Analytical Separations

An Introduction to Chromatography

In general matter do not exist in a pure form but in a ‘mixed’ state.

**CHROMATOGRAPHY**

Chromatography refers to a group of instrumental techniques used for the separation of a mixture of analytes into their individual components.

Chromatography allows the identification and quantification of the components separated, as a function of time.

The objective of chromatographic techniques is to separate mixtures into its components in the smallest of space and in the shortest of time.

In general matter do not exist in a pure form but in a ‘mixed’ state.

- **Pure Substances**: 1) one type of molecule 2) all samples show the same intensive properties
- **Mixture**: 1) made of multiple types of molecules 2) samples may show different intensive properties

What are the substances present and in what quantities?

Requires separation of the mixtures before identification and quantification.

The ‘mixture’ introduced into a tube containing a material - stationary phase - s.p. - (column); s.p. ‘holds’ the analytes tentatively.

Another phase is moved over the s.p.; mobile phase – m.p. (eluent) that elutes the ‘held’ analytes off the s.p.

The ‘analytes’ enters the column at the same time and leaves the column at different times (separation).

A device ‘looks’ at the eluate to detect the eluted components (detector).

The detector response is plotted – chromatogram.

Two types of columns: packed and open tubular.

The objective of chromatographic techniques is to separate mixtures into its components in the smallest of space and in the shortest of time.
In chromatography a mixture is separated by distributing its components between two phases.

The stationary phase remains fixed in a tube the mobile phase moves over the stationary phase carrying the components of the mixture. The stationary phase alters the movement of the components in a mixture; components move slower than the mobile phase.

The components interact with the mobile and/or stationary phases differently due to the differences of their solubility of in the mobile phase and the stationary phase. Components will move at different speeds through the column, enabling the separation of the components in the mixture from one another.

In gas chromatography (GC), the mobile phase is an inert gas such as helium and the m.p. does not take an active role in the separation process.

The stationary phase is generally coated or chemically bonded to an inert support (diatomaceous earth/silica), both in GC and LC.

In chromatographic systems the separation of analytes is initiated with the sample mixture being introduced (injected) into the mobile phase.
Partitioning of an analyte between two phases is a thermodynamic process and is characterized by an equilibrium constant, $K_i$ (function of temperature).

### Partition Coefficient, $K_i$ for analyte $i$,

$$K_i = \frac{C_{i,\text{phase 1}}}{C_{i,\text{phase 2}}}$$

- $C_{i,\text{phase 1}}$: Concentration of analyte $i$ in phase 1
- $C_{i,\text{phase 2}}$: Concentration of analyte $i$ in phase 2

The ‘temporary holding’ of analyte occurs in the stationary phase.

Chromatography, in general involves the partitioning and re-partitioning of analytes between two phases (extraction) many, many times; i.e. multiple ‘extractions’ done during the separation process.

These multiple ‘partitionings’ are achieved by moving one phase (mobile phase) relative to the other (stationary phase); thereby constantly refreshing the system with the mobile phase.

This process enables their separation from each other.

In liquid chromatography (LC), the mobile phase is a solvent, that takes an active role in the partitioning/separation of analytes.

In gas chromatography (GC), the mobile phase is an inert gas such as helium and the m.p. does not take an active role in the separation process.

Each analyte in the mixture partitions to a different degree between the phases, therefore retains differently, depending on the $K$ value of the analyte.

**LC** (Macro-scale column chromatography)

Mobile phase moves faster (m.p. velocity = $u$) than analytes.
Under proper stationary/mobile phase combination, each analyte migrates with a unique velocity, which is less than the mobile phase velocity $u$.

Components in a mixture (analytes) separate from each other as distinct bands because each analyte interact (retain) differently with the stationary phase and the mobile phase.

There are different mechanisms involved depending on the type of interaction implemented e.g. partitioning, adsorption, size exclusion etc.

In GC and LC differential partitioning of the components is the predominant mechanism of separation. Sufficient time is required to establish the partitioning of each component between the mobile and stationary phases.

Moving an analyte through the column results in the broadening of the analyte band – concentration profile.

Low broadening (preferred) leads to superior chromatograms.

