Ch. 25 High Performance Liquid Chromatography (HPLC)

- Most compounds are not sufficiently volatile.
- HPLC uses high pressure to force solvent through closed columns containing very fine particles that give high-resolution separations.

**High Performance Liquid Chromatography (HPLC)**
- Solvent pump
  - High pressure piston pump
- Injection valve
  - Has interchangeable sample loops
- Column
- Detectors
  - Spectroscopic
  - Refractive index
  - Evaporative light scattering
  - Electrochemical

**HPLC Column**
- Steel or plastic; 5-30 cm in length with an inner diameter of 1-5 mm.
- Guard column: containing the same stationary phase as the main column; to protect the main column.
- Increased temperature decreases retention time and improves resolution by hastening diffusion of solutes.

**Particle Sizes and Van Deemter Plot**

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

- H: plate height; \( u \): flow rate; \( A \): Multiple paths (Eddy diffusion); \( B/u \), Longitudinal diffusion (molecular diffusion); \( C \), Equilibration time (resistance to mass transfer).
- Increasing efficiency is equivalent to decreasing H.

**Table 25-1 Performance as a function of particle diameter**

<table>
<thead>
<tr>
<th>Particle size (( d_p )) (( \mu m ))</th>
<th>Retention time (( t )) (min)</th>
<th>Plate number (N)</th>
<th>Required pressure (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>30</td>
<td>25 000</td>
<td>19</td>
</tr>
<tr>
<td>3.0</td>
<td>18</td>
<td>42 000</td>
<td>87</td>
</tr>
<tr>
<td>1.5</td>
<td>9</td>
<td>83 000</td>
<td>700</td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>125 000</td>
<td>2 300</td>
</tr>
</tbody>
</table>

NOTE: Theoretical performance of 10-\( \mu m \) diameter x 30 cm long capillary for minimum plate height for solute with capacity factor \( k' \) = 2 and diffusion coefficient \( D \).
The Stationary Phase

- Highly pure, spherical, microporous particles of silica, Polystyrene (pH 8-12).
- Stationary phase is covalently attached to the polymer.
- Octadecyl (C\textsubscript{18}-ODS) stationary phase is the most common in HPLC. Bulky isobutyl groups are used to protect siloxane (Si-O) bonds from hydrolysis.

Adsorption Chromatography

- In adsorption chromatography, solvent molecules compete with solute molecules for binding sites on the stationary phase sites.
- Elution: Solute is eluted from the column when solvent displaces the solute.

Adsorption Chromatography

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Eluent strength (°)</th>
<th>Eluotropic series</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>0.22</td>
<td>284</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.20</td>
<td>245</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.30</td>
<td>233</td>
</tr>
<tr>
<td>Ethyl ether</td>
<td>0.40</td>
<td>217</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>0.46</td>
<td>210</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.51</td>
<td>210</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.55</td>
<td>210</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.60</td>
<td>223</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>0.70</td>
<td>235</td>
</tr>
</tbody>
</table>

*Eluent strength (°): a measure of the solvent adsorption energy.
*Eluotropic series: ranks solvents by their abilities to displace solutes from a given adsorbent.

Hydrophobic Interaction Chromatography

- Based on the interaction of a hydrophobic stationary phase with a hydrophobic region of a solute such as a protein.
- Eluent strength is increased by decreasing the salt concentration.

Hydrophilic Interaction Chromatography

- Most useful for small molecules that are too polar to be retained by reversed-phase columns.
- Stationary phases for hydrophilic interaction chromatography are strongly polar.
- The mobile phase contains polar organic solvent mixed with water. Eluent strength is increased by increasing the fraction of water in the mobile phase.

The Elute Process

- Normal-phase chromatography:
  - Adsorption chromatography on bare silica
  - Polar stationary phase (e.g. SiO) and a less polar solvent.
  - A more polar the solvent has a higher eluent strength (°).

