HPLC Separation Fundamentals

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Presentation Outline

- Major HPLC modes
- Key Equations
  - Resolution
  - van Deemter
- Common terms & definitions
- Key parameters & conditions that affect them
  - Efficiency, selectivity, and retention
- Role of pressure
  - Sub-2um
- Gradient

Presentation Objectives

Understand physical significance of chromatographic parameters, especially Retention Factor and Resolution

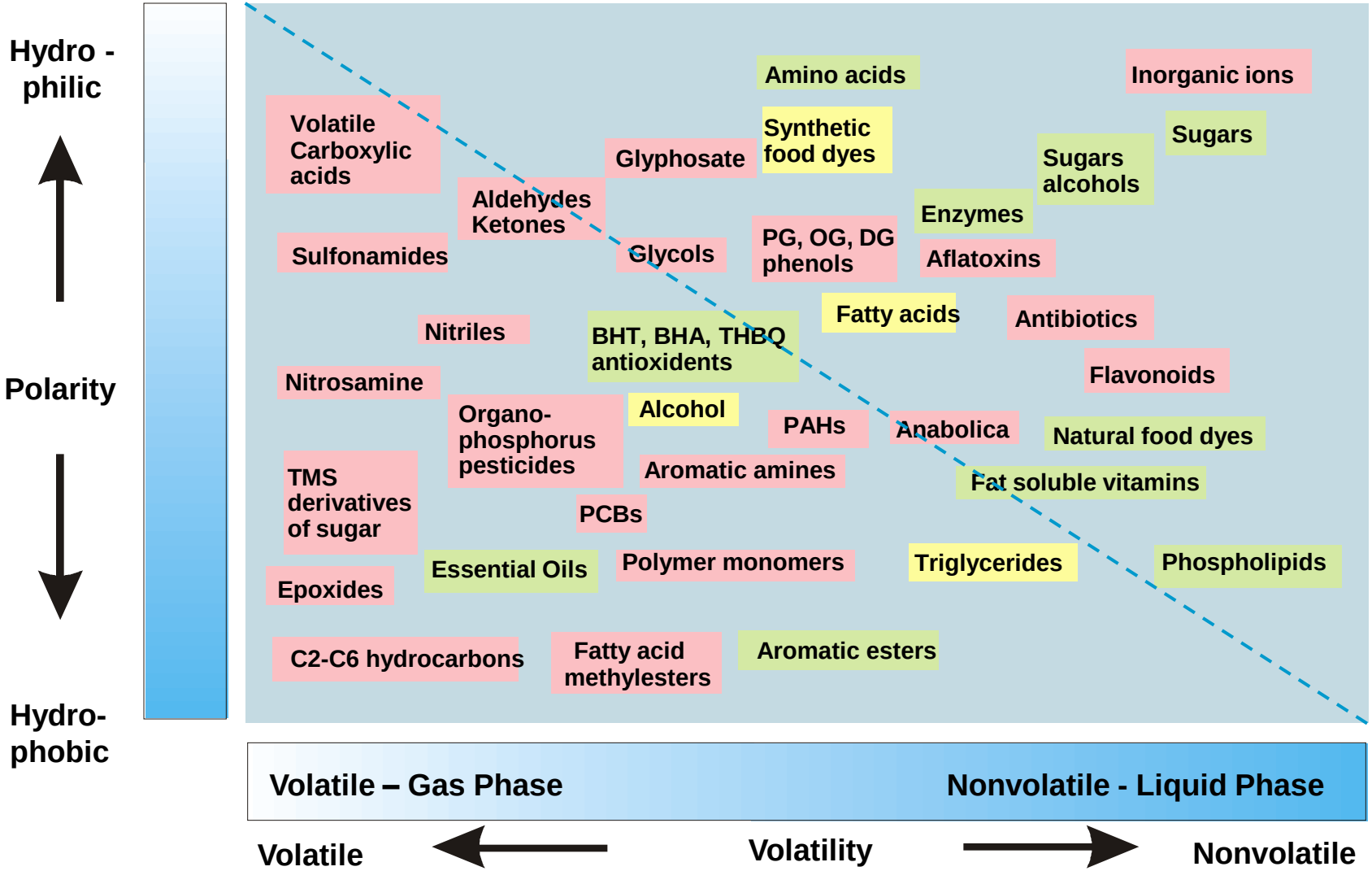
Understand effect of role of Selectivity and Column Efficiency in improving Resolution

Column Selection

Parameters that affect column pressure

Separation occurs in column volumes – not in time

Separation Techniques



Major Separation Modes of HPLC
A Review

There are four major separation modes that are used to separate most compounds:

- Reversed-phase chromatography (most popular)
- Normal-phase (adsorption) chromatography
- Ion exchange chromatography
- Size exclusion chromatography

Normal Phase or Adsorption Chromatography

What is Normal Phase Chromatography (NPLC)?

How does it work?

When would you choose normal phase?

Normal Phase or Adsorption Chromatography

Analyte partitions between mobile phase and stationary phase based upon polarity

Normal Phase or Adsorption Chromatography

Analyte partitions between mp and sp based upon polarity

- **Stationary Phase (column packing) is polar**
  - silica (strongest) > amino > diol > cyano (weakest)
- **Mobile Phase (solvent) is non-polar**
  - hexane, *iso*-octane, methylene chloride, ethyl acetate, etc.
- **More polar compounds are more retained**
- **Retention decreases (faster elution) as polarity of mobile phase increases**

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Reasons to choose normal phase

- **Different from RPLC**
- **Analyze very polar compounds**
- **Sample injection solvent is non-polar (*e.g.*, extraction from water)**
- **Recovery in non-polar solvents is desirable (*e.g.*, prep sep'n)**
- **Isomer separation (steric interaction with stationary phase)**

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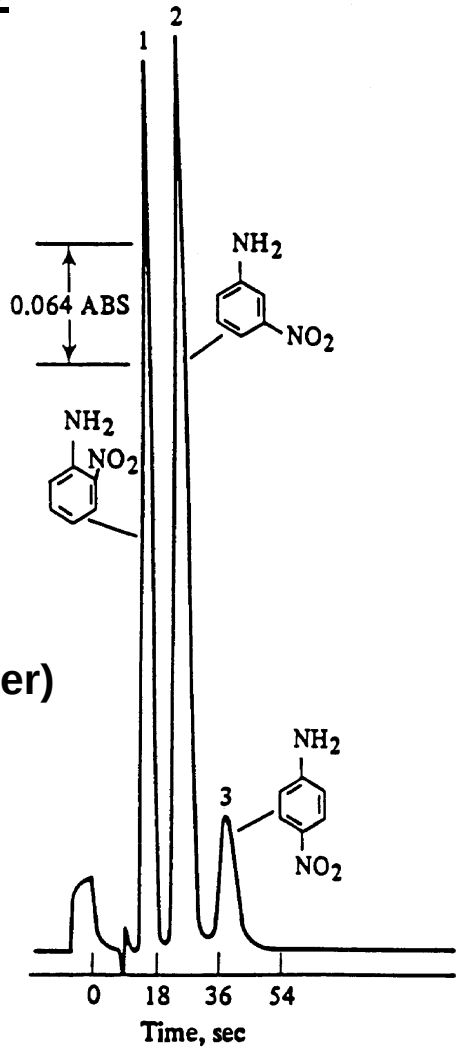
Separation of Nitroanilines on HPLC Column packed with silica gel using hexane (mobile phase component A) mixed with methylene chloride (mobile phase component B)



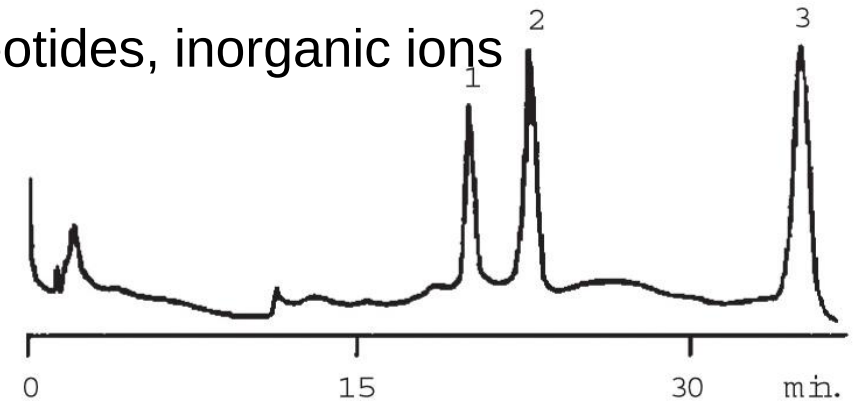
Ion Exchange Chromatography

In ion exchange:

- Electrostatic Interaction of analyte with stationary phase
- Column packing contains ionic groups,
  (*e.g*. sulfonate, tetraalkylammonium)
- Mobile phase is an aqueous buffer (*e.g*. phosphate, formate, etc.)
- Similarities to ion-pair chromatography
- Well suited to the separation of inorganic and organic anions and cations in aqueous solution
- Separations: Proteins, nucleotides, inorganic ions

Basic proteins on strong cation exchanger ($-SO_3^-$)

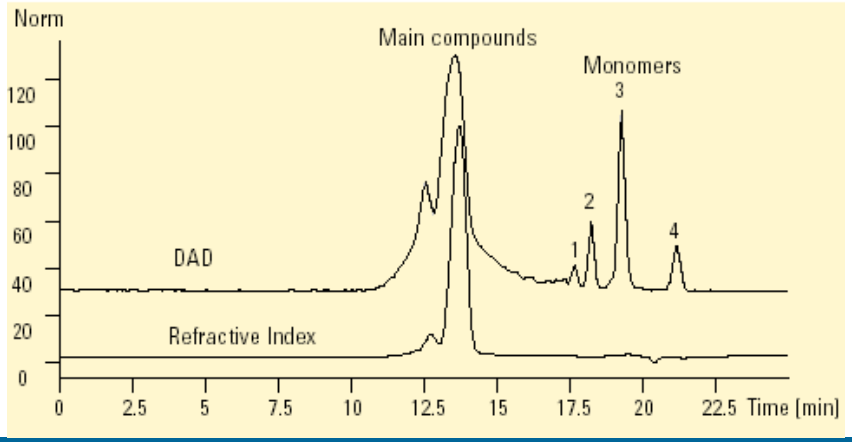
1. **RNA polymerase**
2. **Chymotrypsinogen**
3. **Lysozyme**



