Basic principles of chromatographic separation techniques

Alessandro Barge
Dipartimento di Scienza e Tecnologia del Farmaco,
Università degli Studi di Torino

What is chromatography?

Chromatography is a process which separates chemical species from one another.

The fundamental driving force of chromatography is the chemical equilibrium that results when a species distributes between two phases.
Chromatography is similar to liquid-liquid extraction.

In liquid-liquid extraction, solutes distribute themselves between two immiscible liquid phases until an equilibrium is established. Solutes will also distribute themselves between a liquid and a solid phase. Similarly, vapors establish equilibrium between gas and solid or between gas and liquid phases.

For any particular phase system, the equilibrium concentrations depend primarily on the chemical composition of the solute.

In the chromatographic process species distribute between two immiscible phases.

The rate of migration of each species is determined by its distribution coefficient, $K_d$.

Species which are distributed mainly into the **flowing phase** move **rapidly**.

Species which are distributed mainly in the **stationary phase** move **slowly**.
TLC – thin layer chromatography

• The stationary phase is supported on glass, plastic or aluminium layer
• The flowing phase climbs the layer by capillarity
• We need to use chemicals to reveal solute spot

LOW RESOLUTION TECHNIQUE

Using the same chromatographic condition

\[ R_f = \frac{B}{A} \]

\( R_f \) is a characteristic of the solute
TLC

- Analytical
  - Follow organic reaction
  - Follow Column Chromatography separation
  - First product analysis

- Preparative
  - Isolate small amount of product.
  - Allow to purify solutes which are also very small \( R_f \)

All TLC techniques need chemical detection

---

**Column Chromatography**

- Flowing phase
  - \( R_f \) is replaced by Retention Volume:
  - \( V_f \) is the flowing phase volume required to elute the solute

- Stationary phase
Column Chromatography

Advantages:

Better resolution than TLC
Higher loading than TLC
Detection can be done by TLC on a small amount of eluate

Good preparative chromatography technique

On the basis of nature of the stationary phase we have:

- Normal phase chromatography
- Reverse phase chromatography
- Ion exchange chromatography
- Size exclusion chromatography
Normal phase Column Chromatography

Common solvents
- Hexane/Et₂O
- Hexane/AcOEt
- DCM/MeOH
...

DCM/MeOH/Water
DCM/MeOH/NH₃
DCM/MeOH/Water/NH₃
ACN/Water
...

High performance refers to high speed, high resolution separations. High performance is achieved by using very small diameter (< 20 μ) column packings.

The use of small diameter packings reduces band broadening and gives narrower peaks.

When small particle packings are used, high pressures are required to push the mobile phase (eluent) through the column. HPLC is sometimes called high pressure liquid chromatography.
Flash Chromatography


Rapid Chromatographic Technique for Preparative Separations with Moderate Resolution

W. Clark Still, Michael Kahn, and Abhiraj Mitra

Experimental Section

Chromatography columns and the flow controller valves were assembled as described in the text. The silica gel used was 40-60 um (Davison, 230-400 mesh) silica gel 60 (6). Merck No. 5719799 silicas were distilled prior to use. Thin layer chromatograms (TLC) were run on glass supported silica gel 60 (0.15 mm thick, F. & K. Merck, No. 7660). Flash Chromatography. General Procedure. Fixed a low viscous, easily soluble system (e.g., ethyl acetate/35-40°C petroleum ether)

Automated Flash Chromatography
HPLC instrument

- Two pumps for gradient elution
- Injection port
- Column
- Ultraviolet detector
- Computer for control and display

- 1960: 40µ non-porous, 100-500 psi, 1000 plates/m, columns: 1m
- 1970: 10µ irregular, micro-porous, 1000-2500 psi, 25,000 plates/m, 3.9 x 300mm
- 1980: today, 3.5 - 5µ spherical – micro-porous, 1500-4000 psi, 50,000 - 80,000 plates/m, 3.9 x 300mm
HPLC pump systems

- Isocratic
  - Fixed composition of the flowing phase

- Gradient (high or low pressure gradient)
  - Flowing phase composition changes during the separation
  - Better separation in less time and less solvent
Retention time and capacity factor in HPLC

The retention time $t_r$ is the time required to elute a peak

$t_0$ is the time required to elute an unretained species ($K_d=0$)

$K_d$ is the distribution coefficient $K_d = \frac{[\text{solute}]_{\text{mobile}}}{[\text{solute}]_{\text{stationary}}}$

Retention is often expressed in terms of capacity factor $k'$
Retention time and retention volume in HPLC

Retention time is dependant on eluent flow rate.

Retention volume \( (V_r) \) is the volume of eluent passed through the column at the retention time.

