11 Chromatographic techniques

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11.1 PRINCIPLES OF CHROMATOGRAPHY

11.1.1 Distribution coefficients

The basis of all forms of chromatography is the **distribution** or **partition coefficient** (K_d), which describes the way in which a compound (the **analyte**) distributes between two immiscible phases. For two such phases A and B, the value for this coefficient is a constant at a given temperature and is given by the expression:

$$\frac{\text{concentration in phase A}}{\text{concentration in phase B}} = K_d \qquad (11.1)$$

The term **effective distribution coefficient** is defined as the total amount, as distinct from the concentration, of analyte present in one phase divided by the total amount present in the other phase. It is in fact the distribution coefficient multiplied by the ratio of the volumes of the two phases present. If the distribution coefficient of an analyte between two phases A and B is 1, and if this analyte is distributed between 10 cm^3 of A and 1 cm^3 of B, the concentration in the two phases will be the same, but the total amount of the analyte in phase A will be 10 times the amount in phase B.

All chromatographic systems consist of the **stationary phase**, which may be a solid, gel, liquid or a solid/liquid mixture that is immobilised, and the **mobile phase**, which

may be liquid or gaseous, and which is passed over or through the stationary phase after the mixture of analytes to be separated has been applied to the stationary phase. During the chromatographic separation the analytes continuously pass back and forth between the two phases so that differences in their distribution coefficients result in their separation.

11.1.2 Column chromatography

In column chromatography the stationary phase is packed into a glass or metal column. The mixture of analytes is then applied and the mobile phase, commonly referred to as the **eluent**, is passed through the column either by use of a pumping system or applied gas pressure. The stationary phase is either coated onto discrete small particles (the **matrix**) and packed into the column or applied as a thin film to the inside wall of the column. As the eluent flows through the column the analytes separate on the basis of their distribution coefficients and emerge individually in the **eluate** as it leaves the column.

Basic column chromatographic components

A typical column chromatographic system using a gas or liquid mobile phase consists of the following components:

- *A stationary phase*: Chosen to be appropriate for the analytes to be separated.
- *A column*: In liquid chromatography these are generally 25–50 cm long and 4 mm internal diameter and made of stainless steel whereas in gas chromatography they are 1–3 m long and 2–4 mm internal diameter and made of either glass or stainless steel. They may be either of the **conventional** type filled with the stationary phase, or of the **microbore** type in which the stationary phase is coated directly on the inside wall of the column.
- *A mobile phase and delivery system*: Chosen to complement the stationary phase and hence to discriminate between the sample analytes and to deliver a constant rate of flow into the column.
- *An injector system*: To deliver test samples to the top of the column in a reproducible manner.
- *A detector and chart recorder*: To give a continuous record of the presence of the analytes in the eluate as it emerges from the column. Detection is usually based on the measurement of a physical parameter such as visible or ultraviolet absorption or fluorescence. A peak on the chart recorder represents each separated analyte.
- *A fraction collector*: For collecting the separated analytes for further biochemical studies.

The two forms of column chromatography to be discussed in this chapter are **liquid chromatography** (LC), mainly **high-performance liquid chromatography** (HPLC), and **gas chromatography** (GC).

11.1.3 Analyte development and elution

Analyte development and elution relates to the separation of the mixture of analytes applied to the stationary phase by the mobile phase and their elution from the column. Column chromatographic techniques can be subdivided on the basis of the development and elution modes.

- In **zonal development,** the analytes in the sample are separated on the basis of their distribution coefficients between the stationary and mobile phases. The sample is dissolved in a suitable solvent and applied to the stationary phase as a narrow, discrete band. The mobile phase is then allowed to flow continuously over the stationary phase, resulting in the progressive separation and elution of the sample analytes. If the composition of the mobile phase is constant as in GC and some forms of HPLC, the process is said to be **isocratic elution**. To facilitate separation however, the composition of the mobile phase may be gradually changed, for example with respect to pH, salt concentration or polarity. This is referred to as **gradient elution**. The composition of the mobile phase may be changed continuously or in a stepwise manner.
- In **displacement** or **affinity development that is confined to some forms of HPLC** the analytes in the sample are separated on the basis of their affinity for the stationary phase. The sample of analytes dissolved in a suitable solvent is applied to the stationary phase as a discrete band. The analytes bind to the stationary phase with a strength determined by their affinity constant for the phase. The analytes are then selectively eluted by using a mobile phase containing a specific solute that has a higher affinity for the stationary phase than have the analytes in the sample. Thus, as the mobile phase is added, this agent displaces the analytes from the stationary phase in a competitive fashion, resulting in their repetitive binding and displacement along the stationary phase and eventual elution from the column in the order of their affinity for the stationary phase, the one with the lowest affinity being eluted first.

11.2 CHROMATOGRAPHIC PERFORMANCE PARAMETERS

11.2.1 Introduction

The successful chromatographic separation of analytes in a mixture depends upon the selection of the most appropriate process of chromatography followed by the optimisation of the experimental conditions associated with the separation. Optimisation requires an understanding of the processes that are occurring during the development and elution, and of the calculation of a number of experimental parameters characterising the behaviour of each analyte in the mixture.

In any chromatographic separation two processes occur concurrently to affect the behaviour of each analyte and hence the success of the separation of the analytes from each other. The first involves the basic mechanisms defining the chromatographic

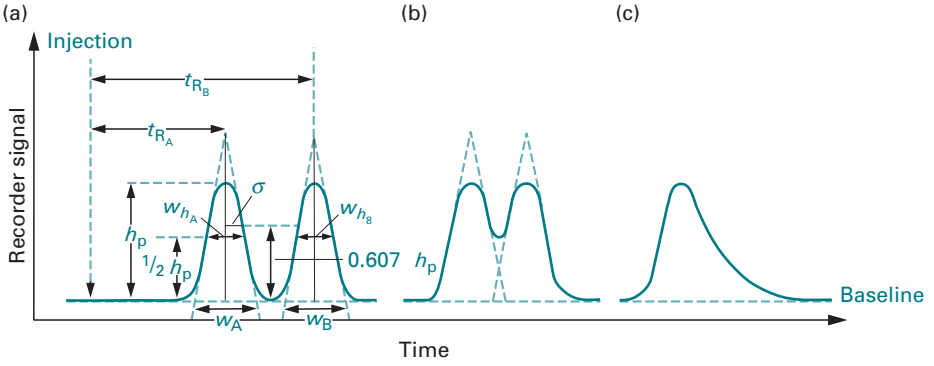

Fig. 11.1 (a) Chromatogram of two analytes showing complete resolution and the calculation of retention times; (b) chromatogram of two analytes showing incomplete resolution (fused peaks); (c) chromatogram of an analyte showing excessive tailing.

process such as adsorption, partition, ion exchange, ion pairing and molecular exclusion. These mechanisms involve the unique kinetic and thermodynamic processes that characterise the interaction of each analyte with the stationary phase. The second general process defines the other processes, such as **diffusion**, which tend to oppose the separation and which result in non-ideal behaviour of each analyte. These processes are manifest as a **broadening** and **tailing** of each analyte band. The analytical challenge is to minimise these secondary processes.

11.2.2 Retention time

A **chromatogram** is a pictorial record of the detector response as a function of **elution volume** or **retention time**. It consists of a series of **peaks** or **bands**, ideally symmetrical in shape, representing the elution of individual analytes, as shown in Fig. 11.1. The retention time t_R for each analyte has two components. The first is the time it takes the analyte molecules to pass through the free spaces between the particles of the matrix coated with the stationary phase. This time is referred to as the **dead time**, t_M. The volume of the free space is referred to as the column **void volume**, V_0. The value of t_M will be the same for all analytes and can be measured by using an analyte that does not interact with the stationary phase but simply spends all of the elution time in the mobile phase travelling through the void volume. The second component is the time the stationary phase retains the analyte, referred to as the **adjusted retention time**, t'_R. This time is characteristic of the analyte and is the difference between the observed retention time and the dead time:

$$t'_R = t_R - t_M \tag{11.2}$$

It is common practice to relate the retention time t_R or t'_R for an analyte to a reference internal standard (Section 11.2.5). In such cases the **relative retention time** is often calculated. It is simply the retention time for the analyte divided by that for the standard.

11.2.3 Retention factor

One of the most important parameters in chromatography is the **retention factor**, k (previously called **capacity factor** and represented by the symbol k'). It is simply the additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase and which, by definition, has a k value of 0. Thus:

$$k = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M} \tag{11.3}$$

It is apparent from this equation that if the analyte spends an equal time in the stationary and mobile phases, its t_R would equal $2t_M$ and its k would be 1, whilst if it spent four times as long in the stationary phase as the mobile phase t_R would equal $5t_M$ so that k would equal $5t_M - t_M / t_M = 4$. Note that k has no units.

If an analyte has a k of 4, it follows that there will be four times the amount of analyte in the stationary phase than in the mobile phase at any point in the column at any time. It is evident, therefore, that k is related to the distribution coefficient of the analyte (equation 11.1), which was defined as the relative concentrations of the analyte between the two phases. Since amount and concentration are related by volume, we can write:

$$k = \frac{t'_R}{t_M} = \frac{M_S}{M_M} = K_d \times \frac{V_S}{V_M} \tag{11.4}$$

where M_S is the mass of analyte in the stationary phase, M_M is the mass of analyte in the mobile phase, V_S is the volume of stationary phase and V_M is the volume of mobile phase. The ratio V_S/V_M is referred to as the **volumetric phase ratio**, β. Hence:

$$k = K_d\beta \tag{11.5}$$

Thus the retention factor for an analyte will increase with both the distribution coefficient between the two phases and the volume of the stationary phase. Values of k normally range from 1 to 10. Retention factors are important because they are independent of the physical dimensions of the column and the rate of flow of mobile phase through it. They can therefore be used to compare the behaviour of an analyte in different chromatographic systems. They are also a reflection of the selectivity of the system that in turn is a measure of its inherent ability to discriminate between two analytes. Such selectivity is expressed by the **selectivity** or **separation factor**, α, which can also be viewed as simply the relative retention ratio for the two analytes:

$$\alpha = \frac{k_A}{k_B} = \frac{K_{d_A}}{K_{d_B}} = \frac{t'_{R_A}}{t'_{R_B}} \tag{11.6}$$

The selectivity factor is influenced by the chemical nature of the stationary and mobile phases. Some chromatographic mechanisms are inherently highly selective. Good examples are affinity chromatography (Section 11.8) and chiral chromatography (Section 11.5.5).

11.2.4 Plate height and resolution

Plate height

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for an analyte to completely equilibrate between the two phases. Each zone is called a **theoretical plate** (of which there are N in total in the column). The length of column containing one theoretical plate is referred to as the **plate height**, H, which has units of length normally in micrometres. The smaller the value of H and the greater the value of N, the more efficient is the column in separating a mixture of analytes. The numerical value of both N and H for a particular column is expressed by reference to a particular analyte. Plate height is simply related to the width of the analyte peak, expressed in terms of its standard deviation σ (Fig. 11.1), and the distance it travelled within the column, x. Specifically:

$$H = \frac{\sigma^2}{x} \tag{11.7}$$

For symmetrical Gaussian peaks, the base width is equal to 4σ and the peak width at the point of inflection, w_i, is equal to 2σ. Hence the value of H can be calculated from the chromatogram by measuring the peak width. The number of theoretical plates in the whole column of length L is equal to L divided by the plate height:

$$N = \frac{L}{H} = \frac{Lx}{\sigma^2} \tag{11.8}$$

If the position of a peak emerging from the column is such that $x = L$, from knowledge of the fact that the width of the peak at its base, w, obtained from tangents drawn to the two steepest parts of the peak, is equal to 4σ (this is a basic property of all Gaussian peaks) hence $\sigma = w/4$ and equation 11.8 can therefore be converted to:

$$N = \frac{L^2}{\sigma^2} = \frac{16L^2}{w^2} \tag{11.9}$$

If both L and w are measured in units of time rather than length, then equation 11.9 becomes:

$$N = 16(t_R/w)^2 \tag{11.10a}$$

Rather than expressing N in terms of the peak base width, it is possible to express it in terms of the peak width at half height ($w_{1/2}$) and this has the practical advantage that this is more easily measured:

$$N = 5.54(t_R/w_{1/2})^2 \tag{11.10b}$$

Equations 11.9 and 11.10 represent alternative ways to calculate the **column efficiency** in theoretical plates. The value of N, which has no units, can be as high as 50 000 to 100 000 per metre for efficient columns and the corresponding value of H can be as little as a few micrometres. The smaller the plate height (the larger the value of N), the narrower is the analyte peak (Fig. 11.2).

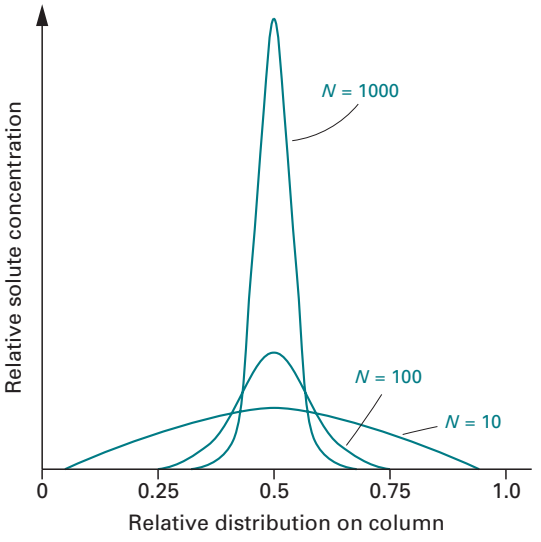

Fig. 11.2 Relationship between the number of theoretical plates (N) and the shape of the analyte peak.

