CHROMATOGRAPHIC METHODS OF SEPARATION

BASIC PRINCIPLES

- All chromatographic separations rely on the differences in interaction between analytes and the two characteristic phases
- **Mobile phase**: carries/transports the analytes
- **Stationary phase**: interacts with the analytes as they are moving through it.

- Analytes that strongly interact with the stationary phase are retained longer, thus **elute** from the column later than those that interact weakly with the stationary phase.
- Analytes separate into bands
- Analytes are detected at the exit of the column and their signals recorded
- Plot: chromatogram
Classification based on the types of mobile and stationary phases and the kinds of equilibria involved in the transfer of solutes between phases

Name based on type of Mobile

**Elution**

- Elution: washing a species through a column by continuous addition of fresh mobile phase
- Mobile phase: eluent
- Partition between mobile and stationary phase
General Classification of Chromatographic Methods

- Classification based on the types of mobile and stationary phases and the kinds of equilibria involved in the transfer of solutes between phases.
- Name based on type of Mobile
  - **Gas Chromatography**
    - Mobile phase: inert gas (helium, nitrogen)
    - Stationary phase: supported liquid (SiO₂ coated with polymer)
    - Analyte must be volatile and thermally stable at working temperatures
    - Detection: flame ionization, thermal conductivity, MS
  - **Liquid-Liquid Chromatography**
    - Mobile phase: liquid
      - Non-polar: normal phase
      - Polar: reversed phase (water/acetonitrile, water/methanol)

Basic Theory

Important Parameters and Variables

- Two basic phenomena
  - Transport/ migration
  - Mass transfer between the two phases
  - Band broadening
- Retention time ($t_R$)
- Peak width
- Resolution
Migration of Solutes

- Effectiveness of separation of two solutes (A and B) depends in part on the relative rates of elution

- Rates of migration are determined by the magnitude of the equilibrium constants for the “reactions” by which the solutes distribute themselves between the mobile and stationary phases

Distribution Constants

- $K_c$: distribution constant
  - partition ratio
  - partition coefficient
- $a_s$: activity in stationary phase
- $a_M$: activity in the mobile phase
- $c_s$: concentration in the stationary phase
- $c_M$: concentration in the mobile phase
- $K_c$ can be manipulated by appropriate choices of mobile phase, stationary phase or both.

  - Linear chromatography:
    - $K_c$ is constant, does not change with solute concentration
    - Gaussian-type peak
    - Retention times independent of amount of analyte injected

\[
A_{\text{mobile}} \leftrightarrow A_{\text{stationary}}
\]

\[
K_c = \frac{(a_A)_S}{(a_A)_M}
\]

\[
K_c = \frac{c_S}{c_M} = \frac{n_s}{n_M} \frac{V_s}{V_M}
\]
Retention Time

- Retention time depends on $K_C$
- $t_M$: time for the unretained species, dead or void time
- $t_S$: time spent in the stationary phase

\[ t_R = t_M + t_S \]
\[ v = \frac{L}{t_R} \]
\[ v = \text{average linear velocity of solute migration} \]
\[ u = \frac{L}{t_M} \]
\[ u = \text{average linear velocity of mobile phase} \]
\[ \bar{v} = u \times \frac{1}{1 + K_C V_S / V_M} \]
Retention/Capacity Factor

- Used to compare migration rates of solutes in columns
- Does not depend on column geometry or volumetric flow rates
- Can be calculated from measured retention times
- For example, for a solute A, the capacity factor $k_A$ is given by:

$$k_A = \frac{K_AV_S}{V_M}$$

$$\nu = u \times \frac{1}{1 + K_AV_S/V_M} = u \times \frac{1}{1 + k_A}$$

$$\frac{L}{t_R} = \frac{L}{t_M} \times \frac{1}{1 + k_A}$$

$$k_A = \frac{t_R - t_M}{t_M}$$

$t_R - t_M$: adjusted retention time

Relative Migration Rates: Selectivity Factor

- The selectivity factor ($\alpha$) compares migration rates

$$\alpha = \frac{K_B}{K_A}$$

$$\alpha = \frac{k_B}{k_A}$$

- For two solutes A and B, B being the more strongly retained species, $\alpha$ is given by:

$$\alpha = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$$
Band Broadening and Column Efficiency

- Band broadening affects the efficiency of the chromatographic column.
- Why do bands become broader as they move down the column?

