IEC with acids or complexing agents as eluents. In clinical laboratories ion exchange has long been employed as the basis for the routine, automated separation of amino acids and other physiologically important amines involved in metabolic disorders and to sequence the structure of biopolymers. Soft, nondenaturing, ion exchange gels are widely used in the large-scale isolation, purification and separation of peptides, proteins, nucleosides and other biological polymers. Metal-loaded ion exchangers and anion exchange chromatography of complexed carbohydrates are well-established separation techniques in carbohydrate chemistry. The combination of pellicular ion exchange columns of low capacity, low concentration eluents with a high affinity for the ion exchange packing, and universal, online detection with a flow-through conductivity detector revolutionized the analysis of inorganic and organic ions in industrial and environmental laboratories. As well as electrostatic interactions, retention in IEC is influenced by hydrophobic sorptive interactions between the sample and stationary phase similar to those in RPC, and size and ionic exclusion effects. Resolution is optimized by choice of the mobile-phase counterion, the ionic strength, pH, temperature, flow rate, and addition of organic modifiers.

In size exclusion chromatography (SEC) retention differences are controlled by the extent to which sample components can diffuse through the pore structure of the stationary phase, as indicated by the ratio of sample molecular dimensions to the distribution of stationary-phase pore size diameters. Since no separation will result under conditions where the sample is completely excluded from the pore volume or can completely permeate the pore volume, the zone capacity of SEC is small compared with that of the other LC techniques. The separation time is predictable for all separations, corresponding (ideally) to a volume of eluent equivalent to the column void volume. No solvent optimization beyond finding a solvent for the sample that is compatible with the stationary phase is required. For synthetic polymers this can result in the use of exotic solvents and high temperatures. SEC is a powerful exploratory method for the separation of unknown samples, since it provides an overall view of sample composition within a predictable time, and is also commonly employed in sample fractionation to isolate components belonging to a defined molecular size range. Analytical separations employ small particles of rigid, polymeric or silica-based gels of controlled pore size to separate samples of different molecular size and to obtain average molecular weights and molecular weight distribution information for polymers.

Fundamentally the retention mechanisms for LC and TLC are identical. TLC is selected over LC when advantage can be taken of the attributes of employing a planar format for the separation. Examples include when a large number of samples requiring minimum sample preparation are to be separated, when post-chromatographic reactions are usually required for detection, or if sample integrity is in question. The use of a disposable stationary phase for TLC allows sample clean-up and separation to be performed simultaneously. Reasons for preferring LC over TLC are its greater separation capacity for mixtures containing more components than can be adequately resolved by TLC; a wider range of stationary phases are available for methods development; a wider selection of detection techniques exist; and automation for unattended operation is more straightforward.

The retention mechanism for MEKC strongly resembles that of RPC with two important differences. Surfactants used to generate the pseudo-stationary phases provide a different type of sorption environment to solvated chemically bonded phases and, therefore, different selectivity. The intrinsic efficiency of MEKC is significantly greater than that of LC and enhances resolution, although the peak capacity is lower owing to the finite migration window for MEKC. A significant number of RPC-type applications are now performed by MEKC, indicating that the method can compete favourably with RPC for some separations. MEKC is inherently a microcolumn technique, providing advantages in coupling to other chromatographic systems and for the analysis of samples only available in small amounts. Disadvantages include sample introduction problems, limited dynamic sample concentration range, and poor limits of detection for trace analysis (because of the very small sample sizes involved). Selectivity optimization is determined largely by the choice of surfactant and the use of mobile- and stationary-phase additives.

