

Concepts in Chromatography

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Outline for Today's Session

1 Introduction To The Technique

Where was chromatography born?
Where and why is chromatography used?
How does it work?

2 Modes Of Chromatography

Reverse Phase
Normal Phase

3 What Makes A Great Separation?

What are goals of a good separation?
Efficiency
Selectivity

4 Mobile Phases

Isocratic v Gradient Methods

5 Conclusions

Mikhail Tswett

Inventor of Chromatography

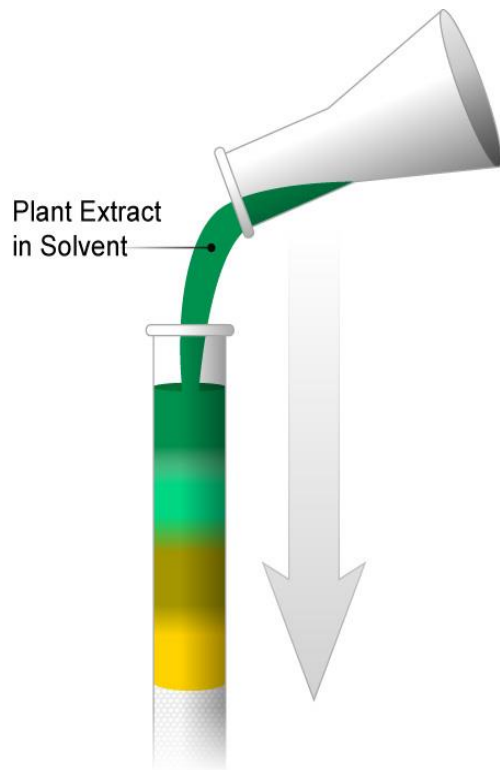
- Russian Botanist
 - May 14th, 1872 – June 26th, 1919
- Invented “liquid-adsorption column chromatography” in 1903 as a technique to separate chlorophylls and carotenoids from plants

Chroma -- color
Graphy -- writing/study of



Tswett's Experiment

- Tall glass open column filled with sand-like particles
- Ground-up plant extract
- Poured into the column and saw colored bands develop as the extract percolated down through the column
- Different compounds had separated



Where is chromatography used today?

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



Drugs



Pesticides



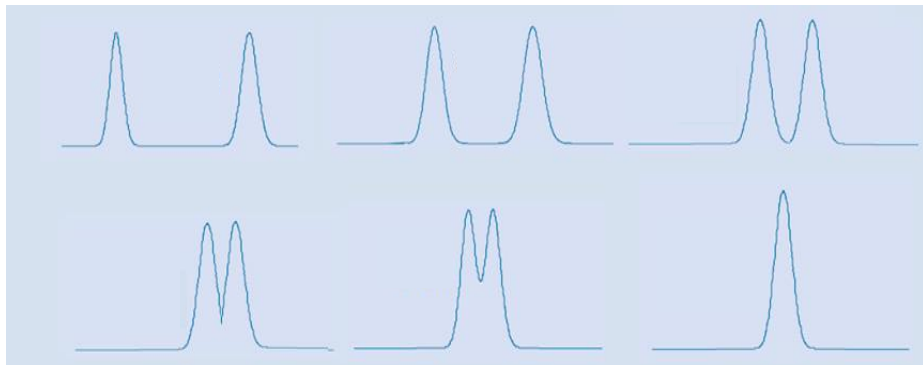
Food



Pollution

Why is chromatography used?

- Separating mixtures
- What is it? (Identification)
- How much is there? (Quantification)



Terminology of Chromatography

■ Resolution

- *Amount of separation between peaks*

■ Hydrophobic

- *Dislikes water*
- *Non-polar*
- *Examples: Oil, fatty acids, steroids*

■ Hydrophilic

- *Likes water*
- *Polar*
- *Examples: Low molecular weight acids, DNA, ionic compounds, +/-charged compounds*

■ Selectivity

- *The ability of the chromatographic system to separate one compound from another and create the elution order of the peaks.*

How Does Chromatography Work?

- We create a separation by changing the *relative speed of each* analyte “band”
 - Competition between the mobile phase and stationary phase

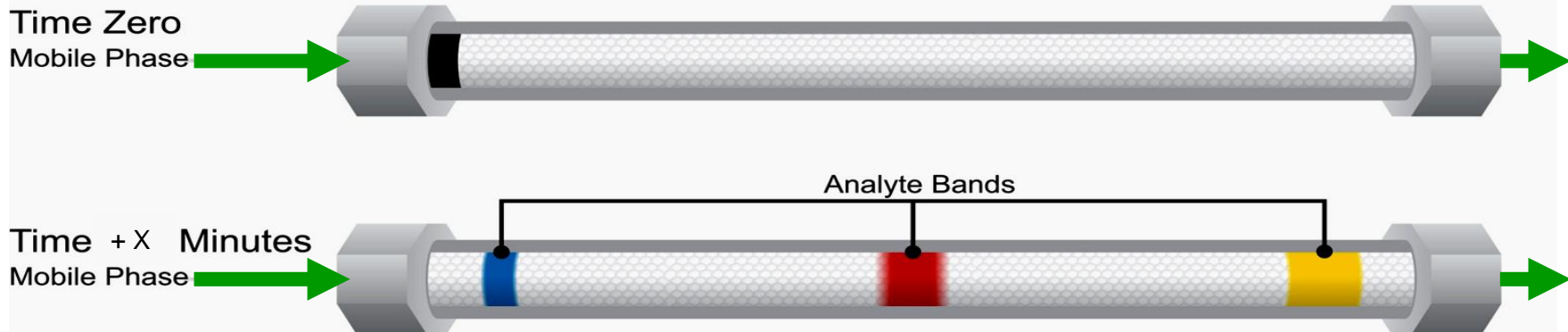
Injected Sample Band (Appears “Black”) (Blue, Red, Yellow)



How Does Chromatography Work?

- We create a separation by changing the relative speed of each analyte band
 - Competition between the mobile phase and stationary phase
- The goal is to separate the Yellow (Polar), Red and Blue (Non-polar) bands on the stationary phase

Injected Sample Band (Appears “Black”) (Blue, Red, Yellow)



Normal-Phase Chromatography

Tswett's 1903 Experiment

Stationary Phase Is **Polar** (Silica)



- Blue is non-polar = likes the non-polar mobile phase best, moves the fastest and comes out **FIRST**
- Red is moderately polar = likes the stationary phase somewhat, and slows down some
- Yellow is very polar = likes the polar stationary phase best, slows down the most and comes out **LAST**

In **NORMAL-PHASE** Chromatography, **POLARS** are Retained

Reversed-Phase Chromatography

The Most Common Mode of Chromatography

Stationary Phase Is **Non-Polar** (C₁₈)



- Yellow is very polar = likes the polar MOBILE PHASE best, moves the fastest, and comes out FIRST
- Red is moderately polar = likes the stationary phase somewhat, and slows down some
- Blue is non-polar = likes the non-polar mobile phase best, moves the fastest and comes out LAST

In Reversed-Phase Chromatography, the NON-POLARS are Retained

First Principles: Modes of Chromatography



Competition between the stationary phase and the mobile phase creates
a separation of compounds in a sample

Typically, the polarity of the mobile phase is OPPOSITE that of the stationary phase

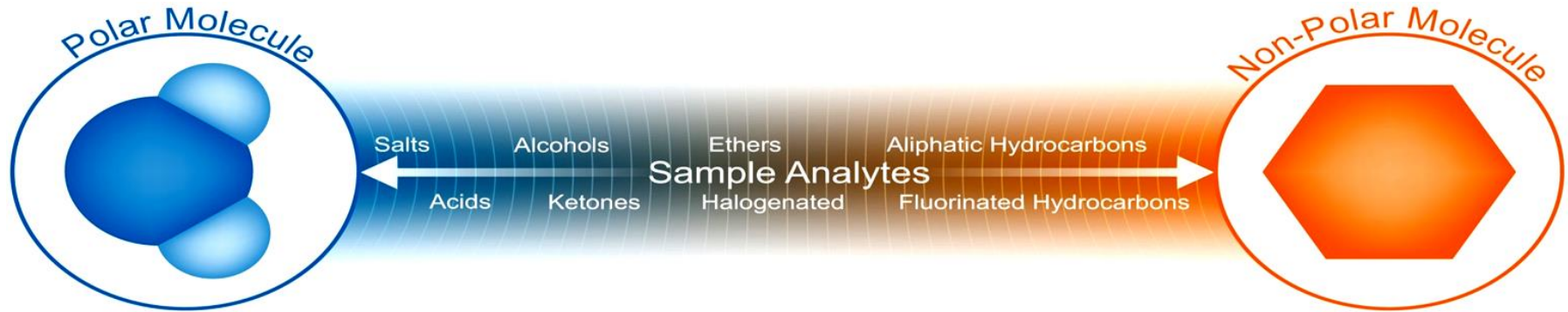


Range of Polarity

- In order to create chromatographic separations, it helps to understand the polarities
 - Sample/analytes
 - Mobile phase
 - Stationary phase (packing material)

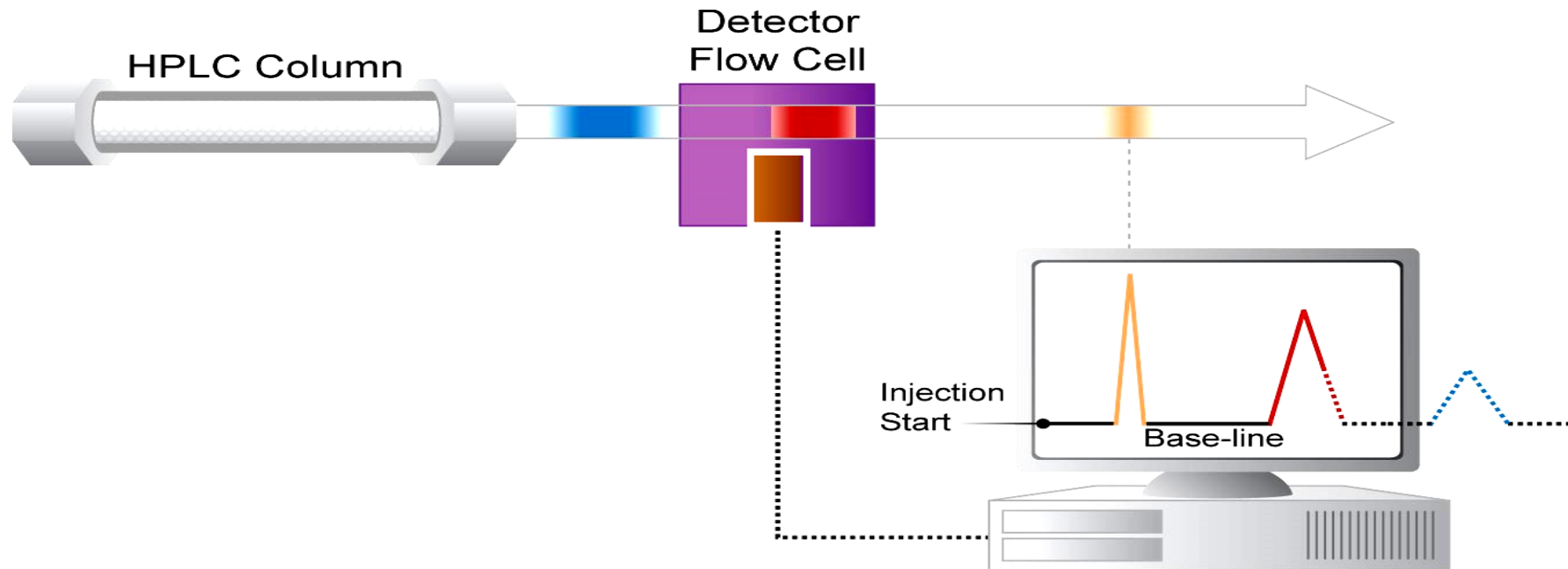


Polarity Scale Compound/Analyte



- When the sample matrix, which is in the moving mobile phase, enters the stationary phase packed into the column, all the different sample compounds (all with different polarities) will either be attracted to the stationary phase, or the mobile phase
- How *fast* the analyte molecule moves through the column is related to how much the molecule “likes/is attracted” to the mobile phase versus the stationary phase

How are Peaks Created/Detected?



What Makes a Great Separation?

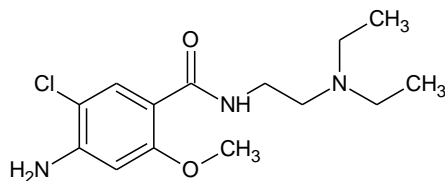
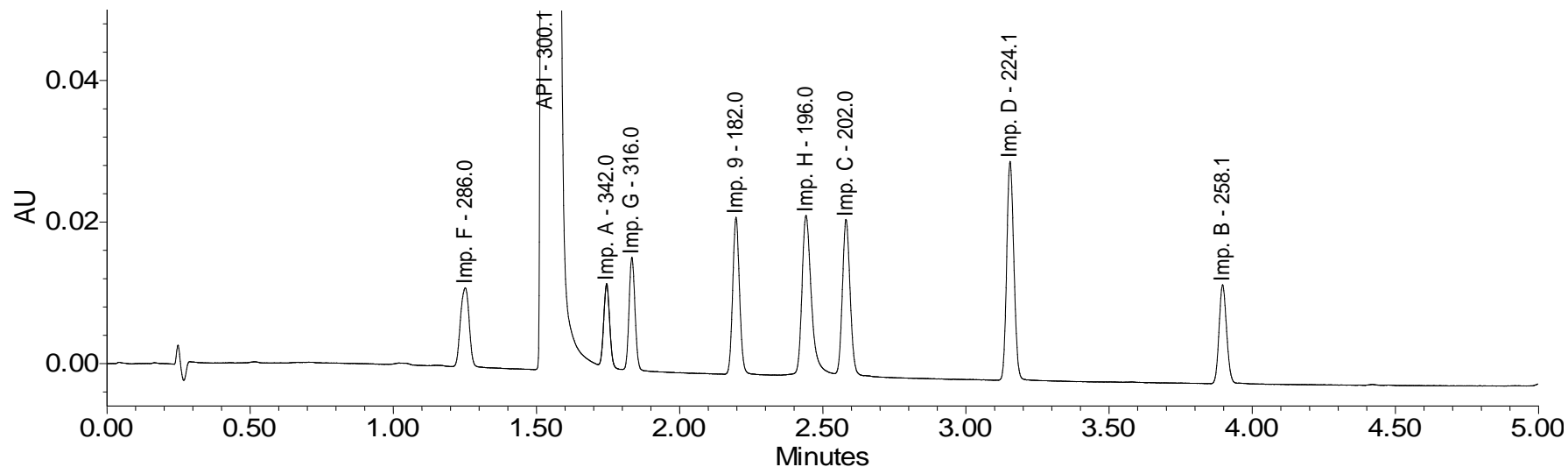
Selectivity

- Tools for Affecting Reverse Phase Method Development
 - pH
 - Column Chemistry
 - Organic Solvents
- Other Factors
 - Temperature
 - Gradient Slope

Efficiency

- The quest for ultra performance
 - How particle technology has evolved over the years
- The advantages of high efficiency columns
- Instrument dispersion and the impact on column considerations
 - Particle, particle size, and dimension impact on methods

Goals for Your Separation



Metoclopramide
 $C_{14}H_{22}ClN_3O_2$
Molecular weight: 299.14 g/mol

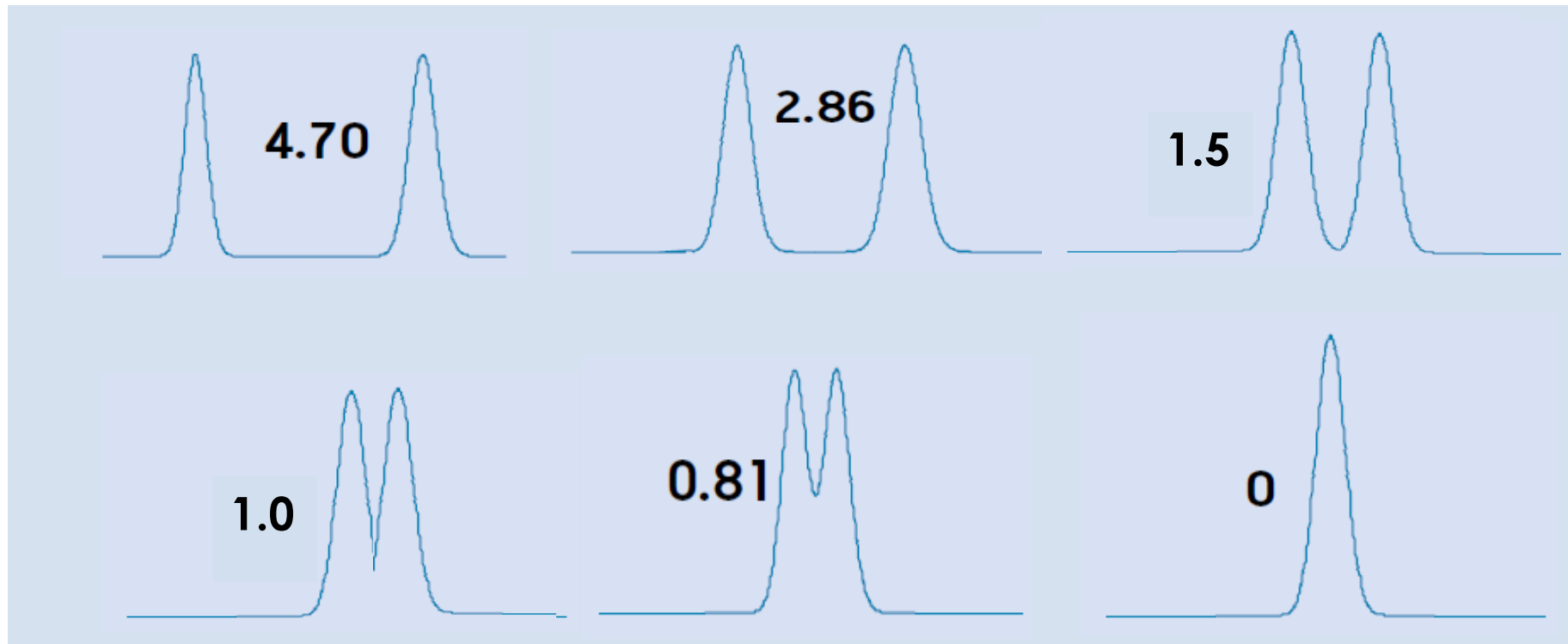
What Makes A Good Separation?

- The most efficient columns produce the sharpest peaks, which gives better separation by minimizing bandspreading.
 - A tight narrow sample “band” is produced when a column’s stationary phase is uniformly packed.
 - The packing material may cause “chemical” bandspreading which broadens the sample band.
- A narrow band of sample produces narrow peaks:
 - Better separation provides more peak resolution
 - Better resolution leads to better quantitation
 - Better sensitivity for lower levels of detection

Why do we need good Resolution?

- *Resolution* is the separation of the peaks in a sample mixture.
- Quantitation requires peaks be separate in order to determine the peak area correctly.
- Purification requires removal of impurities.
- Compound identification is easier if the compounds are well separated.

Resolution & Separation



Why we do Chromatography

We want to pull things apart!

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

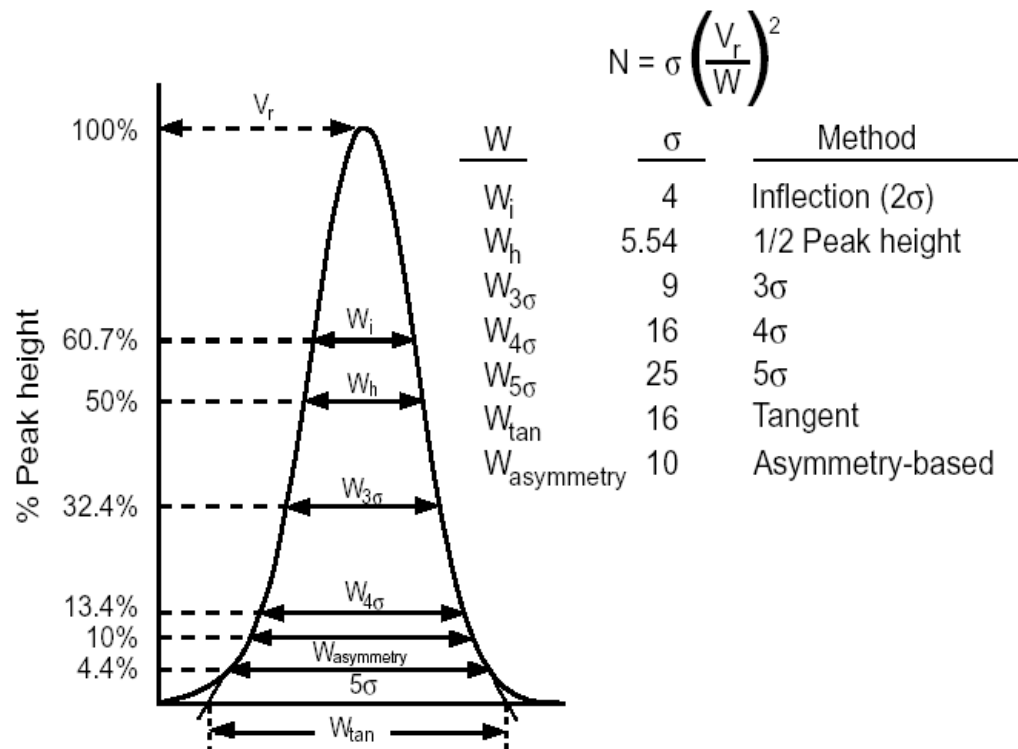
Mechanical Contributions

- Ultra-low dispersion system
- Operate at optimal linear velocity
- Particle morphology
- Small particles
- Well-packed columns

Chemical/Physical Contributions

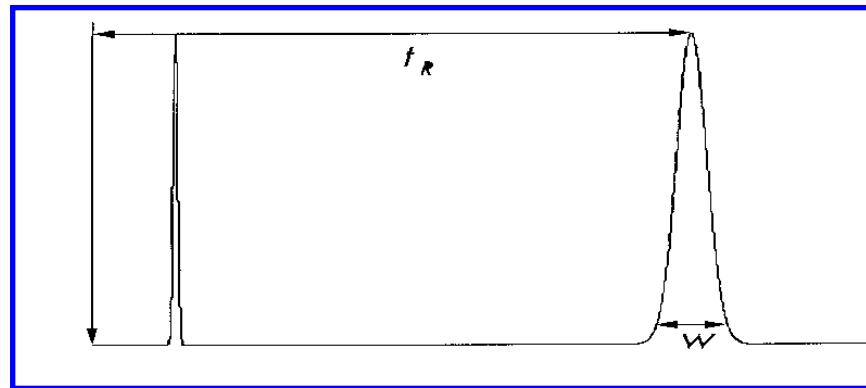
- Complementary bonded phases
- Multiple particle substrates
- Ability to utilize high pH
- Increase retentivity

Theoretical Plates (N)



Column Efficiency – Plate Count

- *Column efficiency* is measured as plate count (N)
- Typical Plate Count Values
 - 12,000 – 15,000 plates for HPLC columns
 - >35,000 for UPLC columns
- The number of plates are measured under specific conditions.



Factors That Affect Plate Count



Mechanical conditions

HPLC instrument used
Tubing and fittings
How well the column was packed



Chemical conditions

Chromatographic conditions (Solvents)
Packing material manufacturing



Flow rate and test compound used

Selectivity

Focus on the right side of the equation

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

Mechanical Contributions

- Ultra-low dispersion system
- Operate at optimal linear velocity
- Particle morphology
- Small particles
- Well-packed columns

Chemical/Physical Contributions

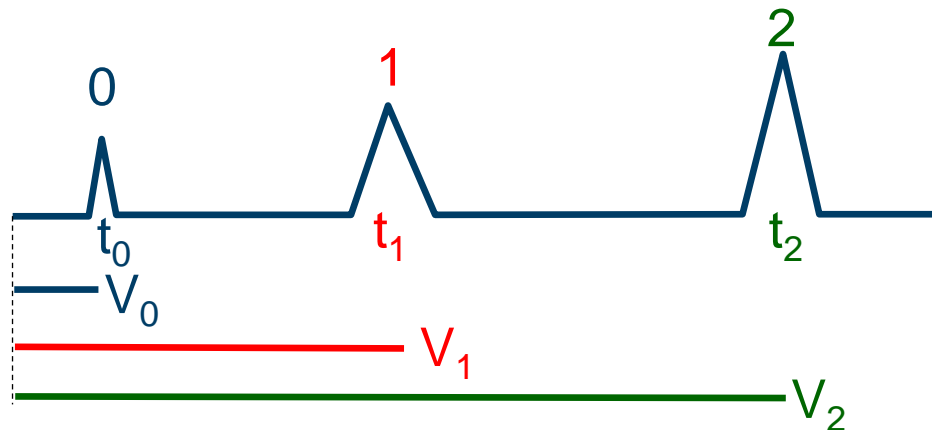
- Complementary bonded phases
- Multiple particle substrates
- Ability to utilize high pH
- Increase retentivity

Why Do We Need Selectivity?

- *Selectivity* is the ability of the column and mobile phase to separate a pair of analytes.