- Rate theory of Chromatography:
  - random-walk mechanism
    - Although the general direction of migration is towards the bottom of the column, random walk is superimposed on the general movement forward.
  - Random motion during migration explains the shape and the breath of chromatographic peaks –
    - Gaussian Distribution around mean retention time.

- Residence time in either phase is irregular-
  - a few particles travel faster because they are accidentally included in the mobile phase most of the time. Some particles lag behind because they are incorporated in the stationary phase for a time longer than the average.
- Width of band/zone is directly related to the residence time and inversely related to the velocity of the mobile phase flow.

Tailing and Fronting

- Tailing: occurs when the distribution constant varies with concentration.
- Fronting: occurs when the amount or sample introduced is too large.
Quantitative Description of Column Efficiency

- Column efficiency is expressed in terms of plate height (H) and plate count (the number of theoretical plates (N)).
- Efficiency increases as N becomes greater and H becomes smaller.
- N and H:
  - From Martin and Synge theory / Plate theory (1941)
  - Chromatographic column similar to distillation column made up of many discrete narrow layers / theoretical plates
  - Equilibrium of the solute between mobile and stationary phase within each theoretical plate
  - Movement: step-wise transfer of equilibrated mobile phase from one plate to the next
- N: few hundred to several hundred thousand
- H: ~ (tenth to 1/10000) mm

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

Definition of Plate Height

- Variance (of the band distribution) per unit length of column (linear distance in cm)
- Length of column that contains a fraction of the analyte that lies between L and L-\( \sigma \)

\[ H = \frac{\sigma^2}{L} \]
Experimental Evaluation of H and N

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

(1) \( \tau = \frac{\sigma}{L} + \frac{t_R}{4} = \frac{W}{4} \)

(2) \( \sigma = \frac{LW}{4t_R} \)

(3) \( H = \frac{\sigma^2}{L} = \frac{LW^2}{16t_R^2} \)

(4) \( N = 16 \left( \frac{t_R}{W} \right)^2 \)

Area of triangle \( \sim 96\% \) of total area

96\% of the area is comprised within \( (\pm 2\sigma) \), \( W = 4\tau \), substitute in (1)

Kinetic Variables Affecting Column Efficiency

**TABLE 26-2** Variables That Influence Column Efficiency

<table>
<thead>
<tr>
<th>Variable</th>
<th>Symbol</th>
<th>Usual Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear velocity of mobile phase</td>
<td>( u )</td>
<td>cm s(^{-1})</td>
</tr>
<tr>
<td>Diffusion coefficient in mobile phase*</td>
<td>( D_M )</td>
<td>cm(^2) s(^{-1})</td>
</tr>
<tr>
<td>Diffusion coefficient in stationary phase*</td>
<td>( D_S )</td>
<td>cm(^2) s(^{-1})</td>
</tr>
<tr>
<td>Retention factor (Equation 26-12)</td>
<td>( k )</td>
<td>unitless</td>
</tr>
<tr>
<td>Diameter of packing particles</td>
<td>( d_p )</td>
<td>cm</td>
</tr>
<tr>
<td>Thickness of liquid coating on stationary phase</td>
<td>( d_f )</td>
<td>cm</td>
</tr>
</tbody>
</table>
Kinetic Variables Affecting Column Efficiency

- Generally, efficiency studies are performed by determining $H$ as a function of mobile-phase velocity

Effect of Mobile Phase: van Deemter Plot
- Minimum $H$ for LC occurs at velocity too low for practical purposes

Theory of Band Broadening
- $A$: Eddy diffusion coefficient, describes multiple path effects
- $B$: Longitudinal diffusion coefficient
- $C_S$ and $C_M$: mass-transfer coefficients for the stationary and mobile phases

$$H = A + \frac{B}{u} + C_S u + C_M u$$

Approximation

![van Deemter plot](image)

