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Trusted Answers

# Making the Leap

## Small Molecule – Biologics

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LC Columns Application Engineer
May 8, 2019

# 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

# HPLC Common Separation Mechanisms

## Small molecules

- Reversed phase*
- Ion exchange
- HILIC
- Normal phase
- Chiral

## Biomolecules

- Reversed phase*
- Ion exchange
- Size exclusion/Gel filtration
- Hydrophobic interaction (HIC)
- HILIC
- Affinity

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



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# Nonpolar and Polar Functional Groups*

## Nonpolar (Hydrophobic)

$-CH_3$
- Methyl

$-(CH_2)_nCH_3$
- Alkyl groups

$-C_6H_5$
- Phenyl groups

## Polar (Hydrophilic)

-COOH
- Carboxyl groups

$-NH_2$
- Amino groups

-OH
- Hydroxyl Groups

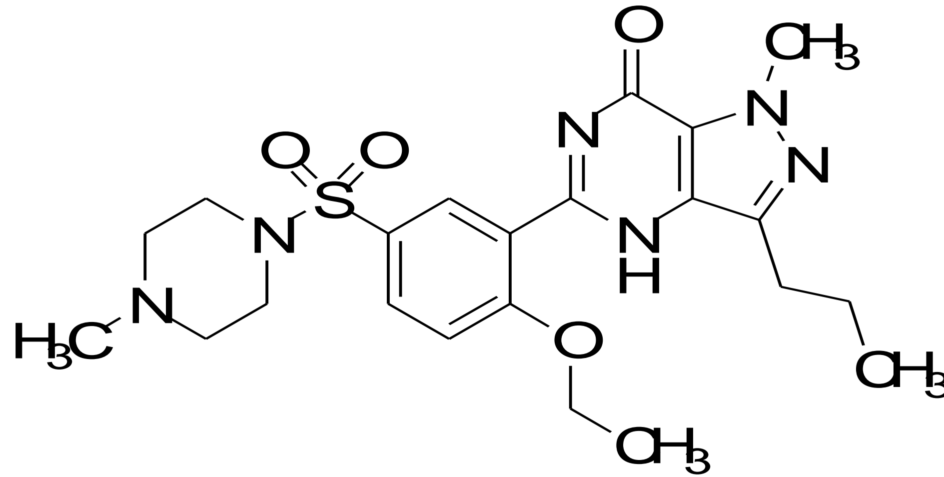
$-CONH_2$
- Amide groups

*Affect solubility and elution order

# Do You Know What This Is?

$C_{22}H_{30}N_6O_4S$

MW=476.6 g/mol




Sildenafil



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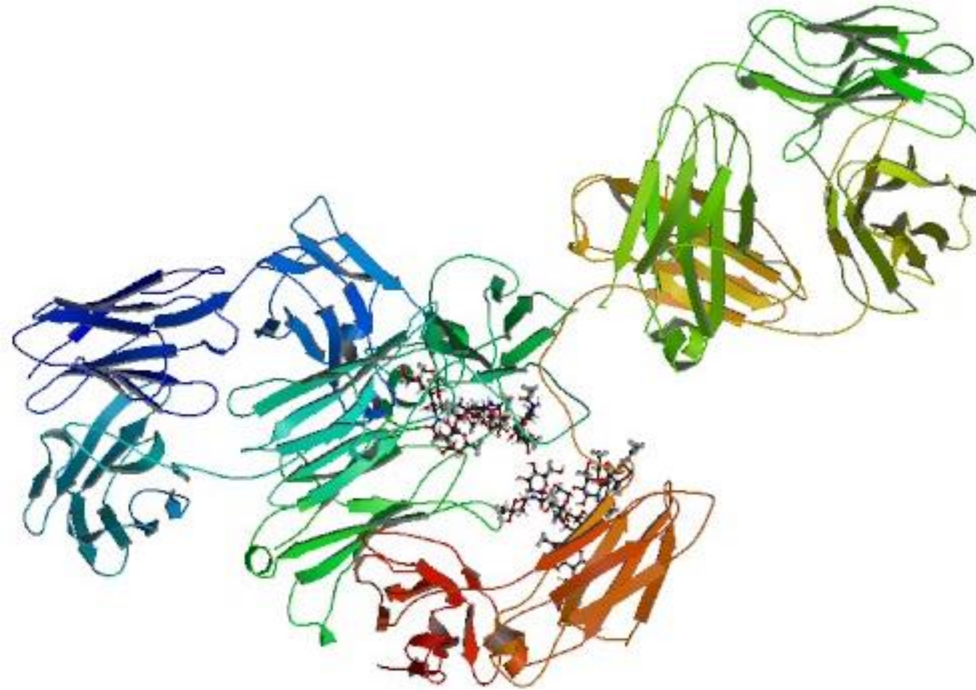
# Do You Know What This Is?

