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# Proteins

*See* **II / CHROMATOGRAPHY / Protein Separation**

# Refractive Index Detectors in Liquid Chromatography

*See* **II / CHROMATOGRAPHY: LIQUID / Detectors: Refractive Index Detectors**

# Size Exclusion Chromatography: Mechanisms

*See* **II / CHROMATOGRAPHY: LIQUID / Mechanisms: Size Exclusion Chromatography**

# Theory of Liquid Chromatography

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Liquid chromatography (LC) involves the separation of the components of a mixture by virtue of differences in the equilibrium distribution of the components between two phases: one which is a liquid (the mobile phase) and moves in a definite direction, the other which is stationary (the stationary phase). Modern LC using very small particles and a relatively high inlet pressure is referred to as high performance liquid chromatography (HPLC).

## Stationary Phase

An advantage of LC over gas chromatography is in the variety of types of stationary and mobile phases which may be used, allowing the separation of such diverse molecular species as pharmaceuticals, agrochemicals, proteins, inorganic and organic ions and biopolymers. The use of a stationary phase with a liquid coated physically onto an inert solid support (partition chromatography) has been superseded by the use of a liquid chemically bonded on to the solid support (bonded-phase chromatography). Ideally, the support does not contribute to the separation process, but its particle size and surface properties have a profound effect on the efficiency of the process.

The term liquid–solid chromatography (LSC) covers a range of techniques:

- adsorption chromatography, when the stationary phase is an active solid (e.g. silica, alumina or a polymer) and separation is based on adsorption affinities between the sample molecules and the surface of the active solid
- ion chromatography, which uses an ion exchange medium
- exclusion chromatography using a stationary phase (e.g. a polymer or porous silica) which separates according to molecular size and shape
- affinity chromatography, which utilizes the unique biological specificity of the analyte and ligand interaction with the stationary phase

Liquid chromatography can be carried out within a tube (column chromatography) or on a flat sheet of material (planar chromatography) such as a sheet of paper (paper chromatography) or a layer of stationary phase coated on to a support, e.g. a glass or plastic sheet (thin-layer chromatography, TLC). In column chromatography, the stationary phase (liquid plus support) can fill the whole inside volume of the tube (packed column) or be concentrated along the inside wall of the tube, leaving an unrestricted path for the mobile phase in the middle of the tube (open tubular or capillary column). The term column will be used in a general sense to apply to both column and planar chromatography.

A significant difference between GC and HPLC is the permeability of columns used. The permeability ($\kappa$) is a measure of flow resistance:

$$\kappa = \eta L \bar{u} / \Delta P = \eta L^2 / \Delta P t_M \qquad [1]$$

where $\eta$ = viscosity, $L$ = column length, $\bar{u}$ = average mobile phase velocity, $\Delta P$ = pressure drop across the column and $t_M$ = residence time for an unretained substance.

Introducing the particle diameter ($d_p$), the flow resistance parameter ($\phi$) is given by:

$$\phi = d_p^2 / \kappa \qquad [2]$$

Permeable columns are the most desirable and typically $\phi$ values are $\sim 500$ for spherical microparticles.

## Mobile Phase

In LC the mobile phase is often called the solvent, but since solid samples have to be made into a solution by dissolution in a sample solvent, the use of the term solvent for the mobile phase may cause confusion. However, in order to avoid solubility problems when the sample is injected on to the column, it is safest, whenever possible, to make the sample solution using the same liquid as is to be used as the mobile phase, in which case no confusion should arise.

The time taken by a sample to pass through the column (the total elution/retention time: $t_R$) is a function of the mobile-phase velocity and the volume of mobile phase required to elute the component from the column, the total retention volume ($V_R$), is given by:

$$V_R = F \times t_R \qquad [3]$$

where $F$ is the volume flow rate of mobile phase measured at the column outlet at ambient temperature ($T_a$) and ambient pressure ($P_a$). Since column temperatures are usually close to ambient, temperature corrections on flow rate are rarely applied. Volume flow rates are measured from the time required to collect a given volume of mobile phase or with an electronic flow meter. Unlike gas chromatography, liquid mobile phases can be considered as incompressible and the flow rate is uniform throughout the column.

