Chromatographic Methods: Basics, Advanced HPLC Methods
Chromatography: Basics

**Chromatography** a physical method for the separation of mixture based on the concept of partition coefficient.

Chromatography involves two phases:
- **Mobile phase**: a liquid/ gas which carries the mixture to be separated.
- **Stationary phase**: through which the mixture is carried by mobile Phase, it can be solid/ liquid.

Separations are carried out based on differences in physical and Chemical properties of constituents of a mixture such as size, shape, mass, charge, boiling point, polarity or chemical affinity.
**Chromatogram:** The visual output of the chromatograph

**Retention time:** The characteristic time a particular analyte takes to pass through the system i.e. from column inlet to the peak maxima

**Peak height:** The distance between the peak maximum and the base line

**Peak width:** The distance between each side of a peak measured at certain height of the peak
Chromatography: principle/Theory

the solutes will elute in order of their increasing **distribution coefficients** with respect to the stationary phase

Plate theory: column is considered to be divided into a number of plates

\[ N = 5.55^* \frac{t_R^2}{w^{1/2}} \]

equilibrium must exist in each plate

\[ X_s = KX_m \]

\((X_m)\); concentration of solute in the mobile phase

\((X_s)\); concentration of solute in the stationary phase

\((K)\); distribution coefficient of the solute between the two phases with reference to the stationary phase

\[ K = \frac{X_s}{X_m} \]
Chromatography: principle/Theory

The change of mass of solute (\(d_m\)) in plate (p) will be

\[
d_m = (X_{m(p-1)} - X_{m(p)})dV
\]

At equilibrium

\[
d_m = v_s dX_{s(p)} + v_m dX_{m(p)}
\]
Chromatography: principle/Theory

\[ X_{m(n)} = X_0 \cdot e^{-\nu} \frac{\nu^n}{n!} \]

Basic elution curve equation it shows that if \((n= \text{no. of theoretical plates})\) is large, the function tends to the Gaussian function.

\[ V_r = V_m + K V_S \]

The \textit{retention volume} depends solely on the distribution coefficient and the volumes of the two phases that are present in the column.

\[ K_A \neq K_B \quad \text{or} \quad V_{S(A)} \neq V_{S(B)} \]

The separation of two solutes depends exclusively on the magnitude of their distribution coefficients \((K_A)\) and \((K_B)\) and the amount of stationary phase available to them, \((V_{(A)})\) and \((V_{(B)})\).
Chromatography
Elution mode

**Isocratic elution:**
The composition of the mobile phase kept constant throughout elution

**Gradient elution:**
The composition of the mobile phase varied during elution
Chromatography
Elution mode

PAH analysis through HPLC

Gradient elution

Isocratic elution

ACN/H₂O 70/30

ACN/H₂O
0-5 50/50
5-20 100/0
20-30 100/0
Chromatography: Types

Based on shape of chromatography (stationary phase)

**Paper chromatography:**
- A paper serves as stationary phase
- Separating and identifying mixtures by colour

**Thin layer chromatography:**
- Thin layer of silica gel, alumina or cellulose adsorbed on an inert substrate

**Column chromatography:**
- The stationary phase is packed in a column
Chromatography: Types
Based on physical state of mobile phase

Gas chromatography:
-Mobile phase is gas like He

Applications: Analytical chemistry, petrochemical, environmental monitoring

Not good for bimolecules e.g. protein due to high heat

Liquid chromatography: Mobile phase is liquid

e.g. high performance liquid chromatography
Chromatography: Types
High performance liquid chromatography

Optimized for rapid high resolution separations
- Very high efficiency HPLC columns with inert packing materials
- Fine particle packing (5µm) providing larger surface for interaction
- HPLC high pressure pumps with very constant flow (6000-10000 psi)
- Unique high accuracy, low dispersion, HPLC sample valves (sub µl - few µl)
- Extremely precise gradient mixers (optional).
- High sensitivity low dispersion HPLC detectors

Applications
quality control, process control, forensic analysis, environmental monitoring and clinical testing
Chromatography: Types

High performance liquid chromatography
# Chromatography: Types

High performance liquid chromatography

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal phase (NP-HPLC)</td>
<td>Polar stationary phase e.g. silica</td>
</tr>
<tr>
<td></td>
<td>Non polar mobile phase e.g. Toluene</td>
</tr>
<tr>
<td></td>
<td>Polar interaction</td>
</tr>
<tr>
<td></td>
<td>Non polar → Polar</td>
</tr>
<tr>
<td>Reverse phase (RP-HPLC)</td>
<td>Non polar stationary phase e.g. C18</td>
</tr>
<tr>
<td></td>
<td>Polar mobile phase such as water</td>
</tr>
<tr>
<td></td>
<td>Hydrophobic interaction</td>
</tr>
<tr>
<td></td>
<td>Polar → Non polar</td>
</tr>
</tbody>
</table>
Chromatography: Types