Peak broadening

A number of processes oppose the formation of a narrow analyte peak thereby increasing the plate height:

- *Application of the sample to the column*: It takes a finite time to apply the analyte mixture to the column, so that the part of the sample applied first will already be moving along the column by the time the final part is applied. The part of the sample applied first will elute at the front of the peak.
- *Longitudinal diffusion*: Fick's law of diffusion states that an analyte will diffuse from a region of high concentration to one of low concentration at a rate determined by the concentration gradient between the two regions and the diffusion coefficient (P) of the analyte. Thus the analyte within a narrow band will tend to diffuse outwards from the centre of the band, resulting in band broadening.
- *Multiple pathways*: The random packing of the particles in the column results in the availability of many routes between the particles for both mobile phase and analytes. These pathways will vary slightly in length and hence elution time. The smaller the particle size the less serious is this problem and in open tubular columns the phenomenon is totally absent, which is one of the reasons why they give shorter elution times and better resolution than packed columns.
- *Equilibration time between the two phases*: It takes a finite time for each analyte in the test sample to equilibrate between the stationary and mobile phases as it passes down the column. As a direct consequence of the distribution coefficient, K_d, some of each analyte is retained by the stationary phase whilst the remainder stays in the mobile phase and continues its passage down the column. This partitioning automatically results in some spreading of each analyte band. Equilibration time, and hence band broadening, is also influenced by the particle size of the stationary phase. The smaller the size, the less time it takes to establish equilibration.

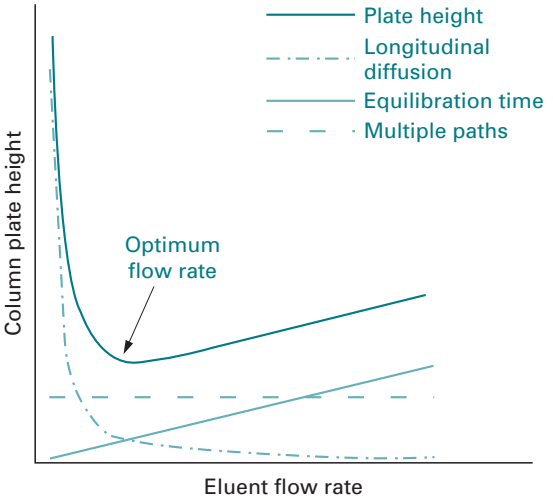

Fig. 11.3 Example of a van Deemter plot. The plot shows that the optimum flow rate for a given column is the net result of the influence of flow rate on longitudinal diffusion, equilibration time and multiple pathways.

Two of these four factors promoting the broadening of the analyte band are influenced by the flow rate of the eluent through the column. Longitudinal diffusion, defined by Fick's law, is inversely proportional to flow rate whilst equilibration time due to the partitioning of the analyte is directly proportional to flow rate. These two factors together with that of the multiple pathways factor determine the value of the plate height for a particular column and, as previously stated, plate height determines the width of the analyte peak. The precise relationship between the three factors and plate height is expressed by the **van Deemter equation** (equation 11.11) which is shown graphically in Fig. 11.3:

$$H = A + \frac{B}{u_x} + Cu_x \qquad (11.11)$$

where u_x is the flow rate of the eluent and A, B and C are constants for a particular column and stationary phase relating to multiple paths, longitudinal diffusion and equilibration time respectively.

Figure 11.3 gives a clear demonstration of the importance of establishing the optimum flow rate for a particular column. Longitudinal diffusion is much faster in a gas than in a liquid and as a consequence flow rates are higher in gas chromatography than in liquid chromatography.

As previously stated, the width of an analyte peak is expressed in terms of the standard deviation σ, which is half the peak width at the point of inflexion ($0.607h_p$, where h_p is the peak height, Fig. 11.1). It can be shown that $\sigma = \sqrt{2Pt_R}$ where P is the diffusion coefficient of the analyte that is a measure of the rate at which the analyte moves randomly in the mobile phase from a region of high concentration to one of lower concentration. It has units of $m^2\ s^{-1}$. Since the value of σ is proportional to the square root of t_R it follows that if the elution time increases by a factor of four the

width of the peak will double. Thus the longer it takes a given analyte to elute, the wider will be its peak. For this reason, increasing the column length is not the preferred way to improve resolution.

Asymmetric peaks

In some chromatographic separations, the ideal Gaussian-shaped peaks are not obtained, but rather asymmetrical peaks are produced. In cases where there is a gradual rise at the front of the peak and a sharp fall after the peak, the phenomenon is known as **fronting**. The most common cause of fronting is overloading the column so that reducing the amount of mixture applied to the column often resolves the problem. In cases where the rise in the peak is normal but the tail is protracted, the phenomenon is known as **tailing** (Fig. 11.1). The probable explanation for tailing is the retention of analyte by a few **active sites** on the stationary phase, commonly on the inert support matrix. Such sites strongly adsorb molecules of the analyte and only slowly release them. This problem can be overcome by chemically removing the sites, frequently hydroxyl groups, by treating the matrix with a silanising reagent such as hexamethyldisilazine. This process is sometimes referred to as **capping**. Peak asymmetry is usually expressed as the ratio of the width of the peak from the centre of the peak at 0.1 h_p.

Resolution

The success of a chromatographic separation is judged by the ability of the system to resolve one analyte peak from another. **Resolution** (R_S) is defined as the ratio of the difference in retention time (Δt_R) between the two peaks (t_{R_A} and t_{R_B}) to the mean (w_{av}) of their base widths (w_A and w_B):

$$R_S = \frac{\Delta t_R}{w_{av}} = \frac{2(t_{R_A} - t_{R_B})}{w_A + w_B} \tag{11.12}$$

When $R_S = 1.0$, the separation of the two peaks is 97.7% complete (thus the overlap is 2.3%). When $R_S = 1.5$ the overlap is reduced to 0.2%. Unresolved peaks are referred to as **fused peaks** (Fig. 11.1). Provided the overlap is not excessive, the analysis of the individual peaks can be made on the assumption that their characteristics are not affected by the incomplete resolution.

Resolution is influenced by column efficiency, selectivity factors and retention factors according to equation 11.13:

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_{av}}\right) \tag{11.13}$$

where k_2 is the retention factor for the longest retained peak and k_{av} is the mean retention factor for the two analytes. Equation 11.13 is one of the most important in chromatography as it enables a rational approach to be taken to the improvement of the resolution between the analytes. For example, it can be seen that resolution increases with $\sqrt{N}$. Since N is linked to the length of the column, doubling the length of the column will increase resolution by $\sqrt{2}$, i.e. by a factor of 1.4 and in general is not the preferred way to improve resolution. Since both retention factors and selectivity factors are linked to retention times and retention volumes, altering the nature of the

two phases or their relative volumes will impact on resolution. Retention factors are also dependent upon distribution coefficients, which in turn are temperature dependent; hence altering the column temperature may improve resolution.

The capacity of a particular chromatographic separation is a measure of the amount of material that can be resolved into its components without causing peak overlap or fronting. Ion-exchange chromatography (Section 11.6) has a high capacity, which is why it is often used in the earlier stages of a purification process.

11.2.5 Qualitative and quantitative analysis

Chromatographic analysis can be carried out on either a qualitative or quantitative basis.

Qualitative analysis

The objective of this approach is to confirm the presence of a specific analyte in a test sample. This is achieved on the evidence of:

- A comparison of the retention time of the peaks in the chromatograph with that of an authentic reference sample of the test analyte obtained under identical chromatographic conditions. Confirmation of the presence of the analyte in the sample can be obtained by spiking a second sample of the test sample with a known amount of the authentic compound. This should result in a single peak with the predicted increase in area.
- The use of either a mass spectrometer or nuclear magnetic resonance (NMR) spectrometer as a detector so that structural evidence for the identity of the analyte responsible for the peak can be obtained.

Quantitative analysis

The objective of this approach is to confirm the presence of a specific analyte in a test sample and to quantify its amount. Quantification is achieved on the basis of peak area coupled with an appropriate calibration graph. The area of each peak in a chromatogram can be shown to be proportional to the amount of the analyte producing the peak. The area of the peak may be determined by measuring the height of the peak (h_P) and its width at half the height (w_h) (Fig 11.1). The product of these dimensions is taken to be equal to the area of the peak. This procedure is time consuming when complex and/or a large number of analyses are involved and dedicated integrators or microcomputers best perform the calculations. These can be programmed to compute retention time and peak area and to relate them to those of a reference standard enabling relative retention ratios and relative peak area ratios to be calculated. These may be used to identify a particular analyte and to quantify it using previously obtained and stored calibration data. The data system can also be used to correct problems inherent in the chromatographic system. Such problems can arise either from the characteristics of the detector or from the efficiency of the separation process. Problems that are attributable to the detector are baseline drift, where the detector signal gradually changes with time, and baseline noise, which is a series of rapid minor fluctuations in detector signal, commonly the result of the operator using too high a detector sensitivity or possibly an electronic fault.

Example 1 CALCULATION OF THE RESOLUTION OF TWO ANALYTES

Question Two analytes A and B were separated on a 25 cm long column. The observed retention times were 7 min 20 s and 8 min 20 s, respectively. The base peak width for analyte B was 10 s. When a reference compound, which was completely excluded from the stationary phase under the same elution conditions, was studied, its retention time was 1 min 20 s. What was the resolution of the two analytes?

Answer In order to calculate the required resolution, it is first necessary to calculate other chromatographic parameters.

(i) The adjusted retention time for A and B based on equation 11.2: $t'_R = t_R - t_M$

For analyte A $t'_R = 440 - 80 = 360$ s

For analyte B $t'_R = 500 - 80 = 420$ s

(ii) The retention factor for A and B based on equation 11.3: $k = t_R/t_M$

For analyte A $k_A = 360/80 = 4.5$

For analyte B $k_B = 420/80 = 5.25$

(iii) The selectivity factor for the two analytes based on equation 11.5:

$\alpha = k_B/k_A$

$\alpha = 5.25/4.5 = 1.167$

(iv) The number of theoretical plates in the column; based on equation 11.10: $N = (t_R/\omega)^2$ for analyte B

$N = (420/10)^2 = 1764$

(v) The resolution of the two analytes based on equation 11.13:

$R_S = (\sqrt{N}/4)[(\alpha - 1)/\alpha)](k'_B/(1 + k_{av})$

gives

$R_s = (\sqrt{1764}/4)(0.167/1.167)(5.25/1 + 4.875) = 1.34$

Discussion From the earlier discussion on resolution, it is evident that a resolution of 1.34 is such as to give a peak separation of greater than 99%. If there were an analytical need to increase this separation it would be possible to calculate the length of column required to double the resolution. Since resolution is proportional to the square root of N, to double the resolution the number of theoretical plates in the column must be increased four-fold, i.e. to $4 \times 1764 = 7056$. The plate height in the column $H = L/N$, i.e. $250/1764 = 0.14$ mm. Hence to get 7056 plates in the column, its length must be increased to $0.14 \times 7056 = 987.84$ mm or 98.78 cm.

Quantification of a given analyte is based on the construction of a calibration curve obtained using a pure, authentic sample of the analyte. The construction of the calibration curve is carried out using the general principles discussed in Section 1.4.6. Most commonly the calibration curve is based on the use of relative peak areas obtained using an internal standard that has been subject to any preliminary extraction procedures adopted for the test samples. The standard must be carefully chosen to have similar physical and structural characteristics to those of the test analyte, and in practice is frequently an isomer or structural analogue of the analyte. Ideally, it should have a retention time close to that of the analyte but such that the resolution is greater than 99.5%.

11.2.6 Sample clean-up

Whilst chromatographic techniques are designed to separate mixtures of analytes, this does not mean that attention need not be paid to the preliminary purification (**clean-up**) of the test sample before it is applied to the column. On the contrary, it is clear that, for quantitative work using HPLC techniques in particular, such preliminary action is essential, particularly if the test analyte(s) is in a complex matrix such as plasma, urine, cell homogenate or microbiological culture medium. The extraction and purification of the components from a cell homogenate is often a complex multi-stage process. For some forms of analysis, for example the analysis of low-molecular-weight organic drugs in biological fluids, sample preparation is relatively easy. **Solvent extraction** is based on the extraction of the analytes from aqueous mixtures using a low-boiling-point water-immiscible solvent such as diethyl ether or dichloromethane. The extract is dried to remove traces of water before it is evaporated to dryness (often under nitrogen or *in vacuo*), the residue dissolved in the minimum volume of an appropriate solvent such as methanol or acetonitrile, and a sample applied to the column. This solvent extraction procedure tends to lack selectivity and is unsatisfactory for protein clean-up and for the HPLC analysis of analytes in the ng cm^{-3} or less range.

The alternative to solvent extraction is **solid phase extraction**, which unlike solvent extraction can be applied to the pre-purification of proteins. The test solution is passed through a small (few millimetres in length) disposable column (cartridge) packed with an appropriate stationary phase similar to those used for HPLC (Section 11.3). These selectively adsorb the analyte(s) under investigation and ideally allow interfering compounds to pass through. For example, for the purification of a mixture of proteins a reversed phase, affinity or ion exchange chromatography cartridge would be selected as these are ideal for desalting or concentrating protein mixtures containing in the range of femto- to picomoles of protein. Once the test solution has been passed through the column, either by simple gravity feed or by the application of a slight vacuum to the receiver vessel, the column is washed to remove traces of contaminants and the adsorbed analyte(s) recovered by elution with a suitable eluent. Complex protein and peptide mixtures can be partially fractionated at this stage by gradient elution. Several commercial forms of this solid phase extraction technique are available, for example Millipore Ziptips™, and the term **pipette tip chromatography** has been coined to describe it.