A very thin segment of test material at the starting point of the column.

Detector: generates a signal proportional to amount of analyte passing through and its response factor.

Moving an analyte through the column results in the broadening of the analyte band – concentration profile.

Low broadening (preferred) leads to superior chromatograms.

A very thin segment of test material at the starting point of the column.

Detector: generates a signal proportional to amount of analyte passing through and its response factor.

Gas Chromatograph

Column packed with the stationary phase through which the mobile phase is moved.

University of Athens
Chromatogram: A graphical plot of the detector signal as a function of time.

Any un-retained substances move at the same velocity as the mobile phase, \( u \).

Chromatographic peaks (from good experimental parameters) take a Gaussian shape.

\[ x \text{- axis: time of run or volume of the mobile phase.} \]

**Types of Chromatography**

(A classification by mechanism of retention)

1. **Partition Chromatography**  
   GC(GLC), LC(LLC)

2. Adsorption Chromatography

3. Ion-exchange Chromatography

4. Molecular Exclusion Chromatography

5. Affinity Chromatography

Partitioning into the phases is controlled by the polarities of the analyte and the phases involved (Like dissolves like) and the volume ratio of the phases.

\[ \frac{m_p}{m_s} = \frac{k}{KV_s} \quad \text{and} \quad \frac{m_i}{m_i + m_m} = \frac{1}{1 + V_v / KV_v} \]
The value of $\sigma$ is a measure of the **broadening** of the peak.

It arises from *in-column* effects and *extra column* effects.

Note: only variances are additive.

Band Broadening (In-column effects)

- Multiple paths taken by analyte molecules;
  - Eddy diffusion
- Longitudinal Diffusion
- Mass Transfer Effects

Band broadening imposes a limitation on the ability to **resolve** consecutive peaks because of the overlap of consecutive analyte bands (i.e. chromatographic peaks).

If there is no broadening the separated regions would come out of the column with region thickness same as at the beginning of the separation process.

Resolution of peaks –

the ability to distinguish two consecutive analyte segments from each other.
Resolution: Defined for two consecutive peaks

\[ R = \frac{2(t_{2e} - t_{1e})}{w_2 + w_1} = \frac{\Delta t_e}{w_{\text{average}}} = \frac{0.589\Delta t_e}{w_{2/2,\text{arg}}} \]

\[ w_{\text{average}} = \frac{w_2 + w_1}{2} \]

For two similar peaks, #1 eluting before #2.
Note: x-axis is in time units, \( t_e \) is the elution time.
(x axis may be expressed as elution volume)

The objective is to separate mixtures into individual components and subsequently identify them.

For quantification R values preferred \( \geq 1.5 \).

\[ R = \frac{2(t_{2e} - t_{1e})}{w_2 + w_1} \]

Enhance retention time difference (Better to optimize)
SELECTIVITY OF THE COLUMN

DECREASE BAND WIDTHS
SELECTIVITY

SELECTIVITY

For separation to occur each analyte in the mixture must leave the column at different (elution) times, \( t_e \).

Since all analytes travel the same length of the column, L, separable analytes would have different migration velocities,

\[ v_i < u \quad \text{and} \quad u = \frac{L}{t_m}. \]

What makes different \( v_i \) values for different analytes?

……. the mechanism involved in this process.

SELECTIVITY

EFFICIENCY

SELECTIVITY

EFFICIENCY

SELECTIVITY

EFFICIENCY
1. **Partition**
2. Adsorption
3. Ion-exchange
4. Molecular Exclusion
5. Affinity

Partition mechanism in a *packed column* is the most complicated system, other columns have less complications.

**Packed column:**
A tube filled uniformly with an inert material (diatomaceous earth) covered uniformly with the stationary phase. Mobile phase is moved over the stationary phase.

\[
K_i = \frac{C_{i,\text{phase}}}{C_{i,\text{phase}}} 
\]

For \( K_i \neq 0 \), analyte disperses in both phases. For \( K_i = 0 \), analyte flushes out, no retention in s.p.

Large \( K_i \) analyte prefers s.p. at equilibrium, and therefore retained in s.p. stronger, vice versa.