Linear flow rates ($\bar{u}$) are measured from the retention time, called the hold-up time, of an unretained substance ($t_M$), i.e. one which moves at the same velocity as the mobile phase:

$$\bar{u} = L / t_M \qquad [4]$$

where $L$ is the column length.

However, whereas in gas chromatography it is reasonable to assume that a substance like nitrogen or helium will have negligible solubility in the stationary phases used, in LC finding a species which is soluble in the mobile phase and will not have at least some retention on the stationary phase – therefore giving too high a hold-up time – often proves difficult. Furthermore, with a porous stationary phase, excluded components (not uncommon with mobile phases with a high water content) do not access the total mobile-phase volume and may actually run ahead of the mobile phase, giving too low a hold-up time.

Porosity can be used to verify the hold-up time. The total porosity ($\varepsilon$) of a column is the volume fraction occupied by the mobile phase:

$$\varepsilon = \frac{V\,(\text{column}) - V\,(\text{packing material})}{V\,(\text{column})} = \frac{4F\,(\text{mL min}^{-1}) t_M\,(\text{s})}{d_c^2\,(\text{mm}) L\,(\text{mm})} \qquad [5]$$

where $F$ = volume flow rate, $d_c$ = column diameter and $L$ = column length. For a totally porous material $\varepsilon \sim 0.75$ and $\sim 0.4$ for porous layer beads.

Thus, values of the hold up time obtained for a given component should give these values according to the type of packing material used. Values $< 0.75$ indicate the molecule is excluded from the pores of the stationary phase, and values $> 0.75$ indicate that retention on the stationary phase has occurred.

Choice of mobile phase is fundamental to the separation in LC. Mixtures of solvent, with up to four components are commonly used, so that as well as the chemical nature of the components, miscibility considerations are important. Physical properties, such as viscosity, volatility, refractive index and UV absorption can also limit the choice of mobile phases.

## Modes of Liquid Chromatography

Analytical LC is always carried out by elution chromatography where the mobile phase is passed continuously through the column and the sample is fed (or injected) into the system as a finite plug. If the conditions for the analysis are optimized, the sample components can be completely separated from each other. Placing a detector at the end of the column, which responds to some property of the sample, produces a trace (the chromatogram) which is a plot of detector response against time.

If the stationary phase is more polar than the mobile phase, the term normal-phase chromatography is used, and if the stationary phase is less polar than the mobile phase the term reversed-phase chromatography is used.

## The General Elution Problem

A typical chromatogram for the separation of a mixture of components which illustrates the characteristics of chromatography, and is often referred to as the general elution problem, is shown in **Figure 1**.

The properties which the chromatogram illustrates and which must be explained by any theory of chromatography are:

- The components of the mixture elute from the column at different times (retention).
- Peak widths increase with retention time (peak shape and broadening).
- The separation of pairs of peaks is not constant (column resolution).

## Chromatographic Retention

Retention parameters are measured in terms of times, mobile-phase volumes or retention factors ($k$) (previously called capacity factors: $k'$). If the flow rate is constant, the volumes are proportional to times, e.g. $t_R$ (time) is analogous to $V_R$ (volume).

If a mixture is chromatographed, the time taken for a component to be eluted from the column, the (total) retention time ($t_R$), is measured from the moment of injection to the appearance of the peak maximum. This, together with the width of the peak measured at the baseline ($w$) or at half peak height ($w_h$), and the elution of an unretained peak, are important parameters in chromatography and are illustrated in **Figure 2**, which shows the separation of a two-component mixture eluting with retention times $(t_R)_1$ and $(t_R)_2$, with an unretained component (retention time, $t_M$).

The retention volume ($V_M$) of an unretained peak (where $V_M = F \times t_M$) is also called the mobile-phase volume and equals the volume (both inter- and intraparticle) available to the mobile phase in the column.