Table 11.1 **Examples of derivatising agents**

Analyte	Reagent
Pre-column	
Ultraviolet detection	
Alcohols, amines, phenols	3,5-Dinitrobenzoyl chloride
Amino acids, peptides	Phenylisothiocyanate, dansyl chloride
Carbohydrates	Benzoyl chloride
Carboxylic acids	1-*p*-Nitrobenzyl-*N*, *N*′-diisopropylisourea
Fatty acids, phospholipids	Phenacyl bromide, naphthacyl bromide
Electrochemical detection	
Aldehydes, ketones	2,4-Dinitrophenylhydrazine
Amines, amino acids	*o*-Phthalaldehyde, fluorodinitrobenzene
Carboxylic acids	*p*-Aminophenol
Fluorescent detection	
Amino acids, amines, peptides	Dansyl chloride, dabsyl chloride, fluoroescamine, *o*-phthalaldehyde
Carboxylic acids	4-Bromomethyl-7-methoxycoumarin
Carbonyl compounds	Dansylhydrazine
Post-column	
Ultraviolet detection	
Amino acids	Phenylisothiocyanate
Carbohydrates	Orcinol and sulphuric acid
Penicillins	Imidazole and mercuric chloride
Fluorescent detection	
Amino acids	*o*-Phthalaldehyde, fluorescamine, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate

Sample derivatisation

Some functional groups, especially hydroxyl, present in a test analyte may compromise the quality of its behaviour in a chromatographic system. The technique of analyte **pre-** or **post-column derivatisation** may facilitate better chromatographic separation and detection by masking these functional groups. Common derivatisation reagents are shown in Table 11.1.

11.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

11.3.1 Principle

It is evident from equations 11.1 to 11.13 that the resolving power of a chromatographic column is determined by a number of factors that are embedded in equation 11.13. This shows that the resolution increases with:

- the number of theoretical plates (N) in the column and hence plate height (H). The value of N increases with column length but there are practical limits to the length of a column owing to the problem of peak broadening (Section 11.2.4);
- the selectivity of the column, α; and
- the retentivity of the column as determined by the retention factor, k.

As the number of theoretical plates in the column is related to the surface area of the stationary phase, it follows that the smaller the particle size of the stationary phase, the greater the value of N, i.e. N is inversely proportional to particle size. Unfortunately, the smaller the particle size, the greater is the resistance to the flow of the mobile phase for a given flow rate. This resistance creates a backpressure in the column that is directly proportional to both the flow rate and the column length and inversely proportional to the square of the particle size. The back-pressure may be sufficient to cause the structure of the matrix to collapse, thereby actually further reducing eluent flow and impairing resolution. This problem has been solved by the development of small particle size stationary phases, generally in the region of 5–10 μm diameter with a narrow range of particle sizes, which can withstand pressures up to 40 MPa. This development, which is the basis of HPLC that was originally and incorrectly referred to as **high-pressure liquid chromatography**, explains why it has emerged as the most popular, powerful and versatile form of chromatography. Larger particle size phases are available commercially and form the basis of **low-pressure liquid chromatography** in which flow of the eluaent through the column is either gravity-fed or pumped by a low pressure pump, often a **peristaltic pump**. It is cheaper to run than HPLC but lacks the high resolution that is the characteristic of HPLC. Many commercially available HPLC systems are available and most are microprocessor-controlled to allow dedicated, continuous chromatographic separations.

Columns

The components of a typical HPLC system are shown in Fig. 11.4. **Conventional columns** used for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressures of up to 50 MPa. The columns are generally 3–25 cm long and approximately 4.6 mm internal diameter to give typical flow rates of 1–3 cm^3 min^{-1}. **Microbore** or **open tubular** columns have an internal diameter of 1–2 mm and are generally 25–50 cm long. They can sustain flow rates of 5–20 mm^3 min^{-1}. Microbore columns have three important advantages over conventional columns:

- reduced eluent consumption due to the slower flow rates;
- ideal for interfacing with a mass spectrometer due to the reduced flow rate; and
- increased sensitivity due to the higher concentration of analytes that can be used.

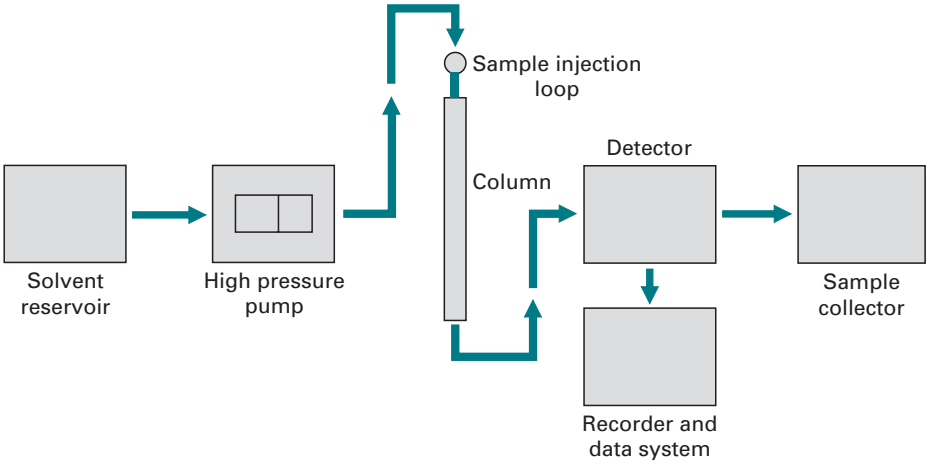

Fig. 11.4 Components of an isocratic HPLC system. For gradient elution two reservoirs and two pumps are used with liquid-phase mixing before entry to the sample injection loop.

Matrices and stationary phases

Two main forms of matrix/stationary phase material are available, based on a rigid solid structure. Both forms involve approximately spherical particles of a uniform size to minimise space for diffusion and hence band broadening to occur. They are made of chemically modified silica or styrene/divinylbenzene copolymers. The two forms are:

- *Microporous supports*: In which micropores ramify through the particles that are generally 5–10 μm in diameter.
- *Bonded phases*: In which the stationary phase is chemically bonded onto an inert support such as silica.

11.3.2 Application of sample

The application of the sample onto an HPLC column in the correct way is a particularly important factor in achieving successful separations. The most common method of sample introduction is by use of a **loop injector** (Fig. 11.5). This consists of a metal loop, of fixed small volume, that can be filled with the sample. The eluent from the pump is then channelled through the loop by means of a valve switching system and the sample flushed onto the column via the loop outlet without interruption of the flow of eluent to the column.

Repeated application of highly impure samples such as sera, urine, plasma or whole blood, which have preferably been deproteinated, may eventually cause the column to lose its resolving power. To prevent this occurrence, a **guard column** is often installed between the injector and the column. This guard column is a short (1–2 cm) column of the same internal diameter and packed with material similar to that present in the analytical column. The packing in the guard column preferentially retains contaminating material and can be replaced at regular intervals.

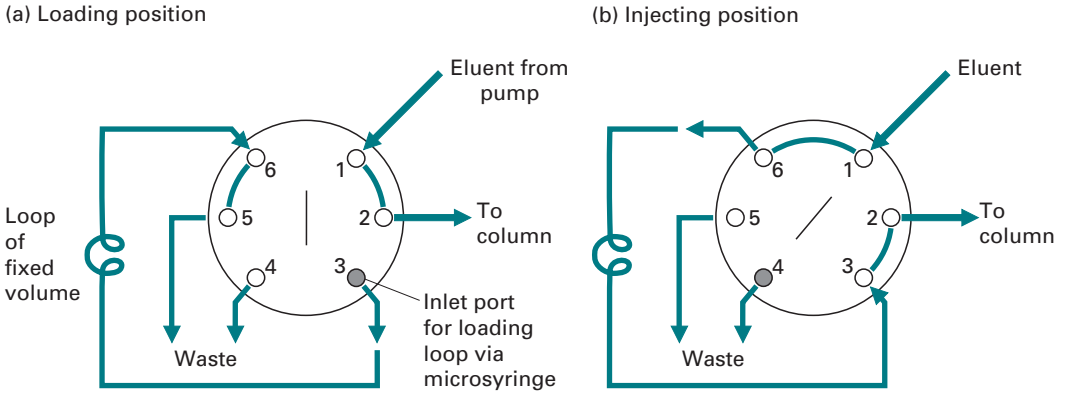

Fig. 11.5 HPLC loop injector. (a) The loop is loaded via port 3 with excess sample going to waste via port 5. In this position the eluent from the pump passes to the column via ports 1 and 2. (b) In the injecting position eluent flow is directed through the loop via ports 1 and 6 and then onto the column.

Mobile phases

The choice of mobile phase to be used in any separation depends on the type of separation to be achieved. Isocratic elution may be made with a single pump, using a single eluent or two or more eluents premixed in fixed proportions. Gradient elution generally uses separate pumps to deliver two eluents in proportions predetermined by a **gradient programmer**. All eluents for use in HPLC systems must be specially purified because traces of impurities can affect the column and interfere with the detection system. This is particularly the case if the detection system is based on the measurement of absorbance changes below 200 nm. Pure eluents for use in HPLC systems are available commercially, but even with these a 1–5 mm microfilter is generally introduced into the system prior to the pump. It is also essential that all eluents be degassed before use otherwise **gassing** (the presence of air bubbles in the eluent) tends to occur in most pumps. Gassing, which tends to be particularly bad for eluents containing aqueous methanol and ethanol, can alter column resolution and interfere with the continuous monitoring of the eluate. Degassing of the eluent may be carried out in several ways – by warming, by stirring vigorously with a magnetic stirrer, by applying a vacuum, by ultrasonication, and by bubbling helium gas through the eluent reservoir.

Pumps

Pumping systems for delivery of the eluent are one of the most important features of HPLC systems. The main features of a good pumping system are that it is capable of outputs of at least 50 MPa and ideally there must be no pulses (i.e. cyclical variations in pressure) as this may affect the detector response. There must be a flow capability of at least 10 cm^3 min^{-1} and up to 100 cm^3 min^{-1} for preparative separations. **Constant displacement pumps** maintain a constant flow rate through the column irrespective of changing conditions within the column. The **reciprocating pump** is the most

commonly used form of constant displacement pump. Such pumps produce small pulses of flow and pulse dampeners are usually incorporated into the system to minimise this pulsing effect. All constant displacement pumps have inbuilt safety cut-out mechanisms so that if the pressure within the column changes from pre-set limits the pump is inactivated automatically.

11.3.3 Detectors

Since the quantity of material applied to an HPLC column is normally very small, it is imperative that the sensitivity of the detector system is sufficiently high and stable to respond to the low concentrations of each analyte in the eluate. The most commonly used detectors are:

- *Variable wavelength detectors*: These are based upon ultraviolet–visible spectrophotometry. These types of detector are capable of measuring absorbances down to 190 nm and can give full-scale deflection (AUFS) for as little as 0.001 absorbance units. They have a detection sensitivity of the order of 5×10^{-10} g cm^{-3} and a linear range of 10^5. All spectrophotometric detectors use continuous flow cells with a small internal volume (typically 8 mm^3) and optical path length of 10 mm which allow the continuous monitoring of the column eluate.
- *Scanning wavelength detectors*: These have the facility to record the complete absorption spectrum of each analyte, thus aiding identification. Such opportunities are possible either by temporarily stopping the eluent flow or by the use of **diode array** techniques, which allow a scan of the complete spectrum of the eluate within 0.01 s and its display as a 3D plot on a VDU screen in real time (Fig. 11.6).
- *Fluorescence detectors*: These are extremely valuable for HPLC because of their greater sensitivity (10^{-12} g cm^{-3}) than UV detectors but they have a slightly reduced linear range (10^4). However, the technique is limited by the fact that relatively few analytes fluoresce. Pre-derivatisation of the test sample can broaden the applications of the technique.
- *Electrochemical detectors*: These are selective for electroactive analytes and are potentially highly sensitive. Two types are available, **amperometric** and **coulometric**, the principles of which are similar. A flow cell is fitted with two electrodes, a stable counter electrode and a working electrode. A constant potential is applied to the working electrode at such a value that, as an analyte flows through the flow cell, molecules of the analyte at the electrode surface undergo either an oxidation or a reduction, resulting in a current flow between the two electrodes. The size of the current is recorded to give the chromatogram. The potential applied to the counter electrode is sufficient to ensure that the current detected gives a full-scale deflection on the recorder within the working analyte range. The two types of detector differ in the extent of conversion of the analyte at the detector surface and on balance amperometric detectors are preferred since they have a higher sensitivity (10^{-12} g cm^{-3} as opposed to 10^{-8} g cm^{-3}) and greater linear range (10^5 as opposed to 10^4).

