Fundamentals and Applications of Chromatography

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Knowles Lab Literature Group Meeting

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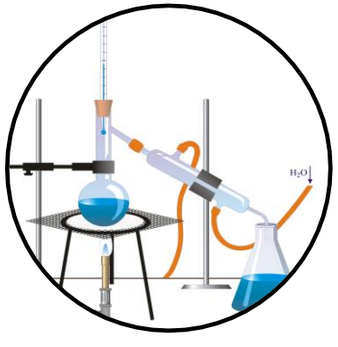
What exactly is chromatography?

Definition

*"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

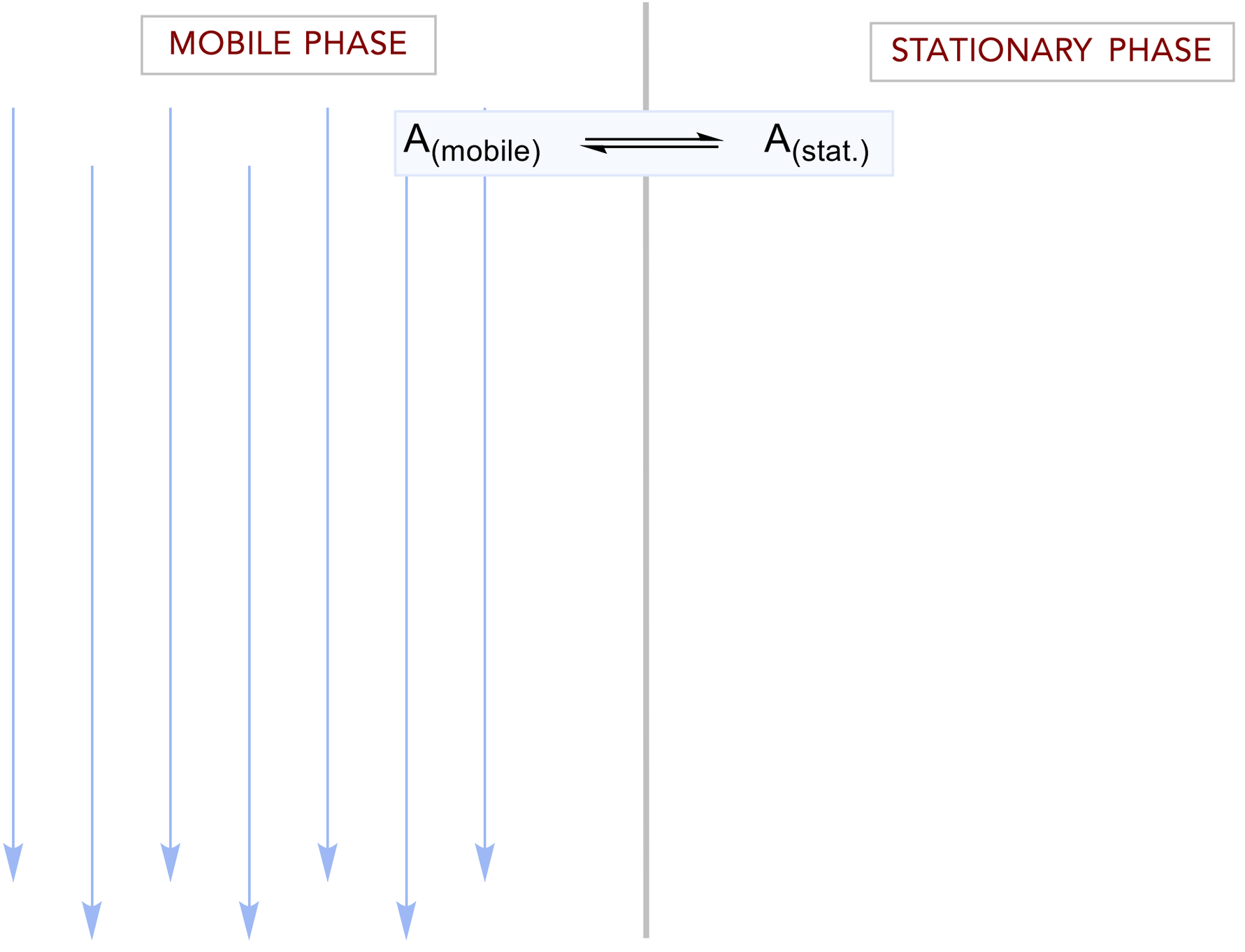
- Parameters affecting separation quality
- The Resolution equation
- The van Deemter equation

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



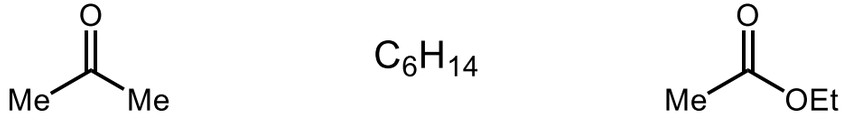
The Key Components of Chromatographic Separation

MOBILE PHASE

STATIONARY PHASE

$A_{(mobile)} \rightleftharpoons A_{(stat.)}$

• Liquid



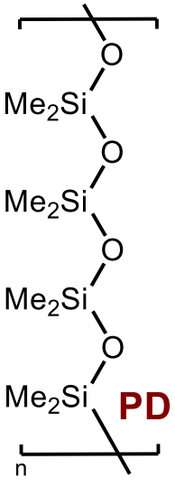
C_6H_{14}

MeOH H_2O MeCN

• Gas

He N_2 H_2

• Liquid



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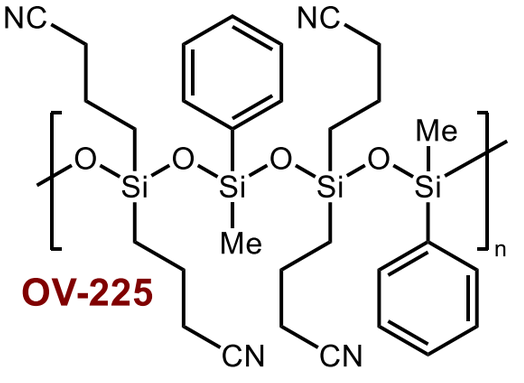
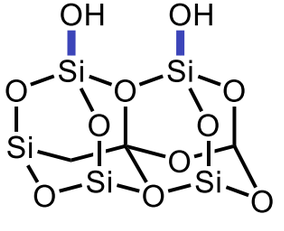


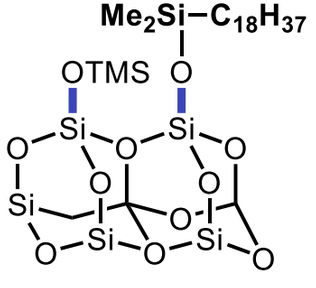
OV-225

• Solid



Al_2O_3

cellulose



Quality of Separation is Measured by Resolution

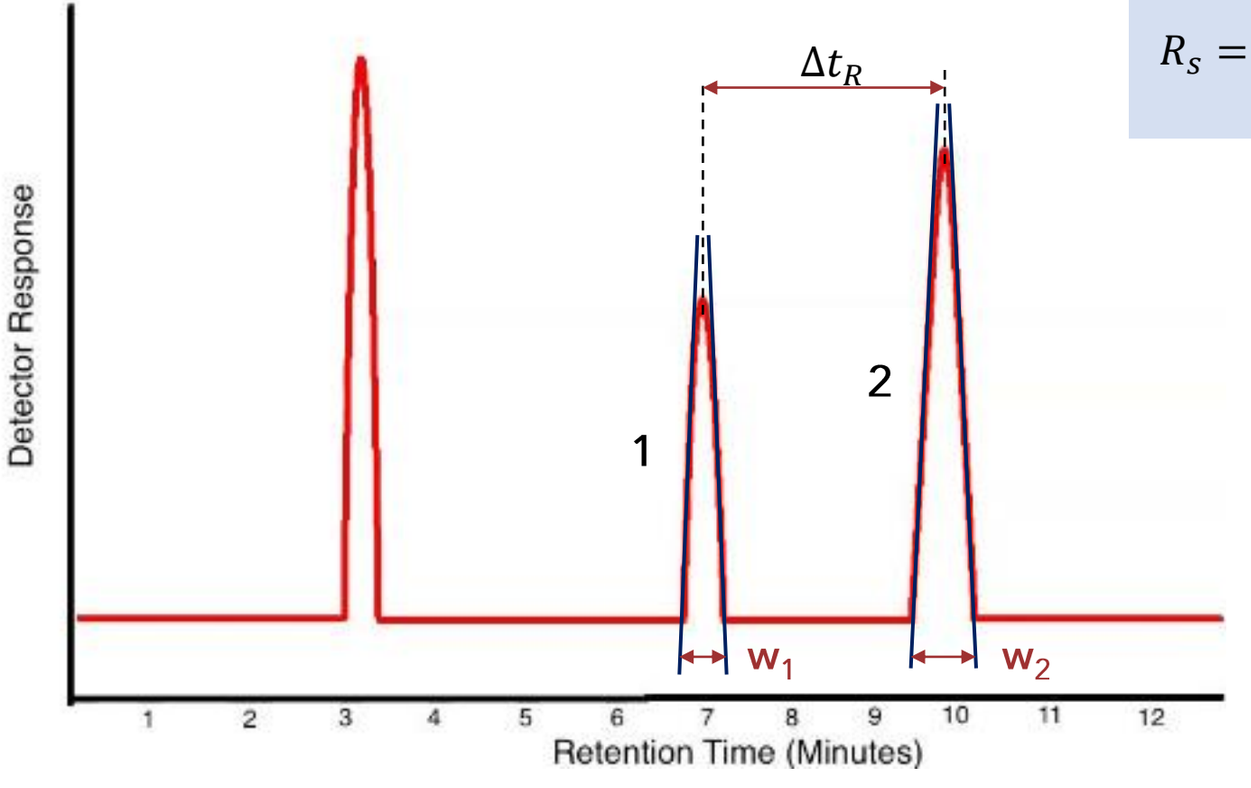
• What are we looking for in an ideal chromatographic separation?

