Fundamentals and Applications of Chromatography

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What exactly is chromatography?

"technique for separating the components of a mixture on the basis of the relative amounts of each solute distributed between a moving fluid stream and a contiguous stationary phase "

—Encyclopedia Britannica

- All separations involve the movement of a compound between two different phases

  - distillation
    - liquid ↔ gas
  - recrystallization
    - solution ↔ solid
  - sublimation
    - solid ↔ gas

- The flowing of one phase relative to the other is the defining feature of chromatographic separations
Overview

Fundamentals and Theory of Chromatography

- Parameters affecting separation quality
  - The Resolution equation
  - The van Deemter equation

Three Common Types of Chromatography

- Gas chromatography
- High-performance liquid chromatography
  - Gel-permeation chromatography

Current Trends in Chromatography Research
The Key Components of Chromatographic Separation

MOBILE PHASE

\[ A_{\text{mobile}} \leftrightarrow A_{\text{stat.}} \]

STATIONARY PHASE
The Key Components of Chromatographic Separation

**MOBILE PHASE**
- Liquid: MeOH, MeCN, C₆H₁₄, MeOEt
- Gas: He, N₂, H₂

**STATIONARY PHASE**
- Liquid: OV-225
- Solid: Al₂O₃, cellulose, Me₂Si-C₁₈H₃₇
Quality of Separation is Measured by Resolution

• What are we looking for in an ideal chromatographic separation?
  
  • Every component of our mixture to elute separately
  
  • Bands of compounds to be narrow and concentrated
  
  • Separation to use a minimum of time and solvent

• We use resolution between chromatogram peaks as a measure of the quality of the separation

\[ R_s = \frac{\text{difference in ret. time}}{\text{average peak width}} = \frac{2\Delta t_R}{(w_1 + w_2)} \]

• resolution improves with larger retention time difference and narrower peaks

• use tangent lines at peak's inflection points to define width
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Many Factors Affect Resolution

- The main considerations for resolution are retention, selectivity, and efficiency

\[ R_s = \frac{k}{k + 1} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4} \]

The Fundamental Resolution Equation

\[ \frac{k}{k + 1} \]  
**Retention term**: describes the retention of a compound relative to an unretained compound  
\[ k \] is the retention factor

\[ \frac{\alpha - 1}{\alpha} \]  
**Selectivity term**: describes ratio of retention factors for adjacent peaks  
\[ \alpha \] is the selectivity factor

\[ \frac{\sqrt{N}}{4} \]  
**Efficiency term**: describes rate of band broadening during separation  
\[ N \] is the number of theoretical plates
Retention is Necessary for Separation

\[ R_s = \frac{k}{k+1} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4} \]

Retention term: describes the retention of a compound relative to an unretained compound

\[ k = \text{retention factor} = \frac{t_R - t_0}{t_0} \]

Void time markers

Reverse phase
- uracil

Normal phase
- tris(tertbutyl)benzene

Totally unretained on stationary phase

Detector Response

Void time marker

Time for solvent to elute

Retention Time (Minutes)

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

Detector Response

Void time marker

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Retention Time (Minutes)

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Retention is Necessary for Separation

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**Retention term**: describes the retention of a compound relative to an unretained compound

\[ k = \text{retention factor} = \frac{t_R - t_0}{t_0} \]

- Essentially gives number of column volumes to elute given compound
- \(2 < k < 3\) is ideal
- \(k > 10\) indicates overly strong retention (wastes time and causes band broadening)
Retention is Necessary for Separation

\[ R_s = \frac{k}{k+1} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4} \]

Retention term: describes the retention of a compound relative to an unretained compound

\[ k = \text{retention factor} = \frac{t_R - t_0}{t_0} \]

- To modify a compound’s \( k \):
  - change the stationary phase
  - change the mobile phase
  - alter the pH of the mobile phase (for ionizable analytes)
Selectivity has the Biggest Effect on Resolution

\[ R_s = \frac{k}{k + 1} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4} \]

**Selectivity term:** \( \alpha \) describes ratio of retention factors \( k \) for adjacent peaks

\[ \alpha = \text{selectivity factor} = \frac{k_2}{k_1} \]

- \( \alpha > 1.1 \) is considered good
- **To modify \( \alpha \) between peaks:**
  - change the stationary phase
  - change the mobile phase
  - alter the pH of the mobile phase (for ionizable analytes)
Selectivity has the Biggest Effect on Resolution

\[ R_s = \frac{k}{k+1} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4} \]

Selectivity term: \( \alpha \) describes ratio of retention factors \( k \) for adjacent peaks

\[ \alpha = \text{selectivity factor} = \frac{k_2}{k_1} \]

