Introduction to Chromatography

Dr. Sana Mustafa
Assistant Professor
Department of Chemistry, Federal Urdu University of Arts, Science & Technology, Karachi.
What is Chromatography?

Derived from the Greek word *Chroma* meaning colour, chromatography provides a way to identify unknown compounds and separate mixtures.
Introduction to Chromatography

Definition

Chromatography is a separation technique based on the different interactions of compounds with two phases, a mobile phase and a stationary phase, as the compounds travel through a supporting medium.

Components:

**mobile phase**: a solvent that flows through the supporting medium

**stationary phase**: a layer or coating on the supporting medium that interacts with the analytes

**supporting medium**: a solid surface on which the stationary phase is bound or coated
Milestones in Chromatography

1903 Tswett - plant pigments separated on chalk columns
1931 Lederer & Kuhn - LC of carotenoids
1938 TLC and ion exchange
1950 reverse phase LC
1954 Martin & Synge (Nobel Prize)
1959 Gel permeation
1965 instrumental LC (Waters)
Applications of Chromatography

- separating mixtures of compounds
- identifying unknown compounds
- establishing the purity or concentration of compounds
- monitoring product formation in the pharmaceutical and biotechnology industries
Types of Chromatography…

- Paper
- Thin layer
- HPLC
- Gas
- Column
Chromatographic Methods Classification
(1) Geometry of the system

- In **column chromatography** the stationary phase is contained in a tube called the column.

- **Planar chromatography**. In this geometry the stationary phase is configured as a thin two-dimensional sheet.
  
  (i) In **paper chromatography** a sheet or a narrow strip of paper serves as the stationary phase.

  (ii) In **thin-layer chromatography** a thin film of a stationary phase of solid particles bound together for mechanical strength with a binder, such as calcium sulfate, is coated on a glass plate or plastic or metal sheet.
(2) Mode of operation

- **Development chromatography.** In terms of operation, in development chromatography the mobile phase flow is stopped before solutes reach the end of the bed of stationary phase. The mobile phase is called the developer, and the movement of the liquid along the bed is referred to as development.

Example of planar development chromatography – TLC or PC

- **Elution chromatography.** This method, employed with columns, involves solute migration through the entire system and solute detection as it emerges from the column. The detector continuously monitors the amount of solute in the emerging mobile-phase stream—the eluate—and transduces the signal, most often to a voltage, which is registered as a peak on a strip-chart recorder.
(3) Retention mechanism

Classification in terms of the retention mechanism is approximate, because the retention actually is a mixture of mechanisms. The main interactions are:

- **hydrophobic** (non-specific), **dipole-dipole** (polar), **ionic**, **special**.
(4) Phases involved

1.) The primary division of chromatographic techniques is based on the type of mobile phase used in the system:

<table>
<thead>
<tr>
<th>Type of Chromatography</th>
<th>Type of Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas chromatography (GC)</td>
<td>gas</td>
</tr>
<tr>
<td>Liquid chromatograph (LC)</td>
<td>liquid</td>
</tr>
</tbody>
</table>
2.) Further divisions can be made based on the type of stationary phase used in the system:

## Gas Chromatography

<table>
<thead>
<tr>
<th>Name of GC Method</th>
<th>Type of Stationary Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas-solid chromatography</td>
<td>solid, underivatized support</td>
</tr>
<tr>
<td>Gas-liquid chromatography</td>
<td>liquid-coated support</td>
</tr>
<tr>
<td>Bonded-phase gas chromatography</td>
<td>chemically-derivatized support</td>
</tr>
</tbody>
</table>

![Gas Chromatography Diagram](attachment:diagram.png)

- **Flow controller**
- **Injector port**
- **Column oven**
- **Column**
- **Detector**
- **Recorder**
- **Carrier gas**
**Liquid Chromatography**

<table>
<thead>
<tr>
<th>Name of LC Method</th>
<th>Type of Stationary Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption chromatography</td>
<td>solid, underivatized support</td>
</tr>
<tr>
<td>Partition chromatography</td>
<td>liquid-coated or derivatized support</td>
</tr>
<tr>
<td>Ion-exchange chromatography</td>
<td>support containing fixed charges</td>
</tr>
<tr>
<td>Size exclusion chromatography</td>
<td>porous support</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>support with immobilized ligand</td>
</tr>
</tbody>
</table>

![Diagram of liquid chromatography](attachment:chart.png)
5) Principle of separation

i. Adsorption chromatography.

