Making the Leap

Small Molecule – Biologics

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LC Columns Application Engineer
June 26, 2018

Outline

- Small and/or biomolecules; Similarities/differences
- Column Choice
- Things to Consider
- Method Conditions
- Troubleshooting

Small and/or Biomolecule; Similarities/Differences

Define

Types of chromatography

- Similar
- Different

Sample types

- Simple
- Complex

Functional groups

- Polarity

Small v. Biomolecules

Small Molecules

Size – MW <1000 (although some may be somewhat larger)

Structure – Simple

Drugs, pesticides, chemicals…

Manufacturing – Chemical synthesis, predictable process

Characterization – straightforward

Stability – Stable

Biologics

Size – Mostly >1000 with some exceptions

Structure – Diverse; need to consider chemistry, biology, and biochemistry

MAbs, aggregates, charge variants, conjugates, peptides, amino acids, nucleic acids

Manufacturing – living cells; “”dirty” (early) process with similar components; difficult to control

Characterization – complex molecular makeup; heterogeneity

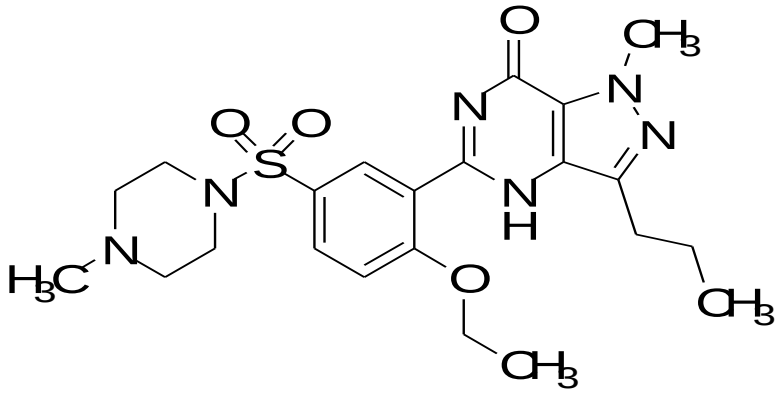
Stability – Sensitive to external conditions; Temp,

pH, Shearing, Folding, Glycoforms

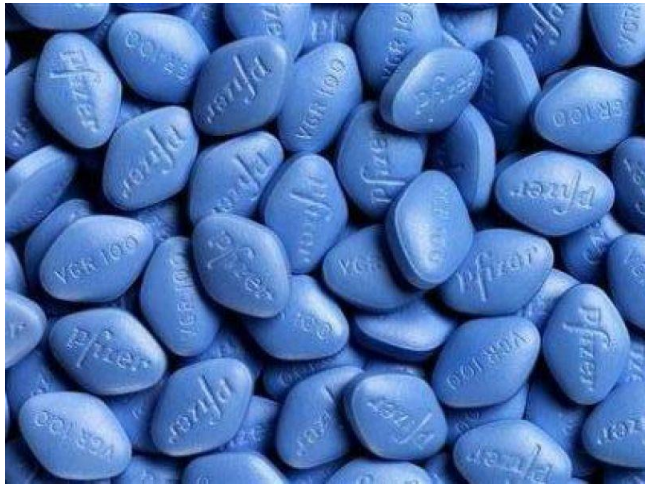
Do you know what this is?

$C_{22}H_{30}N_6O_4S$

MW=476.6 g/mol



Sildenafil



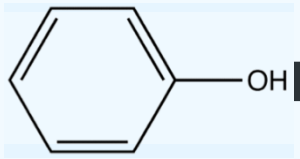
Polarity of Functional Groups – Nonionic

Affect Solubility and Elution Order

Functional Group	Polarity
	

Polarity of Functional Groups

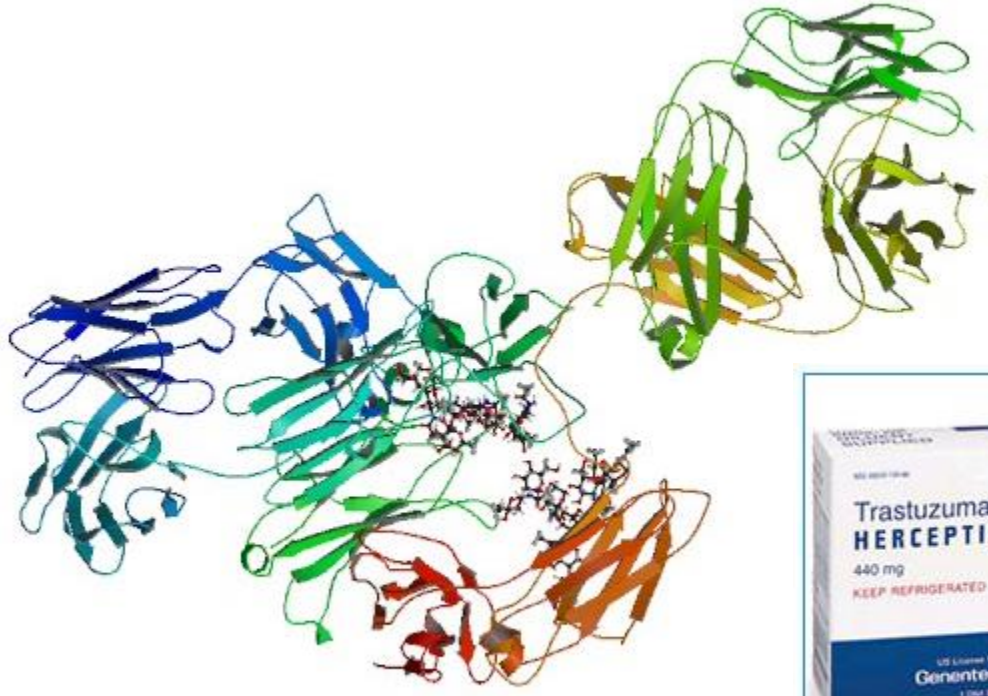
Affect Solubility and Elution Order

Functional Group	Polarity
	

Do you know what this is ?

$C_{6470}H_{10012}N_{1726}O_{2013}S_{42}$

MW = 145421.50 g/mol



HPLC Common Separation Mechanisms

Small Molecules

- Reversed Phase*
- Ion Exchange
- HILIC
- Normal Phase
- Chiral

Biomolecules – Intact Protein

- Reversed Phase*
- Ion Exchange
- Size Exclusion/Gel Filtration
- Affinity
- Hydrophobic Interaction (HIC)

*Of the many different HPLC separation mechanisms and column types, reverse phase is commonly used for both small molecules and biomolecules

Choosing the Right Column …

Sorbent characteristics
- –Particle size
- –Plate count
- –Back pressure
- –Pore size
- –Bonding chemistry
  - Small Molecule – C18, C8
  - Proteins – C4, C8

Column characteristics
- –Internal diameter
- –Length
- –Material

Instrument compatibility

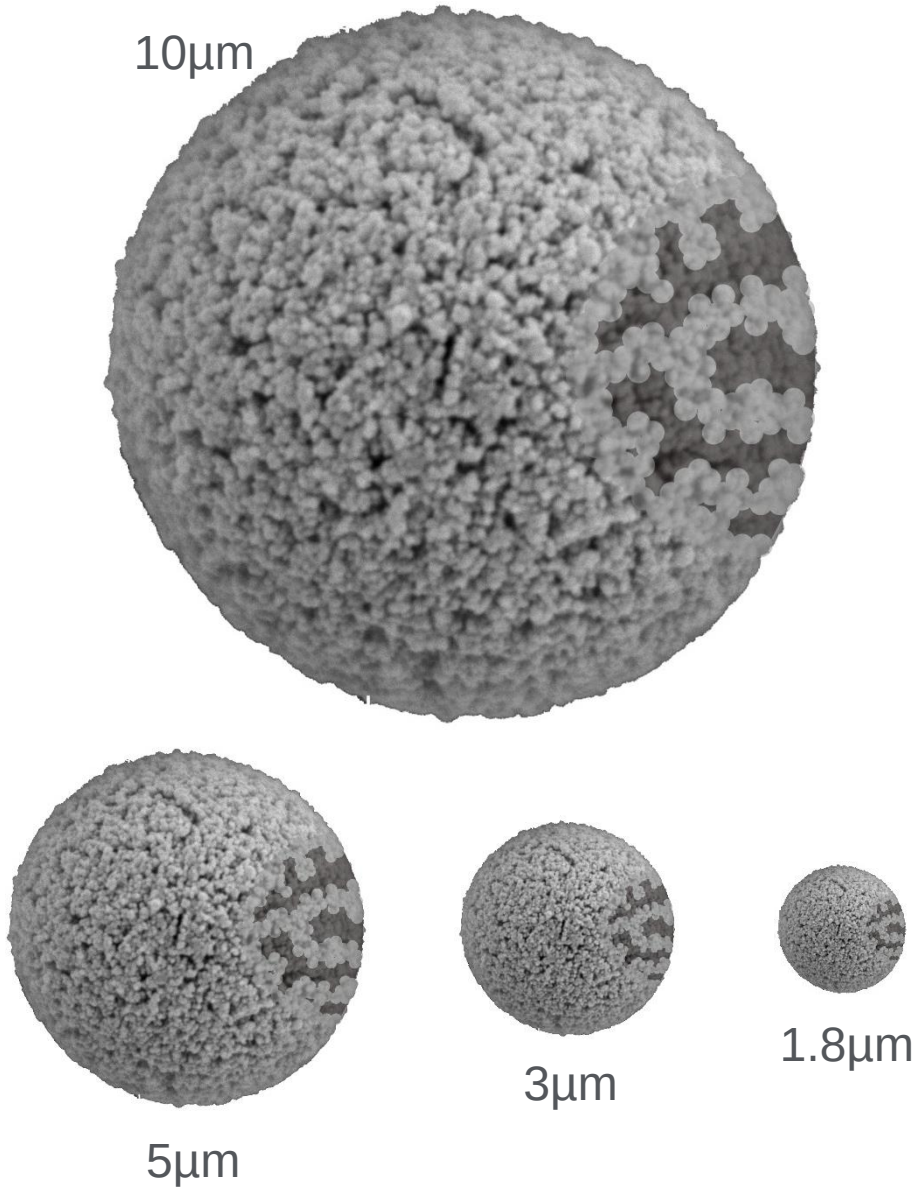
Particle Size

Diameter

Plate count

Back pressure

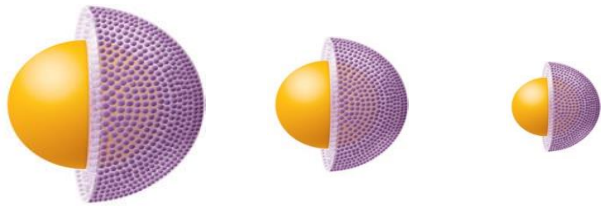
Diameter (µm)	Reduced Plate Height	Plate Count	Pressure
1.8	5.04	198000	380 bar
3	8.4	119000	140 bar
5	14	71000	50 bar
10	28	36000	12 bar



Agilent InfinityLab Poroshell 120 Portfolio Small Molecules & Peptides

start here

Best all around	Best for low pH mobile phases	Best for high pH mobile phases	Best for alternative selectivity	Best for polar Analytes	Best for Chiral
InfinityLab Poroshell **EC-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **SB-C18** 2.7 µm	InfinityLab Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **Bonus-RP** 2.7 µm	InfinityLab Poroshell **HILIC** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **Chiral-V** 2.7 µm
InfinityLab Poroshell **EC-C8** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **SB-C8** 2.7 µm	InfinityLab Poroshell **HPH-C8** 2.7 µm, 4 µm	InfinityLab Poroshell **PFP** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **HILIC-Z** 2.7 µm	InfinityLab Poroshell **Chiral-T** 2.7 µm
			InfinityLab Poroshell **Phenyl-Hexyl** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **HILIC-OH5** 2.7 µm	InfinityLab Poroshell **Chiral-CD** 2.7 µm
			InfinityLab Poroshell **SB-Aq** 2.7 µm		InfinityLab Poroshell **Chiral-CF** 2.7 µm
			InfinityLab Poroshell **EC-CN** 2.7 µm		