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

MW = 145421.50 g/mol



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# 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

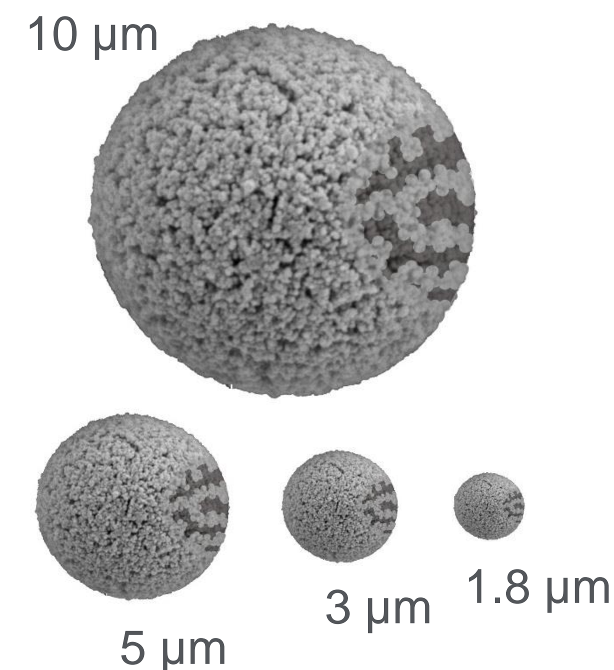
Instrument compatibility

# Particle Size

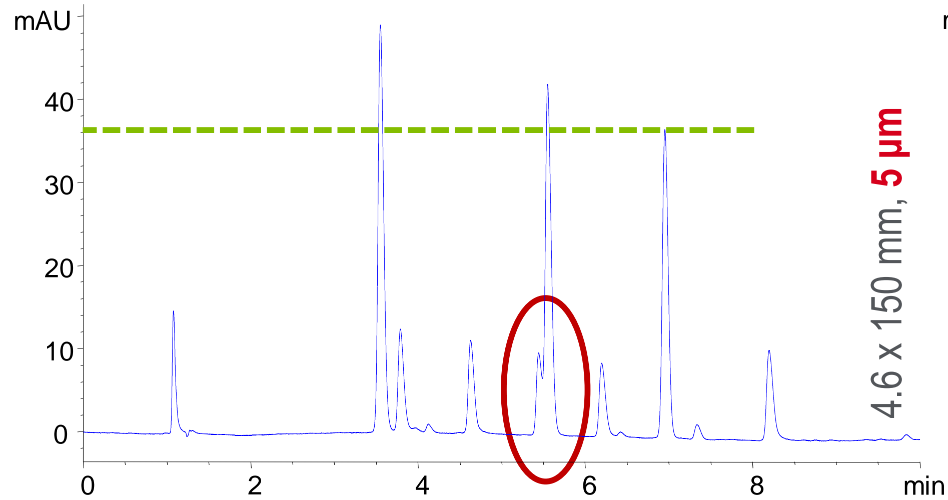
- Diameter
- Plate count
- Back pressure

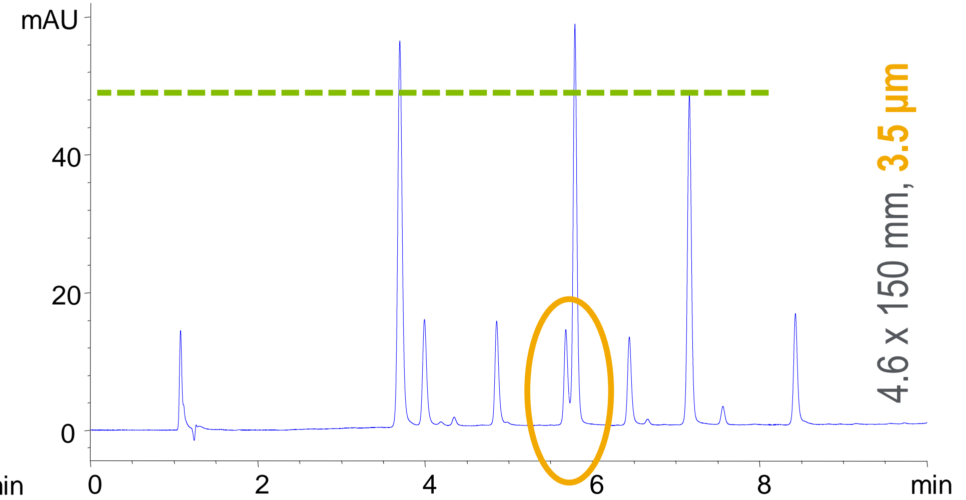
| Column Length (mm) | Resolving Power N(5 µm) | Resolving Power N(3.5 µm) | Resolving Power N(1.8 µm) | Typical Pressure* Bar (1.8 µm) |
|---|---|---|---|---|
| 150 | 12,500 | 21,000 | 32,500 | 724 |
| 100 | 8,500 | 14,000 | 24,000 | 420 |
| 75 | 6000 | 10,500 | 17,000 | 320 |
| 50 | 4,200 | 7,000 | 12,000 | 210 |
| 30 | N.A. | 4,200 | 6,500 | 126 |
| 15 | N.A. | 2,100 | 2,500 | 55 |