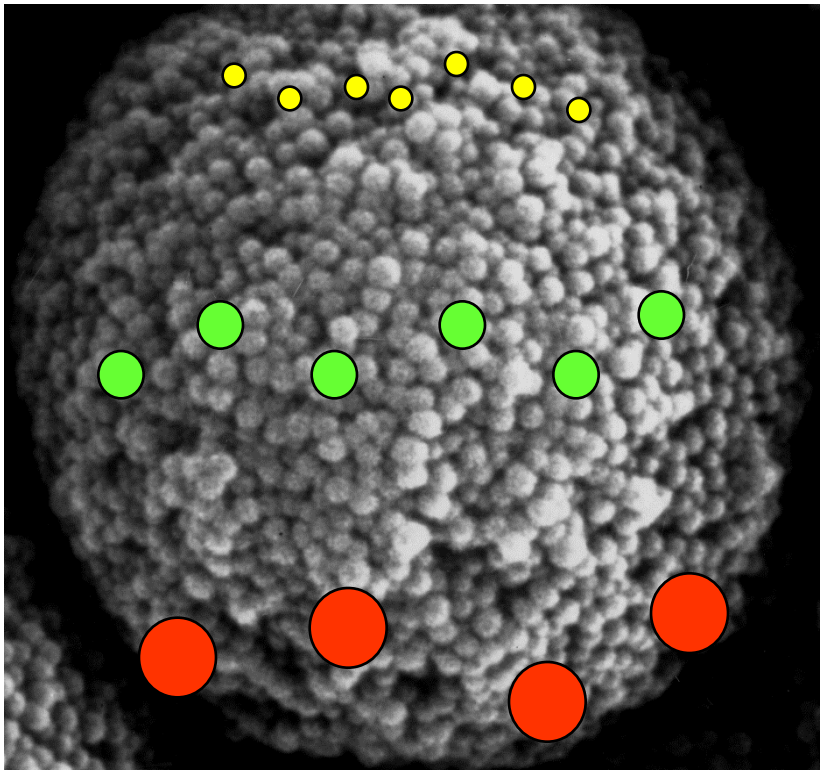
Size Exclusion Chromatography (SEC)

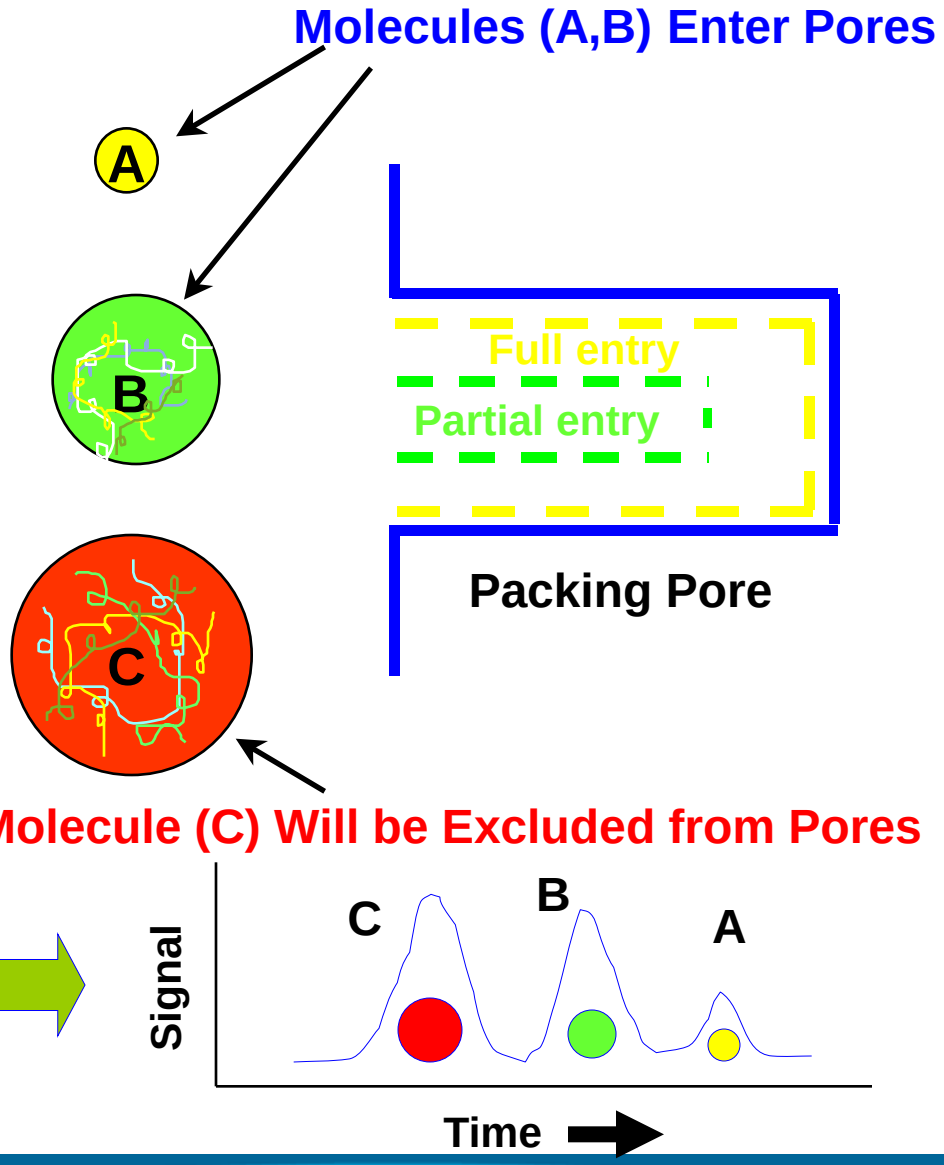
- There are two modes:
  - non-aqueous SEC [sometimes termed Gel Permeation Chromatography (GPC)]
  - aqueous SEC [sometimes referred to as Gel Filtration Chromatography (GFC)]
- No interaction between the sample compounds and packing material
  - Molecules diffuse into pores of a porous medium
  - Molecules are separated depending on their size relative to the pore size
    - ✓ molecules larger than the pore opening do not diffuse into the particles while molecules smaller than the pore opening enter the particle and are separated
    - ✓ large molecule elute first, smaller molecules elute later
- The mobile phase is chosen mainly to dissolve the analyte
- Used mainly for polymer characterization and for analysis of proteins.

Gel Permeation Chromatogram of Polybutadiene polymer on non-aqueous SEC (GPC) column;
The monomers elute after the polymer.
Column: PLgel mixed-D gel
Mobile phase: Tetrahydrofuran (THF)



Mechanism of SEC





Molecules must freely enter and exit pores to be separated. Largest molecules elute first, followed by intermediate size molecules and finally the smallest molecules elute last.

Reversed-Phase Chromatography (RPC)

Principle: Partition of analytes between polar mobile phase and non-polar stationary phase

- Nonpolar (nonspecific) interactions of analyte with hydrophobic (or lipophilic) stationary phase:
  - C18, C8, Phenyl, C3, etc.
- Different sorption affinities between analytes results in their separation
  - More polar analytes are less retained
  - Analytes with larger hydrophobic part are retained longer
- Mobile phase: water (buffer) + water-miscible organic solvent
  e.g. MeOH, ACN
- Can be used for non-polar, polar, ionizable and ionic molecules
- Gradient elution is often used for analysis of compounds with large differences in polarity

Chromatography Terms are All Around Us

But what do they mean…..

Gradient Steepness

Efficiency

Plates

Particle Size

Peak Capacity

3.5µm

Gradient Retention

RRLC

Peak Shape

Resolution

1.8µm

Retention Factor

Selectivity

UPLC

Tailing Factor

Rapid Resolution HT

RRHT

Rapid Resolution

Some Basic Chromatography Parameters

- **Retention Factor (*k*), Capacity Factor (*k'*)**
- **Selectivity or Separation Factor (α)**
- **Column Efficiency as Theoretical Plates (N)**
- **Resolution (R_s)**

Definition of Resolution

$$R_s = \frac{t_{R-2} - t_{R-1}}{(w_2 + w_1)/2} = \frac{\Delta t_R}{\bar{w}}$$

Resolution is a measure of the ability to separate two components

Resolution …

Determined by 3 Key Parameters – Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

$$R_S = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k + 1)}$$

N = Column Efficiency – Column length and particle size

α = Selectivity – Mobile phase and stationary phase

***k* = Retention Factor** – Mobile phase strength

Retention Factor (*k*), Capacity Factor (*k'*)

Chromatographic Separation is an Equilibrium Process

Sample Partitions between Stationary Phase and Mobile Phase

$$K = C_s/C_m$$

Compound moves through the column only while in mobile phase.

Separation occurs in Column Volumes.
(Flow is volume/time)

Retention Factor (*k*), Capacity Factor (*k'*)

$K = C_s/C_m \Rightarrow\Rightarrow \boxed{k = \frac{t_R - t_0}{t_0}}$

k is measure of number of column volumes required to elute compound.

Fundamental, dimensionless parameter that describes the retention.

***k* = 1 to 20 - OK; *k* = 3 to 10 - Better; *k* = 5 to 7 - Ideal**

Selectivity (α)

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

α is measure relative difference in retention

Selectivity (α)

$$\alpha = \frac{k_2}{k_1} = \frac{(t_{R2} - t_0)/t_0}{(t_{R1} - t_0)/t_0}$$

α is measure relative difference in retention

Selectivity (α)

$$\alpha = \frac{k_2}{k_1} = \frac{(t_{R2} - t_0)}{(t_{R1} - t_0)}$$

α is measure relative difference in retention

Selectivity (α)

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

α is measure relative difference in retention

By definition k_2 is more retained component;
k_1 is less retained component, so α is always ≥ 1

To obtain separation, α must be > 1

Column Efficiency (N)

N - Number of theoretical plates – This is one case where more is better! "Plates" is a term inherited from distillation theory. For LC, it is a measure of the relative peak broadening (or peak width) for an analyte in a separation – **w**

$$N = 16\left[\frac{t_R}{w}\right]^2$$

A Number of Theoretical Plates

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$$N = 16\left[\frac{t_R}{w}\right]^2 \quad \text{or} \quad N = \frac{L}{H}$$

Column length

HETP

A Number of Theoretical Plates

Resolution …

Determined by 3 Key Parameters – Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

$$R_S = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k + 1)}$$

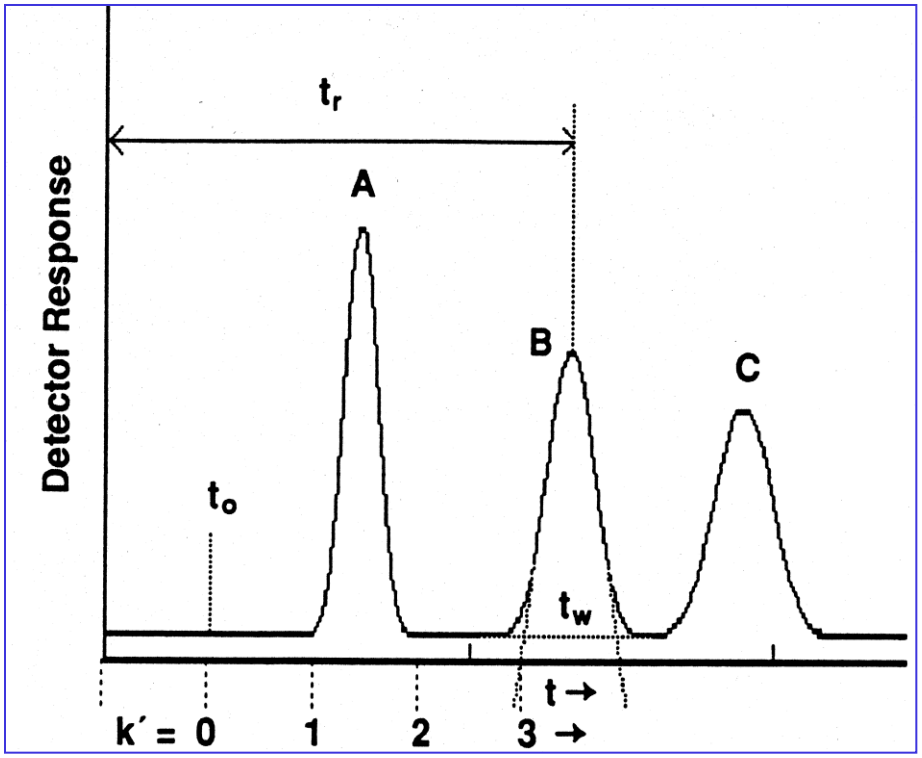
N = Column Efficiency – Column length and particle size