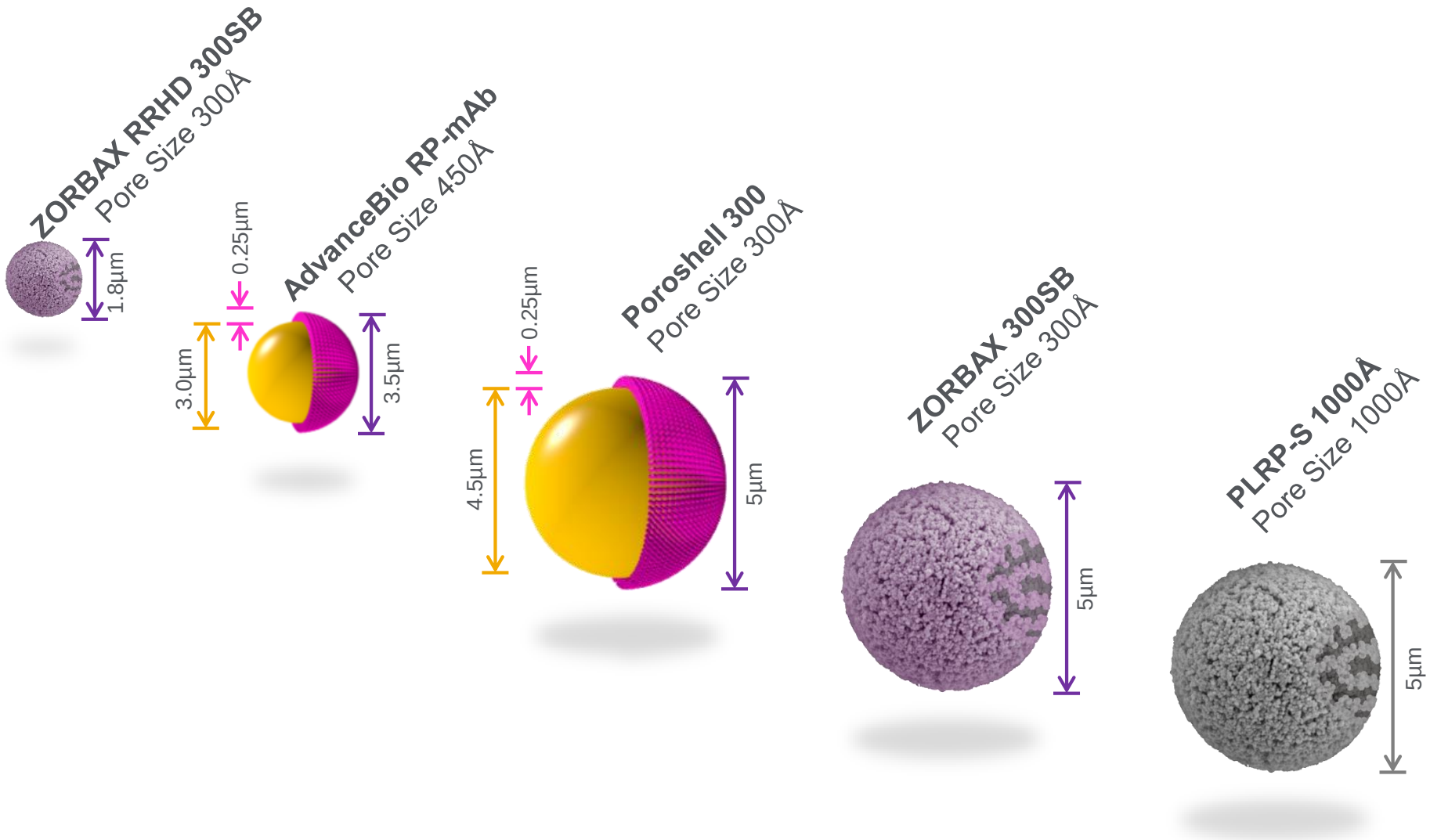


4µm **2.7µm** **1.9µm**

Reversed Phase Chemistries

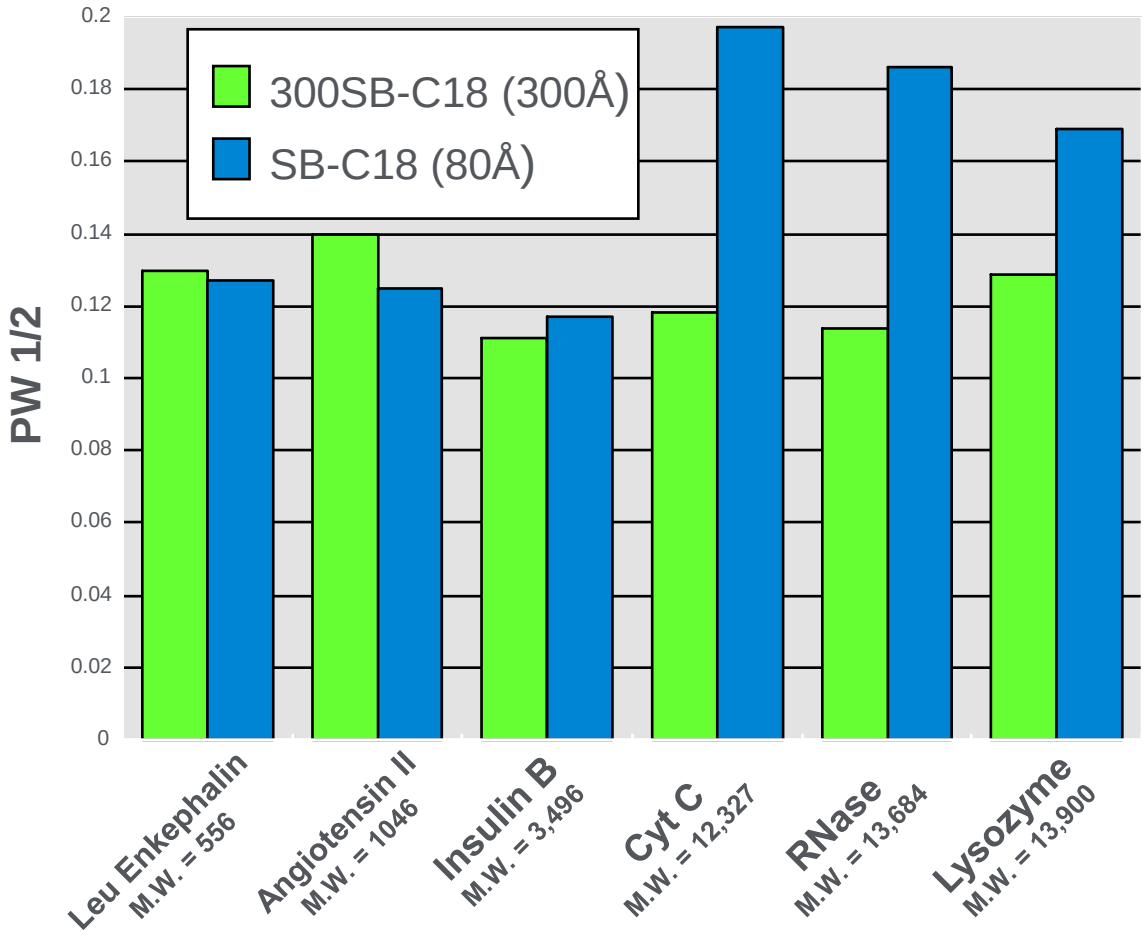
Columns for Intact Protein Analysis

No “One size fits all” Column …



Pore Size & Molecular Size Effect on Peak Width

Gradient Separations



000970P2.PPT

Choose Column Configuration for Application

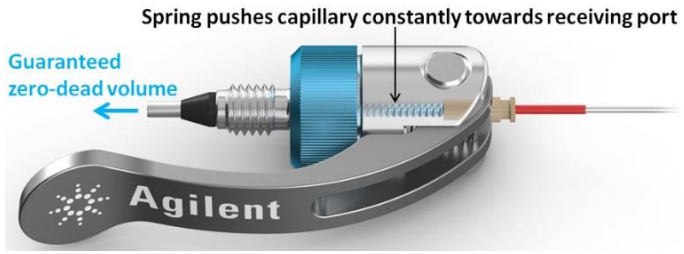
Column Type	I.D. (mm)	Lengths (mm)	Particle Sizes (mm)	Flow Rate Ranges	Applications
Capillary	0.3, 0.5	35 – 250	≤5	1 – 10 mL/min	Max sensitivity LC/MS
MicroBore	1.0	30 – 150	≤5	30 – 60 mL/min	Higher sensitivity LC/MS
Narrow Bore	2.1	15 – 150	1.8 – 5	0.1 – 0.3 mL/min	High sensitivity LC/MS
Solvent Saver	3.0	100 - 250	1.8 – 5	0.3 – 1.0 mL/min	Analytical
Analytical	4.6	15 – 250	1.8 – 5	1 – 4 mL/min	Analytical
Semi-prep	9.4	50 – 250	≥5	4 – 10 mL/min	Small scale prep (mg)
Preparative	>21.2	50 – 250	≥5	20 – 100 mL/min	Large scale prep

Things to Consider

Instrument

van Deemter

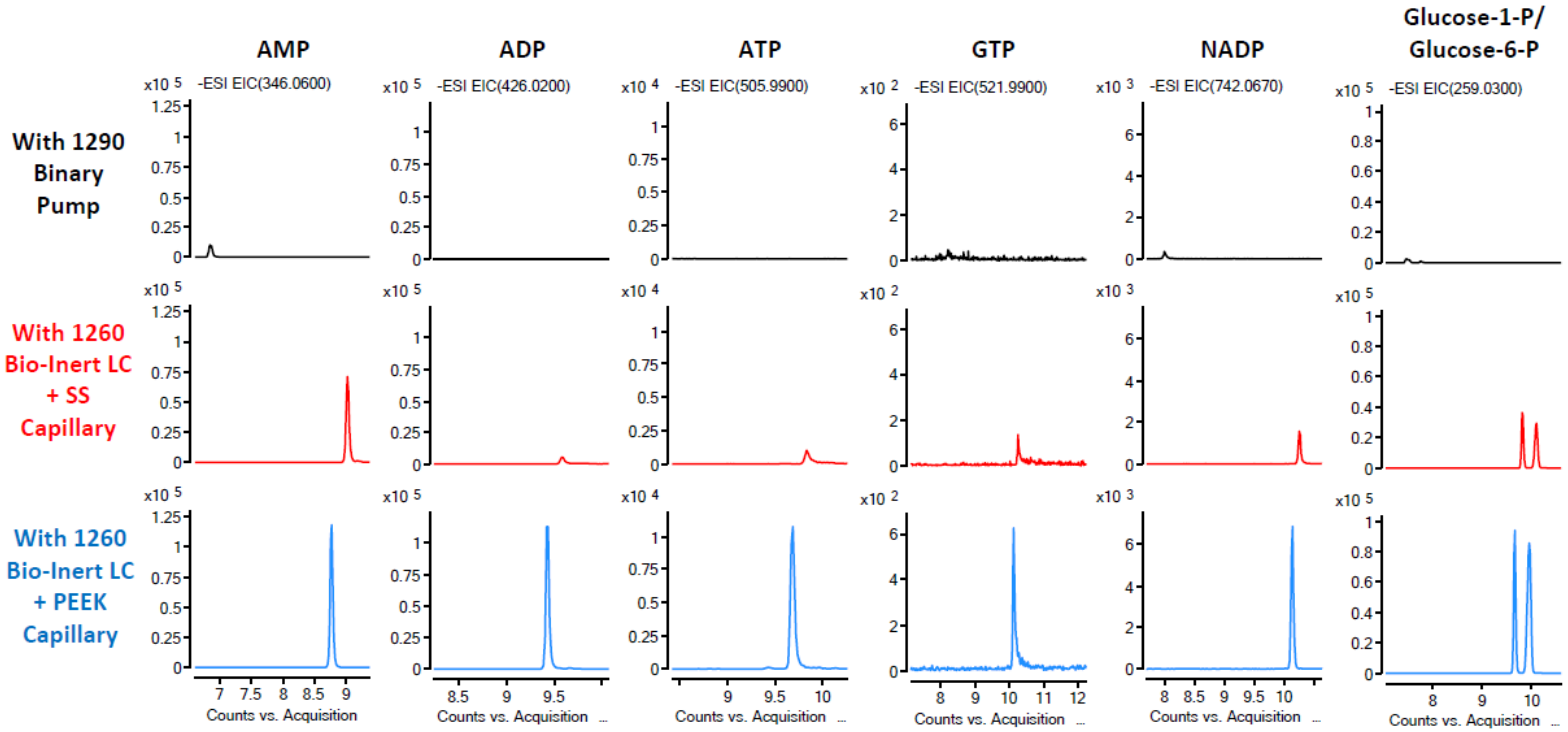
Recovery



Quick Connect fitting

Column and Instrument Materials

Nucleotide Phosphates on AdvanceBio MS Spent Media (HILIC stationary phase in PEEK lined SS hardware)