- Changing selectivity gives the most resolution improvement
- This is why the identity of the stationary and mobile phases is so important
**Efficiency Measures Rate of Band Broadening**

\[ R_s = \frac{k}{k+1} \times \frac{\alpha - 1}{\alpha} \times \sqrt{\frac{N}{4}} \]

**Efficiency term:** highest for bands that stay narrow and symmetric even at long retention times

\[ N = \text{theoretical plates} = 16 \left( \frac{t_{R,1}}{w_1} \right)^2 \]

Generally, \( 100 < N < 25,000 \)

- Bands naturally widen as solutes take various paths through stationary phase

**Best ways to improve column efficiency**

- Decrease particle size and increase uniformity
- Increase column length
  \( \Delta t_R \propto L \quad \text{width}^{1/2} \propto L \)
van Deemter Equation

Jan van Deemter
1918-2004

\[ HETP = A + \frac{B}{u} + C \cdot u \]

Relates separation efficiency to mobile phase flow velocity \( u \)

\( HETP \)  Height Equivalent to Theoretical Plate: distance corresponding to one theoretical plate

\( A \)  Eddy diffusion: describes channeling through non-ideal packing (i.e., polydisperse mobile phase)

\( B \)  Longitudinal diffusion: describes unavoidable diffusion of compound along length of column

\( C \)  Resistance to mass transfer: inversely proportional to analyte’s equilibration rate b/w phases

\( u \)  Flow rate: nothing fancy here — \( u = \) Length of column / void time
van Deemter Equation Graphically

\[ HETP = A + \frac{B}{u} + C \cdot u \]

- Eddy diffusion term

- Results from analytes taking multiple different paths through column (channeling)

- Lots of channeling leads to poor separation by way of broad bands

- Minimized by having well-packed columns with small, uniformly shaped stationary phase particles
van Deemter Equation Graphically

$HETP = A + \frac{B}{u} + C \cdot u$

- Longitudinal diffusion term
- Arises from thermal diffusion of analyte
- The longer the analyte spends on column, the greater effect $B$ has
- Not much else can be done to avoid this

Flow rate that maximizes separation efficiency
van Deemter Equation Graphically

\[ HETP = A + \frac{B}{u} + C \cdot u \]

- Resistance to mass transfer term

\[ A_{\text{(mobile)}} \leftrightarrow A_{\text{(stat.)}} \]

- Want analyte to equilibrate fully between phases

- If flow rate is too high, then equilibrium artificially biased towards \( A_{\text{(mobile)}} \)

- Faster equilibration allows faster flow rate to be used → flatter C section
"The General Elution Problem"

How do we simultaneously achieve both high resolution and reasonable run times?

- **Isocratic high strength**: Poor resolution
  - Eluent Strength vs. Time

- **Isocratic low strength**: Excessive time, band broadening
  - Eluent Strength vs. Time

- **Gradient strength**: Balances resolution and run time
  - Eluent Strength vs. Time
  - Amenable to all forms of chromatography

Example: 50300SPLIT30

50 ºC → 30 ºC/min → 300 ºC
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Current Trends in Chromatography Research
Gas Chromatography (GC)

- “Gas” indicates that the mobile phase is a gas

**Common GC column classes**

- **capillary column**
- **liquid stationary phase**
- **porous solid support**
- **porous solid support coated w/liquid stationary phase**

**Wall-coated**  **Porous layer**  **Support-coated**  **Packed**

Common for gaseous analytes
Mobile and Stationary Phases for GC

MOBILE PHASE

- He, H₂, and N₂ are the most common mobile phases
- Equilibration between phases is slowest in N₂ (heaviest gas), so flow rate needs to be slower than for He or H₂

STATIONARY PHASE

LEAST POLAR

squalane

PDMS

MOST POLAR

OV-225

OV-17

most common

PEG
Common Detectors for GC

**Flame Ionization Detector (FID, what our GC has)**

- Prized for a low detection limit and a large linear response range (10^7 orders of magnitude)
- Can detect anything combustible
- Not amenable to preparative GC

**Mass Spectrometry (GCMS)**

- Good for essentially any analyte
- Gives lots of info on complex mixtures
- Incredibly sensitive detection
High-Performance Liquid Chromatography (HPLC)

“High-pressure LC?” — Nope!