Supercritical fluids have solvating properties that are intermediate between those of gases and liquids. In addition, supercritical fluids are compressible so that their density and solvating power can be varied by changing external parameters, such as pressure and temperature. This feature is unique to supercritical fluids and represents a major approach to selectivity optimization. Temperature not only affects density, but may also influence the vapour pressure of low molecular weight solutes, promoting some GC-like character to the retention mechanism. The most common mobile phase is carbon dioxide, a relatively nonpolar fluid. More-polar fluids, such as water, ammonia or methanol, tend to have unfavourable critical constants or are highly corrosive to column or instrument components, limiting their use. Mixed mobile phases can be used to vary selectivity, such as carbon dioxide–methanol mixtures, but miscibility problems and high critical constants for the mixed mobile phases may restrict the range of properties available. SFC can provide faster separations than LC, but it is more restricted than LC in the choice of mobile phases and retention mechanisms to vary selectivity. SFC is compatible with most detection options available for both GC and LC. All practical applications of SFC occur significantly above ambient temperature, which is unsuitable for the separation of some thermally labile compounds and most biopolymers. Supercritical fluids such as carbon dioxide are unable to mask active sites on typical column packings, resulting in unsatisfactory separations of polar compounds owing to adsorption, which produces unacceptable peak shapes and poor resolution. However, SFC finds applications in many areas where GC and LC are unsatisfactory, for example in the separation of middle molecular weight compounds, low molecular weight synthetic polymers, fats and oils, enantiomers, and organometallic compounds.

Instrumentation

Modern chromatographic methods are instrumental techniques in which the optimal conditions for the separation are set and varied by electromechanical devices controlled by a computer external to the column or layer. Separations are largely automated with important features of the instrumentation being control of the flow and composition of the mobile phase, provision of an inlet system for sample introduction, column temperature control, online detection to monitor the separation, and display and archiving of the results. Instrument requirements differ significantly according to the needs of the method employed. Unattended operation is usually possible by automated sample storage or preparation devices for time-sequenced sample introduction.

Gas Chromatography

For GC a supply of gases in the form of pressurized cylinders is required for the carrier gas and perhaps also for the detector, for operating pneumatic valves, and for providing automatic cool-down by opening the oven door. To minimize contamination, high purity gases are used combined with additional purification devices. Each cylinder is fitted with a two-stage pressure regulator for coarse pressure and flow control. Fine tuning is achieved using metering valves or by electronic pressure control combining electromechanical devices with sensors to compensate automatically for changes in ambient conditions. The column oven is generally a forced air circulation thermostat heated resistively and capable of maintaining a constant temperature or of being programmed over time. The detector and sample inlet are generally thermostated separately in insulated metal blocks heated by cartridge heaters. The most common method of introducing samples into a GC inlet is by means of a microsyringe (pyrolysis, headspace and thermal desorption devices can be considered specialized sample inlets). For packed-column injection a small portion of (liquid) sample is introduced by microsyringe through a silicone septum into a glass liner or the front portion of the column, which is heated and continuously swept by carrier gas. The low sample capacity and carrier gas flow

rates characteristic of narrow-bore open-tubular columns require more sophisticated sample-introduction techniques based on sample splitting or solvent elimination and refocusing mechanisms.

The principal methods of detection are varied, conveniently grouped under the headings of gas-phase ionization devices, bulk physical property detectors, optical detection and electrochemical devices. Further classification is possible based on the nature of the detector response – universal, selective or specific. The flame ionization detector and thermal conductivity detector are examples of (near) universal detectors; the flame photometric detector, thermionic ionization detector and atomic emission detector are element-selective detectors; and the photoionization detector and electron capture detector are structure-selective detectors. GC coupling to mass spectrometry and IR spectroscopy is straightforward and widely utilized for automated structure identification as well as detection. Detection in the gas phase is a favourable process and GC detectors are among the most sensitive and versatile by virtue of the range of mechanisms that can be exploited.