*Different* \( K_i \) *values* of different analytes makes different analytes to be *retained differently* on s.p. Thus they moves through the column at *different velocities*, resulting in *different* \( t_i \); *separation*.

**Plate Theory of Band Broadening**
*In-column effects; Packed column*

Partitioning of an analyte between the two phases is characterized by its partition coefficient, \( K_i \).

In a column many ‘partitions’ of the analyte occurs as the analyte moves thro’ the column.

Each partitioning/equilibrium ‘region’ is referred to as a *plate*. *Equilibrium thermodynamics invoked.*
Plate theory: Each equilibration ‘region’ is a theoretical plate.

Number of theoretical plates; \( N = \frac{L}{H} \)

HETP: Height equivalent of a theoretical plate. The ‘length’ of an equilibrium ‘region’ in the column; plate height.

The smaller the HETP, more compact the peak be. And also the higher would be the number of equilibrations in the column (N).

Higher number of N (theoretical plates) ensures smaller HETPs, compact peaks thus better separations.

For a column of length L, \( N = \frac{L}{H} \)

Number of theoretical plates (N) is a measure of the compactness of eluting peaks.

N measures the EFFICIENCY of a column.

Major objective: increase efficiency of columns.

To increase N is to decrease H. \( H = \frac{L}{N} \)

A measure of the “effective length of an equilibrium region” is termed the height equivalent of the theoretical plate, HETP, H.

Decreasing the peak width is an integral part of method development in chromatography.

Smaller H values (i.e. larger N values) generate smaller peak widths.

Ideal peaks are Gaussian in shape.

Characterized by \( \sigma \).

Narrow peaks are generated by more EFFICIENT columns; with larger number of theoretical plates.
Efficiency of column can be calculated using the data of a peak.

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

\[
N = 5.55 \left( \frac{t_r}{w_{b/2}} \right)^2
\]

\[
N = 16 \left( \frac{t_r}{w_{b}} \right)^2
\]

\[
H = \frac{\sigma^2}{L}
\]

For asymmetric peaks,

\[
N = \frac{41.7 \left( t_r / w_{0.1} \right)^2}{A / B + 1.25}
\]

\[
w_{0.1} = A + B
\]

The **capacity factor** \( k' \) (retention factor);

There are two primary column-related factors that affect capacity factor: volume of the stationary phase and the volume of the mobile phase.

The other important factor is the **partition coefficient of the analyte**.

\[
k' = \frac{n_{i,s}}{n_{i,m}} = \frac{C_{i,s}V_{bn}}{C_{i,m}V_{m}} = \frac{C_{i,s}V_i}{C_{i,m}V_m} = \left( \frac{K_iV_i}{V_m} \right) = \frac{K_i}{\beta}
\]

The **capacity factor**

Expression that measures the degree of retention of an analyte relative to an un-retained peak, where \( t_b \) is the retention time for the sample peak and \( t_m \) is the retention time for an un-retained peak. Thus the higher the capacity factor, the longer the retention time.

Any retention shifts (capacity factor) are due to the changing column phase ratio or the changing the chemistry of partitioning.
The **relative selectivity** is directly related to how different analyte molecules **interacts** with the phases involved in the column.

If the analyte strongly interacts with the stationary phase, it is because of strong **“intermolecular forces”** of the analyte with the stationary phase. It is a function of the structure of **both the analyte molecule and the phases**, especially the stationary phase.

If the analyte – s.p. structures are similar, then these attractive forces for one another are strong. If attractive forces are weak, then analyte to stationary phase attraction is weak, and retention is less.