- pressure determined with 60:40 MeOH/water, 1ml/min, 4.6mm ID

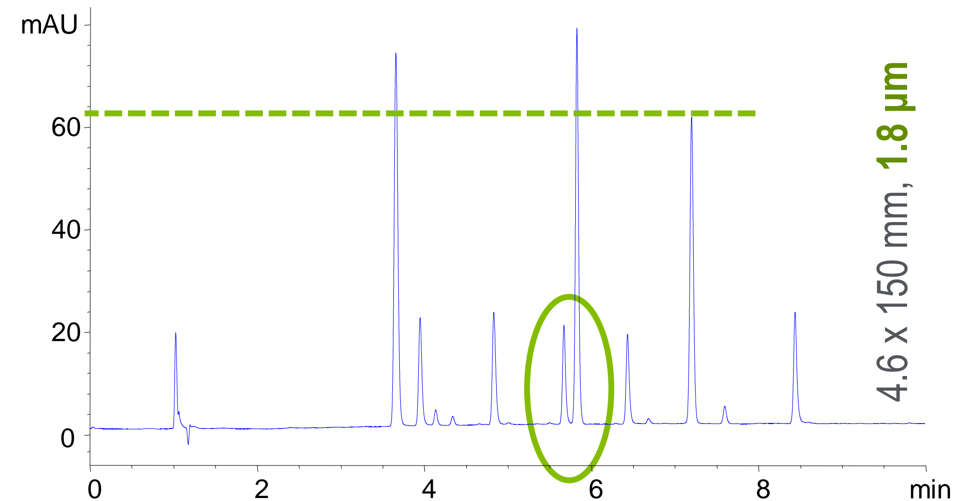


# Increase Resolution
## No Run Time Increase!

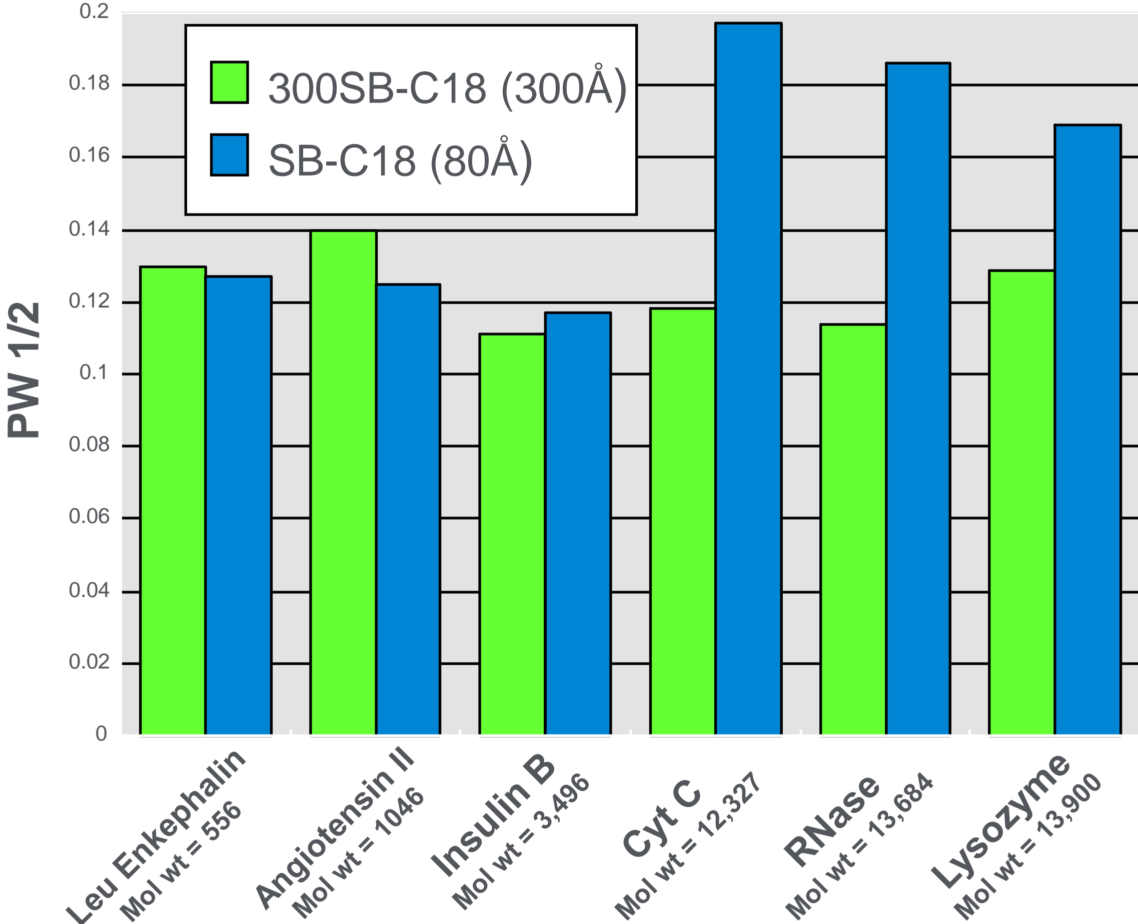


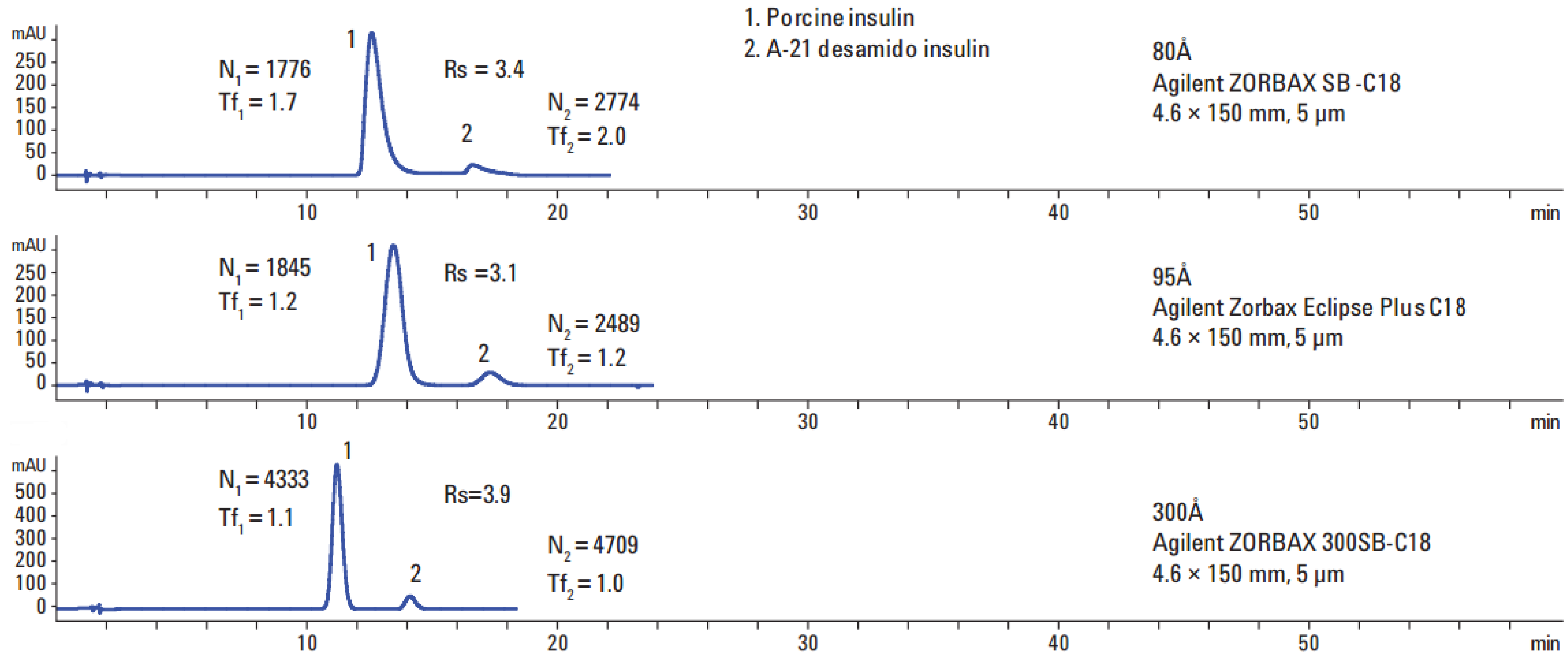

- **Increased** resolution due to reduced peak width
- **Increased** peak height due to reduced peak width gives increased sensitivity



# Pore Size and Molecular Size Effect on Peak Width



# Pore Size:
# Effect on the efficiency of a large molecule




*Chromatograms on 4.6 × 150 mm, 5 µm columns with different pore size.*

# Columns for intact protein analysis

## No “one size fits all” column …

| Particle | Diameter | Diffusion Distance | Pore Size | Matrix | Porosity |
|---|---|---|---|---|---|
| ZORBAX RRHD 300SB | 1.8µm | 0.9µm | 300Å | Silica | 100% (fully porous) |
| AdvanceBio RP-mAb | 3.5µm | 0.25µm (3.0µm solid core) | 450Å | Silica | 37% (ultra thin shell) |
| Poroshell 300 | 5µm | 0.25µm (4.5µm solid core) | 300Å | Silica | 27% (ultra thin shell) |
| ZORBAX 300SB | 5µm | 2.5µm | 300Å | Silica | 100% (fully porous) |
| PLRP-S 1000Å | 5µm | 2.5µm | 1000Å | Polymer | 100% (fully porous) |