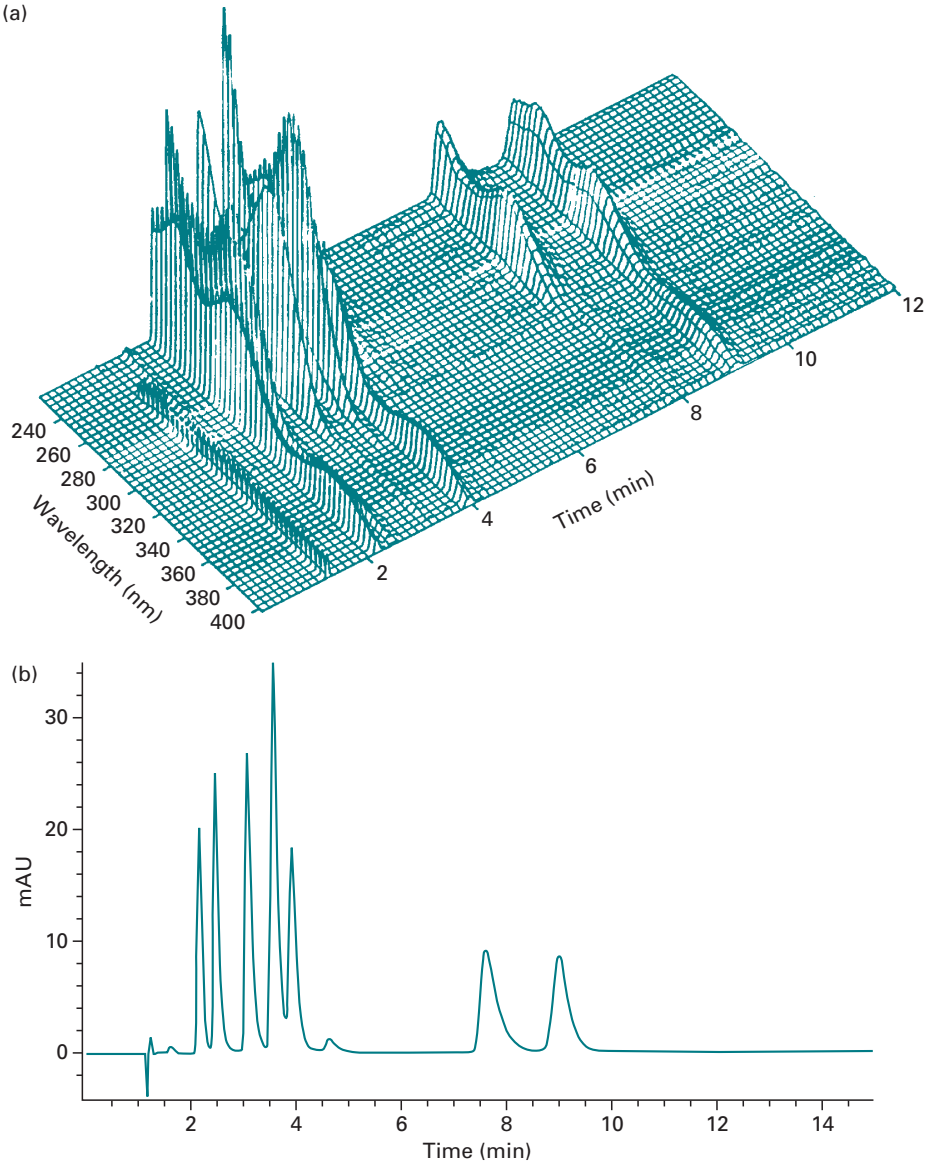

Fig. 11.6 Separation by HPLC of the dihydropyridine calcium channel blocker lacidipine and its metabolites. Column: ODS Hypercil. Eluent: methanol/acetonitrile/water (66%, 5%, 29% by volume) acidified to pH 3.5 with 1% formic acid. Flow rate: 1 cm^3 min^{-1}. Column temperature: 40 °C. (a) As recorded by a diode array detector and (b) by an ultraviolet detector. (Reproduced by permission of GlaxoSmithKline, UK.)

For reduction reactions the working electrode is normally mercury, and for oxidative reactions carbon or a carbon composite.

- *Mass spectrometer detectors*: These enable the analyte to be detected and its structure determined simultaneously. The technical problems associated with the logistics of removing the bulk of the mobile phase before the sample is introduced

into the mass spectrometer have been resolved in a number of ways that are discussed in detail in Chapter 9. Analytes may be detected by total ion current (TIC) (Section 9.4.1) or selected ion monitoring (SIM) (Section 9.5.6). An advantage of mass spectrometry detection is that it affords a mechanism for the identification of overlapping peaks. If there is a suspicion that a large peak is masking a smaller peak then the presence of a minor analyte can be confirmed by selected ion monitoring provided that the minor and major analytes have a unique molecular ion or fragment ion.

- *NMR spectrometer detectors*: These give structural information about the analyte that is complementary to that obtained via HPLC–MS.
- *Refractive index detectors*: These rely on a change in the refractive index of the eluate as analytes emerge from the column. The great advantage is that they will respond to any analyte in any eluent, changes in refractive index being either positive or negative. Their limitation is the relatively modest sensitivity (10^{-7} g cm^{-3}) but they are commonly used in the analysis of carbohydrates.
- *Evaporative light-scattering detectors* (ELSD): These rely on the vaporisation of the eluate, evaporation of the eluent and the quantification of the analyte by light scattering. The eluate emerging from the column is combined with a flow of air or nitrogen to form an aerosol; the eluent is then evaporated from the aerosol by passage through an evaporator and the emerging dry particles of analyte irradiated with a light source and the scattered light detected by a photodiode. The intensity of the scattered light is determined by the quantity of analyte present and its particle size. It is independent of the analyte's spectroscopic properties and hence does not require the presence of a chromophoric group or any prior derivatisation of the analyte. It can quantify analytes in flow rates of up to 5 cm^3 min^{-1}. Appropriate calibration gives good, stable quantification of the analyte with no baseline drift. It is an attractive method for the detection of fatty acids, lipids and carbohydrates.

The sensitivity of ultraviolet absorption, fluorescence and electrochemical detectors can often be increased significantly by the process of derivatisation, whereby the analyte is converted pre- or post-column to a chemical derivative. Examples are given in Table 11.1.

Ultra-performance liquid chromatography (UPLC)

As was pointed out earlier, the resolution of a mixture of analytes increases as the particle size of the stationary phases decreases, but such a decrease leads to a high back-pressure from eluent flow. The technological solution to this problem represented by HPLC has recently been advanced by the development of new stationary phases of less than 2 μm diameter by the Waters Corporation. The particles of 1.7 μm diameter are made of 'Bridged Ethylsiloxane Silica Hybrid' (BEH)™ and are available in a range of forms suitable for various applications (Fig. 11.7). Back-pressures of up to 150 MPa are generated and this necessitated the development of special pumps, columns and detectors capable of operating in a pulse-free way at these high pressures. The instrumentation available under the trade name of ACQUITY™

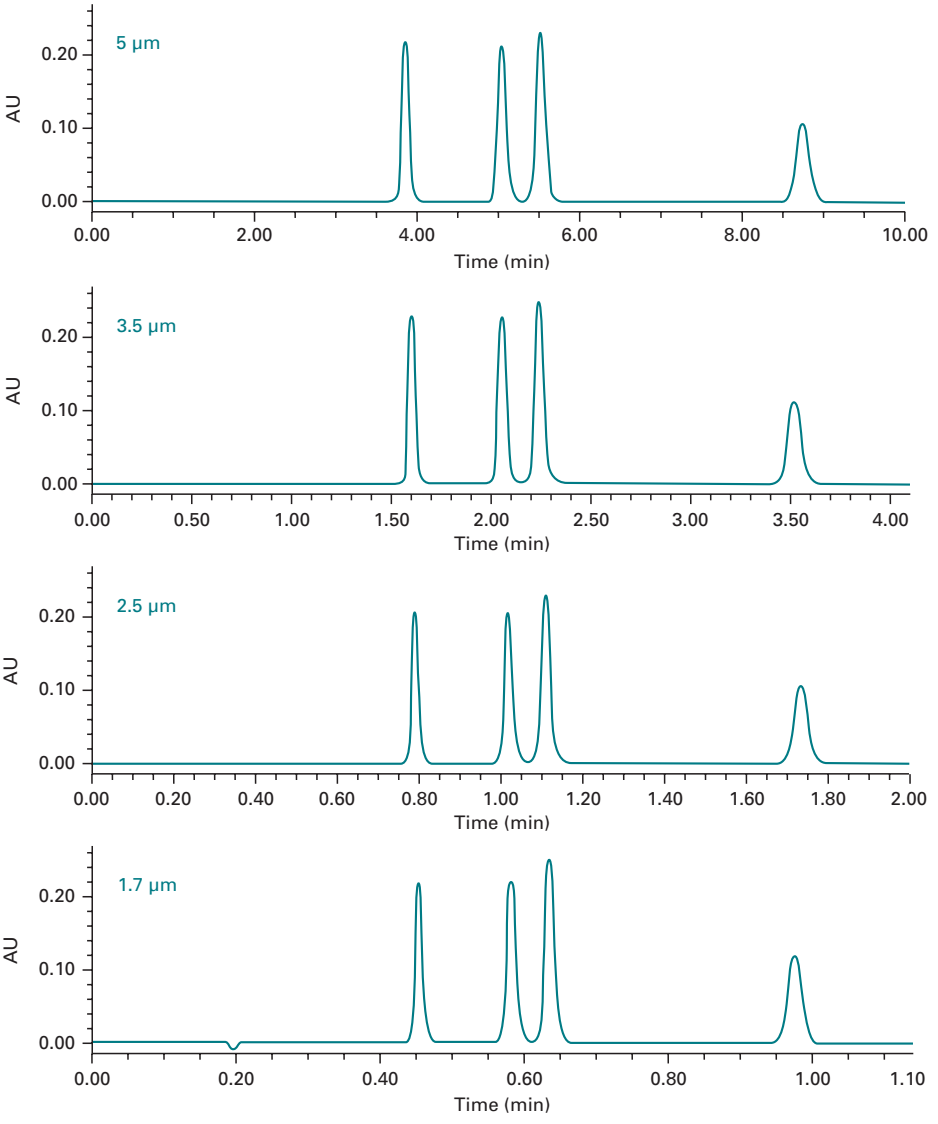

Fig 11.7 Chromatograms showing the influence of particle size on the resolution and elution time of four analytes in an HPLC system demonstrating the time advantages of the pressure-tolerant sub-2 µm ACQUITY UPLC system. (Reproduced by permission of Waters Corporation, UK.)

and the term UPLC are both registered to Waters. The system operates up to 10 times faster than conventional HPLC and chromatograms are routinely complete in less than 5 min. Whereas conventional HPLC peaks last up to 10 s, UPLC peaks may last 1 s so detectors have to respond ultra-fast to define the peak. The technology is now widely used in drug discovery and metabolite identification by the pharmaceutical industry.

11.3.4 Perfusion chromatography

The high resolution achieved by HPLC is based on the use of small diameter particles for the stationary phase. However, this high resolution is achieved at the cost of the generation of high pressures, relatively low flow rates and the constraints the high pressure imposes on the instrumentation. **Perfusion chromatography** overcomes some of these limitations by the use of small particles (10–50 μm diameter) that have channels of approximately 1 μm diameter running through them that allow the use of high flow rates without the generation of high pressures. The high flow rates result in small plate heights (Section 11.2.4) and hence high resolution in very short separation times. The particles are made of polystyrene-divinylbenzene and are available under the trade name POROS. Two types of pore are available: **through pores** that are long (up to 8000 Å) and **diffusive pores** that are shorter (up to 1000 Å). The stationary phase is coated onto the particles, including the surface of the pores. The eluent perfuses through the pores allowing the analyte to equilibrate rapidly with the stationary phase. By comparison, the microporous particles used in HPLC have a much smaller diameter pore, hence the greater back-pressure. All the forms of stationary phase used for the various forms of chromatography are available for perfusion chromatography. The technique uses the same type of instrumentation as HPLC. Protein separations in as short a time as 1 min can be achieved.

11.4 ADSORPTION CHROMATOGRAPHY

11.4.1 Principle

This is the classic form of chromatography, which is based upon the principle that certain solid materials, collectively known as **adsorbents**, have the ability to hold molecules at their surface. This adsorption process, which involves weak, non-ionic attractive forces of the van der Waals' and hydrogen-bonding type, occur at specific **adsorption sites**. These sites have the ability to discriminate between types of molecules and are occupied by molecules of either the eluent or of the analytes in proportions determined by their relative strength of interaction. As eluent is constantly passed down the column, differences in these binding strengths eventually lead to the separation of the analytes.

Silica is a typical adsorbent. It has silanol (Si-OH) groups on its surface, which are slightly acidic, and can interact with polar functional groups of the analyte or eluent. The **topology** (arrangement) of these silanol groups in different commercial preparations of silica explains their different separation properties. Other commonly used adsorbents are alumina and carbon.

In general, an eluent with a polarity comparable to that of the most polar analyte in the mixture is chosen. Thus, alcohols would be selected if the analytes contained hydroxyl groups, acetone or esters would be selected for analytes containing carbonyl groups, and hydrocarbons such as hexane, heptane and toluene for analytes that are predominantly non-polar. Mixtures of solvents are commonly used in the context of gradient elution.

11.4.2 Hydroxylapatite chromatography

Crystalline hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$) is an adsorbent used to separate mixtures of proteins or nucleic acids. One of the most important applications of hydroxylapatite chromatography is the separation of single-stranded DNA from double-stranded DNA. Both forms of DNA bind at low phosphate buffer concentrations but as the buffer concentration is increased single-stranded DNA is selectively desorbed. As the buffer concentration is increased further, double-stranded DNA is released. This behaviour is exploited in the technique of Cot analysis (Section 5.3.4). The affinity of double-stranded DNA for hydroxylapatite is so high that it can be selectively removed from RNA and proteins in cell extracts by use of this type of chromatography.