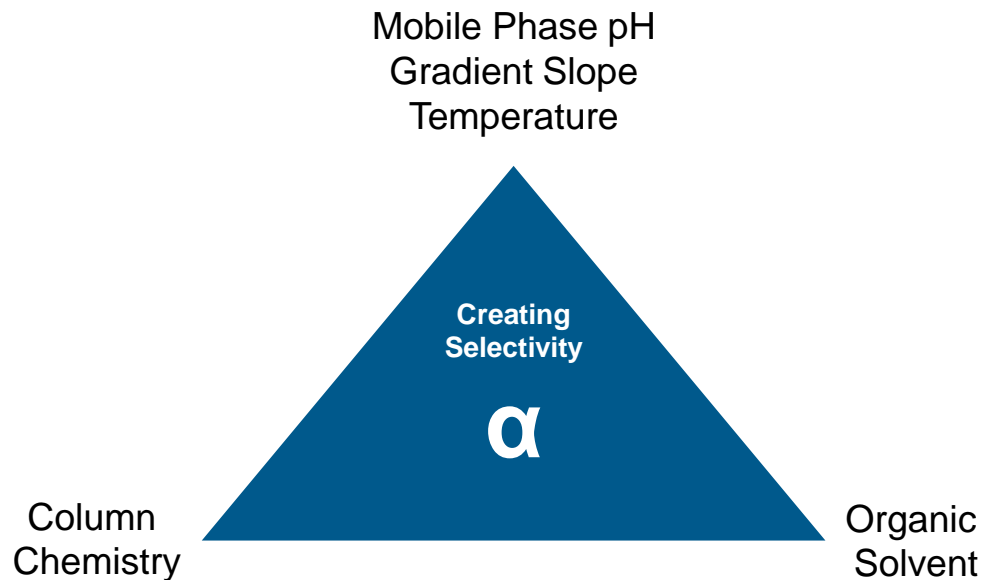
Selectivity Equation

$$\alpha = \frac{k_2}{k_1} \quad k_2 = \frac{V_2 - V_0}{V_0} \quad k_1 = \frac{V_1 - V_0}{V_0}$$

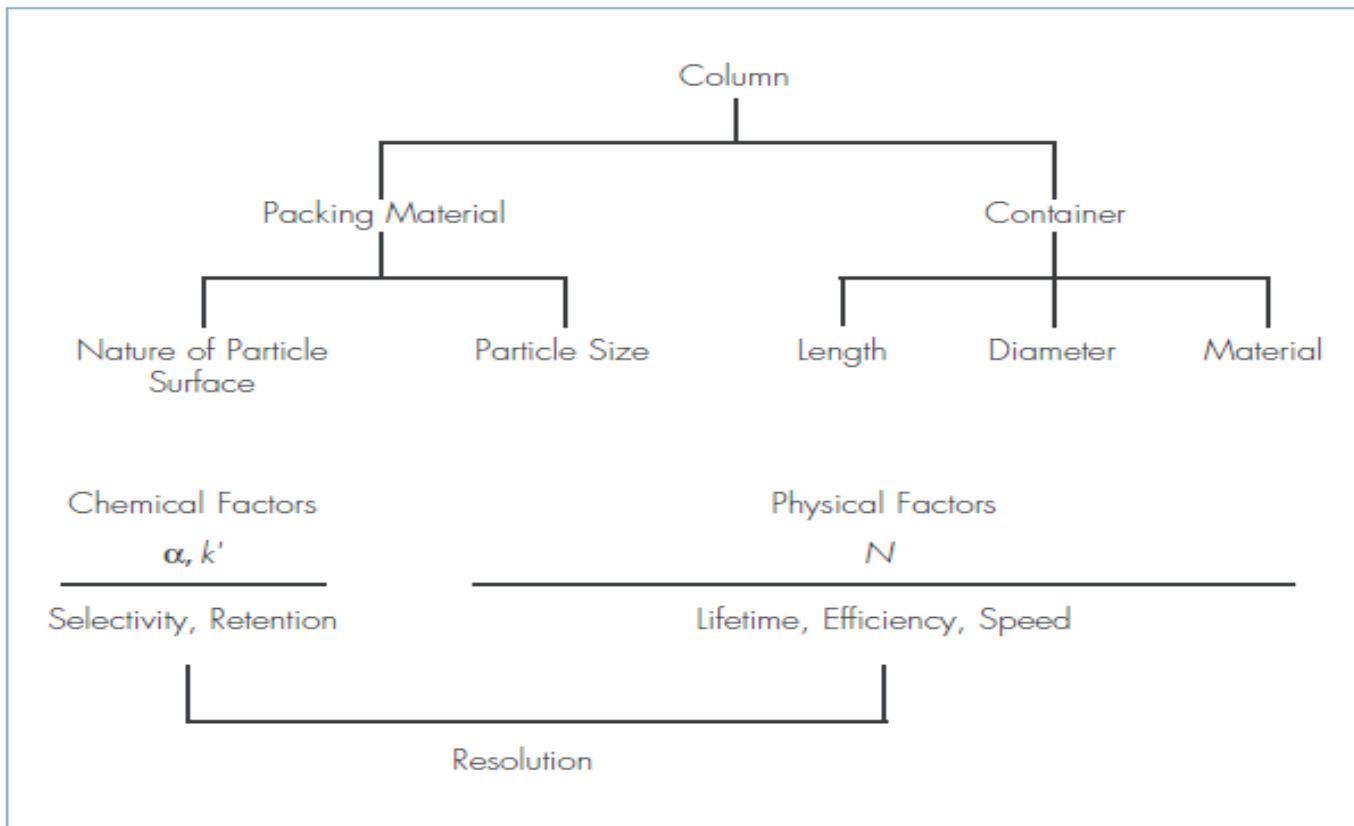


Chemical Factors That Affect Selectivity

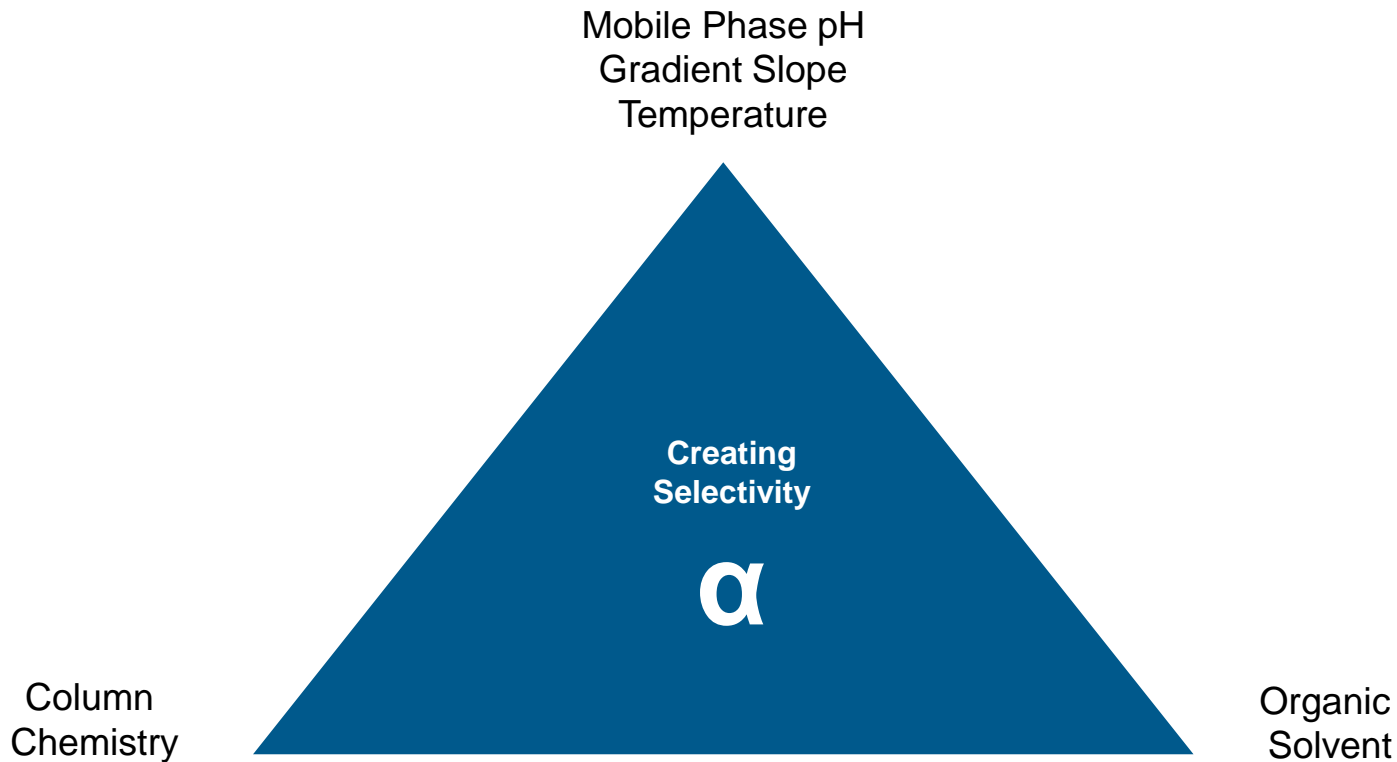
$$\frac{\alpha - 1}{\alpha} \frac{k}{k + 1}$$



Columns Components



Creating Selectivity: Reversed-Phase Method Development



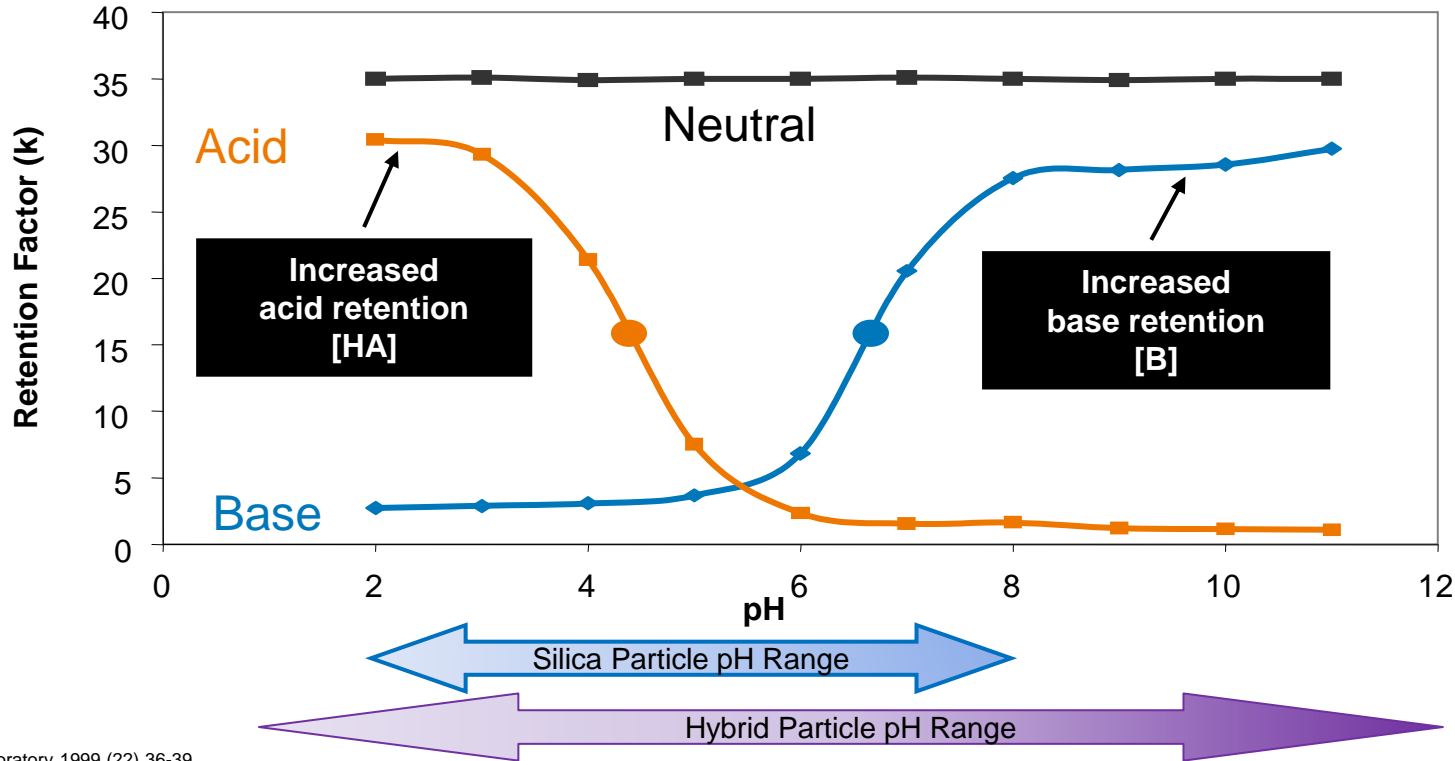
- pKa
 - *pH* at which the molecules of the analyte in solution are 50% ionized (charged) and 50% are un-ionized

± 2 Rule

*If you adjust the pH
± 2 pH units from the pKa,
you will make ~100%
of the molecules
either ionized or un-ionized*

Dependence of Retention on pH:

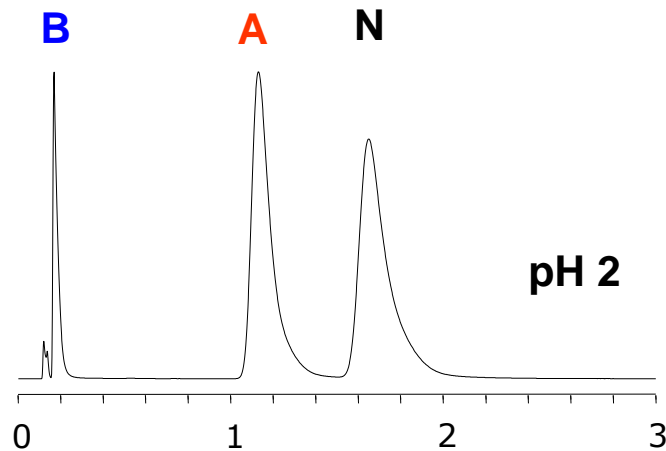
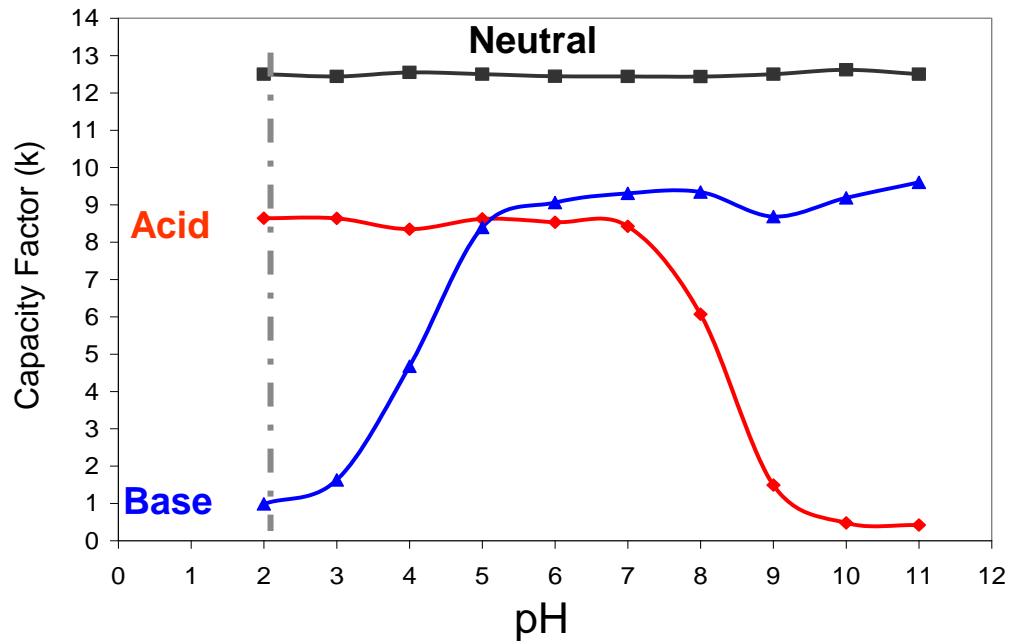
Reversed-Phase Retention Map



Neue et. al. American Laboratory 1999 (22) 36-39.

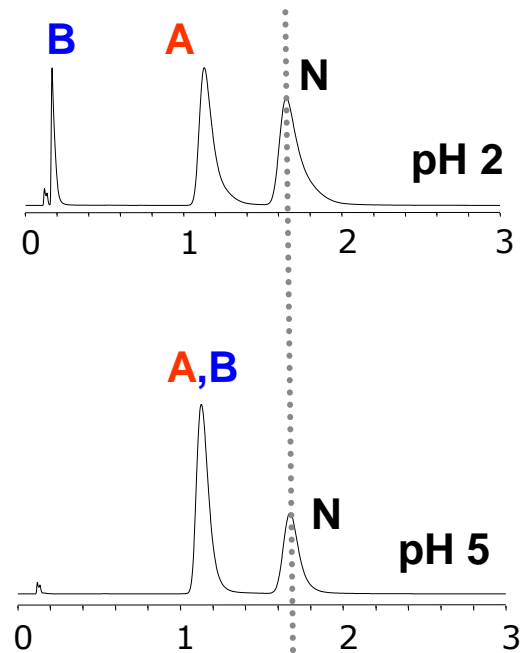
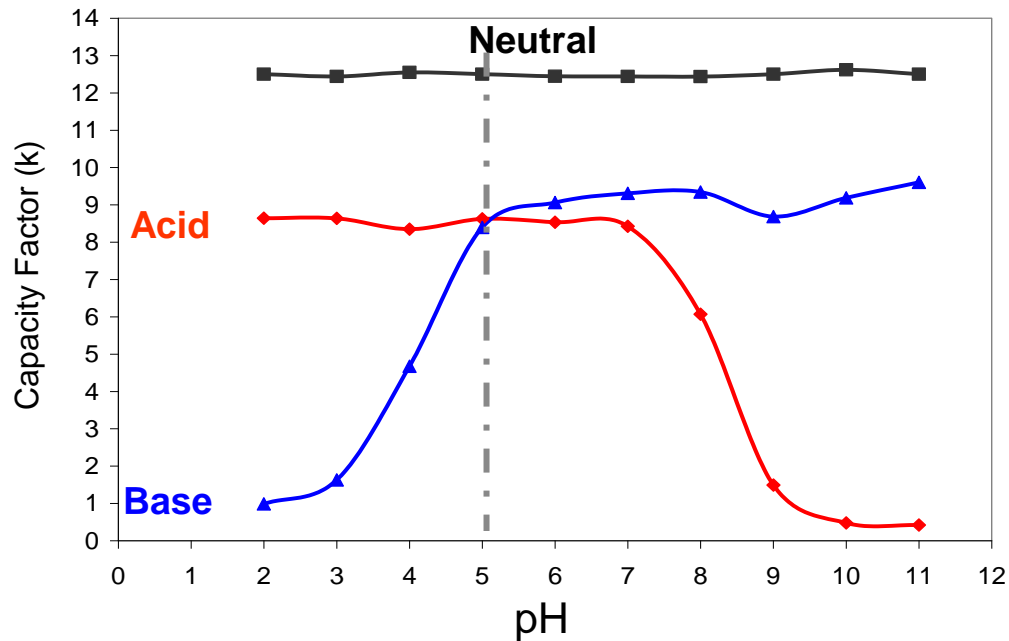
Relating Retention Maps to Chromatography

Reversed-Phase Retention Map



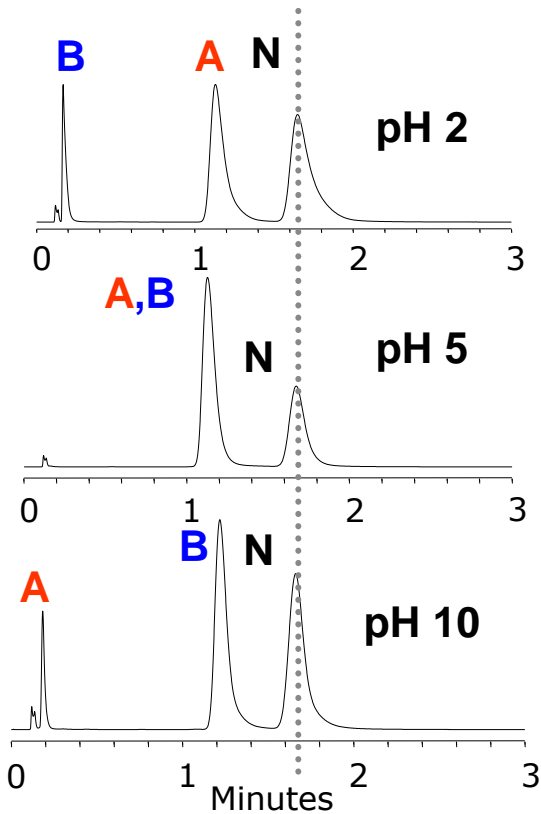
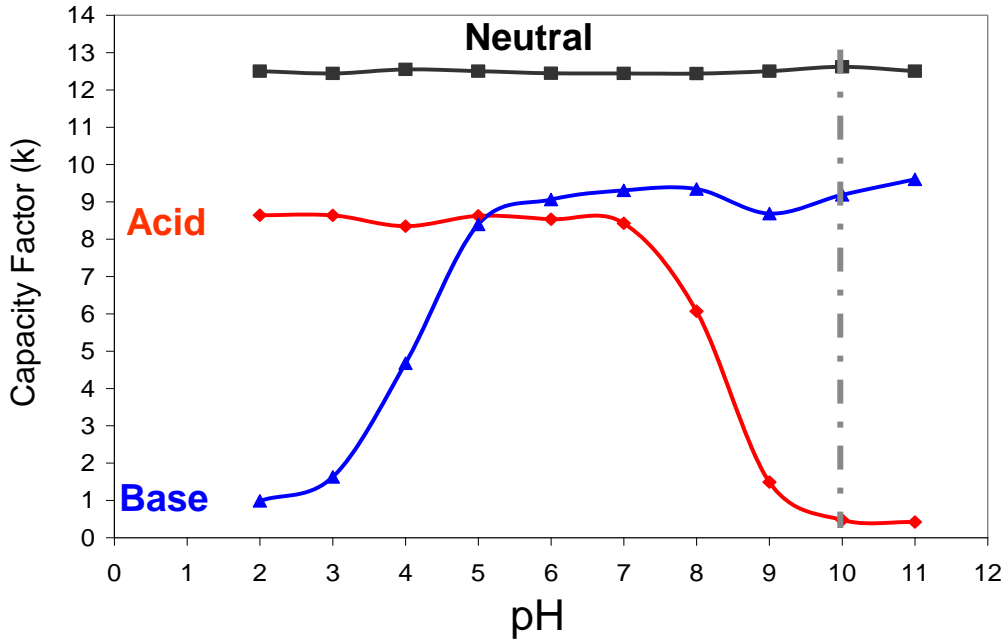
Relating Retention Maps to Chromatography