The adjusted retention time/volume ($t'_R/V'_R$) is the total elution time/volume minus the retention

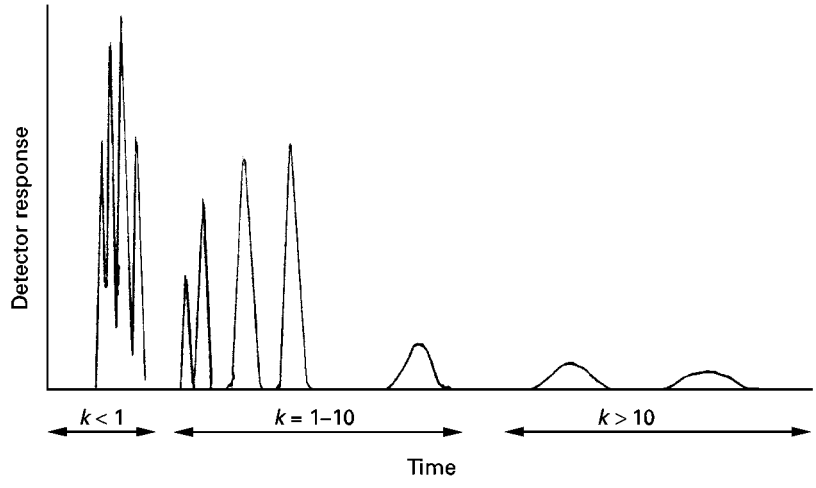


**Figure 1** The general elution problem.

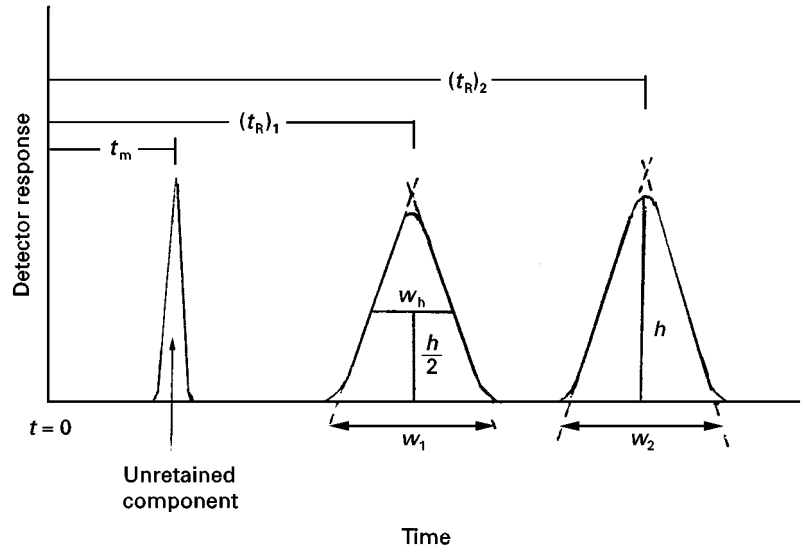


**Figure 2** Separation of a two-component mixture showing retention parameters. See text for details.

time/volume of the mobile phase:

$$t'_R = t_R - t_M; \quad V'_R = V_R - V_M \qquad [6]$$

The unretained peak, which has no affinity for the stationary phase and does not exhibit exclusion, passes through the column at the same speed as the mobile phase. A substance which shows affinity for the stationary phase moves through the column more slowly than the mobile phase and is said to be retained. The ratio of the two velocities is known as the retardation factor ($R$):

$$R = \frac{\text{rate of movement of retained peak}}{\text{rate of movement of mobile phase}} \qquad [7]$$

A retained component spends time in both the mobile phase ($t_M$) and the stationary phase ($t_S$) and retention time $t_R$ is given by:

$$t_R = t_M + t_S \qquad [8]$$

The time spent in the stationary phase is dependent on the distribution coefficient ($K_c$) such that $t_S = K_c V_S$. If $C_S$ and $C_M$ are the concentrations of a component in the stationary phase and mobile phase respectively, the distribution constant is given by:

$$K_c = C_S/C_M \qquad [9]$$

The rate of movement of a component through the column is inversely proportional to the distribution constant, i.e. a substance with a high concentration in the stationary phase (a high distribution coefficient) moves slowly through the column. Components of a mixture are, therefore, separated only if their distribution coefficients differ. Using volumes rather than times we can write:

$$V_R = V_M + K_c V_S \quad \text{or} \quad V'_R = K_c V_S \qquad [10]$$

which is the fundamental equation for chromatography, neglecting the effects of nonlinearity of the sorption isotherm and band broadening.