• 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



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

• 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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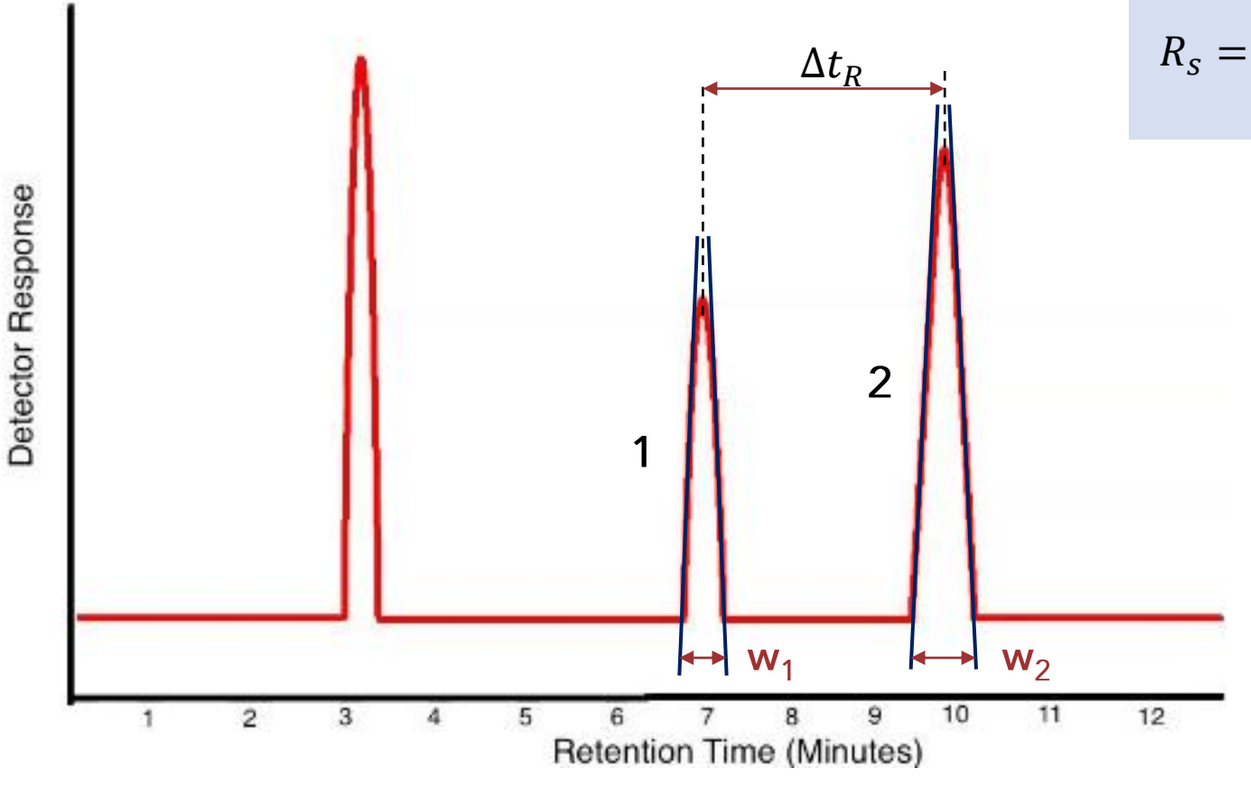
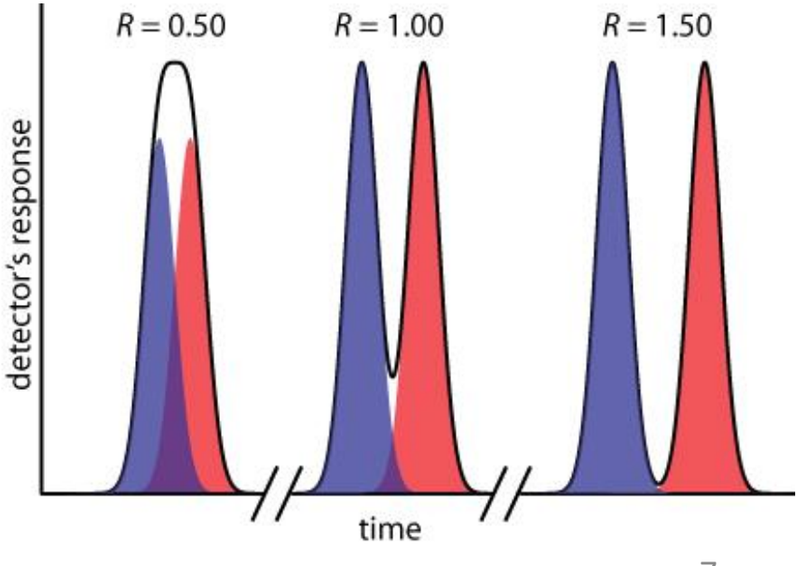




Many Factors Affect Resolution

- 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

α is the selectivity factor

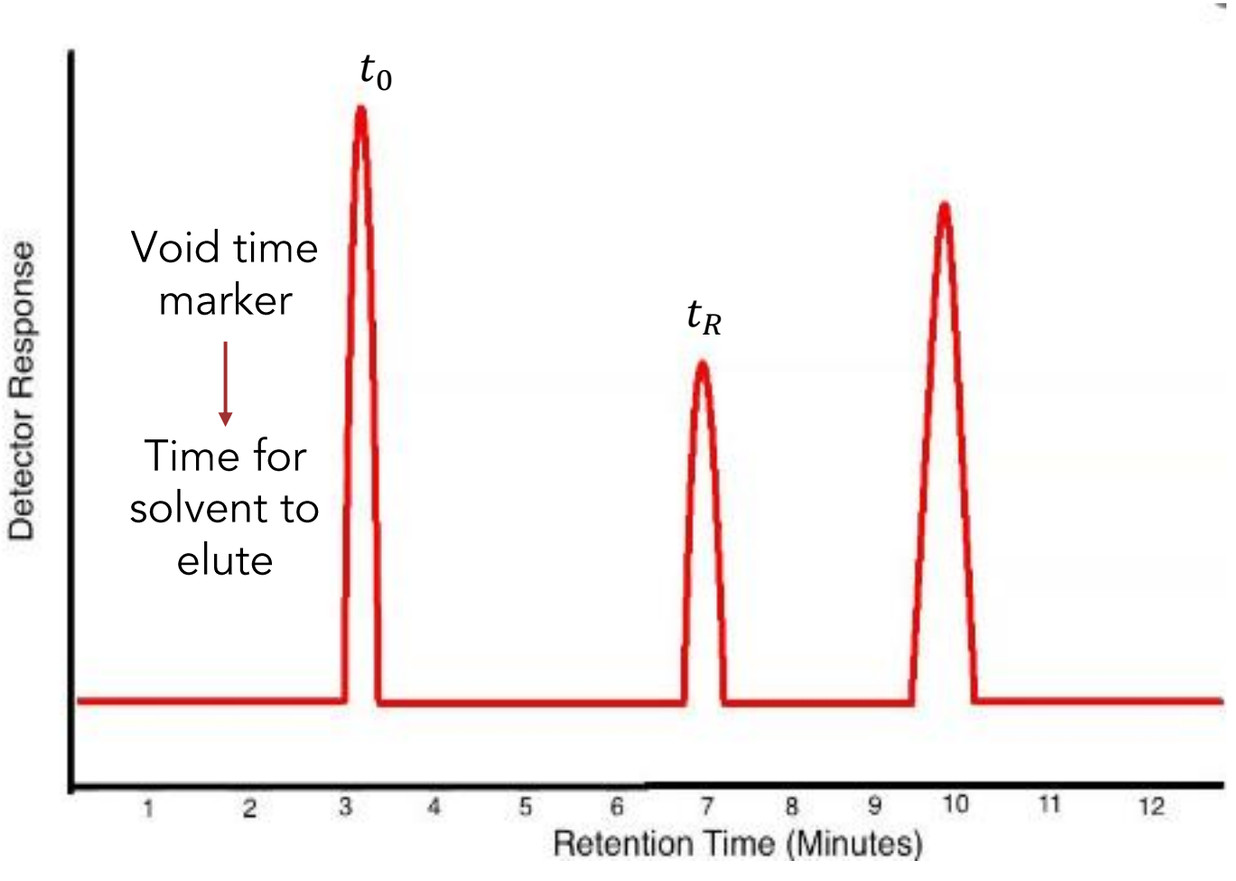
$\frac{\sqrt{N}}{4}$ **Efficiency term:** describes rate of band broadening during separation

N is the number of theoretical plates

Retention is Necessary for Separation

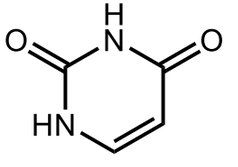
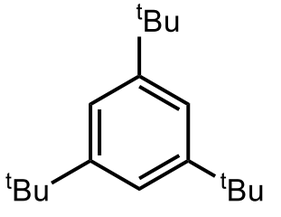
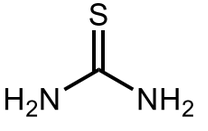
$$R_s = \frac{\boldsymbol{k}}{\boldsymbol{k+1}} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4}$$

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



$$k = retention\ factor = \frac{t_R - t_0}{t_0}$$

Void time markers

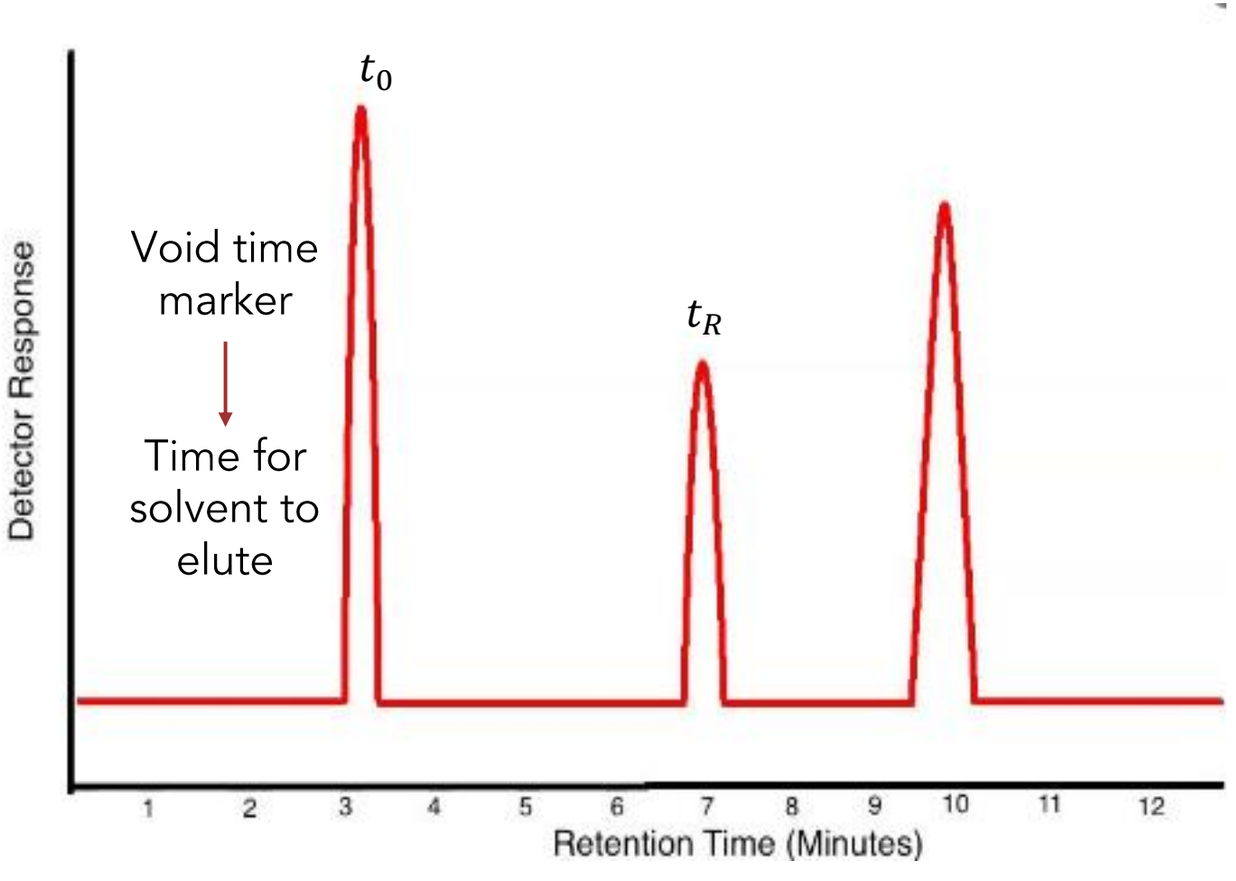
Reverse phase	Normal phase
 uracil	 tris(tertbutyl)benzene
 thiourea	

Totally unretained on stationary phase

Retention is Necessary for Separation

$$R_s = \frac{\boldsymbol{k}}{\boldsymbol{k+1}} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4}$$