- Smaller particle diameter for sharper peaks and increased resolution, but higher operating pressures
- Superficially porous particles for sharper peaks and lower operating pressures
- Polymeric particles for increased pH stability and reduced non-specific interactions
- **Pore size should be large enough to accommodate the molecule of interest**

# Bonding Chemistry

Sorbent characteristics

- Particle size
- Plate count
- Back pressure
- Pore size
- Bonding chemistry
  – Small Molecule – C18, C8
  – Proteins – C3, C4, C8

Column characteristics

- Internal diameter
- Length
- Material

Instrument compatibility



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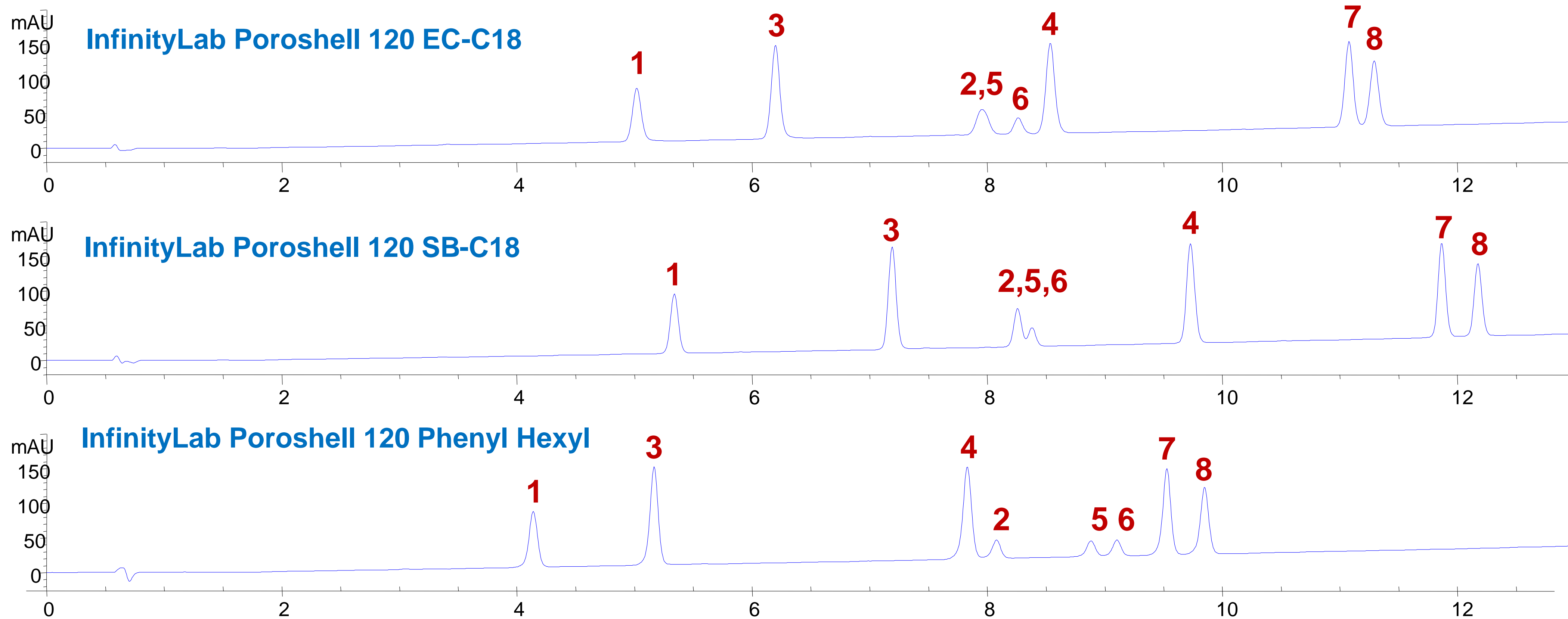
# Selectivity Differences Across InfinityLab Poroshell Bonded Phases




1. Hydrocortisone 2. B Estradiole, 3. Andostadiene 3. 17 dione, 4. Testosterone 5. Ethyestradione 6. Estrone 7. Norethindone acetate 8. Progestreone

*40-80 % Methanol in 14 min, DAD 260, 80 nm 0.4 ml/min, 2.1 x 100 mm column, 40 C, 0.1% Formic Acid in Water and Methanol, Agilent 1260 Method Development Solution*



