1
General Concepts

1.1
Introduction

The concept of separating sample components in a column was first developed in 1903 by Mikhail Tswett, who introduced the term chromatography in 1906. Unfortunately, his contemporaries showed little interest for the idea and almost 30 years went by before scientists in Germany rediscovered the principle of column liquid chromatography (LC). Then, in 1943 Arne Tiselius (in Sweden) classified chromatography into three modes: frontal, elution, and displacement. The elution mode actually became synonymous with almost all chromatography, but in recent years the displacement mode has attracted new interest, particularly in the separation of proteins.

In the years immediately prior to and during the Second World War, the principles of ion exchange chromatography (IEC) and liquid–liquid partition chromatography began to develop into crude technical solutions. Then after the war, in the early 1950s, the new technique of thin layer chromatography (TLC) came to light and gradually improved the partition principles used in paper chromatography. A. Martin and R.L.M. Synge (in the United Kingdom) received the Nobel Prize in 1952 for the invention of partition chromatography. Martin with James had also developed gas–liquid chromatography at this time. Gas chromatography (GC) was readily accepted by research chemists at the major oil companies, who understood the large potential of this technique and participated in developing the new instrumentation.

Size exclusion chromatography (SEC) was developed in Sweden by Porath and Flodin with dextrin materials (1959), by Hjertén with polyacrylamide (1961) and agarose (1964) materials, and by Moore in the United States with polystyrene–divinylbenzene (PS-DVB) materials (1964).

Supercritical fluid chromatography was demonstrated as early as 1962, but it did not receive much interest until the technology was improved more than 20 years later.

The introduction of open tubular columns into gas chromatography revolutionized GC, first with glass capillaries in the 1970s and then with fused silica columns in the 1980s. A similar revolution started with the gradual development of new

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Table 1.1 Properties of chromatographic techniques.

Technique	Mobile phase	Driving force	Stationary phase
GC	Gas	Gas pressure/flow	Solids, liquid films
HPLC	Liquid	Pump flow	Solvated solids
SFC	Supercritical fluid	Pump flow	Solids, liquid films
TLC	Liquid	Capillary forces	Solids
EC	Liquid	Electric field	Solids
MEKC	Liquid	Electric field	Micelles

columns and instrumentation in liquid chromatography. With columns filled with small particles, the high-pressure liquid chromatography of the 1970s–1980s was later renamed high-performance liquid chromatography (HPLC).

Gel electrophoresis (GE) was developed in the 1940s, while capillary electrophoresis appeared 40 years later. Then chromatography with electric potential-driven liquid flow also developed into micellar electrokinetic chromatography (MEKC) and electrochromatography (EC), both with capillary columns. Electrophoresis, thus, is not a chromatographic technique, since there is no stationary phase, except in MEKC and EC.

To date, HPLC has become the dominating chromatographic technique, with capillary GC being second only to it (for the more volatile analytes). Both GC and HPLC are mature separation techniques today; however, HPLC is still being developed toward faster and more efficient separations and also partially toward miniaturized columns, particularly for applications in the life science area. The majority of the other techniques already mentioned are niche techniques today, but still important for a relatively smaller number of users compared to HPLC and GC. Electric potential-driven techniques have an added opportunity for new technology on microchips.

Some of the properties of the chromatographic techniques are shown in Table 1.1.

1.2 Migration and Retention

1.2.1 General

In a chromatographic system, the sample is introduced in a small volume at the inlet of a column or another carrier of the stationary phase. The mobile phase transports the sample components in contact with the stationary phase throughout the column.

Due to different interactions between the sample components and the stationary phase, the sample components migrate through the system at different speeds and elute from the column with different retention times.

The retention time is defined as the time between the sample introduction and the elution from the column.

At the end of the column, a detector provides a signal for all eluting components (universal detection) or for a limited number (selective detection).

In a sample with many components, some compounds will coelute, partly or completely, depending on the complexity of the sample and the peak capacity of the column.

With mass spectrometric detection, even coeluting components can be identified.

