1

General aspects of chromatography

Chromatography, the process by which the components of a mixture can be separated, has become one of the primary analytical methods for the identification and quantification of compounds in the gaseous or liquid state. The basic principle is based on the concentration equilibrium of the components of interest, between two immiscible phases. One is called the stationary phase, because it is immobilized within a column or fixed upon a support, while the second, called the mobile phase, is forced through the first. The phases are chosen such that components of the sample have differing solubilities in each phase. The differential migration of compounds lead to their separation. Of all the instrumental analytical techniques this hydrodynamic procedure is the one with the broadest application. Chromatography occupies a dominant position that all laboratories involved in molecular analysis can confirm.

1.1 General concepts of analytical chromatography

Chromatography is a physico-chemical method of separation of components within mixtures, liquid or gaseous, in the same vein as distillation, crystallization, or the fractionated extraction. The applications of this procedure are therefore numerous since many of heterogeneous mixtures, or those in solid form, can be dissolved by a suitable solvent (which becomes, of course, a supplementary component of the mixture).

A basic chromatographic process may be described as follows (Figure 1.1):

1. A vertical hollow glass tube (the *column*) is filled with a suitable finely powdered solid, the *stationary phase*.
2. At the top of this column is placed a small volume of the sample mixture to be separated into individual components.

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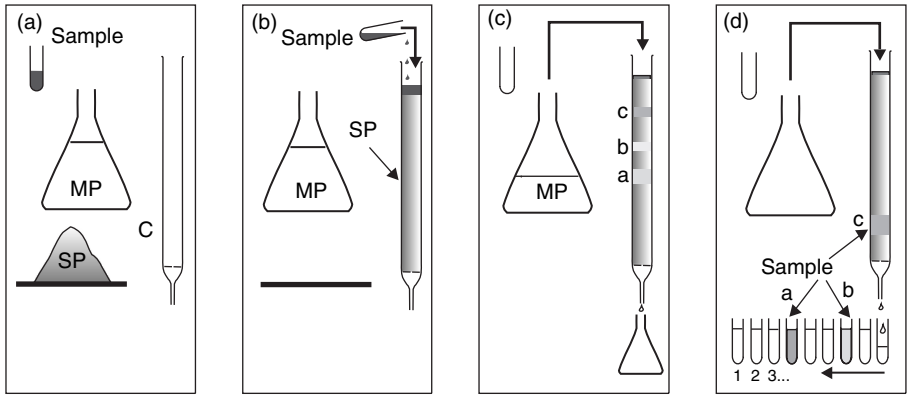

Figure 1.1 *A basic experiment in chromatography*. (a) The necessary ingredients (C, column; SP, stationary phase; MP, mobile phase; and S, sample); (b) introduction of the sample; (c) start of elution; (d) recovery of the products following separation.

3. The sample is then taken up by continuous addition of the *mobile phase*, which goes through the column by gravity, carrying the various constituents of the mixture along with it. This process is called *elution*. If the components migrate at different velocities, they will become separated from each other and can be recovered, mixed with the mobile phase.

This basic procedure, carried out in a column, has been used since its discovery on a large scale for the separation or purification of numerous compounds (*preparative column chromatography*), but it has also progressed into a stand-alone *analytical technique*, particularly once the idea of measuring the migration times of the different compounds as a mean to identify them had been conceived, without the need for their collection. To do that, an optical device was placed at the column exit, which indicated the variation of the composition of the eluting phase with time. This form of chromatography, whose goal is not simply to recover the components but to control their migration, first appeared around 1940 though its development since has been relatively slow.

The identification of a compound by chromatography is achieved by comparison: To identify a compound which may be A or B, a solution of this unknown is run on a column. Next, its *retention time* is compared with those for the two reference compounds A and B previously recorded using the same apparatus and the same experimental conditions. The choice between A and B for the unknown is done by comparison of the retention times.

In this experiment a true separation had not been effected (A and B were pure products) but only a comparison of their times of migration was performed. In such an experiment there are, however, three unfavourable points to note: the procedure is fairly slow; absolute identification is unattainable; and the physical contact between the sample and the stationary phase could modify its properties, therefore its retention times and finally the conclusion.

This method of separation, using two immiscible phases in contact with each other, was first undertaken at the beginning of the 20th century and is credited to botanist Michaël Tswett to whom is equally attributed the invention of the terms *chromatography* and *chromatogram*.

The technique has improved considerably since its beginnings. Nowadays chromatographic techniques are piloted by computer software, which operate highly efficient miniature columns able to separate nano-quantities of sample. These instruments comprise a complete range of accessories designed to assure reproducibility of successive experiments by the perfect control of the different parameters of separation. Thus it is possible to obtain, during successive analyses of the same sample conducted within a few hours, recordings that are reproducible to within a second (Figure 1.2).

The essential recording that is obtained for each separation is called a *chromatogram*. It corresponds to a two-dimensional diagram traced on a chart paper or a screen that reveals the variations of composition of the eluting mobile phase as it exits the column. To obtain this document, a sensor, of which there exists a great variety, needs to be placed at the outlet of the column. The detector signal appears as the ordinate of the chromatogram while time or alternatively elution volume appears on the abscissa.

■ The identification of a molecular compound only by its retention time is somewhat arbitrary. A better method consists of associating two different complementary methods, for example, a chromatograph and a second instrument on-line, such as a

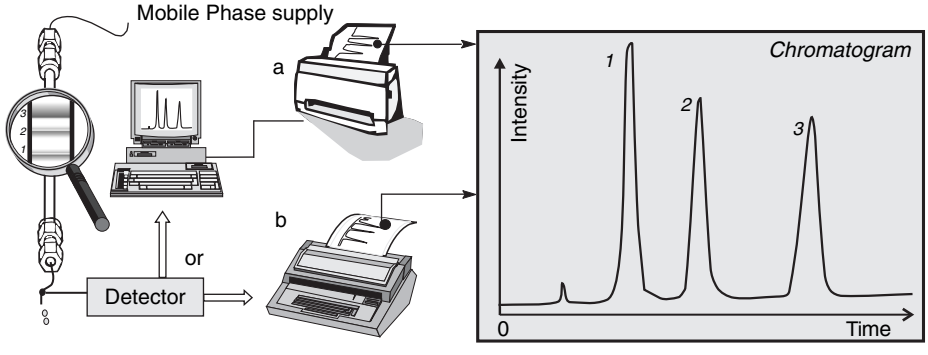

Figure 1.2 *The principle of analysis by chromatography*. The chromatogram, the essential graph of every chromatographic analysis, describes the passage of components. It is obtained from variations, as a function of time, of an electrical signal emitted by the detector. It is often reconstructed from values that are digitized and stored to a microcomputer for reproduction in a suitable format for the printer. (a). For a long time the chromatogram was obtained by a simple chart recorder or an integrator (b). Right, a chromatogram illustrating the separation of a mixture of at least three principal components. Note that the order of appearance of the compounds corresponds to the relative position of each constituent on the column.

mass spectrometer or an infrared spectrometer. These hyphenated techniques enable the independent collating of two different types of information that are independent (time of migration and 'the spectrum'). Therefore, it is possible to determine without ambiguity the composition and concentration of complex mixtures in which the concentration of compounds can be of the order of nanograms.

1.2 The chromatogram

The *chromatogram* is the representation of the variation, with time (rarely volume), of the amount of the analyte in the mobile phase exiting the chromatographic column. It is a curve that has a baseline which corresponds to the trace obtained in the absence of a compound being eluted. The separation is complete when the chromatogram shows as many *chromatographic peaks* as there are components in the mixture to be analysed (Figure 1.3).