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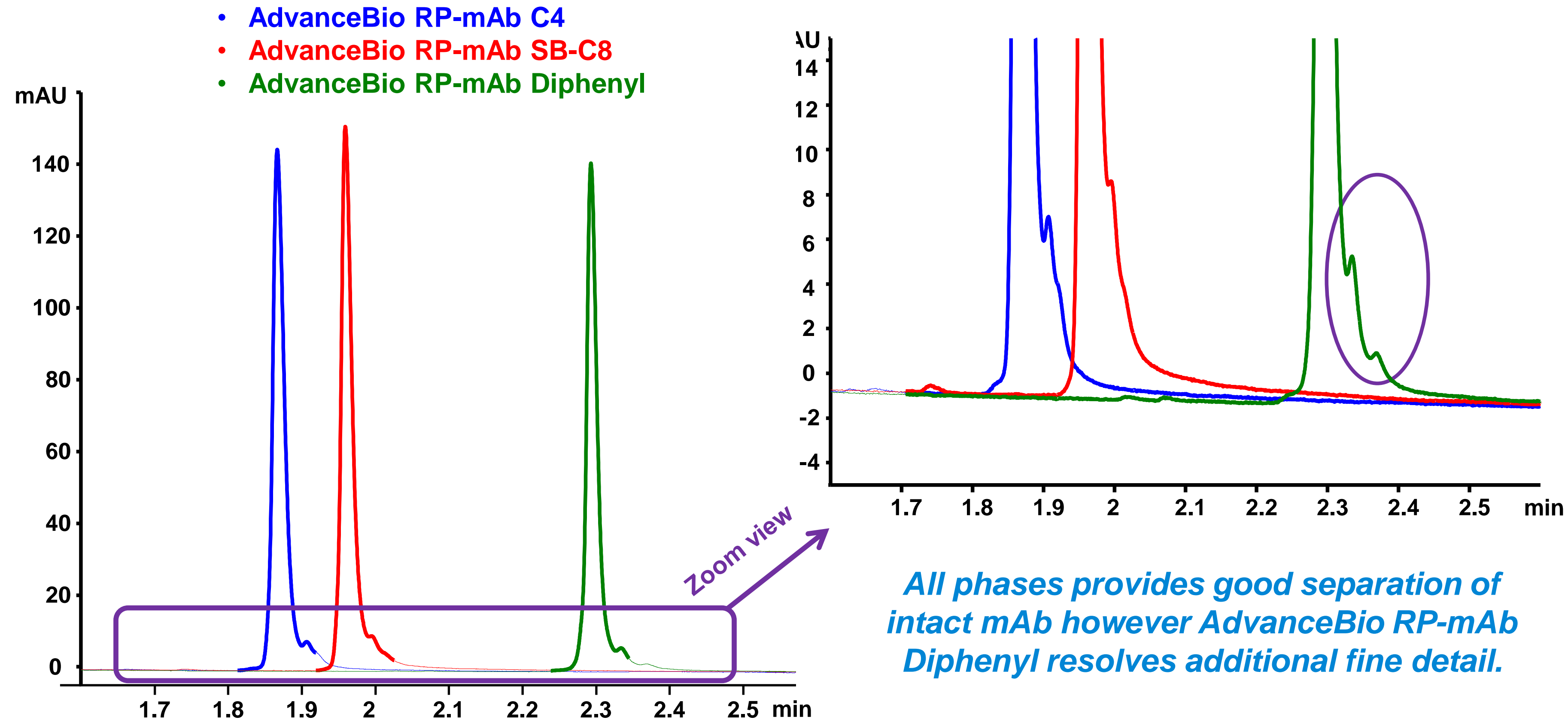
# Bonding Chemistry