1.2.2 Mobile and Stationary Phases

The sample components (solutes) can interact directly with components of the mobile phase, except in gas chromatography where there are no such interactions and the mobile phase is simply a carrier gas for the sample components.

When the stationary phase is a solid, often with polar surface groups, and the mobile phase is either a gas (in GC) or an organic solvent (in LC), the separation principle is based on adsorption, and the term adsorption chromatography can be used. Other not so commonly used terms are gas–solid chromatography and liquid–solid chromatography. The adsorption forces include dispersion interactions, dipolar interactions, acid–base interactions, complexation, and so on.

In gas chromatography, the stationary phase can also be a liquid, where the separation principle is based on partition between the two phases. This was also the case formerly in liquid chromatography, but after the introduction of chemically bonded stationary phases into HPLC, the stationary phase cannot be described as a liquid anymore.

1.2.3 Chromatograms

When the sample components are separated and detected by a detector connected to the outlet of the column and the signals from the detector are visualized as a function of time, a chromatogram is obtained, as shown in Figure 1.1.

In a chromatogram, the elution time is found at the x-axis, while the y-axis constitutes the size of the detector signal.

Depending on the conditions, the separation of the sample components as well as the time of analysis can be adjusted, as shown in Figure 1.2.

With isocratic elution (constant composition of the mobile phase), the peak width will increase with increasing elution time. This cannot be seen clearly in Figure 1.2b as the elution mode is gradient elution (changing composition of the mobile phase).

1.2.4 Retention Factor

At any given time during the migration through the system, there is a distribution of molecules of each component between the two phases:

$$n_s/n_m,$$

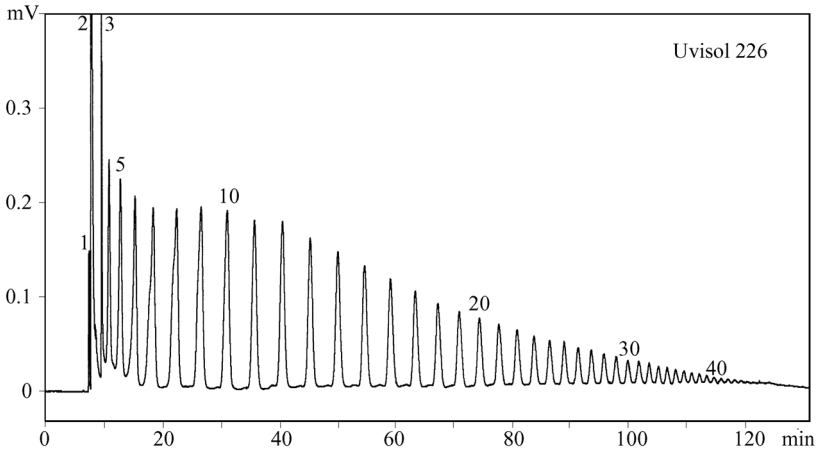

Figure 1.1 Chromatogram of polymeric amines separated by gradient elution in HPLC.