**Resolution** between consecutive peaks

\[
R = \frac{\sqrt{N}}{4} \left( \frac{\alpha_{2,1} - 1}{\alpha_{2,1}} \right) \left( \frac{k_2'}{1 + \alpha_{avg}} \right) = \frac{\sqrt{N}}{4} \left( \frac{t_b}{t_a} - 1 \right)
\]

**Efficiency**  Relative retention  Capacity factor

**Velocity of Migration of Analyte (i) Band:**

\[
v_i = f(u, K_i, \beta) \quad k_i' = \text{capacity factor} \quad \beta = \text{phase ratio; } V_m/V_s
\]

\[
k_i = \frac{K_i V_a}{V_m}
\]

\[
u = \text{linear velocity m.p.} \quad V_a = \text{volume of s.p. column} \quad V_m = \text{volume of m.p. column} \quad K_i = \text{partition coefficient of analyte i in the sp/mp system.}
\]

\[
v_i = \frac{1}{K_i V_m} + 1
\]

**Resolution between two (consecutive) peaks is related as:**

\[
R = \frac{\Delta t_r}{w_{arg}} = \frac{\Delta t_r}{4\sigma_{arg}} = \frac{0.589\Delta t_r}{w_{1/2}}
\]

\[
R \propto \sqrt{N} \quad R \propto \sqrt{\frac{L}{H}}
\]

Because HETP, H, is an intensive property, by changing the physical length of the column alone, resolution can be increased.

In general, capillary columns (open-tubular) provide higher resolutions than packed columns (GC).

Plate theory assumes instantaneous attainment of partitioning; that is the instantaneous equilibration of analytes in a ‘plate’.
Rate Theory of Band Broadening
(In-column effects; Packed column)

- Multiple paths taken by analyte molecules, Eddy diffusion
- Longitudinal Diffusion
- Mass Transfer Effects

Extra-column broadening ~constant

\[
H = \frac{\sigma^2}{L} = \frac{\sigma_{\text{column}}^2}{L} + \sigma_{\text{detector}}^2
\]

The band broadening arises due to several reasons, and any model explaining in-column band broadening must consider the realistic features in the column; *kinetic and thermodynamic* factors. Each band broadening factor would contribute to \( \sigma_{\text{column}} \).

1. Eddy Diffusion (multiple path):

   Packed particles are uneven, ‘pseudo-spheres’; of average size \( d_p \). Larger particles generate a larger range of multiple paths (packed columns); \( H_{\text{eddy}} = \lambda d_p = A \)

   Practically, add extra-column contribution to \( A \).

2. Longitudinal Diffusion:

   Diffusion: Natural tendency for matter to move from high concentration regions to lower concentration regions.

   Occurs in both m.p. and s.p. (more in m.p. in GC)

   Longitudinal broadening relates – *inversely* to the mobile phase velocity (i.e. \( u \)); *directly* to the diffusion coefficient (time of residence of analyte in the m.p. column \( t=L/u \)) (diffusion broadening in s.p.–small in GC, so ignored);

   \[
   H_{\text{det}} = \frac{2\gamma D_u}{u} \frac{B}{u} \quad u = \text{linear velocity (} u \text{)}
   \]
Analyte plug broadens with time, always.

3. Mass Transfer Effects:

Ideal equilibration must allow enough time to reach that state, in reality it is not the case because the m.p. is in constant motion; and equilibration is not instantaneous.

That is to say complete equilibration does not occur fast enough; partial (non) - equilibrium system.

a. Stationary phase, \( H_{stat} \)
b. Mobile phase, \( H_{mob} \)

3 a. Stationary phase

“An incomplete equilibrium” due to a time lag between molecules in the s.p. relative to those in m.p.

Diffusion

A plug of analyte diffuses out to produce a Gaussian curve of distribution of concentration. The \( \sigma \) of it, is given by the Einstein's Law;

\[
\sigma^2 = 2Dt
\]

\[ t = \frac{L}{u} ; \text{longitudinal diffusion in m.p.} \]

\[
\sigma^2 = 2D\frac{L}{u} ; \text{ by substitution}
\]

HETP from diffusion: \( H_D = \frac{\sigma^2}{L} = \frac{2D}{u} = \frac{2yD}{u} = \frac{B}{u} \)

\[ \gamma = 0.6 \text{ packed} \]
\[ \gamma = 1 \text{ capillary} \]

Mass Transfer Effects:

Ideal equilibration must allow enough time to reach that state, in reality it is not the case because the m.p. is in constant motion; and equilibration is not instantaneous.