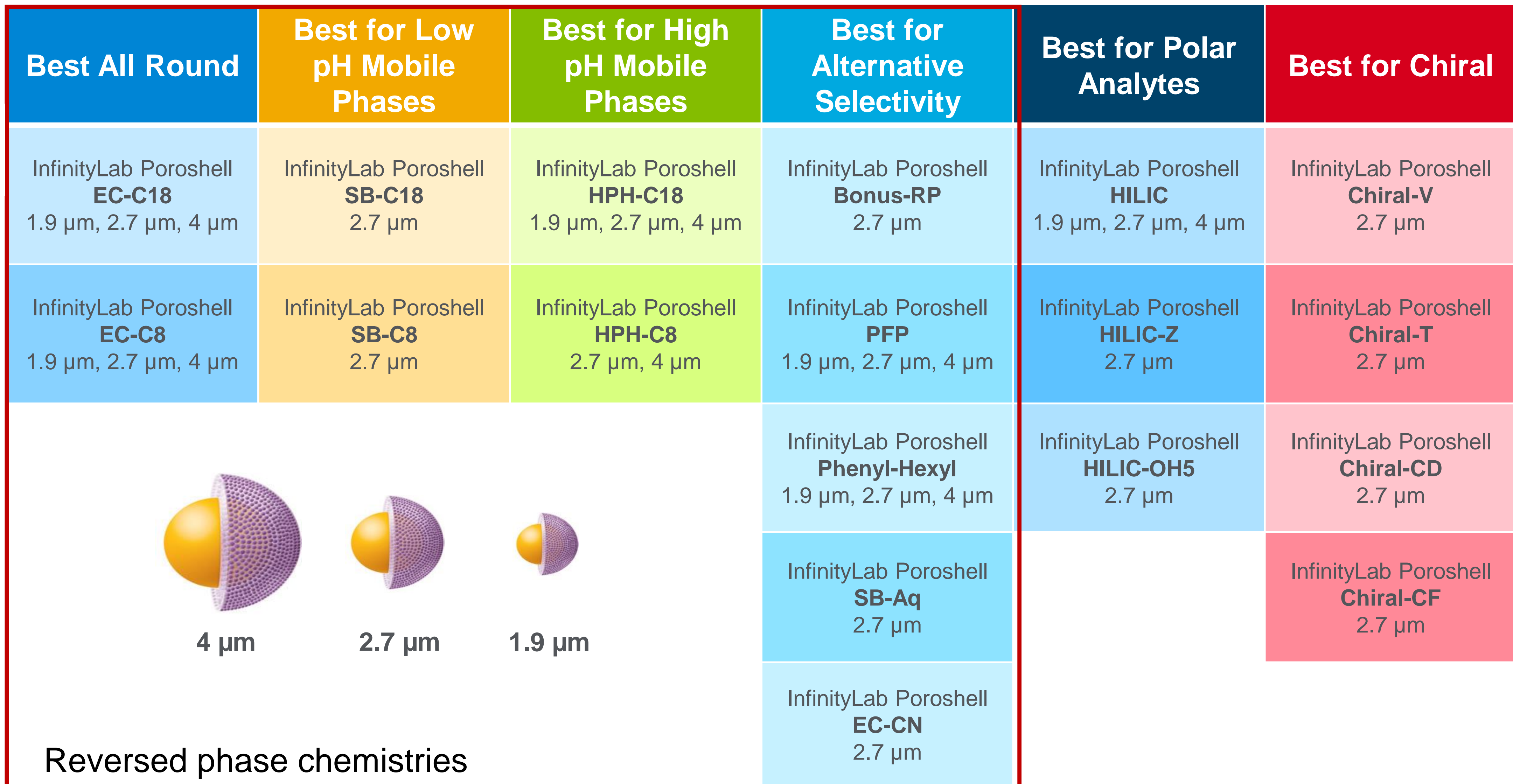
- AdvanceBio RP-mAb C4 provides a sharp peak and resolves fine detail in less than 2-minutes
- AdvanceBio RP-mAb Diphenyl resolves additional fine detail




***All phases provides good separation of intact mAb however AdvanceBio RP-mAb Diphenyl resolves additional fine detail.***

Method Parameters
Column: 2.1 x 100 mm
Mobile phase A:
0.1% TFA in water:IPA (98:2)
Mobile phase B:
IPA:acetonitrile:MPA* (70:20:10)
Flow rate: 1.0 mL/min,
Temperature: 80 °C
Gradient: 10-58% B in 4 min,
Injection: 5 µL
Sample: Herceptin IgG1
Temperature: 80 °C
Detection: UV @ 254 nm

# Agilent InfinityLab Poroshell 120 Portfolio Small Molecules and Peptides



| Best All Round | 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 | | |

# Agilent Biomolecule Columns

| Agilent Biomolecule HPLC Columns | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Titer Determination** | **Aggregate Analysis** | **Intact Purity & PTM Analysis** | | **Sequence Variant & PTM Analysis** | **Charge Variant Analysis** | **Glycan Analysis** | **Amino Acid / Cell Culture Media Analysis** | |
| Affinity | Size Exclusion | Reverse Phase >150 Å | Hydrophobic Interaction | Reverse Phase < 150 Å | Ion Exchange | Hydrophilic Interaction | Reverse Phase < 150 Å | Hydrophilic Interaction |
| Bio-Monolith Protein A | AdvanceBio SEC | PLRP-S | AdvanceBio HIC | AdvanceBio Peptide Plus | Bio mAb | AdvanceBio Glycan Mapping | AdvanceBio Amino Acid Analysis (HpH) | AdvanceBio MS Spent Media |
| Bio-Monolith Protein G | Bio SEC-3 | AdvanceBio RP mAb | | AdvanceBio Peptide Mapping | Bio IEX (SAX, WAX, SCX, WCX) | ZORBAX RRHD 300-HILIC 1.8 µm | ZORBAX AAA | |
| | Bio SEC-5 | ZORBAX RRHD 300 Å, 1.8 µm | | | PL SCX, SAX | | | |
| | | ZORBAX 300SB | | | Bio-Monolith (QA, DEAE, SO3) | | | |
| | | Poroshell 300 | | | | | | |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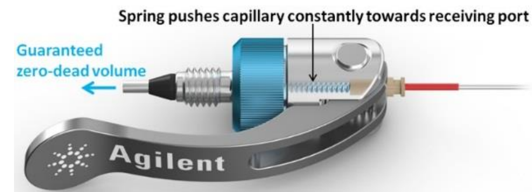
# Column Configuration
# Which is best for your application

| Column Type | id (mm) | Length (mm) | Particle Size (mm) | Flow Rate Range | Applications |
|---|---|---|---|---|---|
| Capillary | 0.3, 0.5 | 35 – 250 | $\leq$5 | 1 – 10 mL/min | Max sensitivity LC/MS |
| MicroBore | 1.0 | 30 – 150 | $\leq$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 | $\geq$5 | 4 – 10 mL/min | Small scale prep (mg) |
| Preparative | >21.2 | 50 – 250 | $\geq$5 | 20 – 100 mL/min | Large scale prep |