11.4.3 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) was developed to purify proteins by exploiting their surface hydrophobicity which is a measure of their dislike of binding molecules of water. Groups of hydrophilic amino acid residues are scattered over the surface of proteins in a way that gives characteristic properties to each protein. In aqueous solution, these hydrophilic regions on the protein are covered with an ordered layer of water molecules that effectively mask the hydrophobic groups of the proteins the majority of which are in the interior of the folded molecule. These hydrophobic groups can, however, be exposed by the addition of salt ions, which preferentially take up the ordered water molecules. The exposed hydrophobic regions can then interact with each other by weak van der Waals' forces causing protein–protein aggregation. In HIC, the presence of hydrophobic groups such as butyl, octyl and phenyl attached to a matrix facilitates protein–matrix interaction rather than facilitating protein–protein interaction. Commercial materials include Phenyl Sepharose and Phenyl SPW, both for low-pressure HIC, and Poly PROPYL Aspartamide, Bio-Gel TSK Phenyl and Spherogel TSK Phenyl for HPLC HIC.

Since HIC requires the presence of salting-out compounds such as ammonium sulphate to facilitate the exposure of the hydrophobic regions on the protein molecule, it is commonly used immediately after fractionation of protein mixtures with ammonium sulphate. To maximise the process, it is advantageous to adjust the pH of the protein sample to that of its isoelectric point. Once the proteins have been adsorbed to the stationary phase, selective elution can be achieved in a number of ways, including the use of an eluent of gradually decreasing ionic strength or of increasing pH (this increases the hydrophilicity of the protein) (Fig. 11.8) or by selective displacement by a displacer that has a stronger affinity for the stationary phase than has the protein. Examples include non-ionic detergents such as Tween 20 and Triton X-100, aliphatic alcohols such as 1-butanol and ethylene glycol, and aliphatic amines such as 1-aminobutane. HIC has many similarities with reversed-phase HPLC (RPC) but has two advantages over it. The first is that it uses aqueous elution conditions that minimise protein denaturation whereas RPC requires non-polar solvents for elution. The second is that it has a higher capacity.

The technique of immobilised artificial membrane chromatography (IAM) resembles HIP and uses phosphotidylcholine-based stationary phases. It is widely used in

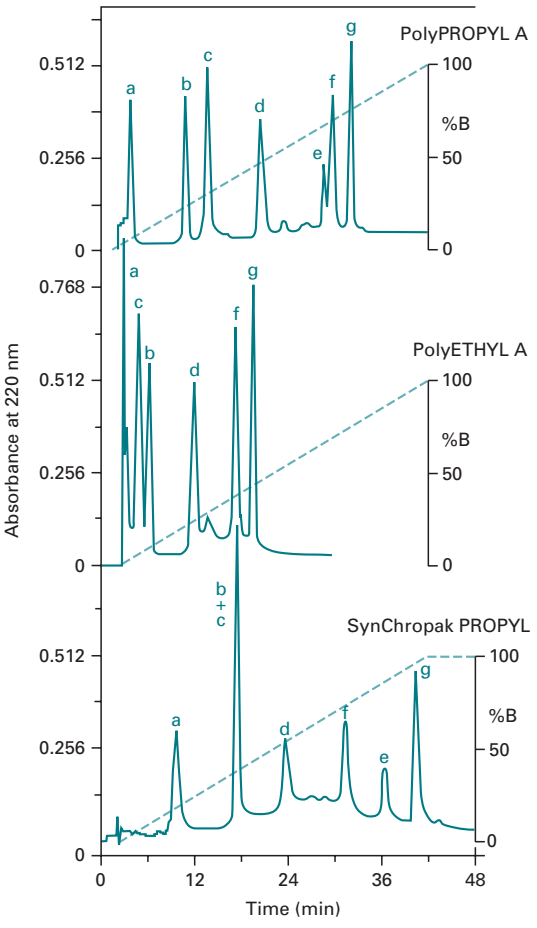

Fig. 11.8 Chromatogram of a mixture of proteins separated by hydrophobic interaction chromatography using different stationary phases. A linear gradient elution program was used changing from 0 to 100% mobile phase B in 40 min. Mobile phase A: 1.8 M ammonium sulphate + 0.1 M potassium phosphate pH 7.0. Mobile phase B: 0.1 M potassium phosphate pH 7.0. Elution was monitored at 220 nm. (Reproduced with permission from K. Benedek (2003) High-performance interaction chromatography, in *HPLC of Peptides and Proteins: Methods and Protocols*, Methods in Molecular Biology No. 251, M.-I. Aguilar (ed.), Humana Press, Totowa, NJ.)

the pharmaceutical industry to predict the ability of candidate drugs to be absorbed and distributed since they are good models of cell membranes. The technique uses a phospholipid-coated filter disc and is configured in 96-well format with an ultraviolet plate reader as a detection system.

11.5 PARTITION CHROMATOGRAPHY

11.5.1 Principle

Like other forms of chromatography, partition chromatography is based on differences in retention factor, k, and distribution coefficients, K_d, of the analytes using

liquid stationary and mobile phases. It can be subdivided into **liquid–liquid chromatography**, in which the liquid stationary phase is attached to a supporting matrix by purely physical means, and **bonded-phase liquid chromatography**, in which the stationary phase is covalently attached to the matrix. An example of liquid–liquid chromatography is one in which a water stationary phase is supported by a cellulose, starch or silica matrix, all of which have the ability to physically bind as much as 50% (w/v) water and remain free-flowing powders. The advantages of this form of chromatography are that it is cheap, has a high capacity and has broad selectivity. Its disadvantage is that the elution process may gradually remove the stationary phase, thereby altering the chromatographic conditions. This problem is overcome by the use of bonded phases and this explains their more widespread use. Most bonded phases use silica as the matrix, which is derivatised to immobilise the stationary phase by reaction with an organochlorosilane.

11.5.2 Normal-phase liquid chromatography

In this form of partition chromatography, the stationary phase is polar and the mobile phase relatively non-polar. The most popular stationary phase is an alkylamine bonded to silica. The mobile phase is generally an organic solvent such as hexane, heptane, dichloromethane or ethyl acetate. These solvents form an **elutropic series** based on their polarity. Such a series in order of increasing polarity is as follows:

n-hexane < cyclohexane < trichloromethane < dichloromethane < tetrahydrofuran < acetonitrile < ethanol < methanol < ethanoic acid < water

The order of elution of analytes is such that the least polar is eluted first and the most polar last. Indeed, polar analytes generally require gradient elution with a mobile phase of increasing polarity, generally achieved by the use of methanol or dioxane. The main applications of normal-phase liquid chromatography are its use to separate analytes that have low water solubility and those that are not amenable to reversed-phase liquid chromatography.

11.5.3 Reversed-phase liquid chromatography

In this form of liquid chromatography, which has many similarities with hydrophobic interaction chromatography, the stationary phase is non-polar and the mobile phase relatively polar, hence the name reversed-phase. By far the most commonly used type is the bonded-phase form, in which alkylsilane groups are chemically attached to silica. Butyl (C_4), octyl (C_8) and octadecyl (C_{18}) silane groups are most commonly used (Table 11.2). The mobile phase is commonly water or aqueous buffers, methanol, acetonitrile or tetrahydrofuran, or mixtures of them. The organic solvent is referred to as an **organic modifier**. Reversed-phase liquid chromatography differs from most other forms of chromatography in that the stationary phase is essentially inert and only non-polar (hydrophobic) interactions are possible with analytes.

Table 11.2 **Examples of silica bonded phases for reversed-phase HPLC**

Product	Particle size	Pore size (Å)
μBondapak octadecyl	10 μm	70
μBondapak phenyl	10 μm	125
μBondapak CN	10 μm	125
μBondapak NH_2	10 μm	80
Zorbax octadecyl	6 μm	70
Zorbax octyl	6 μm	70
Zorbax NH_2	6 μm	70
Discovery octyl	5 μm	180
Supelcosil LC-octadecyl	5 μm	120
Supelcosil LC-301 methyl	5 μm	300
Supelcosil LC-308 octyl	5 μm	300

Note: 1 Å = 0.1 nm.

Reversed-phase HPLC is probably the most widely used form of chromatography mainly because of its flexibility and high resolution. It is widely used to analyse drugs and their metabolites, insecticide and pesticide residues, and amino acids, peptides and proteins. Octadecylsilane (ODS) phases bind proteins more tightly than do octyl- or methylsilane phases and are therefore more likely to cause protein denaturation because of the more extreme conditions required for the elution of the protein.

11.5.4 Ion-pair reversed-phase liquid chromatography

Although the separation of some highly polar analytes, such as amino acids, peptides, organic acids and the catecholamines, is not possible by reversed-phase chromatography, it is sometimes possible to achieve such separations by one of two approaches:

- *Ion suppression*: The ionisation of the analytes is suppressed by using a mobile phase with an appropriately high or low pH thus giving the molecules greater hydrophobic character. For weak acid analytes, for example, an acidified mobile phase would be used.
- *Ion-pairing*: A **counter ion** that has a charge opposite to that of the analytes to be separated is added to the mobile phase so that the resulting ion-pair has sufficient hydrophobic, lipophilic character to be retained by the non-polar stationary phase of a reversed-phase system. Thus, to aid the separation of acidic analytes, which would be present as their conjugate anions, a quaternary alkylamine ion such as tetrabutylammonium would be used as the counter ion, whereas for the separation

of bases, which would be present as cations, an alkyl sulphonate such as sodium heptanesulphonate would be used:

$$\underset{\text{carboxylic acid anion}}{RCOO^-} + \underset{\text{counter cation}}{R'_4N^+} \rightleftharpoons \underset{\text{ion-pair}}{[RCOO^-N^+R'_4]}$$

$$\underset{\text{conjugate acid of weak base}}{RN^+H_3} + \underset{\text{counter anion}}{R'SO_3^-} \rightleftharpoons \underset{\text{ion-pair}}{[RH_3N^{+-}O_3SR']}$$

The technique can also be applied to proteins. The addition of trifluoroacetic acid (TFA) suppresses the ionisation of exposed groups on the protein surface giving it greater hydrophobic character. In practice, the success of the ion-pairing approach is variable and somewhat empirical. The size of the counter ion, its concentration and the pH of the solution are all factors that may profoundly influence the outcome of the separation.

Octyl- and octadecylsilane-bonded phases are used most commonly in conjunction with a water/methanol or water/acetonitrile mobile phase. One of the advantages of ion-pair reversed-phase chromatography is that if the sample to be resolved contains a mixture of non-ionic and ionic analytes, the two groups can be separated simultaneously because the ion-pair reagent does not affect the chromatography of the non-ionic species.

11.5.5 Chiral chromatography

This form of chromatography allows mixtures of enantiomers (mirror image forms, denoted either as D or L or as S or R) to be resolved. One of these techniques is based on the fact that diastereoisomers, which are optical isomers that do not have an object–image relationship, have different physical properties even though they contain identical functional groups. They can therefore be separated by conventional chromatographic techniques, most commonly reversed-phase chromatography. The diastereoisomer approach requires that the enantiomers contain a function group that can be derivatised by a chemically and optically pure **chiral derivatising agent** (CDA) that converts them to a mixture of diastereoisomers:

$$\underset{\text{mixture of enantiomers}}{(R+S)} + \underset{\text{chiral derivatising agent}}{R'} \rightleftharpoons \underset{\text{mixture of diastereoisomers}}{RR' + SR'}$$

Examples of CDAs include the R or S form of the following:

For amines	*N*-trifluoroacetyl-1-prolylchloride, α-phenylbutyric anhydride
For alcohols	2-Phenylpropionyl chloride, 1-phenylethylisothiocyanate
For ketones	2,2,2-Trifluoro-1-pentylethylhydrazine
For aliphatic and alicyclic acids	1-Menthol, desoxyephedrine

An alternative approach to the resolution of enantiomers is to use a chiral mobile phase. In this technique a transient diastereomeric complex is formed between the enantiomers and the chiral mobile phase agent. Examples of chiral mobile phase agents include albumin, α_1-acid glycoprotein, α, β- and γ-cyclodextrins, camphor-10-sulphonic acid and *N*-benzoxycarbonylglycyl-L-proline, all of which are used with a reversed-phase chromatographic system. For example, this technique has been used to show that cannabidiol, one of the main components of marijuana, consists of (+) and (−) forms only one of which is physiologically active.

The most successful approach to chiral chromatography, however, has been the use of a chiral stationary phase. This is based upon the principle that the need for a three-point interaction between the stationary phase (working as a **chiral discriminator**) and the enantiomer would allow the resolution of racemic mixtures due to the different spatial arrangement of the functional groups at the chiral centre in the enantiomers. The cyclodextrins are cyclic oligosaccharides that have an open truncated conical structure 6 to 8 Å (0.6 to 0.8 nm) wide at their base. Their inner surface is predominantly hydrophobic, but secondary hydroxyl groups are located around the wide rim of the cone. β-Cyclodextrin has seven glucopyranose units and contains 35 chiral centres and α-cyclodextrin has six glucopyranose units, 30 chiral centres and is smaller than β-cyclodextrin. Collectively they are referred to as **chiral cavity phases** because they rely on the ability of the enantiomer to enter the three-dimensional cyclodextrin cage while at the same time presenting functional groups and hence the chiral centre for interaction with hydroxyl groups on the cone rim.

11.6 ION-EXCHANGE CHROMATOGRAPHY

11.6.1 Principle

This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an **ion exchanger**, and analyte. It is frequently chosen for the separation and purification of proteins, peptides, nucleic acids, polynucleotides and other charged molecules, mainly because of its high resolving power and high capacity. There are two types of ion exchanger, namely **cation** and **anion exchangers**. Cation exchangers possess negatively charged groups and these will attract positively charged cations. These exchangers are also called **acidic ion exchangers** because their negative charges result from the ionisation of acidic groups. Anion exchangers have positively charged groups that will attract negatively charged anions. The term **basic ion exchangers** is also used to describe these exchangers, as positive charges generally result from the association of protons with basic groups.