A: 10 mM Ammonium Formate pH 6.8 in water, B: acetonitrile + 10 mM Ammonium Formate pH 6.8, 95-30% B in 10 minutes, 0.25 mL/min, 0.2 µL injection (5 ng each on column), MS Source: ESI-, m/z 191.02, 346.06, 426.02, 505.99, 521.99, 742.067, 743.067, 259.03

Instrument Considerations

Bioinert system

- **100% Bio-inert**
  - Precious sample does not touch metal surfaces
  - pH range 1-13 (short-term 14)
  - 2 M salt, 8 M urea
  - No stainless steel in mobile phase flow path
  - New capillary technology

- Metal clad PEEK capillary design
- Mechanical interlock, molded tip
- 600 bar

Phosphoric acid passivation

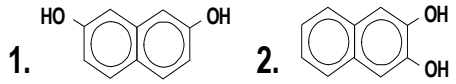
- Improve both peak shape and sensitivity
  - Run 90:10 acetonitrile:water with 0.5% (v/v) phosphoric acid overnight through Channel B, column, and MS nebulizer

Acid Wash Can Improve Peak Shape

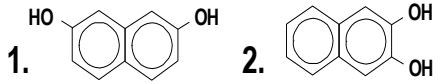
Metal sensitive compounds can chelate

1% H_3PO_4 is used on SB columns, 0.5 % on endcapped columns and system

Before Acid Wash



After 1% H_3PO_4 Acid Wash



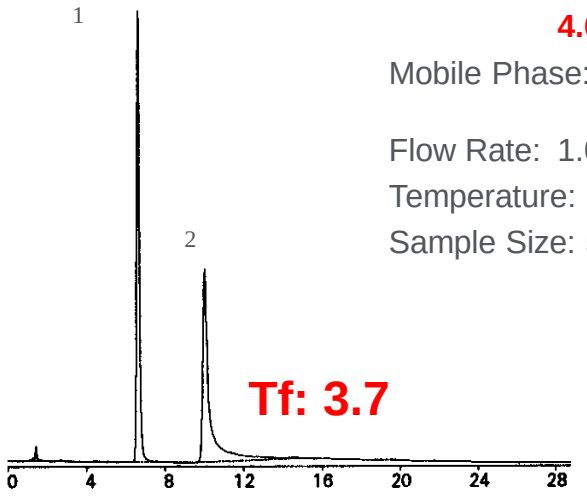
Columns: ZORBAX SB-Phenyl 4.6 x 150 mm

Mobile Phase: 75% 25mM Am phosphate
25% ACN

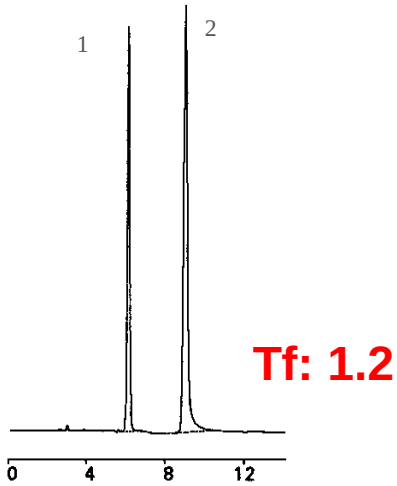
Flow Rate: 1.0 mL/min.

Temperature: RT

Sample Size: 5 uL



Tf: 3.7



Tf: 1.2

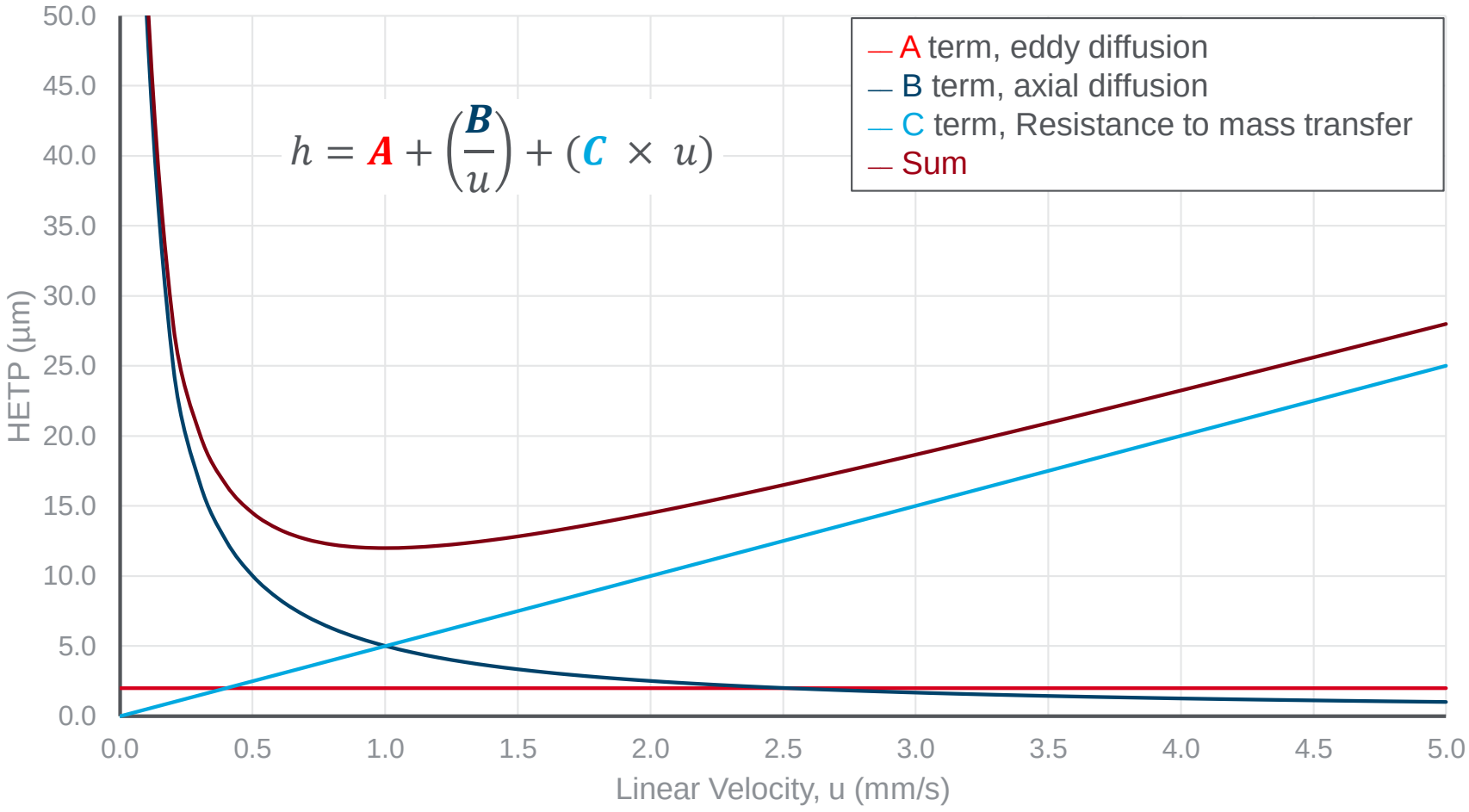
•***Hint*: Look for lone pair of electrons on "O" or "N" which can form 5 or 6 membered ring with metal**

Factors Affecting Plate Count
van Deemter Equation

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

L: Column length

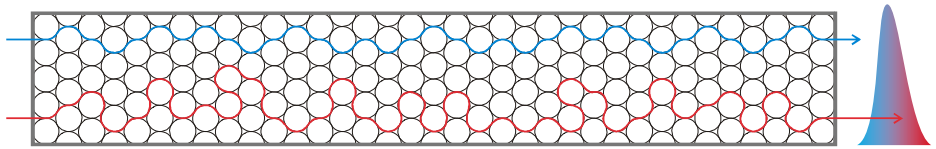
H: HETP



A, B, and *C* are constants for a particular compound and set of experimental conditions as flow rate is varied

Factors Affecting Plate Count

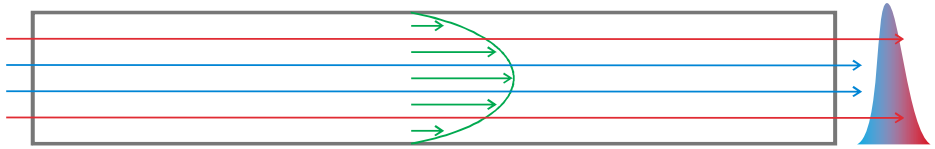
A term Eddy diffusion (multiple path effect)



$w_{eddy} \sim \lambda\, d_p$ λ: Quality of column packing

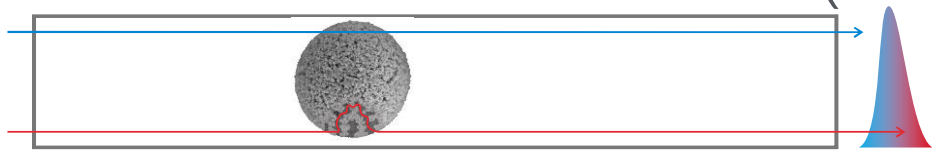
B term Molecular diffusion

Increase in peak width due to self-diffusion of the analyte



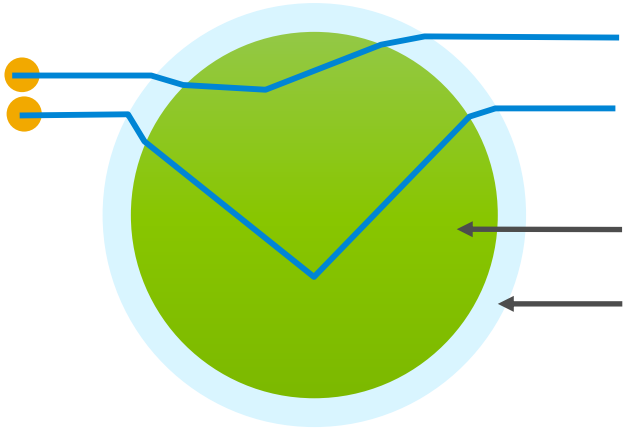
C term Mass transfer (within particle)

$w_C \sim d_p^2$



van Deemter Equation – C term
“Resistance to Mass Transfer”