Reversed-Phase Retention Map

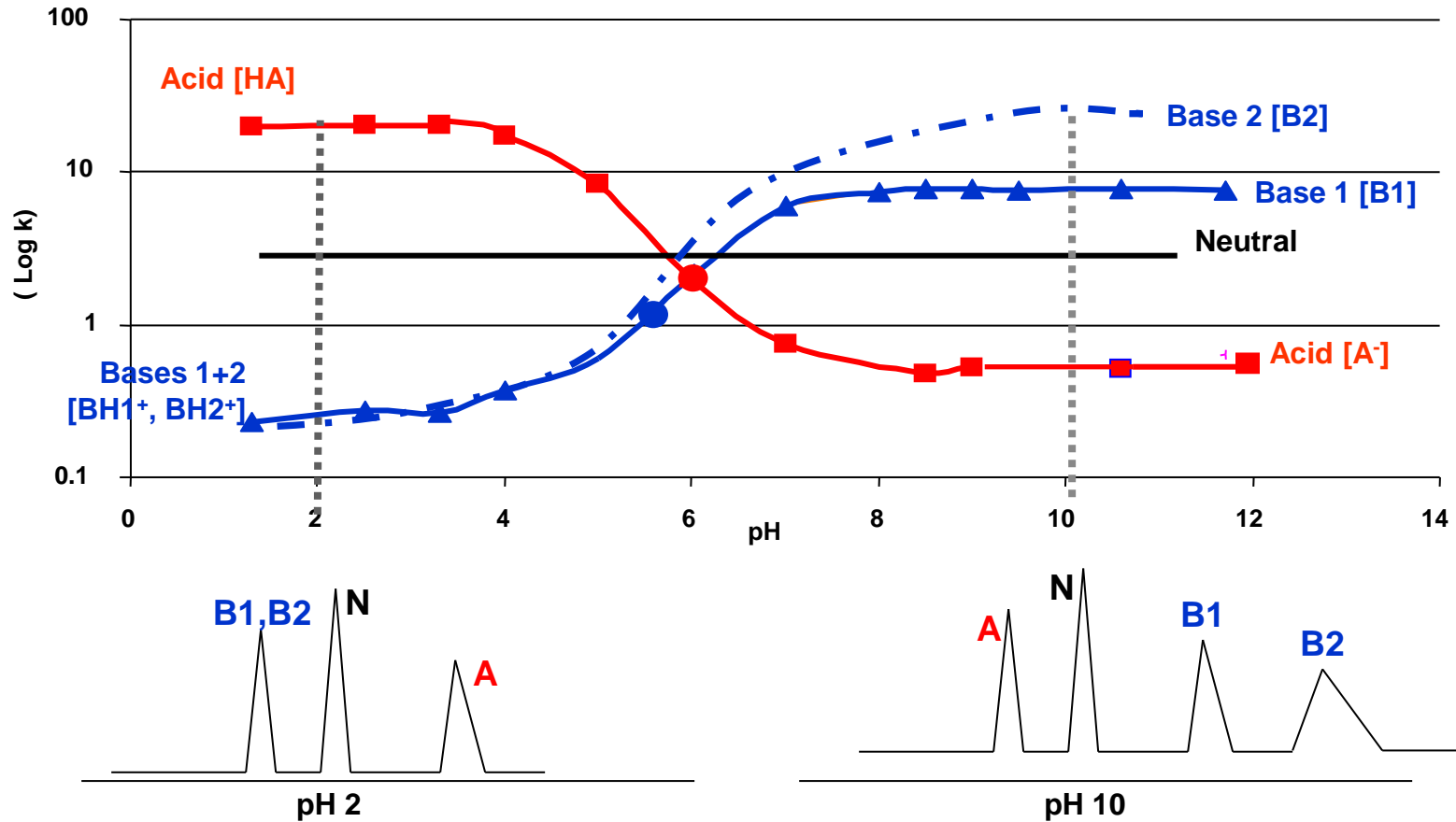


Relating Retention Maps to Chromatography

Reversed-Phase Retention Map

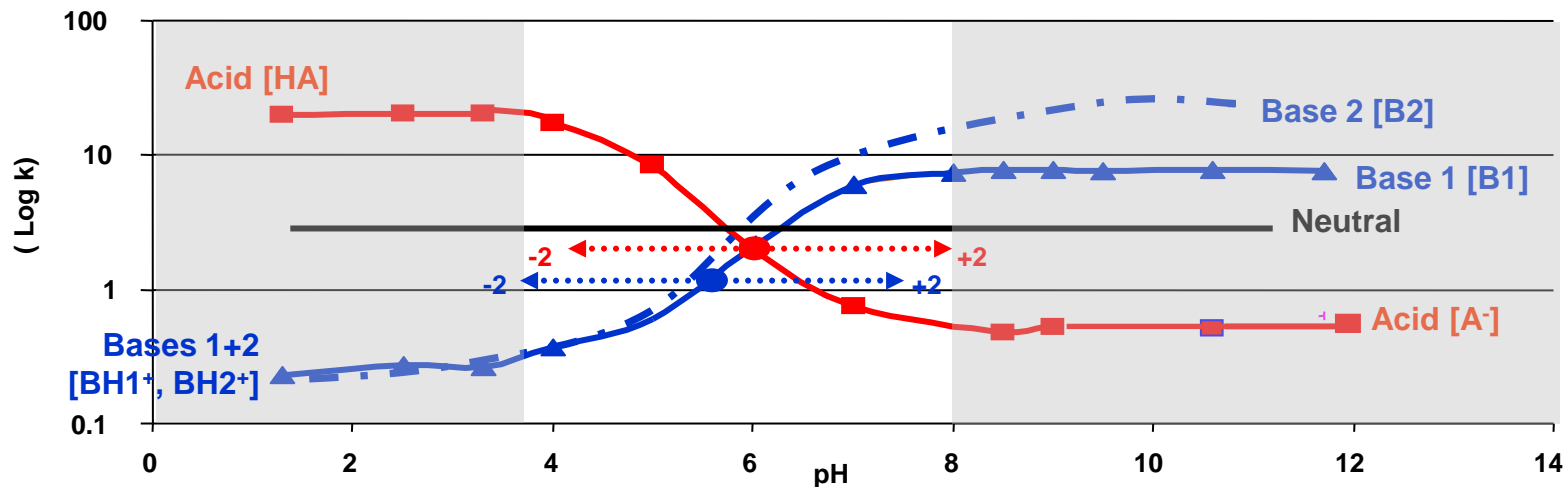


Using pH to Create Separations



Good Reproducibility

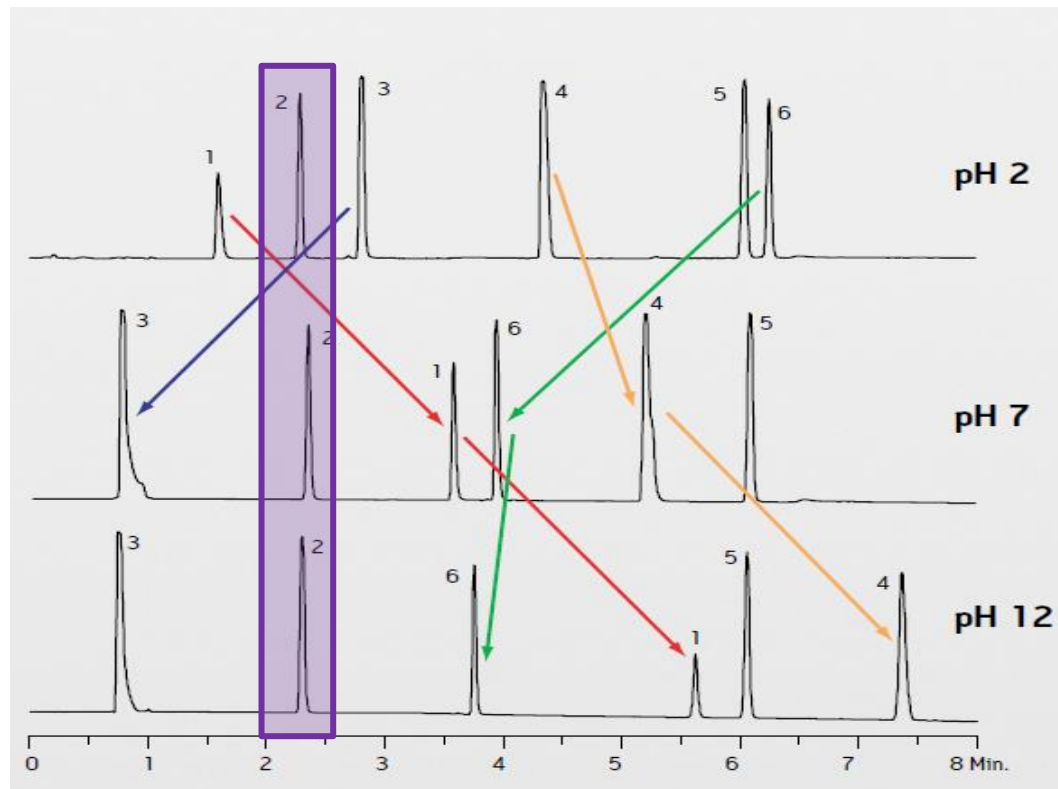
± 2 Units from Analyte pK_a Values



Improved retention reproducibility due to flattening of curves
Large pH change – Small retention change

The Importance of Mobile Phase pH:

Rapid Method Development



- Using a wide mobile phase pH range is an effective approach to change compound selectivity
- Increase selectivity for:
 - Acids (Green (6)/Blue (3))
 - Bases (Red (1)/Yellow (4))
- Neutrals (Peak 2) are largely unaffected by mobile phase pH

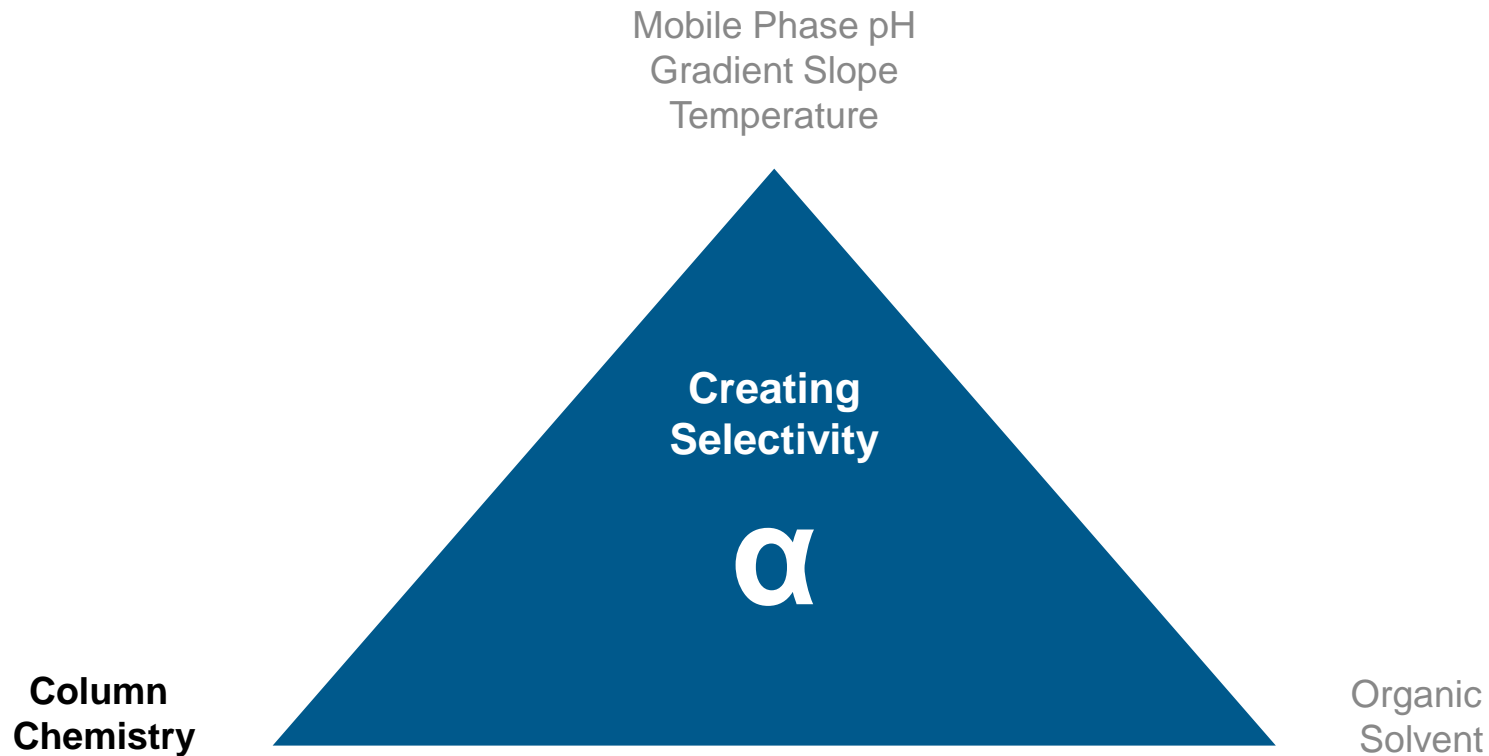
Practical pH Range Guidance for Method Development

- For new methods development, create aqueous buffers at:
 - **pH 2-3 for acidic** analytes
 - **pH 9-10 for basic** analytes

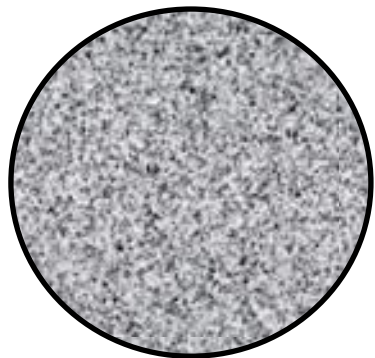
- At these pH's, when mixed with organic solvents:
 - **pH 2-3 Acidic** analytes - un-ionized, increased retention
 - Basic analytes are less retained
 - **pH 9-10 for basic** analytes - un-ionized, increased retention
 - Acidic analytes are less retained

- **Note:** *If operating above **pH 8**, make sure the column is packed with high pH tolerant hybrid particles!*

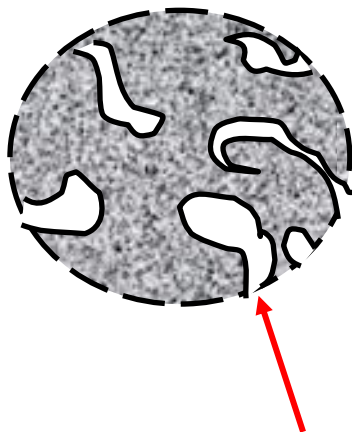
Creating Selectivity: Reversed-Phase Method Development



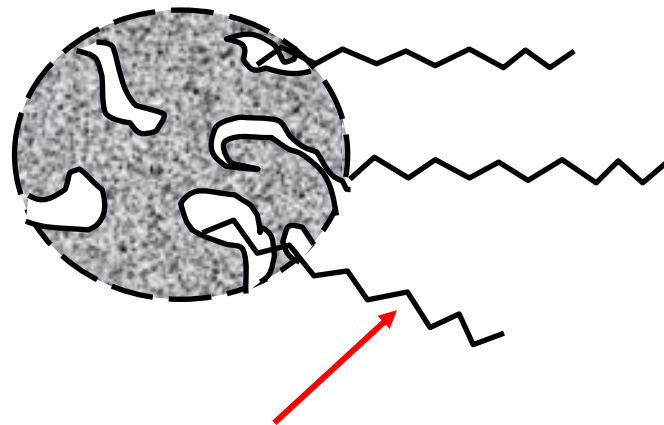
Packing Material



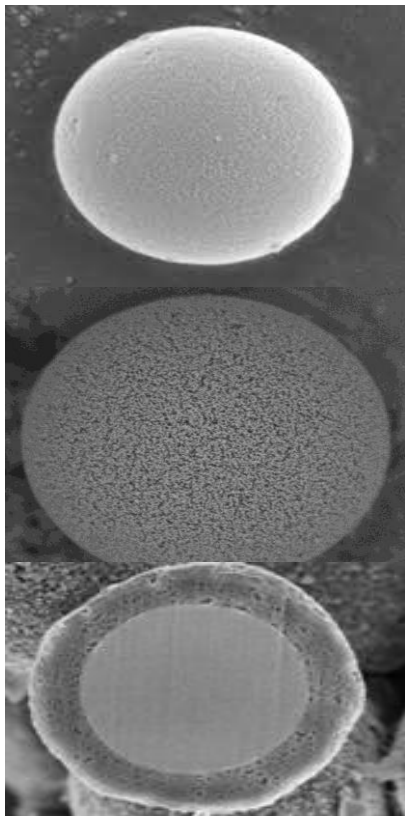
Particle



Pores



Bonded phase



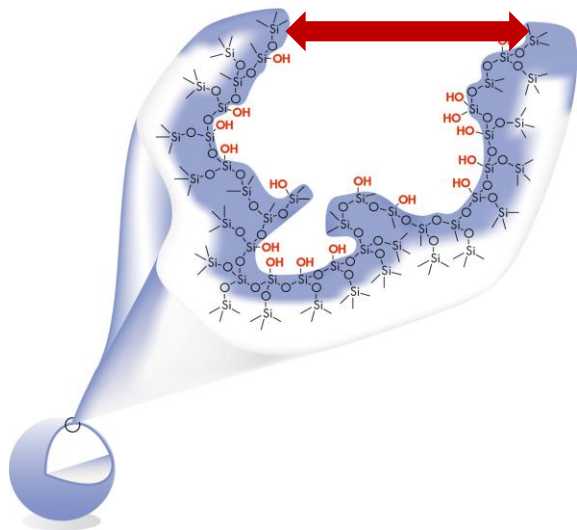
Non-porous

Fully porous

Superficially porous

What are important attributes for chromatography?

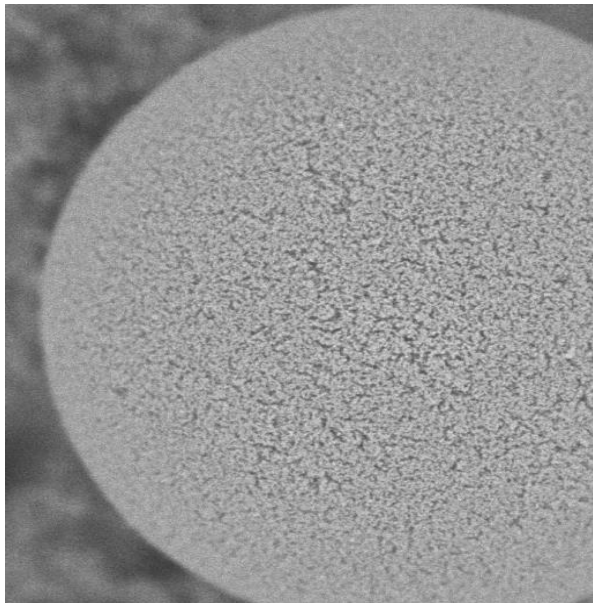
- Pore Diameter (**PD**)
- Pore Volume (**PV**)
- Surface Area (**SA**)
- Particle Composition
- Particle Size
- Particle Surface Charge
- Particle Morphology
- Ligand/Ligand Density



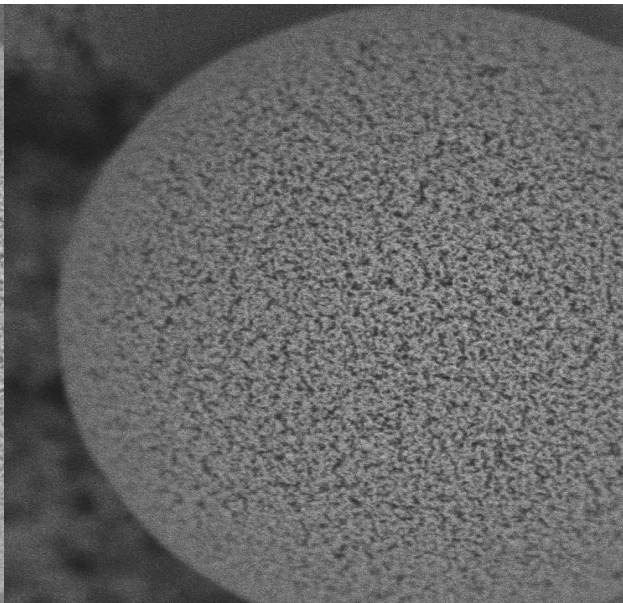
Analyte MW	Pore Size Recommendation
< 3,000	60 -130 Å (6 -13 nm)
3,000 – 10,000	125-200 Å (12.5-20 nm)
>10,000	300 – 1,000 Å (30 -100 nm)
Very Large	Non-porous

Pore Diameter

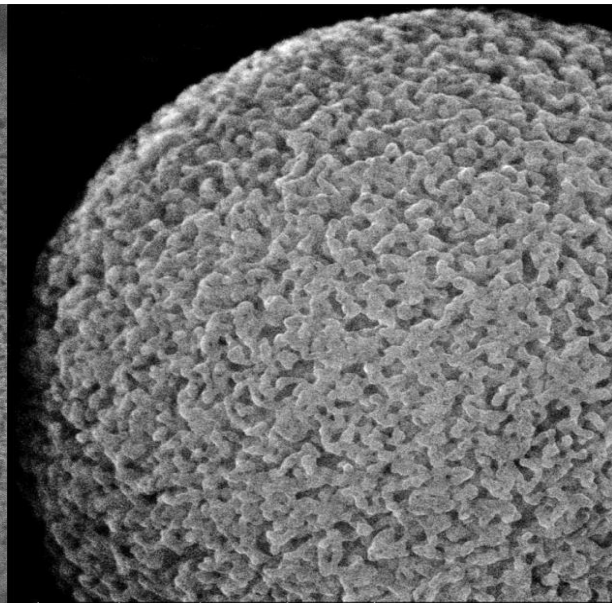
125 Å



200 Å

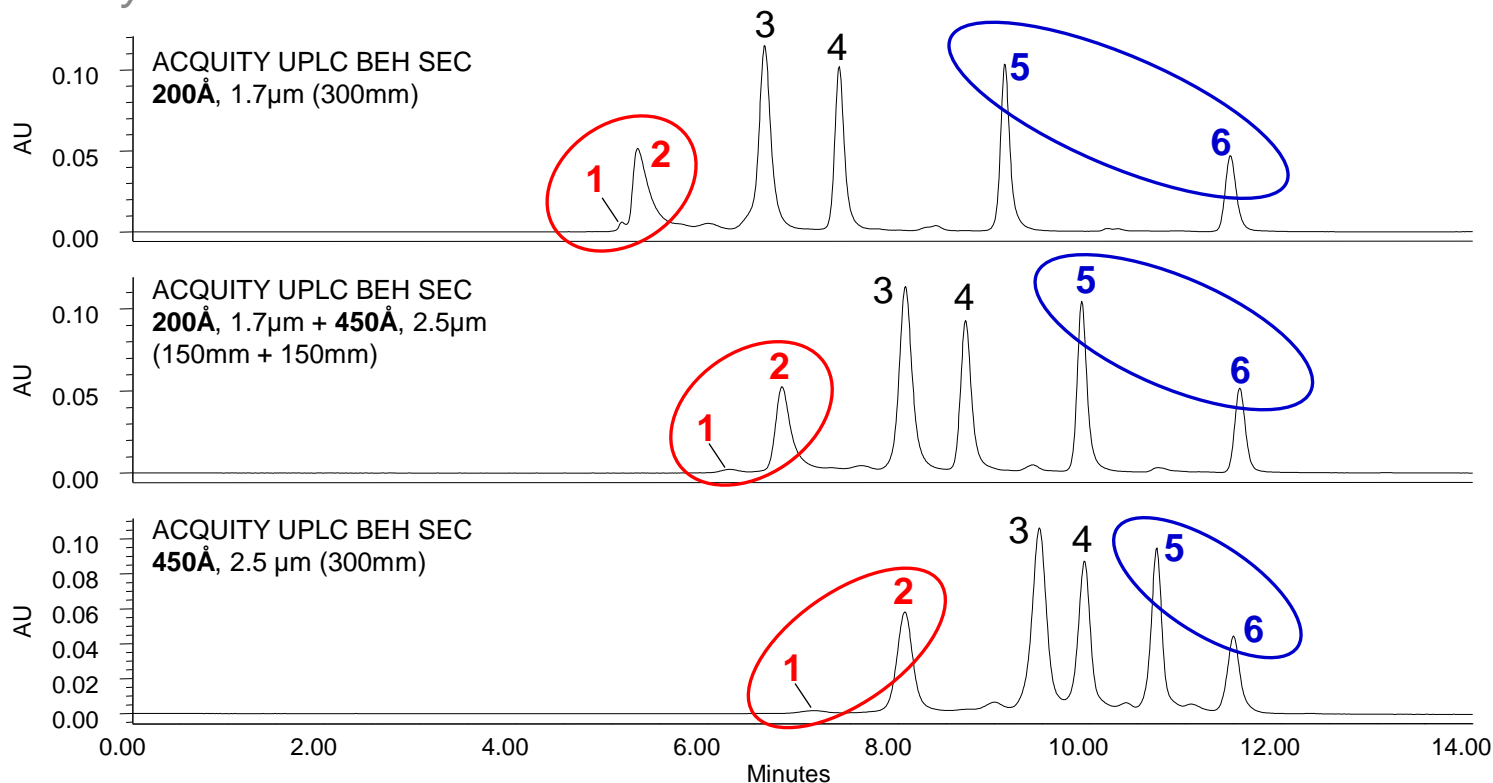


450 Å



The Importance of Pore Diameter

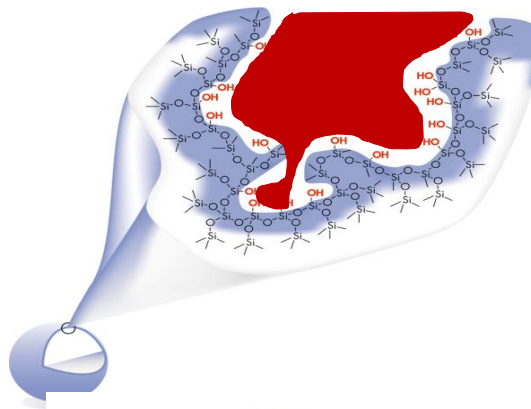
Analyte Selectivity/Resolution



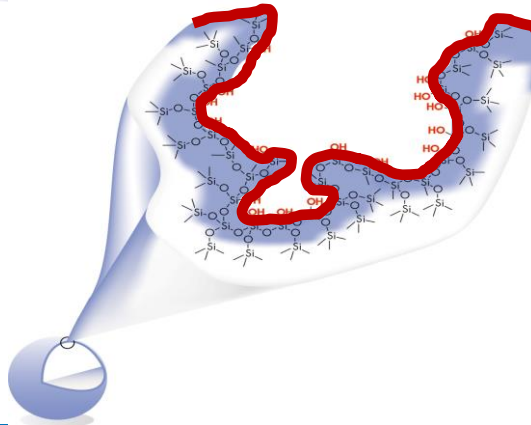
1. Thyroglobulin Dimer (1,340 KDa), 2. Thyroglobulin (667 KDa), 3. IgG (150 KDa), 4. BSA (66 KDa), 5. Myoglobin (17 KDa), 6. Uracil (112 Da)

What are important attributes for chromatography?