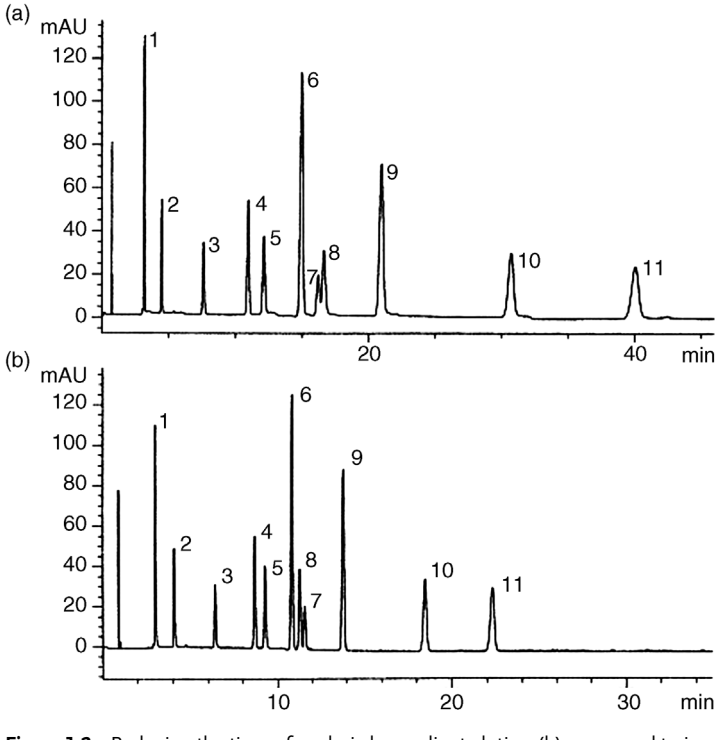

Figure 1.2 Reducing the time of analysis by gradient elution (b) compared to isocratic elution with constant mobile phase composition (a). (From Ref. [7], with permission.)

where n_s and n_m are the number of molecules in the stationary and mobile phases, respectively, at a given time. When n_s is much larger than n_m, the migration is very slow and the analyte elutes with high *retention*. In Figure 1.2, compound **11** has the highest retention:

$k = n_s/n_m$ is called the *retention factor*.

Info-box 1.1

k is the recommended symbol by IUPAC for describing the retention of a compound; it is independent of flow rate, column dimensions, and so on [1].

If one component migrates through the column in the mobile phase only, with no interactions with the stationary phase, the migration time is called t_M. An analyte with interactions with the stationary phase will be retained and will elute at t_R:

$$t_R = t_M + t_M k = t_M(1 + k).$$

The t_M can be determined by injecting a component known to have no interactions with the stationary phase.

From Equation 1.1, we can obtain a method for measuring k:

$$k = (t_R - t_M)/t_M. \tag{1.1}$$

Time units can also be replaced with volume units:

$$V_R = V_M(1 + k).$$

1.3 Band Broadening

A sample is injected in a limited volume at the column inlet. If there were no band broadening, the volume or the width of the band would be exactly the same at the point of detection. Unfortunately, this is not the case. In all chromatographic systems, there is band broadening (Figure 1.2), caused by different physical processes.

In the columns, the following processes can occur:

- Eddy diffusion
- Longitudinal diffusion in the mobile phase
- Resistance to mass transfer: in the mobile phase, stationary phase, and stagnant mobile phase

If the distribution of each band is assumed to be a Gaussian distribution, the extent of band broadening can be expressed by the column efficiency N:

$$N = (t_R/\sigma)^2,$$

where t_R is the retention time and σ^2 is the band variance in time units (σ is the standard deviation of the Gaussian distribution).

Another expression for the band broadening in a column with length L is the plate height H:

$$H = L/N,$$

where H is measured in micrometer.

Since H is a function of the variance, individual contributions to band broadening can be expressed as individual contributions to the plate height.

1.3.1 Eddy Diffusion

Eddy diffusion occurs due to the presence of multiple channels of different widths and lengths in porous structures. Large inhomogeneous particles cause large contributions to band broadening of eddy diffusion. In a packed column, the size of the eddy diffusion is proportional to the particle size. A wide range of particle size also increases the eddy diffusion.

The main contribution of eddy diffusion to the plate height is

$$H = C_e d_p,$$

where d_p is the particle diameter of one-size particles and C_e is a constant.

In open tubular columns, there is no eddy diffusion.

Info-box 1.2

In liquid chromatography, eddy diffusion is responsible for a major part of the band broadening in the column. Since eddy diffusion is a combination of diffusion and convection, the term eddy dispersion might be more correct than eddy diffusion. Contributions to eddy dispersion come from column internal diameter, column length, and column packing efficiency besides particle size and homogeneity [2].

1.3.2 Longitudinal Diffusion

Longitudinal diffusion in the mobile phase is due to the natural tendency of a compound in a concentrated band to diffuse into less concentrated zones. The contribution of longitudinal band broadening is proportional to the diffusion constant. Since the diffusion velocity in gases is about 10^4 times higher than the diffusion in liquids, this contribution to band broadening is much more important in GC than in HPLC.