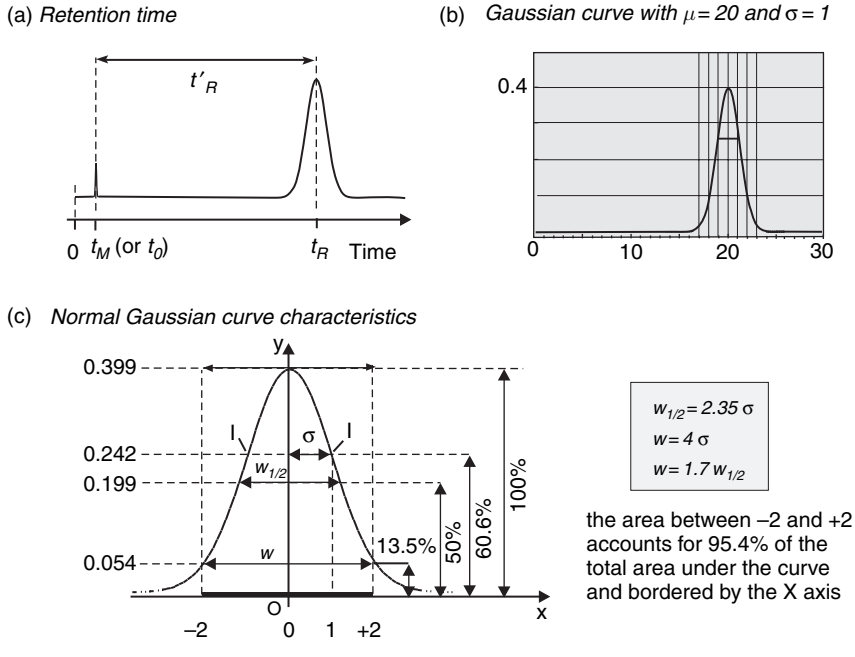

Figure 1.3 *Chromatographic peaks.* (a) The concept of retention time. The hold-up time t_M is the retention time of an unretained compound in the column (the time it took to make the trip through the column); (b) Anatomy of an ideal peak; (c) Significance of the three basic parameters and a summary of the features of a Gaussian curve; (d) An example of a real chromatogram showing that while travelling along the column, each analyte is assumed to present a Gaussian distribution of concentration.

(d) *Comparaison between a true chromatrogram and normal Gaussian-shaped peaks*

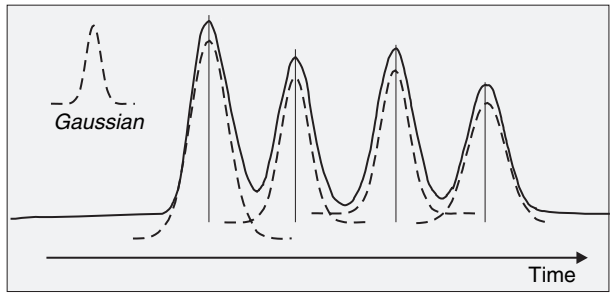

Figure 1.3 (*Continued*)

A constituent is characterized by its *retention time* t_R, which represents the time elapsed from the sample introduction to the detection of the peak maximum on the chromatogram. In an ideal case, t_R is independent of the quantity injected.

A constituent which is not retained will elute out of the column at time t_M, called the *hold-up time* or *dead time* (formerly designated t_0). It is the time required for the mobile phase to pass through the column.

The difference between the retention time and the hold-up time is designated by the *adjusted retention time* of the compound, t'_R.

If the signal sent by the sensor varies linearly with the concentration of a compound, then the same variation will occur for the area under the corresponding peak on the chromatogram. This is a basic condition to perform quantitative analysis from a chromatogram.

1.3 Gaussian-shaped elution peaks

On a chromatogram the perfect elution peak would have the same form as the graphical representation of the law of Normal distribution of random errors (Gaussian curve 1.1, cf. Section 22.3). In keeping with the classic notation, μ would correspond to the retention time of the eluting peak while σ to the standard deviation of the peak (σ^2 represents the *variance*). y represents the signal as a function of time x, from the detector located at the outlet of the column (Figure 1.3).

This is why ideal elution peaks are usually described by the probability density function (1.2).

$$y = \frac{1}{\sigma\sqrt{2\pi}} \cdot \exp\left[-\frac{(x-\mu)^2}{2\sigma^2}\right] \tag{1.1}$$

$$y = \frac{1}{\sqrt{2\pi}} \cdot \exp\left[-\frac{x^2}{2}\right] \tag{1.2}$$

This function is characterized by a symmetrical curve (maximum for $x = 0$, $y = 0.3999$) possessing two inflection points at $x = +/- 1$ (Figure 1.3), for which the ordinate value is 0.242 (being 60.6 per cent of the maximum value). The width of the curve at the inflection points is equal to 2σ, ($\sigma = 1$).

In chromatography, $w_{1/2}$ represents the width of the peak at half-height ($w_{1/2} = 2.35\sigma$) and σ^2 the *variance* of the peak. The width of the peak 'at the base' is labelled w and is measured at 13.5 per cent of the height. At this position, for the Gaussian curve, $w = 4\sigma$ by definition.

Real chromatographic peaks often deviate significantly from the Gaussian ideal aspect. There are several reasons for this. In particular, there are irregularities of concentration in the injection zone, at the head of the column. Moreover, the speed of the mobile phase is zero at the wall of the column and maximum in the centre of the column.

The observed asymmetry of a peak is measured by two parameters, the *skewing factor a* measured at 10 per cent of its height and the *tailing factor TF* measured at 5 per cent (for the definition of these terms, see Figure 1.4):

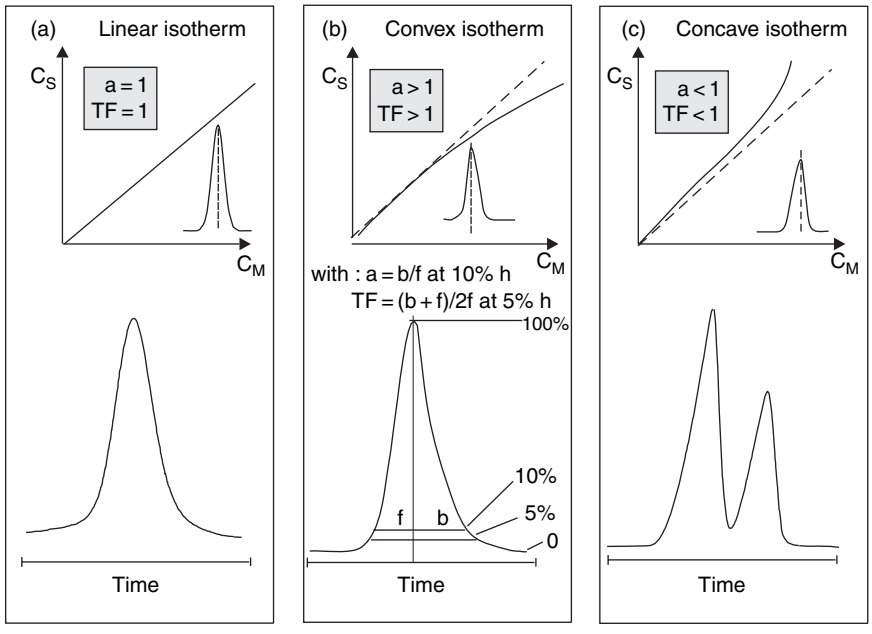

Figure 1.4 *Distribution isotherms.* (a) The ideal situation corresponding to the invariance of the concentration isotherm. (b) Situation in which the stationary phase is saturated – as a result of which the ascent of the peak is faster than the descent (skewing factor greater than 1); (c) The inverse situation : the constituent is retained too long by the stationary phase, the retention time is therefore extended and the ascent of the peak is slower than the descent apparently normal. For each type of column, the manufacturers indicate the capacity limit expressed in ng/compound, prior to a potential deformation of the corresponding peak. The situations (a), (b) and (c) are illustrated by authentic chromatograms taken out from liquid chromatography technique.

$$a = \frac{b}{f} \tag{1.3}$$

$$TF = \frac{b+f}{2f} \tag{1.4}$$

1.4 The plate theory

For half a century different theories have been and continue to be proposed to model chromatography and to explain the migration and separation of analytes in the column. The best known are those employing a statistical approach (stochastic theory), the theoretical plate model or a molecular dynamics approach.