11.6.2 Materials and applications

Matrices used include polystyrene, cellulose and agarose. Functional ionic groups include sulphonate ($-SO_3^-$) and quaternary ammonium ($-N^+R_3$), both of which are

Table 11.3 **Examples of commonly used ion exchangers**

Type	Functional groups	Functional group name	Matrices
Weakly acidic (cation exchanger)	$-COO^-$	Carboxy	Agarose
	$-CH_2COO^-$	Carboxymethyl	Cellulose
			Dextran
			Polyacrylate
Strongly acidic (cation exchanger)	$-SO_3^-$	Sulpho	Cellulose
	$-CH_2SO_3^-$	Sulphomethyl	Dextran
	$-CH_2CH_2CH_2SO_3^-$	Sulphopropyl	Polystyrene
			Polyacrylate
Weakly basic (anion exchanger)	$-CH_2CH_2N^+H_3$	Aminoethyl	Agarose
	$-CH_2CH_2N^+H$ $\vert$ $(CH_2CH_3)_2$	Diethylaminoethyl	Cellulose
			Dextran
			Polystyrene
			Polyacrylate
Strongly basic (anion exchanger)	$-CH_2N^+(CH_3)_3$	Trimethylaminomethyl	Cellulose
	$-CH_2CH_2N^+(CH_2CH_3)_3$	Triethylaminoethyl	Dextran
	$-CH_2N^+(CH_3)_2$ $\vert$ CH_2CH_2OH	Dimethyl-2-hydroxyethyl-aminomethyl	Polystyrene
	$-CH_2CH_2N^+(CH_2CH_3)_2$ $\vert$ $CH_2CH(OH)CH_3$	Diethyl-2-hydroxypropyl-aminoethyl	

strong exchangers because they are totally ionised at all normal working pH values, and carboxylate ($-COO^-$) and diethylammonium ($-HN^+(CH_2CH_3)_2$), both of which are termed **weak exchangers** because they are ionised over only a narrow range of pH values. Examples are given in Table 11.3. Bonded phase ion exchangers suitable for HPLC, containing a wide range of ionic groups, are commercially available. Porous varieties are based on polystyrene, porous silica or hydrophilic polyethers, and are particularly valuable for the separation of proteins. They have a particle diameter of 5–25 µm. Most HPLC ion exchangers are stable up to 60 °C and separations are often carried out at this temperature, owing to the fact that the raised temperature

decreases the viscosity of the mobile phase and thereby increases the efficiency of the separation.

Choice of exchanger

The choice of the ion exchanger depends upon the stability of the test analytes, their relative molecular mass and the specific requirements of the separation. Many biological analytes, especially proteins, are stable within only a fairly narrow pH range so the exchanger selected must operate within this range. Generally, if an analyte is most stable below its isoionic point (giving it a net positive charge) a cation exchanger should be used, whereas if it is most stable above its isoionic point (giving it a net negative charge) an anion exchanger should be used. Either type of exchanger may be used to separate analytes that are stable over a wide range of pH values. The choice between a strong and weak exchanger also depends on analyte stability and the effect of pH on analyte charge. Weak electrolytes requiring a very low or high pH for ionisation can be separated only on strong exchangers, as they only operate over a wide pH range. In contrast, for strong electrolytes, weak exchangers are advantageous for a number of reasons, including a reduced tendency to cause protein denaturation, their inability to bind weakly charged impurities and their enhanced elution characteristics. Although the degree of cross-linking of an exchanger does not influence the ion-exchange mechanism, it does influence its capacity. The relative molecular mass and hence size of the proteins in the sample therefore determines which exchanger should be used.

Eluent pH

The pH of the buffer selected as eluent should be at least one pH unit above or below the isoionic point of the analytes. In general, cationic buffers such as Tris, pyridine and alkylamines are used in conjunction with anion exchangers, and anionic buffers such as acetate, barbiturate and phosphate are used with cation exchangers. The precise initial buffer pH and ionic strength should be such as just to allow the binding of the analytes to the exchanger. Equally, a buffer of the lowest ionic strength that effects elution should initially be used for the subsequent elution of the analytes. This ensures that initially the minimum numbers of contaminants bind to the exchanger and that subsequently the maximum number of these impurities remains on the column. If, however, gradient elution is to be used, the initial conditions chosen are such that the exchanger binds all the test analytes at the top of the column.

Elution

Gradient elution is far more common than isocratic elution. Continuous or stepwise pH and ionic strength gradients may be employed but continuous gradients tend to give better resolution with less peak tailing. Generally with an anion exchanger, the pH gradient decreases and the ionic strength increases, whereas for cation exchangers both the pH and ionic gradients increase during the elution.

11.7 MOLECULAR (SIZE) EXCLUSION CHROMATOGRAPHY

11.7.1 Principle

This chromatographic technique for the separation of molecules on the basis of their molecular size and shape exploits the **molecular sieve** properties of a variety of porous materials. The terms **exclusion** or **permeation chromatography** or **gel filtration** describe all molecular separation processes using molecular sieves. The general principle of exclusion chromatography is quite simple. A column of microparticulate cross-linked copolymers generally of either styrene or divinylbenzene and with a narrow range of pore sizes is in equilibrium with a suitable mobile phase for the analytes to be separated. Large analytes that are completely excluded from the pores will pass through the interstitial spaces between the particles and will appear first in the eluate. Smaller analytes will be distributed between the mobile phase inside and outside the particles and will therefore pass through the column at a slower rate, hence appearing last in the eluate (Fig. 11.9).

The mobile phase trapped by a particle is available to an analyte to an extent that is dependent upon the porosity of the particle and the size of the analyte molecule. Thus, the distribution of an analyte in a column of cross-linked particles is determined solely by the total volume of mobile phase, both inside and outside the particles, that is available to it. For a given type of particle, the distribution coefficient, K_d, of a particular analyte between the inner and outer mobile phase is a function of its molecular size. If the analyte is large and completely excluded from the mobile phase within the particle, $K_d = 0$, whereas, if the analyte is sufficiently small to gain complete access to the inner mobile phase, $K_d = 1$. Due to variation in pore size between individual particles, there is some inner mobile phase that will be available and some that will not be available to analytes of intermediate size; hence K_d values vary between 0 and 1. It is this complete variation of K_d between these two limits that makes it possible to separate analytes within a narrow molecular size range on a given particle type.

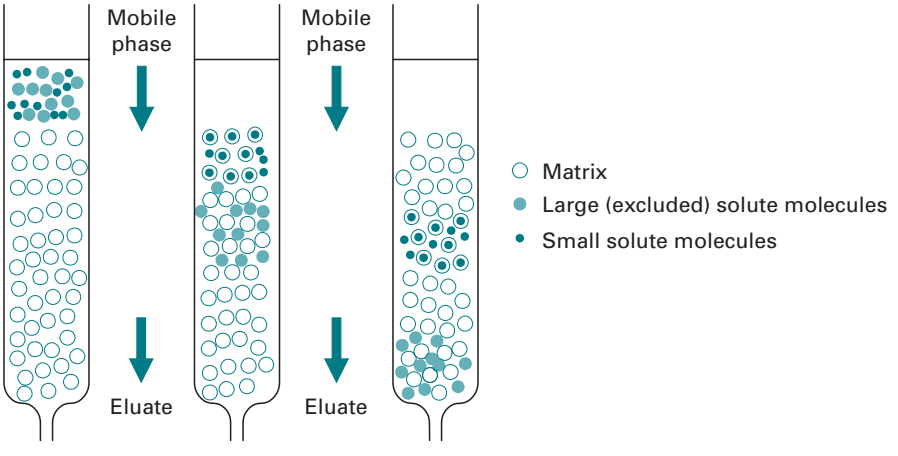

Fig. 11.9 Separation of different size molecules by exclusion chromatography. Large excluded molecules are eluted first in the void volume.

Example 2 ESTIMATION OF RELATIVE MOLECULAR MASS

Question The relative molecular mass (M_r) of a protein was investigated by exclusion chromatography using a Sephacryl S300 column and using aldolase, catalase, ferritin, thyroglobulin and Blue Dextran as standard. The following elution data were obtained.

	Retention volume	
	M_r	V_r (cm^3)
Aldolase	158 000	22.5
Catalase	210 000	21.4
Ferritin	444 000	18.2
Thyroglobulin	669 000	16.4
Blue Dextran	2 000 000	13.6
Unknown		19.5

What is the approximate M_r of the unknown protein?

Answer A plot of the logarithm of the relative molecular mass of individual proteins versus their retention volume has a linear section from which it can be deduced that the unknown protein with a retention volume of 19.5 cm^3 must have a relative molecular mass of 330 000.

For two analytes of different relative molecular mass and K_d values, K'_d and K''_d, the difference in their elution volumes, V_S, can be shown to be:

$$V_S = (K'_d - K''_d)V_i \qquad (11.14)$$

where V_i is the inner volume within the particle available to a compound whose $K_d = 1$.

In practice, deviations from ideal behaviour, for example owing to poor packing of the column, make it advisable to reduce the sample volume below the value of V_S because the ratio between sample volume and inside particle volume affects both the sharpness of the separation and the degree of dilution of the sample.

The stationary phases for exclusion separations are generally based on silica, polymethacrylate or polyvinyl acetate or chloride or on cross-linked dextran or agarose (Table 11.4). All are available in a range of pore sizes. They are generally used where the eluent is an organic system. The supports for affinity separations are similar to those for exclusion separations.

11.7.2 Applications

Purification

The main application of exclusion chromatography is in the purification of biological macromolecules by facilitating their separation from larger and smaller molecules.

Table 11.4 **Stationary phases commonly used for exclusion chromatography**

Polymer	Trade name	Fractionation range[a] ($M_r \times 10^{-3}$)
Low-pressure liquid chromatography		
Dextran	Sephadex	
	G-10	<0.7
	G-25	1–5
	G-50	1.5–30
	G-100	4–150
	G-200	5–600
Dextran, cross-linked	Sephacryl	
	S-100	1–100
	S-200	5–250
	S-300	10–1500
	S-400	20–8000
Agarose	Sepharose	
	6B	10–4000
	4B	60–20 000
	2B	70–40 000
Polyacrylamide	Bio-Gel	
	P-2	0.1–1.8
	P-6	1–6
	P-30	2.5–40
	P-100	5–100
	P-300	60–400
High-performance liquid chromatography		
Polyvinyl chloride	Fractogel	
	TSK HW-40	0.1–10
	TSK HW-55	1–700
	TSK HW-65	50–5000
	TSK HW-75	500–50 000

Table 11.4 (*cont.*)

Polymer	Trade name	Fractionation range[a] ($M_r \times 10^{-3}$)
Dextran linked to cross-linked agarose	Superdex	
	75	3–70
	200	10–600

Note: [a]Determined for globular proteins. The range is approximately the same for single-stranded nucleic acids and smaller for fibrous proteins and double-stranded DNA.

Viruses, enzymes, hormones, antibodies, nucleic acids and polysaccharides have all been separated and purified by use of appropriate gels or glass granules.

Relative molecular mass determination

The elution volumes of globular proteins are determined largely by their relative molecular mass (M_r). It has been shown that, over a considerable range of relative molecular masses, the elution volume or K_d is an approximately linear function of the logarithm of M_r. Hence the construction of a calibration curve, with proteins of a similar shape and known M_r, enables the M_r values of other proteins, even in crude preparations, to be estimated (See Example 2, p. 463).

Solution concentration

Solutions of high M_r substances can be concentrated by the addition of dry Sephadex G-25 (coarse). The swelling gel absorbs water and low M_r substances, whereas the high M_r substances remain in solution. After 10 min the gel is removed by centrifugation, leaving the high M_r material in a solution whose concentration has increased but whose pH and ionic strength are unaltered.

Desalting

By use of a column of, for example, Sephadex G-25, solutions of high M_r compounds may be desalted, i.e. removed from contaminants such as salts, detergents, lipids and chaotropic agents. The high M_r compounds move with the void volume, whereas the low M_r compounds are distributed between the mobile and stationary phases and hence move slowly. This method of desalting is faster and more efficient than dialysis. Applications include removal of phenol from nucleic acid preparations, ammonium sulphate from protein preparations and salt from samples eluted from ion-exchange chromatography columns.

11.8 AFFINITY CHROMATOGRAPHY

11.8.1 Principle

Separation and purification of analytes by affinity chromatography is unlike most other forms of chromatography and such techniques as electrophoresis and

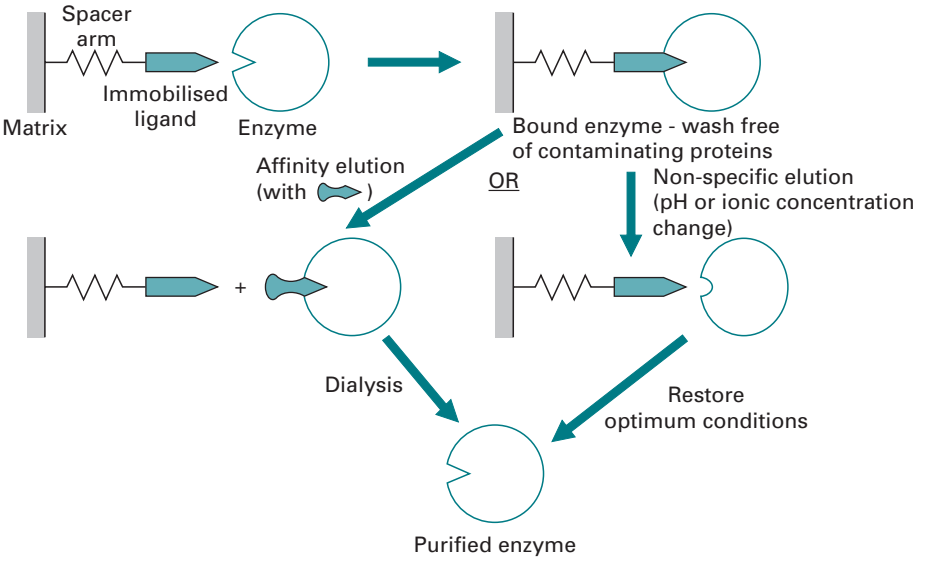

Fig. 11.10 Principle of purification of an enzyme by affinity chromatography.

centrifugation in that it does not rely on differences in the physical properties of the analytes. Instead, it exploits the unique property of extremely specific biological interactions to achieve separation and purification. As a consequence, affinity chromatography is theoretically capable of giving absolute purification, even from complex mixtures, in a single process. The technique was originally developed for the purification of enzymes, but it has since been extended to nucleotides, nucleic acids, immunoglobulins, membrane receptors and even to whole cells and cell fragments.