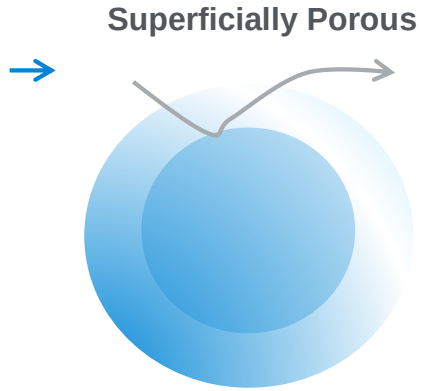
$$w_C \sim d_p^2$$

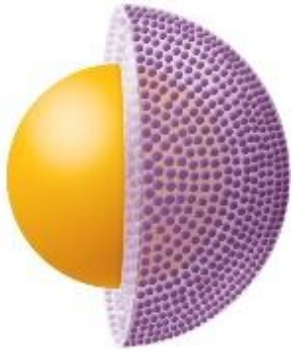


Different diffusion paths

Porous particle

Stationary layer of mobile phase

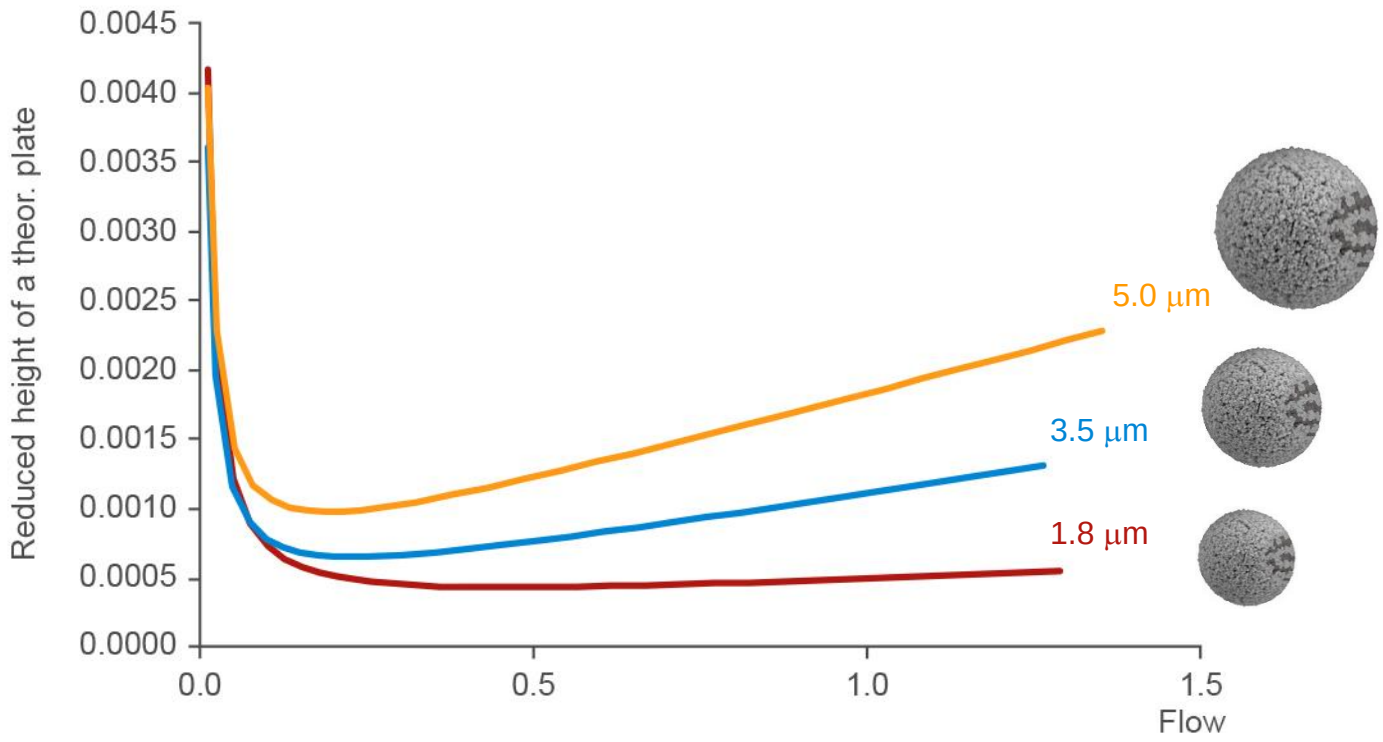




Poroshell is made of a solid core with a porous outer layer. Reduces C term.

Van Deemter Equation
Measured for Different Particle Sizes

- Small particles = lower heights of theoretical plates ∴higher separation efficiency
- For smaller particles, separation efficiency suffers less when increasing the flow

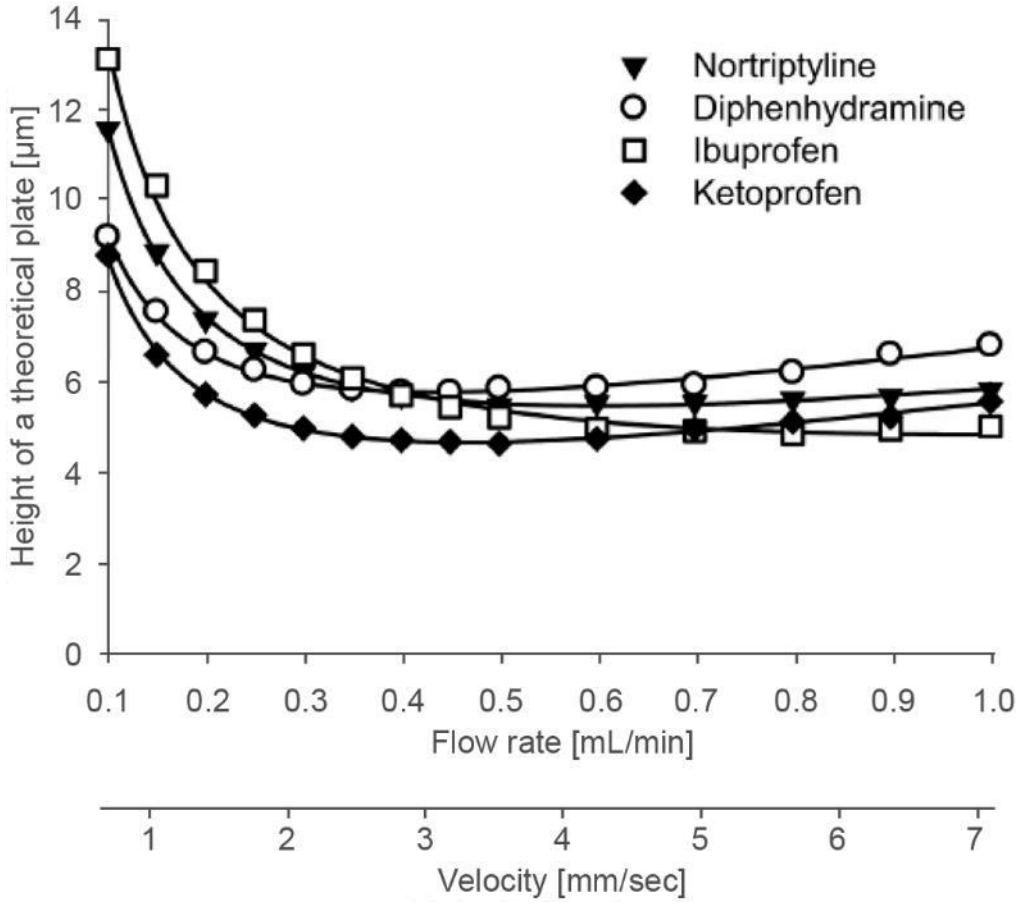


➢ **Point where minimun is reached, is the "optimal" flow rate at which maximal plate number is reached**

van Deemter Equation
Curves for Different Analytes

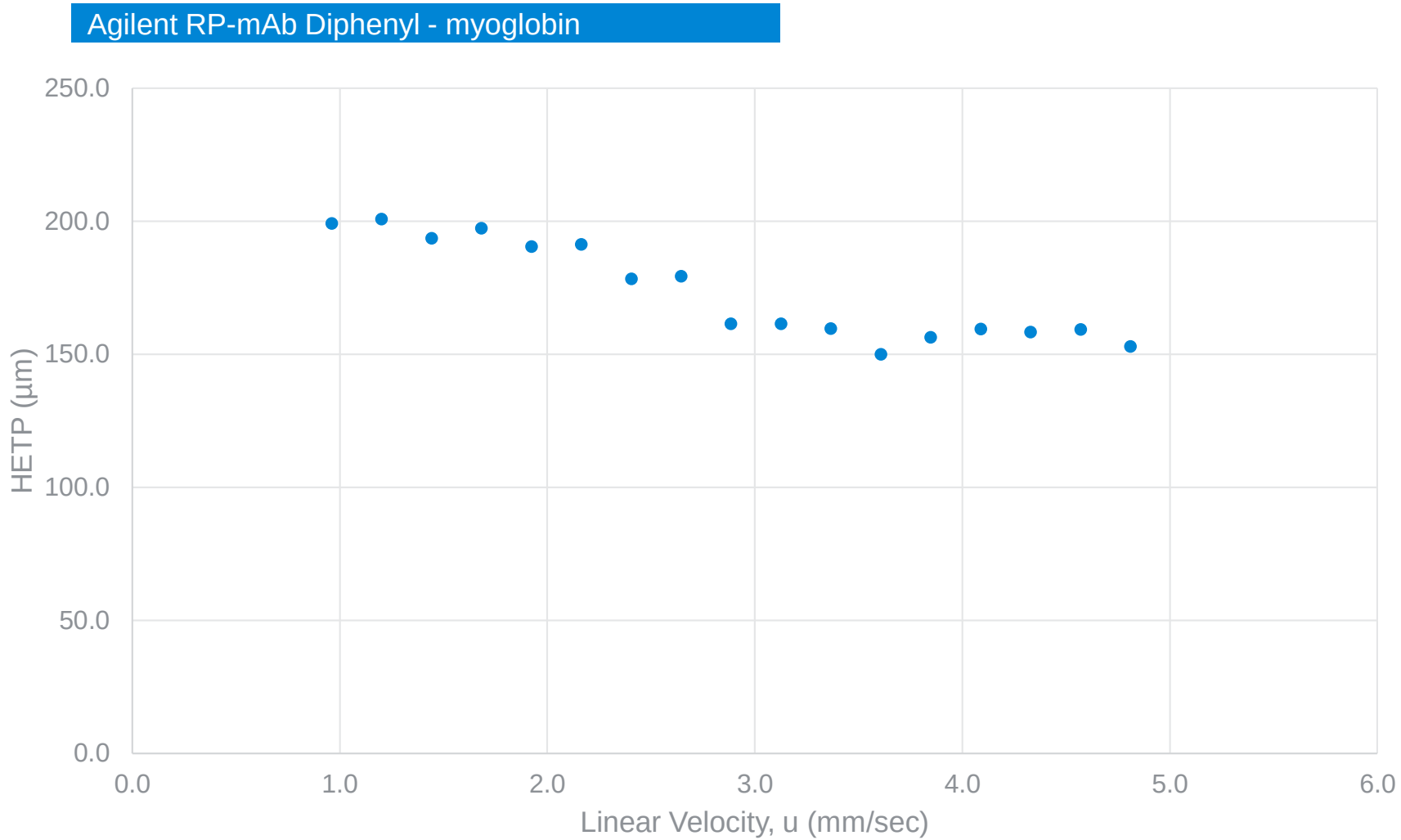
- van Deemter equation for isocratic runs only
- Compound and instrument specific
- Even for sub-2-µm particles not horizontal
- Optimum flow rate depends on compound

Are proteins like small molecules ?



P. Petersson et al (AZ), J.Sep.Sci, 31, 2346-2357, ***2008***

Van Deemter Plots



- Are proteins like small molecules ? … NO !

Slower Diffusion of Large Molecules Broadens Peaks at High Flow

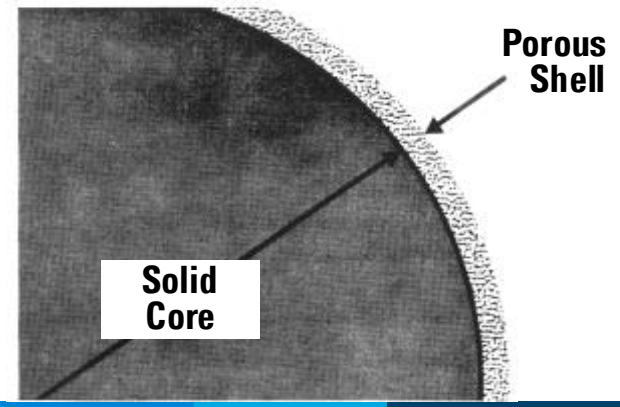
So… decrease diffusion time for macromolecules!

