Applications of LC in Bio-Analysis

A. From Gravity-Flow LC to HPLC

- Early LC
  - Glass columns 1 to 5 cm diameter, and 50 to 500 cm long
  - Stationary Phase Particle size: 150 to 200 μm
  - Flow rates: tenths of ml per minute
  - Long separation times
- HPLC (High Performance Liquid Chromatography)
  - Particle size: 3 to 10 μm
  - Pressurized flow
Effects of Particle Size

- Mass transfer term in the van Deemter equation

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

**Analyte:** N, N'-dimethyl-p-aminobenzene

**Mobile phase (hexane, n-methyl chloride, isopropyl alcohol)**

**Column dimension:** 30 cm x 2.4 mm

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Extracolumn Band Broadening

- **Where**
  - Open tubes in
    - injection system,
    - detector region,
    - piping connecting various components

- **Why?**
  - Differences in flow rates between layers of liquid adjacent to the wall and the center of the tube

- **How much**

\[ H_{ex} = \frac{\pi D_M^2 u}{24 D_M} \]
Application of LC for Bioanalysis

- Proteins in Complex Matrices
  - Separation (from other cellular components)
  - Isolation (from other proteins)
  - Purification
- Chromatographic Techniques
  - Reversed Phase Chromatography
    - hydrophobicity
  - Ion Exchange Chromatography
    - charge
  - Size Exclusion Chromatography:
    - size and form
  - Affinity Chromatography
    - Specific interactions

Block Diagram of a typical HPLC
B. Reversed Phase Liquid Chromatography

B.1 Stationary Phase
- Porous silica: uniform and mechanically sturdy
- Average size (1.5 to 10 µm)
  - Most common size 3 and 5 µm
- Surface of Silica is hydrolyzed with HCl to silanol groups
  - Surface contain approximately 8 µmol OH/m²
  - Surface area 100 to 400 m²/g
- Silanol groups are reacted with organochlorosilanes to form siloxanes
  - Due to steric hindrance coverage is only 4 mol/m²
  - Siloxanes surfaces must be capped
  - Reaction with chlorotrimethylsilane
- R chain length: from 2 to 18
- Most popular stationary phases: n-octadecyl ODS (C18) and octyl silane (C8)
- Pore size ~ 10 nm

B-2 Mobile Phase
- Polar
  - Aqueous buffer + acetonitrile (CH₃CN) or methanol (CH₃OH)
- Aqueous buffer (pH 2-8) (~20 mM)
  - ammonium, phosphate or carbonate based.
- Ion pairing reagents used to increase the hydrophobicity of charged analytes
  - Anionic ion pairing reagent: trifluoroacetic acid (TFA)
  - Cationic ion pairing reagent: tetraalkyl ammonium salts
- Pumping Pressure: 300-400 atm
B-3 Detection

- UV
  - 254 nm for peptides
  - 280 nm for proteins
- Diode Array Detector (DAD)
- Fluorescence
- ESI-MS
- IR
- Refractive Index

- Ideal Detector
  - Adequate sensitivity
  - Good reproducibility and stability
  - Linear response to solutes
  - Short response time independent of flow rates
  - High reliability and ease of use
  - Similar response towards all solutes or highly predictable and selective response towards one or more classes of substances
  - Nondestructive

C. Ion Exchange Chromatography

- Separation according to overall charge
- Competitive interaction between salt ions and charged sample molecule for the charged functional group on the stationary phase
- Elution: increase concentration of salt of the mobile phase or change pH
### C-1 Stationary phase

- Agarose or cellulose beads with covalently attached groups
- Anion exchangers: positively charged groups
  - Diethyl aminoethyl \([-(CH_2)_2-^+NH(CH_2CH_3)_2]\) (DEAE) (pH = 4-8)
- Cation exchangers: negatively charged groups
  - Carboy methyl (CM) (\(-CH_2-COO^-\))(pH = 4-8)

### C-2 Elution

- pH of mobile phase determines the charge on the protein
- Buffer concentration kept low (10 to 20 mM) to minimize competition with buffer ions
- Buffer commonly used: phosphate and acetate salts
- Desorption of protein: increase ionic strength or change pH of mobile phase
- Use of salt gradient is common
- pH gradient also used
D. Affinity Chromatography