α = Selectivity – Mobile phase and stationary phase

***k* = Retention Factor** – Mobile phase

Chromatographic Profile

Equations Describing Factors Controlling R_S



Retention Factor

$$k = \frac{(t_R - t_0)}{t_0}$$

Selectivity

$$\alpha = k_2 / k_1$$

Theoretical Plates-Efficiency

$$N = 16(t_R / t_W)^2$$

$$= 5.54(t_R / W_{1/2})^2$$

Chromatographic Terms

Resolution

The distance between two neighboring peaks

R = 1.5 is baseline resolution

R = 2 is highly desirable during method development

$$R_s = 2 \frac{(t_{R2} - t_{R1})}{(w_1 + w_2)}$$

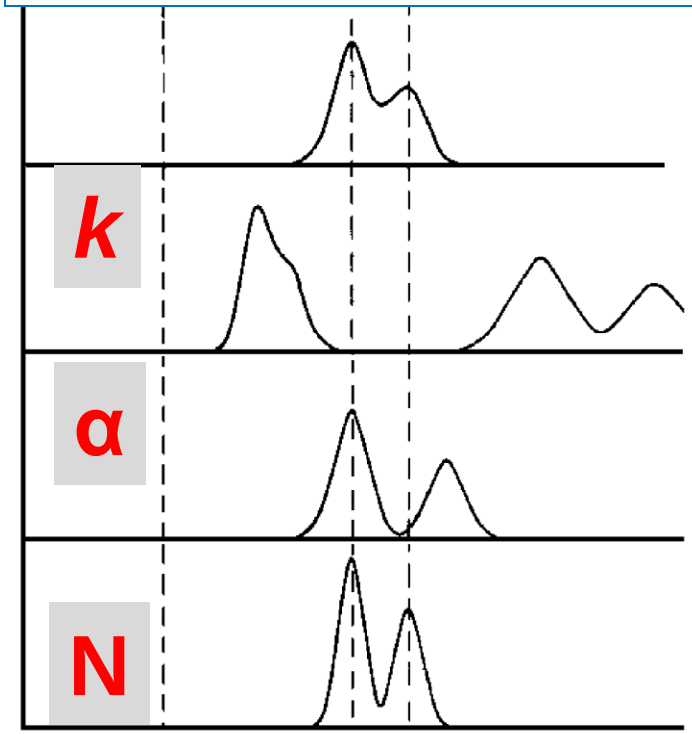
Resolution is increased by increased separation

Resolution is affected by how fat or wide the peaks are, so thin is better!

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

Factors that Improve Resolution

$$R_S = \frac{\sqrt{N}}{4} \frac{(\alpha-1)}{\alpha} \frac{k}{(k+1)}$$

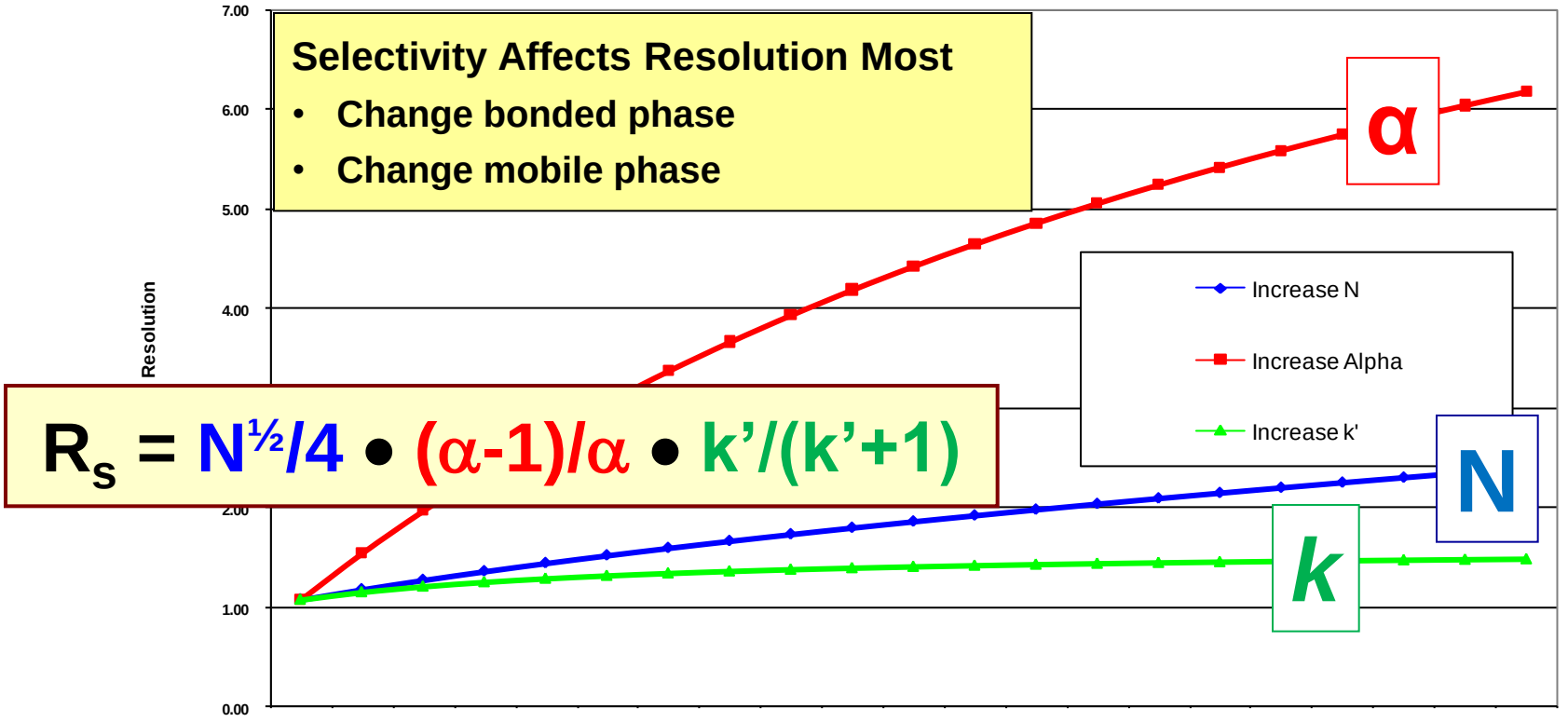


Increase retention

Change relative peak position

Reduce peak Width

Resolution as a Function of Selectivity, Column Efficiency, or Retention

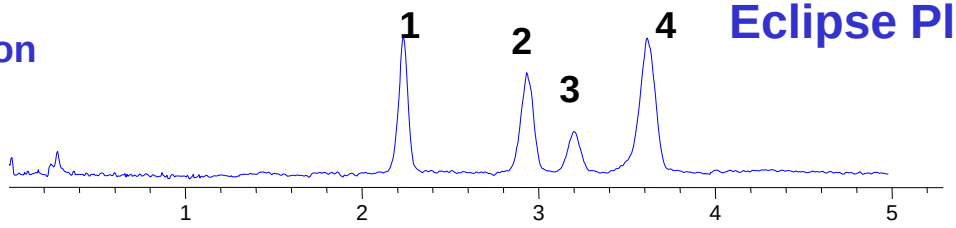


$$R_s = N^{1/2}/4 \bullet (\alpha-1)/\alpha \bullet k'/(k'+1)$$

Plates:	5000	10000	15000	20000	
Alpha:	1.10	1.35	1.60	1.85	2.1
k':	2.0	4.5	7.0	9.5	12.0

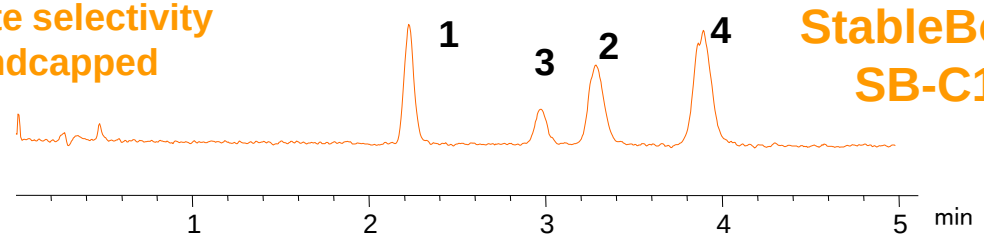
Similar Stationary Phases May Give Different Selectivity

1st choice
Best Resolution & Peak Shape



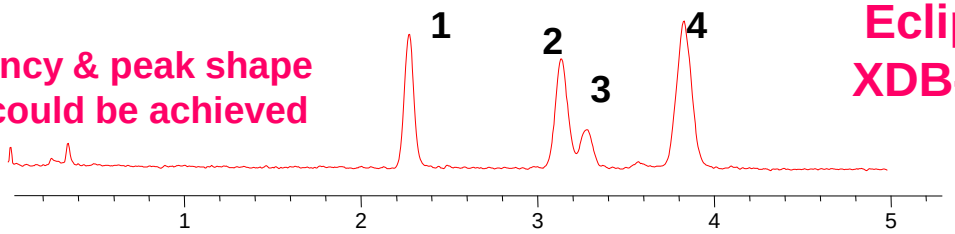
Eclipse Plus C18

2nd choice
Good alternate selectivity due to non-endcapped



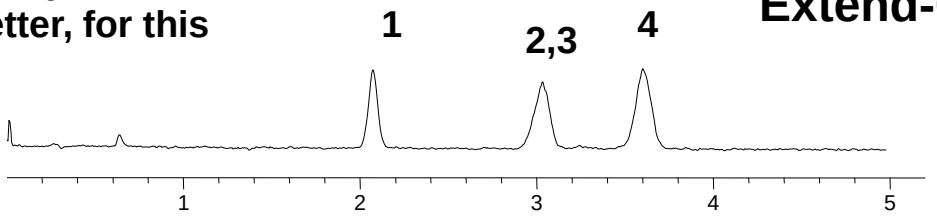
StableBond SB-C18

3rd choice
Good efficiency & peak shape
Resolution could be achieved



Eclipse XDB-C18

4th choice
Resolution not likely, Other choices better, for this separation.