To explain the mechanism of migration and separation of compounds on the column, the oldest model, known as Craig's *theoretical plate model* is a static approach now judged to be obsolete, but which once offered a simple description of the separation of constituents.

Although chromatography is a dynamic phenomenon, Craig's model considered that each solute moves progressively along a sequence of distinct static steps. In liquid–solid chromatography this elementary process is represented by a cycle of adsorption/desorption. The continuity of these steps reproduces the migration of the compounds on the column, in a similar fashion to that achieved by a cartoon which gives the illusion of movement through a sequence of fixed images. Each step corresponds to a new state of equilibrium for the *entire* column.

These successive equilibria provide the basis of *plate theory* according to which a column of length L is sliced horizontally into N fictitious, small plate-like discs of same height H and numbered from 1 to n. For each of them, the concentration of the solute in the mobile phase is in equilibrium with the concentration of this solute in the stationary phase. At each new equilibrium, the solute has progressed through the column by a distance of one disc (or plate), hence the name *theoretical plate theory*.

The *height equivalent to a theoretical plate* (HETP or H) will be given by equation (1.5):

$$H = \frac{L}{N} \tag{1.5}$$

This employs the polynomial approach to calculate, for a given plate, the mass distributed between the two phases present. At instant I, plate J contains a total mass of analyte m_{T} which is composed of the quantity m_{M} of the analyte that has just arrived from plate $J-1$ carried by the mobile phase formerly in equilibrium at instant $I-1$, to which is added the quantity m_{S} already present in the stationary phase of plate J at time $I-1$ (Figure 1.5).

$$m_{\mathrm{T}}(I, J) = m_{\mathrm{M}}(I-1, J-1) + m_{\mathrm{S}}(I-1, J)$$

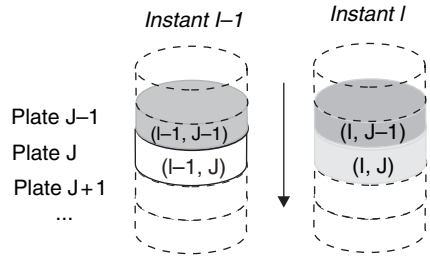

Figure 1.5 *Schematic of a column cross-section.*

If it is assumed for each theoretical plate that: $m_S = Km_M$ and $m_T = m_M + m_S$, then by a recursive formula, m_T (as well as m_M and m_S), can be calculated. Given that for each plate the analyte is in a concentration equilibrium between the two phases, the total mass of analyte in solution in the volume of the mobile phase V_M of the column remains constant, so long as the analyte has not reached the column outlet. So, the chromatogram corresponds to the mass in transit carried by the mobile phase at the $(N+1)th$ plate (Figure 1.6) during successive equilibria. This theory has a major fault in that it does not take into account the dispersion in the column due to the diffusion of the compounds.

■ The plate theory comes from an early approach by Martin and Synge (Nobel laureates in Chemistry, 1952), to describe chromatography by analogy with distillation

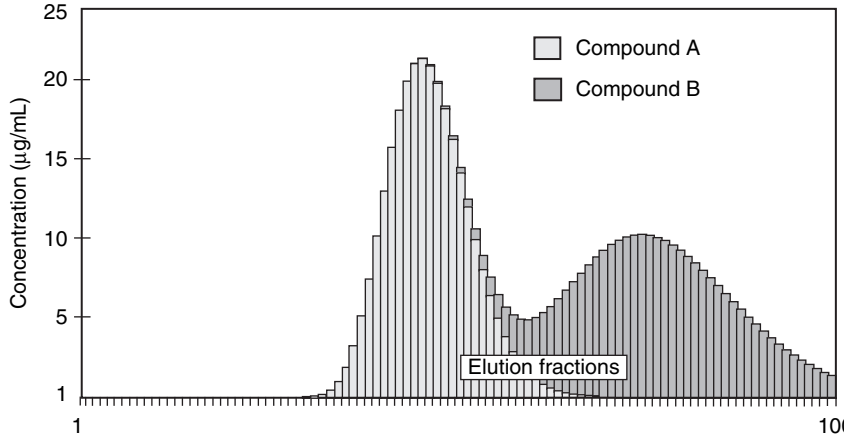

Figure 1.6 *Theoretical plate model.* Computer simulation, aided by a spreadsheet, of the elution of two compounds A and B, chromatographed on a column of 30 theoretical plates ($K_A = 0.6$; $K_B = 1.6$. $M_A = 300\,\mu g$; $M_B = 300\,\mu g$).The diagram represents the composition of the mixture at the outlet of the column after the first 100 equilibria. The graph shows that application of the model gives rise to a non-symmetrical peak (Poisson summation). However, taking account of compound diffusion and with a larger number of equilibriums, the peaks look more and more like a Gaussian distribution.

and counter current extraction as models. This term, used for historical reasons, has no physical significance, in contrast to its homonym which serves to measure the performances of a distillation column.

The *retention time* t_R, of the solute on the column can be sub-divided into two terms: t_M (hold-up time), which cumulates the times during which it is dissolved in the mobile phase and travels at the same speed as this phase, and t_S the cumulative times spent in the stationary phase, during which it is immobile. Between two successive transfers from one phase to the other, it is accepted that the concentrations have the time to re-equilibrate.

■ In a chromatographic phase system, there are at least three sets of equilibria: solute/mobile phase, solute/stationary phase and mobile phase/stationary phase. In a more recent theory of chromatography, no consideration is given to the idea of molecules immobilized by the stationary phase but rather that were simply slowed down when passing in close proximity.

1.5 Nernst partition coefficient (K)

The fundamental physico-chemical parameter of chromatography is the equilibrium constant K, termed the *partition coefficient*, quantifying the ratio of the concentrations of each compound within the two phases.

$$K = \frac{C_S}{C_M} = \frac{\text{Molar concentration of the solute in the stationary phase}}{\text{Molar concentration of the solute in the mobile phase}} \qquad (1.6)$$

Values of K are very variable since they can be large (e.g. 1000), when the mobile phase is a gas or small (e.g. 2) when the two phases are in the condensed state. Each compound occupies only a limited space on the column, with a variable concentration in each place, therefore the true values of C_M and C_S vary in the column, but their ratio is constant.

Chromatography and thermodynamics. Thermodynamic relationships can be applied to the distribution equilibria defined above. K, (C_S/C_M), the equilibrium constant relative to the concentrations C of the compound in the mobile phase (M) and stationary phase (S) can be calculated from chromatography experiments. Thus, knowing the temperature of the experiment, the variation of the standard free energy $\Delta G°$ for this transformation can be deduced:

$$C_M \Leftrightarrow C_S \qquad \Delta G° = -RT \ln K$$

In gas chromatography, where K can be easily determined at two different temperatures, it is possible to obtain the variations in standard enthalpy $\Delta H°$ and entropy $\Delta S°$ (if it is accepted that the entropy and the enthalpy have not changed):

$$\Delta G° = \Delta H° - T\Delta S°$$

The values of these three parameters are all negative, indicating a spontaneous transformation. It is to be expected that the entropy is decreased when the compound moves from the mobile phase to the stationary phase where it is fixed. In the same way the Van't Hoff equation can be used in a fairly rigorous way to predict the effect of temperature on the retention time of a compound. From this it is clear that for detailed studies in chromatography, classic thermodynamics are applicable.

$$\frac{\mathrm{d}\ln K}{\mathrm{d}T} = \frac{\Delta H}{RT^2}$$

1.6 Column efficiency

1.6.1 Theoretical efficiency (number of theoretical plates)

As the analyte migrates through column, it occupies a continually expanding zone (Figure 1.6). This linear dispersion σ_1 measured by the variance σ_1^2 increases with the distance of migration. When this distance becomes L, the total column length, the variance will be:

$$\sigma_L^2 = H \cdot L \tag{1.7}$$

Reminding the plate theory model this approach also leads to the value of the height equivalent to one theoretical plate H and to the number N, of theoretical plates ($N = L/H$).