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



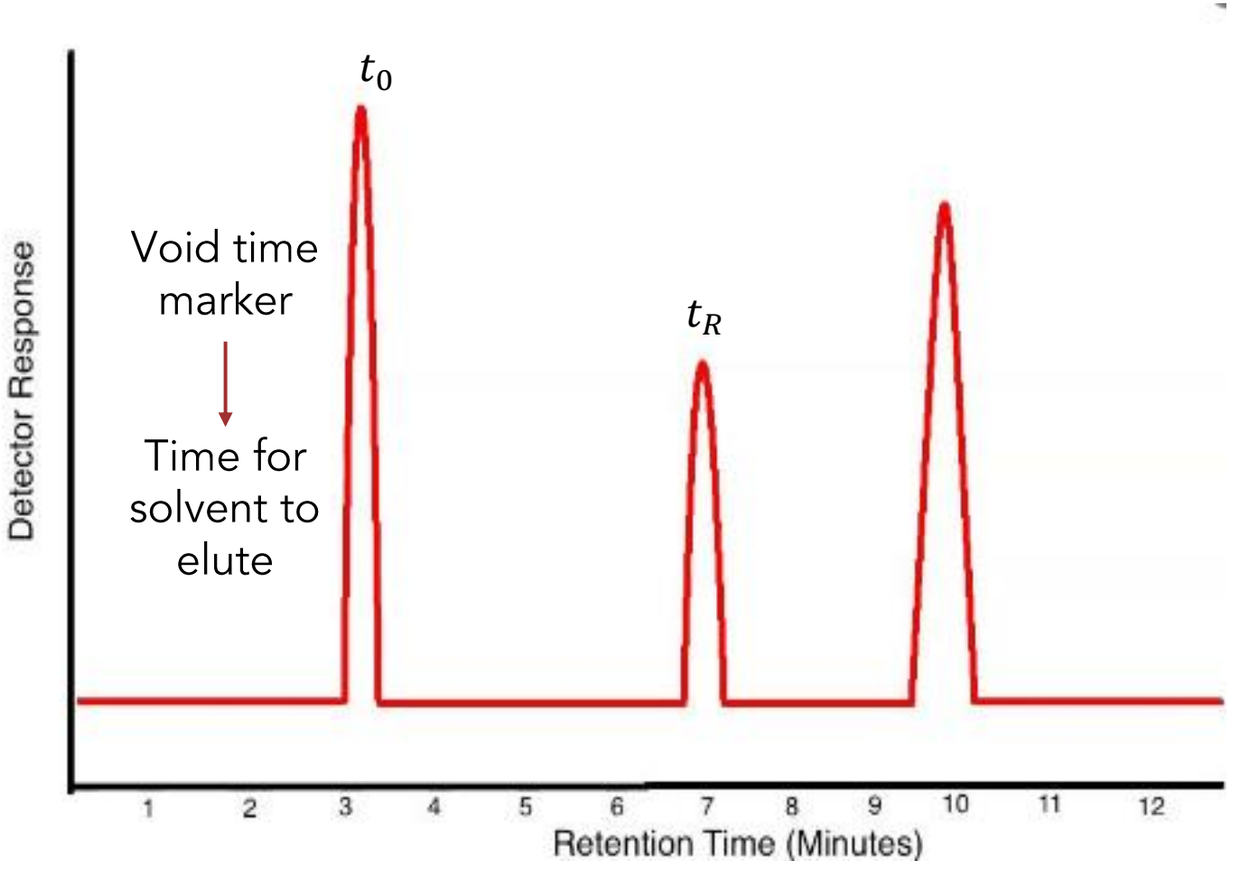
$$k = retention\ factor = \frac{t_R - t_0}{t_0}$$

- 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

$$R_s = \frac{\boldsymbol{k}}{\boldsymbol{k+1}} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4}$$

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



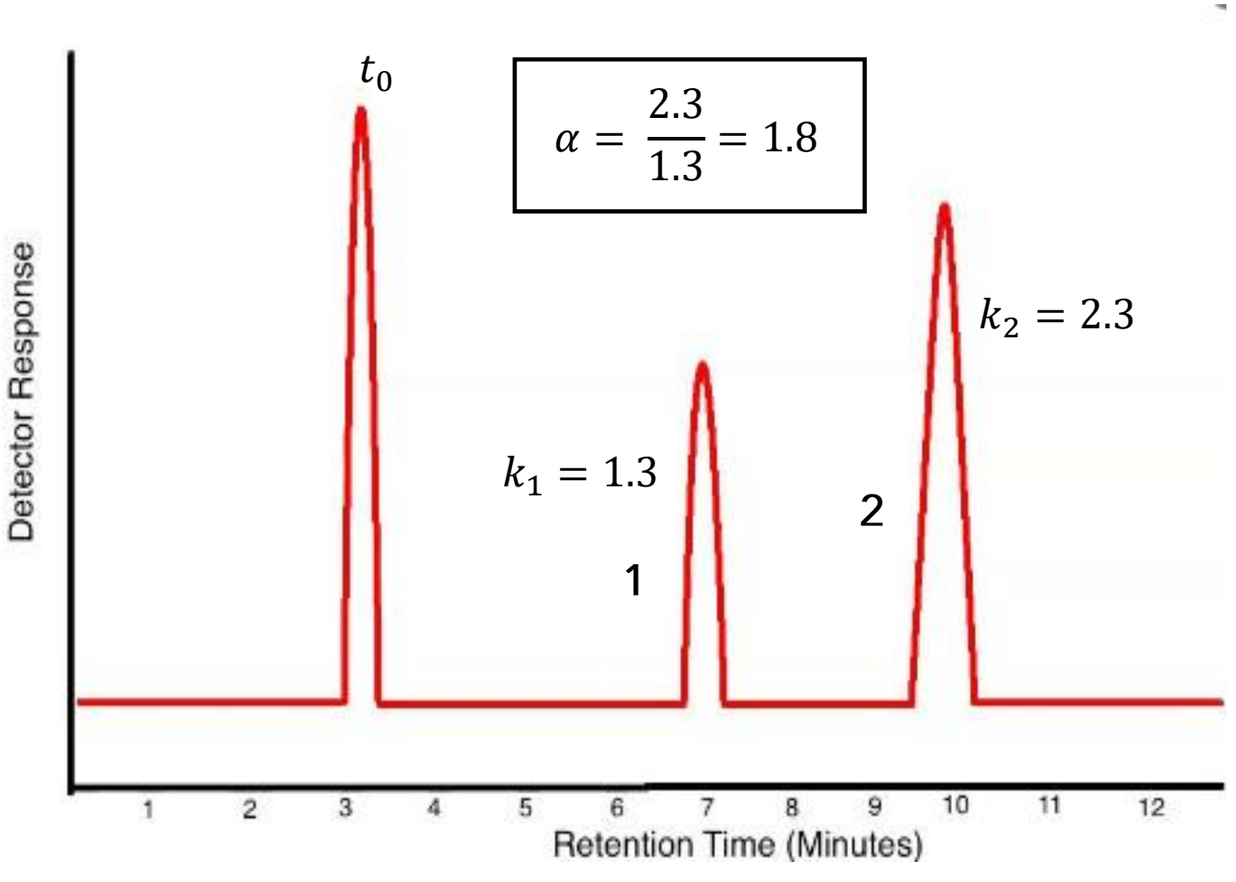
$$k = retention\ factor = \frac{t_R - t_0}{t_0}$$

- <u>To modify a compound's $\boldsymbol{k}$</u>:
- 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

$$R_s = \frac{k}{k+1} \times \frac{\boldsymbol{\alpha - 1}}{\boldsymbol{\alpha}} \times \frac{\sqrt{N}}{4}$$

Selectivity term: α describes ratio of retention factors k for adjacent peaks



$$\alpha = selectivity\ factor = \frac{k_2}{k_1}$$

- α > 1.1 is considered good

- <u>To modify $\boldsymbol{\alpha}$ between peaks</u>:

- 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

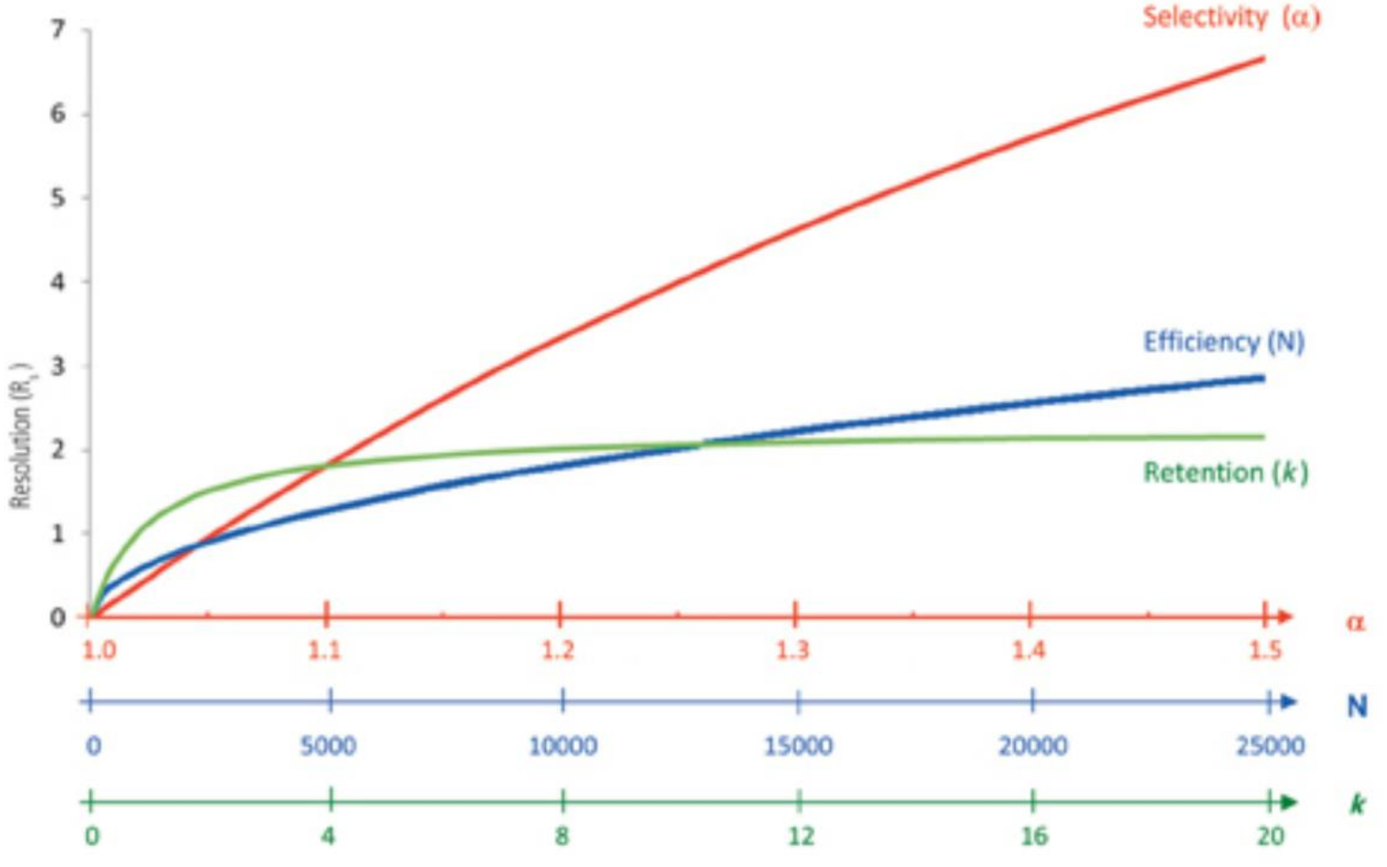
$$R_s = \frac{k}{k+1} \times \frac{\boldsymbol{\alpha - 1}}{\boldsymbol{\alpha}} \times \frac{\sqrt{N}}{4}$$