The retention volume of a unretained component is equal to \( V_m \), the volume of mobile phase in the column.

\[
V_r = t_r F \quad \text{and} \quad V_m = t_m F
\]

\[
V_r = V_m + K_d V_s
\]

\( F \) is flow rate
\( V_s \) is the stationary phase volume

Column efficiency

Column efficiency refers to peak width. An efficient column gives narrow peaks making it easier to separate sample components.

Efficiency is a function of:
- column length
- Particle size
- Flow rate

Changing these parameters affects the pressure drop across the column. Column length and flow rate also affect the retention time.
Column efficiency

Efficiency is measured in terms of the number of theoretical plates $N$

$$N = 16 \left( \frac{t_r}{W} \right)^2$$

The height equivalent to a theoretical plate (HETP) is given by

$$HETP = \frac{L}{N}$$

$L$ = column length

Van Deemter equation
Selectivity

The selectivity parameter $\alpha$ is a measure of peak spacing.

Selectivity is measured from retention:

$$\alpha = \frac{t_{r2} - t_0}{t_{r1} - t_0} = \frac{k'_2}{k'_1}$$

Resolution

The objective of chromatography is the separation of component mixtures.

Resolution is the term used to quantitatively describe how well the objective was met.

$$R_s = \frac{t_{r2} - t_{r1}}{\frac{1}{2}(w_1 + w_2)}$$
Resolution

The objective of chromatography is the separation of component mixtures.

Resolution is the term used to quantitatively describe how well the objective was met.

\[
R_s = \frac{(1/4) (\alpha-1) \sqrt{N} \left( \frac{k^*}{1 + k^*} \right)}{
\text{Selectivity Factor} \quad \text{Efficiency Factor} \quad \text{Retention Factor}}
\]
Short time…. High resolution!!!

- Thiourea - 0.430
- toluene - 1.034
- propylbenzene - 1.742
- butylbenzene - 2.413
- hexylbenzene - 5.058

No. of components: 5
Complete Separation: 6.00 min

- Thiourea - 0.046
- toluene - 0.088
- propylbenzene - 0.137
- butylbenzene - 0.182
- hexylbenzene - 0.360

UPLC™
No. Of components: 5
Complete Separation: 0.60 min
Separation purposes

- Qualitative analysis
- Quantitative analysis

Qualitative analysis: Based on retention time
- Relevant parameters: k’

Quantitative analysis: Based on evaluation of peak area
- Relevant parameters: k’, α, R_s
Preparative separation

Optimization of column loading

Optimization of solvent volume and experimental time

Optimization of $k'$, $\alpha$, and $R_s$ for the peak of interest

$k'$ should have the lowest value compatible with separation

$\alpha$ and $R_s$ should have the highest values compatible with separation

Normal phase chromatography

Normal phase refers to the use of polar column packings and low polarity eluents

Separation of low to moderate polarity compounds

Samples which have little solubility in aqueous eluents are candidates for normal phase chromatography. Normal phase is often successful at separating geometric and positional isomers.

Normal phase packings

Bare adsorbent: Silica gel and alumina

Bonded phases: silica or polymer supports onto which polar functional groups such as -NH$_2$ or -CN have been chemically bound.

Eluents:

Weak solvent: Hexane

Strong solvents: Methyl-tert-butyl ether, Methylene chloride, Acetonitrile, Methanol
Column and solvent selection

**Reversed phase chromatography**

Over 75% of all HPLC separations are carried out on reversed phase columns.

The reversed phase is a good choice for mixtures with different numbers, types or locations of alkyl functional groups. It is also suitable for samples with different types of polar functional groups.

### Reversed phase packings

- Silica bonded C18 groups, C8 groups, Phenyl groups
- Bare polystirenic resins

### Eluents:

- Weak solvent: Water
- Strong solvents: Methanol, Acetonitrile, isopropanol, THF

### Solvent additives

<table>
<thead>
<tr>
<th>Weak</th>
<th>Strong</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>CH$_3$CN</td>
</tr>
<tr>
<td>Water/TFA 0.1%</td>
<td>CH$_3$CN/TFA 0.1%</td>
</tr>
<tr>
<td>Water/C$_3$F$_7$COOH</td>
<td>CH$_3$CN/C$_3$F$_7$COOH</td>
</tr>
<tr>
<td>AcONH$_4$ 7 mM, pH=7</td>
<td>CH$_3$CN</td>
</tr>
<tr>
<td>AcONH$_4$ 4 mM, pH=4</td>
<td>CH$_3$CN</td>
</tr>
<tr>
<td>CF$_3$COCOONH$_4$ 4mM pH=4</td>
<td>CH$_3$CN</td>
</tr>
<tr>
<td>NH$_3$ 7 mM</td>
<td>CH$_3$CN</td>
</tr>
</tbody>
</table>

Not surprisingly, the reversed phase is the chromatographer's first choice when sample structure is unknown.
Column and solvent selection

**Ion Pair chromatography**

Ion pair chromatography is a technique for the separation of ionizable organic compounds on reversed phase columns.