Therefore (Figure 1.7), any chromatogram that shows an elution peak with the temporal variance σ^2 permits the determination of the *theoretical efficiency N* for

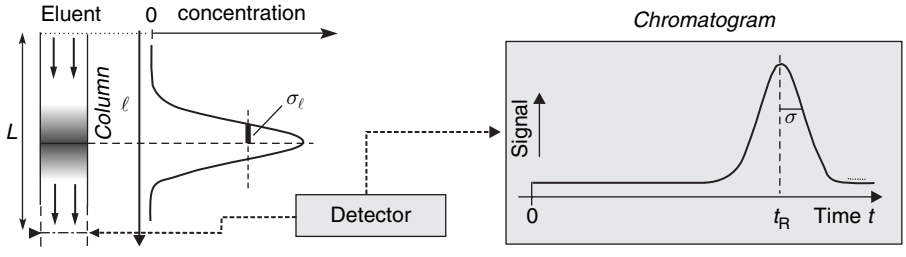

Figure 1.7 *Dispersion of a solute in a column and its translation on a chromatogram.* Left, graph corresponding to the isochronic image of the concentration of an eluted compound at a particular instant. Right, chromatogram revealing the variation of the concentration at the outlet of the column, as a function of time. t_R and σ are in the same ratio as L and σ_L. In the early days the efficiency N was calculated from the chromatogram by using a graduated ruler.

the compound under investigation (1.8), and by deduction, of the value of H knowing that $H = L/N$;

$$N = \frac{L^2}{\sigma_L^2} \quad \text{Or} \quad N = \frac{t_R^2}{\sigma^2} \tag{1.8}$$

If these two parameters are accessible from the elution peak of the compound, just because t_R and σ are in the same ratio as that of L to σ_L.

On the chromatogram, σ represents the half-width of the peak at 60.6 per cent of its height and t_R the retention time of the compound. t_R and σ should be measured in the same units (time, distances or eluted volumes if the flow is constant). If σ is expressed in units of volume (using the flow), then 4σ corresponds to the 'volume of the peak', that contains around 95 per cent of the injected compound. By consequence of the properties of the Gaussian curve ($w = 4\sigma$ and $w_{1/2} = 2.35\sigma$), Equation 1.9 results. However, because of the distortion of most peaks at their base, expression 1.9 is rarely used and finally Equation 1.10 is preferred.

N is a relative parameter, since it depends upon both the solute chosen and the operational conditions adopted. Generally a constituent is selected which appears towards the end of the chromatogram in order to get a reference value, for lack of advance knowledge of whether the column will successfully achieve a given separation.

$$N = 16\frac{t_R^2}{w^2} \tag{1.9}$$

$$N = 5.54\frac{t_R^2}{w_{1/2^2}} \tag{1.10}$$

1.6.2 Effective plates number (real efficiency)

In order to compare the performances of columns of different design for a given compound – or to compare, in gas chromatography, the performances between a capillary column and a packed column – more realistic values are obtained by replacing the *total retention time* t_R, which appears in expressions 1.8–1.10, by the *adjusted retention time* t'_R which does not take into account the *hold-up time* t_M spent by any compound in the mobile phase ($t'_R = t_R - t_M$) The three preceding expressions become:

$$N_{\text{eff}} = \frac{t'^2_R}{\sigma^2} \tag{1.11}$$

$$N_{\text{eff}} = 16\frac{t'^2_R}{w^2} \tag{1.12}$$

$$N_{\text{eff}} = 5.54 \frac{t'^{2}_{R}}{w_{1/2}{}^{2}} \tag{1.13}$$

Currently it is considered that these three expressions are not very useful.

1.6.3 Height equivalent to a theoretical plate (HETP)

The *equivalent height of a theoretical plate* H, as already defined (expression 1.5), is calculated for reference compounds to permit a comparison of columns of different lengths. H does not behave as a constant, its value depends upon the compound chosen and upon the experimental conditions.

For a long time in gas chromatography an adjustment value called the effective height of a theoretical plate H_{eff} was calculated using the true efficiency.

This corresponds to the Equation 1.14;

$$H_{\text{eff}} = \frac{L}{N_{\text{eff}}} \tag{1.14}$$

In chromatography, in which the mobile phase is a liquid and the column is filled with spherical particles, the adjusted height of the plate h, is often encountered. This parameter takes into account the average diameter d_{m} of the particles. This eliminates the effect of the particle size. Columns presenting the same ratio (length of the column)/(diameter of the particles) will yield similar performances.

$$h = \frac{H}{d_{\text{m}}} = \frac{L}{N d_{\text{m}}} \tag{1.15}$$

1.7 Retention parameters

Hold-up times or volumes are used in chromatography for various purposes, particularly to access to retention factor k. and thermodynamic parameters. Only basic expressions are given below.

1.7.1 Retention times

The definition of retention times, *hold-up time*, t_{M}, *retention time*, t_{R} and *adjusted retention time*, t'_{R}, have been given previously (paragraph 1.2).

1.7.2 Retention volume (or elution volume) V_R

The *retention volume* V_R of an analyte represents the volume of mobile phase necessary to enable its migration throughout the column from the moment of entrance to the moment in which it leaves. To estimate this volume, different methods (direct or indirect) may be used, that depend of the physical state of the mobile phase. On a standard chromatogram with time in abscissa, V_R is calculated from expression 1.16, if the flow rate F is constant,

$$V_R = t_R \cdot F \tag{1.16}$$

The volume of a peak, V_{peak} corresponds to that volume of the mobile phase in which the compound is diluted when leaving the column. It is defined by:

$$V_{peak} = w \cdot F \tag{1.17}$$

1.7.3 Hold-up volume (or dead volume) V_M

The volume of the mobile phase in the column (known as the dead volume), V_M, corresponds to the accessible interstitial volume. It is often calculated from a chromatogram, provided a solute not retained by the stationary phase is present. The dead volume is deduced from t_M and the flow rate F:

$$V_M = t_M \cdot F \tag{1.18}$$

Sometimes, in the simplest cases, the volume of the stationary phase designated by V_S can be calculated by subtracting the dead volume V_M from the total internal volume of the empty column.

1.7.4 Retention (or capacity) factor k

When a compound of total mass m_T is introduced onto the column, it separates into two quantities: m_M, the mass in the mobile phase and m_S, the mass in the stationary phase. During the solute's migration down the column, these two quantities remain constant. Their ratio, called the *retention factor k*, is constant and independent of m_T:

$$k = \frac{m_S}{m_M} = \frac{C_S}{C_M} \cdot \frac{V_S}{V_M} = K \frac{V_S}{V_M} \tag{1.19}$$

The retention factor, also known as the *capacity factor k*, is a very important parameter in chromatography for defining column performances. Though it does

not vary with the flow rate or the column length, k is it not a constant as it depends upon the experimental conditions. For this reason it is sometimes designated by k' rather than k alone.