In adsorption chromatography the stationary-phase volume is replaced by the surface area ($A_S$) of the stationary phase, and the distribution coefficient is changed to the adsorption coefficient ($K_A$).

An alternative expression (the retention factor: $k$) for the distribution of a sample component is in terms of the relative number of moles ($n$) of a component in the stationary and mobile phases, such that:

$$k = n_S/n_M = K_c \cdot (V_S/V_M) \qquad [11]$$

$$R = \frac{\text{amount of solute in the mobile phase}}{\text{amount of solute in mobile + stationary phases}} \qquad [12]$$

$$\text{or} \quad R = n_M/(n_M + n_S)$$

$$= 1/(1 + k) \qquad [13]$$

Substituting the retention factor into the equation:

$$V_R = V_M + K_C V_S \quad \text{gives} \quad V_R = V_M(1 + k) \qquad [14]$$

or using retention times:

$$t_R = t_M(1 + k) \quad [15]$$

and on rearrangement:

$$k = (t_R - t_M)/t_M \quad [16]$$

This last expression is widely used as a simple way of expressing retention from values easily measured from the chromatogram, and without the need to measure flow rates.

Since:

$$t_M = L/\bar{u} \quad [17]$$

we can write:

$$t_R = \frac{L}{\bar{u}}(1 + k) \quad [18]$$

Hence the retention time is directly proportional to the column length and inversely proportional to the linear flow rate of the mobile phase.

## Peak Shape and Broadening

The variation of solute concentration in the stationary phase with solute concentration in the mobile phase, at constant temperature, is known as the sorption isotherm. Simple chromatographic theory assumes a linear isotherm relationship, i.e. the distribution coefficient is constant. Under these conditions the retention time is independent of sample concentration and the peak moves with a constant speed. Given a peak profile with plug-shape distribution on injection, this shape should be maintained as the peak passes through the column to emerge at the exit. However, because of longitudinal diffusion in the direction of flow, the peak takes on a Gaussian distribution. If the isotherm relationship is nonlinear (e.g. Langmuir or anti-Langmuir), the distribution coefficient is not constant but varies with solute concentration and there is a distribution of solute velocities across the peak, which is described as tailing or fronting. This relationship between isotherm shape and peak shape is illustrated in **Figure 3**.

The width of a chromatographic peak is a function of the column efficiency, expressed as the plate number ($N$), calculated from the following equations depending on the value used for the peak width (Figure 2):

$$N = (V_R/\sigma)^2 = (t_R/\sigma)^2 = 16(t_R/w_b)^2 = 5.545\,(t_R/w_h)^2 \quad [19]$$

where $\sigma$ is the standard deviation of the Gaussian peak.

The column length divided by the plate number gives the plate height or height equivalent to a theoretical plate ($H$) and normalizes the plate number for column length: $H = L/N$. The concept of plates in chromatographic theory (the plate theory) is by analogy with the distillation process and represents a notional length of the column in which the solute molecules reach a distribution equilibrium. Thus,