Increase the Diffusion Rate

- Elevated operating temperature -- Works with StableBond
- Decreased solvent viscosity -- Helps but changes elution

Decrease the Diffusion Distance

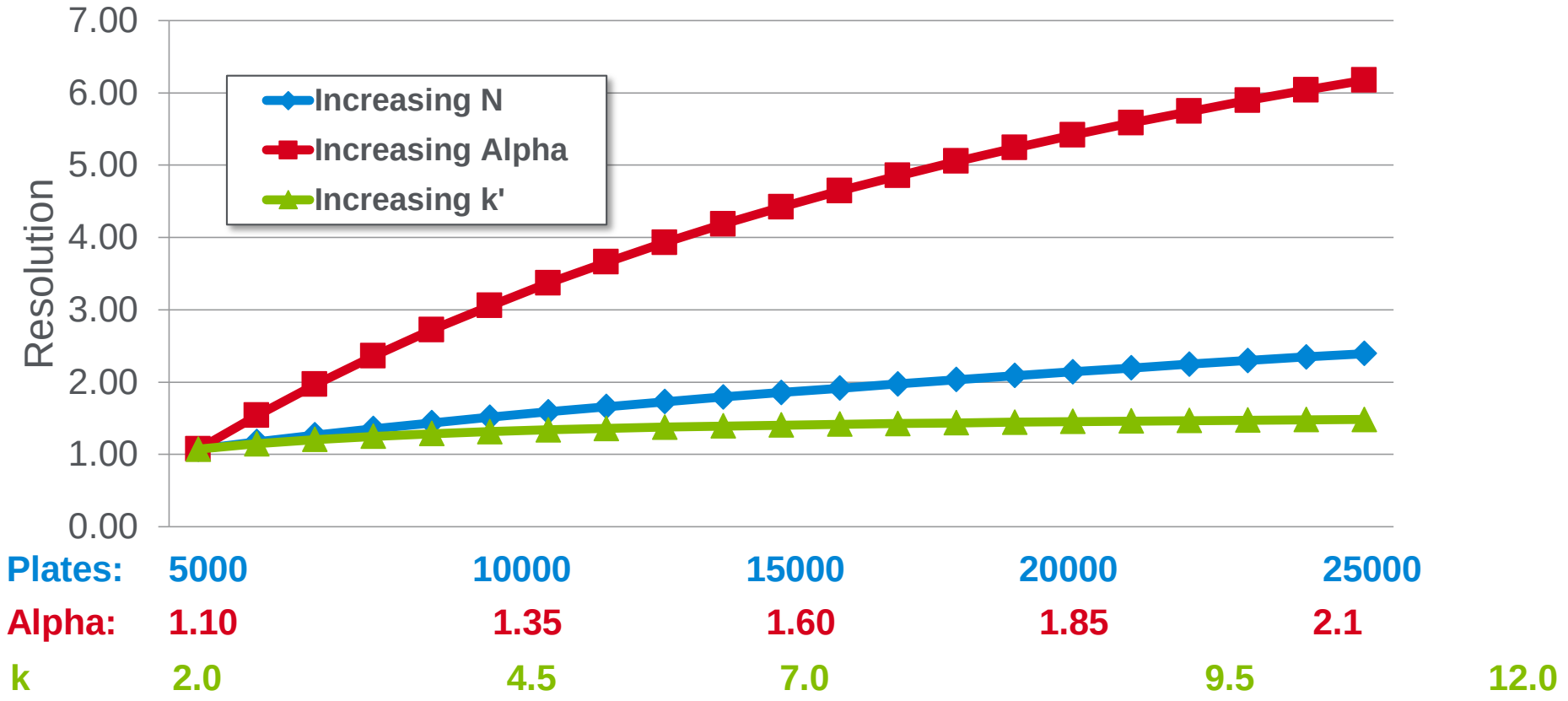
- Small particles (<2-um) – Increased back pressure
- Limit diffusion distance into a particle!



Resolution
Factors that Affect It

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution Efficiency Selectivity Retention



Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

} **Typical Analytical Method Development Parameters**

Resolution Relationship for Gradient Elution

$$R \approx \frac{\sqrt{N}}{4} \alpha \; k^*$$

k^* - represents the fact that k changes constantly during a gradient

$$k^* = \frac{87 \; t_g \; F}{S \; (\Delta\%B) \; V_m}$$

$\Delta\%B$ = difference between initial and final % B values

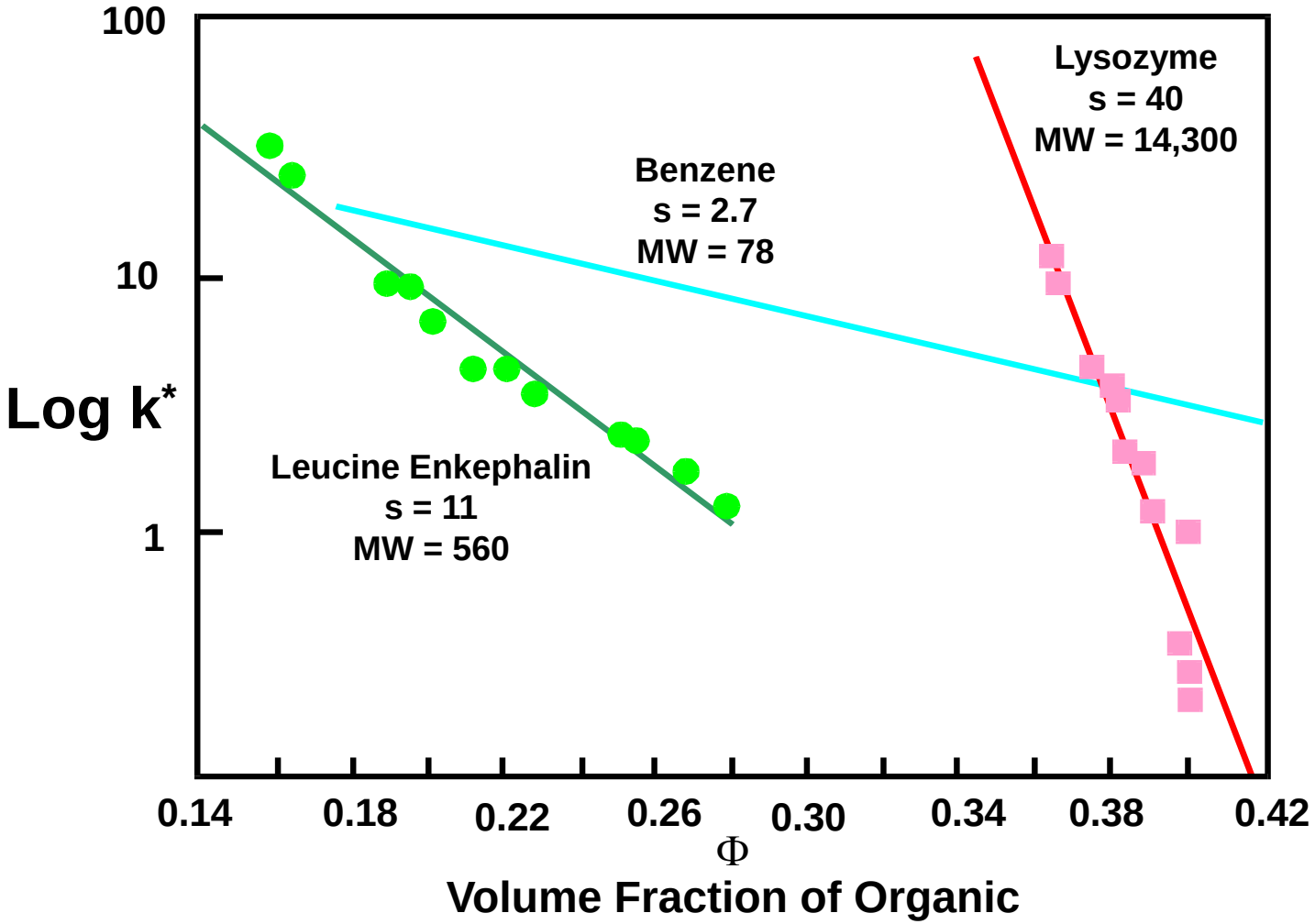
S = Constant that changes with MW:
4 for 100 - 500 Da; 10 < S < 1000 for peptides and proteins

F = flow rate (mL/min)

t_g = gradient time (min)

V_m = column void volume (mL)

Larger Molecules v. Small Molecules
More Sensitive to Changes in % Organic



- **Lysozyme is 15X more sensitive to changes in organic modifier than benzene**
- **4X more sensitive than leucine enkephalin.**

Method Conditions

Mobile phase

- Organic Modifier
- Additives

pH

Temperature

Sample

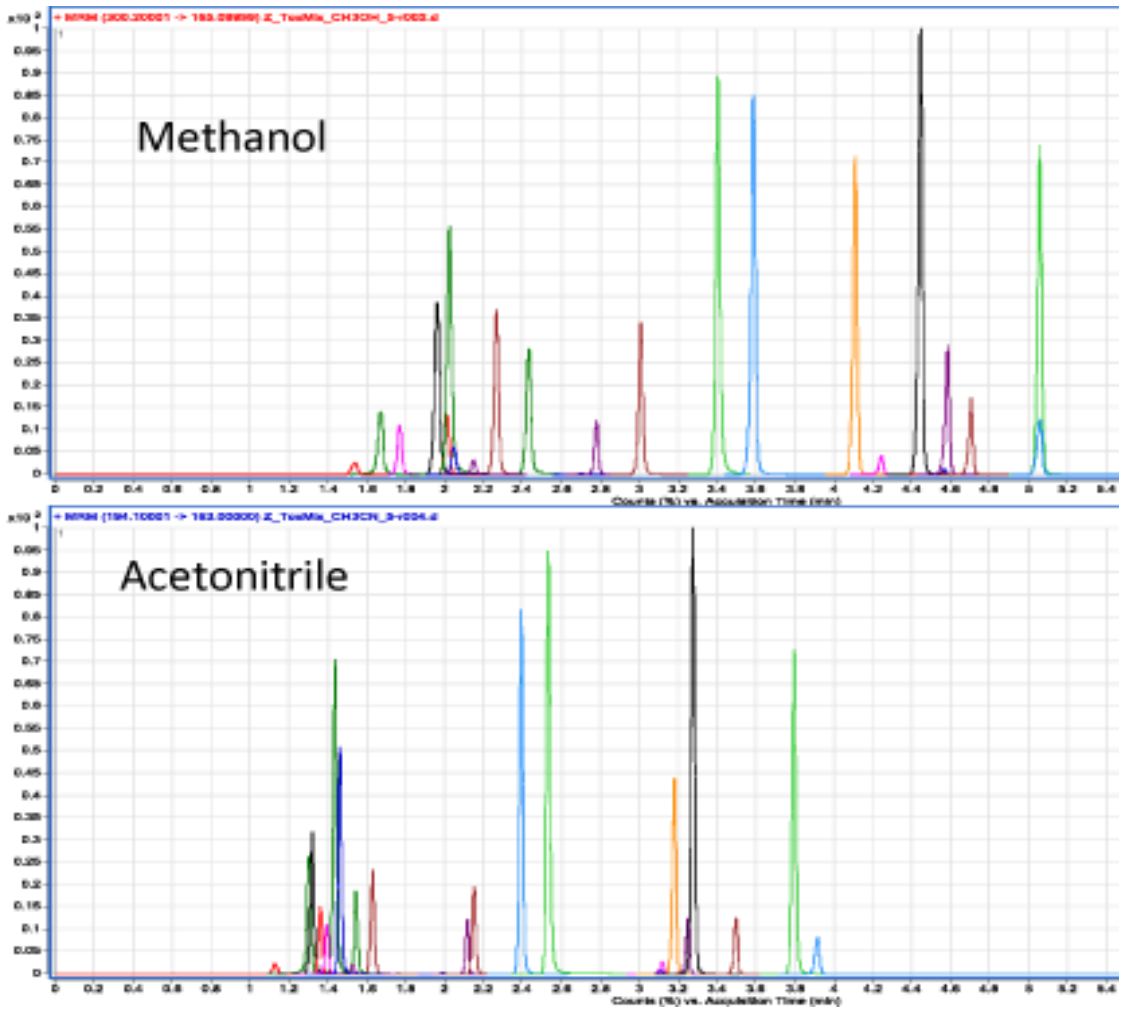
Exploring Organic Modifiers – Small Molecules

Why?