Chromatography in which separation is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid.
i. **Partition chromatography.**

Chromatography in which separation is based mainly on differences between the solubility of the sample components in the stationary phase (gas chromatography), or on differences between the solubilities of the components in the mobile and stationary phases (liquid chromatography).
Ion-exchange chromatography.

Chromatography in which separation is based mainly on differences in the ion-exchange affinities of the sample components. Anions like SO₃⁻ or cations like N(CH₃)₃⁺ are covalently attached to stationary phase, usually a resin.
Molecular exclusion chromatography. A separation technique in which separation mainly according to the hydrodynamic volume of the molecules or particles takes place in a porous non-adsorbing material with pores of approximately the same size as the effective dimensions in solution of the molecules to be separated.
Affinity chromatography. The particular variant of chromatography in which the unique biological specificity of the analyte and ligand interaction is utilized for the separation.
The affinity chromatography

Common for all types of affinity chromatography is that an affinity ligand specific for a binding site on the target molecule, is coupled to an inert chromatography matrix.

Under suitable binding conditions this affinity matrix will bind molecules according to its specificity only. All other sample components will pass through the medium unadsorbed (Fig.1.1).

After a wash step the adsorbed molecules are released and eluted by changing the conditions towards dissociation or by adding an excess of a substance that displaces the target molecule from the affinity ligand.
The development of an affinity chromatography method boils down to:

1. Finding a ligand specific enough to allow step elution.
2. Finding conditions for safe binding and release within the stability window of the target molecule and the ligand.
Theory of Chromatography

1.) Typical response obtained by chromatography (i.e., a chromatogram):

Where:
- $t_R$ = retention time
- $t_M$ = void time
- $W_b$ = baseline width of the peak in time units
- $W_h$ = half-height width of the peak in time units
Note: The separation of solutes in chromatography depends on two factors:

(a) a difference in the retention of solutes (i.e., a difference in their time or volume of elution
(b) a sufficiently narrow width of the solute peaks (i.e., good efficiency for the separation system)

Peak width & peak position determine separation of peaks

A similar plot can be made in terms of elution volume instead of elution time. If volumes are used, the volume of the mobile phase that it takes to elute a peak off of the column is referred to as the *retention volume* \(V_R\) and the amount of mobile phase that it takes to elute a non-retained component is referred to as the *void volume* \(V_M\).
2.) Solute Retention:

A solute’s retention time or retention volume in chromatography is directly related to the strength of the solute’s interaction with the mobile and stationary phases.

Retention on a given column pertain to the particulars of that system:
- size of the column
- flow rate of the mobile phase

\[
\text{average migration rate } \quad \bar{v} = \frac{\text{L}}{t_R} \quad \text{column length}
\]

Capacity factor \((k')\): more universal measure of retention, determined from \(t_R\) or \(V_R\).

\[
k' = \frac{(t_R - t_M)}{t_M}
\]

or

\[
k' = \frac{(V_R - V_M)}{V_M}
\]

capacity factor is useful for comparing results obtained on different systems since it is independent on column length and flow-rate.
The value of the capacity factor is useful in understanding the retention mechanisms for a solute, since the fundamental definition of $k'$ is:

$$k' = \frac{\text{moles A}_{\text{stationary phase}}}{\text{moles A}_{\text{mobile phase}}}$$

$k'$ is directly related to the strength of the interaction between a solute with the stationary and mobile phases.

Moles $A_{\text{stationary phase}}$ and moles $A_{\text{mobile phase}}$ represents the amount of solute present in each phase at equilibrium.

Equilibrium is achieved or approached at the center of a chromatographic peak.

When $k'$ is $\leq 1.0$, separation is poor
When $k'$ is $> 30$, separation is slow
When $k'$ is $= 2-10$, separation is optimum
A simple example relating $k'$ to the interactions of a solute in a column is illustrated for partition chromatography:

$$K_D$$

A (mobile phase) $\Leftrightarrow$ A (stationary phase)

where: $K_D$ = equilibrium constant for the distribution of A between the mobile phase and stationary phase

Assuming local equilibrium at the center of the chromatographic peak:

$$k' = \frac{[A]_{\text{stationary phase}} \cdot \text{Volume}_{\text{stationary phase}}}{[A]_{\text{mobile phase}} \cdot \text{Volume}_{\text{mobile phase}}}$$

$$k' = \frac{\text{Volume}_{\text{stationary phase}}}{\text{Volume}_{\text{mobile phase}}} K_D$$

