Column chromatography

By-

Dr. Ekta Khare

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 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 4mm internal diameter and made of stainless steel.
- Whereas in gas chromatography they are 13m long and 24mm 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.

… Basic column chromatographic components

- **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 usuallybased 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.

Analyte development and elution

- 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 sampleis 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 sampleanalytes.
- 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.

… Analyte development and elution

- 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.

CHROMATOGRAPHIC PERFORMANCE PARAMETERS

- 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 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.

... CHROMATOGRAPHIC PERFORMANCE PARAMETERS

- **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.
- 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:

$$t'_R = t_R - t_M$$

... Chromatographic performance parameters

Retention factor

- One of the most important parameters in chromatography is the retention factor, 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:

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

- 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.

Retention factor

- 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, 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:

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

- 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 VM is the volume of mobile phase.
- The ratio V_S/V_M is referred to as the volumetric phase ratio, b. Hence:

$$k = K_d \beta$$

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

- 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 (hP) and its width at half the height (wh) (Fig 11.1).
- The product of these dimensions is taken to be equal to the area of the peak.
- 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.

… Quantitative analysis

- Quantification of a given analyte is based on the construction of a calibration curve obtained using a pure, authentic sample of the analyte.
- 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%.