It is generally preferred over ion exchange because it offers higher efficiency and greater control over selectivity.

Ion pair chromatography differs from reversed phase in that the eluent contains a hydrophobic counter ion called an *ion pairing agent*.

It is widely believed that ion pairing agents adsorb onto the stationary phase to form the equivalent of an ion exchange stationary phase.

---

**Phase packings**

- Silica bonded: C18 groups, C8 groups,
- Eluents:
  - Weak solvent: Water buffer with ion pairing agents
  - Strong solvents: Methanol, Acetonitrile

---

**Column and solvent selection**

**Ion Pair chromatography**

**Ion pairing agents**

Buffers are used to keep the sample compound ionized. Buffer concentrations of 0.02 - 0.20 M are typically used.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>1.1 – 3.1</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.2 – 8.2</td>
</tr>
<tr>
<td>Acetate</td>
<td>3.8 – 5.8</td>
</tr>
<tr>
<td>Borate</td>
<td>8.2 – 10.2</td>
</tr>
</tbody>
</table>

---

**Phases packings**

- Silica bonded: C18 groups, C8 groups,
- Eluents:
  - Weak solvent: Water buffer with ion pairing agents
  - Strong solvents: Methanol, Acetonitrile
### Column and solvent selection

#### Ion Pair chromatography

<table>
<thead>
<tr>
<th>Ion pairing agents</th>
<th>Phase packings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion pairing agents are added at concentration of 0.005 to 0.5 M.</td>
<td>Silica bonded C18 groups, C8 groups,</td>
</tr>
<tr>
<td><strong>For an anionic sample:</strong></td>
<td>Eluents:</td>
</tr>
<tr>
<td>Tetrabutylammonium hydrogen sulfate</td>
<td>Weak solvent: Water buffer with ion pairing agents</td>
</tr>
<tr>
<td>Tetrabutylammonium phosphate</td>
<td>Strong solvents: Methanol Acetonitrile</td>
</tr>
<tr>
<td>Cetyltrimethylammounium bromide</td>
<td></td>
</tr>
<tr>
<td>Triocetyl amine</td>
<td></td>
</tr>
<tr>
<td><strong>For a cationic sample:</strong></td>
<td></td>
</tr>
<tr>
<td>Sodium octylsulfonate</td>
<td></td>
</tr>
<tr>
<td>Sodium dodecylsulfate</td>
<td></td>
</tr>
</tbody>
</table>

#### Ion exchange chromatography

<table>
<thead>
<tr>
<th>Ion exchange phase packings</th>
<th>Eluents:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer bound: Ammonium salt</td>
<td>Weak solvent: Water buffer low concentration</td>
</tr>
<tr>
<td>Sulfonate salt</td>
<td>Strong solvent: Water buffer high concentration acid or basic solution</td>
</tr>
</tbody>
</table>

The unique selectivity of ion exchange chromatography is most useful for the separation of *inorganic ions*. It is also useful for the separation of *proteins, peptides and amino acids*.

The retention in ion exchange is controlled by the *pH* and *ionic strength* of the eluent rather than its organic solvent content.

High capacity ion exchange packings are especially useful for preparative separations.
Column and solvent selection

Size exclusion chromatography

Size exclusion chromatography (SEC) is used for samples which contain high molecular weight compounds and for samples whose components are significantly different in molecular size. SEC is sometimes used for purified metal complexes from salts.

Size exclusion phase packings
Cross-linked sugar (sepharose, dextrane, …)

Eluents:
Water or buffer.
In preparative conditions water or volatile buffer are preferred.

Sample preparation

The sample must first be dissolved or diluted in a suitable solvent.

Concentrations up to about 5 mg/ml are typical for analytical separations.

Preparative separations use higher concentrations.

Sample solvents which produce a detector response should be avoided because they introduce large peaks which may interfere with the analysis.

Sample solvents which are strong eluents should be avoided because they can cause band broadening or band splitting.

In general, the eluent strength of the sample solvent should be no greater than that of the eluent. Thus the best sample solvent is often the eluent itself.

Sample solution must be filtered on a 0.45µm filter.
Sample preparation

Peak Distortion due to Solvent Choice

HPLC Analysis: Effect of Sample Solvent

Sample in MeOH

Sample in HPLC Mobile Phase (9.1% TFA, 4% ACN and 5% MeOH in Water)

Something more about solvents

Equal Strength Eluents

MeOH ACN THF

Ternary Gradient
Init: 5% ACN, 11% MeOH / Water
Final: 25% ACN, 25% MeOH / Water

Binary Gradient
Init: 14% MeOH / Water
Final: 58% MeOH / Water

Binary Gradient
Init: 10% ACN / Water
Final: 40% ACN / Water
Applying the same gradient with both organic solvents, sample elutes later with methanol than with acetonitrile. Changing the organic solvent may improve peak shape due to the additional interaction with the sample.