Liquid Chromatography

Modern LC employs columns with small particle sizes and high packing density requiring high pressures for operation at useful mobile-phase velocities. Syringe-type or single- or multiple-head reciprocating piston pumps are commonly used to provide the operating pressures needed in configurations that depend on the design of the solvent-delivery system. A single pump is sufficient for isocratic operation. A single pump and electronically operated proportioning valves can be used for continuous variation of the mobile-phase composition (gradient elution) or, alternatively, independent pumps in parallel (commonly two) are used to pump different solvents into a mixing chamber. Between the pump and sample inlet may be a series of devices (check valves, pulse dampers, mixing chambers, flow controllers, pressure transducers, etc.) that correct or monitor pump output to ensure that a homogeneous, pulseless liquid flow is delivered to the column at a known pressure and volumetric flow rate. These devices may be operated independently of the pump or in a feedback network that continuously updates the pump output. Mobile-phase components are stored in reservoir bottles with provision for solvent degassing, if this is required for normal pump and detector operation. Loop-injection valves situated close to the head of the column are universally used for sample introduction. This allows a known volume of sample to be withdrawn at ambient conditions, equivalent to the volume of the injection loop, and then inserted into the fully pressurized mobile-phase flow by a simple rotation of the valve to change the mobile-phase flow paths. Although most separations are performed at room temperature, either the column alone or the whole solvent-delivery system may be thermostated to a higher temperature when this is desirable or required for the separation. The separation is monitored continuously on the low pressure side of the column using several bulk physical property, photometric, or electrochemical detectors fitted with microvolume flow cells.

Common detection principles are UV absorbance, fluorescence, refractive index and amperometry. Coupling to MS and IR spectroscopy is becoming more common, as is online coupling to nuclear magnetic resonance (NMR) spectrometers. Detection is a more difficult aspect in the condensed phase and neither the variety nor operating characteristics of LC detectors compare favourably with GC detectors, although they allow a wide range of sample types to be analysed routinely. Special materials are used in the fabrication of biocompatible and corrosion-resistant instruments for the separation of biopolymers and for ion chromatography. Individual pumps can handle solvent delivery requirements over a decade range or so of flow rates. The diversity of column diameters used in modern LC for analysis and preparative-scale applications demands flow rates that vary from a few μL per minute to tens of litres per minute. Consequently, instruments are designed for efficient operation within a particular application range and are not universal with respect to column selection. Furthermore, analytical detectors tend to be designed with sensitivity as the main concern and preparative-scale detectors for capacity, such that the two are generally not interchangeable even when the same detection principle is employed. For preparative-scale work some form of automated sample fraction collection is necessary and economy of operation may dictate incorporation of an integrated mobile-phase recycle feature.

Supercritical Fluid Chromatography

Instrumentation for SFC is a hybrid of components used in GC and LC modified to meet the requirements of operation with a compressible fluid. The mobile phase is typically carbon dioxide (with or without modifier) contained in a pressurized cylinder and delivered to the pump in liquid form. Syringe pumps or cooled reciprocating piston pumps modified for pressure control are commonly used. A high precision

pressure transducer is installed between the pump and sample inlet for programming the inlet pressure or fluid density during the course of a separation. Simultaneous measurement of the column temperature and pressure control allows constant density or density programming under computer control if the appropriate isotherms are known or can be approximated. Two pumps are generally used to generate mobile-phase composition gradients comprising liquid carbon dioxide and an organic solvent. Loop-injection valves similar to LC are the most convenient devices for sample introduction. The column oven is usually a forced air circulation thermostat similar to those used in GC. The full range of flame-based detectors used in GC can be used with only slight reoptimization as well as the main optical detectors used in LC, after modification for high pressure operation. A unique feature of the chromatograph is a restrictor required to maintain constant density along the column and to control the linear velocity of the fluid through the column. Orifice-type restrictors are usually placed between the column and detector for flame-based detectors and back-pressure regulators after the detector flow cell for optical detectors.

MEKC and CEC

MEKC and CEC employ the same instruments as used for capillary electrophoresis with the addition of overpressure capability for the buffer reservoirs when used for CEC. The separation capillary is terminated in two buffer reservoirs containing the high voltage electrodes that provide the electric field to generate the flow of mobile phase. The buffer reservoirs can be moved into place pneumatically and sequenced automatically to introduce a sample vial for sample introduction or a run buffer vial for separation. The column area is thermostated to maintain a constant temperature. A miniaturized optical detector positioned between the buffer reservoirs is commonly used for on-column detection. Some form of interlock mechanism is used to prevent operator exposure to the high voltages, up to 30–50 kV, typically used. A high level of automation is achieved under computer control and unattended operation is generally possible.