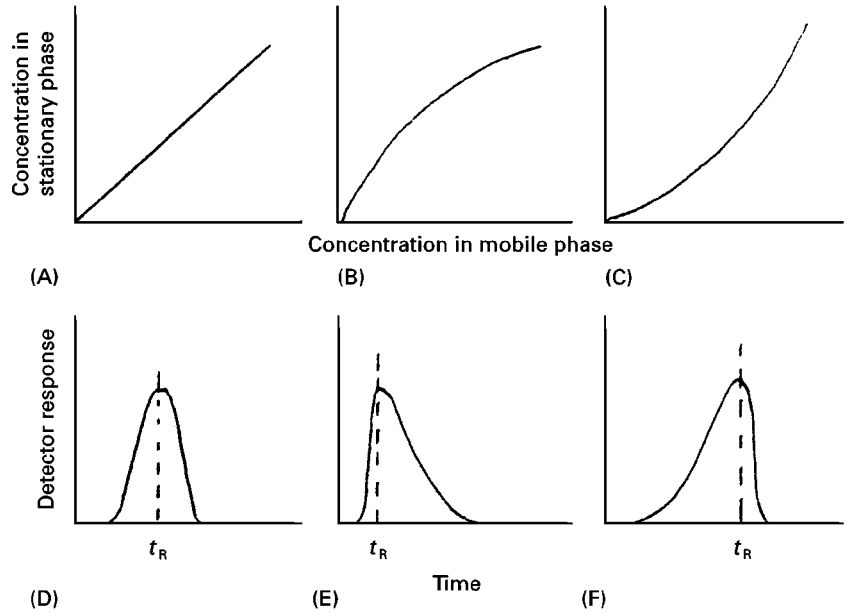


**Figure 3** Isotherm shape and their effect on peak shape and retention times. (A) Linear; (B) Langmuir; (C) anti-Langmuir; (D) Gaussian; (E) tailing; (F) fronting.

a large number of theoretical plates corresponds to an efficient column.

Consideration of the chromatographic process as controlled by equilibrium gives a satisfactory explanation of chromatographic retention in term of the distribution coefficients, but in considering band broadening a different approach is required – the rate theory of chromatography. This was first applied by van Deemter, Klinkenberg and Zuiderweg to gas chromatography, but has been extended to include LC. As the solute band passes through the column the band width increases and the solute is diluted by the mobile phase. Although the process of fluid flow is complex, three main contributions to band broadening (i.e. to the variance ($\sigma^2$) of the Gaussian peak) may be recognized: the multipath effect (formally called eddy diffusion), molecular diffusion and mass transfer.

#### The Multipath Effect (the *A* term)

Molecules flowing through a packed bed of stationary phase will take paths of different lengths resulting in a small difference in retention times. This has the effect of broadening the band by an amount dependent on the particle diameter ($d_p$), such that:

$$A = 2\lambda d_p \qquad [20]$$

The packing constant ($\lambda$) is an empirical term depending on the shape (spherical or irregular) of the packing material and the packing efficiency, and reaches a minimum value $\cong 0.5$. For open tubular columns there is no $A$ term.

#### Longitudinal Molecular Diffusion (the *B* term)

Solute molecules diffuse in a longitudinal direction (i.e. along the column axis) according to Fick's law of diffusion. The amount of band spreading is directly proportional to the coefficient of diffusion ($D_M$) of the solute molecules in the mobile phase, and inversely proportional to the mobile-phase flow rate. An obstruction factor ($\psi$) is introduced to account for the restricted diffusion in a packed bed.

Hence:

$$B = 2\psi D_M/\bar{u} \qquad [21]$$

Longitudinal molecular diffusion is only significant in LC if small ($<10$ μm) stationary-phase particles are used at low mobile phase velocities and with a relatively high solute diffusion coefficient.

#### Mass Transfer (the *C* terms)

In LC, band broadening due to mass transfer is a complex process involving both the stationary and mobile phases. Two processes are responsible for band broadening in the stationary phase (stationary phase mass transfer: $C_s$). The first of these processes involves the finite rate of mass transfer across the mobile-phase/stationary-phase interface. At the head of the column the solute is distributed between the stationary and mobile phases according to the value of the distribution coefficient. As the band moves down the column, solute at the leading edge of the band is continually meeting new stationary phase, into which it dissolves. To maintain the equilibrium, solute will dissolve from the trailing edge of the band out of the stationary phase back into the mobile phase. Because this process is not instantaneous the band is broadened. The second process involves the statistical distribution of the rates of diffusion of individual molecules in the stationary phase, resulting in small differences in the time that individual molecules spend in the stationary phase. A fast-moving mobile mass sweeps the zone more rapidly through the column and accentuates the band broadening as does a greater film thickness ($d_f$) of stationary phase. A higher rate of solute diffusion in the stationary phase ($D_S$) will decrease the band broadening so that:

$$C_s = q \cdot \frac{k}{(1+k)^2} \frac{d_f^2 \bar{u}}{D_S} \qquad [22]$$

where $q$ is a configuration factor depending on the nature of the stationary phase.

In adsorption chromatography, the $C_s$ term is expressed in terms of the adsorption/desorption kinetics of the solute molecules on the stationary phase.