The technique requires that the material to be isolated is capable of binding reversibly to a specific ligand that is attached to an insoluble matrix:

$$\underset{\text{macromolecule}}{\text{M}} + \underset{\substack{\text{ligand}\\(\text{attached}\\\text{to matrix})}}{\text{L}} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \underset{\text{complex}}{\text{ML}}$$

Under the correct experimental conditions, when a complex mixture containing the specific compound to be purified is added to the immobilised ligand, generally contained in a conventional chromatography column, only that compound will bind to the ligand. All other compounds can therefore be washed away and the compound subsequently recovered by displacement from the ligand (Fig. 11.10). The method requires a detailed preliminary knowledge of the structure and biological specificity of the compound to be purified so that the separation conditions that are most likely to be successful may be carefully planned. In the case of an enzyme, the ligand may be the substrate, a competitive reversible inhibitor or an allosteric modifier. The conditions chosen would normally be those that are optimal for enzyme–ligand binding. Since the success of the method relies on the reversible formation of the complex and on the numerical values of the first-order rate constants k_{+1} and k_{-1}, as the

enzyme is added progressively to the insolubilised ligand in a column, the enzyme molecules will be stimulated to bind and a dynamic situation develops in which the concentration of the complex and the strength of the binding increase.

11.8.2 Materials and applications

Matrix

An ideal matrix for affinity chromatography must have the following characteristics:

- possess suitable and sufficient chemical groups to which the ligand may be covalently coupled, and be stable under the conditions of the attachment;
- be stable during binding of the macromolecule and its subsequent elution;
- interact only weakly with other macromolecules to minimise non-specific adsorption;
- exhibit good flow properties.

In practice, particles that are uniform, spherical and rigid are used. The most common ones are the cross-linked dextrans and agarose, polyacrylamide, polymethacrylate, polystyrene, cellulose and porous glass and silica.

Ligand

The chemical nature of a ligand is dictated by the biological specificity of the compound to be purified. In practice it is sometimes possible to select a ligand that displays **absolute specificity** in that it will bind exclusively to one particular compound. More commonly, it is possible to select a ligand that displays **group selectivity** in that it will bind to a closely related group of compounds that possess a similar in-built chemical specificity. An example of the latter type of ligand is 5′AMP, which can bind reversibly to many NAD^+-dependent dehydrogenases because it is structurally similar to part of the NAD^+ molecule. It is essential that the ligand possesses a suitable chemical group that will not be involved in the reversible binding of the ligand to the macromolecule, but which can be used to attach the ligand to the matrix. The most common of such groups are $-NH_2$, $-COOH$, $-SH$ and $-OH$ (phenolic and alcoholic).

To prevent the attachment of the ligand to the matrix interfering with its ability to bind the macromolecule, it is generally advantageous to interpose a **spacer arm** between the ligand and the matrix. The optimum length of this spacer arm is six to ten carbon atoms or their equivalent. In some cases, the chemical nature of this spacer is critical to the success of separation. Some spacers are purely hydrophobic, most commonly consisting of methylene (CH_2) groups; others are hydrophilic, possessing carbonyl (CO) or imido (NH) groups. Spacers are most important for small immobilised ligands but generally are not necessary for macromolecular ligands (e.g. in immunoaffinity chromatography, Section 11.8.3) as their binding site for the mobile macromolecule is well displaced from the matrix. Several supports of the agarose, dextran and polyacrylamide type are commercially available with a variety of spacer arms and ligands pre-attached ready for immediate use. Examples of ligands are given in Table 11.5. Glutathione Sepharose High Performance is an agarose support used

Table 11.5 **Examples of group-specific ligands commonly used in affinity chromatography**

Ligand	Affinity
Nucleotides	
5′-AMP	NAD^+-dependent dehydrogenases, some kinases
2′5′-ADP	$NADP^+$-dependent dehydrogenases
Calmodulin	Calmodulin-binding enzymes
Avidin	Biotin-containing enzymes
Fatty acids	Fatty-acid-binding proteins
Heparin	Lipoproteins, lipases, coagulation factors, DNA polymerases, steroid receptor proteins, growth factors, serine protease inhibitors
Proteins A and G	Immunoglobulins
Concanavalin A	Glycoproteins containing α-D-mannopyranosyl and α-D-glucopyranosyl residues
Soybean lectin	Glycoproteins containing *N*-acetyl-α-(or β)-D-galactopyranosyl residues
Phenylboronate	Glycoproteins
Poly(A)	RNA containing poly(U) sequences, some RNA-specific proteins
Lysine	rRNA
Cibacron Blue F3G-A	Nucleotide-requiring enzymes, coagulation factors

for the isolation any GST-tagged cloned protein. It is possible to remove the GST tag in a one-step process by adding PreScission™ Protease to the matrix, as it will be bound to the column and remove the tag as the protein is eluted.

Practical procedure

The procedure for affinity chromatography is similar to that used in other forms of liquid chromatography. The buffer used must contain any cofactors, such as metal ions, necessary for ligand–macromolecule interaction. Once the sample has been applied and the macromolecule bound, the column is eluted with more buffer to remove non-specifically bound contaminants. The purified compound is recovered from the ligand by either **specific** or **non-specific elution**. Non-specific elution may be achieved by a change in either pH or ionic strength. pH shift elution using dilute acetic acid or ammonium hydroxide results from a change in the state of ionisation of groups in the ligand and/or the macromolecule that are critical to ligand–macromolecule binding. A change in ionic strength, not necessarily with a concomitant change in pH, also causes elution due to a disruption of the ligand–macromolecule interaction; 1M NaCl is frequently used for this purpose.

Applications

Many enzymes and other proteins, including receptor proteins and immunoglobulins, have been purified by affinity chromatography. The application of the technique is limited only by the availability of immobilised ligands. The principles have been extended to nucleic acids and have made a considerable contribution to developments in molecular biology. Messenger RNA, for example, is routinely isolated by selective hybridisation on poly(U)-Sepharose 4B by exploiting its poly(A) tail. Immobilised single-stranded DNA can be used to isolate complementary RNA and DNA. Whilst this separation can be achieved on columns, it is usually performed using single-stranded DNA immobilised on nitrocellulose filters. Immobilised nucleotides are useful for the isolation of proteins involved in nucleic acid metabolism.

11.8.3 Immunoaffinity chromatography

The use of antibodies as the immobilised ligand has been exploited in the isolation and purification of a range of proteins including membrane proteins of viral origin. Monoclonal antibodies may be linked to agarose matrices by the cyanogen bromide technique. Protein binding to the immobilised antibody is achieved in neutral buffer solution containing moderate salt concentrations. Elution of the bound protein quite often requires forceful conditions because of the need to disrupt the very tight ionic or hydrophobic binding with the antibody ($K_d = 10^{-8}$ to 10^{-12} M) and this may lead to protein denaturation. Examples of elution procedures include the use of high salt concentrations with or without the use of detergent and the use of urea or guanidine hydrochloride, both of which cause protein denaturation. The use of some other chaotropic agents (ions or small molecules that increase the water solubility of non-polar substances) such as thiocyanate, perchlorate and trifluoroacetate or lowering the pH to about 3 may avoid denaturation. Organic solvents such as acetonitrile can also be used to disrupt the hydrophobic interaction.

11.8.4 Metal chelate chromatography (immobilised metal affinity chromatography)

This is a special form of affinity chromatography in which an immobilised metal ion such as Cu^{2+}, Zn^{2+}, Hg^{2+} or Cd^{2+} or a transition metal ion such as Co^{2+}, Ni^{2+} or Mn^{2+} is used to bind proteins selectively by reaction with imidazole groups of histidine residues, thiol groups in cysteine residues and indole groups in tryptophan residues sterically available on the surface of the proteins. The immobilisation of the protein involves the formation of a coordinate bond that must be sufficiently stable to allow protein attachment and retention during the elution of non-binding contaminating material. The subsequent release of the protein can be achieved either by simply lowering the pH or by the use of complexing agents such as EDTA. Most commonly the metal atom is immobilised by attachment to an iminodiacetate- or tris(carboxymethyl)-ethylenediamine-substituted agarose. Nickel or cobalt immobilised metal affinity chromatography is commonly used to isolate and purify His-tagged proteins

(Section 6.7.1). The cobalt commercial product called Dynabeads Myone TALON™ has the practical advantage that it only binds adjacent histidines or histidines in certain arrangements and is commonly used for the isolation of recombinant proteins.

11.8.5 Dye–ligand chromatography

A number of triazine dyes that contain both conjugated rings and ionic groups fortuitously have the ability to bind to some proteins. The term pseudo-ligand has therefore been used to describe the dyes. It is not possible to predict whether a particular protein will bind to a given dye as the interaction is not specific but is thought to involve interaction with ligand-binding domains via both ionic and hydrophobic forces. Dye binding to proteins enhances their binding to materials such as Sepharose 4B and this is exploited in the purification process. The attraction of the technique is that the dyes are cheap, readily coupled to conventional matrices and are very stable. The most widely used dye is Cibacron Blue F3G-A. Dye selection for a particular protein purification is empirical and is made on a trial-and-error basis. Attachment of the protein to the immobilised dye is generally achieved at pH 7 to 8.5. Elution is most commonly brought about either by a salt gradient or by affinity (displacement) elution.

11.8.6 Covalent chromatography

This form of chromatography has been developed specifically to separate thiol(–SH)-containing proteins by exploiting their interaction with an immobilised ligand containing a disulphide group. The principle is illustrated in Fig. 11.11. The most commonly used ligand is a disulphide 2′-pyridyl group attached to an agarose matrix such as Sepharose 4B. On reaction with the thiol-containing protein, pyridine-2-thione is released. This process can be monitored spectrophotometrically at 343 nm, thereby allowing the adsorption of the protein to be followed. Once the protein has been attached covalently to the matrix, non-thiol-containing contaminants are eluted and unreacted thiopyridyl groups removed by use of 4 mM dithiothreitol or mercaptoethanol. The protein is then released by displacement with a thiol-containing compound such as 20—50 mM dithiothreitol, reduced glutathione or cysteine. The matrix is regenerated by reaction with 2,2′-dipyridyldisulphide. The method has been used successfully for many proteins but its use is limited by its cost and the rather difficult regeneration stage. It can, however, be applied to very impure protein preparations.

11.9 GAS CHROMATOGRAPHY

11.9.1 Principle

The principles of gas chromatography (GC) are similar to those of HPLC but the apparatus is significantly different. It exploits differences in the partition coefficients

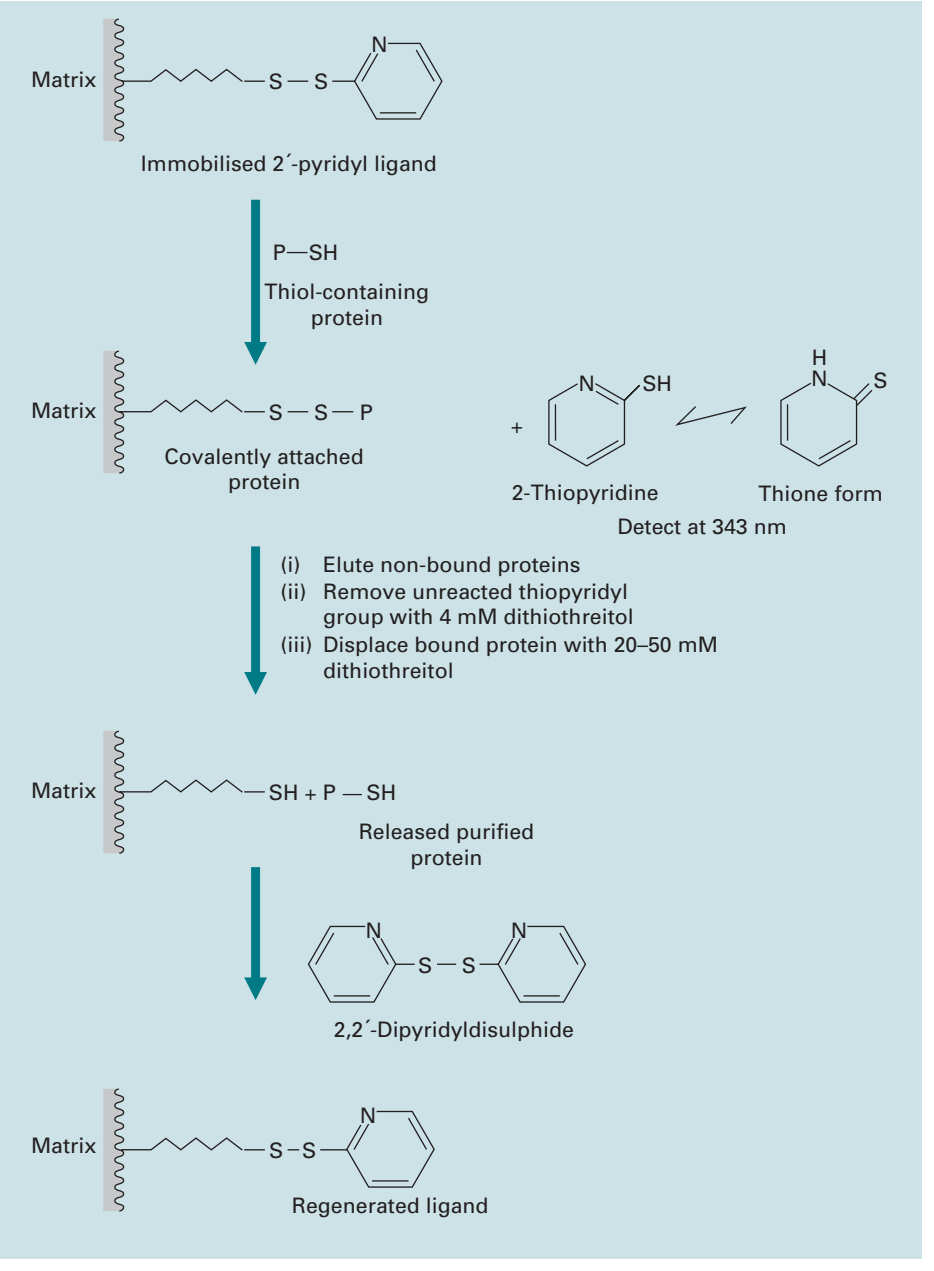

Fig. 11.11 Principle of purification of a protein (P-SH) by covalent chromatography.