As $K_D$ increases, interaction of the solute with the stationary phase becomes more favorable and the solute’s retention ($k'$) increases.
Separation between two solutes requires different $K_D$’s for their interactions with the mobile and stationary phases

since $\Delta G = -RT \ln K_D$

peak separation also represents different changes in free energy
3.) Efficiency:

Efficiency is related experimentally to a solute’s peak width.
- an efficient system will produce narrow peaks
- narrow peaks → smaller difference in interactions in order to separate two solutes

Efficiency is related theoretically to the various kinetic processes that are involved in solute retention and transport in the column
- determine the width or standard deviation (σ) of peaks

Estimate σ from peak widths, assuming Gaussian shaped peak:

\[ W_b = 4\sigma \]
\[ W_h = 2.354\sigma \]

Dependent on the amount of time that a solute spends in the column (k’ or tR)
**Number of theoretical plates (N):** compare efficiencies of a system for solutes that have different retention times

\[ N = \left( \frac{t_R}{\sigma} \right)^2 \]

or for a Gaussian shaped peak

\[ N = 16 \left( \frac{t_R}{W_b} \right)^2 \]

\[ N = 5.54 \left( \frac{t_R}{W_h} \right)^2 \]

The larger the value of \( N \) is for a column, the better the column will be able to separate two compounds.

- the better the ability to resolve solutes that have small differences in retention
- \( N \) is independent of solute retention
- \( N \) is dependent on the length of the column
Plate height or height equivalent of a theoretical plate (H or HETP): compare efficiencies of columns with different lengths:

\[ H = \frac{L}{N} \]

*Note:* \( H \) simply gives the length of the column that corresponds to one theoretical plate.

\( H \) can be also used to relate various chromatographic parameters (e.g., flow rate, particle size, etc.) to the kinetic processes that give rise to peak broadening:

**Why Do Bands Spread?**

- a. Eddy diffusion
- b. Mobile phase mass transfer
- c. Stagnant mobile phase mass transfer
- d. Stationary phase mass transfer
- e. Longitudinal diffusion
Rate Theory of Chromatography

\[ H = H_L + H_S + H_M + H_{SM} \]

- \( H \) = height equivalent to theoretical plate (as in Plate Theory)
- \( H_L \) = contribution due to longitudinal diffusion
- \( H_S \) = stationary phase mass transfer contribution
- \( H_M \) = diffusion associated with mobile phase effects
- \( H_{SM} \) = diffusion into or mass transfer across a stagnant layer of mobile phase (neglect)

\[ H = B/\mu + C\mu + A \]

van Deemter Equation A, B & C are coefficients, \( \mu \) = velocity
a.) *Eddy diffusion* – a process that leads to peak (band) broadening due to the presence of multiple flow paths through a packed column.

As solute molecules travel through the column, some arrive at the end sooner than others simply due to the different path traveled around the support particles in the column that result in different travel distances.

Longer path arrives at end of column after (1).
b.) **Mobile phase mass transfer** – a process of peak broadening caused by the presence of different flow profile within channels or between particles of the support in the column.

A solute in the center of the channel moves more quickly than solute at the edges, it will tend to reach the end of the channel first leading to band-broadening.

The degree of band-broadening due to eddy diffusion and mobile phase mass transfer depends mainly on:

1) the size of the packing material
2) the diffusion rate of the solute
c.) **Stagnant mobile phase mass transfer** – band-broadening due to differences in the rate of diffusion of the solute molecules between the mobile phase outside the pores of the support (flowing mobile phase) to the mobile phase within the pores of the support (stagnant mobile phase).

Since a solute does not travel down the column when it is in the stagnant mobile phase, it spends a longer time in the column than solute that remains in the flowing mobile phase.

The degree of band-broadening due to stagnant mobile phase mass transfer depends on:

1) the size, shape and pore structure of the packing material
2) the diffusion and retention of the solute
3) the flow-rate of the solute through the column
**d.) Stationary phase mass transfer** – band-broadening due to the movement of solute between the stagnant phase and the stationary phase.

Since different solute molecules spend different lengths of time in the stationary phase, they also spend different amounts of time on the column, giving rise to band-broadening.