Band broadening in the mobile phase also results from two different processes. Moving mobile phase mass transfer ($C_M$) results from frictional forces which modify the laminar flow profile across a channel between two particles, resulting in a higher flow velocity in the centre of the channel. Stagnant mobile-phase mass transfer ($C_M^*$) is the result of slow diffusion of solute molecules in and out of the pores of a porous stationary phase. The overall mobile-phase mass transfer can be represented by the expression:

$$C_M = f(d_p^2, d_c^2) \cdot \bar{u}/D_M \qquad [23]$$

where $d_p$ is the particle diameter and $d_c$ is the column diameter, $D_M$ the solute diffusion coefficient in the mobile phase and $\bar{u}$ is the linear velocity.

Giddings, recognizing that molecules are free to diffuse from one flow path into another, introduced the idea of 'coupling' the multipath term ($A$) and the mobile-phase mass transfer ($C_M$) so that the variation of the plate height ($H$) with $\bar{u}$ is then given by:

$$H = B/\bar{u} + C_s\bar{u} + C_M^*\bar{u} + \{1/A + 1/C_M \cdot \bar{u}\}^{-1} \qquad [24]$$

The contribution of the various terms to the total plate height is illustrated in **Figure 4**.

### Extra-Column Band Broadening

So far, we have only considered band-broadening processes within the chromatographic column itself but, in assessing the overall performance of the system, the instrument as a whole is important. Thus, the injection system, detector and connecting tubing all contribute to the overall peak shape. The objective for injection is to get the sample on to the column in as narrow a plug as possible. Slow transfer of the sample from the injector to the column causes peak broadening and peak tailing. Large dead volumes in the detector can lead to remixing of components and deterioration of the separation as well as dilution of the sample peaks, reducing detection limits. The peak broadening in an open tube (radius $r$ and length $L$), volume flow rate $F$, and for a solute with diffusion coefficient $D_M$ is given by:

$$\pi r^4 FL/24D_M \qquad [25]$$

In particular, short lengths ( $< 30$ cm) of narrow-bore ( $\sim 0.01$ in) connecting tubing should be used.

## Column Resolution

Chromatographic separation is only achieved when there is a difference in the distribution coefficients of two components, i.e. the molecular interactions (dispersion forces, dipole interactions and hydrogen-bonding forces) between the sample molecules and the stationary phase are sufficiently different. More fundamentally it is the free energies of distribution $\Delta(\Delta G^\theta)$ of the components of a mixture which must differ. It can be shown that:

$$\Delta(\Delta G^\theta) = -RT \ln \alpha = -RT \ln [(K_c)_2/(K_c)_1] \quad [26]$$

A stationary phase which produces a large degree of separation is said to have high selectivity. The separation of two components (1 and 2) is expressed by the relative retention ($\alpha$):

$$\alpha = t'_{R(2)}/t'_{R(1)} = V'_{R(2)}/V'_{R(1)} = k_{(2)}/k_{(1)} = K_{C(2)}/K_{C(1)} \qquad [27]$$

If one of the pair is a standard substance, the symbol used for relative retention is $r$. Having achieved a separation, it is necessary to prevent remixing of the components and the ability to achieve this is a function of the column efficiency, as measured by the plate number. The combined effects of stationary-phase selectivity and column efficiency are expressed in the peak resolution ($R_s$) of the column:

$$R_s = \frac{(t_R)_2 - (t_R)_1}{(w_1 + w_2)/2} \qquad [28]$$

A value of $R_s = 1.5$ is normally considered to represent baseline separation for Gaussian-shaped peaks. To achieve the maximum peak resolution, both high selectivity and column efficiency (giving narrow bands) are required.