The contribution of the longitudinal diffusion to the plate height is

$$H_l = c_l D_m / u,$$

where D_m is the diffusion coefficient in the mobile phase, c_l is a constant, and u is the linear mobile phase flow rate.

1.3.3 Resistance to Mass Transfer

Resistance to mass transfer describes the band broadening caused by transporting the analytes by diffusion and convection from one phase to the other.

Resistance to mass transfer is, in general, inversely proportional to the diffusion constants in either phase.

In the mobile phase, there is an additional link to eddy diffusion. The contribution to the plate height can be described by band broadening taking place in the mobile phase, stagnant mobile phase, and stationary phase.

Resistance to mass transfer in the mobile phase

a) *In an open tubular column*

$$H_m = c_m d_c^2 u / D_m,$$

where d_c is the column internal diameter, u is the linear flow rate, and

$$c_m = (1 + 6k + 11k^2)/96(1 + k)^2.$$

b) *In a packed column*

$$H_m = c_{mp} d_p^2 u / D_m,$$

where d_p is the particle diameter and u is the linear flow rate (measured in mm s^{-1}).

In packed columns, H_m should be coupled with the eddy diffusion and the coupled term $H_{me} = 1/(1/H_e + 1/H_m)$.

Note: The plate height in a packed column is independent of the column inner diameter.

Resistance to mass transfer in the stagnant mobile phase (in a packed column)

$$H_{stm} = c_{stm} d_p^2 u / D_m,$$

where c_{stm} is a constant and the other parameters are as before.

Resistance to mass transfer in the stationary phase

$$H_s = c_s d_f^2 u / D_s,$$

where d_f is the film thickness, c_s is a constant, and the other parameters are as before.With thin films, H_s is small and can be neglected. In gas chromatography, this is the case for columns with a film thickness of 0.25 μm or less.

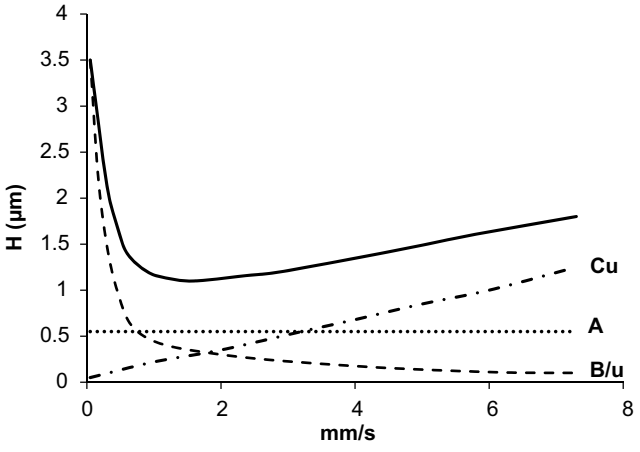

Figure 1.3 Van Deemter plot of plate height (H) as a function of linear flow.

1.3.4
Combined Band Broadening in a Column

The van Deemter equation combines the different contributions in a simplified equation:

$$H = A + B/u + Cu, \tag{1.2}$$

where A is eddy diffusion, B is the longitudinal diffusion in the mobile phase, and C is the resistance to mass transfer in the stationary phase (in GC) or in both the phases (LC).

The contribution of stagnant mobile phase is valid only with large particles and is therefore usually neglected in the van Deemter equation for high-performance systems.

The contribution of resistance to mass transfer in the mobile phase can be neglected in GC, but not in HPLC.

The van Deemter equation can be visualized by plotting the contributions to the plate height as a function of the linear mobile phase velocity (Figure 1.3).

In open tubular columns, there is no eddy diffusion and the band broadening can be expressed by the Golay equation:

$$H = 2D_m/u + f_g d_c^2 u/D_m + f_s d_f^2 u/D_s, \tag{1.3}$$

where $f_g = (1 + 6k + 11k^2)/96(1 + k)^2$, $f_s(2/3)k/(1 + k)^2$, and the other parameters are as before.