- Pore Diameter (**PD**)
- Pore Volume (**PV**)
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Pore Volume



Surface Area

Phase Ratio: Pore Volume and the Surface Area Contributions

$$\text{Phase Ratio} = (1 - \varepsilon_e) \left(\frac{A_s}{V_p + \frac{\varepsilon_e}{\delta_{sk}}} \right)$$

Specific Surface Area (A_s)

Specific Pore Volume (V_p)

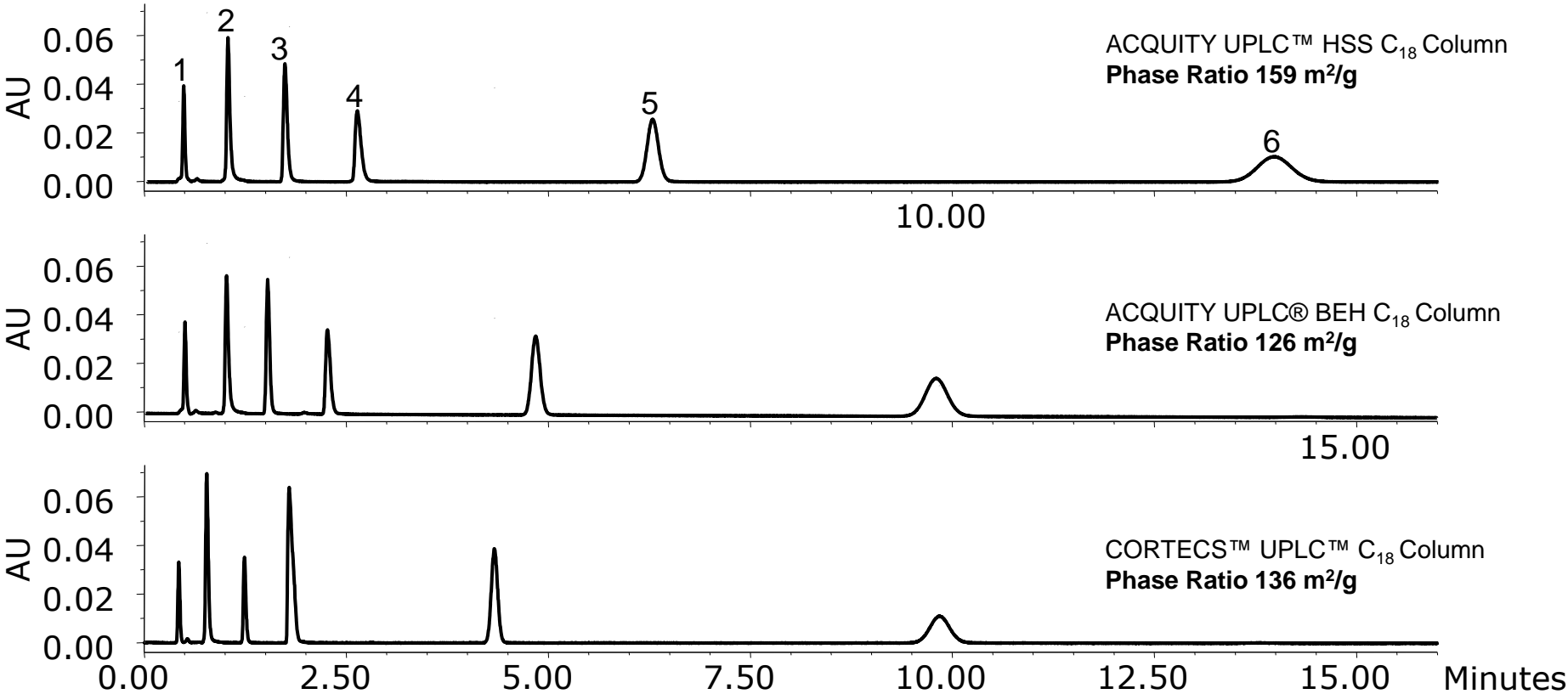
External or Interstitial Porosity (ε_e) = 0.39

Skeletal Density (δ_{sk}) = 2.2 g/cm³ silica

Skeletal Density (δ_{sk}) = 2.02 g/cm³ BEH

Base Particle	HSS	BEH	CORTECS™
Morphology	Fully porous	Fully porous	Solid-Core $\rho = 0.7$
Composition	silica	hybrid	silica
Pore Volume (V_p)	0.70 cm³/g	0.70 cm³/g	0.26 cm³/g
Pore Size	100 Å	130 Å	90 Å
Surface Area (A_s)	230 m²/g	185 m²/g	100 m²/g
Phase Ratio	159 m²/cm³	126 m²/cm³	136 m²/cm³

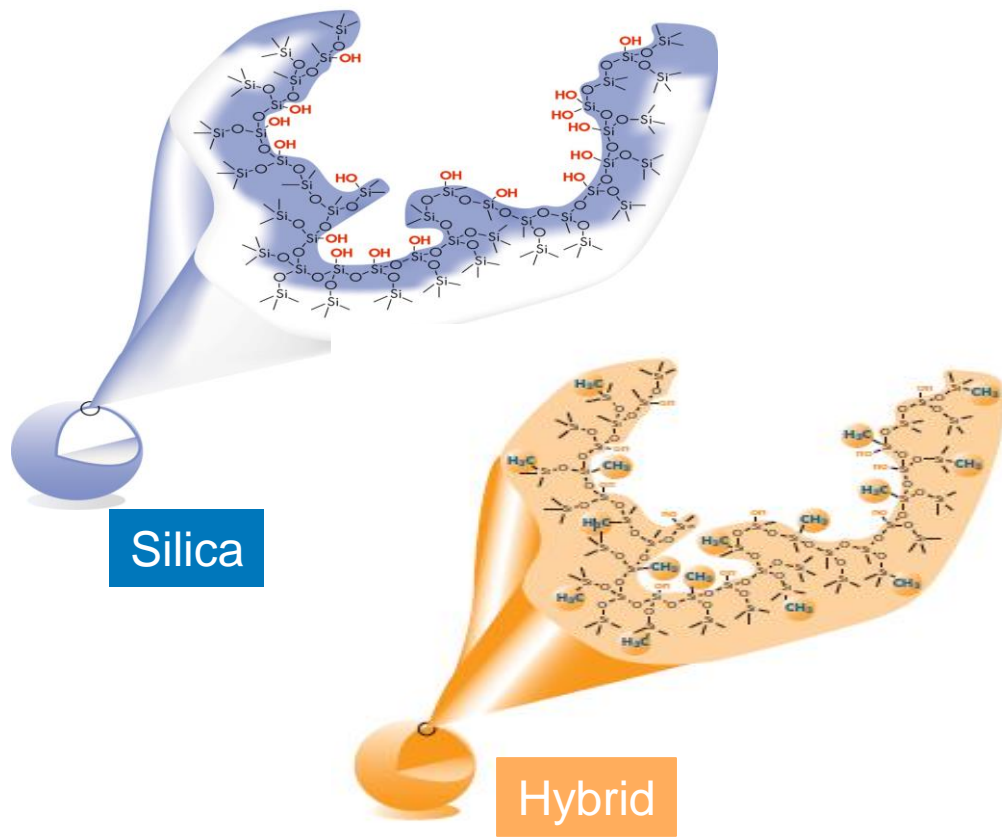
Dependence of Phase Ratio on Retention



1) Uracil 2) Pyrenesulfonic Acid 3) Promethazine 4) Amitriptyline 5) Butylparaben 6) Naphthalene. Conditions: ACN/ 15.4 mM Ammonium Formate pH 3 (35/65); 0.25 mL/min; 30 °C; 2.1x50 mm

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Particle Composition

	Silica	Polymeric	Hybrid
Key Attributes	<ul style="list-style-type: none"> • pH range (2 – 8) 	<ul style="list-style-type: none"> • pH range (1 – 14) 	<ul style="list-style-type: none"> • pH range (2 – 12)
Advantage	<ul style="list-style-type: none"> • Strong • Rigid • Most widely used packing material for HPLC 	<ul style="list-style-type: none"> • pH stable in acids and bases • Synthesized with controlled pore size 	<ul style="list-style-type: none"> • Strong and rigid • pH stable in acids and bases • Synthesized with controlled pore size • Symmetrical peak shape
Disadvantages	<ul style="list-style-type: none"> • Dissolves at basic pH (pH>8) • Silanol activity results in peak tailing in many basic analytes 	<ul style="list-style-type: none"> • Not very rigid • Collapses under excessive pressure 	<ul style="list-style-type: none"> • None of the silica or polymeric packing materials disadvantages
Application Area	<ul style="list-style-type: none"> • Normal phase • Reversed phase 	<ul style="list-style-type: none"> • Gel permeation • Size exclusion 	<ul style="list-style-type: none"> • Reversed phase • HILIC

What are important attributes for chromatography?

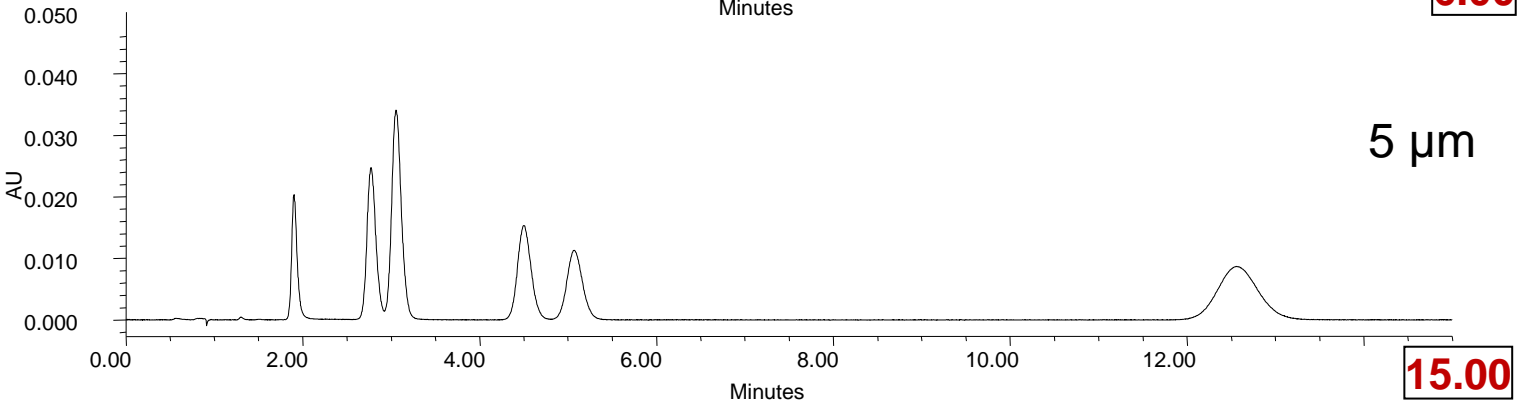
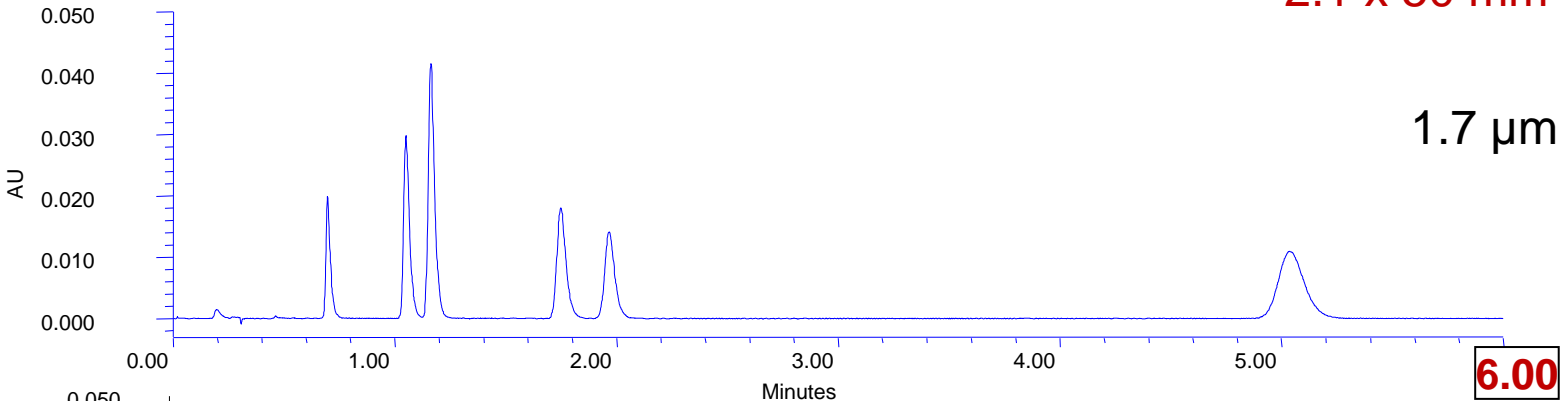
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Smaller Particle Advantage

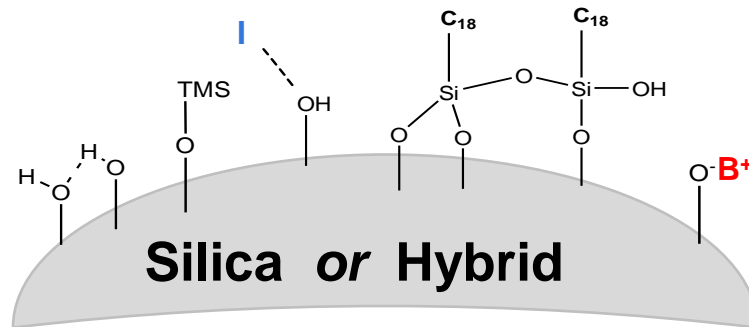
Resolution, Speed, Sensitivity

2.1 x 50 mm columns



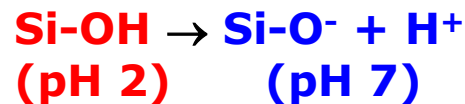
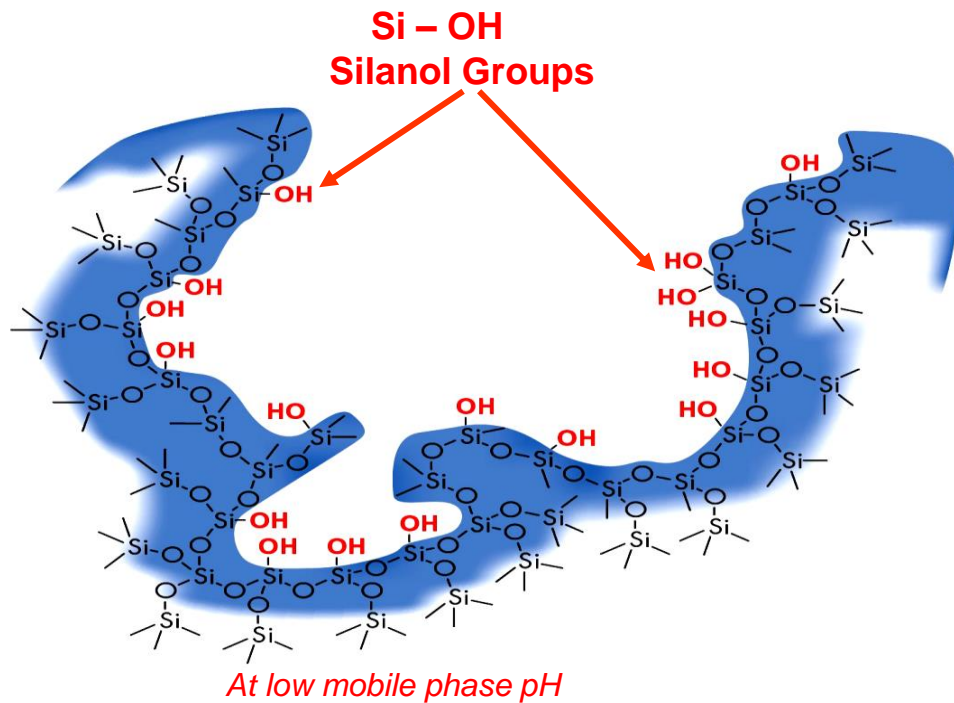
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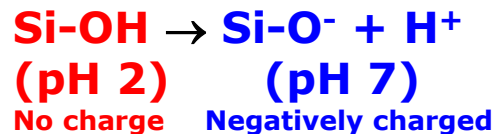
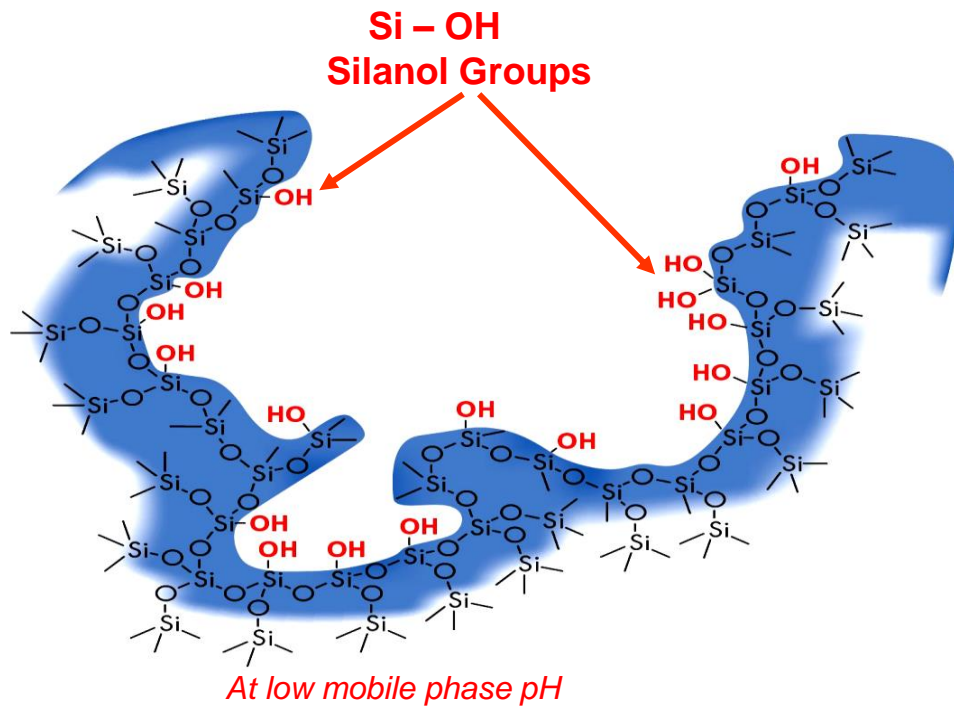
Unbonded Silica Gel Particles

Pore Surface - Silanol Groups



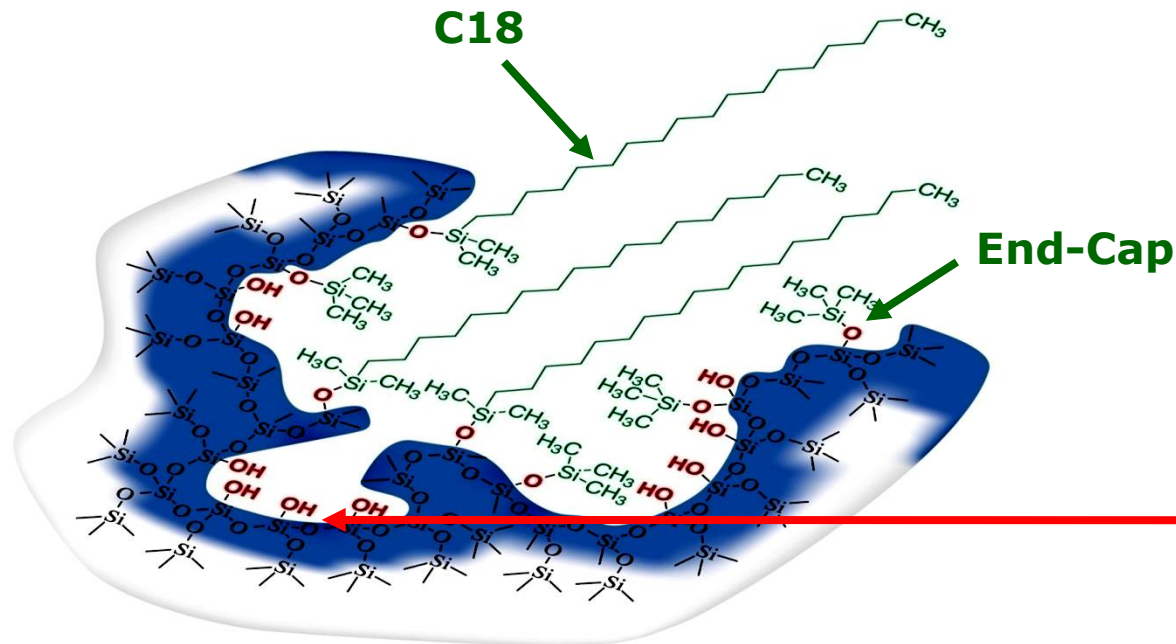
- Surface silanols are acidic sites
 - Low pH mobile phase
 - Uncharged and behave as weak acids
 - Increased pH mobile phase
 - Silanols de-protonate and become negatively charged

Unbonded Silica Gel Particle: Pore Surface - Silanol Groups



- Strong interaction between ionized surface silanols (Si-O^-) and basic analytes (+)
- Creates potential for cation exchange retention mechanism in the column

C18 Bonded and “Fully End-Capped” High Purity Silica Gel Pore



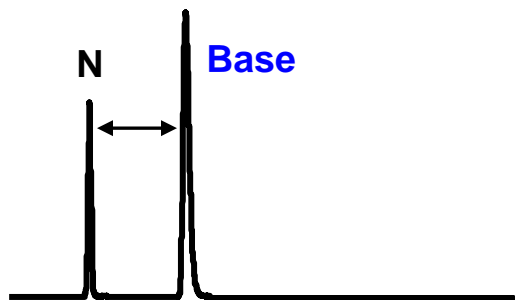
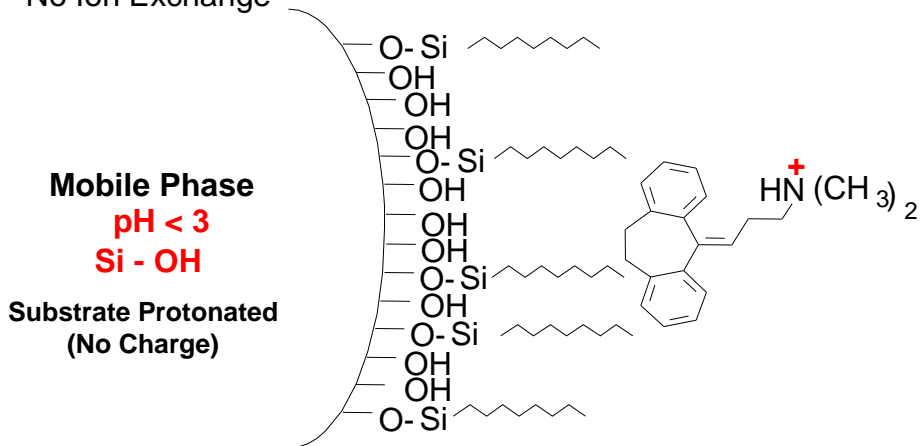
~ 50% of surface
silanols remain due to
steric hindrance

Difficulty bonding silanols in
micro-pores

Mixed Mode Retention on LC Column

Reversed-Phase Interaction with Ligands

No Ion Exchange



Mixed Mode Retention on LC Column

Reversed-Phase Interaction with Ligands

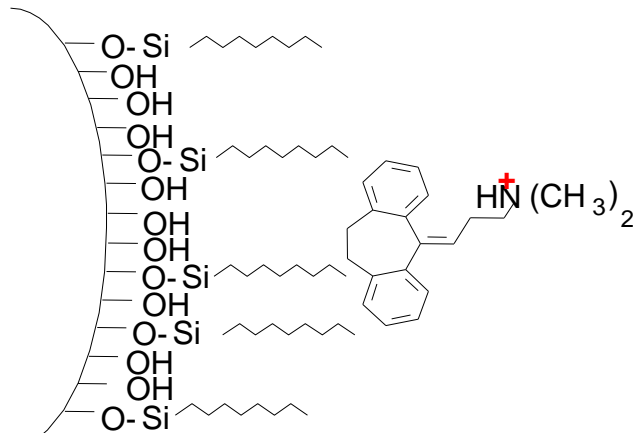
No Ion Exchange

Mobile Phase

pH < 3

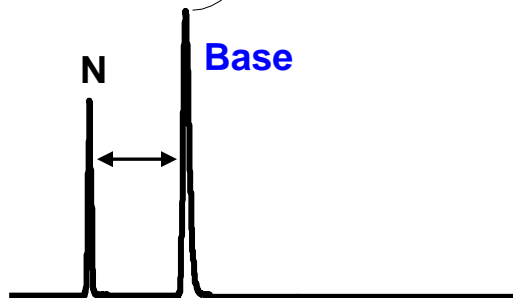
Si - OH

Substrate Protonated
(No Charge)



N

Base



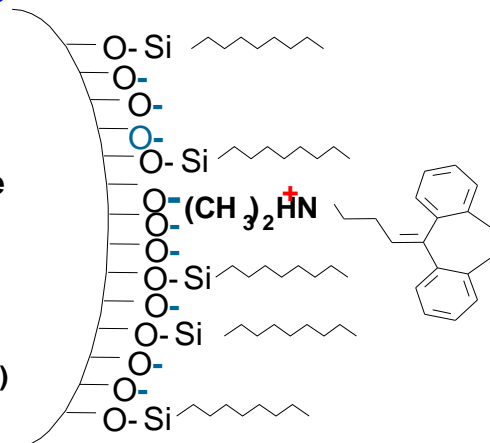
Weak Cation Exchange & Reversed-Phase High Silanol Activity

Mobile Phase

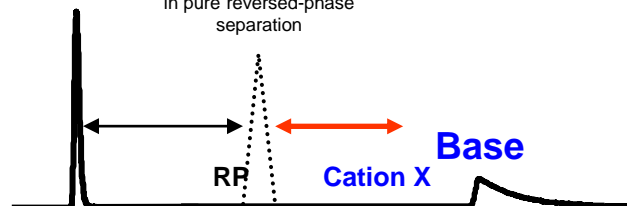
pH > 5

Si - O⁻

Substrate
De-protonated
(Negative Charge)

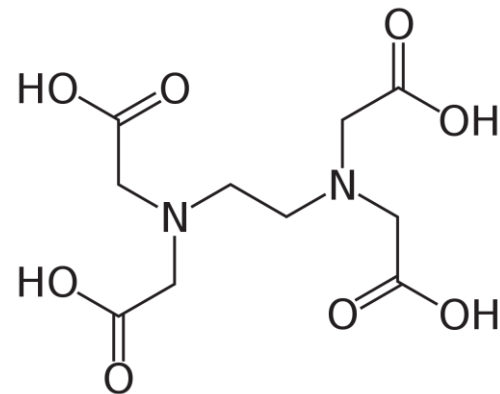
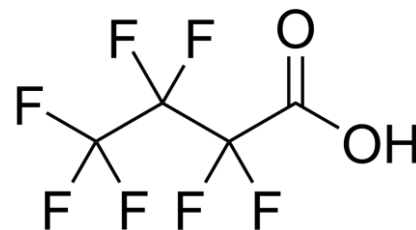


Where base peak should be
in pure reversed-phase
separation

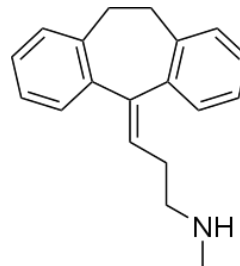
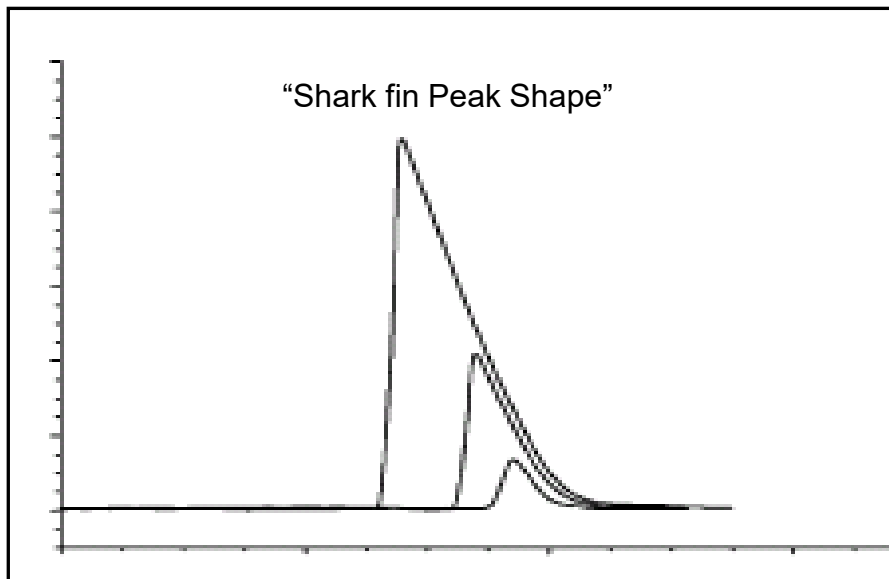


Why are/were modifiers added to mobile phases?