Selectivity term: α describes ratio of retention factors k for adjacent peaks

$$\alpha = selectivity\ factor = \frac{k_2}{k_1}$$

- 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

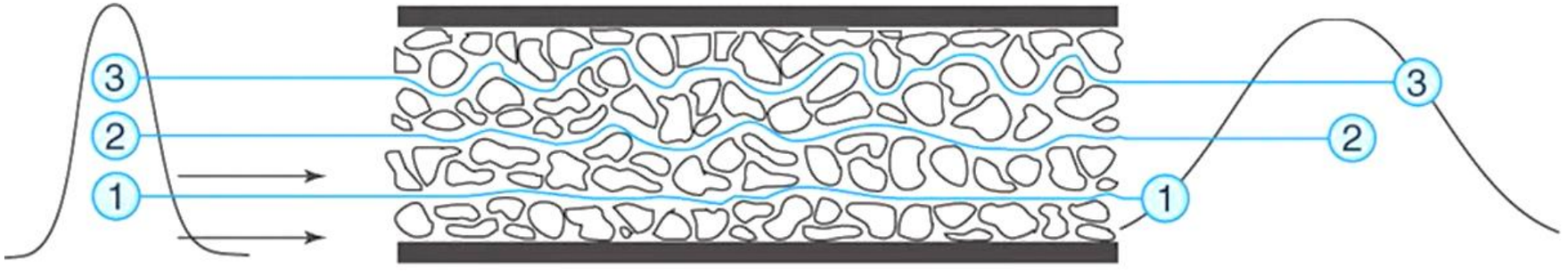
$$R_s = \frac{k}{k+1} \times \frac{\alpha - 1}{\alpha} \times \frac{\sqrt{N}}{4}$$

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

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

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

$\Delta t_R \propto L$ $\quad$ $width^{1/2} \propto L$

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

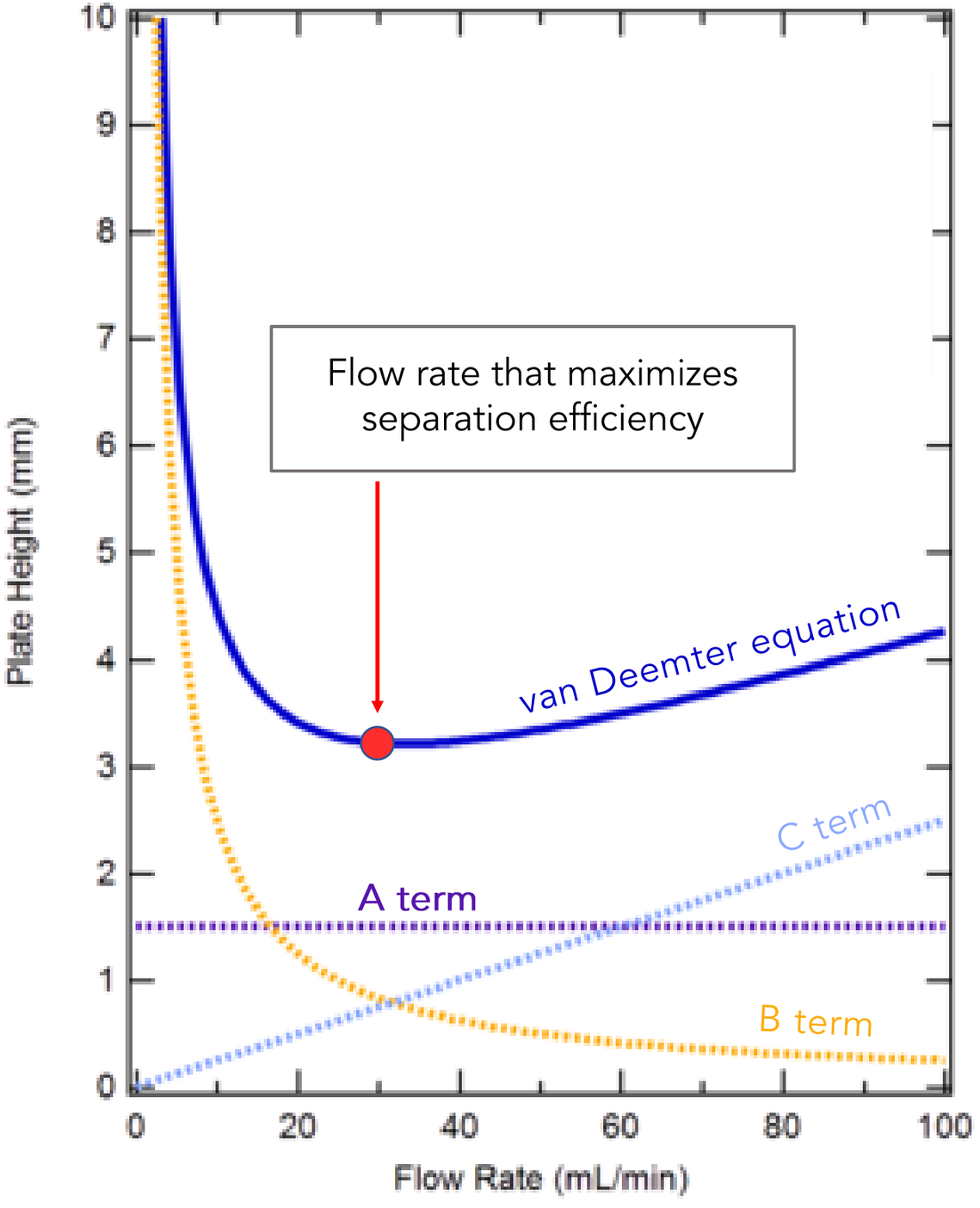
A **Eddy diffusion:** describes channeling through non-ideal packing (i.e., polydisperse mobile phase)

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



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

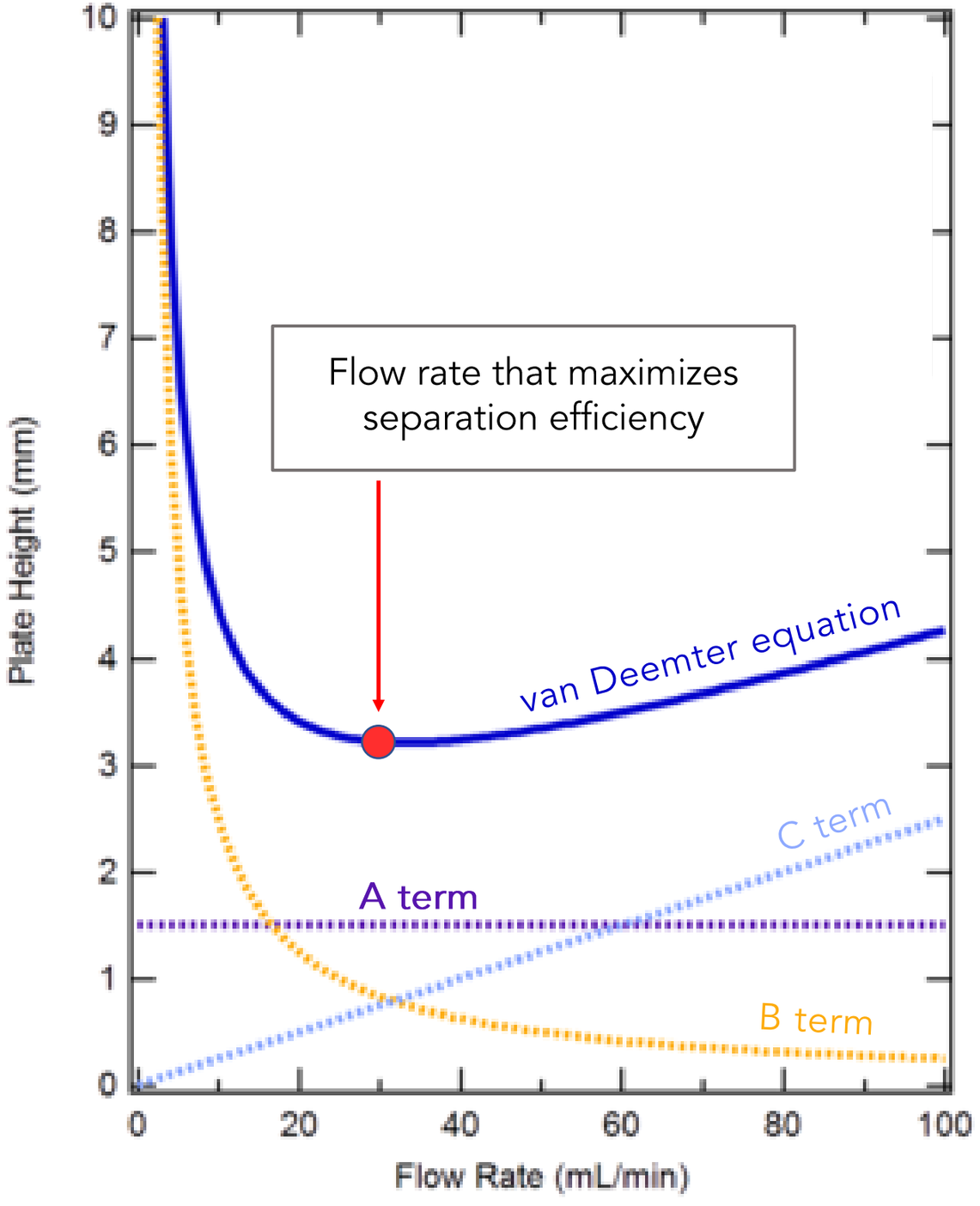
- **Eddy diffusion term**

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

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

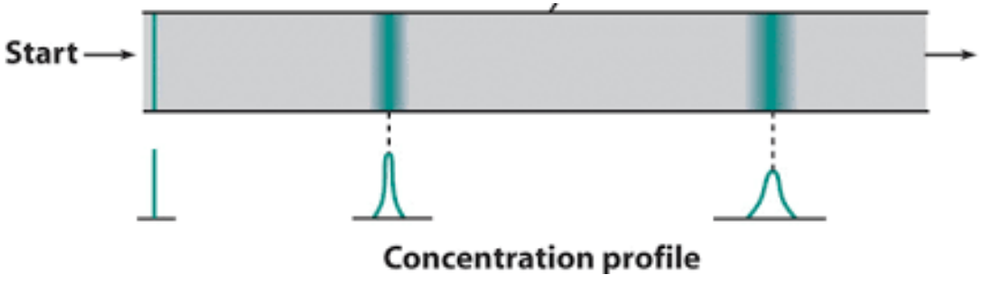
- Minimized by having well-packed columns with small, uniformly shaped stationary phase particles