The Golay equation is one of the most prominent equations in modern chromatography.

Info-box 1.3

For a deeper understanding of the contributions to band broadening, see Ref. [3].

1.3.5
Band Broadening outside the Column

Band broadening also takes place in the injector, in the connecting tubing to the column, in the connection to the detector, in the detector, and sometimes due to slow electronics in the detector or the data system. In the connecting tubing, the band broadening is caused by the parabolic flow profile in a tube, caused by friction at the walls.

The contribution to band broadening, of the flow in an empty tube, measured as variance is

$$\sigma^2 = \pi r^2 L/24 D_m F,$$

where r is the tube radius, L is the length, D_m is the diffusion coefficient, and F is the volumetric flow rate.

This is equivalent to the contribution to the plate height:

$$H = r^2 u/24 D_m, \tag{1.4}$$

where u is the linear velocity L/t.

1.4
Measuring Column Efficiency

1.4.1
Plate Numbers

The column efficiency, as mentioned in Section 1.3, can be expressed by the plate number N and defined as

$$N = (t_R/\sigma)^2, \tag{1.5}$$

where t_R is the retention time and σ is the standard deviation, assuming that the distribution of molecules of a component within a peak can be characterized by a Gaussian distribution.

From a chromatogram (Figure 1.4), we can measure either manually or by the data system both the retention time and the peak width. The peak width at half height is often used, but if there is peak tailing, it is not a good representation of the real peak width.

One alternative is the width at the baseline between the tangents to the inflexion points at 0.607 h of a Gaussian peak.

At half height, the peak width is 2.354σ, and at the base between the tangents, $w = 4\sigma$.

Replacing σ as a function of w results in measurements of column efficiency as

$$N = 5.54(t_R/w)^2 \quad \text{(at half height)}, \tag{1.6}$$

$$N = 16(t_R/w)^2 \quad \text{(at the base)}, \tag{1.7}$$

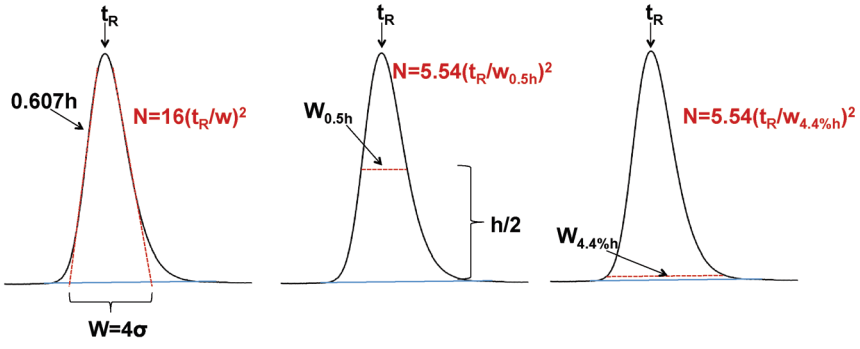

Figure 1.4 Peak widths between tangents at baseline, at half height, and at 4.4% of height.

or

$$N = 25(t_R/w)^2 \quad (\text{at } 4.4\% \text{ of peak height}). \tag{1.8}$$

The number of plates is calculated by measuring both retention time and peak width in time units.

Longer columns result in higher plate numbers ($N = L/H$).

Measurements of column efficiency in LC must always be performed with isocratic elution at constant temperature and the sample dissolved in the mobile phase.

Gradient elution can be used for measuring peak capacity, but never for measuring plate numbers.

1.4.2 Coupling Columns

Coupling two (or more) columns in series makes a longer separation system. However, one should be aware of the fact that if a good column is connected with a mediocre column, the plate numbers cannot be added. If the retention factor is equal in both the columns, the total plate number N_t is

$$N_t = 2^2/\left(\frac{1}{N_1}+\frac{1}{N_2}\right). \tag{1.9}$$

Thus, if two columns with $N_1 = 10\,000$ and $N_2 = 1000$ plates are connected, the total plate number will be only about 3600. Short guard columns will not reduce the total peak numbers, since the peak broadening in the short column is limited.