Extend-C18

Mobile phase: (69:31) ACN: water
Flow 1.5 mL/min.
Temp: 30 °C
Detector: Single Quad ESI positive mode scan
Columns: RRHT 4.6 x 50 mm 1.8 um

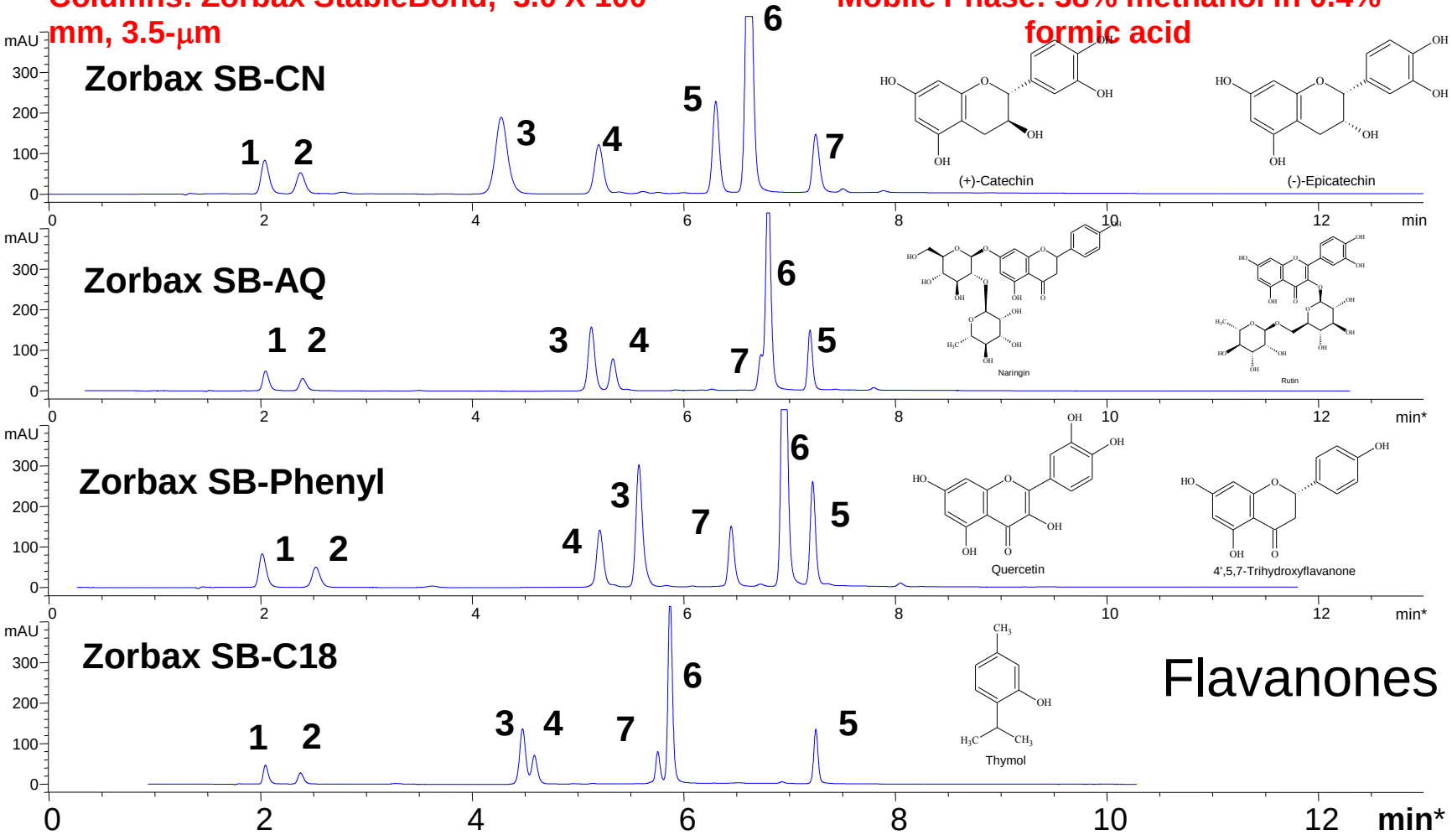
Sample:
1. anandamide (AEA)
2. Palmitoylethanolamide (PEA)
3. 2-arachinoylglycerol (2-AG)
4. Oleoylethanolamide (OEA)

Multiple bonded phases for most effective method development.
Match to one you're currently using.

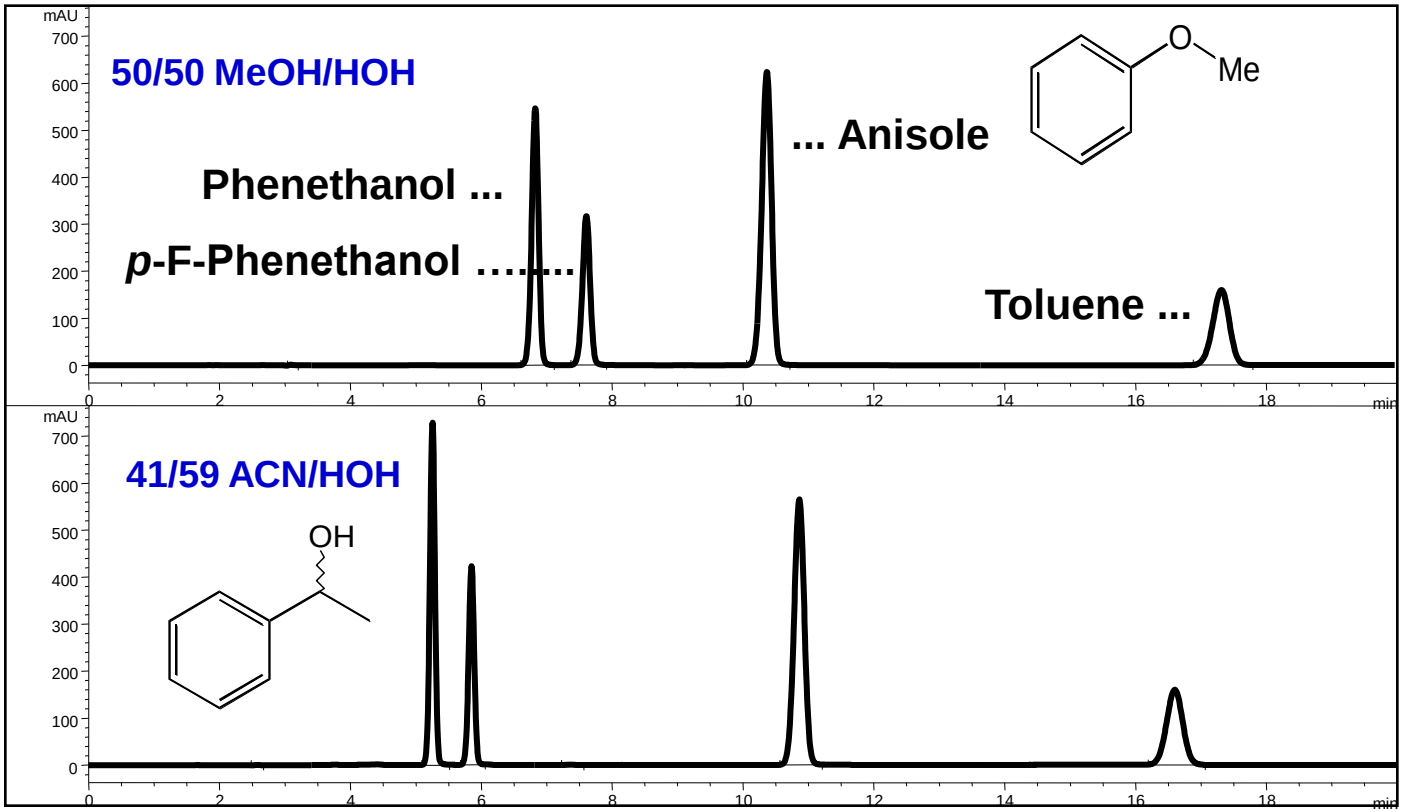
Different Stationary Phases May Give Significantly Different Selectivity

Columns: Zorbax StableBond, 3.0 X 100 mm, 3.5-µm

Mobile Phase: 38% methanol in 0.4% formic acid



Different Mobile Phases May Give Different Selectivity



ZORBAX® SB-C18 4.6 x 250 mm
1 mL/min, 40°C, 225 nm

If α Has the Most Impact, Why Focus on N? It's Easy – Very Predictable, Brute Force

High plate number (N) provides:

- **Sharp and narrow peaks**
- **Better detection**
- **Peak capacity to resolve complex samples**

But…

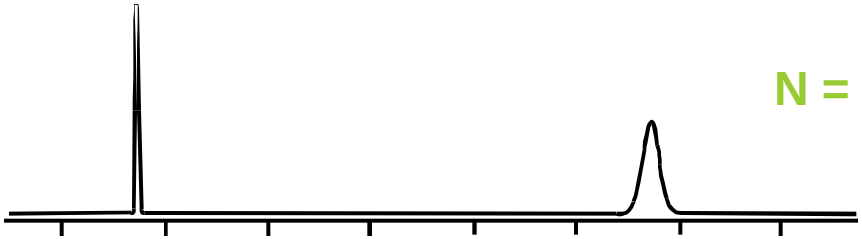
- Resolution increases only with the square root of the plate number.
- Plate number increase is limited by experimental conditions (analysis time, pressure)

Selectivity (α) helps best but… Is difficult to predict, so method development is slower (experience helps, model retention)

Note: Software supported, optimization for separation of multi-component mixtures can reduce method development time (ChromSword, DryLab)

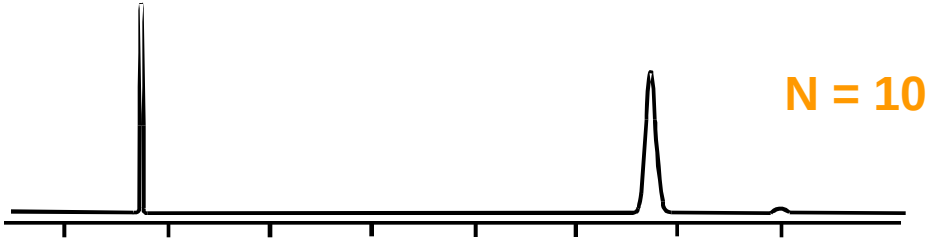
Column Efficiency (N) Increases with Decreasing Particle Size (d_p)