van Deemter Equation Graphically



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

- Longitudinal diffusion term

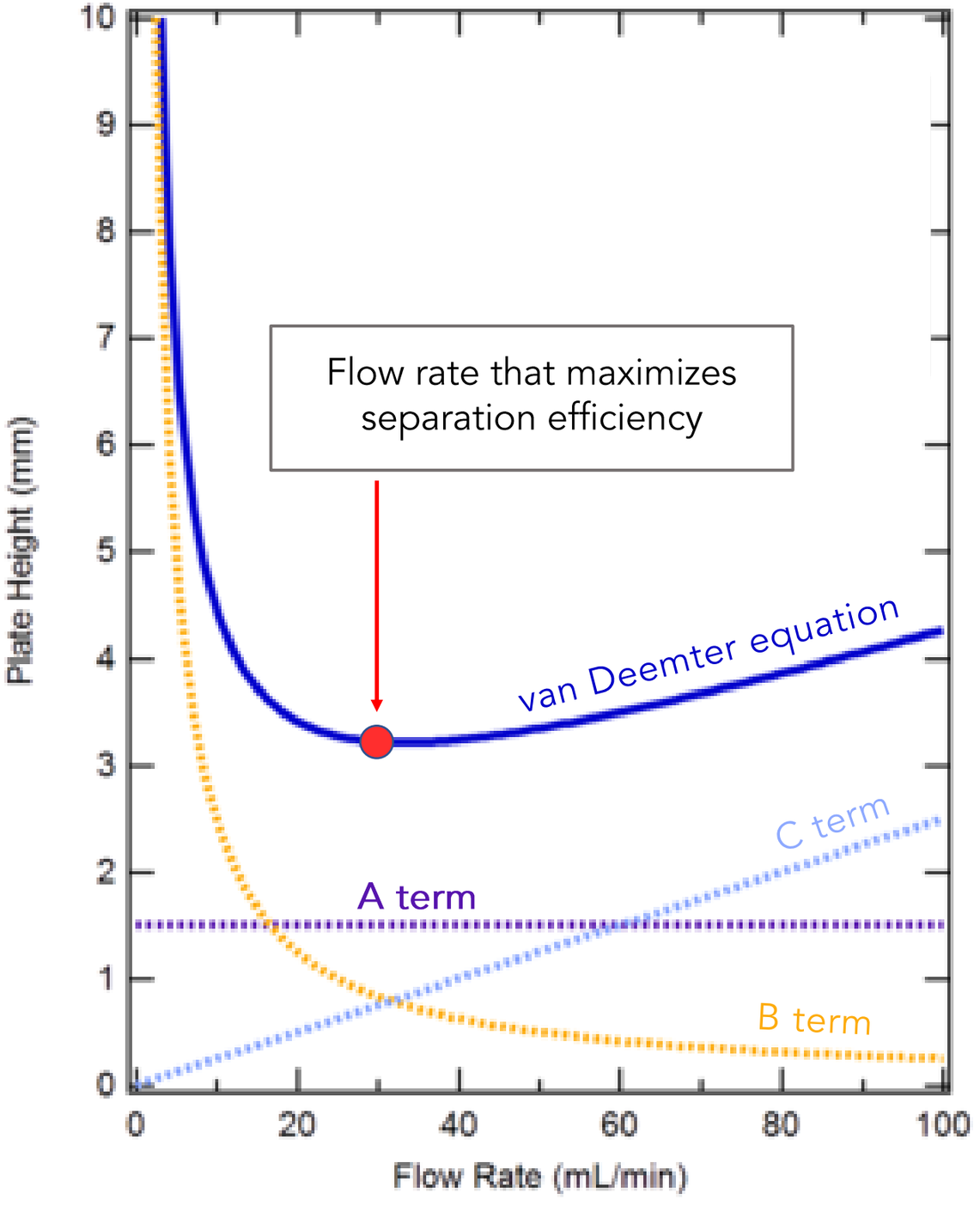


- Arises from thermal diffusion of analyte

- The longer the analyte spends on column, the greater effect B has

- Not much else can be done to avoid this

van Deemter Equation Graphically



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

- **Resistance to mass transfer term**

$A_{(mobile)} \rightleftharpoons A_{(stat.)}$

- Want analyte to equilibrate fully between phases

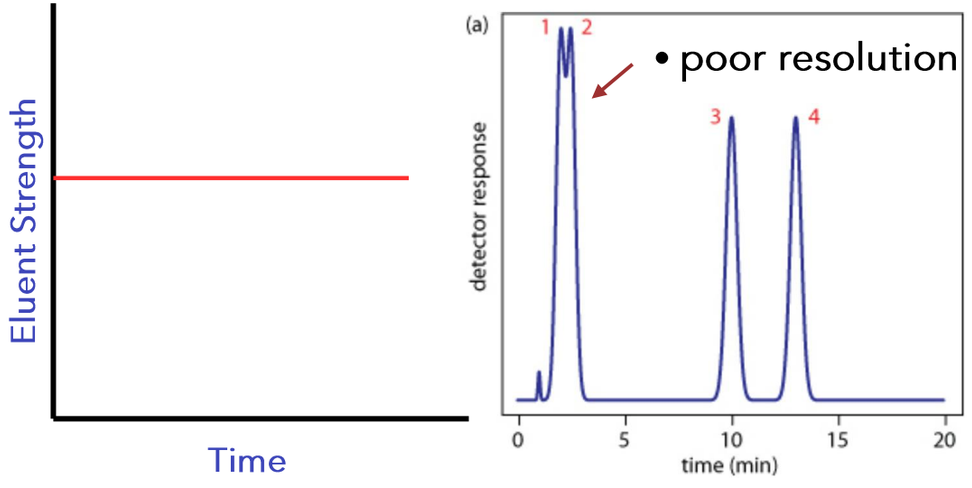
- If flow rate is too high, then equilibrium artificially biased towards $A_{(mobile)}$

- Faster equilibration allows faster flow rate to be used → flatter C section

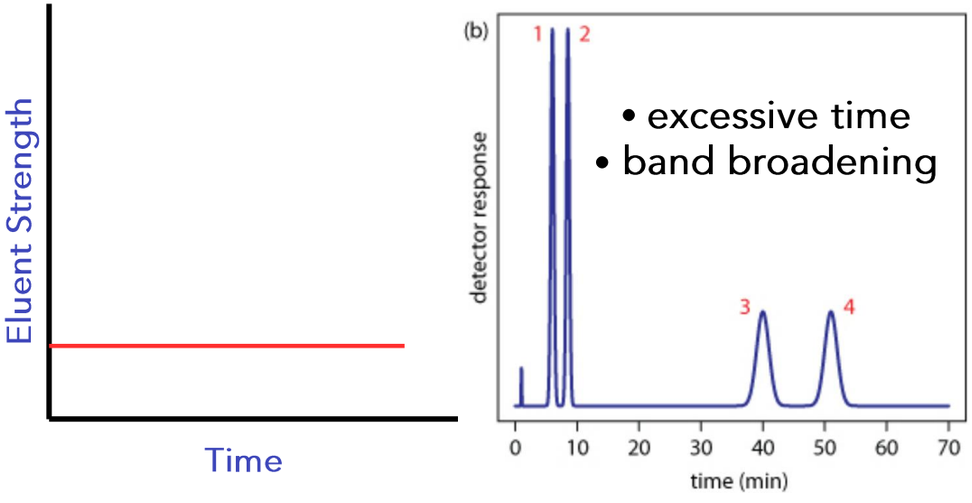
"The General Elution Problem"

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

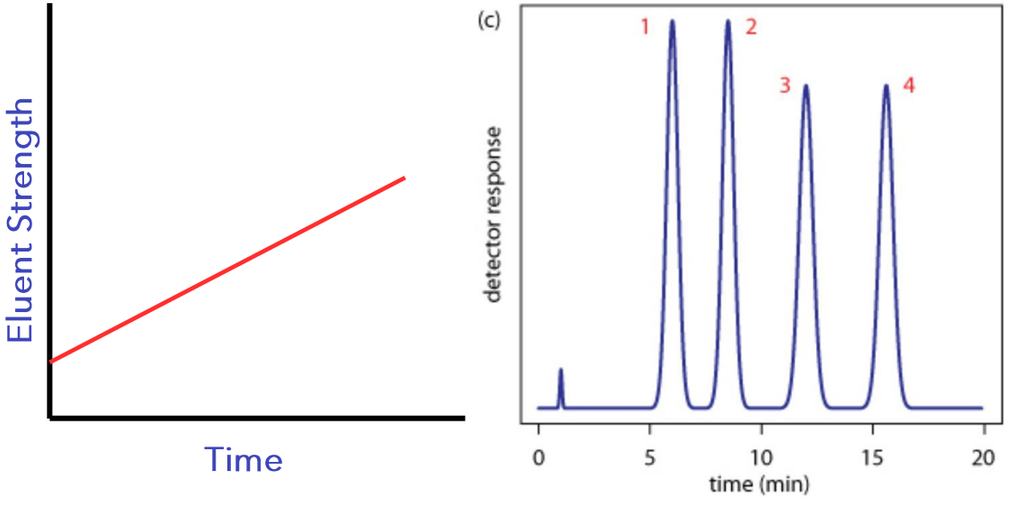
Isocratic high strength



Isocratic low strength



Gradient strength



- balances resolution and run time
- amenable to all forms of chromatography

Example: 50300SPLIT30

50 °C $\xrightarrow{30\ °C/min}$ 300 °C

Overview

Fundamentals and Theory of Chromatography

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

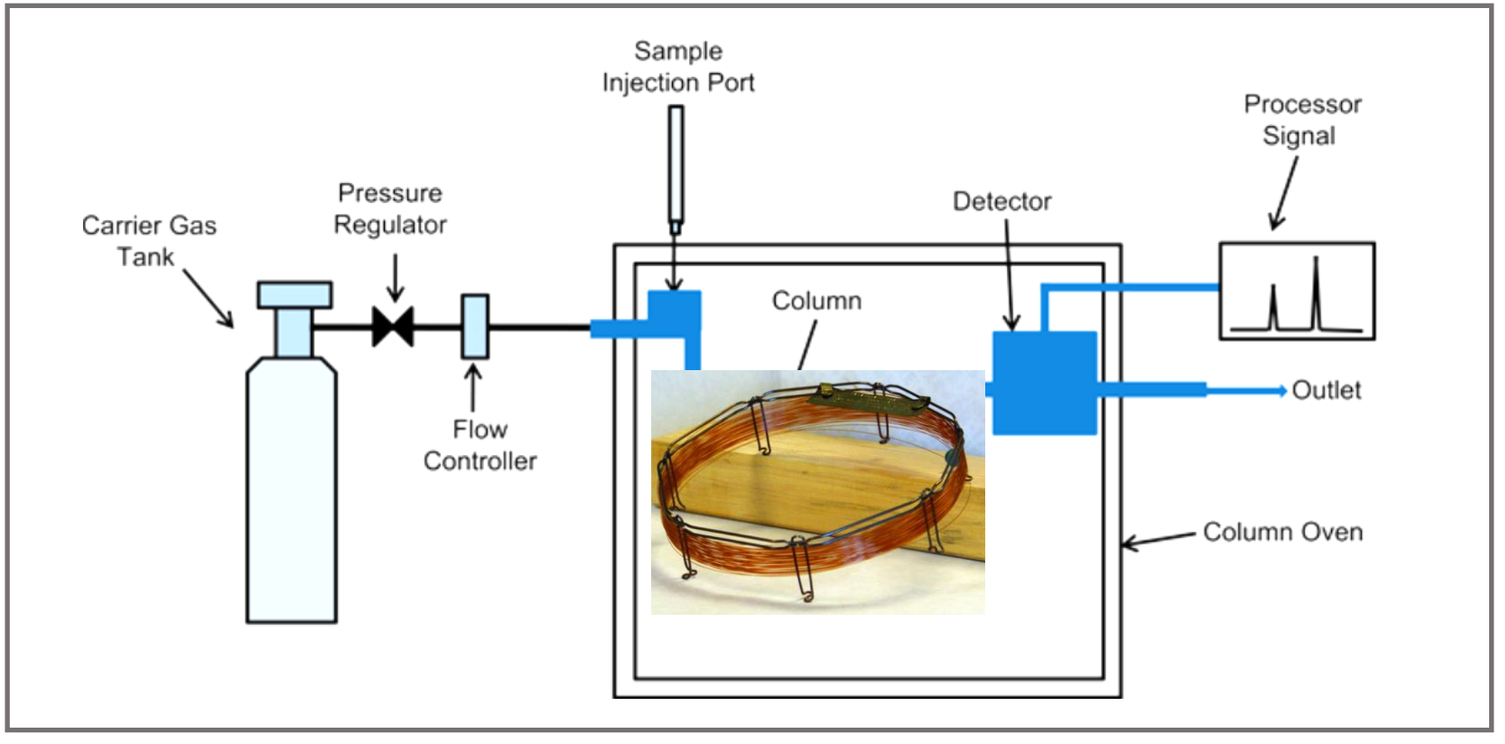
Three Common Types of Chromatography

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

Current Trends in Chromatography Research

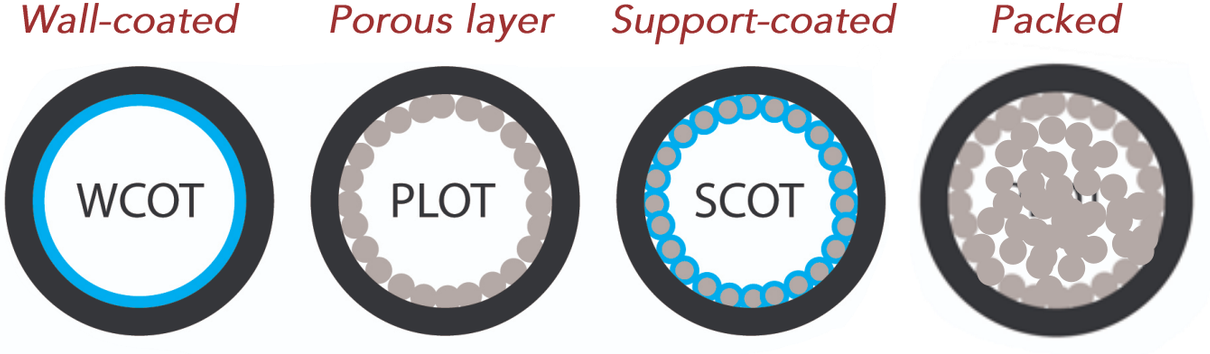
Gas Chromatography (GC)