![Theory of Band Broadening](image)
Theory of Band Broadening

• Multipath term A: Eddy Diffusion
  – the multitude of pathways available for a molecule
  – Different Lengths of pathways lead to different residence time in the column for same molecule
  – Not significant at low velocities where ordinary diffusion effectively averages effects of eddy diffusion
  – Stagnant pools of mobile phase add slow the exchange process

\[ H = A + \frac{B}{u} + C_A u + C_M u \]

Theory of Band Broadening

• Longitudinal diffusion term B/u
  – Molecules diffuse form region of high concentration to regions of low concentration
  – Rate proportional to concentration differences and to diffusion coefficient \( D_M \) of the species.
  – Migration from center to either side (opposed to the direction of flow)
  – Important in GC, less significant in LC
• The Stationary-Phase Mass-Transfer Term $C_s u$
  – For immobilized liquid stationary phase
  – The mass transfer coefficient is directly proportional to the square of the thickness of the film on the support particle ($d_f$) and inversely proportional to the diffusion coefficient $D_s$ of the solute in the film.
  – Reduces the average frequency at which the analyte reach the liquid-liquid interface where transfer to the mobile phase occur
  – With thick film, molecules must travel father to reach the surface and with smaller diffusion coefficients, they travel slower → slower rate of mass transfer and increase in plate height.

• Mobile-Phase Mass-Transfer Term $C_M u$.
  – $C_M$ is inversely proportional to the diffusion coefficient of the analyte in the mobile phase $D_M$.
  – for packed column is proportional to the square of the particle diameter of the packing material ($d_p$)
TABLE 26-3 Processes That Contribute to Band Broadening

<table>
<thead>
<tr>
<th>Process</th>
<th>Term in Equation 26-23</th>
<th>Relationship to Column* and Analyte Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple flow paths</td>
<td>$A$</td>
<td>$A = 2\lambda d_p$</td>
</tr>
<tr>
<td>Longitudinal diffusion</td>
<td>$B/u$</td>
<td>$B/u = \frac{2\gamma D_M}{u}$</td>
</tr>
<tr>
<td>Mass transfer to and from stationary phase</td>
<td>$C_S u$</td>
<td>$C_S u = \frac{f(k)d_t^2}{D_S} u$</td>
</tr>
<tr>
<td>Mass transfer in mobile phase</td>
<td>$C_M u$</td>
<td>$C_M u = \frac{f'(k)d_p^2}{D_M} u$</td>
</tr>
</tbody>
</table>

$\lambda$ and $\gamma$: constants depending on quality of packing
Optimization of Column Performance

- Reduce band broadening
- Alter relative migration rates of solutes
- Reduce separation time

- Zone broadening is increased by kinetic variables that increase plate height
- Migration rates are varied by changing variables that affect retention and selectivity factors

Resolution

- How far apart two bands are relative to their widths
- Quantitative measure of the ability of the column to separate two analytes

\[
R_S = \frac{\Delta Z}{W_A + W_B} = \frac{2\Delta Z}{W_A + W_B} = \frac{2(t_R^B - t_R^A)}{W_A + W_B}
\]

\[
R_S = \frac{\sqrt{N}}{4} \left( \frac{k}{1+k} \right)
\]

- \( k \): average of \(-k_A\) and \(-k_B\)
- \( \alpha \to 1 \)
Variables that Affect Column Performance

- Kinetic factors (1\textsuperscript{st} term)
  - Related to $N$
- Thermodynamic factors (2\textsuperscript{nd} and 3\textsuperscript{rd} terms)
  - 2\textsuperscript{nd} term: depends solely on properties of the solutes for a given mobile-phase and stationary-phase combination
  - Third term: depends on properties of both the solute and the column
- $\alpha$, $k$, $N$ or $H$

General Elution Problem

- Optimization ($k \sim 1$ to 5) for solutes with shorter retention times, generally leads to very long retention time for the other solutes and excessive broadening
- Solution: decrease $k$ during the separation
  - Gradient elution (as opposed to Isocratic elution)
- In GC: temperature gradient is applied
Application of LC for Bio-analysis