5um



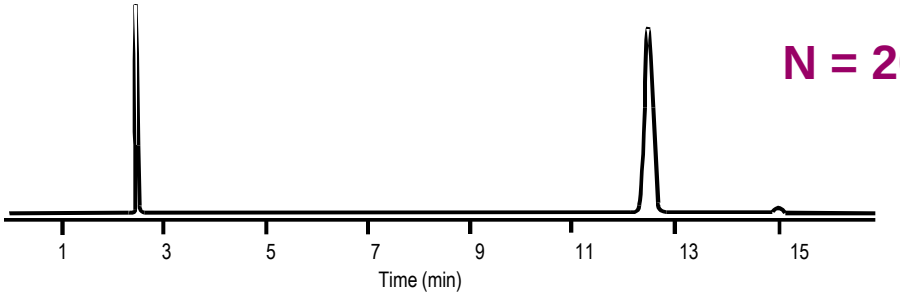
N = 5000 theoretical plates

3.5um



N = 10,000 theoretical plates

1.8um



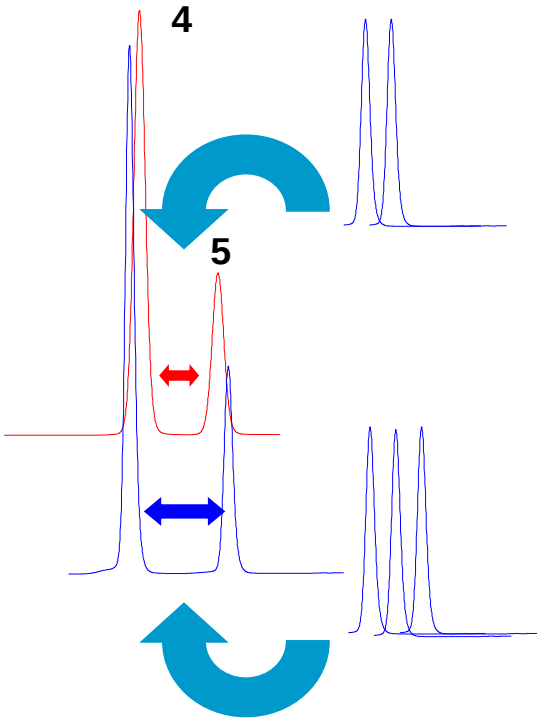
N = 20,000 theoretical plates

Peak Capacity

Better Peak Capacity – More Peaks Resolved

Peak capacity is the number of peaks that can be separated (at a specified resolution, example R=1) by a given system (think column length, particle size) in a given amount of time.

It is another measure of efficiency.



5µm

2 peaks fit

1.8µm

3 peaks fit
1/3 More!!

R_s = +50%

Decreasing Particle Size

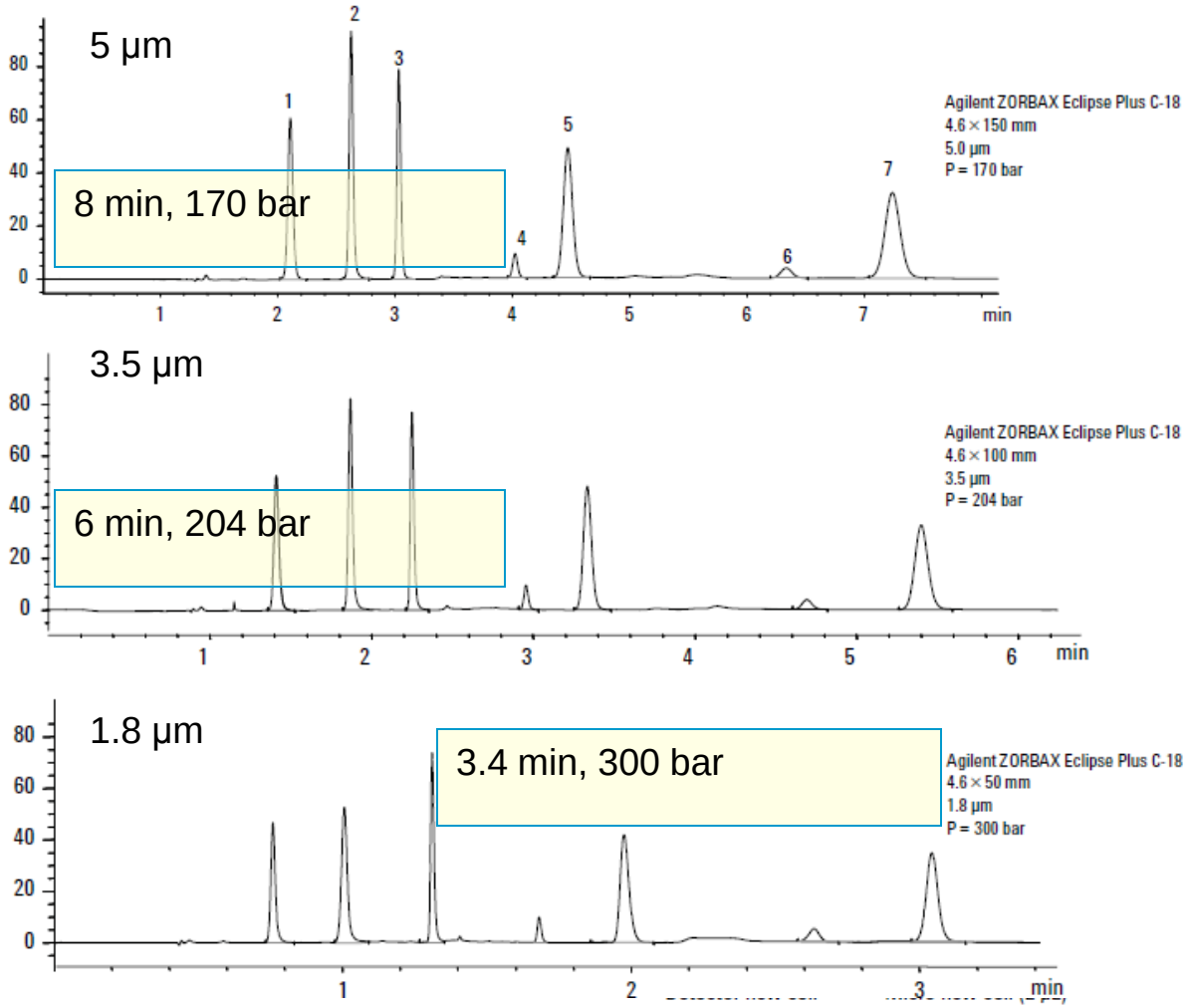


Table 2. Chromatographic Conditions

LC	Agilent 1200 SL
Mobile phase A	25 mM NaH_2PO_4 pH = 2.5
Mobile phase B	Methanol
Flow rate	1.00 mL/min
Column compartment temperature	35 °C
Detection	220 nm, no Reference
Response time	0.05 s
Injection volume	Adjusted for column size: 5 µm, 5 µL 3.5 µm, 3.3 µL 1.8 µm, 1.7 µL
Detector flow cell	Micro flow cell (2 µL)

Table 3. Gradients for Equivalent k*

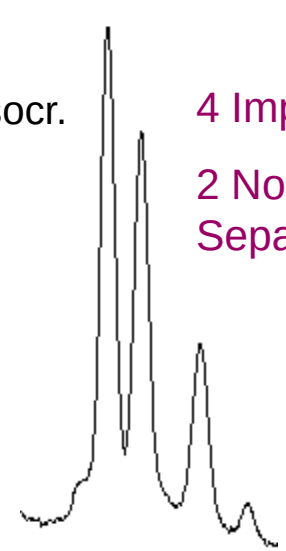
%B	5 µm	3.5 µm	1.8 µm
1	0.00 min →	0.00 min →	0.00 min
12	1.50 min	1.00 min	0.50 min
30	1.53 min	1.03 min	0.51 min

Smaller Particle Size Columns Improve Resolution – But Pressure Increases

Up to 60% higher resolution than in conventional HPLC

Customer Example Isocr. Impurity Method

Zoom of critical time range @ 7min



4 Impurities

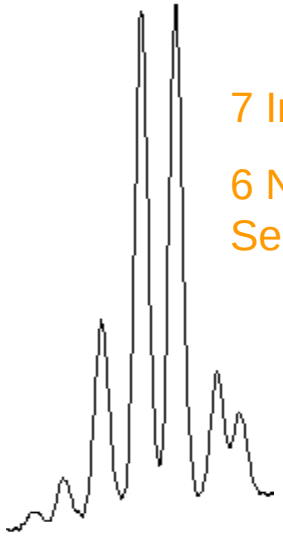
2 Not Baseline Separated!

4.6 x 150, 5um

93 bar

N = 7,259

Rs = 1.15



7 Impurities

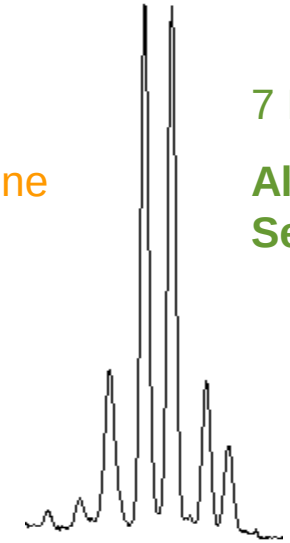
6 Not Baseline Separated!

4.6 x 150, 3.5um

165 bar

N = 14,862

Rs = 1.37



7 Impurities

All 7 Baseline Separated!

4.6 x 150, 1.8um

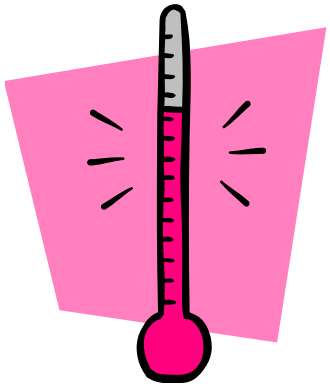
490 bar

N = 28,669

Rs = 1.80 (+57%)

Higher Temperature as an Aid to Method Development and Faster Operation

Higher Temperature:



Temperature should always be considered as a parameter during method development

Decreases Mobile Phase Viscosity

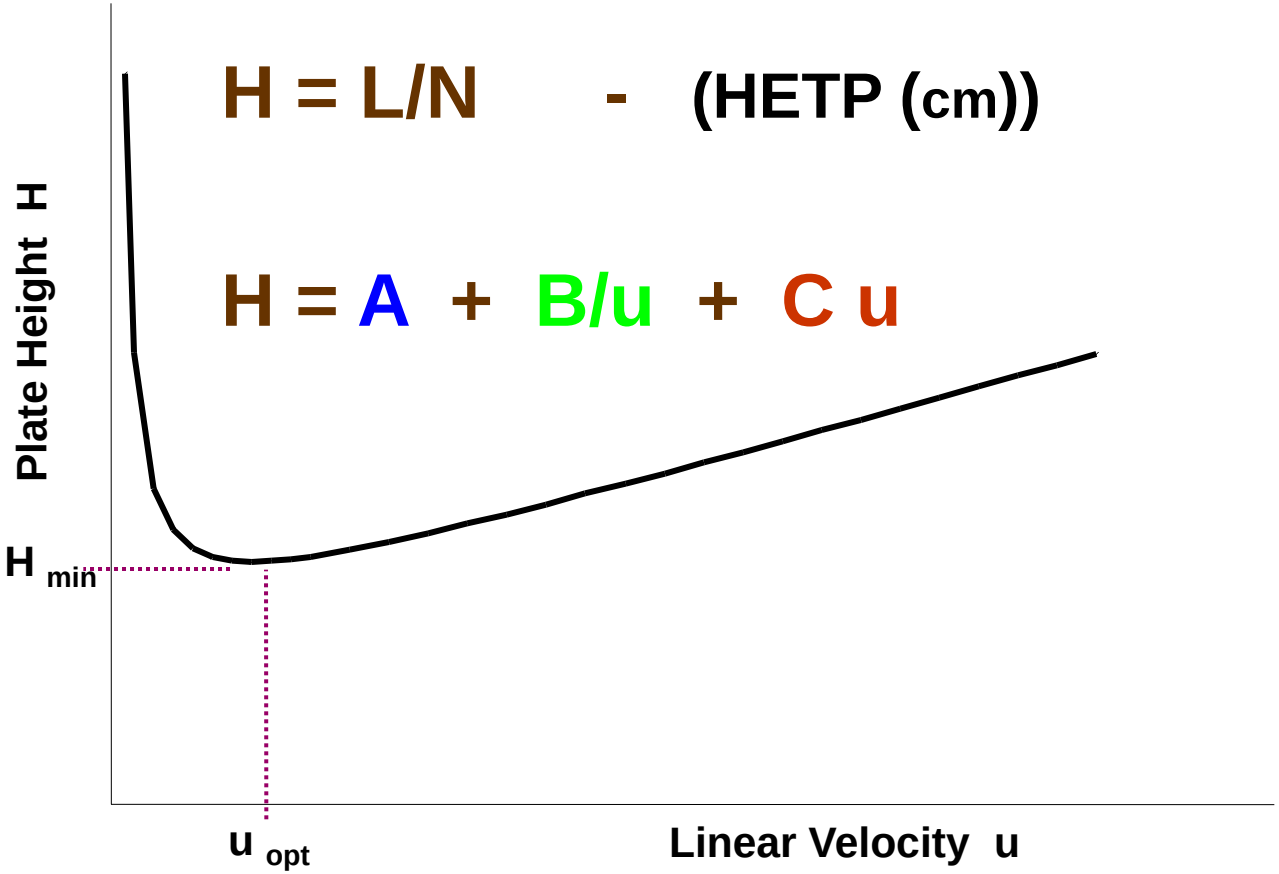
- Lowers backpressure – allows for higher flow rates, faster separations, greater efficiency and use of sub 2-micron columns

Provides more rapid mass transfer:

- Improves Efficiency – enhances resolution
- Decreases analysis time – faster separations with no loss in resolution

Can change selectivity – optimize resolution

Effect of Flow Rate on Column Efficiency
The van Deemter Equation



The smaller the plate height, the higher the plate number and the greater the chromatographic resolution

Effect of Flow Rate on Column Efficiency

Eddy Diffusion

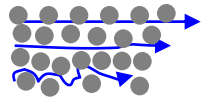
$H = L/N$

$H = \underline{A}$

Plate Height H

H_{min}

Eddy Diffusion



Linear Velocity u

Effect of Flow Rate on Column Efficiency
Longitudinal Diffusion

$H = L/N$

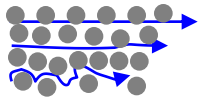
$H = A + B/u$

Plate Height H

H_{min}

Longitudinal diffusion

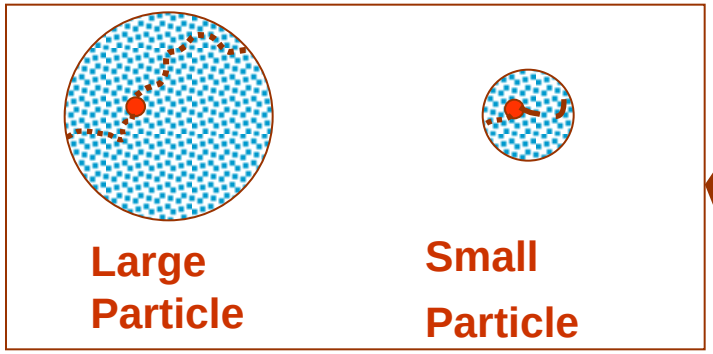
Eddy Diffusion



Linear Velocity u

Effect of Flow Rate on Column Efficiency

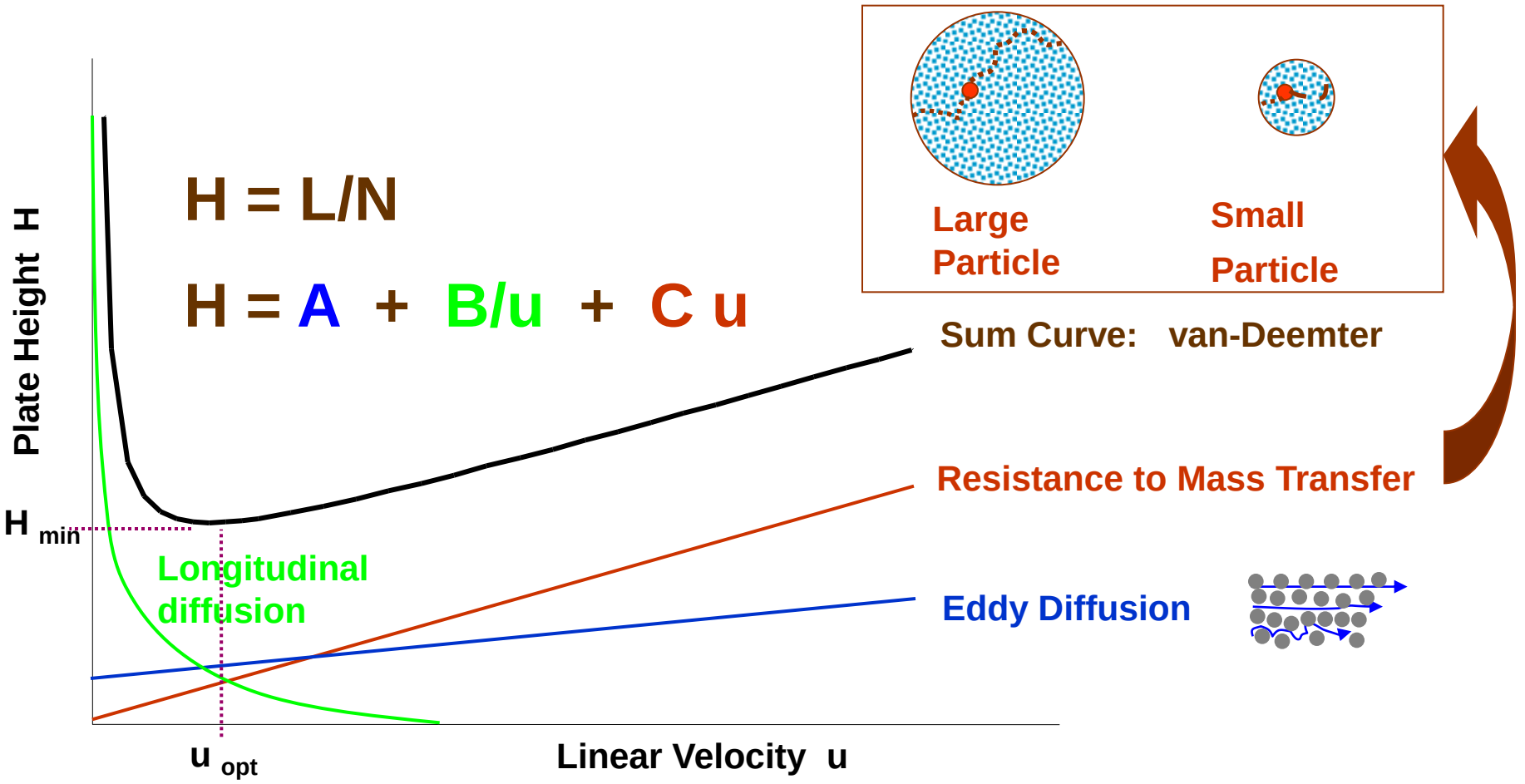
Resistance to Mass Transfer



$$H = L/N$$

$$H = A + B/u + C\,u$$

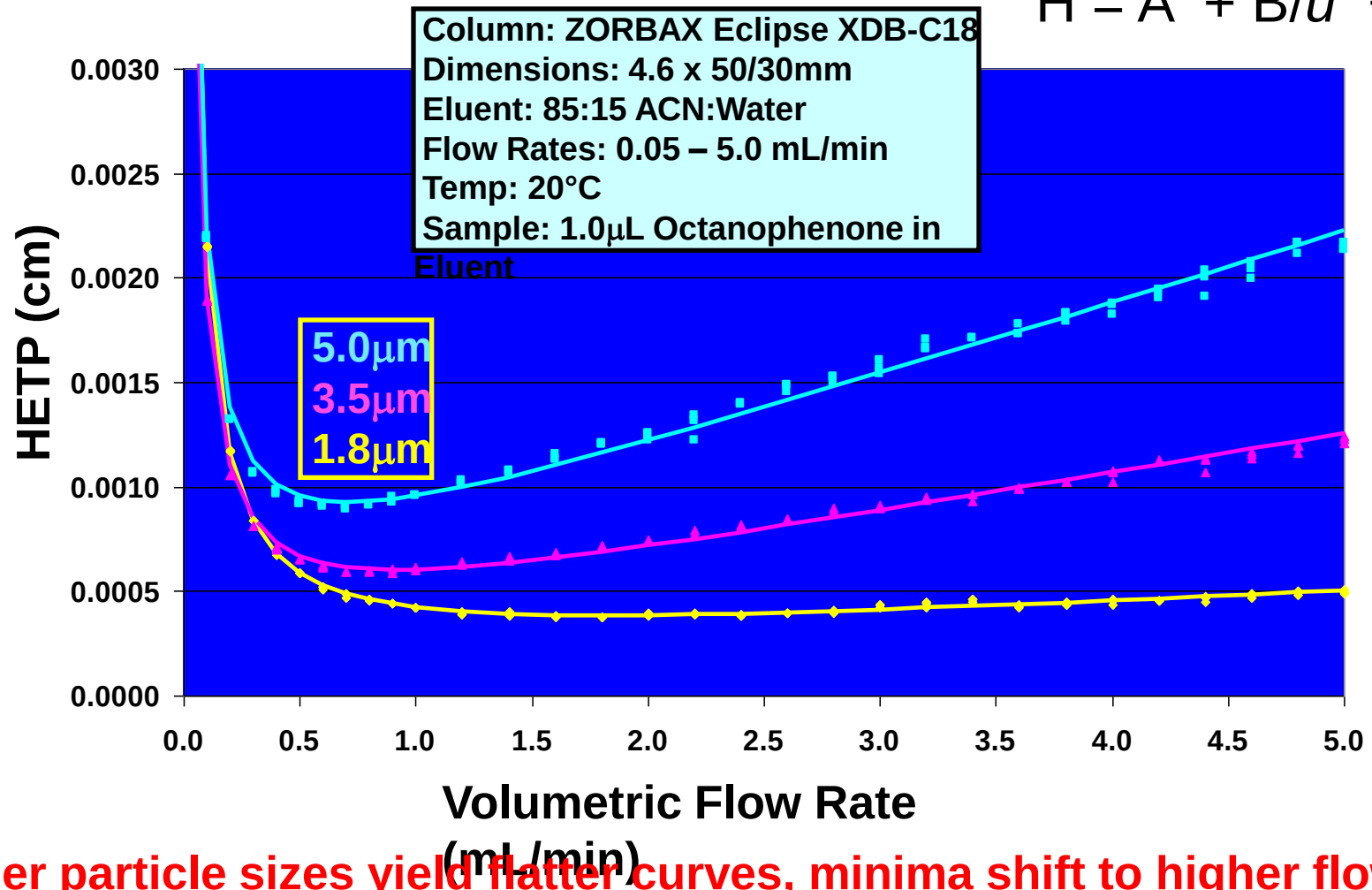
Putting it Together
The van Deemter Equation