- Reverse-phase chromatography:
  - Nonpolar stationary phase and more polar solvent.
  - A less polar solvent has a higher eluent strength.
  - More common, less sensitive to polar impurities (such as water) in the eluent.
Isocratic and Gradient Elution

Isocratic elution
- Is performed with a single solvent (or constant mixture)

Gradient elution
- Increasing amount of solvent B are added to solvent A to create a continuous gradient; B (organic) and A (aqueous).
- Increased eluent strength (more polar) is required to elute more strongly retained solutes.
- In a reverse-phase separation, less polar solvent to elute more strongly retained solutes; more polar solvent to increase resolution.

Gradient Elution

HPLC Solvents

• Very pure HPLC-grade
• To prevent degradation of columns with impurities and to minimize detector background signals from contaminants.
• Filter and degas or purging with gas (sparging).
• Organic solvents for normal-phase chromatography should be 50% saturated with water.
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- Detectors
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**High-Pressure Piston Pump**

- The quality of an HPLC pump is measured by how steady and reproducible a flow it can produce.
- A fluctuating flow rate can create detector noise that obscures weak signals.

**Injection Valve**

- Injection valve has interchangeable sample loops, each of which holds a fixed volume.
- Different sizes of loops can hold volumes that range from 2 - 1000 μL.
- Use syringe to load sample (blunt needle)
- Filter sample (0.5 μm)

**Ideal Detector**

- Adequate sensitivity
- Good stability and reproducibility
- A linear response to solutes: over several orders of magnitude
- Wide temperature range
- Short response time independent of flow rate
- High reliability and ease of use
- Similarity in response toward all solutes
- Nondestructive

**Commercial HPLC Detectors**

<table>
<thead>
<tr>
<th>Detector</th>
<th>Approximate limit of detection (ng)</th>
<th>Used with gradient?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet</td>
<td>0.1-1</td>
<td>Yes</td>
</tr>
<tr>
<td>Refractive index</td>
<td>100-1,000</td>
<td>No</td>
</tr>
<tr>
<td>Evaporative light-scattering</td>
<td>0.1-1</td>
<td>Yes</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>0.01-1</td>
<td>No</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>0.001-0.01</td>
<td>Yes</td>
</tr>
<tr>
<td>Nitrogen (N2 + NO + NO2)</td>
<td>0.3</td>
<td>Yes</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.5-1</td>
<td>No</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>0.1-1</td>
<td>Yes</td>
</tr>
<tr>
<td>Fourier transform infrared</td>
<td>1,000</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Table 25-3: Comparison of commercial HPLC detectors* [25 TH] [5.9L Edition]

**Photodiode Array UV-Visible Detector**

- A UV detector with a flow-through cell is the most common HPLC detector (many solutes absorb UV light).
- Usually a double-beam device: one beam passes through the sample cell and the other beam is a reference beam.
- Simple systems use the intense 254 nm emission of a Hg lamp. Deuterium, xenon, or tungsten lamps with interference filters are often used too.
UV-visible Absorption Cell

- The cell is a typical, Z-shape, flow-through cell
- Typical cell volumes are 1-10 L (to minimize extra column band broadening, the volume of such a cell should be kept as small as possible).

Refractive Index Detector

- Response nearly to all solutes
- Detection limit is low; sensitive to changes in pressure and temperature and pressure.
- Useless in gradient elution because it is impossible to match exactly the sample and the reference while the solvent composition is changing.

Criteria for Adequate Separation

- Capacity factor ($k'$): 0.5-20
  - Too small ‣ the first peak is distorted by the solvent front
  - Too great ‣ run takes too long
- Minimum resolution: 1.5
  > 2 is better in case of small changes of conditions
- Keep pressure in operating conditions (usually ~ 15 MPa or 150 bar or 2200 psi)
- Peaks should be symmetric

HPLC Analysis

- Determine the goal of the analysis
- Select a method of sample preparation
- Choose a detector
- Select solvent

Advantages: Complex mixtures; Robust for multiple samples; Can analyze polar compounds; High resolution and great reproducibility;
Disadvantages: Complexity; Low sensitivity for some compounds; Cost.