This parameter takes into account the ability, great or small, of the column to retain each compound. Ideally, k should be superior to one but less than five, otherwise the time of analysis is unduly elongated.

An experimental approach of k can be as follows:
Suppose the migration of a compound in the column. Recalling Craig's model, each molecule is considered as passing alternately from the mobile phase (in which it progresses down the column), to the stationary phase (in which it is immobilized). The average speed of the progression down the column is slowed if the time periods spent in the stationary phase are long. Extrapolate now to a case which supposes n molecules of this same compound (a sample of mass m_T). If we accept that at each instant, the ratio of the n_S molecules fixed upon the stationary phase (mass m_S) and of the n_M molecules present in the mobile phase (mass m_M), is the same as that of the times (t_S and t_M) spent in each phase for a single molecule, the three ratios will therefore have the same value:

$$\frac{n_S}{n_M} = \frac{m_S}{m_M} = \frac{t_S}{t_M} = k$$

■ Take the case of a molecule which spends 75 per cent of its time in the stationary phase. Its average speed will be four times slower than if it rested permanently in the mobile phase. As a consequence, if 4 µg of such a compound has been introduced onto the column, there will be an average of 1 µg permanently in the mobile phase and 3 µg in the stationary phase.

Knowing that the retention time of a compound t_R is such that $t_R = t_M + t_S$, the value of k is therefore accessible from the chromatogram ($t_S = t'_R$); see Figure 1.7:

$$k = \frac{t'_R}{t_M} = \frac{t_R - t_M}{t_M} \tag{1.20}$$

This important relation can also be written:

$$t_R = t_M(1 + k) \tag{1.21}$$

Bearing in mind the relations (1.16) and (1.18), the retention volume V_R of a solute can be written :

$$V_R = V_M(1 + k) \tag{1.22}$$

or

$$V_R = V_M + KV_S \tag{1.23}$$

This final expression linking the experimental parameters to the thermodynamic coefficient of distribution K, is valid for the ideal chromatography.

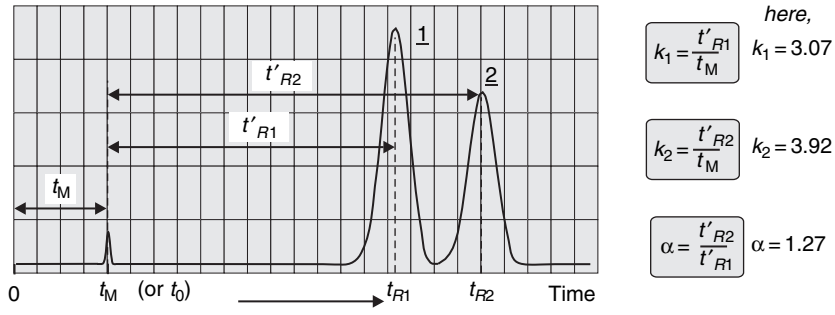

Figure 1.8 *Retention factors and separation factor between two compounds*. Each compound has its own retention factor. On this figure, the separation factor is around 1.3. The separation factor is also equal to the ratio of the two retention factors. α alone is not enough to determine whether the separation is really possible.

1.8 Separation (or selectivity) factor between two solutes

The separation factor α, (1.24) enables the comparison of two adjacent peaks 1 and 2 present in the same chromatogram (Figure 1.8). Using Equations 1.20 and 1.19, it can be concluded that the separation factor can be expressed by Equation 1.25.

By definition α is greater than unity (species 1 elutes faster than species 2):

$$\alpha = \frac{t'_{R(2)}}{t'_{R(1)}} \tag{1.24}$$

or

$$\alpha = \frac{k_2}{k_1} = \frac{K_2}{K_1} \tag{1.25}$$

For non-adjacent peaks the *relative retention factor r*, is applied, which is calculated in a similar manner to α.

1.9 Resolution factor between two peaks

To quantify the separation between two compounds, another measure is provided by the *resolution factor R*. Contrary to the selectivity factor which does not take into account peak widths, the following expression is used to calculate R between two compounds 1 and 2 (Figure 1.9):

$$R = 2\frac{t_{R(2)} - t_{R(1)}}{w_1 + w_2} \tag{1.26}$$

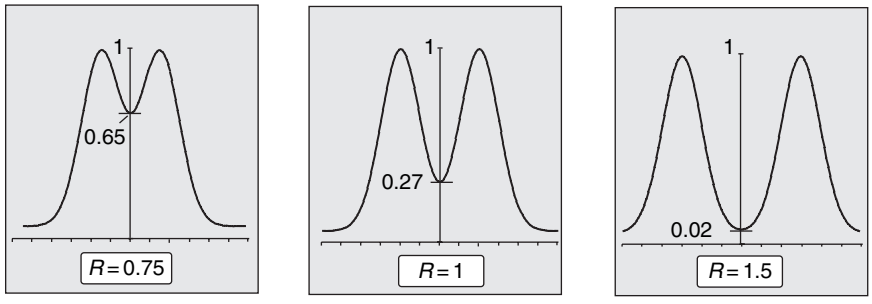

Figure 1.9 *Resolution factor*. A simulation of chromatographic peaks using two identical Gaussian curves, slowly separating. The visual aspects corresponding to the values of R are indicated on the diagrams. From a value of R = 1.5 the peaks can be considered to be baseline resolved, the valley between them being around 2 per cent.

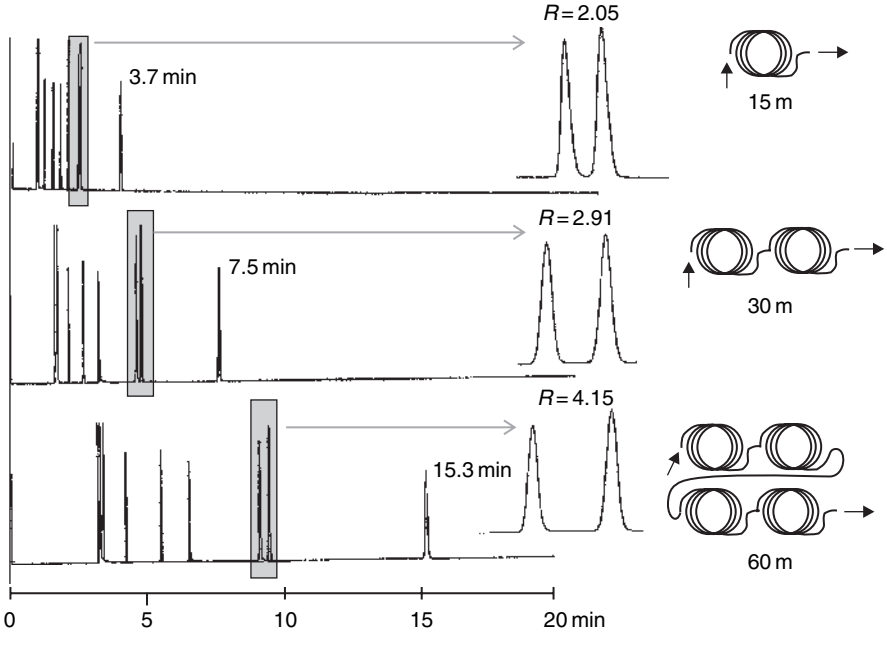

Figure 1.10 *Effect of column length on the resolution*. Chromatograms obtained with a GC instrument illustrating that by doubling the length of the capillary column, the resolution is multiplied by 1.41 or $\sqrt{2}$ (adapted from a document of SGE Int. Ltd).

Other expressions derived from the preceding ones and established with a view to replacing one parameter by another or to accommodate simplifications may also be employed to express the resolution. Therefore expression 1.27 is used in this way.