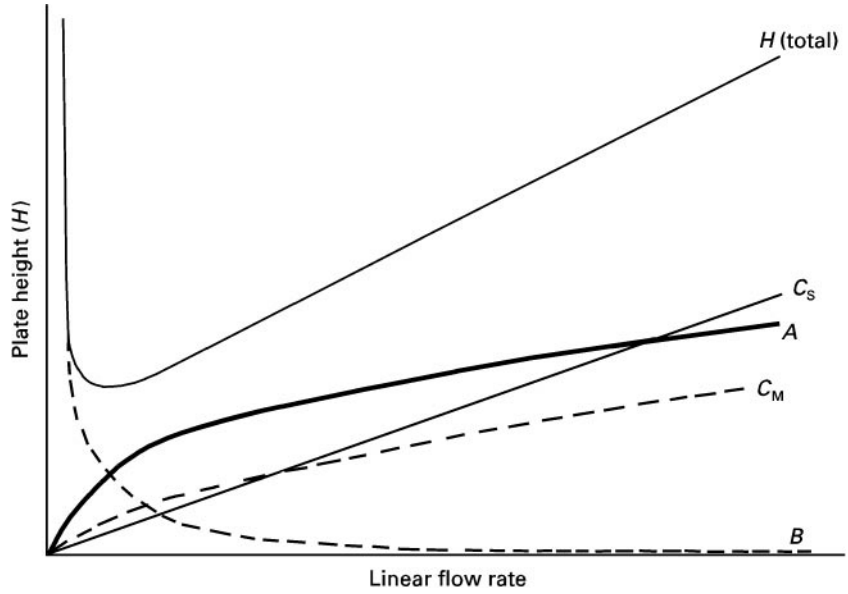


**Figure 4** The van Deemter plot for LC and the variation of the terms $A$, $B$, $C_s$ and $C_M$ with flow rate. See text for details.

Increased resolution can always be achieved by an increase in column length since the peak separation ($\Delta t_R$) is proportional to the distance of migration down the column, but peak width is only proportional to the square root of the migration distance. The penalty for this, however, is longer retention times and an increased inlet pressure of mobile phase.

The Purnell equation shows how peak resolution is related to the retention factor ($k$), the plate number ($N$) and the relative retention ($\alpha$):

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{k_2}{1 + k_2} \qquad [29]$$

where the subscript 2 refers to the second peak.

Conditions for obtaining maximum values of the plate number have already been discussed. In LC the relative retention is governed by the nature of the mobile phase as well as the stationary phase. In order to obtain a satisfactory separation, a balance must be achieved between the interactions (dispersion forces, dipole–dipole interactions, dipole-induced–dipole interactions, H-bonded forces), represented in **Figure 5** by an interaction triangle.

If the interactions between the sample and stationary phase are too strong, the retention times will be excessively long, whereas if the interactions between sample and mobile phase are too strong, retention times will be too short. Modifications to the mobile phase (e.g. the addition of ion-pairing agents, chiral molecules) may also be used to change stationary-phase–mobile-phase interactions.

The interactions are maximized in the concept of 'like has an affinity for like'. Thus, for a sample which contains predominantly nonpolar species, a nonpolar stationary/mobile phase will optimize the dispersion forces, and polar interactions will be absent. For polar samples a polar stationary/mobile phase will maximize both dipole–dipole interactions and dipole-induced–dipole interactions and the effect of dispersion forces will be reduced. At least a partial separation can be achieved with $\alpha$ values as low as 1.01, but values in the range 1.5–3.0 are preferable but above values of about 5.0 little additional resolution is achieved.

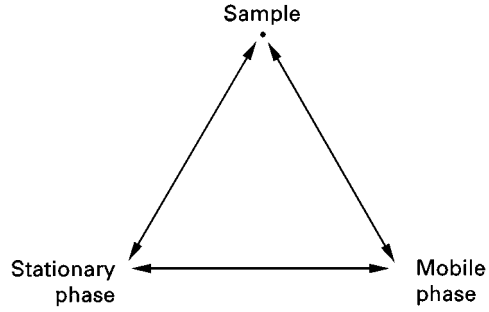


**Figure 5** The interaction triangle in LC.

Peak resolution increases rapidly with increasing $k$ values, but at values $> 10$ the term $k_2/(1 + k_2) \rightarrow 1$ and the term plays no further part in the resolution. The use of $k$ values $< 1$ gives very short retention times and poor resolution, so that the optimum for $k$ is between 1 and 10. The retention equation $t_R = L/\bar{u}(1 + k)$ shows that retention times are a function of both the mobile-phase velocity ($\bar{u}$) and the retention factor.

In LC, temperature has little effect on retention, because of the relatively small values of the enthalpies of solution involved. The van't Hoff equation describes the change in equilibrium constant with temperature and if the phase ratio ($V_S/V_M$) is independent of temperature we can also write for the retention factor:

$$\frac{d \ln k}{dT} = \frac{\Delta H}{RT^2} \qquad [30]$$

where $\Delta H$ is the enthalpy of solution (or adsorption) from the mobile phase to the stationary phase.