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

EDTAHFBA

Problem: Basic Compounds Have High Tailing When Using Formic Acid



1.25, 0.25, and 0.05 µg nortriptyline

4.6 mm x 150 mm column.²

Mobile Phase Composition:

A= 0.02M **formic acid** in water pH 2.75

B= acetonitrile – 0.04M **formic acid** in water 50:50 (v/v)

1. A. Mendez et al. / J. Chromatogr. A 986 (2003) 33–44

2. D.V. McCalley / J. Chromatogr. A 1075 (2005) 57–64

Charged Surface Hybrid (CSH) Particles

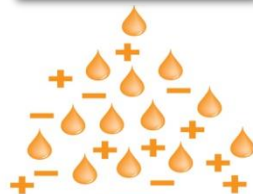
Step 1



Unbonded BEH Particle

Start with the rugged, ultra-efficient, ethylene bridged hybrid (BEH) particle

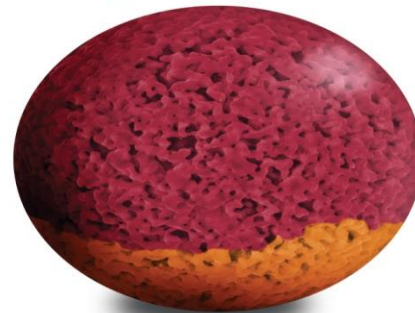
Step 2



Apply Controlled Surface Charge

Add reproducible low-level charge to particle surface

Step 3

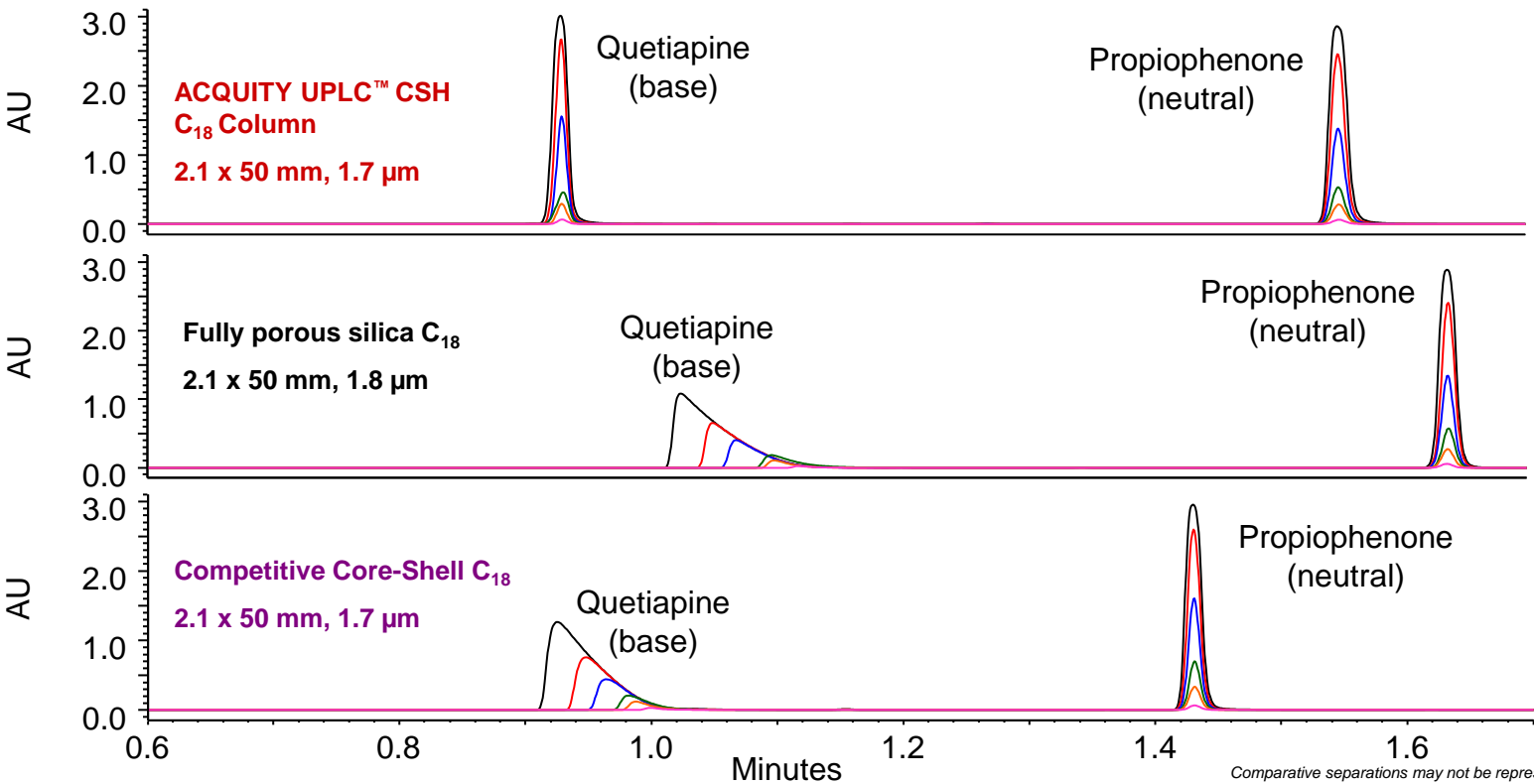


Bond and End Cap

Functionalize with appropriate bonded phase chemistry

Benefits of CSH Technology

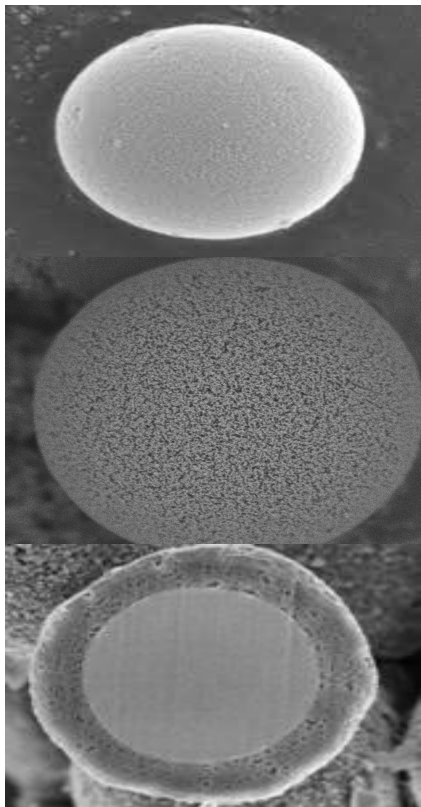
Loading Comparison in Formic Acid



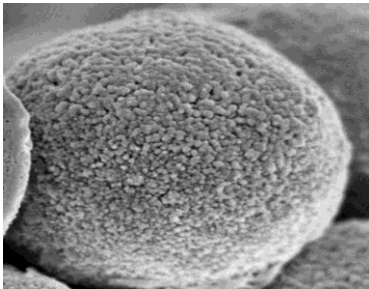
Comparative separations may not be representative of all applications

What are important attributes for chromatography?

- Pore Diameter (**PD**)
- Pore Volume (**PV**)
- Surface Area (**SA**)
- Particle Composition
- Particle Size
- Particle Surface Charge
- Particle Morphology
- Ligand/Ligand Density



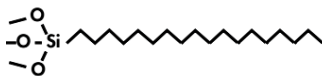
**Smooth
Surface**



**Rough
Surface**

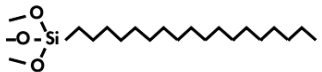
What are important attributes for chromatography?

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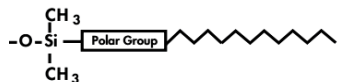
Full Coverage C₁₈

General purpose, balance non-polar retention for acids, bases, and neutrals



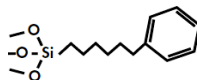
Mid Coverage C₁₈

Balanced retention for polar and non-polar compounds



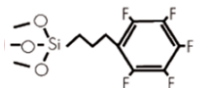
Embedded Polar C₁₈

Different selectivity, with improved peak shape for basic compounds



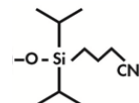
Phenyl Hexyl

Different selectivity especially for aromatic compounds



Pentafluorophenyl Propyl

Different selectivity especially for basic compounds



Cyano

Different selectivity especially for polar molecules



Amide (**HILIC Phase**)

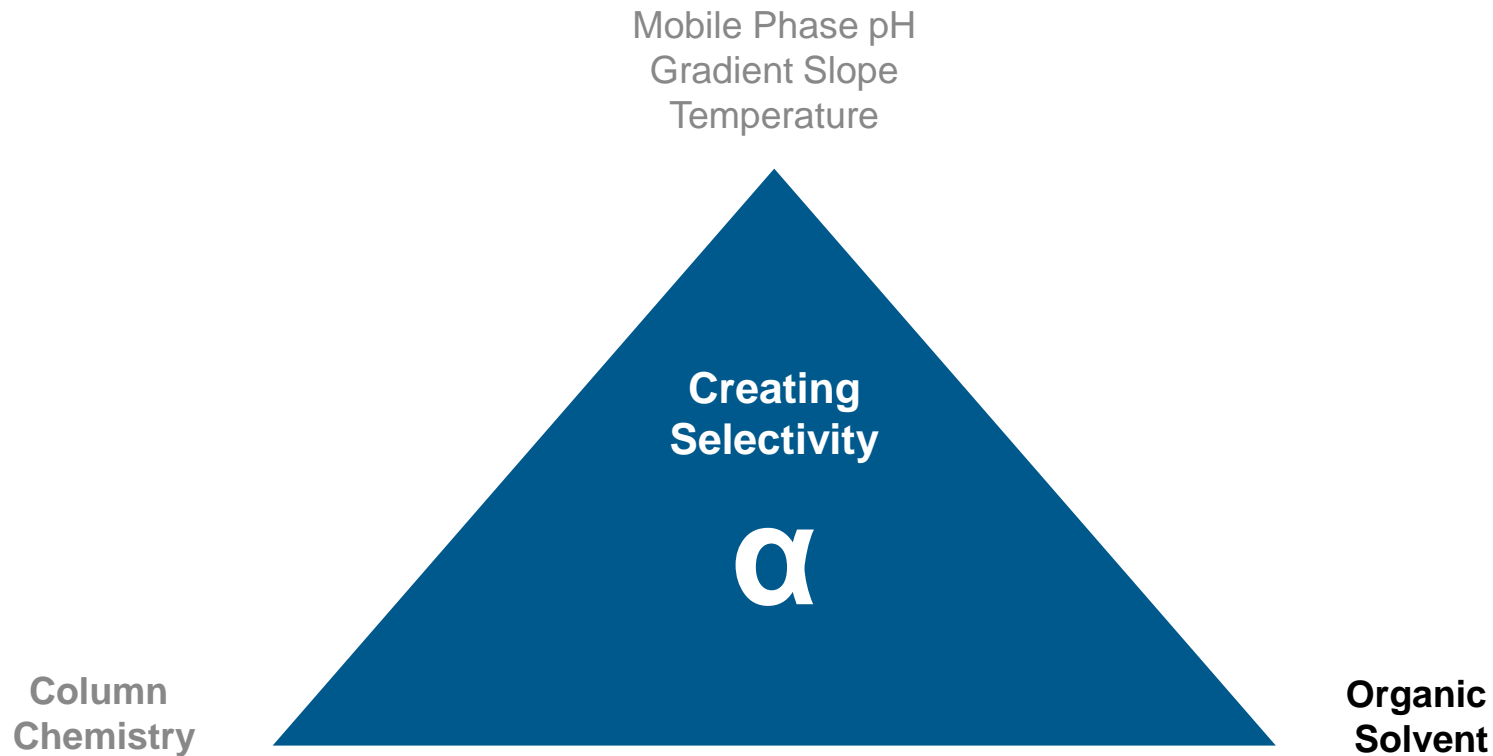
Different selectivity for polar basic/polar acid molecules

Summary:

Reversed-Phase Column Chemistries

- Important column chemistry attributes
 - Ligands and particle substrates
 - Reversed-phase and mixed mode behavior
- Consider pH limitations of columns

Creating Selectivity: Reversed-Phase Method Development



LC Solvent Characteristics

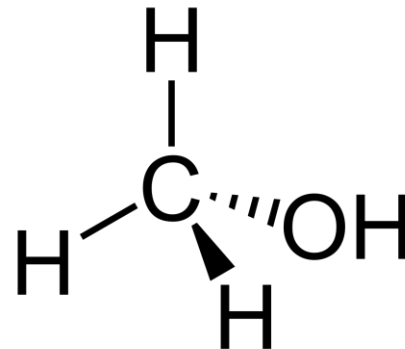
Solvent	Relative Elution Strength*	Comments
Methanol	1.0	Lower toxicity
Acetonitrile	3.1	Low viscosity
Tetrahydrofuran	3.7	Check for peroxides before concentrating
IPA	8.3	High viscosity
Acetone	8.8	High UV cutoff
Ethyl Acetate	High	Low solubility in water
Methylene Chloride	High	Least soluble in water

*High Purity Guide, Burdick & Jackson Laboratories, Inc. Solvent Properties of Common Liquids; L.R. Snyder, J. Chromatogr. 92, 223 (1974); J. Chromatogr. Sci., 16, 223 (1978).

Organic Solvent Considerations

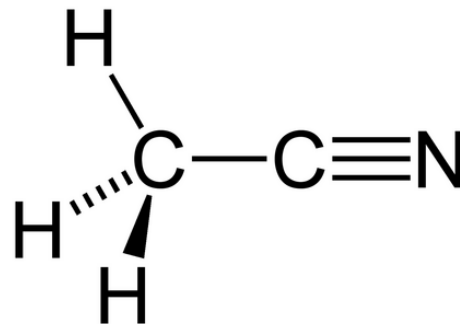
■ Methanol

- Protic solvent
 - C-OH group
 - Hydrogen bond donor and acceptor (polar interaction)
- More polar - Weaker reverse phase elution solvent
- More viscous – Lower flow rate ceiling

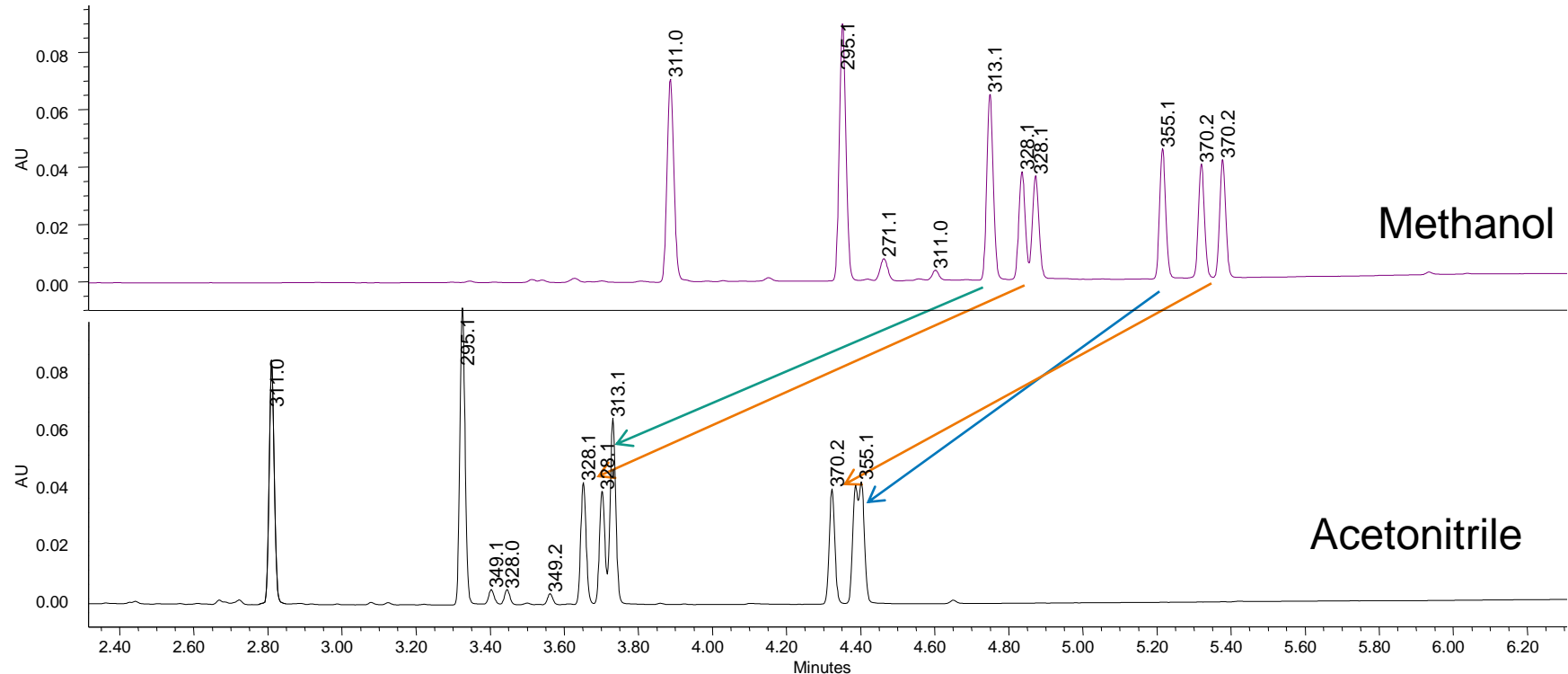


■ Acetonitrile

- Aprotic solvent
 - C≡N group
 - Hydrogen bond acceptor only (polar interaction)
 - Pi-Pi electron interactions
- Less polar - Stronger reverse phase elution solvent
- Less viscous – Higher flow rate ceiling



Norgestimate and Ethinyl Estradiol with related substances



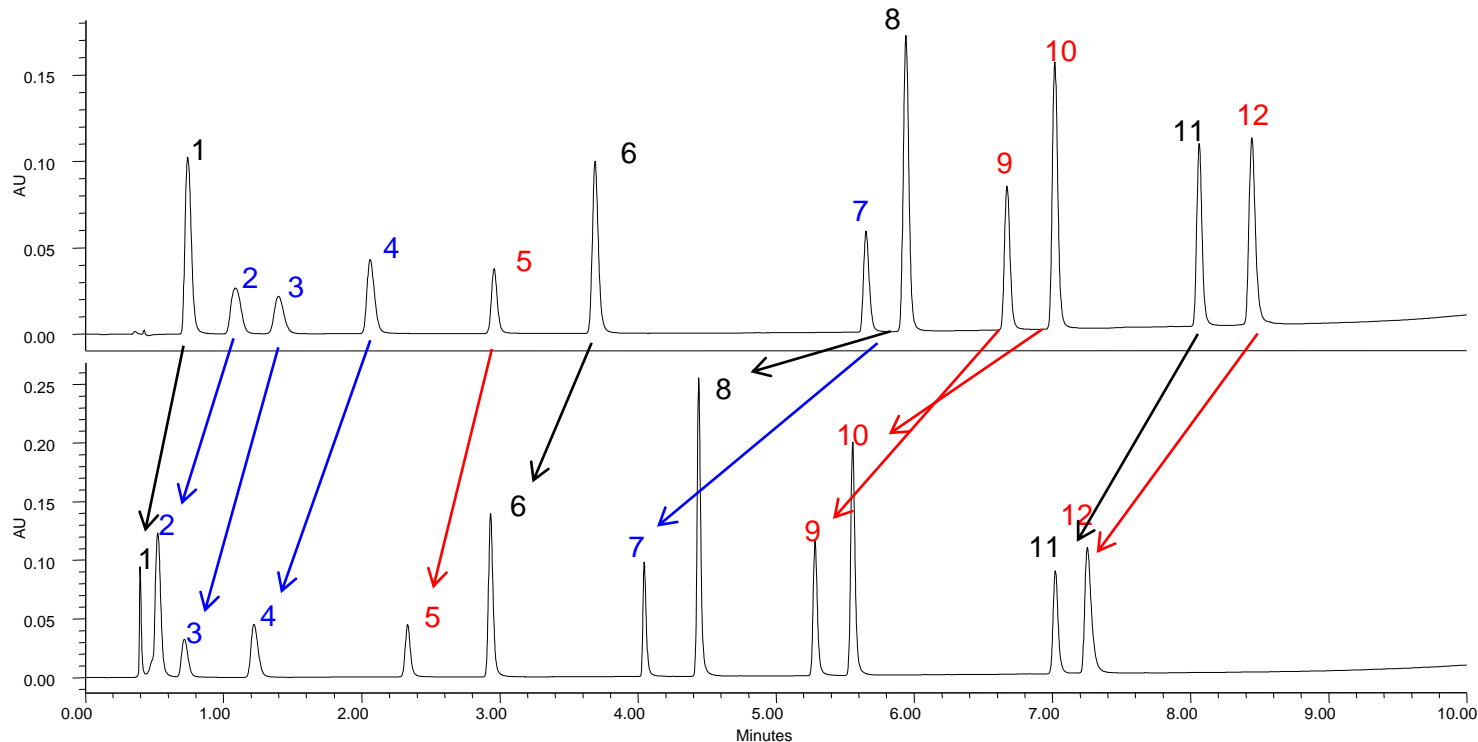
Effects of Solvent on Separation: Changes in Retention

Separation on a XSelect™ CSH C₁₈ 3.0 x 50 mm 2.5 μm

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Bases, Acids, Neutrals

1. Thymine
2. Amiloride
3. Doxylamine
4. Pindolol
5. 2-Acetamidophenol
6. Acetanilide
7. Imipramine
8. Demoxepam
9. Suprofen
10. Ketoprofen
11. Hexanophenone
12. Diflunisal



Methanol
0.1% Formic Acid

Acetonitrile
0.1% Formic Acid

Effects of Solvent on Separation: Changes in Selectivity

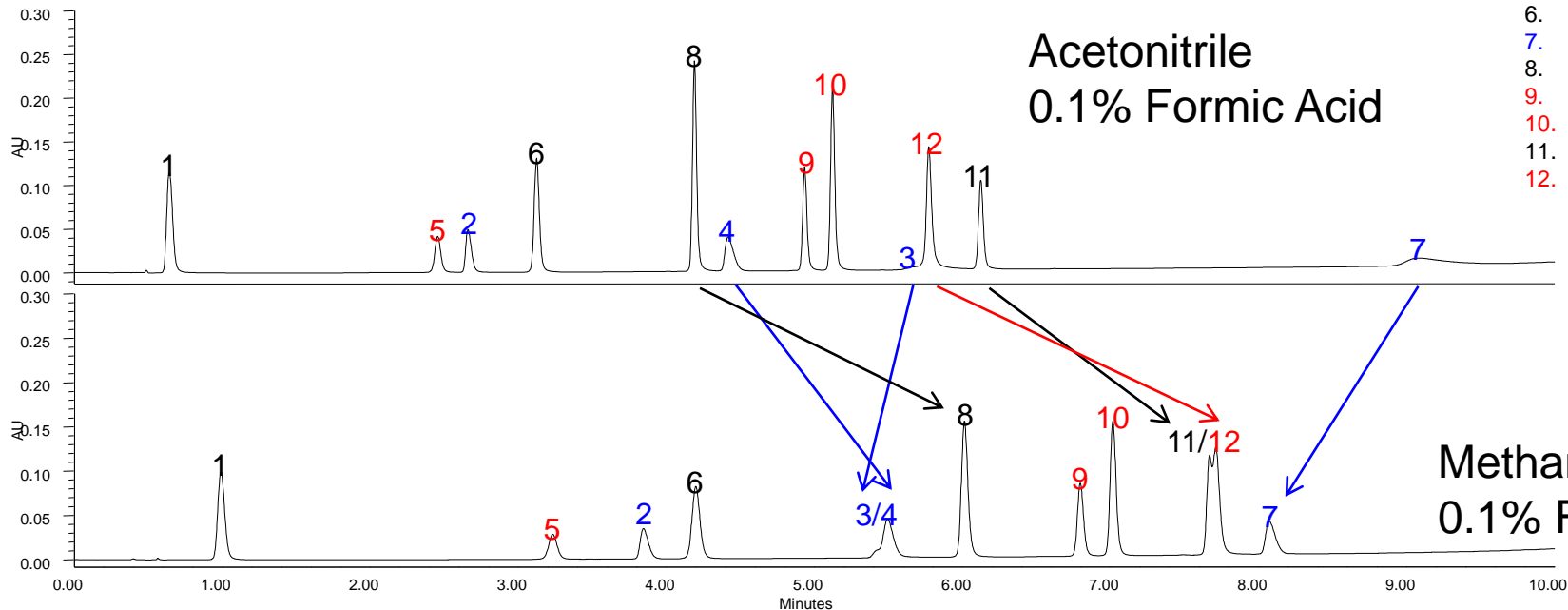
Separation on a XSelect HSS PFP 3.0 x 50 mm 2.5 µm

Bases, Acids, Neutrals

1. Thymine
2. Amiloride
3. Doxylamine
4. Pindolol
5. 2-Acetamidophenol
6. Acetanilide
7. Imipramine
8. Demoxepam
9. Suprofen
10. Ketoprofen
11. Hexanophenone
12. Diflunisal

Acetonitrile
0.1% Formic Acid

Methanol
0.1% Formic Acid



Mobile Phase Recommendations



Mobile phase is important for best chromatography and lowest baseline noise



Use the highest purity organic solvents, water and additives

LC/MS-grade is generally better quality than HPLC-grade

Some of the best solvents have certificates of analysis available

Some LC/MS-grade solvents are prefiltered to 0.2 micron

- **DO NOT** filter these solvents. The filters are “dirtier” than the solvents.