That is to say complete equilibration does not occur fast enough; partial (non) - equilibrium system.

a. Stationary phase, \( H_{stat} \)
b. Mobile phase, \( H_{mob} \)

3 a. Stationary phase

“An incomplete equilibrium” due to a time lag between molecules in the s.p. relative to those in m.p.
3 b. Mobile phase

Larger number of streams, smaller diffusion coefficients and faster carrier gas velocities decrease broadening.

\[
H_{\text{mob}} = \frac{k^2 d_p^2}{96(1+k)^2 D_m} \frac{u}{u_{\text{optimum}}} = C_{\text{mob}} u
\]

For packed column

\[
C_{\text{mob}} = \frac{1+6k + 11k^2}{24(k+1)^2} \frac{r^2}{D_m}
\]

For open-tubular column

Diffusion coefficient \(D_m\) is very sensitive to temperature changes; \(D_m\) increases with temperature.

Overall: Van Deemter Equation:

\[
H_{\text{column}} = A + \frac{B}{u} + (C_{\text{stat}} + C_{\text{mob}}) u
\]

\[
H_{\text{column}} = A + \frac{B}{u} + Cu
\]

Expressions for capillary (WCOT, SCOT, PLOT) differs from the packed column, however the trends are the same. See text.

Smaller \(d_p\) \(\rightarrow\) lesser # differing multiple paths lengths \(\rightarrow\) \(H\) smaller; accomplished in capillary GC (open tubular) \(d_p \approx d_s\).

\[
H_{\text{column}} = \lambda d_p + \frac{2\gamma D_m}{u} + \left(\frac{qk^2 d_p^2}{(k+1)^2 D_s} + \frac{1+6k + 11k^2}{24(k+1)^2} \frac{r^2}{D_m}\right) u
\]

(packed column)

Diffusion coefficient \(D_m\) is very high in GC; equilibration fast; capillary column - s.p. coated on surface, m.p. small allows - faster equilibration possible!
Effect of Temperature

Retention time reduces; faster equilibration. Flow rate needs raising.

Later eluting peaks widen; always.

\[ \sigma = \sqrt{2D\tau} = \frac{2DL}{u} \]

Asymmetric bands:

The partition coefficient is a constant for a given ‘column’, temperature, analyte combination; but,

1. If the injections overloads the column, the s.p. over saturates making the above assumption fail, \( C_s \) larger than expected.

2. If the ‘inert’ support is not actually inert and interacts attractively with the analytes (defective regions in the column) the ‘assumption’ fails too, \( C_s \) smaller than expected.

Fronting due to overloading, it is alleviated by using diluted samples. The number of ‘sites’ available for analyte to partition in the s.p. is less than needed.

Tailing can be (mostly) is because of adsorption on the s.p.

To avoid tailing the active sites on the silica particles must be ‘deactivated’ - silanization.

Surface hydroxyl groups often are the active sites forms H-bonds with polar solutes.

\[
\text{HMDS} = (\text{CH}_3)_3\text{SiNHSi(CH}_3)_3
\]

\[ X = \text{NHSi(CH}_3)_3 \]
<table>
<thead>
<tr>
<th>Property</th>
<th>Packed</th>
<th>Open tubular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column length, L</td>
<td>2.4 m</td>
<td>100 m</td>
</tr>
<tr>
<td>Linear gas velocity</td>
<td>8 cm/s</td>
<td>16 cm/s</td>
</tr>
<tr>
<td>Plate height for methyl oleate</td>
<td>0.53 mm</td>
<td>0.34 mm</td>
</tr>
<tr>
<td>Capacity factor, k', for methyl oleate</td>
<td>58.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Theoretical plates, N</td>
<td>3 290</td>
<td>294 000</td>
</tr>
<tr>
<td>Resolution of methyl stearate and methyl oleate</td>
<td>1.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Retention time of methyl oleate</td>
<td>28.8 min</td>
<td>38.5 min</td>
</tr>
</tbody>
</table>

* Methyl stearate (CH₃(CH₂)₁₇CO₂H) and methyl oleate (CH₃(CH₂)₇CH=CH(CH₂)₇CO₂H) were separated on columns with polyethylene glycol succinate stationary phase at 180°C.

source: L. S. Lier, Introduction to Open Tubular Columns (Norwalk, CT: Perkin-Elmer Corp., 1979).