The main use of temperature control is to increase the reproducibility of retention times, and to increase column efficiency through the effect of temperature on viscosity and diffusion. The first choice to be made is, therefore, the choice of chromatographic mode (normal phase or reversed phase) and the stationary phase. In practice, the reversed-phase mode can be used for samples with a wide range of polarities and is usually the mode of choice. Bonded stationary phases with a hydrocarbon chain attached to the silica surface (e.g. Si–$C_{18}$) are widely used; the polarity of the phase may be modified by introducing functional groups (e.g. –CN, –$NH_2$) into the chain.

To select the mobile phase, the concept of solvent strength and polarity is utilized. A strong solvent is one which causes a sample to elute rapidly from the column. Various measures of solvent strength are used:

- solvent strength parameter ($E^\circ$), based on the adsorption energies of the solvent on alumina
- solvent polarity parameter ($P'$), based on experimental solubility data which reflects the proton acceptor, proton donor and dipole interactions of the solvent molecule
- the Hildebrand solubility parameter ($\delta$), which measures dispersion and dipole interactions, and hydrogen acceptor and donor properties.

Figure 1 for the general elution problem also shows values of $k$ for different zones of the chromatogram. With low $k$ values ($k < 1$) the peaks are eluted too rapidly and there is no time for separation. With high $k$ values ($k > 10$) elution times are long, the peaks are

broad and the peaks are over-resolved. This problem can be corrected using the technique of gradient elution which is analogous to temperature programming in gas chromatography. Assuming that the chromatogram in Figure 1 was obtained isocratically using a binary mixture of 50% methanol–water, it would be possible to choose a lower solvent strength mixture (e.g. 25% methanol–water) and then increase the methanol content (say to 75%) over a given period of time. This would have the effect of increasing the $k$ values for the early peaks and decreasing the $k$ values for the later peaks, the object being to get all peaks in the optimum region, where $k$ is between 1 and 10. Modern computer-controlled liquid chromatographs have the facility to use isocratic periods and linear and nonlinear gradient programs with multiple ramps to give better control over $k$ values.

The retention equation also indicates that a similar effect could be achieved using the analogous technique of flow programming and changing the mobile-phase flow rate. Having optimized the retention factors by gradient elution, it may still be necessary to alter the selectivity of the system in order to achieve a complete separation. Using the solvent strength parameters, solvents can be classified into eight groups according to their proton acceptor, proton donor and dipole properties and a triangular graph constructed. Solvents are selected from the different classes in order to maximize differences in their properties (bearing in mind the need for solvent compatability, e.g. miscibility).

For the solvent polarity parameter the value of $P'$ for a binary mixture ($AB$) is given by:

$$P'_{AB} = \phi_A P'_A + \phi_B P'_B \qquad [31]$$

where $\phi_A$ and $\phi_B$ are volume fractions of $A$ and $B$.

From this relationship it is possible to calculate mixtures of solvents having the same solvent strengths, e.g. 45% methanol–water, 52% acetonitrile–water, 37% tetrahydrofuran–water all have the same solvent strength and would give the same retention factors ($k$), but because of their different proton acceptor/donor and dipole properties they would give different selectivities ($\alpha$ values).

A satisfactory separation is achieved when all three terms in the Purnell equation are optimized.

## Future Developments

The theory of LC is well established but a greater understanding of the complexities of fluid flow may lead to improved column performance. Developments in the understanding of the interactions between solute molecules and stationary/mobile phases and the preparation of new stationary phases (e.g. chiral phases) will lead to new applications. Developments in the field of capillary electrophoresis may erode some of the traditional fields of application of LC, e.g. in the analysis of ionic compounds. Developments in instrumentation with new coupled techniques are also a possibility. The most likely area for further development is in the area of data handling and instrument control using the newer breed of computers.

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# Ultraviolet and Visible Detection in Liquid Chromatography

*See* **II / CHROMATOGRAPHY: LIQUID / Detectors: Ultraviolet and Visible Detection**