It is also useful to relate the resolution to the efficiency, the retention factor and the separation factors of the two solutes (expression 1.28, obtained from 1.26 when $w_1 = w_2$). The chromatograms on Figure 1.10 present an experimental verification.

$$R = 1.177 \frac{t_{R(2)} - t_{R(1)}}{\delta_1 + \delta_2} \tag{1.27}$$

$$R = \frac{1}{4}\sqrt{N_2} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1 + k_2} \tag{1.28}$$

$$R = \frac{\sqrt{N}}{2} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2 - k_1}{k_1 + k_2 + 2} \tag{1.29}$$

1.10 The rate theory of chromatography

In all of the previous discussion and particularly in the plate theory, the velocity of the mobile phase in the column and solute diffusion are, perhaps surprisingly, never taken into account. Of all things, the speed should have an influence upon the progression of the analytes down the column, hence their dispersion and by consequence, upon the quality of the analysis undertaken.

Rate theory is a more realistic description of the processes at work inside a column which takes account of the time taken for the solute to equilibrate between the two phases. It is the dynamics of the separation process which is concerned. The first kinetic equation *for packed columns in gas phase chromatography* was proposed by Van Deemter.

1.10.1 Van Deemter's equation

This equation is based on a Gaussian distribution, similar to that of plate theory. Its simplified form, proposed by Van Deemter in 1956, is well known (expression 1.30). The expression links the plate high H to the average linear velocity of the mobile phase $\bar{u}$ in the column (Figure 1.11).

$$H = A + \frac{B}{\bar{u}} + C\bar{u} \tag{1.30}$$

The three experimental basic coefficients A, B and C are related to diverse physico-chemical parameters of the column and to the experimental conditions. If H is expressed in cm, A will also be in cm, B in cm^2/s and C in s (where velocity is measured in cm/s).

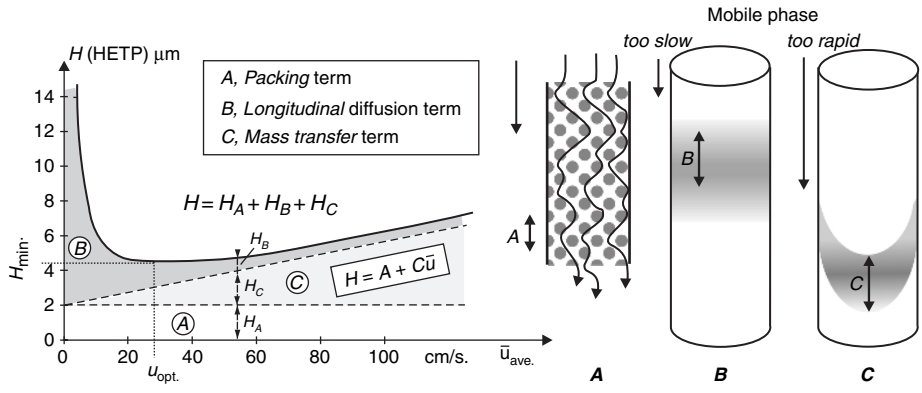

Figure 1.11 *Van Deemter's curve in gas chromatography with the domains of parameters A, B and C indicated.* There exists an equation similar to that of Van Deemter that considers temperature: $H = A + B/T + CT$.

This equation reveals that there exists an *optimal flow rate* for each column, corresponding to the minimum of H, which predicts the curve described by Equation 1.30.

The loss in efficiency as the flow rate increases is obvious, and represents what occurs when an attempt is made to rush the chromatographic separation by increasing the pressure upon the mobile phase.

However, intuition can hardly predict the loss in efficiency that occurs when the flow rate is too slow. To explain this phenomenon, the origins of the terms A, B and C must be recalled . Each of these parameters represents a domain of influence which can be perceived on the graph (Figure 1.11).

The curve that represents the Van Deemter equation is a hyperbola which goes through a minimum ($H_{\min}$) when:

$$\bar{u}_{\text{opt}} = \sqrt{\frac{B}{C}} \tag{1.31}$$

Packing related term $A = 2\lambda . d_p$

Term A is related to the flow profile of the mobile phase passing through the stationary phase. The size of the particles (diameter d_p), their dimensional distribution and the uniformity of the packing (factor characteristic of packing λ) can all be the origin of flow paths of different length which cause broadening of the solute band and improper exchanges between the two phases. This results in turbulent or *Eddy diffusion*, considered to have little importance in liquid chromatography and absent for WCOT capillary columns in GC (Golay's equation without term A, cf. paragraph 1.10.2). For a given column, nothing can be done to reduce the A term.

Gas (mobile phase) term $B = 2\gamma D_G$

Term B, which can be expressed from D_G, the diffusion coefficient of the analyte in the gas phase and λ, the above packing factor, is related to the *longitudinal molecular diffusion* in the column. It is especially important when the mobile phase is a gas.

This term is a consequence of the entropy which reminds us that a system will tend spontaneously towards the maximum degrees of freedom, chaos, just as a drop of ink diffuses into a glass of water into which it has fallen. Consequently, if the flow rate is too slow, the compounds undergoing separation will mix faster than they will migrate. This is why one never must interrupt, even temporarily, a chromatography once underway, as this puts at risk the level of efficiency of the experiment.

Liquid (stationary phase) term $C = C_G + C_L$

Term C, which is related to the *resistance to mass transfer* of the solute between the two phases, becomes dominant when the flow rate is too high for an equilibrium to be attained. Local turbulence within the mobile phase and concentration gradients slow the equilibrium process ($C_S \Leftrightarrow C_M$). The diffusion of solute between the two phases is not instantaneous, so that it will be carried along out of equilibrium. The higher the velocity of mobile phase, the worse the broadening becomes. No simple formula exists which takes into account the different factors integrated in term C. The parameter C_G is dependent upon the diffusion coefficient of the solute in a gaseous mobile phase, while the term C_L depends upon the diffusion coefficient in a liquid stationary phase. Viscous stationary phases have larger C terms.

■ In practice, the values for the coefficients of A, B and C in Figure 1.11 can be accessed by making several measurements of efficiency for the same compound undergoing chromatography at different flow rates, since flow and average linear speed are related. Next the hyperbolic function that best satisfies the experimental values can be calculated using, by preference, the method of multiple linear regression.

1.10.2 Golay's equation

A few years after Van Deemter, Golay proposed a modified relationship reserved to capillary columns used in gas phase chromatography. There is no A term because there is no packing in a capillary column (see paragraph 2.5.2).

$$H = \frac{B}{\bar{u}} + C_L\bar{u} + C_G\bar{u} \tag{1.32}$$

Expression 1.33 leads to the minimum value for the HETP for a column of radius r, if the retention factor of the particular compound under examination is known.

The *coating efficiency* can then be calculated being equal to 100 times the ratio between the value found using expression 1.33 and that deduced from the efficiency ($H = L/N$) obtained from the chromatogram.

$$H_{\text{theo.min}} = r\sqrt{\frac{1 + 6k + 11k^2}{3(1+k)^2}} \tag{1.33}$$

1.10.3 Knox's equation

Another, more recent equation, the Knox equation, is applicable to various types of liquid chromatography and includes the adjusted height h:

$$h = A\bar{u}^{1/3} + \frac{B}{\bar{u}} + C\bar{u} \tag{1.34}$$

1.11 Optimization of a chromatographic analysis

Analytical chromatography is used essentially in quantitative analysis. In order to achieve this effectively, the areas under the peaks must be determined with precision, which in turn necessitates well-separated analytes to be analysed. A certain experience in chromatography is required when the analysis has to be optimized, employing all available resources in terms of apparatus and software that can simulate the results of temperature modifications, phases and other physical parameters.