The degree of band-broadening due to stationary phase mass transfer depends on:

1. the retention and diffusion of the solute
2. the flow-rate of the solute through the column
3. the kinetics of interaction between the solute and the stationary phase
e.) Longitudinal diffusion – band-broadening due to the diffusion of the solute along the length of the column in the flowing mobile phase.

The degree of band-broadening due to longitudinal diffusion depends on:

1) the diffusion of the solute
2) the flow-rate of the solute through the column
**Van Deemter equation:** relates flow-rate or linear velocity to H:

\[
H = A + \frac{B}{\mu} + C\mu
\]

where:

- \(\mu\) = linear velocity (flow-rate \times V_m/L)
- \(H\) = total plate height of the column
- \(A\) = constant representing eddy diffusion & mobile phase mass transfer
- \(B\) = constant representing longitudinal diffusion
- \(C\) = constant representing stagnant mobile phase & stationary phase mass transfer

One use of plate height (H) is to relate these kinetic processes to band broadening to a parameter of the chromatographic system (e.g., flow-rate).

This relationship is used to predict what the resulting effect would be of varying this parameter on the overall efficiency of the chromatographic system.

**Number of theoretical plates\((N)\)**  
\[
(N) = 5.54 \left(\frac{t_R}{W_h}\right)^2
\]

peak width \((W_h)\)

\[
H = \frac{L}{N}
\]
Plot of van Deemter equation shows how $H$ changes with the linear velocity (flow-rate) of the mobile phase

$$H = A + \left( \frac{B}{\mu} \right) + (C \times \mu)$$

Optimum linear velocity ($\mu_{\text{opt}}$) - where $H$ has a minimum value and the point of maximum column efficiency:

$$\mu_{\text{opt}} = \frac{B}{C}$$

$\mu_{\text{opt}}$ is easy to achieve for gas chromatography, but is usually too small for liquid chromatography requiring flow-rates higher than optimal to separate compounds.
4.) Measures of Solute Separation:

**separation factor \( (\alpha) \)** – parameter used to describe how well two solutes are separated by a chromatographic system:

\[
\alpha = \frac{k_2'}{k_1'}
\]

\[
k' = \frac{(t_R - t_M)}{t_M}
\]

where:

- \( k_1' \) = the capacity factor of the first solute
- \( k_2' \) = the capacity factor of the second solute,
  with \( k_2' \cong k' \)

A value of \( \alpha \cong 1.1 \) is usually indicative of a good separation.

Does not consider the effect of column efficiency or peak widths, only retention.
resolution \( (R_s) \) – resolution between two peaks is a second measure of how well two peaks are separated:

\[
R_s = \frac{t_{r2} - t_{r1}}{(W_{b2} + W_{b1})/2}
\]

where:

\( t_{r1}, W_{b1} = \) retention time and baseline width for the first eluting peak
\( t_{r2}, W_{b2} = \) retention time and baseline width for the second eluting peak

\( R_s \) is preferred over \( \alpha \) since both retention \( (t_r) \) and column efficiency \( (W_b) \) are considered in defining peak separation.

\( R_s \geq 1.5 \) represents baseline resolution, or complete separation of two neighboring solutes \( \rightarrow \) ideal case.

\( R_s \geq 1.0 \) considered adequate for most separations.
Example

☐ What is the resolution of two Gaussian peaks of identical width (3.27 s) and height eluting at 67.3 s and 74.9 s, respectively?

☐ ANS: Resolution = 2.32
Example

- Substance A and B were found to have Retention time of 16.40 and 17.63 min, respectively, on a 30.0cm column. An unretained species passed through the column in 1.30 min. The peak widths (at base) for A and B were 1.11 and 1.21 min, respectively. Calculate:
  - The column resolution
  - The average number of plates in the column
  - The plate height
  - The length of column required for resolution of 1.5.
  - The time required to elute substance B on the longer column
  - The plate height required for a resolution of 1.5 on the original 30 cm column and in the original time
VARIABLE AFFECT COLUMN PERFORMANCE

Retention factor (k’), selectivity factor (α) and theoretical plates (N), HETP (H)

- Variation in N
- Variation in H
- Variation in Retention factor
- Variation in selectivity factor
Application of chromatography

- Qualitative analysis
- Quantitative analysis
  - Analysis based on peak height
  - Analysis based on peak area
    - Calibration and standards
    - The internal standard method
    - The area normalization method