Buffered mobile phases enhance retention and mass loading. There is a high risk of breakthrough and retention loss leading to recovery problems when buffers are left out!
UV Detector set-up

Diode array:
- multiple wavelength selection
- 3D chromatogram can be acquired

Single wavelength UV detector:
- generally set on 190 – 220 nm
- if the compound of interest shows an absorption peak in a well defined spectrum region the detector can be set on this wavelength

LC-MS
API Ionisation Techniques

- ESI and APcI differ in...
  - How ions are generated
    - ESI - solution phase ionization
    - APcI - gas phase ionization
  - Analyte compatibility
    - ESI - polar compounds and large biomolecules
    - APcI - less polar, smaller compounds (relative to those ionized by ESI) that have some volatility
  - Flow rate compatibility
    - ESI - 0.001 to 1 mL/min
    - APcI - 0.2 to 2 mL/min
<table>
<thead>
<tr>
<th>Technique</th>
<th>Flow Rate (ml/min)</th>
<th>MW Range</th>
<th>Species Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESI</td>
<td>0.001 – 0.3</td>
<td>&lt;200,000 Da</td>
<td>(M+H)^+ (M-H)^- (M+nH)^n+</td>
</tr>
<tr>
<td>APcI</td>
<td>0.2 – 2.0</td>
<td>&lt;1000 Da</td>
<td>(M+H)^+ (M-H)^-</td>
</tr>
</tbody>
</table>

Electrospray Ionisation Theory

As droplets evaporate, the electric field increases and ions move towards the surface.
Electrospray Ionisation

- Probe held at ~3 kV
- Desolvation of spray using N₂ gas
- Cone ~ 10-100 V
- Ion evaporation
Recognising Multiply Charged Ions

- Mass spectrometers operate on the basis of mass-to-charge ratio (m/z). Mass assignments are normally made assuming a single charge per ion (i.e. m/z = m)

- Single charge Mass = (M+H)
- Double charge Mass = \(\frac{1}{2} (M+2H)\)
- n charge Mass = \(\frac{1}{n} (M+nH)\)

- Isotopes of doubly charged ions are separated by 0.5 Da
Atmospheric Pressure Chemical Ionisation (APcI)

- Low molecular weight (<1000 Da)

- Singly charged species

- In-source fragmentation can occur, even at low cone voltages - caused by increased temperature

- Mobile phase can be non-polar (normal-phase chromatography)
APcI Theory

- The probe is heated to aid desolvation and a gaseous vapour forms.
- Mobile phase vapour enters the source and solvent ions react with ions formed by the corona discharge pin to produce reactive reagent ions.
- Analyte molecules react with these reagent ions and usually gain or lose a hydrogen (protonation or deprotonation) for positive or negative ion mode.
- The ions then pass into the Z-Spray source and are analysed as in ESI mode.

Positive or Negative?

<table>
<thead>
<tr>
<th>Basic Compounds (-NH₂)</th>
<th>Acidic Compounds (-CO₂H, -OH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M+H)⁺</td>
<td>(M-H)⁻</td>
</tr>
</tbody>
</table>

Linuron  +ve ion  Ibuprofen  -ve ion
pH Considerations

**Positive ion mode** - Analysis of basic compounds
Lower pH with an acid
  e.g. Formic or acetic acid

**Negative ion mode** - Analysis of acidic compounds
Raise pH with a base
  e.g. Ammonium hydroxide/Ammonia soln.

Commonly Used Solvents and Additives

**Solvents**
- Water
- Acetonitrile
- Methanol
- Isopropanol

**Additives**
- Acetic acid
- Formic acid
- Ammonium hydroxide
- Ammonium acetate*  

* Salt concentrations should be kept to 10 mM or less.
Solvents and Additives to be Used with Discretion

- TFA - Used with Proteins and Peptides
  - Will suppress (to some extent) positive ion electrospray at levels > 0.1%.
  - Will greatly suppress negative ion electrospray.
- TEA
  - Readily ionized to give an intense (M + H)^+ ion at m/z 102.
  - Will suppress positive ion electrospray of less basic compounds. May enhance negative ion electrospray of less basic compounds.
- THF
  - 100% THF is highly flammable.
  - Should not be used with APCI if air is being used as the nebuliser gas.

Unsuitable Solvents and Buffers

- Non-volatile salts (phosphate, borate, citrate, etc.)
- Surface active agents/detergents (suppress ionisation)
- Inorganic acids (sulphuric acid, phosphoric acid etc.)