■ In gas phase chromatography, the separations can be so complex that it can be difficult to determine in advance whether the temperature should be increased or decreased. The choice of column, its length, its diameter, the stationary phase composition and the phase ratio (V_M/V_S) as well as the parameters of separation (temperature and flow rate), are amongst the factors which interact with each other.

The *resolution* and the *elution time* are the two most important dependent variables to consider. In all optimizations, the goal is to achieve a sufficiently complete separation of the compounds of interest in the minimum time, though it should not be forgotten that time will be required to readjust the column to the initial conditions to be ready for the next analysis. Chromatography corresponds, in fact, to a slow type of analysis. If the resolution is very good then optimization consists to save time in the analysis. This can be done by the choice of a shorter column – recalling that the resolution varies with the square root of the column length (cf. the parameter N of formula 1.28 and Figure 1.10).

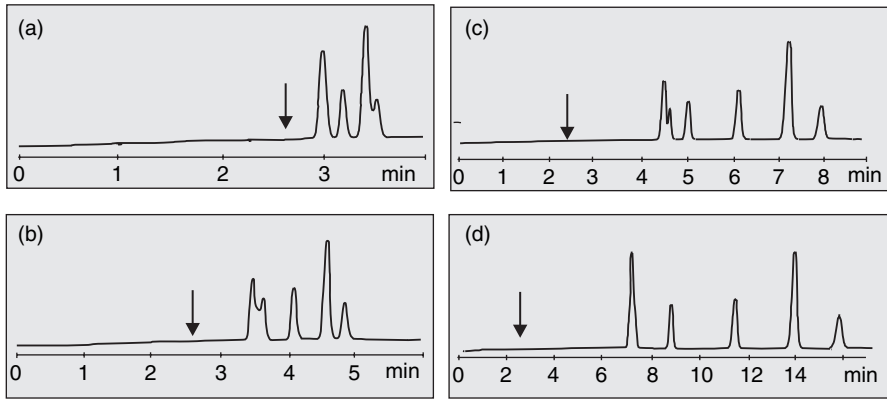

Figure 1.12 *Chromatograms of a separation*. The mobile phase in each trace is a binary mixture water/acetonitrile: (a) 50/50; (b) 55/45; (c) 60/40; (d) 65/35. The arrow indicates the dead time t_M (min) *(J.W. Dolan, LC-GC Int., 1994 7(6), 333)*.

Figure 1.12 shows the optimization of a separation, by liquid chromatography, of a mixture of aromatic hydrocarbons. In this case, optimization of the separation has been carried out by successive modifications of the composition of the mobile phase. Note that by optimizing the sequence in this manner, the cycle time of analysis increases.

If only certain compounds present in a mixture are of interest, then a selective detector can be used which would detect only the desired components. Alternately, at the other extreme, attempts might be made to separate the largest number of compounds possible within the mixture.

Depending upon the different forms of chromatography, optimization can be more or less rapid. In gas phase chromatography optimization is easier to achieve than in liquid chromatography in which the composition of mobile phase must

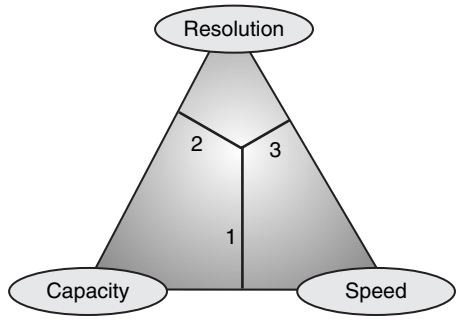

Figure 1.13 *The chromatographer's triangle*. The shaded areas indicate the domain corresponding to analytical chromatography based principally upon the five parameters K, N, k, α and R.

be considered: software now exists that can help in the choice of mobile phase composition. Based upon certain hypotheses (Gaussian peaks), the areas of poorly defined peaks can be found.

The chromatographer must work within the limits bound by a triangle whose vertices correspond to three parameters which are in opposition: the *resolution*, the *speed* and the *capacity* (Figure 1.13). An optimized analytical separation uses the full potential of the selectivity which is the most efficient parameter. In the chromatographer's triangle shown, the optimized conditions are close to the vertex of resolution.

1.12 Classification of chromatographic techniques

Chromatographic techniques can be classified according to various criteria: as a function of the *physical nature* of the phases; of the *process* used; or by the *physico-chemical phenomena* giving rise to the Nernst distribution coefficient K. The following classification has been established by consideration of the physical nature of the two phases involved (Figure 1.14).

1.12.1 Liquid phase chromatography (LC)

This type of chromatography, in which the mobile phase is a liquid belongs to the oldest known form of the preparative methods of separation. This very broad category can be sub-divided depending on the retention phenomenon.

Liquid/solid chromatography (or adsorption chromatography)

The stationary phase is a solid medium to which the species adhere through the dual effect of physisorption and chemisorption. The physico-chemical parameter involved here is the *adsorption coefficient*. Stationary phases have made much progress since the time of Tswett, who used calcium carbonate or inulin (a very finely powdered polymer of ordinary sugar).

Ion chromatography (IC)

In this technique the mobile phase is a buffered solution while the solid stationary phase has a surface composed of ionic sites. These phases allow the exchange of their mobile counter ion with ions of the same charge present in the sample. This type of separation relies on *ionic distribution coefficients*.

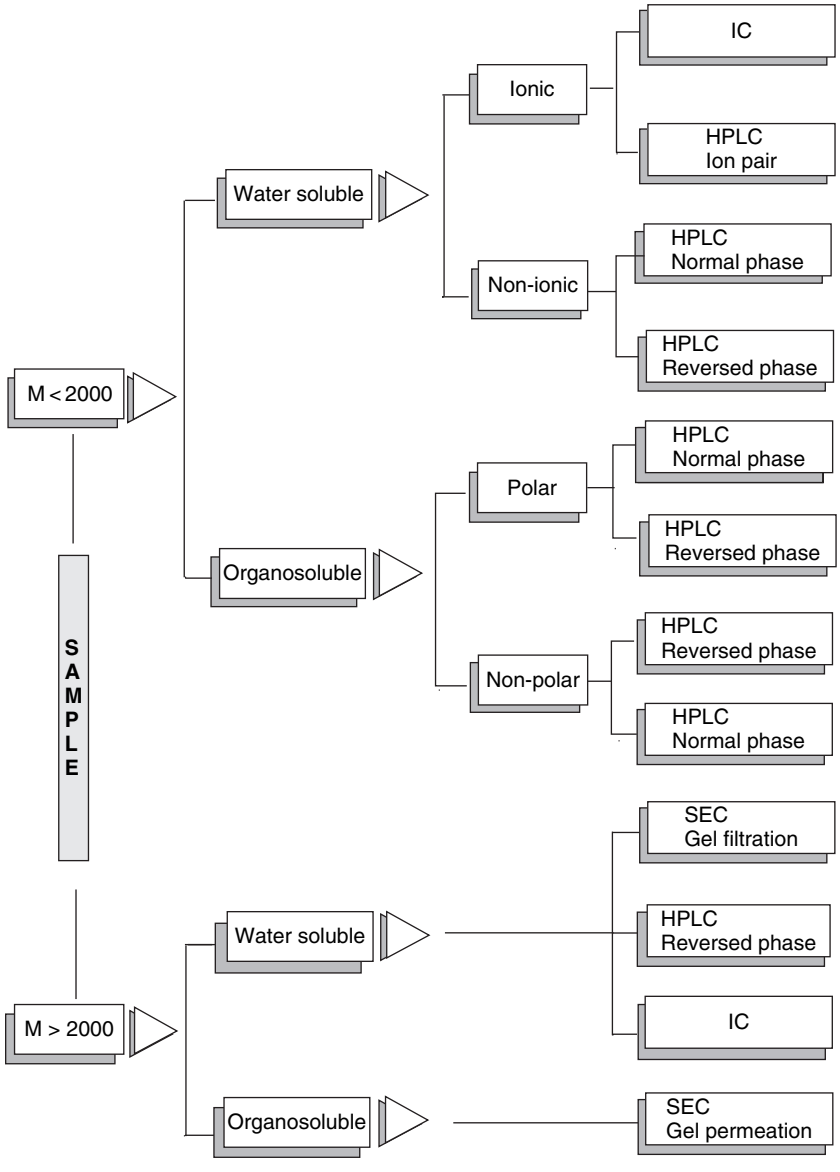

Figure 1.14 *Selection guide for all of the different chromatographic techniques with liquid mobile phases.* The choice of technique is chosen as a function of the molar mass, solubility and the polarity of the compounds to be separated.