- Given other forms of chromatography using pressurized mobile phases, “high-performance” is now preferred

- The required operating pressure is a function of numerous parameters

\[
P = \frac{\eta L u}{K^0 \pi r^2 d^2}
\]

- Increasing the solvent viscosity, column length, or flow rate linearly increases pressure

- Column diameter and particle size have big impact
  - Decrease particle diameter by half and pressure increases by 4x
Mobile and Stationary Phases for Chiral HPLC

**MOBILE PHASE**
- Both normal and reverse phase, but reverse phase is most common
- AcOH and TEA are common pH modifiers

Hexanes/iPrOH  Hexanes/CHCl₃  Hexanes/EtOAc  H₂O/MeCN  H₂O/MeOH

**STATIONARY PHASE**
- A broad array of chirality sources are used on commercial stationary phases

- **Cellulose-derivatives**
  - Often supported in 5 µm silica particles
  - The basis for OD-H columns

- **Pirkle-type phases**
  - π-acid/π-base binding sites
  - Usually have H-bond donor too

- **Ligand-exchange chromatography**
  - Often used for D- and L- amino acids
  - Mobile phase often contains NH₃

![Cellulose derived](image)

![Pirkle-type phases](image)

![Ligand-exchange chromatography](image)
Mobile and Stationary Phases for Chiral HPLC

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  - Hexanes/iPrOH
  - Hexanes/CHCl₃
  - Hexanes/EtOAc
- AcOH and TEA are common pH modifiers
  - H₂O/MeCN
  - H₂O/MeOH

**STATIONARY PHASE**

- A broad array of chirality sources are used on commercial stationary phases
  - Inclusion complexes
  - Take advantage of differential complexation between analyte isomers and stationary phase
    - Cyclodextrins are the most common, but crown ethers have also been used
Size-Exclusion Chromatography (SEC)

Gel-permeation chromatography is specifically SEC with an organic eluent

- Larger hydrodynamic radius
- Smaller hydrodynamic radius

- Separation is based on the analyte’s hydrodynamic radius
- Hydrodynamic radius includes the solvent shell around analyte
- Not a problem if analyte is similar to calibration standards
Mobile and Stationary Phases for SEC

MOBILE PHASE

- Both normal and reverse phase are available
- Reverse phase most common for biomolecules

STATIONARY PHASE

- In general, SEC stationary phases try to minimize chemical interactions with analyte so size becomes defining feature
  - Tune size of pores in stationary phase to select for size range of analytes in sample

- Reverse-phase stationary phases
  - common for biomolecule purifications, especially proteins
  - have lower mechanical strength than silica-based phases, so low flow rates must be used

- Crosslinked agarose
  - difunctional electrophiles (e.g., epichlorohydrin) used to crosslink

S6FF

6%-crosslinked agarose
Sepharose 6FF column

3,6-anhydrogalactose

galactose
Mobile and Stationary Phases for SEC

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**STATIONARY PHASE**

- In general, SEC stationary phases try to **minimize chemical interactions** with analyte so size becomes defining feature
  - Tune size of pores in stationary phase to select for size range of analytes in sample

- **Normal-phase stationary phases**
  - mainly used for synthetic polymer analysis and purification
  - porous crosslinked polymers are the most common stationary phase class

![PS-co-PDVB particles](image)

![Poly(styrene-co-divinylbenzene)](image)

- relatively unfunctionalized so limits chemical interactions
- produced mainly from emulsion polymerization with polymeric porogens

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Current Trends in Chromatography Research
Brief History of Chromatography

1880-1900
Columns of charcoal or limestone found to fractionate crude petroleum

1900
Mikhail Tsvet separates plant pigments on CaCO₃ with ether/EtOH and coins "chromatography" (color writing)

1941
Archer Martin & Richard Synge invent partition chromatography (hydrated SiO₂ used to separate amino acids)

1949
Martin & Anthony James invent gas chromatography

1969
Waters corporation commercializes first HPLC system, the ALC100 HPLC

2004-present
Invention of ultra-high-pressure LC and further increases in pressure limits

Improvements to capacity, detector sensitivity, and instrument automation
A Principle Focus: Accommodating Smaller Packing

- Minimize Eddy diffusion term
- Minimize band broadening
- Maximize efficiency (theo. plates)

\[ P = \frac{\eta L u}{K^0 \pi r^2 d^2} \]

- Best systems today handle ~1500 bar, but 5000 bar is the goal

- Pressure demands increase rapidly as particle size decreases → tests limits of pumps and instrumentation

• Current approaches to overcoming pressure issue

- Develop stationary phases that can withstand temperatures >200 °C (limit is currently around 80 °C)
  - Invent narrower columns that can still be reliably packed with stationary phase
  - Develop new stationary phase morphologies, such as core-shell particles

  - Core prevents analyte from getting “stuck” in packing
  - Remarkably reduce Eddy diffusion
  - 3 µm core-shell particles outcompete 2 µm fully porous particles
  - Core can be made of thermally conductive Au to counteract friction

A Few Main Takeaways

- Resolution encapsulates the quality of a separation
  \[ R_s = \frac{k}{k+1} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4} \]

- Selectivity term has biggest effect on resolution
  - Modify eluent or stationary phase first

- Longitudinal diffusion is always occurring, so don’t let analytes just sit on column

- Picking too high a flow rate risks interfering with phase equilibration

- Trial and error is always part of the game