- "Gas" indicates that the mobile phase is a gas



- Common GC column classes

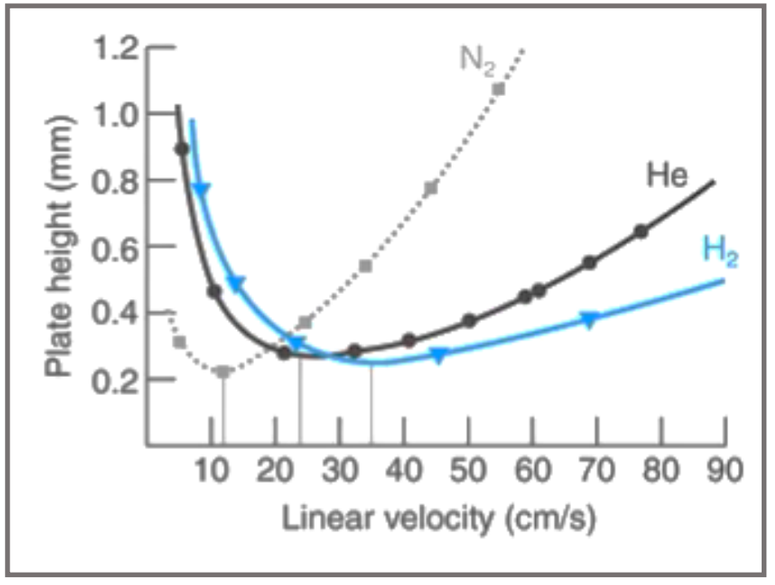
capillary column
liquid stationary phase
porous solid support
porous solid support coated w/liquid stationary phase



Common for gaseous analytes

Mobile and Stationary Phases for GC

MOBILE PHASE

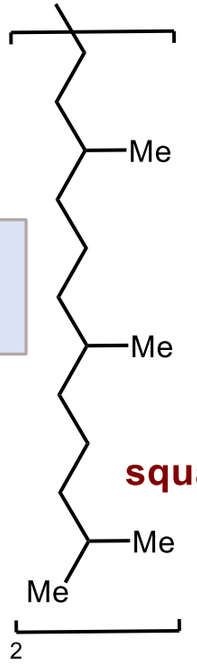


- He, H_2, and N_2 are the most common mobile phases

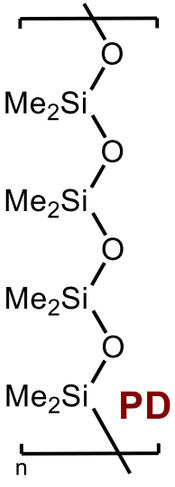
- Equilibration between phases is slowest in N_2 (heaviest gas), so flow rate needs to be slower than for He or H_2

STATIONARY PHASE

LEAST POLAR



squalane



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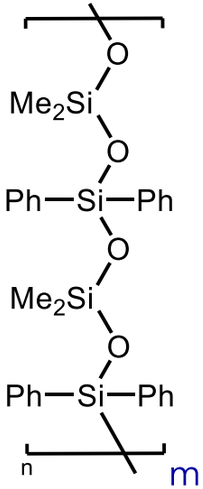
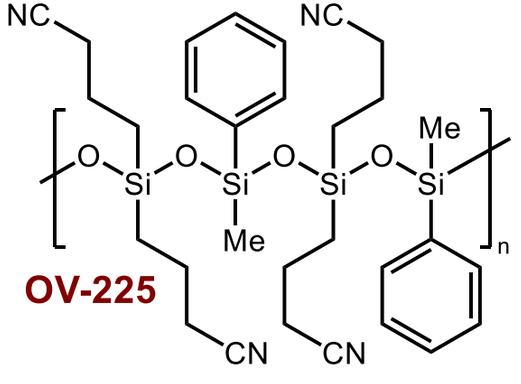
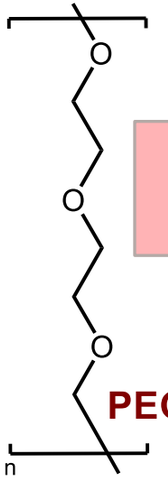


OV-17

most common



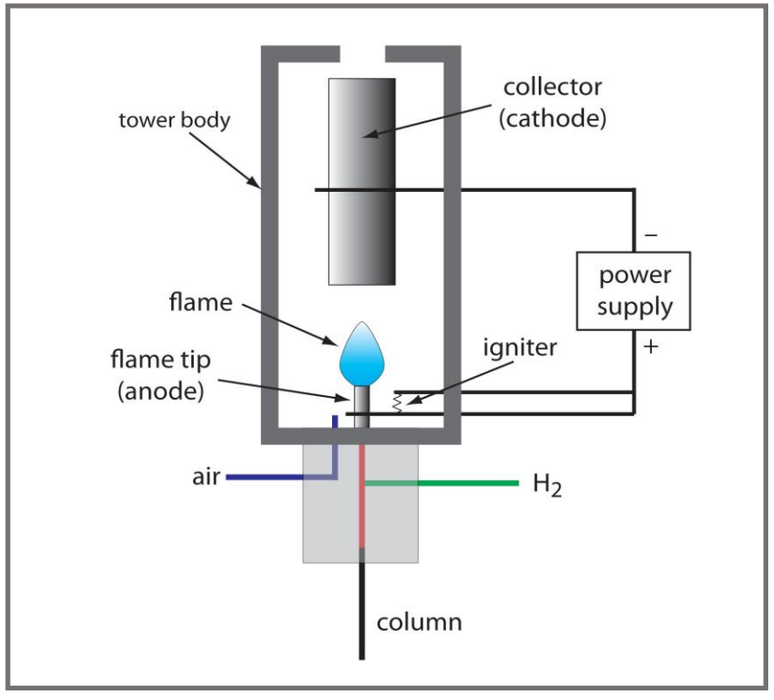
OV-225



PEG

MOST POLAR

Common Detectors for GC

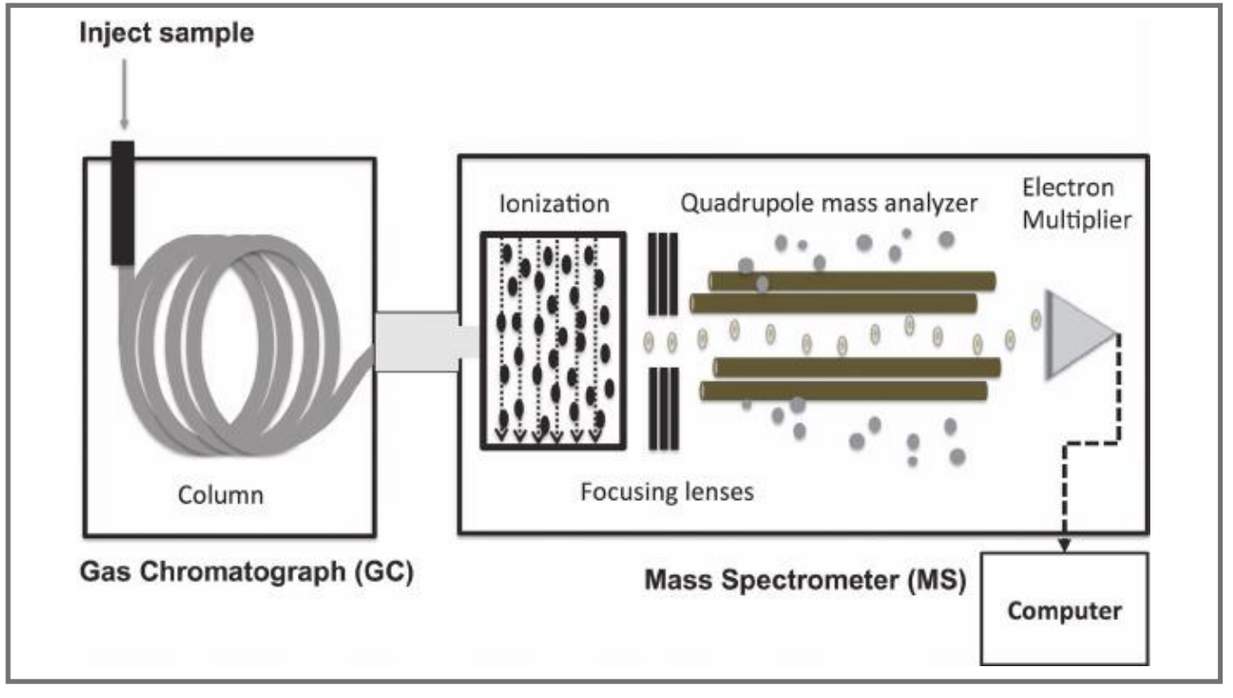


Flame Ionization Detector (FID, what our GC has)

- 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)

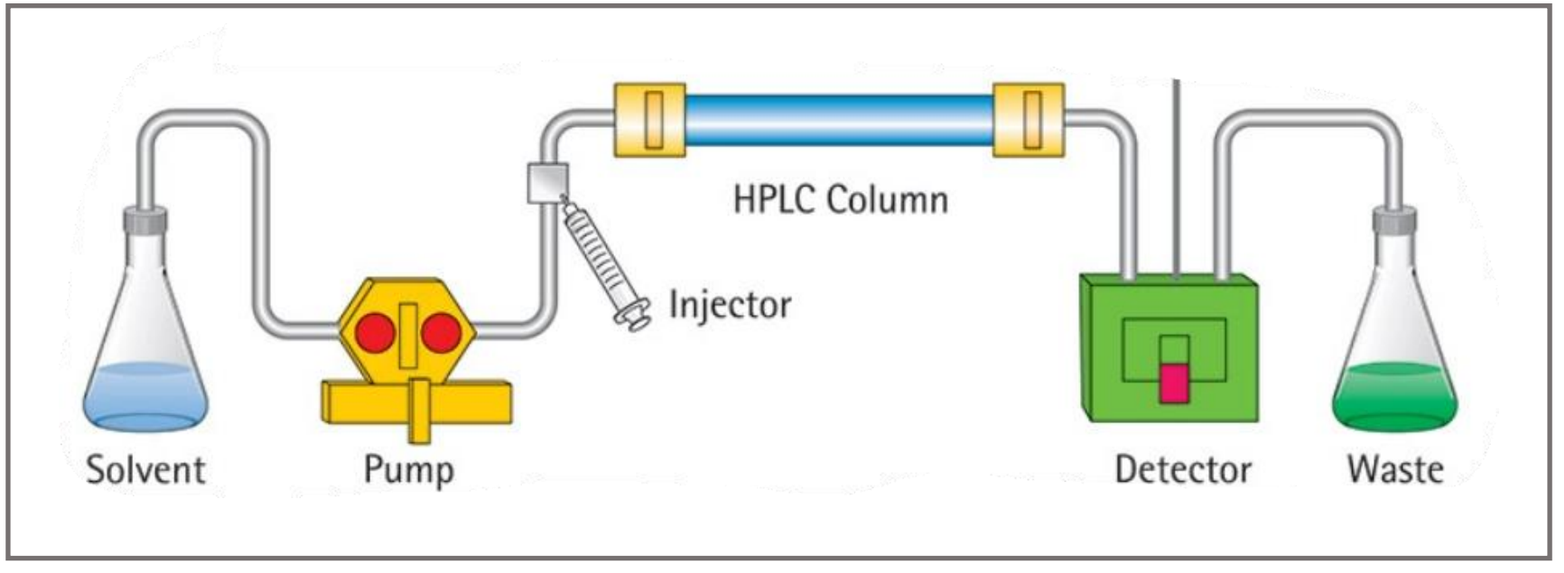
- Good for essentially any analyte
- Gives lots of info on complex mixtures
- Incredibly sensitive detection