Other important things

ONLY use dedicated bottles and other glassware for HPLC

- **NEVER** wash HPLC glassware in a dishwasher with detergent.

- **ONLY** rinse bottles in mobile phase quality water and solvents

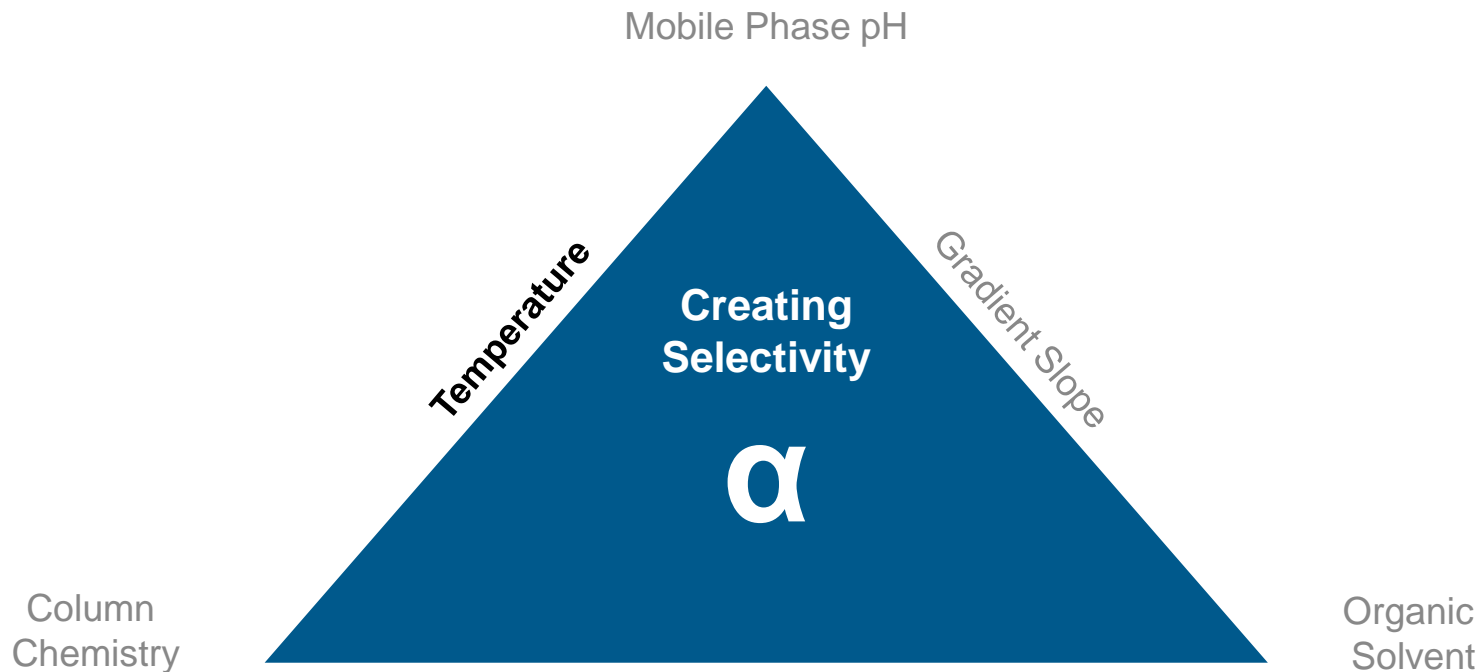
DO NOT top off bottle with more mobile phase.

- Old mobile phase will have changes in concentration (evaporation).

- Empty, clean bottle, refill.

Cover solvent bottles with proper caps, not tape, Parafilm, etc.

Tools for Optimizing Selectivity in Reversed-Phase Method Development

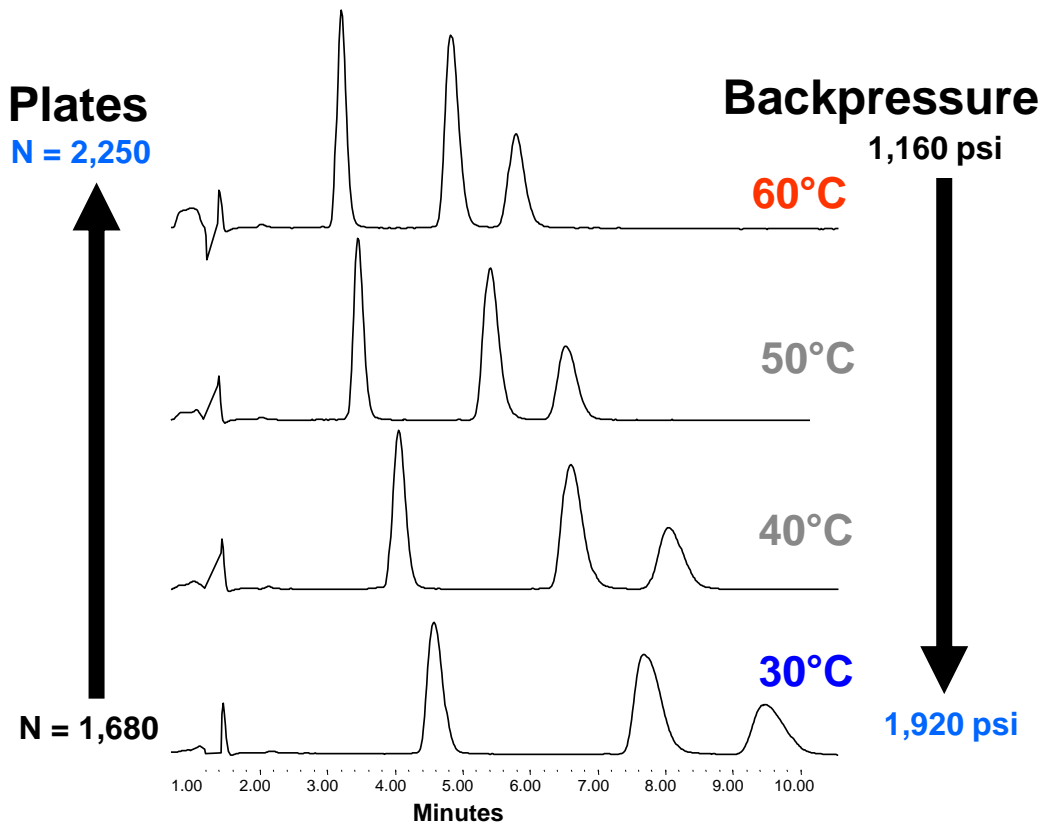


Method Optimization: Influence of Temperature

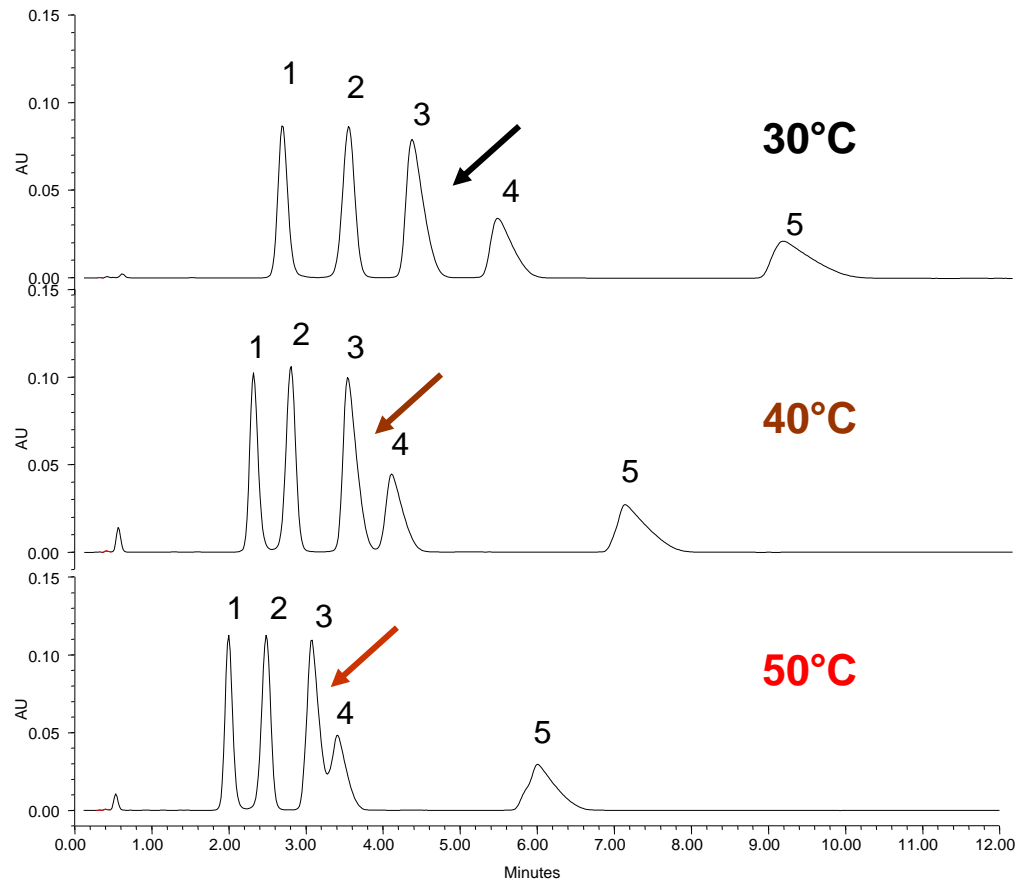
- Reduced mobile phase viscosity
- Lower backpressure
 - If flow rate is held constant
- Improve analyte diffusivity
 - Higher optimal linear velocity
- Changes in retention and selectivity

Effect of Temperature: Isocratic Separations

- Benefits of higher temperature
 - Shorter run time
 - Better sensitivity
 - Sharper peaks (higher efficiency, N)
 - Lower backpressure
- Risks of higher temperature
 - Shorter column lifetime due to accelerated chemical attack



Temperature and Selectivity



- Small, but sometimes significant selectivity changes obtained from temperature changes
- Useful for selectivity fine tuning

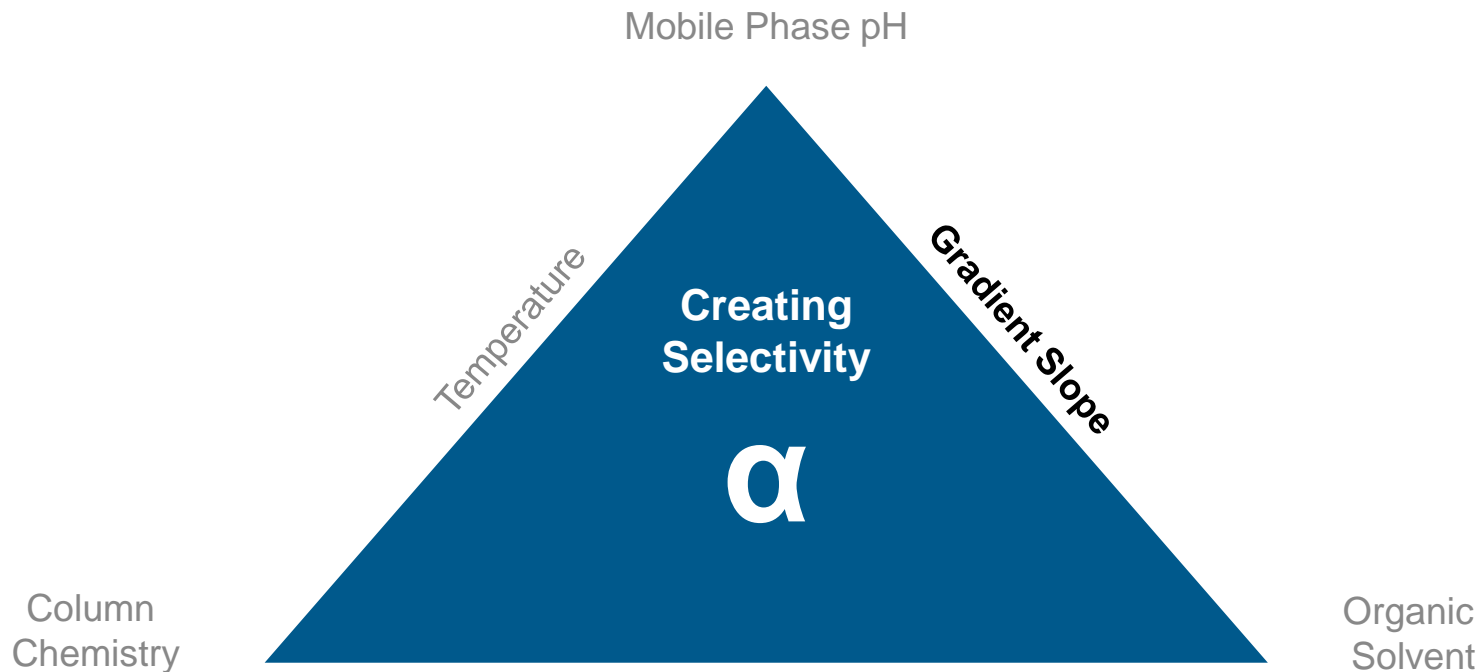
- 1 Triamterene
- 2 Althiazide
- 3 Bumetanide
- 4 Benzthiazide
- 5 Ethacrynic Acid

Temperature Effects Summary

- Elevated temperature
 - Earlier elution for most analytes
 - Reduces system backpressure
 - Enhances sensitivity

- Changing temperature
 - Potential to change the selectivity of the separation
 - Possibility of separating co-eluting peaks

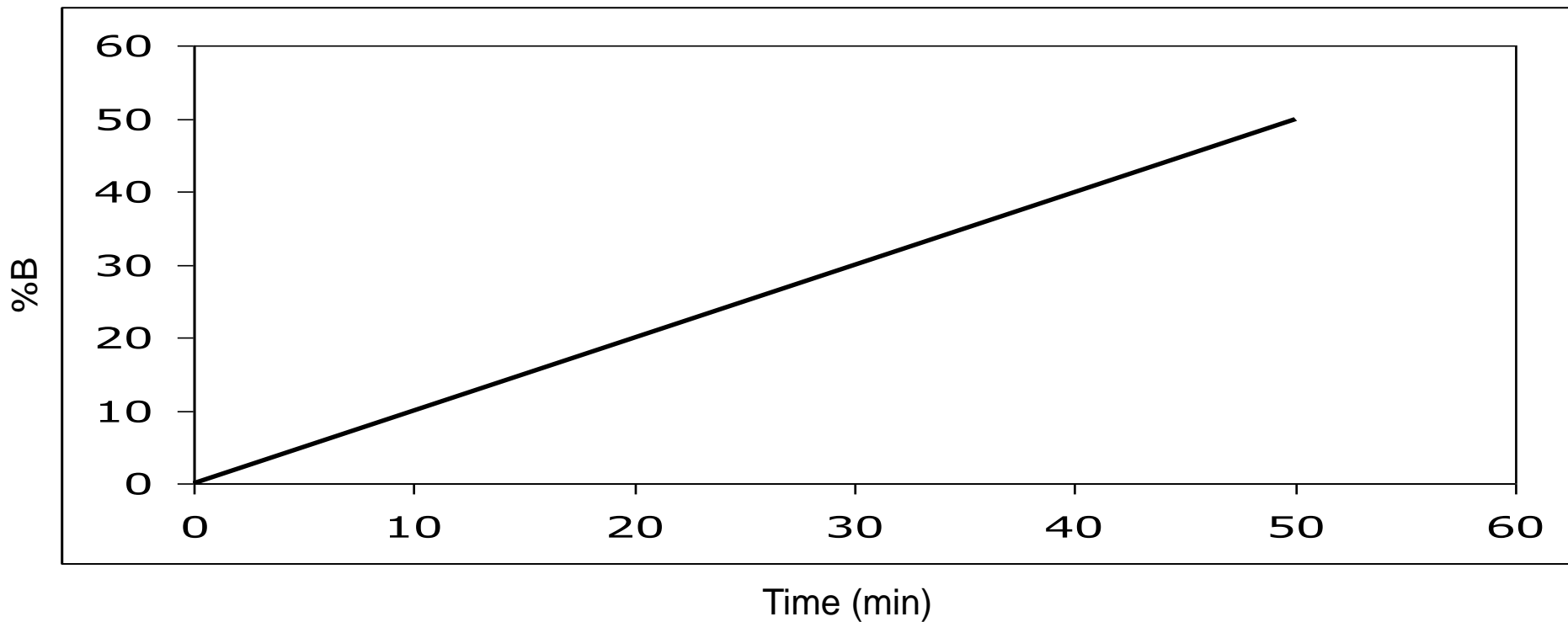
Tools for Optimizing Selectivity in Reversed-Phase Method Development



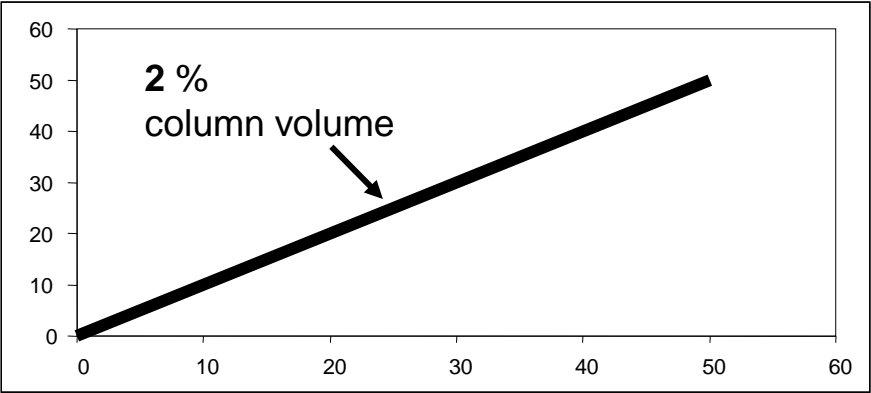
Method Optimization: Gradient Slope

- Shallower gradient slope may improve resolution
 - Decreasing gradient slope will decrease sensitivity
- Steeper gradient slope may compress the peaks and often reduce the resolution
 - Increasing gradient slope will increase sensitivity
- Changing gradient slope is a balance between peak heights relative to resolution
- Changes in retentivity and selectivity

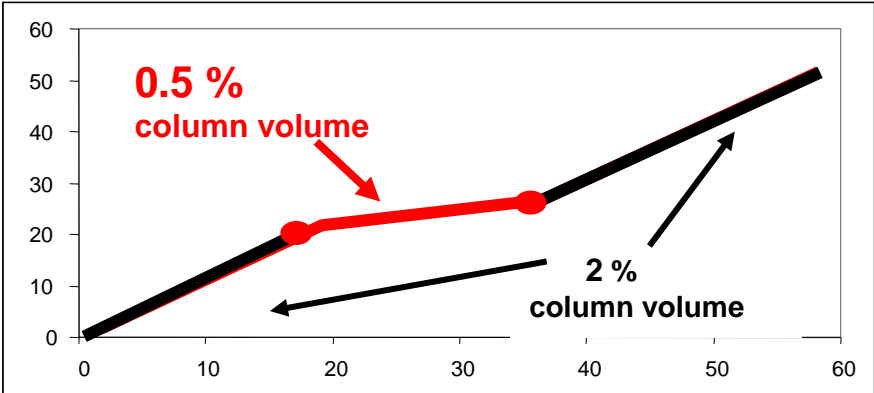
Gradient Slopes



Segmented Gradients



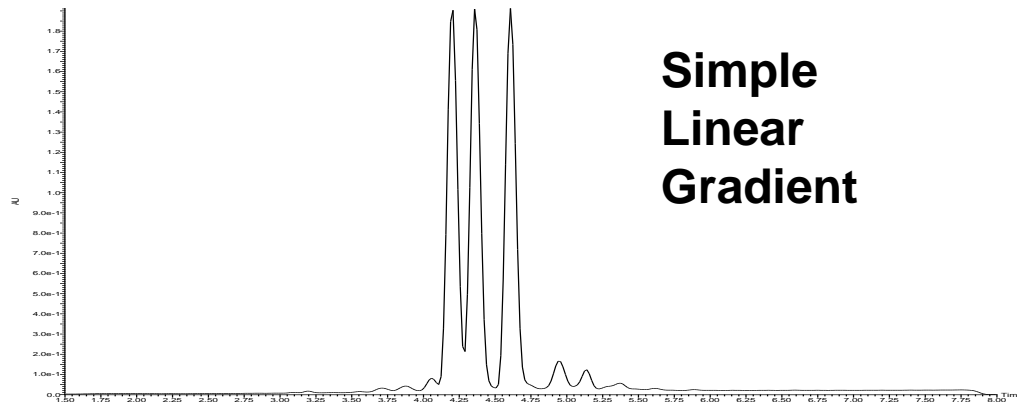
Linear



Segmented Gradient

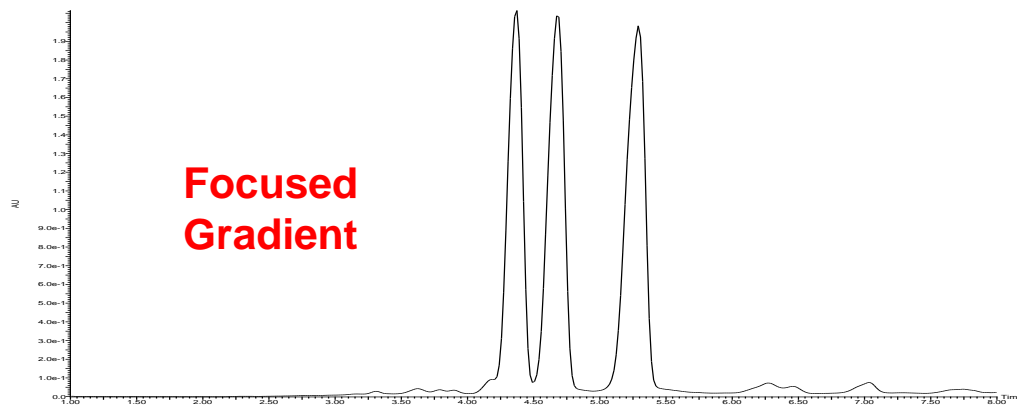
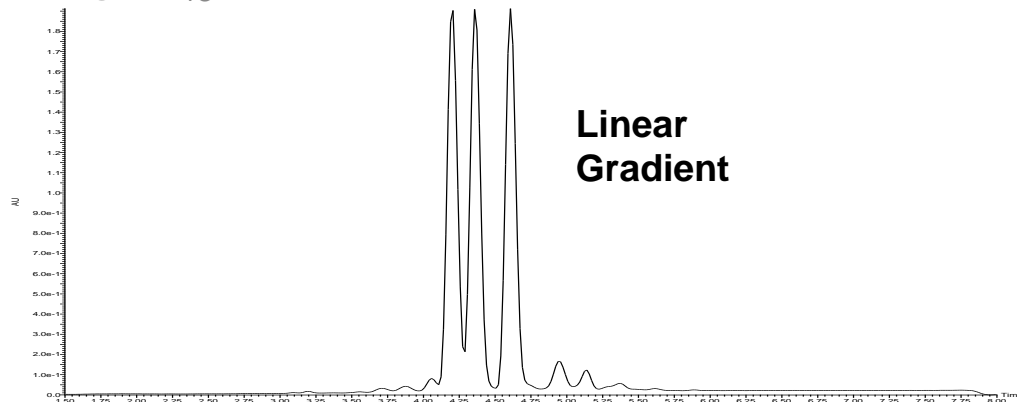
Linear Methanol Gradient at 60°C

XBridge™ C₁₈ column , 5 μm, 4.6 x 50 mm

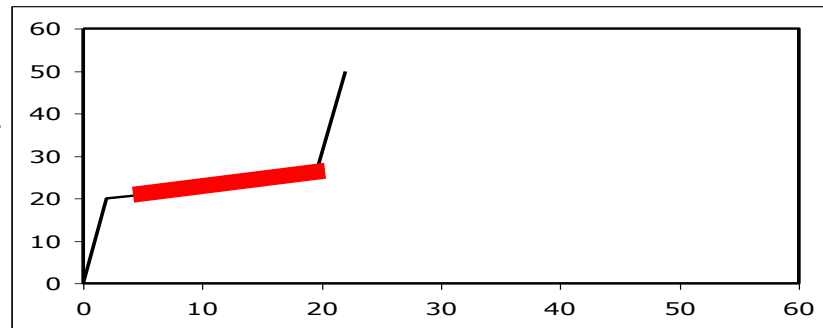


Focused Methanol Gradient at 60°C

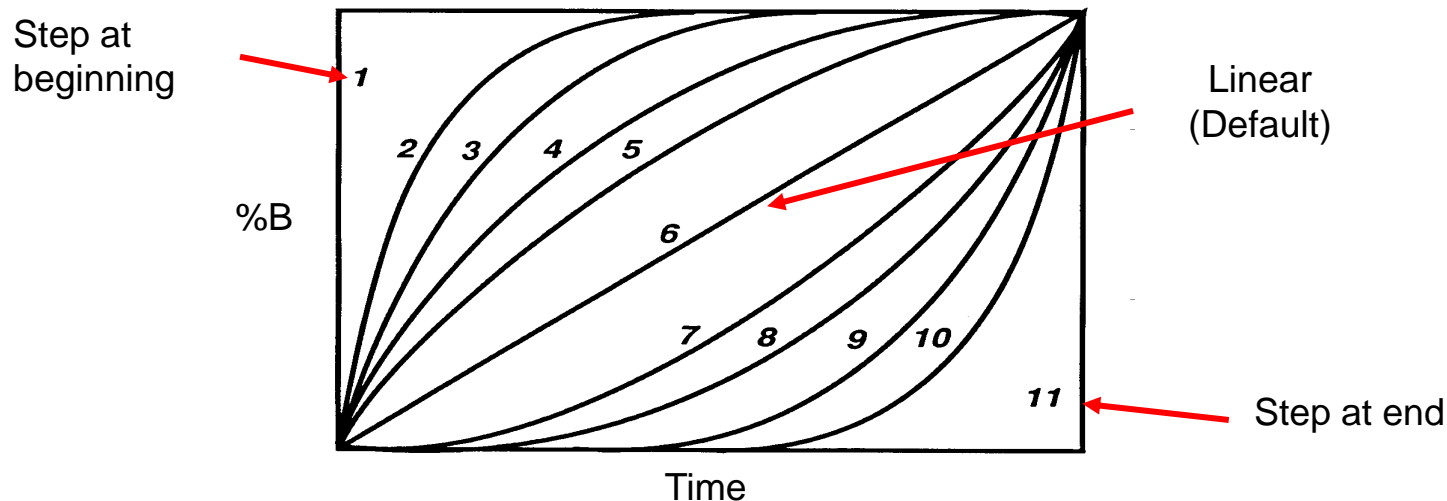
XBridge C₁₈, 5 μm, 4.6 x 50 mm



Apply a **focused gradient** to achieve a reasonable separation



Gradients Do Not Have to Be Linear!