Planar Chromatography

The total automation of sample application, chromatogram development and *in situ* quantitation in planar chromatography has proved difficult. Instead the individual procedures are automated, requiring operator intervention to move the layer from one operation to the next. Samples are typically applied to the layer as spots or narrow bands using low volume dosimeters or spray-on techniques. Application volume, method, location and sample sequence are automated for unattended operation. The chromatogram is obtained by manual development in a number of development chambers of different design, or can be automated such that the conditioning of the layer, the selected solvents for the development, and the development length are preselected and controlled through the use of sensors. For multiple-development techniques the layer can be alternately developed, dried, new solvent introduced and the process repeated with changes in the development length and mobile-phase composition for any or all the programmed development steps. Apparatus for forced-flow development is also available and resembles a liquid chromatograph with the column replaced by the layer sandwiched between a rigid support and a polymeric membrane in an overpressure development chamber to allow external pressure to be used to create the desired mobile-phase velocity.

After development the chromatogram is recorded using scanning or video densitometry. The unique feature compared with detection in column chromatography is that the separation is recorded in space rather than time while in the presence of the stationary phase. The common forms of detection are optical methods based on UV–visible absorption and fluorescence. In mechanical scanning the layer is moved on a translation stage under a slit projecting the image of the monochromatic light source on the layer surface and the light reflected from the surface monitored continuously with a photodiode or similar device. Substances that absorb the light produce a proportional decrease in the intensity of the reflected light that can be related to the amount of sample present (for fluorescence there is a proportionate increase in the amount of light emitted at a wavelength that is longer than the absorbed wavelength). Electronic scanning is not as well developed but involves uniformly illuminating the whole layer and imaging the plate surface onto a video camera, or similar device, to capture and integrate the static image of the absorbing zones.

Conclusion

Many of the important developments in chromatography have already been made, yet the technique continues to evolve by the introduction of new materials that extend the scope of existing methods and through finding new applications. General applications are dominated by the techniques of gas chromatography and column liquid chromatography,

which are the most mature in terms of their evolutionary development, although it is widely recognized that column liquid chromatography still lacks a sensitive and universal detector for general applications. This void may be filled by mass spectrometry, which has made great strides in the last few years towards this goal based on particle-beam interfaces and atmospheric ionization techniques coupled with the development of low cost mass separators. By comparison, thin-layer chromatography and supercritical fluid chromatography have become recognized as techniques with niche applications and are unlikely to supplant gas and column liquid chromatography as the dominant chromatographic methods used in analytical laboratories. The microcolumn techniques of capillary electrophoresis, micellar electrokinetic chromatography, and capillary electrochromatography have quickly established themselves as useful laboratory methods and are likely to become of increasing importance as they complete their evolutionary cycle. In particular, the infant capillary electrochromatography has the potential to replace column liquid chromatography from many of its traditional separation roles, but has yet to reach a state of development to be considered as a routine laboratory technique.

The only thing that is certain about science is uncertainty. Although chromatographic methods are likely to dominate separation science for the first part of the twenty-first century, it would be a foolish person who predicts their form, continuing development, and main applications. Throughout the history of chromatography general approaches have had to adapt to changing needs brought about by dramatic shifts in the focus on different types of applications, and this has a significant impact on the relative importance of the various techniques. However, chromatography should be considered as an holistic approach to separations, and will be better understood and correctly employed if we abandon the current trend to compartmentalize the technique based on specialization in individual subject areas.

See Colour Plate 3.

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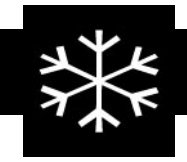
CRYSTALLIZATION



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Introduction

Crystallization from solution is a separation technique where a solid phase is separated from a mother liquor. In contrast to other separation processes, however, the dispersed phase consisting of numerous solid particles also forms the final product, that has to meet the required product specifications. Crystallization can thus also be seen as a technique to obtain solid products, where the crystallization process has to be carefully controlled in order to meet the ever-increasing demands of the customer on particle properties like particle size distribution, crystal shape, degree of agglomeration, caking behaviour and purity. Since the particles must also be easily