The smaller the plate height, the higher the plate number and the greater the chromatographic resolution

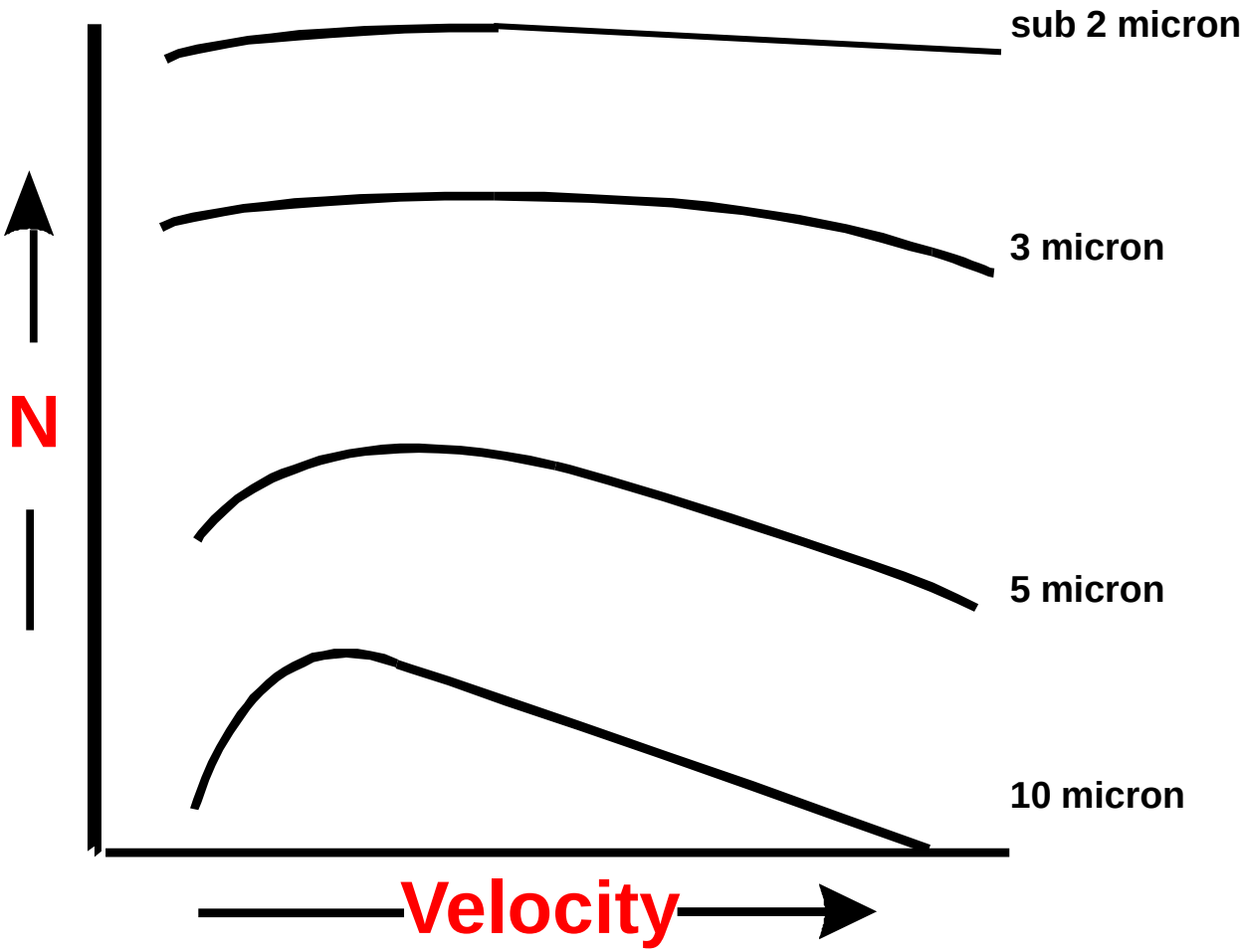
Van Deemter Curve
Effect of Particle Size

$$H = A + B/u + Cu$$



Smaller particle sizes yield flatter curves, minima shift to higher flow rates

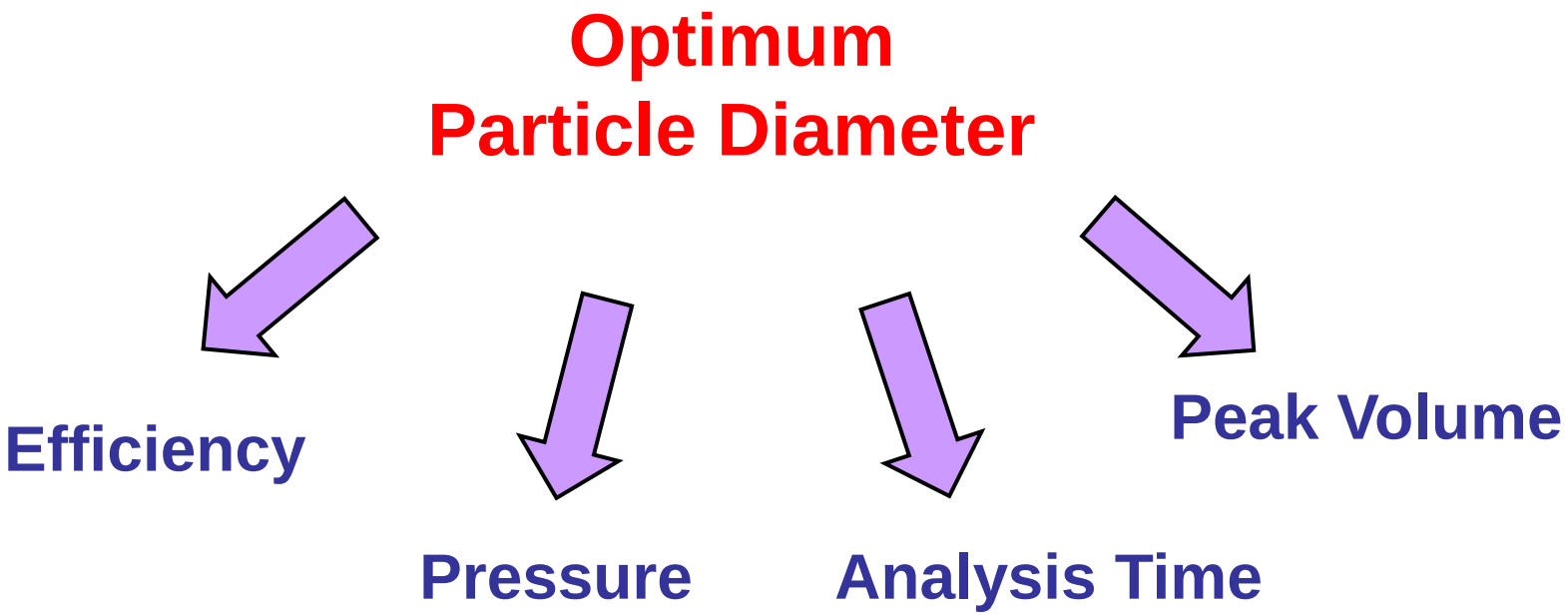
Columns Packed with Smaller Particles Provide Higher Efficiency



$N \propto 1/(d_p)$

$P \propto 1/(d_p)^2$

Key Chromatographic Parameters Affected by Particle Size



What About Pressure?

Pressure Increases with Decreasing Particle Size

Equation For Pressure Drop Across an HPLC Column

$$\Delta P = \frac{\eta \cdot L \cdot v}{\theta \cdot d_p^2}$$

ΔP = Pressure Drop

η = Fluid Viscosity

L = **Column Length**

v = Flow Velocity

d_p = **Particle Diameter**

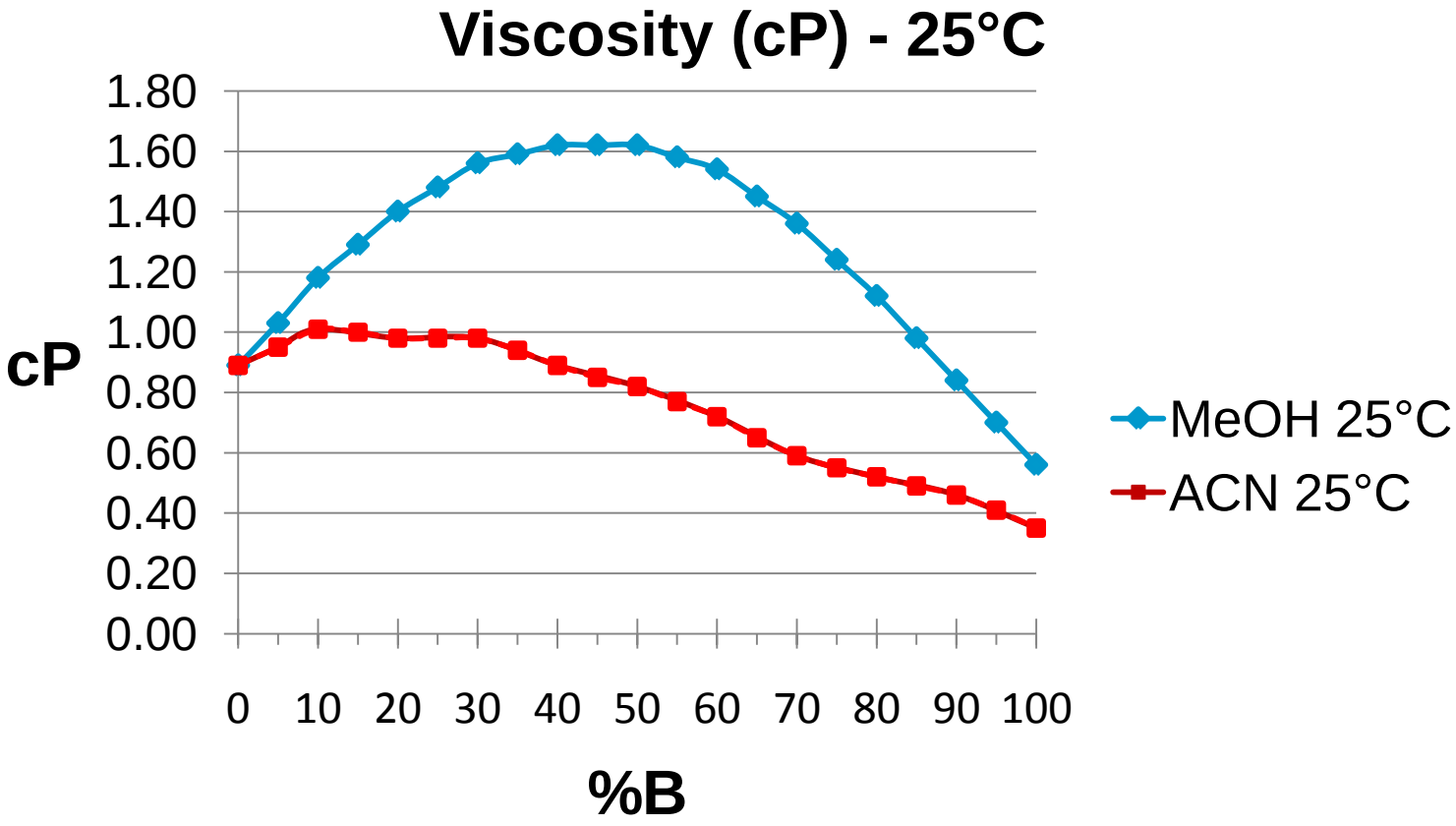
θ = Dimensionless Structural Constant of Order 600 For Packed Beds in LC