Hydrophilic interaction chromatography (HILIC-HPLC)

- A kind of partition chromatography
- Solute equilibrate between a liquid stationary phase and eluent
- Separation based on polar differences
- Can separate acid, base, and neutral molecule in single chromatogram
- Good for separation of very polar compounds such as amino acids, glycopeptides, oligonucleotides, and highly polar natural products
Chromatography: Types
Ion exchange chromatography

Principle: highly charged proteins bind stronger to the column material, so that they elute at higher salt concentrations in the buffer than less charged proteins.

From: www.ucl.ac.uk/~ucbcdb/enzpur/ionX.htm

Foto of phycobiliprotein purification in the lab of H. Küpper on a MonoQ anion exchange column.
Ion pair chromatography

- Ion Pair Chromatography is a method for improving the separation of charged analytes.
- The ion pair reagents comprise of an alkyl chain with an ionizable terminus.

Advantages over ion exchange:

- Simple preparation of buffers
- Wide choice of carbon chain lengths for improved retention and separation
- Significantly reduced separation time
- Simultaneous separation of both ionized and nonionized solutes
- Highly reproducible results
- Improved peak shape

Quaternary Amine (Q-Series) Ion Pair Reagent

![Quaternary Amine (Q-Series) Ion Pair Reagent](image)
Chromatography: Types

Size exclusion chromatography

Principle: Small proteins can enter more of the pores in the column material than large proteins, so that small proteins migrate **slower**
Chromatography: Types

Displacement chromatography

The higher-affinity solutes are preferentially retained near the head of the column, with the lower-affinity solutes moving farther downstream each making pure zones.

A displacer having higher affinity than Sample components will push other Components downstream making a displacement train.

Advantage:
Comparatively more material can be separated High-retention conditions can be achieved without gradient operation.

Disadvantage:
Overlap of zone may occur Difficulty in interpretation on chromatogram Column regeneration.
Chromatography: Types

Affinity chromatography

Based on a highly specific interaction between analyte and stationary phase

![Diagram of affinity chromatography process]

Key:
- Protein of interest
- Ligand
- Ligand attached to polymer bead

Protein mixture is added to column containing a polymer-bound ligand specific for protein of interest.

Solution of ligand

Unwanted proteins are washed through column.

dolly.biochem.arizona.edu/.../methods.html

Foto of TcHMA4 purification in the lab of H. Küpper on an IMAC column
Chromatography: Types

Special techniques

**Fast protein liquid chromatography**
- Also called as fast performance liquid chromatography
- Often used for protein purification
- Operates at low pressure typically less than 5 bar
- Flow rate is relatively high, typically 1-5 ml/min.
- Relatively low cost
- Pressure is not a limitation

**Supercritical fluid chromatography (SFC)**
- Mobile phase is carbon dioxide
- To separate thermally labile molecules
- Separation of chiral compounds

**Chiral column chromatography:**
- Chiral stationary phase
- For separation of enantiomers
Chromatography: Types
Special techniques

Two-dimensional chromatography:
- Two columns with different physicochemical properties are used
- Increased peak capacity
Hyphenated Techniques combine chromatographic and spectral methods to exploit the advantages of both.

Chromatography - Produces pure or nearly pure fractions of chemical components in a mixture.

Spectroscopy – Produces selective information for identification using standards or library spectra.
Hyphenated techniques

LC-Absorbance data

James K. Hardy and The University of Akron http://ull.chemistry.uakron.edu/chemsep/hyphen/
Hyphenated techniques

Liquid chromatography- mass spectrometry (LC-MS)
Hyphenated techniques

HPLC-ICP-MS
Hyphenated techniques

HPLC-ICP-MS

Speciation of As in Plant extract through RP-HPLC (C18) coupled to ICP-MS
Hyphenated techniques

HPLC-ICP-MS-ESI-MS
Hyphenated techniques

HPLC-ESI-MS-ICP-MS
Hyphenated techniques
HPLC-ESI-MS-ICP-MS
Hyphenated techniques

HPLC-ESI-MS-MS

Theoretical peptide fragmentation pattern
The slides can be downloaded from the workgroup homepage

www.uni-konstanz.de → Department of Biology → Workgroups → Küpper lab,

or directly

http://www.uni-konstanz.de/FuF/Bio/kuepper/Homepage/AG_Kuepper_Homepage.html