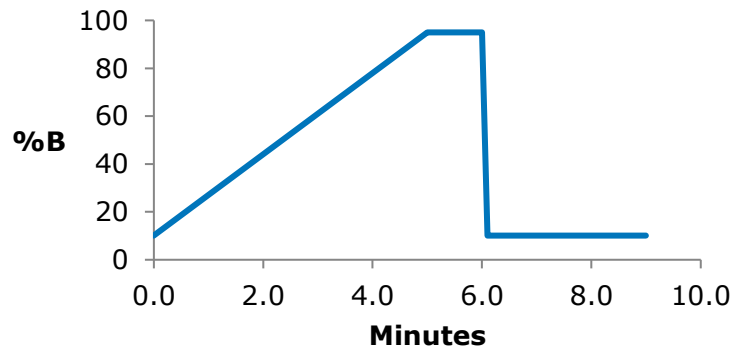


- The CURVE is the program for %B changes
- You program the time when the gradient should reach a certain %B and the curve number
- The time the gradient gets to the column will depend on the system volume

Gradient Programming Examples

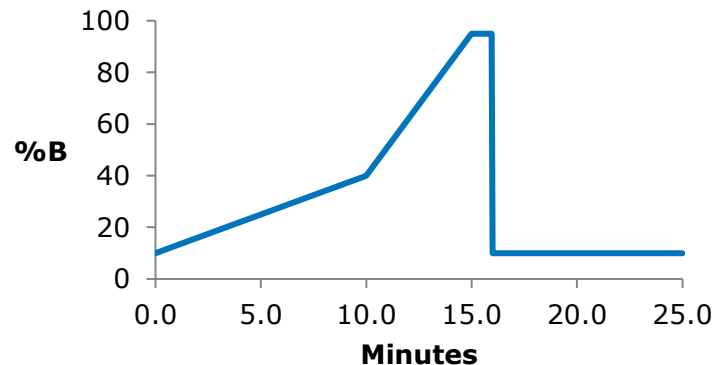
	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	0.600	90.0	10.0	Initial
2	5.00	0.600	5.0	95.0	6
3	6.00	0.600	5.0	95.0	6
4	6.10	0.600	90.0	10.0	6

Run time = 9 minutes



	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	0.250	95.0	5.0	Initial
2	10.00	0.250	60.0	40.0	6
3	15.00	0.250	10.0	90.0	6
4	16.00	0.250	95.0	5.0	11

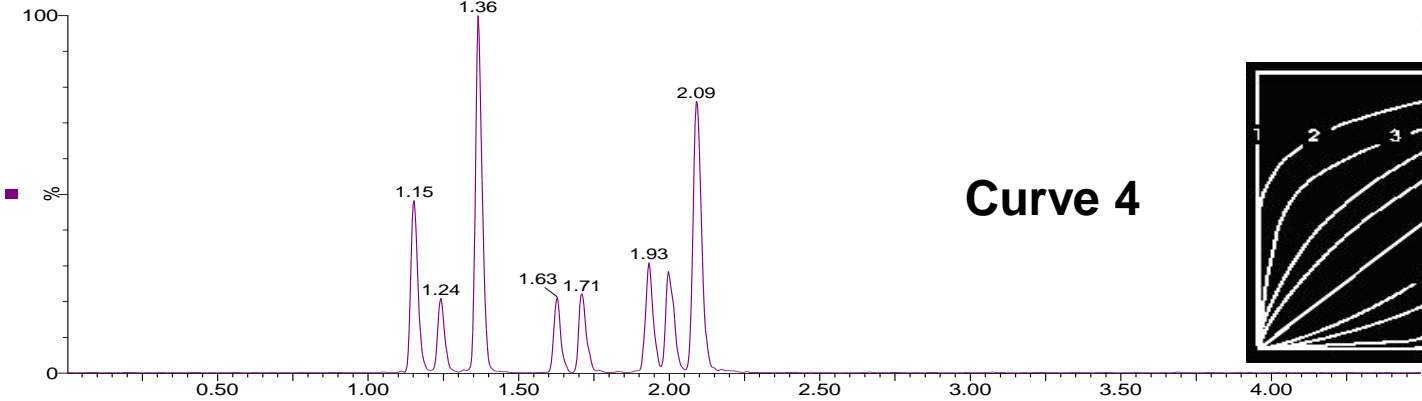
Run time = 25 minutes



Example: Gradient Curves: 2-15% ACN in 4 Minutes

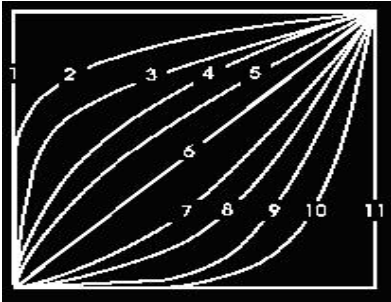
100

25Feb_Mixed50 1ul ACN T3 C4 Sm (Mn, 2x1)

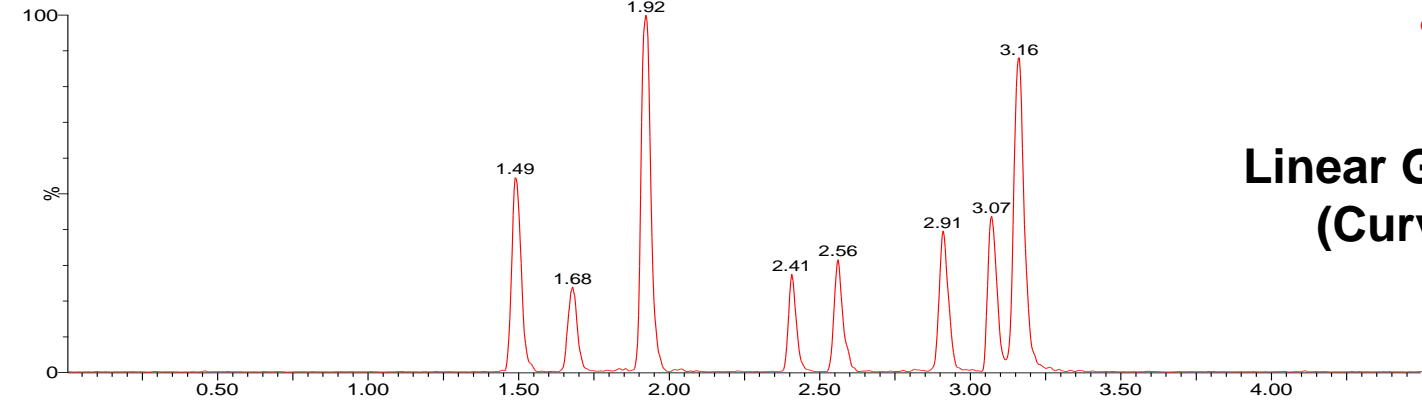


Curve 4

MRM of 8 Channels ES+
TIC
1.04e5



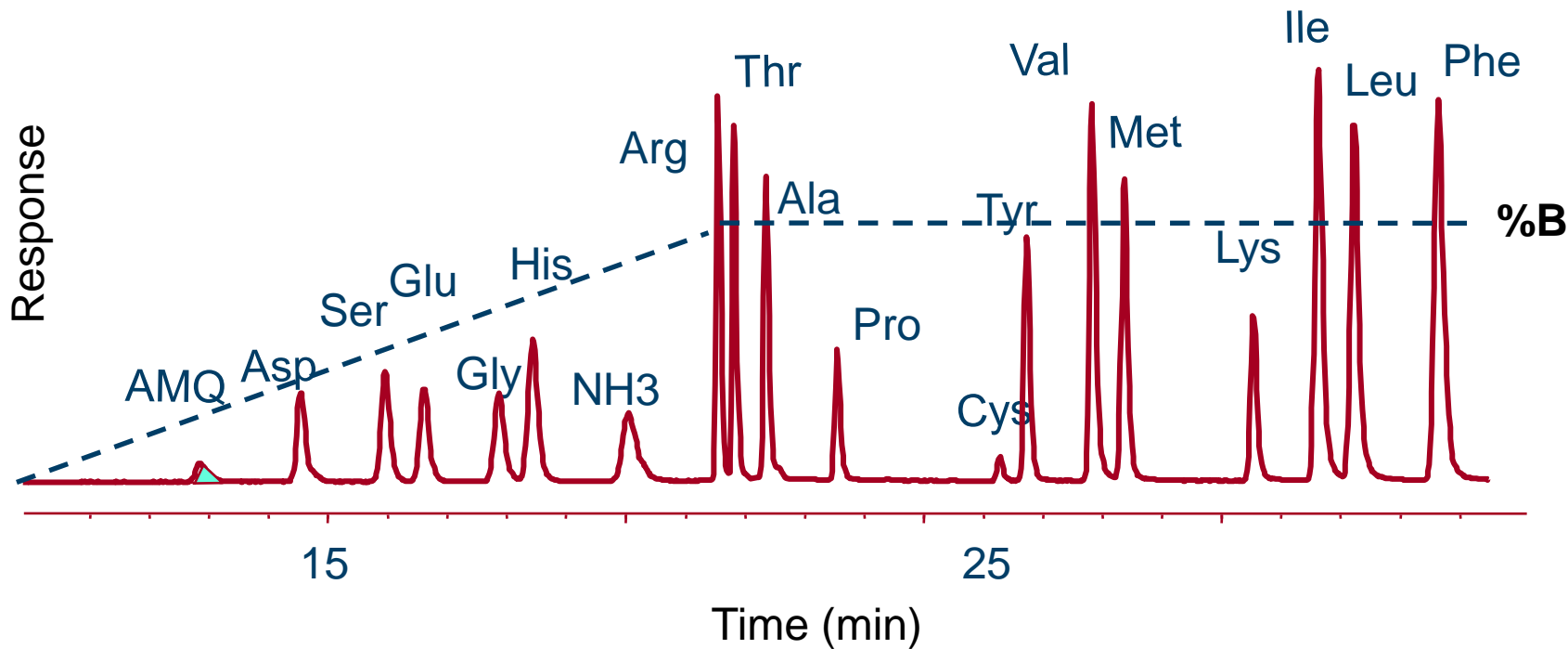
25Feb_Mixed50 1ul ACN T3 Sm (Mn, 2x1)



MRM of 8 Channels ES+
TIC
6.42e4

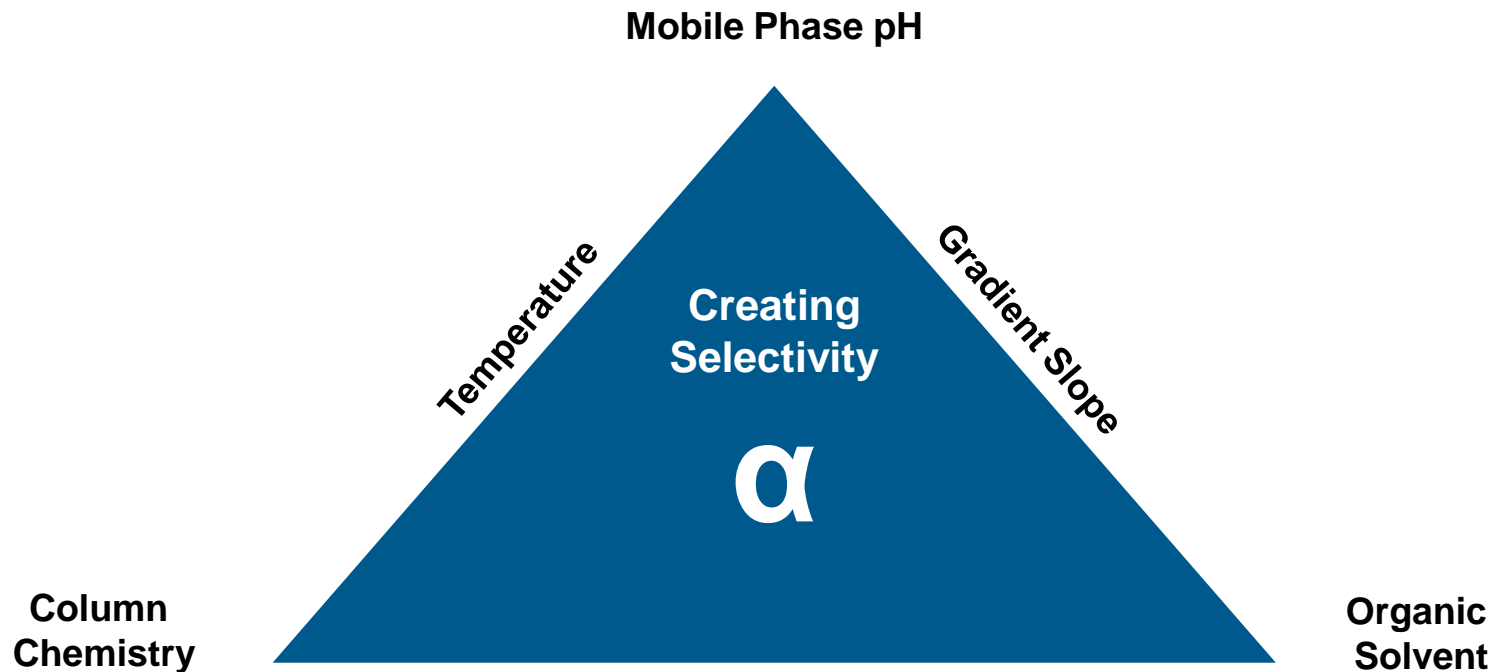
Linear Gradient
(Curve 6)

Gradient Separation – Amino Acid Hydrolysate



- Performing shallow gradients around analytes of interest can improve resolution
 - By segmenting the gradient, it is possible to maintain separations in critical elution zones and to reduce time and solvent consumption during less demanding (or unimportant) segments of the separation
- Changing gradient slope is a balance between peak heights relative to resolution
- Gradients do not have to be linear

Tools for Optimizing Selectivity in Reversed-Phase Method Development



Selectivity

- Tools for Affecting RP Method Development
 - pH
 - Column Chemistry
 - Organic Solvents
- Other Factors
 - Temperature
 - Gradient Slope

Efficiency

- The quest for ultra performance
 - How particle technology has evolved over the years
- The advantages of high efficiency columns
- Instrument dispersion and the impact on column considerations
 - Particle, particle size, and dimension impact on methods

Why we do Chromatography

We want to pull things apart!

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

Mechanical Contributions

- Ultra-low dispersion system
- Operate at optimal linear velocity
- Particle morphology
- Small particles
- Well-packed columns

Chemical/Physical Contributions

- Complementary bonded phases
- Multiple particle substrates
- Ability to utilize high pH
- Increase retentivity

Efficiency

Focus on the left side of the equation

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

Mechanical Contributions

- Ultra-low dispersion system
- Operate at optimal linear velocity
- Particle morphology
- Small particles
- Well-packed columns

Chemical/Physical Contributions

- Complementary bonded phases
- Multiple particle substrates
- Ability to utilize high pH
- Increase retentivity

Column Length and Efficiency

Consider two columns containing the same packing material, same particle size and have same mobile phase, only one is **twice as long**

50mm



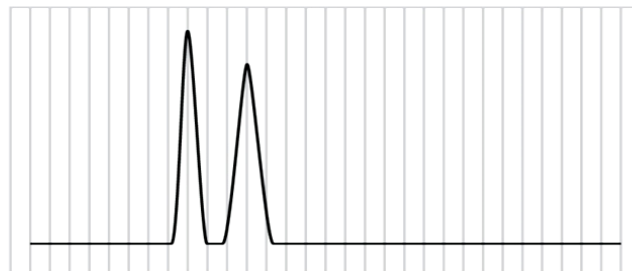
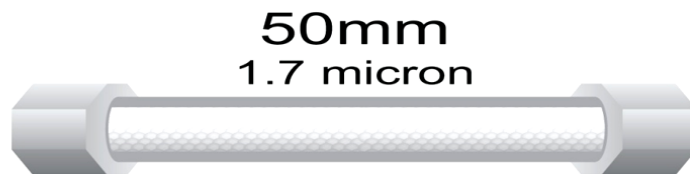
100mm



Particle Size and Efficiency

Consider Two columns containing the same packing material chemistry, the same length and using the same mobile phase.

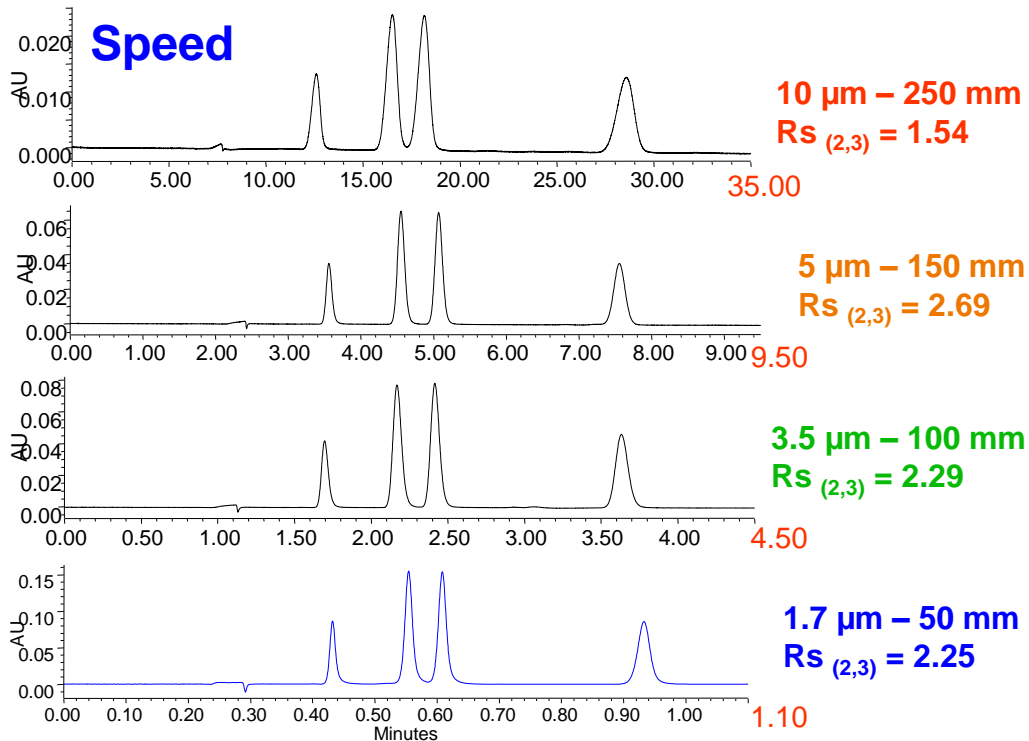
One column has *smaller particles which are a third the size.*