Size exclusion chromatography (SEC)

The stationary phase here is a material containing pores whose dimensions are selected as a function of the size of the species to be separated. This method

therefore uses a form of selective permeability at the molecular level leading to its name, *gel filtration* or *gel permeation* depending on the nature of the mobile phase, which is either aqueous or organic. For this technique, the distribution coefficient is called the *diffusion coefficient*.

Liquid/liquid chromatography (or partition chromatography, LLC)

The stationary phase is an *immobilized* liquid upon an inert and porous material, which has only a mechanical role of support. Impregnation, the oldest procedure for immobilizing a liquid on a porous material, is a method now abandoned because of the elevated risk of washing out the column, which is called *bleeding*.

Liquid/bound phase chromatography

In order to immobilize the stationary phase (generally a liquid polymer), it is preferable to fix it by covalent bonding to a mechanical support. The quality of separation depends upon the *partition coefficient K* of the solute between the two phases, a phenomenon comparable to a liquid-liquid extraction between an aqueous and organic phase in a separating funnel.

1.12.2 Gas phase chromatography (GC)

The mobile phase is an inert gas and as above this form of chromatography can be sub-divided according to the nature of the phase components:

Gas/liquid/ chromatography (GLC)

As indicated above the mobile phase here is a gas and the stationary phase is an immobilized liquid, either by impregnation or by bonding to an inert support which could be, quite simply, the inner surface of the column. This is the technique commonly called *gas phase chromatography (GC)*. The gaseous sample must be brought to its vapour state. It was Martin and Synge who, in 1941, suggested the replacement of the liquid mobile phase by a gas in order to improve the separations. From this era comes the true beginnings of the development of analytical chromatography. Here once again it is the partition coefficient K that is involved.

Gas/solid chromatography (GSC)

The stationary phase is a porous solid (such as graphite, silica gel or alumina) while the mobile phase is a gas. This type of gas chromatography is very effective

for analyses of gas mixtures or of compounds that have a low boiling point. The parameter concerned is the *adsorption coefficient*.

1.12.3 Supercritical fluid chromatography (SFC)

Here the mobile phase is a fluid in its supercritical state, such as carbon dioxide at about 50 °C and at more than 150 bar (15 MPa). The stationary phase can be a liquid or a solid. This technique combines the advantages of those discussed above: liquid/liquid and gas/liquid chromatography.

Problems

1.1 A mixture placed in an Erlenmeyer flask comprises 6 mL of silica gel and 40 mL of a solvent containing, in solution, 100 mg of a non-volatile compound. After stirring, the mixture was left to stand before a 10 mL aliquot of the solution was extracted and evaporated to dryness. The residue weighed 12 mg.

Calculate the adsorption coefficient, $K = C_S/C_M$, of the compound in this experiment.

1.2 The retention factor (or capacity factor), k of a compound is defined as $k = m_S/m_M$, that is by the ratio of the masses of the compound in equilibrium in the two phases. Show, from the information given in the corresponding chromatogram, that the expression used – $k = (t_R - t_M)/t_M$ – is equivalent to this. Remember that for a given compound the relation between the retention time t_R, the time spent in the mobile phase t_M (hold-up or dead time) and the time spent in the stationary phase t_S, is as follows:

$$t_R = t_M + t_S$$

1.3 Calculate the separation factor (or selectivity factor), between two compounds, 1 and 2, whose retention volumes are 6 mL and 7 mL, respectively. The dead volume of the column used is 1 mL. Show that this factor is equal to the ratio of the distribution coefficients K_2/K_1 of these compounds $(t_{R(1)} < t_{R(2)})$.

1.4 For a given solute show that the time of analysis – which can be compared with the retention time of the compound held longest on the column – depends, amongst other things, upon the length of the column, the average

linear velocity of the mobile phase and upon the volumes V_S and V_M which indicate respectively the volume of the stationary and mobile phases.

1.5 Equation (2) is sometimes employed to calculate N_{eff}. Show that this relation is equivalent to the more classical equation (1):

$$N_{eff} = 5.54 \frac{(t_R - t_M)^2}{w_{1/2}^2} \quad (1)$$

$$N_{eff} = N \frac{k^2}{(k+1)^2} \quad (2)$$

1.6 The resolution factor R for two solutes 1 and 2, whose elution peaks are adjacent, is sometimes expressed by equation (1):

$$R = \frac{t_{R(2)} - t_{R(1)}}{w_{1/2(1)} + w_{1/2(2)}} \quad (1)$$

$$R = \frac{1}{4}\sqrt{N_2}\frac{\alpha - 1}{\alpha}\frac{k_2}{1 + k_2} \quad (2)$$

1. Show that this relation is different from the basic one by finding the expression corresponding to the classic equation which uses w_b, the peak width at the baseline.

2. If it is revealed that the two adjacent peaks have the same width at the baseline ($w_1 = w_2$), then show that relation (2) is equivalent to relation (1) for the resolution.

1.7 1. Show that if the number of theoretical plates N is the same for two neighbouring compounds 1 and 2, then the classic expression yielding the resolution, equation (1) below, can be transformed into (2).

$$R = \frac{\sqrt{N}}{2}\frac{k_2 - k_1}{k_1 + k_2 + 2} \quad (1)$$

$$R = \frac{1}{2}\sqrt{N}\frac{\alpha - 1}{\alpha + 1}\frac{\bar{k}}{1 + \bar{k}} \quad (2)$$

2. Show that if $\bar{k} = (k_1 + k_2)/2$ then expressions (1) and (2) are equivalent.

1.8 The effective plate number N_{eff} may be calculated as a function of the separation factor α for a given value of the resolution, R. Derive this relationship.

$$N_{\text{eff}} = 16R^2 \frac{\alpha^2}{(\alpha - 1)^2}.$$

1.9 Consider two compounds for which $t_M = 1\,\text{min}$, $t_1 = 11.30\,\text{min}$ and $t_2 = 12\,\text{min}$. The peak widths at half-height are 10 s and 12 s, respectively. Calculate the values of the respective resolutions using the relationships from the preceding exercise.

1.10 Certain gas phase chromatography apparatus allows a constant gas flow in the column when operating under programmed temperature conditions.

1. What is the importance of such a device?

2. Deduce from the simplified version of Van Deemter's equation for a full column the expression which calculates the optimum speed and which conveys a value for the HETP.

1.11 Explain how the resolution can be expressed in terms of the retention volumes in the following equation:

$$R = \frac{\sqrt{N}}{2} \frac{V_{R(2)} - V_{R(1)}}{V_{R(1)} + V_{R(2)}}$$

1.12 Which parameters contribute an effect to the widths of the peaks on a chromatogram? Is it true that all of the compounds present in a sample and which can be identified upon the chromatogram have spent the same amount of time in the mobile phase of the column? Can it be said that a reduction in the retention factor k of a compound enhances its elution from the column?