between a stationary liquid phase and a mobile gas phase of the volatilised analytes as they are carried through the column by the mobile gas phase. Its use is therefore confined to analytes that are volatile but thermally stable. The partition coefficients are inversely proportional to the volatility of the analytes so that the most volatile elute first. The temperature of the column is raised to 50–300 °C to facilitate analyte

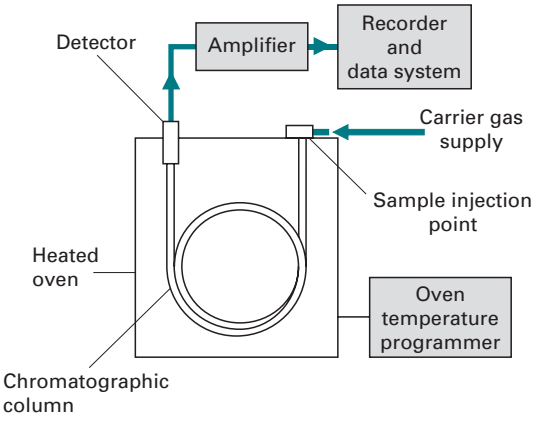

Fig. 11.12 Components of a GC system.

volatilisation. The stationary phase consists of a high-boiling-point liquid material such as silicone grease or wax that is either coated onto the internal wall of the column or supported on an inert granular solid and packed into the column. There is an optimum flow rate of the mobile gas phase for maximum column efficiency (minimum plate height, *H*). Very high resolutions are obtained (equations 11.8 to 11.12) hence the technique is very useful for the analysis of complex mixtures. Gas chromatography is widely used for the qualitative and quantitative analysis of a large number of low-polarity compounds because it has high sensitivity, reproducibility and speed of resolution. Analytically, it is a very powerful technique when coupled to mass spectrometry.

11.9.2 Apparatus and experimental procedure

The major components of a GC system are:

- a column housed in an oven that can be temperature programmed;
- a sample inlet point;
- a carrier gas supply and control; and
- a detector, amplifier and data recorder system (Fig. 11.12).

Columns

These are of two types:

- *Packed conventional columns*: These consist of a coiled glass or stainless steel column 1–3 m long and 2–4 mm internal diameter. They are packed with stationary phase coated on an inert silica support. Commonly used stationary phases include the polyethylene glycols (Carbowax 20M, very polar), methylphenyl- and methylvinylsilicone gums (OV17 and OV101, medium and non-polar respectively),

Apiezon L (non-polar) and esters of adipic, succinic and phthalic acids. β-Cyclodextrin-based phases are available for chiral separations (Section 11.5.5). The most commonly used support is Celite (diatomaceous silica), which because of the problem of support–sample interaction is often treated so that the hydroxyl groups that occur in the Celite are modified. This is normally achieved by silanisation of the support with such compounds as hexamethyldisilazane. The support particles have a large surface area and an even size, which, for the majority of practical applications, ranges from 60–80 mesh (0.25 mm) to 100–120 mesh (0.125 mm) (Section 11.3.1). The smaller the particle size and the thinner the coating the less band spreading occurs.

- *Capillary (open tubular) columns*: These are made of high-quality fused quartz and are 10–100 m long and 0.1–1.0 mm internal diameter. They are of two types known as **wall-coated open tubular** (WCOT) and **support-coated open tubular** (SCOT), also known as **porous layer open tubular** (PLOT) columns, for adsorption work. In WCOT columns the stationary phase is thinly coated (0.1–5 μm) directly onto the walls of the capillary whilst in SCOT columns the support matrix is bonded to the walls of the capillary column and the stationary phase coated onto the support. Commonly used stationary phases include polyethylene glycol (CP wax and DB wax, very polar) and methyl and phenyl-polysiloxanes (BP1, non-polar; BP10, medium polar). They are coated onto the supporting matrix to give a 1% to 25% loading, depending upon the analysis. The capacity of SCOT columns is considerably higher than that of WCOT columns.

The operating temperature for all types of column must be compatible with the stationary phase chosen for use. Too high a temperature results in excessive **column bleed** owing to the phase being volatilised off, contaminating the detector and giving an unstable recorder baseline. The working temperature range is chosen to give a balance between peak retention time and resolution. Column temperature is controlled to $\pm 0.1\ ^{o}C$. Analyte partition coefficients are particularly sensitive to temperature so that analysis times may be regulated by adjustment of the column oven, which can be operated in one of two modes:

- *Isothermal analysis*: Here a constant temperature is employed.
- *Temperature programming*: The temperature is gradually increased to facilitate the separation of compounds of widely differing polarity or M_r. This, however, sometimes results in excessive bleed of the stationary phase as the temperature is raised, giving rise to baseline variation. Consequently some instruments have two identical columns and detectors, one set of which is used as a reference. The currents from the two detectors are opposed, hence, assuming equal bleed from both columns, the resulting current gives a steady baseline as the column temperature is raised. The choice of phase for analysis depends on the analytes under investigation and is best chosen after reference to the literature.

Application of sample

The majority of non- and low-polar compounds are directly amenable to GC, but other compounds possessing such polar groups as $-OH$, $-NH_2$ and $-COOH$ are generally

retained on the column for excessive periods of time if they are applied directly. Poor resolution and peak tailing usually accompany this excessive retention (Section 11.2.4). This problem can be overcome by derivatisation of the polar groups. This increases the volatility and effective distribution coefficients of the compounds. Methylation, silanisation and perfluoracylation are common derivatisation methods for fatty acids, carbohydrates and amino acids.

The test sample is dissolved in a suitable solvent such as acetone, heptane or methanol. Chlorinated organic solvents are generally avoided as they contaminate the detector. For packed and SCOT columns the sample is injected onto the column using a microsyringe through a septum in the injection port attached to the top of the column. Normally 0.1 to 10 mm^3 of solution is injected. As there is only a small amount of stationary phase present in WCOT columns, only very small amounts of sample may be applied to the column. Consequently a **splitter system** has to be used at the sample injection port so that only a small fraction of the injected sample reaches the column. The remainder of the sample is vented to waste. The design of the splitter is critical in quantitative analyses in order to ensure that the ratio of sample applied to the column to sample vented is always the same. It is common practice to maintain the injection region of the column at a slightly higher temperature (+20 to 50 °C) than the column itself as this helps to ensure rapid and complete volatilisation of the sample. Sample injection is automated in many commercial instruments as this improves the precision of the analysis.

Mobile phase

The mobile phase consists of an inert gas such as nitrogen for packed columns or helium or argon for capillary columns. The gas from a cylinder is pre-purified by passing through a variety of molecular sieves to remove oxygen, hydrocarbons and water vapour. It is then passed through the chromatography column at a flow rate of 40–80 $cm^3\ min^{-1}$. A **gas-flow controller** is used to ensure a constant flow irrespective of the back-pressure and temperature of the column.

11.9.3 Detectors

Several types of detector are in common use in conjunction with GC:

- *Flame ionisation detector* (FID): This responds to almost all organic compounds. It has a minimum detection quantity of the order of 5×10^{-12} g s^{-1}, a linear range of 10^7 and an upper temperature limit of 400 °C. A mixture of hydrogen and air is introduced into the detector to give a flame, the jet of which forms one electrode, whilst the other electrode is a brass or platinum wire mounted near the tip of the flame (Fig. 11.13). When the sample analytes emerge from the column they are ionised in the flame, resulting in an increased signal being passed to the recorder. The carrier gas passing through the column and the detector gives a small background signal, which can be offset electronically to give a stable baseline.

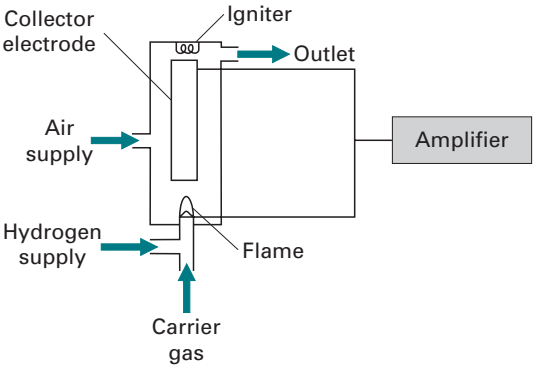

Fig. 11.13 GC flame ionisation detector. The tip of the flame forms the anode and the collector electrode the cathode.

$$\text{analyte} + H_2 + O_2 \longrightarrow \text{combustion products} + H_2O + \text{ions} + \text{radicals} + \text{electrons}$$

$$\sum(\text{ions})^- + \sum(\text{electrons})^- \longrightarrow \text{current}$$

- *Nitrogen–phosphorus detector* (NPD) (also called a **thermionic detector**): This is similar in design to an FID but has a crystal of a sodium salt fused onto the electrode system, or a burner tip embedded in a ceramic tube containing a sodium salt or a rubidium chloride tip. The NPD has excellent selectivity towards nitrogen- and phosphorus-containing analytes and shows a poor response to analytes possessing neither of these two elements. Its linearity (10^5) and upper temperature limit (300 °C) are not quite as good as an FID but its detection limit (10^{-14} g s^{-1}) is better. It is widely used in organophosphorus pesticide residue analysis.
- *Electron capture detector* (ECD): This responds only to analytes that capture electrons, particularly halogen-containing compounds. This detector is widely used in the analysis of polychlorinated compounds, such as the pesticides DDT, dieldrin and aldrin. It has a very high sensitivity (10^{-13} g s^{-1}) and an upper temperature limit of 300 °C but its linear range (10^2 to 10^4) is much lower than that of the FID. The detector works by means of a radioactive source (^{63}Ni) ionising the carrier gas and releasing an electron that gives a current across the electrodes when a suitable voltage is applied. When an electron-capturing analyte (generally one containing a halogen atom) emerges from the column, the ionised electrons are captured, the current drops and this change in current is recorded. The carrier gas most commonly used in conjunction with an ECD is nitrogen or an argon +5% methane mixture.
- *Flame photometric detector*: This exploits the fact the P- and S-containing analytes emit light when they are burned in a FID-type detector. This light is detected and quantified. The detection limit is of the order of 1.0 pg for P-containing compounds and 20 pg for S-containing compounds.
- *Rapid scanning Fourier transform infrared detector*: This records the infrared spectrum of the emerging analytes and can give structural as well as quantitative information about the analyte. Any analyte with an infrared spectrum can be detected with a detection limit of about 1 ng.

- *Mass spectrometer detector*: This is a universal detector that gives a mass spectrum of the analyte and therefore gives both structural and quantitative data. Its detection limit is less than 1 ng per scan. Analytes may be detected by a **total ion current** (TIC) (Section 9.4.1) trace that is non-selective, or by **selected ion monitoring** (SIM) (Section 9.5.11) that can be specific for a selected analyte. In cases where authentic samples of the test compounds are not available for calibration purposes or in cases where the identity of the analytes is not known, a mass spectrometer is the best means of detecting and identifying the analyte. Special separators are available for removing the bulk of the carrier gas from the sample emerging from the column prior to its introduction in the mass spectrometer (Section 9.3).

Modern GC systems are controlled by dedicated microcomputers capable of automating and optimising the experimental conditions, recording the calibration and test retention data and carrying out statistical analysis of it and displaying the outputs in colour graphics in real time. They are capable of carrying out both qualitative and quantitative analysis on a similar basis to that of LC.

11.10 SUGGESTIONS FOR FURTHER READING

Niessen, W. M. A. (2007). *Liquid Chromatography–Mass Spectrometry*, 3rd edn. Boca Raton, FL: CRC Press. (A definitive guide with particular emphasis on applications in proteomics, drug discovery, food safety and environmental monitoring.)

Pyell, U. (ed.) (2006). *Electrokinetic Chromatography: Theory, Instrumentation and Applications*. New York: John Wiley. (A comprehensive coverage of the techniques and its most recent applications.)

Zachariou, M. (ed.) (2007). *Affinity Chromatography: Methods and Protocols*. Totowa, NJ: Humana Press. (A detailed account of recent applications of this important method of protein purification.)