1.4.3 Plate Height

The other measure for the band broadening as mentioned in Section 1.3 is the plate height H:

$$H = L/N. \tag{1.10}$$

The plate height is also called the height equivalent to a theoretical plate (HETP).

In both the well-packed HPLC columns and the capillary GC, plate heights of 5–10 μm and 0.2–0.25 mm, respectively, should be obtained.

1.4.3.1 Reduced Plate Height

Reduced plate height h is another way of expressing column efficiency.

In a packed column, $h = H/d_p$.

In the well-packed HPLC columns, reduced plate heights of 2–3 or even less than 2 can be obtained.

In an open tubular column, $h = H/d_c$, where d_c is the column inner diameter.

In capillary GC, reduced plate heights of less than 1 can be obtained.

Reduced plate height allows us to compare the efficiency of columns with different particle sizes or different internal diameters (in open capillary columns).

Info-box 1.4

A more thorough treatment of reduced parameters can be found in Ref. [4].

1.4.4
Effective Plate Number

Effective plate number N_{eff} is actually a measure of the band broadening in the stationary phase and is calculated by using adjusted retention times:

$$t'_R = (t_R - t_M).$$

At high k, there is little difference between plate numbers and effective plate numbers. Effective plate numbers are mostly used in gas chromatography.

1.4.5
Asymmetry

Most chromatographic peaks cannot be characterized with a perfect Gaussian distribution and some peaks are tailing, while others are fronting. Peak asymmetry can be measured as the asymmetry factor, at 10% of the peak height (Figure 1.5). Well-made columns are expected to have an asymmetry factor A_s as close to 1 as possible.

1.5
Resolution

The resolution R_S of two closely eluting bands is defined as the difference between the band centers divided by the average bandwidth, measured in the same

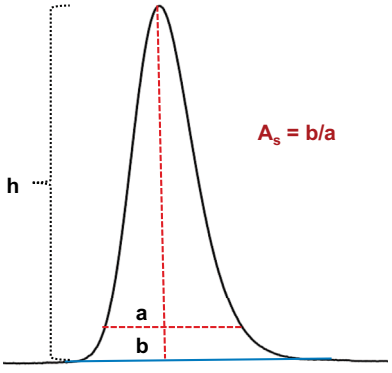

Figure 1.5 Measurement of the asymmetry of a chromatographic peak, where *h* is the peak height and *a* and *b* are measured at 10% of *h*.

units (Figure 1.6):

$$R_s = 2(t_2 - t_1)/(w_1 + w_2).$$

When $R_S = 1.5$, there is baseline separation between the two peaks and when $R_S = 1$, there is still only 2% overlap (with perfect Gaussian peaks), as shown in Figure 1.6.

The resolution of two peaks is determined by three variables: the retention factor (k), the plate number (N), and the separation factor (α).

The separation factor (selectivity) $\alpha = k_2/k_1$ (where $k_2 > k_1$).

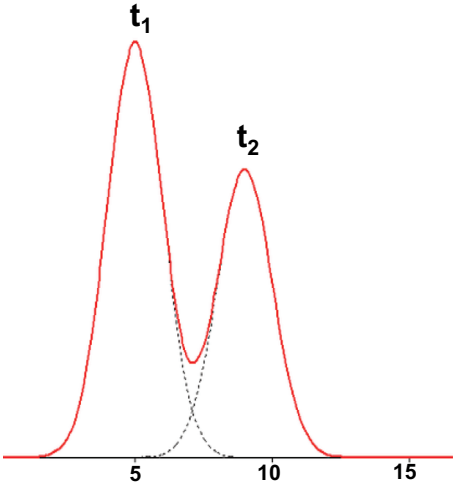

Figure 1.6 Chromatogram of two partially resolved peaks.