- ✓ It's easy – ACN & MeOH are readily available
- ✓ Works on any bonded phase – optimize separation no matter the column choice

MeOH – Higher pressure, generally better peak shape with bases, Protic solvent

Acetonitrile – Aprotic, wider UV window, stronger than MeOH



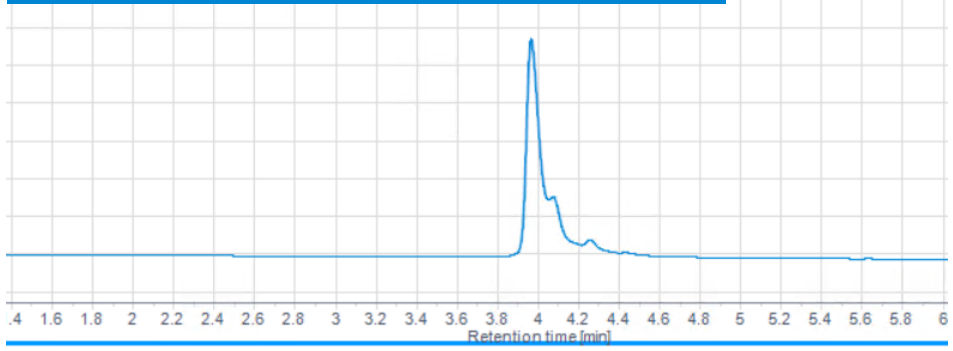
"Fast Analysis of Illicit Drug Residues on Currency using Agilent Poroshell 120", Anne E. Mack, James R. Evans and William J. Long, September 2010, 5990-6345EN.

Mobile Phase: Organic Modifier

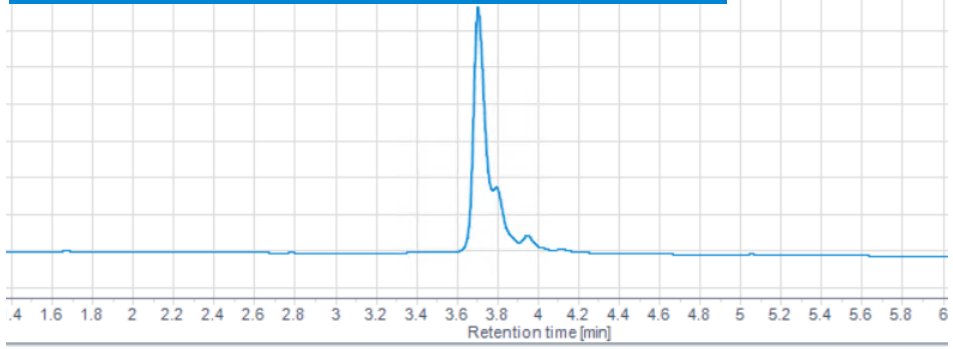
Columns	2.1x50mm
Mobile phase	A: water:IPA 98:2 + 0.1% TFA B: IPA:ACN:MPA 70:20:10
Temperature	80 °C
Flow rate	0.5 mL/min
Gradient	25 – 45% B in 10 min

Columns	2.1x50mm
Mobile phase	A: 0.1% TFA in water B: 0.08% TFA in ACN
Temperature	80 °C
Flow rate	0.5 mL/min
Gradient	25 – 45% B in 10 min

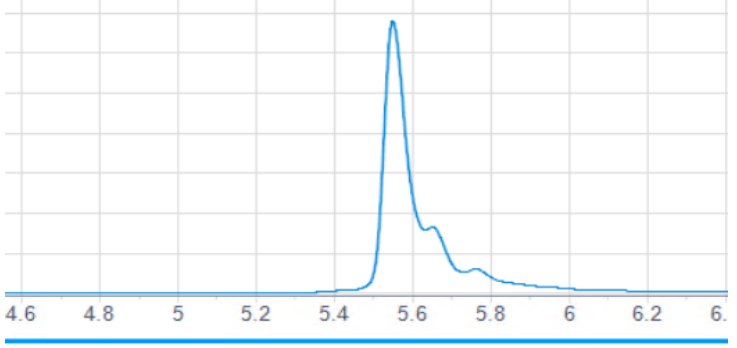
AdvanceBio RP-mAb Diphenyl



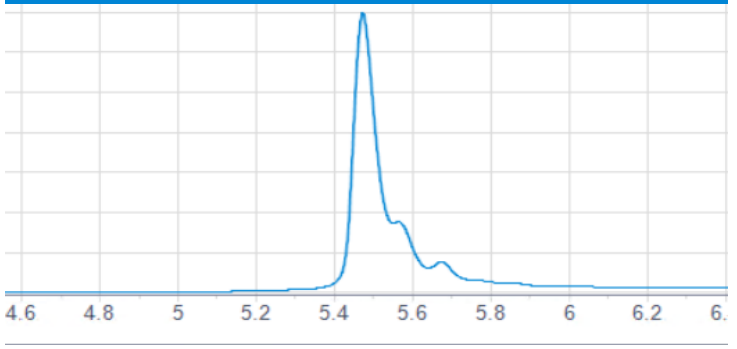
Polyphenyl column (other vendor)



AdvanceBio RP-mAb Diphenyl



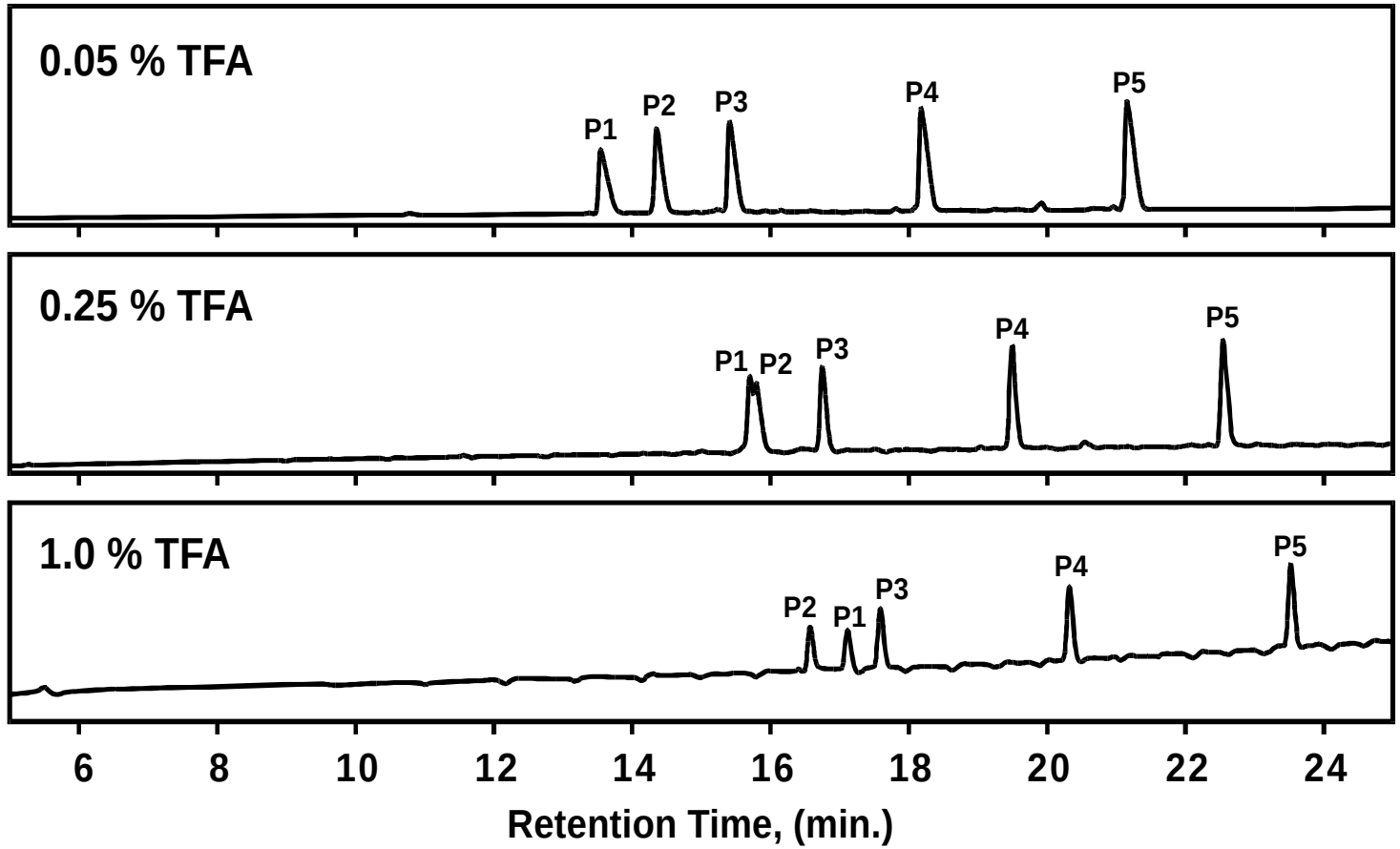
Polyphenyl column (other vendor)



TFA Concentration

Affect on Reversed-Phase Peptide Separation

Conditions: Column: Zorbax 300SB-C8, 4.6 x 150 mm, Mobile Phase: A= H_2O and TFA, B= ACN and TFA, Gradient: 0-30% B in 30 min., Flow: 1 mL/min.,Temp.: 40°C, Detect.: UV-254 nm, Sample: 6 µL injection volume, Peptide Standards S1-S5, decapeptides differing slightly in hydrophobicity