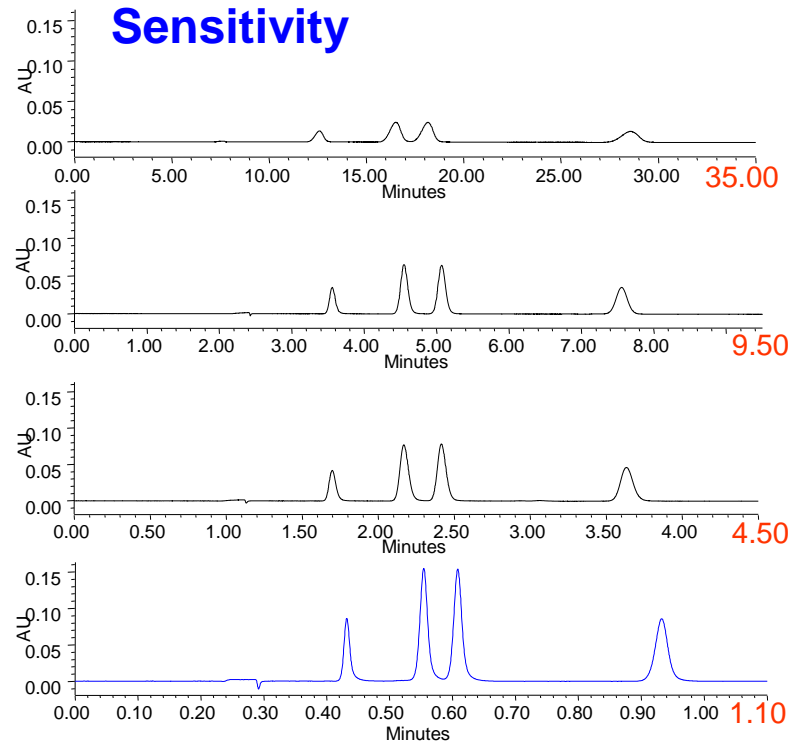
Efficiency does not Impact Selectivity

Increasing Efficiency Improves Speed and Sensitivity

Speed

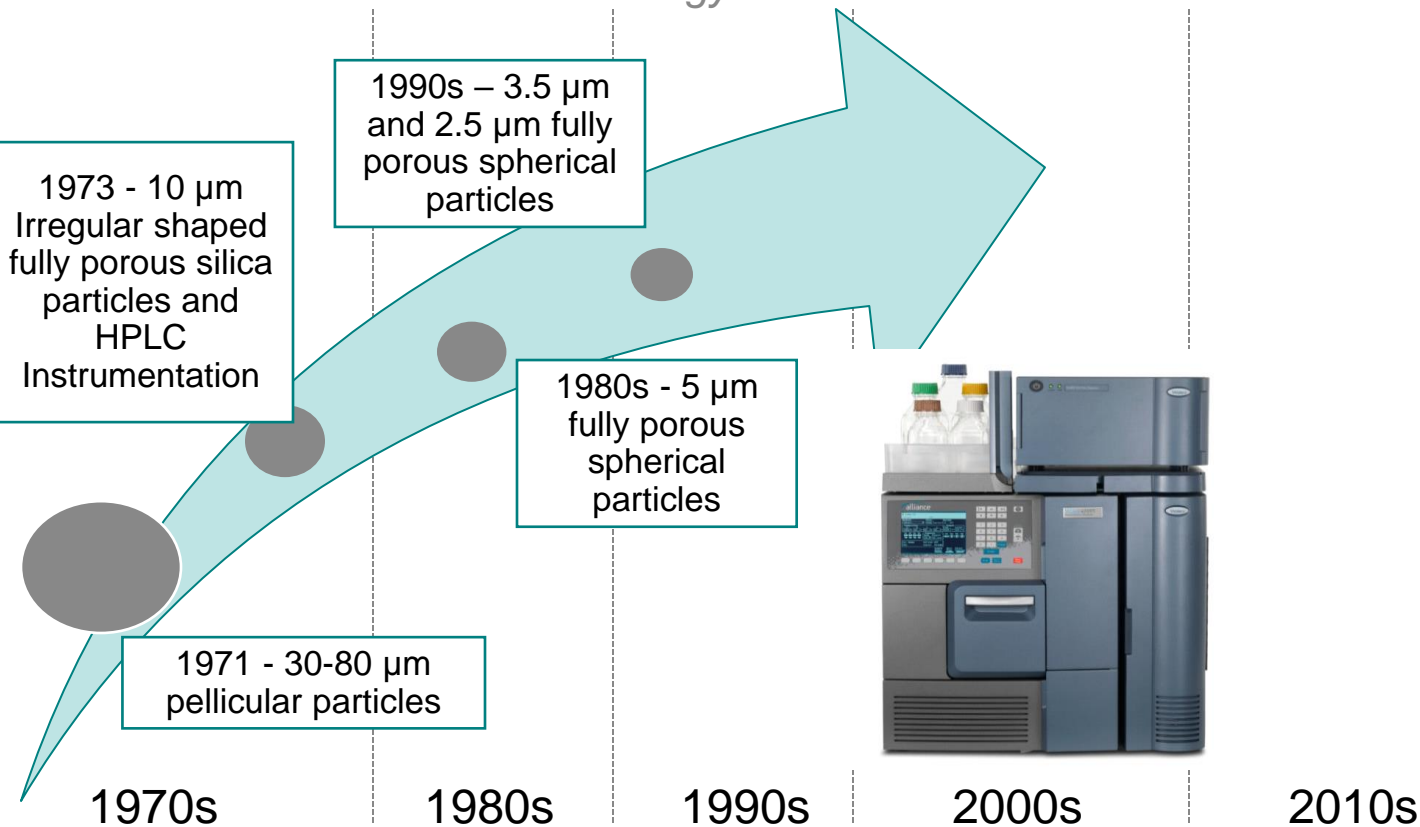


Sensitivity



The Quest for Ultra Performance

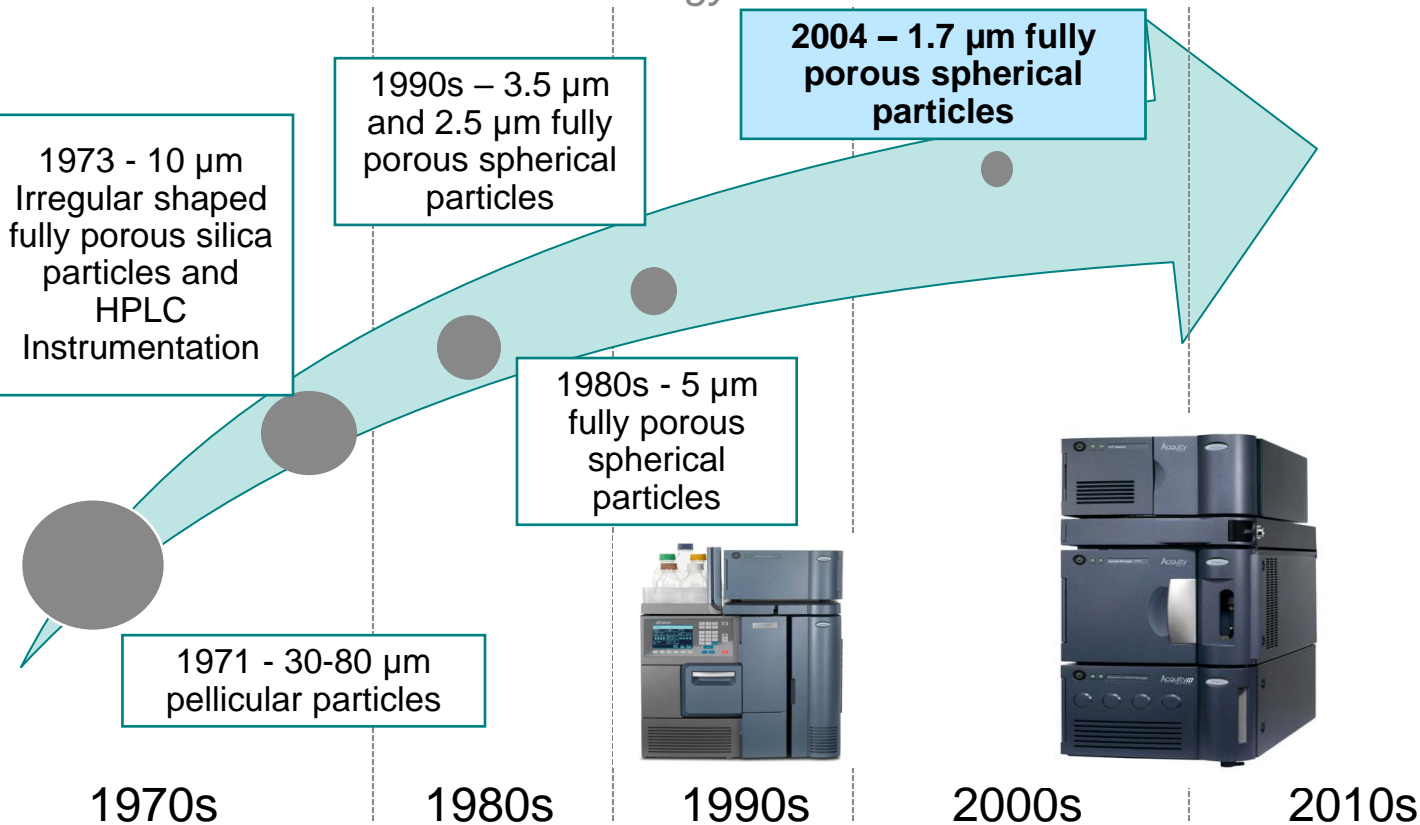
Advancements in Particle Technology



Efficiency

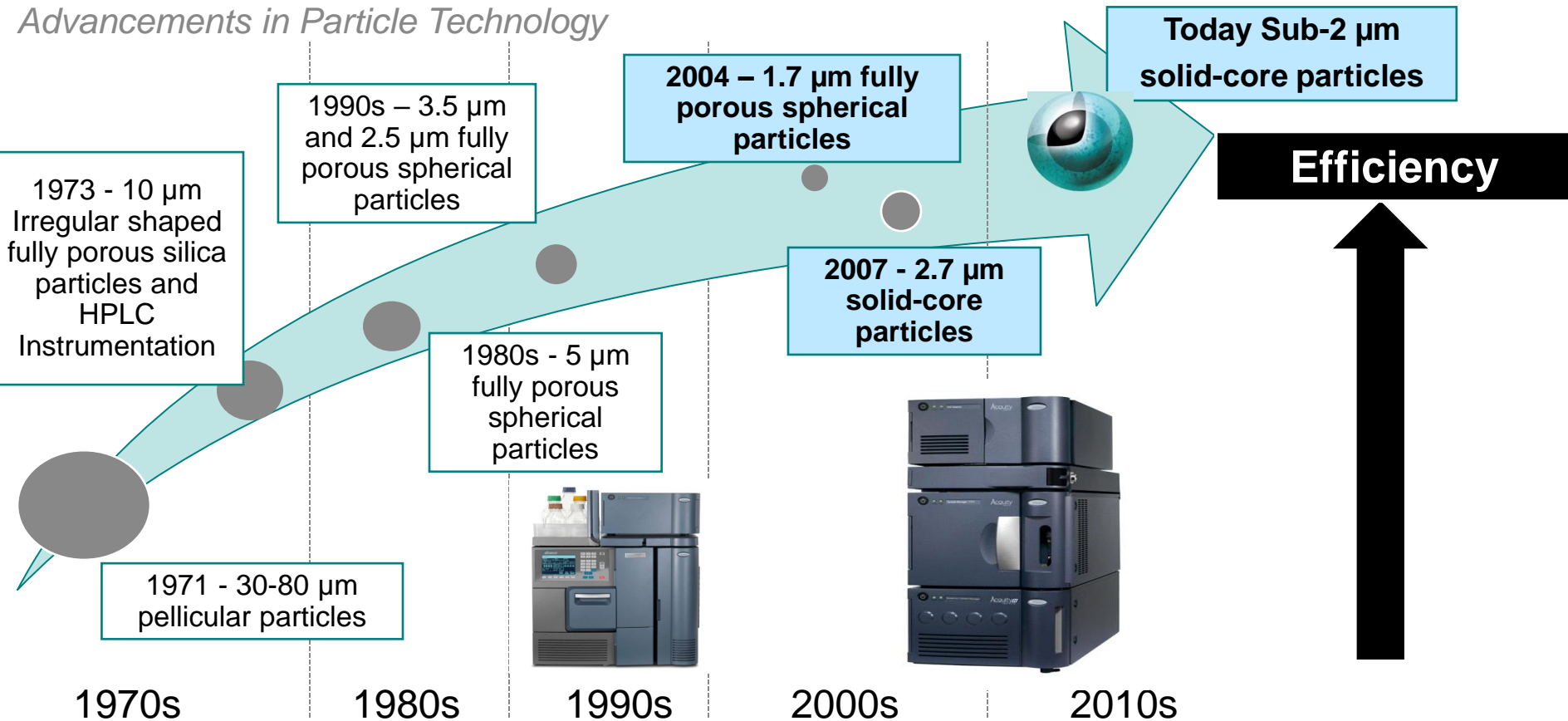
The Quest for Ultra Performance

Advancements in Particle Technology



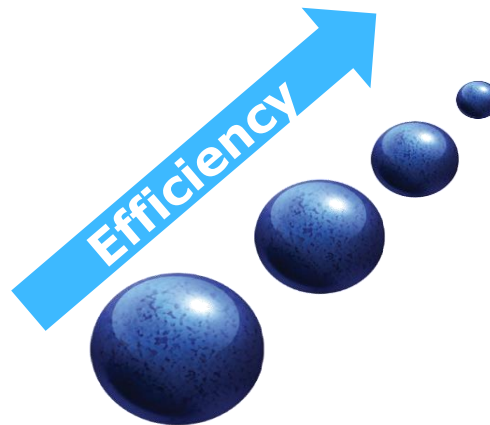
The Quest for Ultra Performance

Advancements in Particle Technology

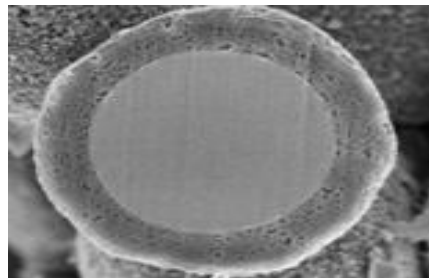


Increasing Column Efficiency

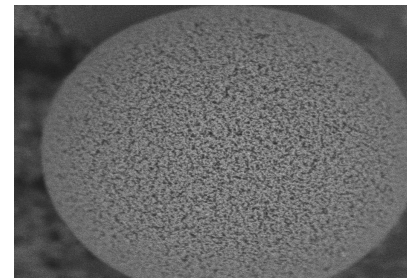
- Two common ways to increase the columns efficiency:
 1. Decrease the particle size
 2. Use column packed with Solid-core particles



Solid-Core Particle

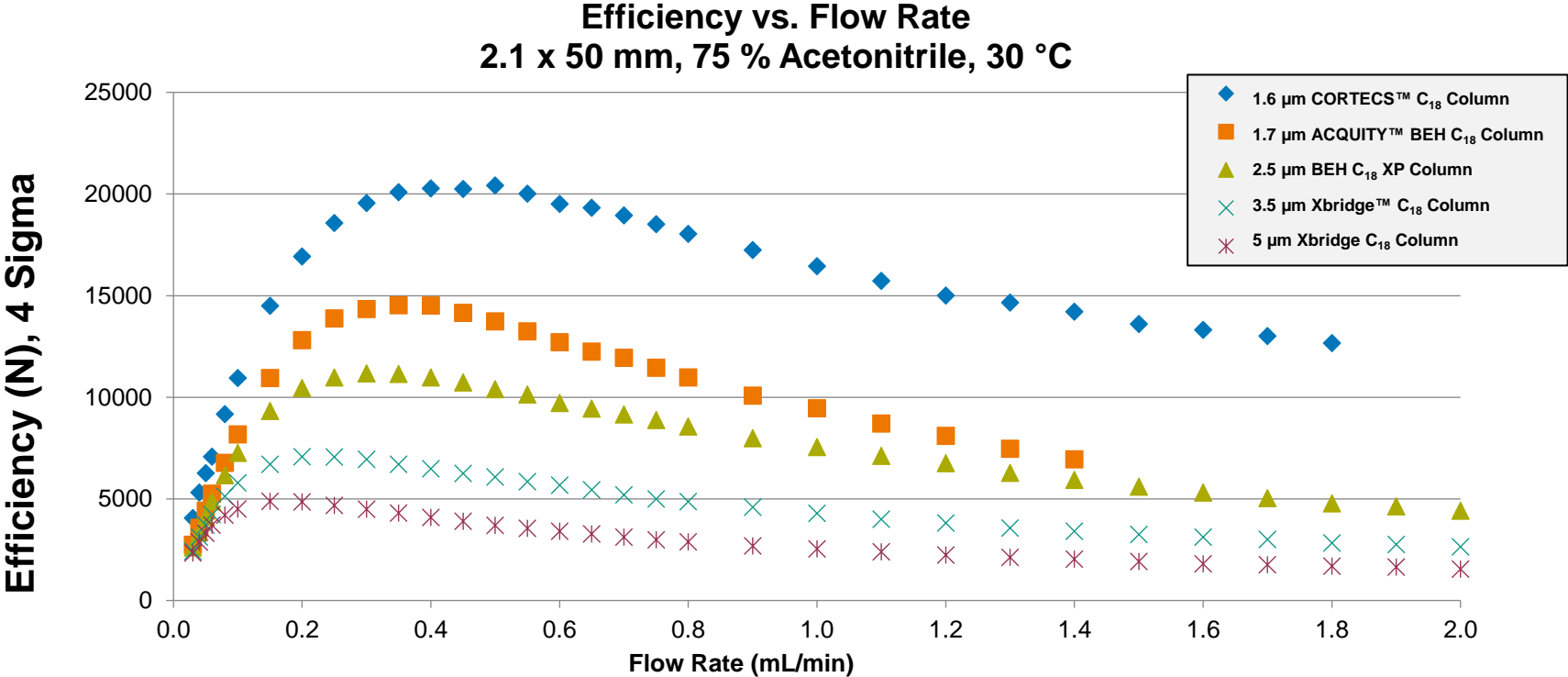


Fully Porous Particle



Levels of Efficiency

Fully porous and Solid Core Columns



The Advantage of High Efficiency Particles

Improved Productivity

$$N \propto \frac{L}{d_p}$$

4.6 x 100 mm, 3.5 μm Column:

$$\frac{L}{d_p} = \frac{100,000 \mu\text{m}}{3.5 \mu\text{m}} = \underline{28,571}$$



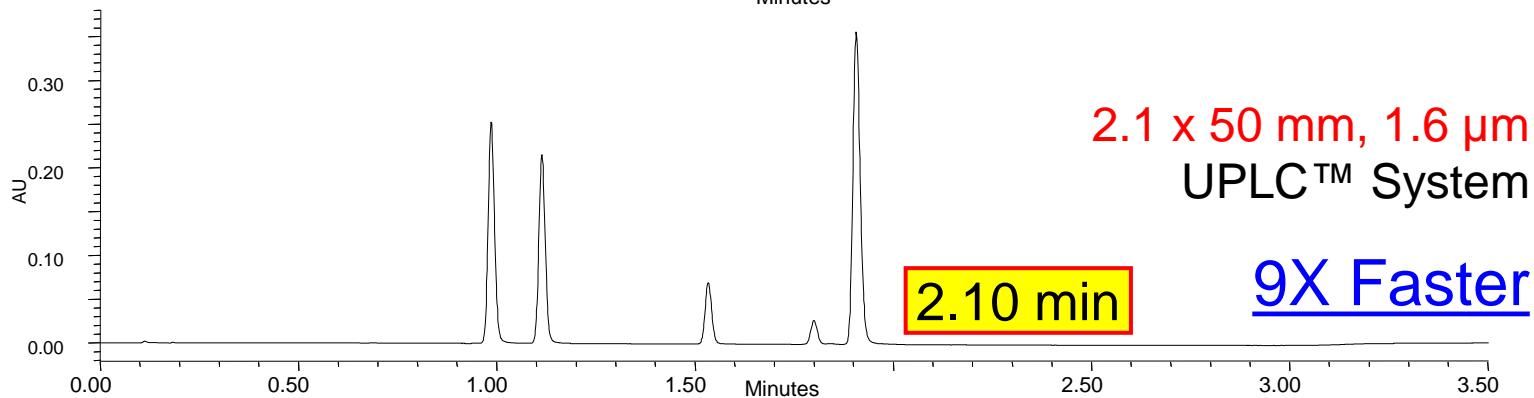
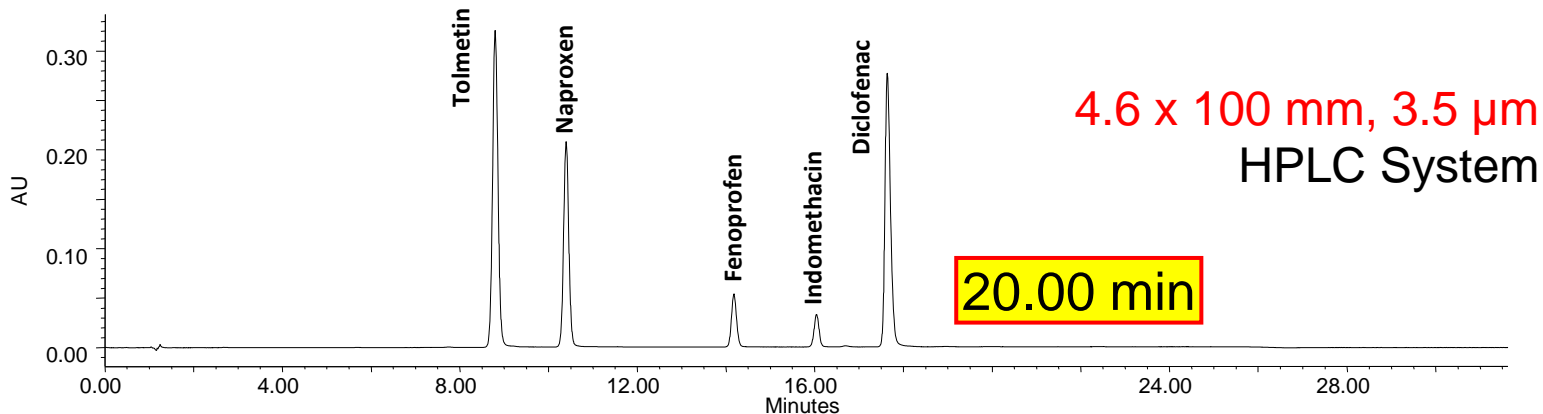
2.1 x 50 mm, 1.7 μm solid-core Column:

$$\frac{L}{d_p} = \frac{50,000 \mu\text{m}}{1.7 \mu\text{m}} = \underline{29,412}$$



The Advantage of High Efficiency Particles

Improved Productivity



Selecting Proper Efficiency Based on Application Difficulty

Application Difficulty	Example	Suggested Efficiency Range	Column Length mm	Particle Size µm	Efficiency
Extremely Difficult	Complex Matrix, Metabolite Identification	> 85,000	250 150 150 Solid Core	3.5 1.7 1.6	71,400 88,200 114,700
Difficult	Impurity Profile Degradation Study	> 50,000	250 150 100 100 Solid Core	5 2.5 1.7 1.6	50,000 60,000 58,800 76,400
Moderate Challenging	Related Compound Assay	> 30,000	300 150 100 75 50 50 Solid Core	10 5 3.5 2.5 1.7 1.6	30,000 30,000 28,500 30,000 29,400 38,200
Easy	Few Peaks, Well Separated (Fast) Content Uniformity, Dissolution	> 15,000	75 50 30 30 Solid Core	5 2.5 1.7 1.6	15,000 20,000 17,600 22,900

Challenges in Using Smaller Particles

- As the column dispersion get smaller the influence of system dispersion increases
 - More significant for small narrow bore columns
 - More significant on weakly retained analytes compared to strongly retained analytes
- Instruments must be able to handle the additional back pressure smaller particles generate when operated at their optimal linear velocity.

Higher Backpressure \neq Performance

Instrument (System) Dispersion

What is it & Where is it

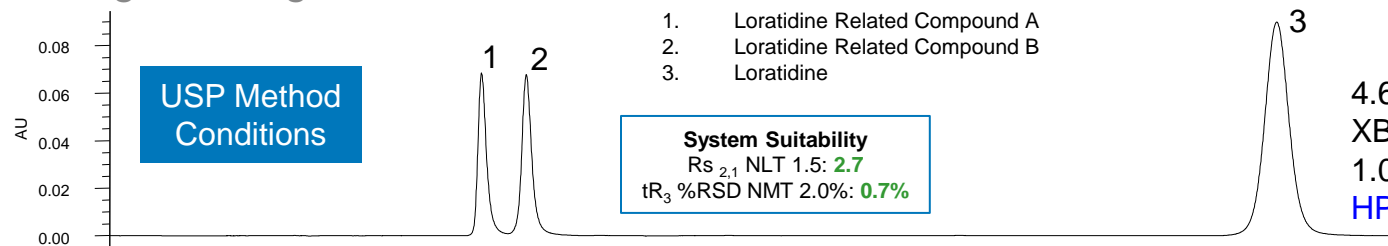


- Instrument dispersion is the broadening of the analytical band due to the instrument's flow path volume
 - It's part of all LC systems, and varies significantly depending on the configuration

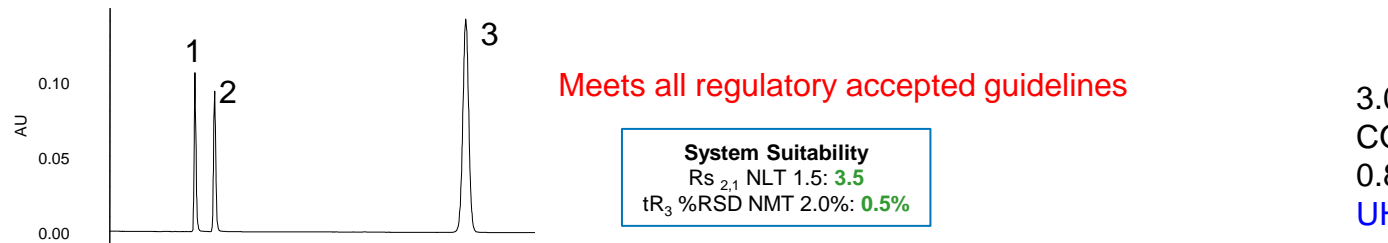
- Any place where the analytical band “moves” adds to the instrument's dispersion
 - Injector
 - Tubing
 - Pre-column
 - Post-column
 - Oven design
 - Flow cell volume

Using Efficiency to Increase Speed while Maintaining Resolution

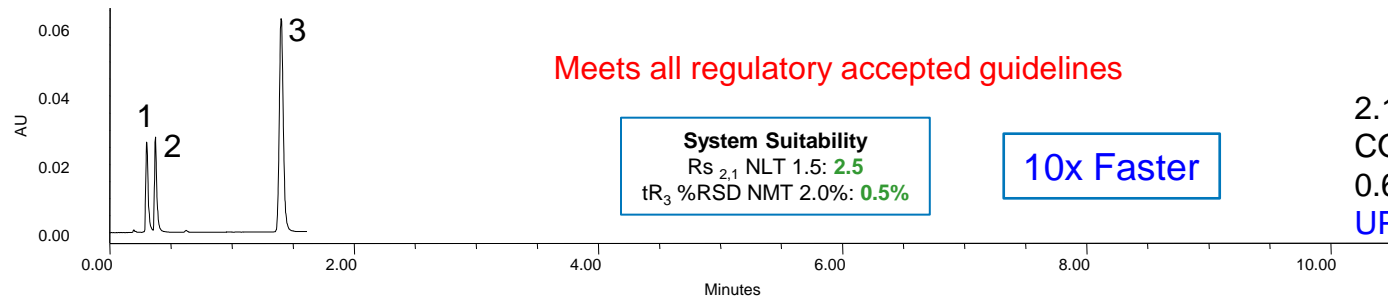
Putting it All Together



4.6 x 150 mm
XBridge BEH C₈ Column **5 μm**
1.00 mL/min
HPLC



3.0 x 100 mm
CORTECS C₈ Column **2.7 μm**
0.80 mL/min
UHPLC



2.1 x 50 mm
CORTECS UPLC C₈ Column **1.6 μm**
0.60 mL/min
UPLC

Why we do Chromatography?

We want to pull things apart!

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

Mechanical Contributions

- Ultra-low dispersion system
- Operate at optimal linear velocity
- Particle morphology
- Small particles
- Well-packed columns

Chemical/Physical Contributions

- Complementary bonded phases
- Multiple particle substrates
- Ability to utilize high pH
- Increase retentivity

An abstract graphic featuring a complex network of interconnected nodes and lines, resembling a molecular structure or a data network. The nodes are represented by small circles in various shades of blue and grey, connected by thin, light blue lines. The background is a gradient of blue, transitioning from a lighter shade on the left to a darker shade on the right. A horizontal band of solid dark blue is positioned across the middle of the image, serving as a backdrop for the text.

Waters

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