The resolution can be described by the equation

$$R_S = \frac{1}{4}(\alpha - 1)\sqrt{N}\frac{k}{(1+k)} \tag{1.11}$$

for two closely eluting compounds.

This is a fundamental equation in chromatography. The resolution of two peaks can be improved by increasing α, N, or k.

1.5.1 Increasing the Resolution

If two compounds are coeluting with small k, the easiest way of improving the resolution is to increase the retention (increasing k). In GC, this is obtained by reducing the column temperature. In HPLC, this is usually obtained by reducing the solvent strength of the mobile phase.

The most effective way to increase α is often by changing the stationary phase or the mobile phase (the latter not in GC).

Note that connecting two columns of equal quality and size will double the plate number, but the resolution will only increase by $\sqrt{2}$.

1.6 Peak Capacity

The peak capacity (n_c) is a measure of the number of compounds that can be theoretically resolved in a column:

$$n_c = (t_x - t_1)/w_{av}, \tag{1.12}$$

where t_1 is the retention time of the first eluting peak, t_x is the retention time of the last eluting peak, and w_{av} is the average peak width at the base ($w = 4\sigma$) or at the half peak height ($w = 2.354\sigma$).

With gradient elution, w_{av} is approximately equal for all peaks.

An alternative measure for peak capacity replaces $(t_x - t_1)$ with the gradient time. With few peaks in the front and at the end of the chromatogram, this method results in higher measured numbers for peak capacity.

In LC, the theoretical peak capacity of one column can vary from less than 20 to more than 1000. The latter can be obtained in long columns with extreme efficiency. In capillary GC, the numbers can be even higher.

1.7 Two-Dimensional Systems

In two-dimensional (2D) chromatography, two separation modes with different separation properties are coupled together in order to increase the ability to separate

many components as in thin layer chromatography, gel electrophoresis, gas chromatography, and liquid chromatography.

In thin layer chromatography and in gel electrophoresis, it is performed in two directions on one plate. For macromolecules such as proteins, 2D GE consists of one separation by size and one separation by pI (the pH at which the analyte has no net charge).

In both GC and LC, separation is performed with two columns. In the comprehensive 2D LC × LC, every fraction from the first column is separated in the second column. In the heart-cut 2D LC–LC, only one fraction or a few fractions are transferred to the second column.

When two columns are coupled together in a two-dimensional system (GC × GC or LC × LC), the theoretical peak capacity of the system is

$$n_{2D} = n_1 \cdot n_2,$$

where n_1 and n_2 are the peak capacities for columns 1 and 2, respectively.

This is correct only if the two columns are 100% orthogonal (separating according to different principles), if there is sufficient sampling frequency (small fraction width) in the first dimension, if there is no band broadening in the connection, and if the full separation space in the second column is used. This is never the case, which means that the actual peak capacity of a 2D system is always lower than the theoretical peak capacity. The optimum sampling frequency is obtained by splitting each peak in the first dimension into four fractions. This ensures that a separation that has been obtained in the first column will not be lost in the second column. The disadvantage of high sampling frequency is that each analyte will be found in several fractions.

Info-box 1.5

Two-dimensional chromatography is described in more detail in Refs [5,6].

1.8 Increased Performance

Increased performance is usually related to higher speed, higher plate numbers, improved resolution, or higher sensitivity, although all these cannot be improved at the same time.

Higher speed will reduce the time of analysis, but will increase the backpressure and may reduce the plate numbers. In packed columns, small particles are preferred for high-speed purposes, since the van Deemter curve is more flat for small particles.

Smaller particles in a packed column will increase the number of plates, but will also increase the backpressure.

Longer columns will give higher plate numbers, but will increase the time of analysis and give higher backpressure.

Reduced column inner diameter will increase the efficiency of open tubular columns, but not of packed columns.

Thin films of stationary phase increase efficiency in GC.

Increased temperature may be beneficial for the efficiency and peak shape and give lower backpressure in LC (but higher backpressure in GC).

The maximum peak capacity is obtained with two-dimensional systems, at the cost of simplicity and some robustness.

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