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

$$P = \frac{\eta L u}{K^o \pi r^2 d^2}$$

- 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

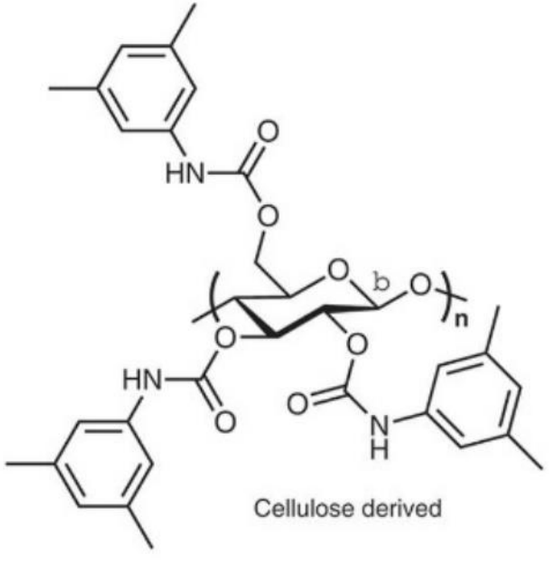
Hexanes/iPrOH Hexanes/$CHCl_3$ Hexanes/EtOAc | H_2O/MeCN H_2O/MeOH

STATIONARY PHASE

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

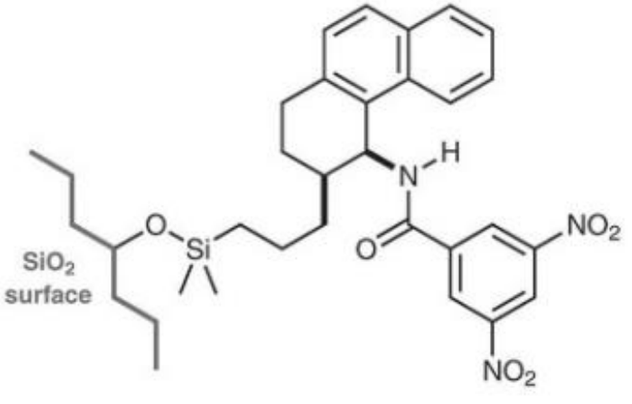
• Cellulose-derivatives

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



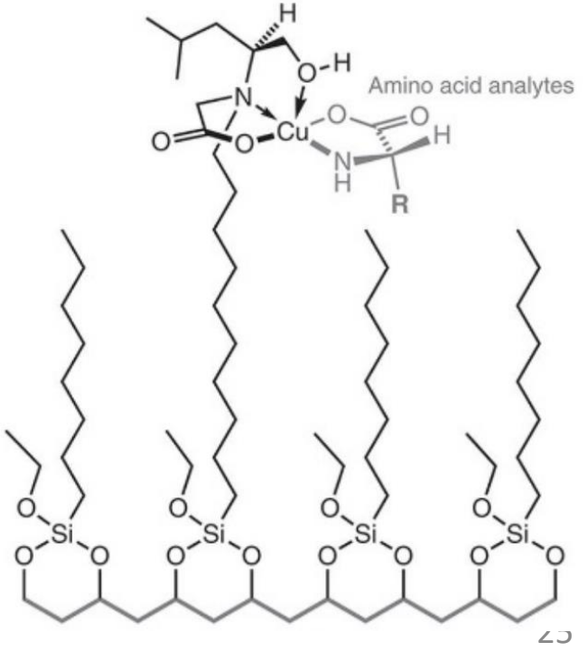
• Pirkle-type phases

- 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 NH_3



Mobile and Stationary Phases for Chiral HPLC

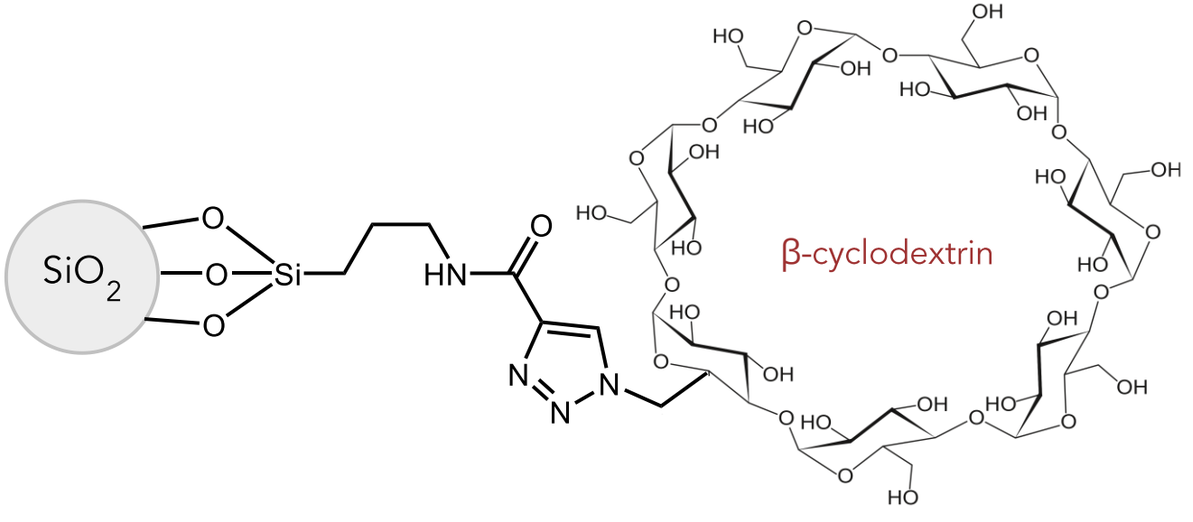
MOBILE PHASE

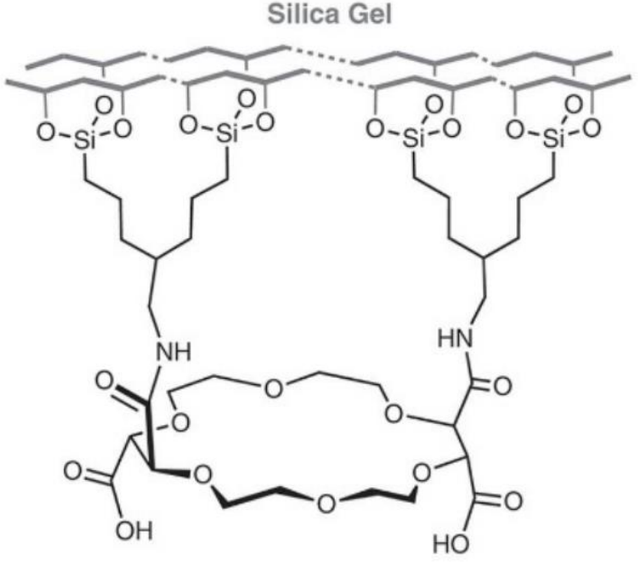
- Both normal and reverse phase, but reverse phase is most common
- AcOH and TEA are common pH modifiers

Hexanes/iPrOH Hexanes/$CHCl_3$ Hexanes/EtOAc | H_2O/MeCN H_2O/MeOH

STATIONARY PHASE

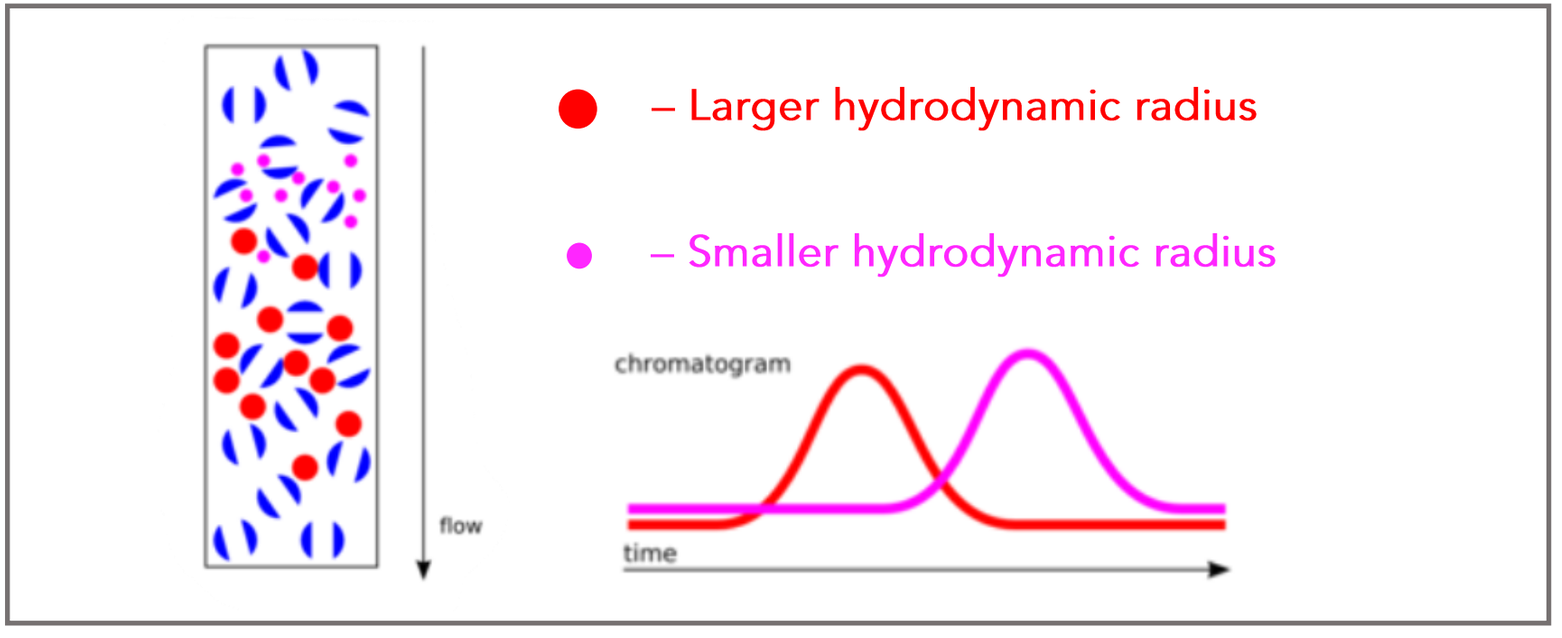
- A broad array of chirality sources are used on commercial stationary phases
- Inclusion complexes
- Take advantage of differential complexation between analyte isomers and stationary phase
- Cyclodextrins are the most common, but crown ethers have also been used