D-1 Stationary Phase

• Highly specific and selective

• Molecular recognition groups (affinity ligands) covalently attached to agarose or cellulose beads
• Affinity ligand binds selectively and reversibly the analyte
• Mechanism of retention: interaction with highly specialized molecular recognition systems which are attached to the stationary phase
• Type of molecular recognition systems:
  – Antigen
  – Enzymes-coenzyme
  – Receptor protein-Hormone
  – Single strand of oligonucleotide

D-2 Mobile Phase

• Two distinct roles
  – Support the strong binding of the analyte molecule to the ligand
  – Weaken and eliminate the analyte-ligand interaction
D-3 Sample Elution

- Sample introduction
- Adsorption
- Washing
  - Removes components of the sample that are non-specifically bound
- Desorption
  - pH change
  - Denaturing agents such as urea
  - Organic solvent
- Specific desorption
  - Add a species that binds the analyte more strongly than the ligand on the stationary phase

D-4 Examples of Affinity Ligands

- Group-specific ligands
  - Bind similar analyte
- Monospecific ligand
  - Show affinity for only one analyte

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<th>Binding Partners for Affinity Chromatography</th>
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<td>Hormone</td>
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</table>
E. Size Exclusion Chromatography

- Gel permeation (GPC): non-aqueous solution: for the separation of polymers
- Gel filtration: aqueous solution
- Separation according to: size and shape
- Stationary phase: porous gel or agarose beads
  - Diameter: 10-40 µm
- Mobile phase
  - acts just as a solvent
  - Aqueous buffer of ionic strength of 50 to 100 mM

E-1 Elution Profile

- Total Exclusion
  - Large molecules of diameter larger than the pores sizes are unretained (excluded) and elute with the solvent
- Total Permeation
  - Small molecules of size much smaller than the pores are retained longest on the column, and elute together (total permeation)
- Selective Permeation
  - Range of separation: 2kDa and 200 kDa depending on pore size and size distribution. molecules are separated according to their sizes
Analytes elute between \( V_0 \) and \( V_0+V_i \)

\[
V_R = t_R \times F
\]

\( V_r \): retention – volume

\( F \): volumetric – flow

\[
V_r = V_0 + V_i + V_0
\]

\( V_r \): total – volume – of – the – column

\( V_r' \): gel – particles – volume

\( V_s' \): intrinsic – volume – of – solvent – inside – the – pores

\( V_0' \): volume – of – free – solvent – outside – pores

\[
V_R = V_0 + KV_i
\]

\[
K = (V_R - V_0)/V_i
\]

What is the value of \( K \) (distribution constant) for the molecules excluded?
What is the value of \( K \) small molecules with maximum permeation?

E-2 Applications

- Separation of proteins from low molecular weight compounds
- Separation of biomolecules according to size
- Can be used to determine molecular weights (calibration necessary)
- Can be used for quantitative determinations
- Higher capacity than gel electrophoresis
Recent trends

- Shorter and smaller columns
- Shorter time of analysis
- Small sample
- Decreased solvent consumption

HisTrap™ HP Columns

- Optimized for high-performance purification of histidine-tagged proteins.
- Ni Sepharose High Performance prepacked in convenient HiTrap 1-ml and 5-ml columns.
- Negligible Ni2+ leakage.
- High binding capacity, at least 40 mg/ml medium.
- Compatible with different additives, such as - Reducing agents (e.g. DTT, TCEP, 2-MEA). - Denaturing agents (urea, Gua-HCl). - Detergents (e.g. Tween, Triton).
- Simple operation with a syringe, pump, or chromatographic system such as ÄKTAdesign or FPLC System.
GE Healthcare Science

- For purification of histidine-tagged* proteins using different kinds of metal ions. For optimizing purification of (histidine)6-tagged protein purification when Ni²⁺ is not the best choice of metal ion. Iminodiacetic acid coupled to Sepharose High Performance. Prepacked with Chelating Sepharose High Performance coupled to iminodiacetic acid for purifying proteins with exposed histidine groups via metal ion complex formation.

TECHNICAL SPECIFICATIONS
- Chelating group: Iminodiacetic acid
- Chelating group coupling linkage: Ether
- Matrix: Highly cross-linked agarose, 6%
- Average particle size: 34 µm
- Metal ion capacity: » 23 µmol Cu²⁺/ml medium
- Binding capacity: » 12 mg (histidine)6-tagged protein
- (Mr 27 600)/ml medium
- Spacer arm: 7-carbon
- pH stability: 3-13 (long term), 2-14 (short term)