- ✓ Many parameters influence column pressure
- ✓Particle size and column length are most critical
- ✓Long length and smaller particle size mean more resolution and pressure
- ✓ We can now handle the pressure

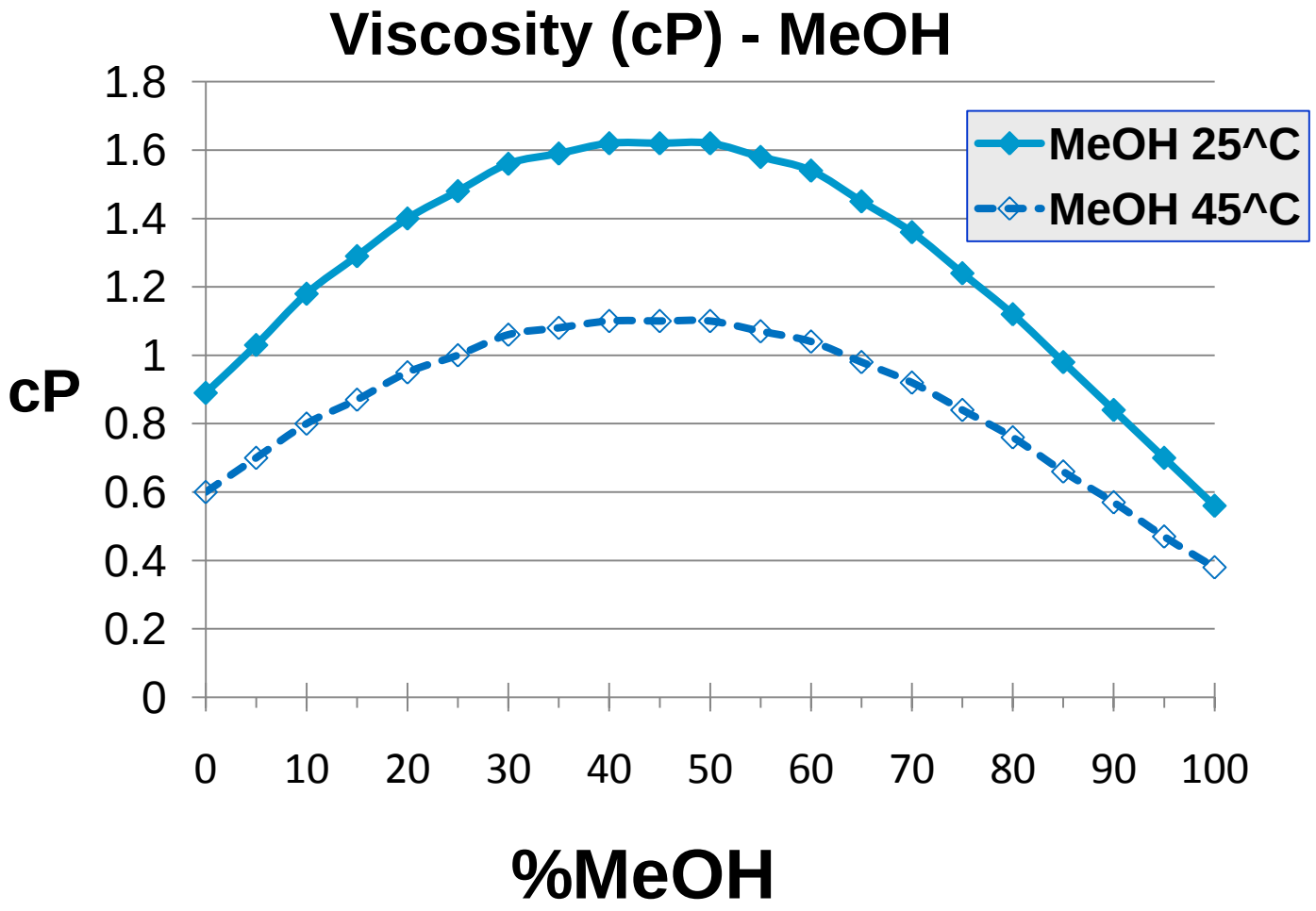
Mobile Phase
How Does it Impact Pressure

1. Solvent viscosity – lower viscosity results in lower pressure
   - **Acetonitrile < Methanol** The difference between MeOH and ACN can be dramatic and is the first thing to change if lower pressure is needed.
   - **Water < Buffer** While buffers increase viscosity, the organic selected is more critical. Make sure the buffer is soluble in the organic at all points in the run (gradient).
2. % of organic solvent – there is a pressure maximum and minimum for organic:aqueous mobile phases and it differs depending on the organic
   - A 2.1 x 100mm column can be used with ACN below 400 bar, especially with slightly elevated temperature.
   - But with MeOH you will need the 600 bar LC systems for almost all MeOH water mobile phases.

Comparison of Effect of Water/ACN and Water/MeOH on Viscosity



Comparison of Effect of Temperature on Viscosity



Gradient Retention (k*)

Selectivity in gradient elution is determined by the gradient retention factor

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

$$k^* = \frac{t_g F}{S \Delta\Phi V_m}$$

$\Delta\Phi$ = change in volume fraction of B solvent
S = constant
F = flow rate (mL/min.)
t_g = gradient time (min.)
V_m = column void volume (mL)

- **S ≈ 4–5 for small molecules**
- **10 < S < 1000 for peptides and proteins**

In gradient separation the effective value of k (k*) for different bands will be about the same.

This Relationship Says that to Keep Relative Peak Position in the Chromatogram Unchanged

Any Decrease in	**Can be Offset by a Proportional**
Column length	Decrease in $\mathbf{t_G}$ or F Increase in $\Delta\Phi$
Column volume (i.d.)	Decrease in t_G or $\mathbf{F^2}$ Increase in $\Delta\Phi$
$\Delta\Phi$ (same column)	Decrease in $\mathbf{t_G}$ or F

$$k^* = \frac{t_G \bullet F}{S \bullet \Delta\Phi \bullet Vm}$$

Two Chromatograms Both Having the Same Gradient Steepness with Respect to Column Volumes

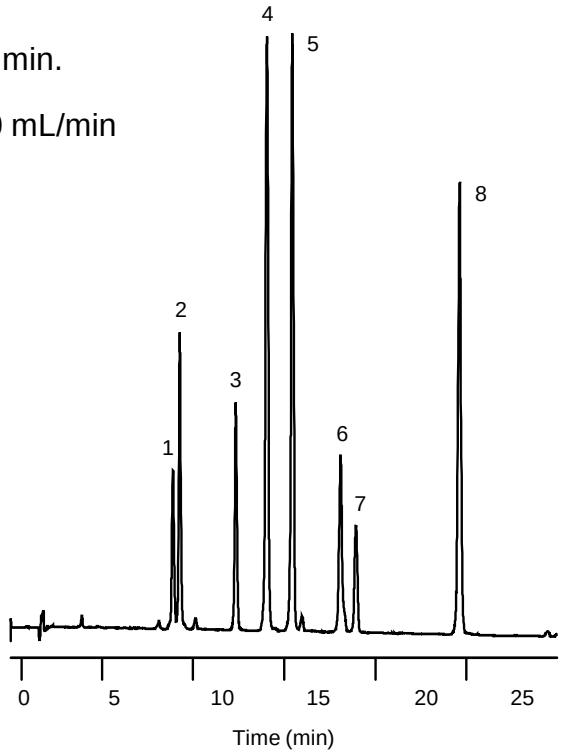
Sample: 1. Tebuthiuron 2. Prometon 3. Prometryne 4. Atrazine 5. Bentazon 6. Propazine 7. Propanil 8. Metolachlor

Column: **StableBond SB-C8 4.6 x 150 mm, 5 µm**

Gradient Time: 30 min.

Flow Rate: 1.0 mL/min

Analysis Time: 24 min

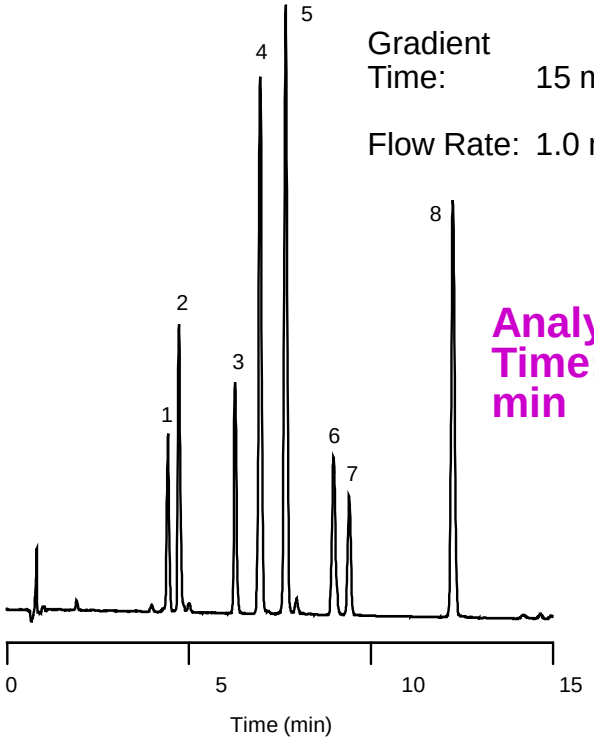


Column: **Rapid Resolution StableBond SB-C8 4.6 x 75 mm, 3.5 µm**

Gradient Time: 15 min.

Flow Rate: 1.0 mL/min

Analysis Time: 12 min



Presentation Objectives

Understand physical significance of chromatographic parameters, especially Retention Factor and Resolution

Understand effect of role of Selectivity and Column Efficiency in improving Resolution

Column Selection

Parameters that affect column pressure

Separation occurs in column volumes – not in time

Summary

HPLC is a powerful analytical tool

Most applications are Reversed-Phase

It's important to understand what the terms mean
- Hopefully they help understand how the separation works

Key Equations
- Resolution (R_s) – Many parameters can affect resolution
- Retention Factor (*k*)- Separation occurs in column volumes
- van Deemter – Effect if particle size, flow rate on N

Pressure does not affect separation - it is a result of chromatographic conditions

Separation occurs in column volumes

THANK YOU !

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