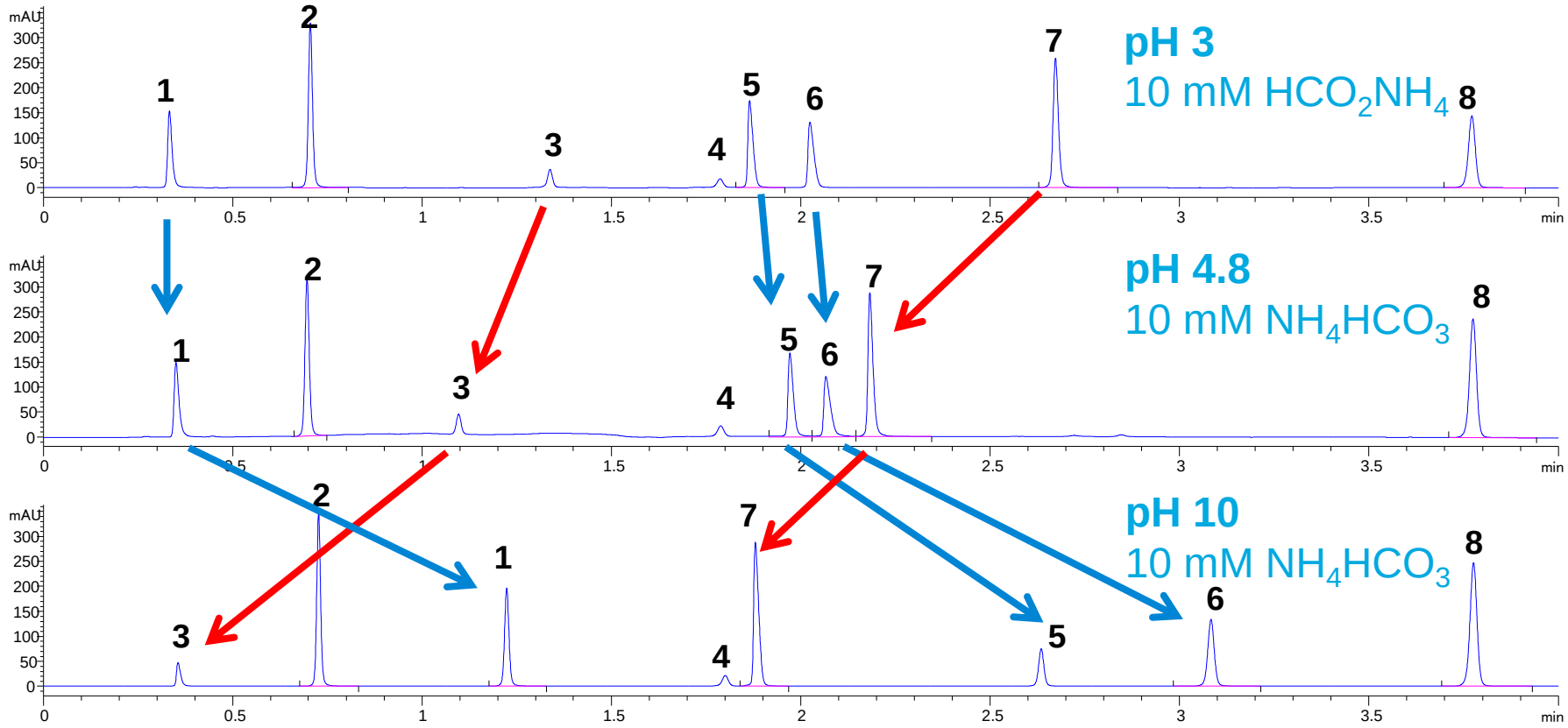


Selectivity Can be Controlled by Changing pH

1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone

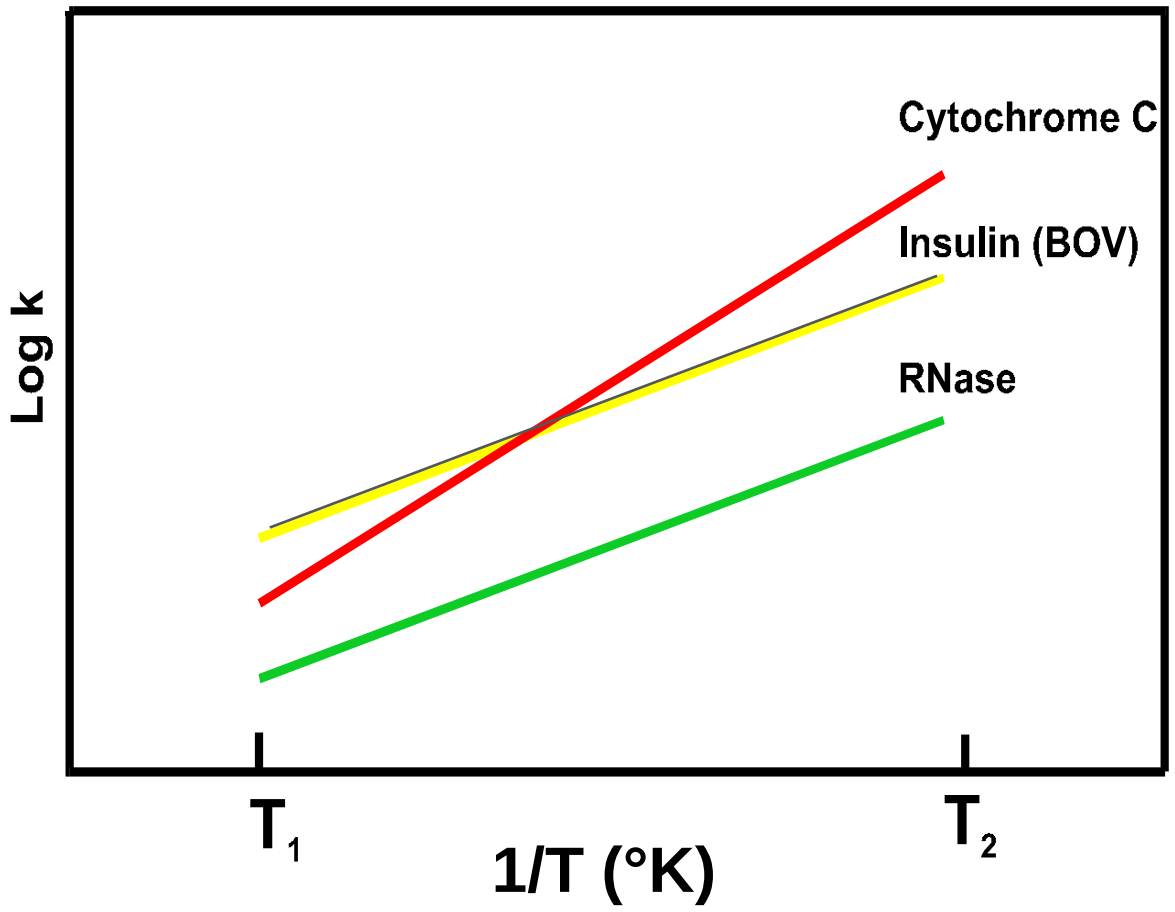
Time	% Buffer	% MeCN
0	10	90
5	90	10
7	10	90
2 ml/min		254 mn

Poroshell HPH-C18 4.6 x 50 mm, 2.7 µm



Increasing Temperature

- Reduces Analysis Time
- May Change Selectivity



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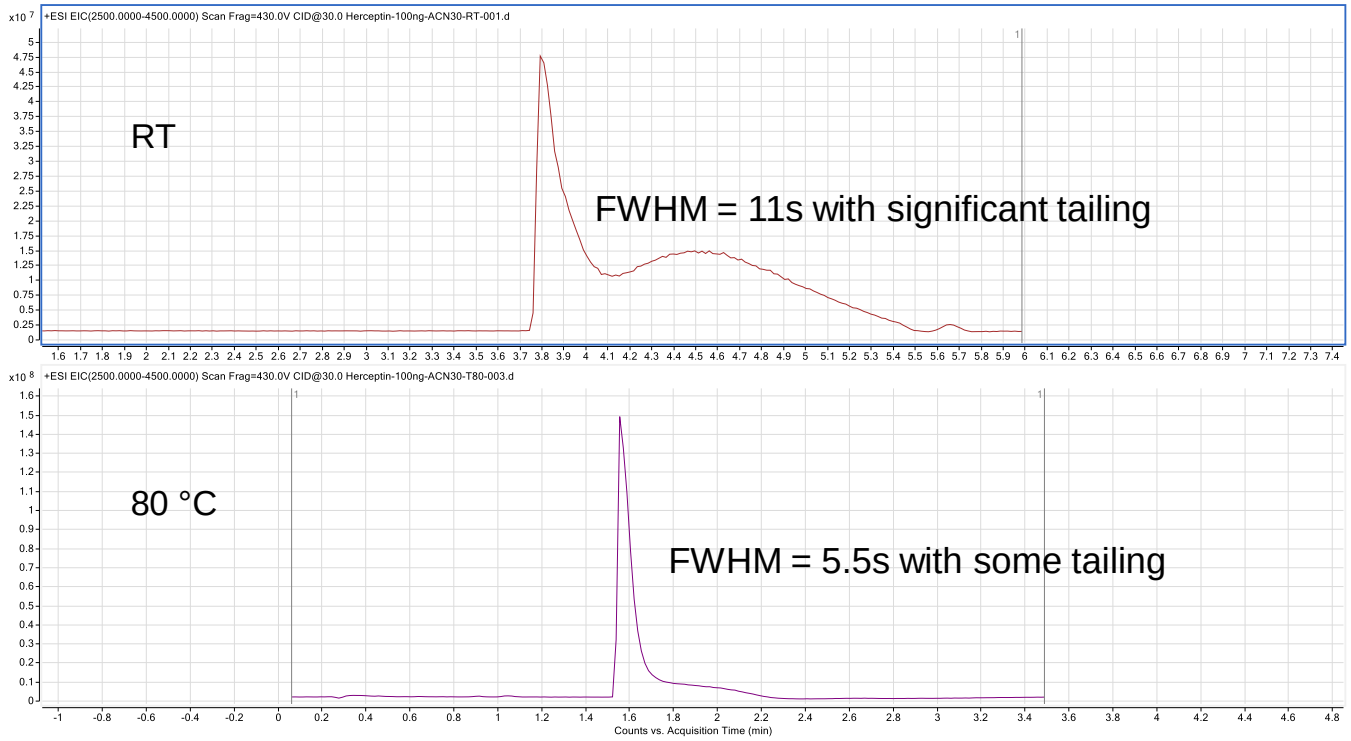
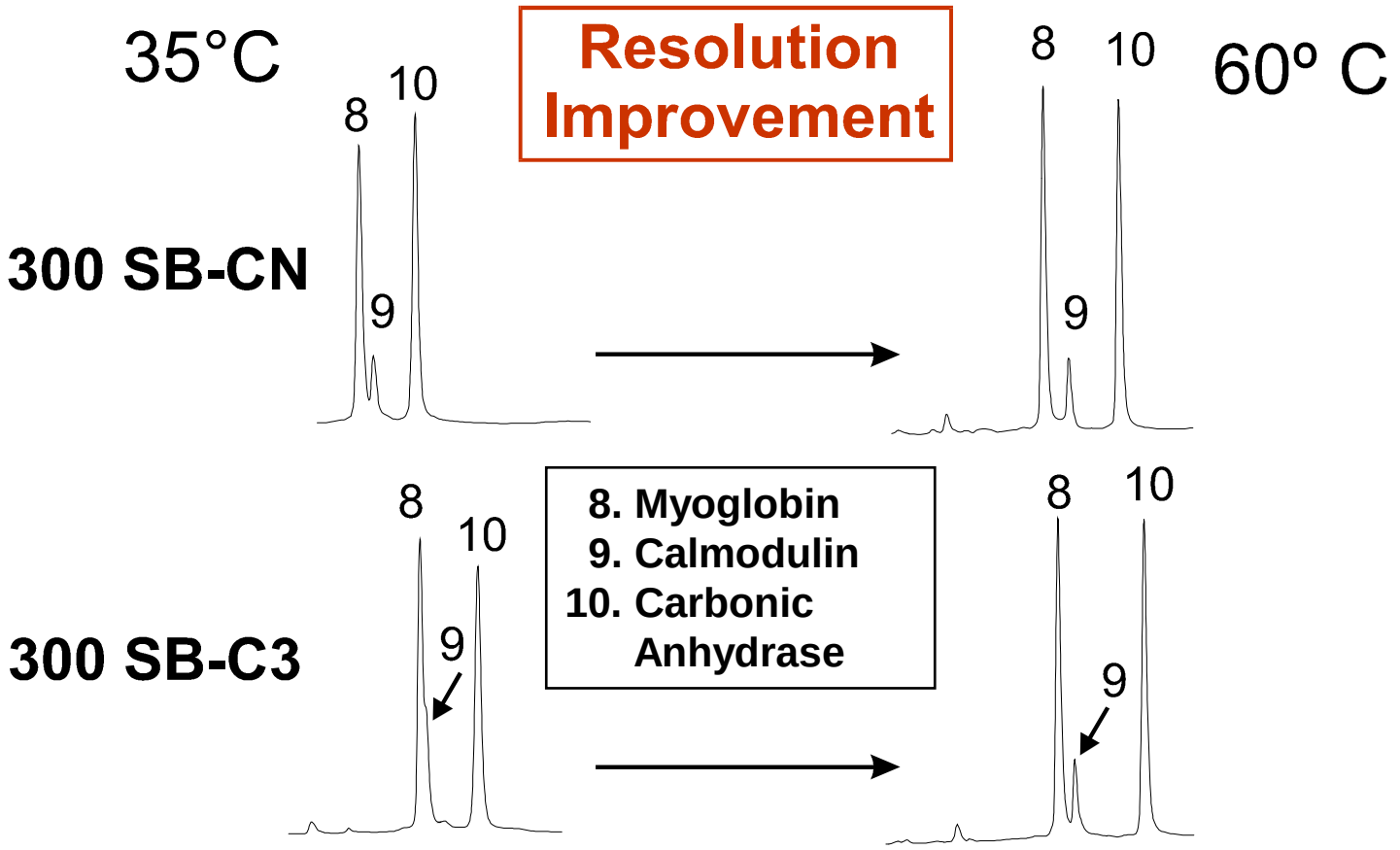
Chromatography Optimization: Column Temperature (Herceptin)