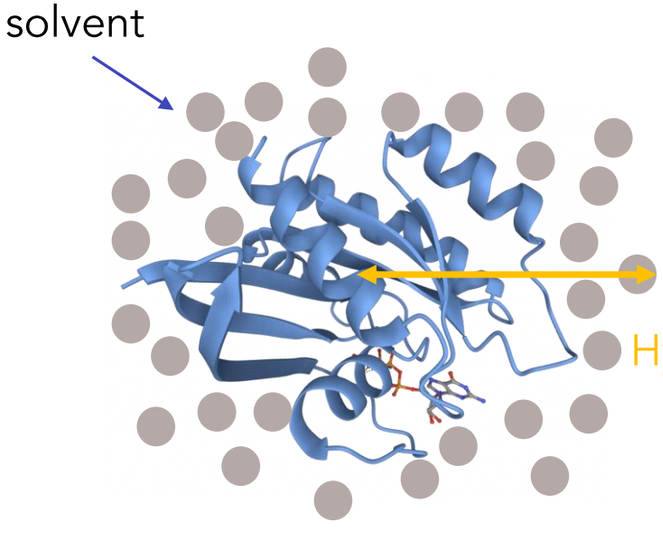




Size-Exclusion Chromatography (SEC)

Gel-permeation chromatography is specifically SEC with an organic eluent





- 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

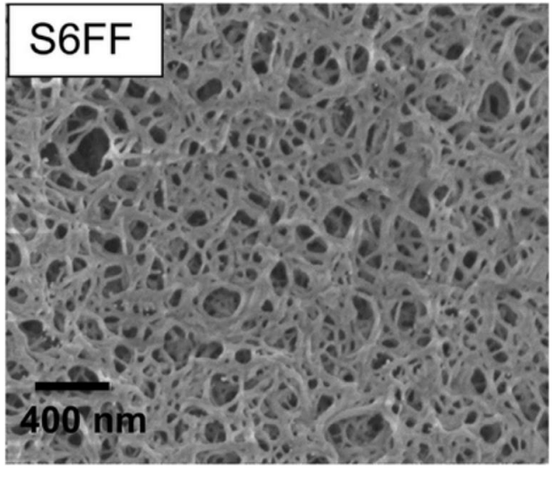
MOBILE PHASE

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

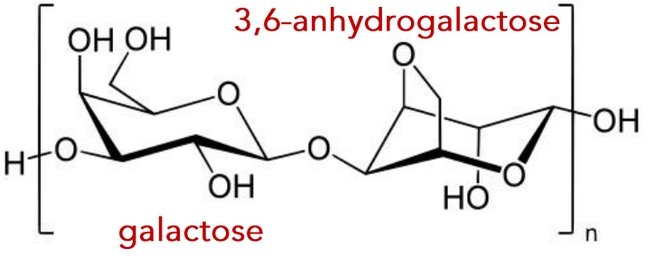
STATIONARY PHASE

- 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



6%-crosslinked agarose
Sepharose 6FF column



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

Mobile and Stationary Phases for SEC

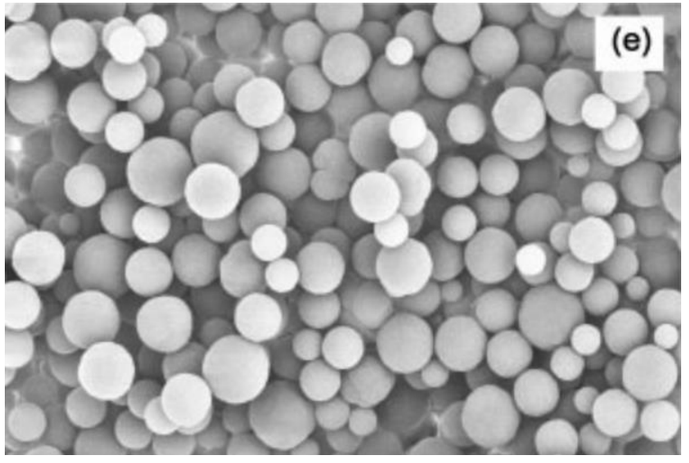
MOBILE PHASE

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

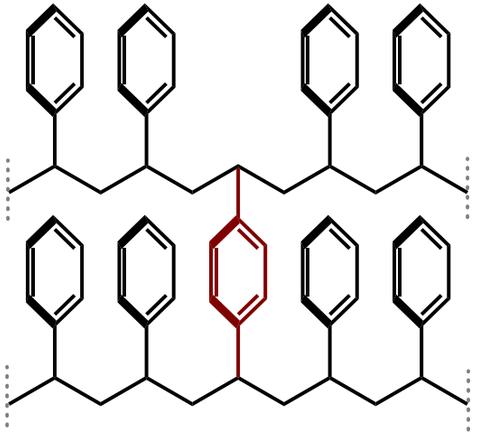
STATIONARY PHASE

- 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

- 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



Poly(styrene-*co*-divinylbenzene)

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

Choe, S. et al. *J. Poly. Sci. A – Poly Sci.* **2004**, *42*, 835–845.

Overview

Fundamentals and Theory of Chromatography

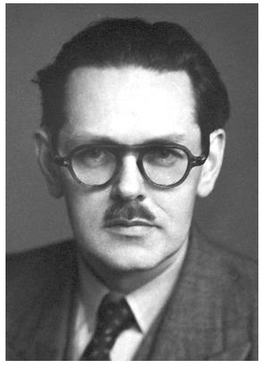
- Parameters affecting separation quality
- The Resolution equation
- The van Deemter equation

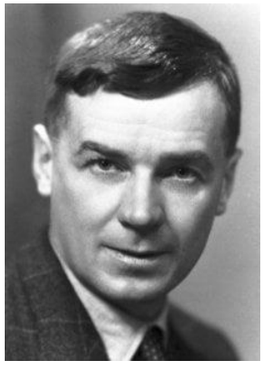
Three Common Types of Chromatography

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

Current Trends in Chromatography Research

Brief History of Chromatography





Waters™

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

1900
Mikhail Tsvet separates plant pigments on $CaCO_3$ with ether/EtOH and coins "*chromatography*" (color writing)

1941
Archer Martin & Richard Synge invent partition chromatography (hydrated SiO_2 used to separate amino acids)

1949
Martin & Anthony James invent gas chromatography

1969
Waters corporation commercializes first HPLC system, the ALC100 HPLC

1970-2000
Improvements to capacity, detector sensitivity, and instrument automation

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

A Principle Focus: Accommodating Smaller Packing

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

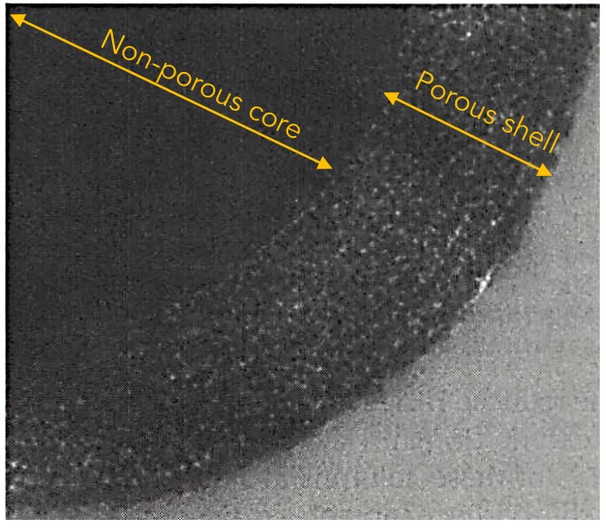
$$P = \frac{\eta L u}{K^o \pi r^2 \boldsymbol{d^2}}$$

- 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

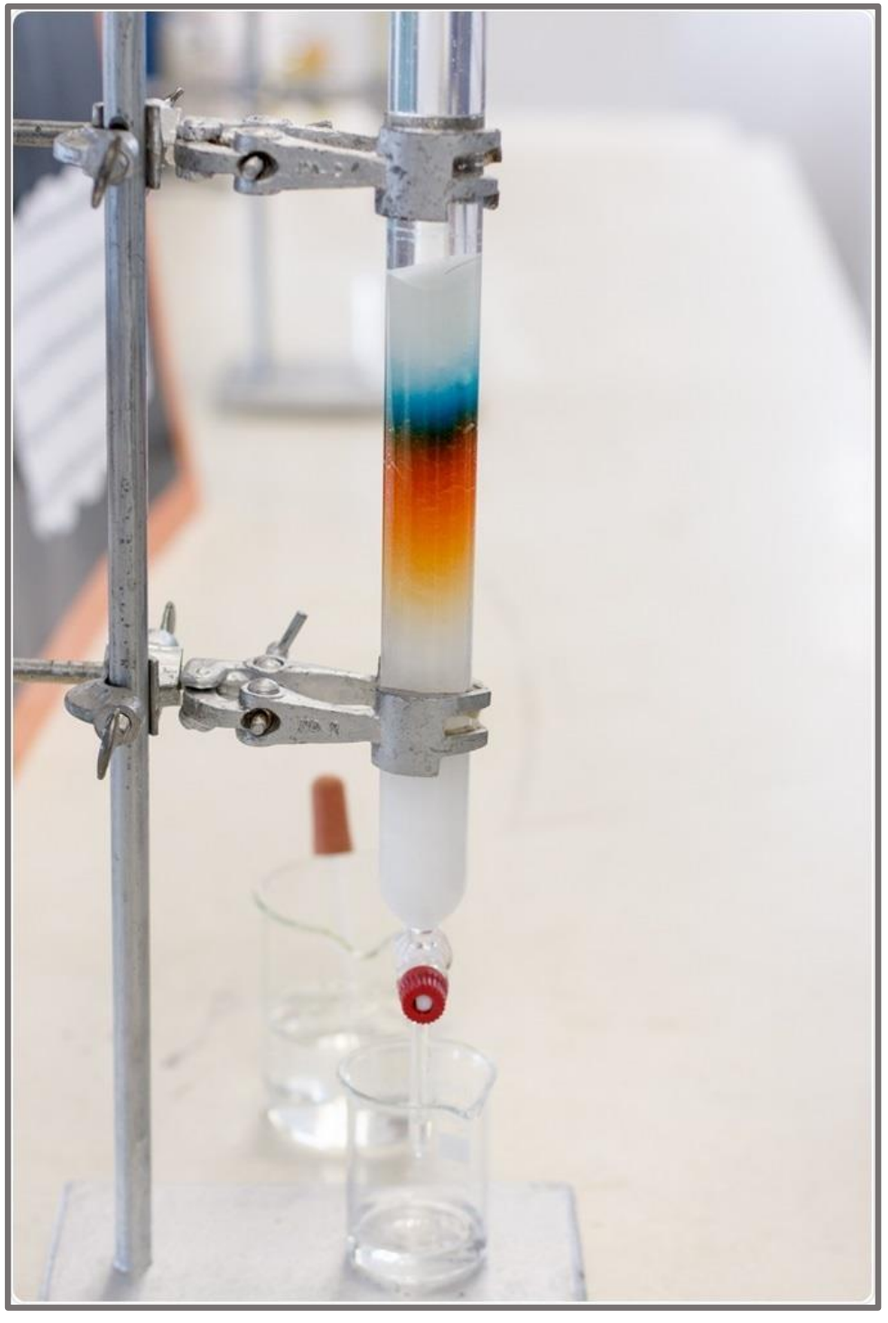
- Develop stationary phases that can withstand temperatures >200 ^{o}C (limit is currently around 80 ^{o}C)
- Invent narrower columns that can still be reliably packed with stationary phase
- Develop new stationary phase morphologies, such as **core-shell particles**



- 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

Gritti, F., Guiochon, G., *J. of Chromat.* **2012**, *1228.*

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