Zorbax 300 diphenyl Column, 2.1× 50 mm (or 100mm), 1.8µm, 0.5ml/min

Using Temperature to Improve Resolution



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Protein and Peptides
Selected Sample Solvents and Applications

Solvent	Application	Comments
0.05-5% TFA in Water	General	Effective solubilization of many samples
6 M Guanidine, buffered at pH 6-8	General	Very good for many proteins and peptides
5-80% Acetic Acid or Formic Acid; 0.1-0.5M Perchloric Acid	Peptides	Frequently used to extract peptides from tissues, precipitating many proteins and cellular debris
6 M Urea/ 5% Acetic aid	Hydrophobic Peptides, Proteins	Useful for membrane proteins, fragments, aggregating systems
Water-Miscible Organic Solvents: Acetonitrile, Methanol, THF, Dioxane, DMSO; +/- TFA; +/- Water	Hydrophobic Peptides, Polypeptides	Limit injection volume to avoid problems; add water, as possible, to improve volume tolerance; acidify with TFA as required

Troubleshooting

Recovery

- Molecule size
- Metals

Detection

- Chromophore
- MS

Column cleaning

What Affects Recovery?

Chain length

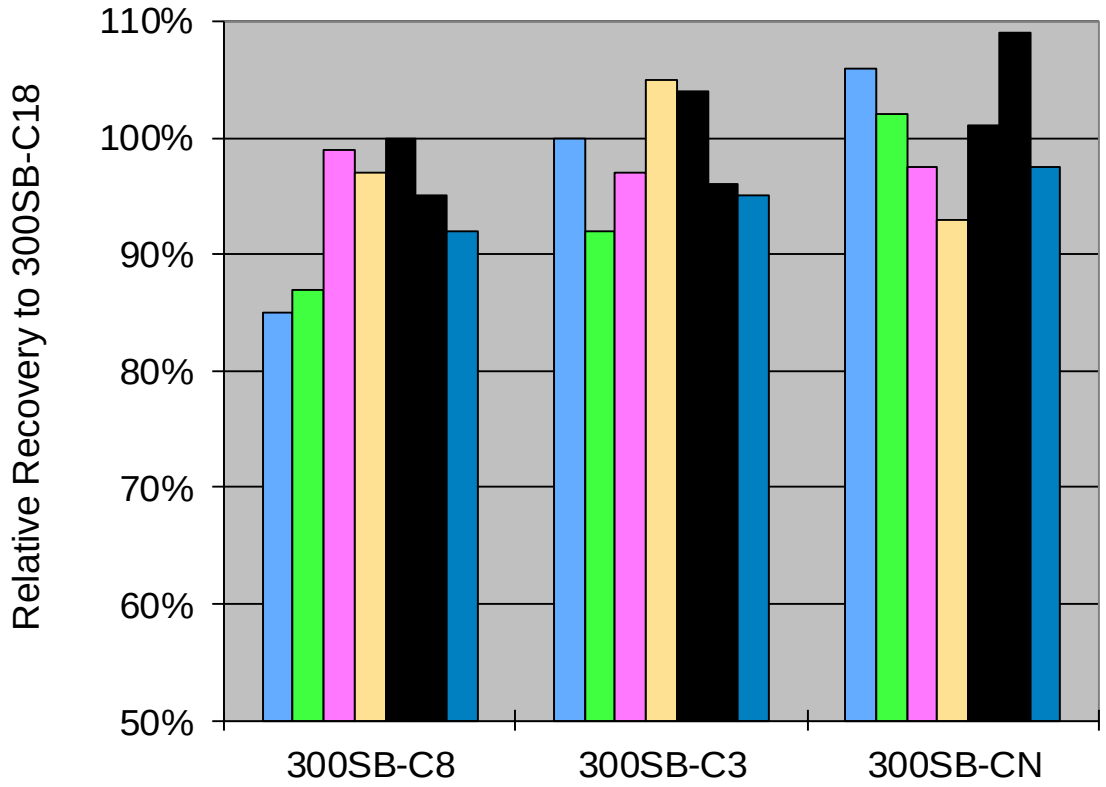
Hydrophobicity / Aggregation

Mobile Phase

Solubility of sample

Size

Recovery of Polypeptides from ZORBAX 300SB Columns



Columns:	4.6 x 150 mm
Mobile Phase:	5 - 40% B in 20 min. A: 0.1% TFA / Water B: 0.1% TFA / ACN
Flow Rate:	1 mL / min.
Temperature:	60°C
Sample:	4 µg each protein 25 µL injection

001007P1.PPT

Proteins and Mass Spec

Complications/Challenges

Aggregation

Degradation

Folding/conformational changes

Size

Poor fragmentation within collision cell (QQQ)

Poor solubility in organics

MP additives

PTMs; oxidation, isomerization, deamidation

- Little or no difference in mass

Multiple glycosylation sites

- Difficult to interpret/deconvolute

Things to do

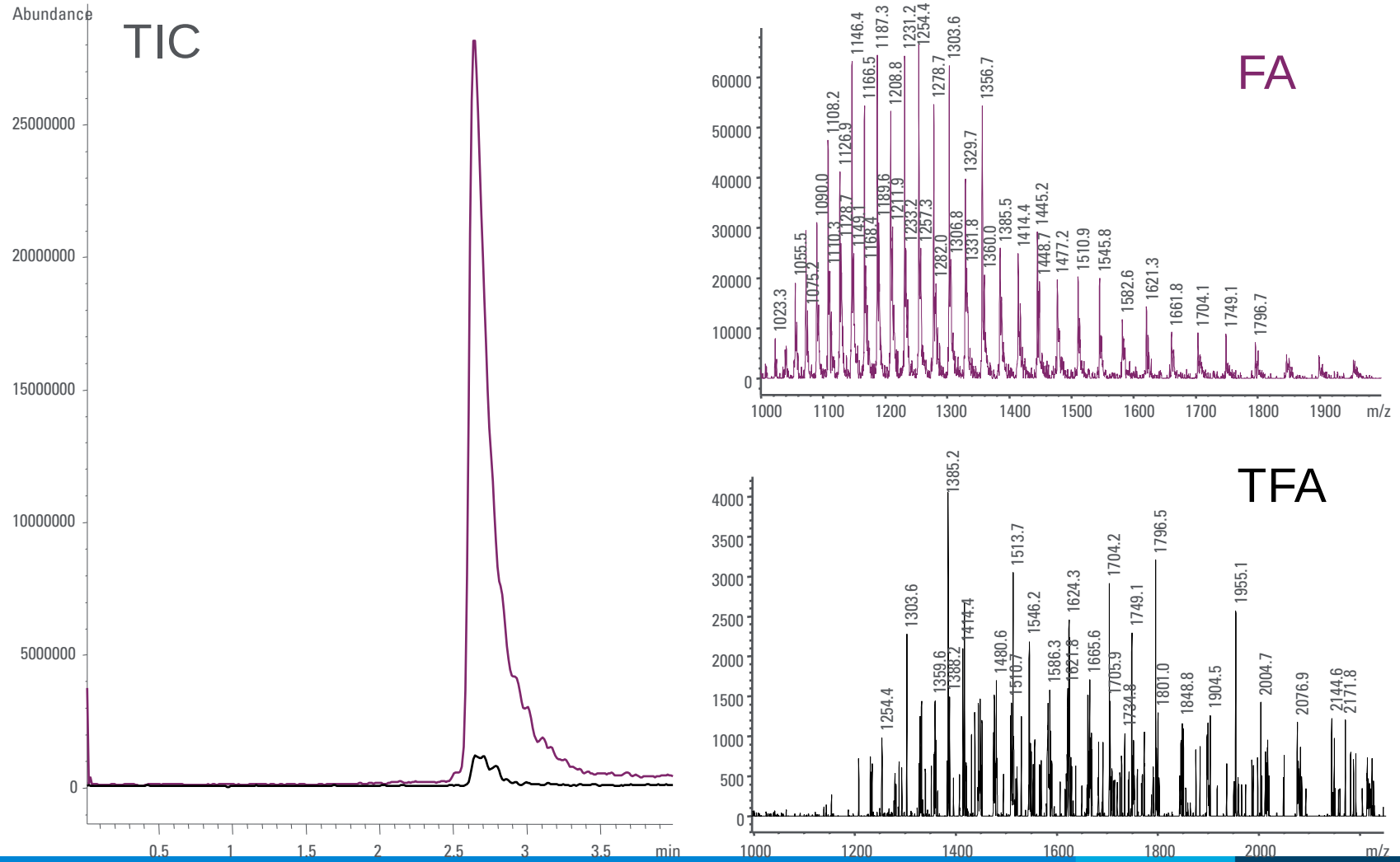
Denature

- Chaotropes: Guanidine, Urea
- Reductants: DTT, BME, Ascorbic Acid
- Temperature

Digestion

Derivatize

Effect of Modifier on MS Response: 10 pmol BSA



Column Cleaning – Small Molecules

Flush with stronger solvents than your mobile phase Make sure detector is taken out of flow path

Reversed-Phase Solvent Choices in Order of Increasing Strength
Use at least 10 x V_m of each solvent for analytical columns

1. Mobile phase without buffer salts (water/organic)
2. 100% Organic (MeOH or ACN)
3. Is pressure back in normal range?
4. If not, discard column or consider more drastic conditions:
   75% Acetonitrile:25% Isopropanol, then
5. 100% Isopropanol
6. 100% Methylene Chloride*
7. 100% Hexane*

* When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.

Column Cleaning Suggestions - Biomolecules

Routine

Each Run

- Run out gradient

Daily

- Stronger protocol

Periodic

- Back flush (if column permits)

Storage

- ≥50% Organic

Additional

General : IPA ramp 0-100% and hold (1/2 flow rate)

Lipids and small hydrophobics : 100% MeCl

Synthetic Peptides : General followed by 100% MeCl

More drastic : Plug flow (repetitive injections) of 3M Guanidine in 50% IPA

Silica based RP columns only

Prior to using either Hexane or Methylene Chloride the column must be flushed with Isopropanol and again before returning to your reversed-phase mobile phase.

Summary

Chromatography of biomolecules is often different from small molecules

- Chemistry, Biology, Biochemistry
  - Temperature
  - pH
  - Shearing
  - Folding, glycoforms, heterogeneity

Structural diversity

- Proteins
- Nucleic Acids
- Conjugates, complexes

Instrumentation

Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 Option 3, Option 3:

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS Columns and Supplies

Option 3 for Sample Preparation, Filtration and QuEChERS

Option 4 for Spectroscopy Supplies

gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

Resources for Support

- Agilent University http://www.agilent.com/crosslab/university
- Tech support http://www.agilent.com/chem/techsupport
- Resource page http://www.agilent.com/chem/agilentresources
  - Quick Reference Guides
  - Catalogs, Column User guides
  - Online Selection Tools, How-to Videos
- InfinityLab Supplies Catalog (5991-8031EN)
- Your local FSE and Specialists
- Youtube – Agilent Channel
- Agilent Service Contracts