# Things to Consider

Instrument

van Deemter




Quick Connect fitting

https://www.agilent.com/en/products/liquid-chromatography/lc-supplies/capillaries-fittings/infinitylab-fittings

https://www.agilent.com/en/products/liquid-chromatography/lc-supplies/infinitylab-flex-bench-family/infinitylab-flex-bench-family

# Chelation and "Sticky" Compounds

Stainless steel has active sites that bind to certain classes of polar molecules*

- **Most active molecules:**
  - Phosphorylated metabolites and
  - Organophosphates and phosphonic acids
  - Di- and tri- carboxylic acids and similar chelating acids

- **Commonly seen in:**
  - Pesticide analysis (glyphosate, AMPA, glufosinate)
  - Fermentation (citric acid cycle, organic acid monitoring)
  - Metabolomics (Nucleotides, sugar phosphates, citric acid cycle)

*See appendix for instructions on dealing with these interactions

# Instrument (and column) Considerations

## Bio-inert 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

## InfinityLab Deactivator Additive

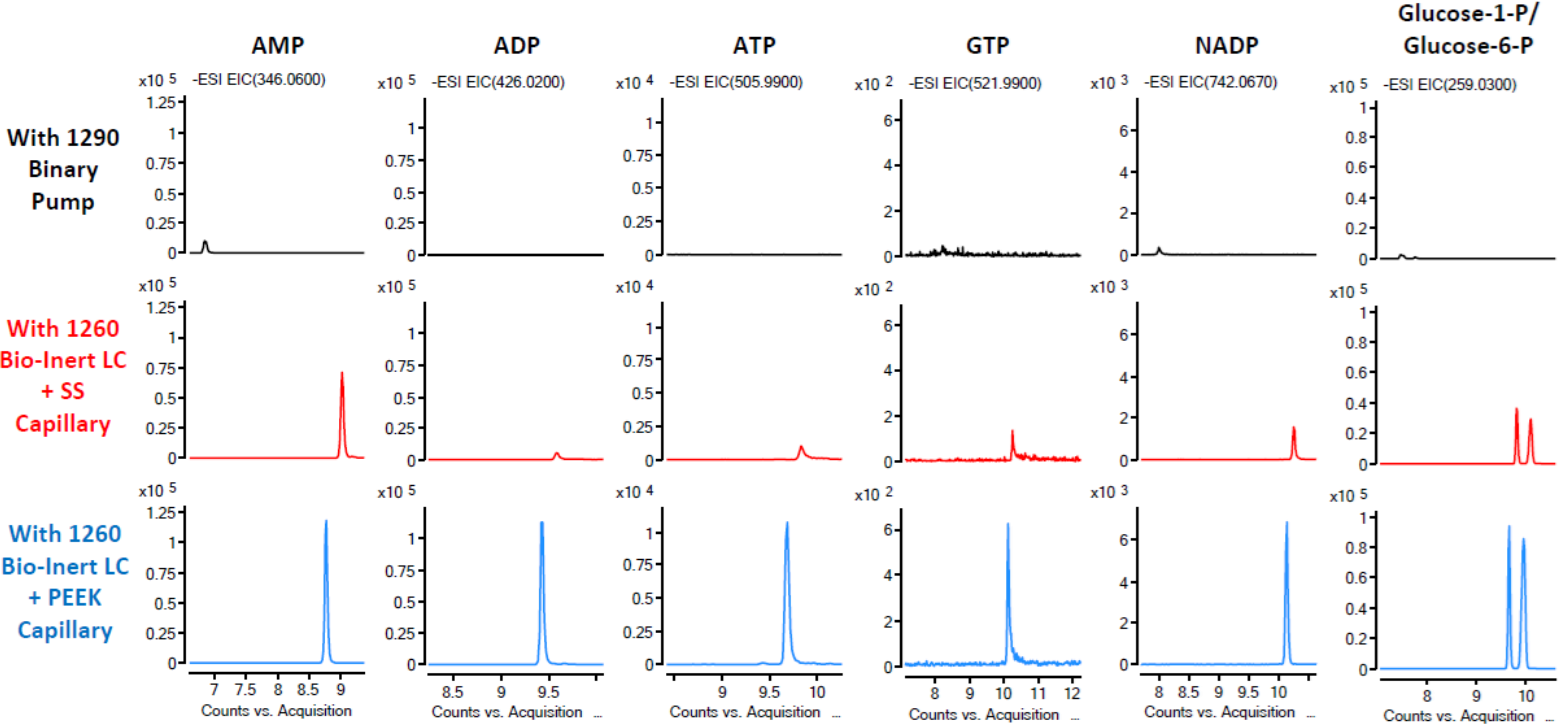
- Use after phosphoric acid passivation

## PEEK-lined columns

- HILIC-Z

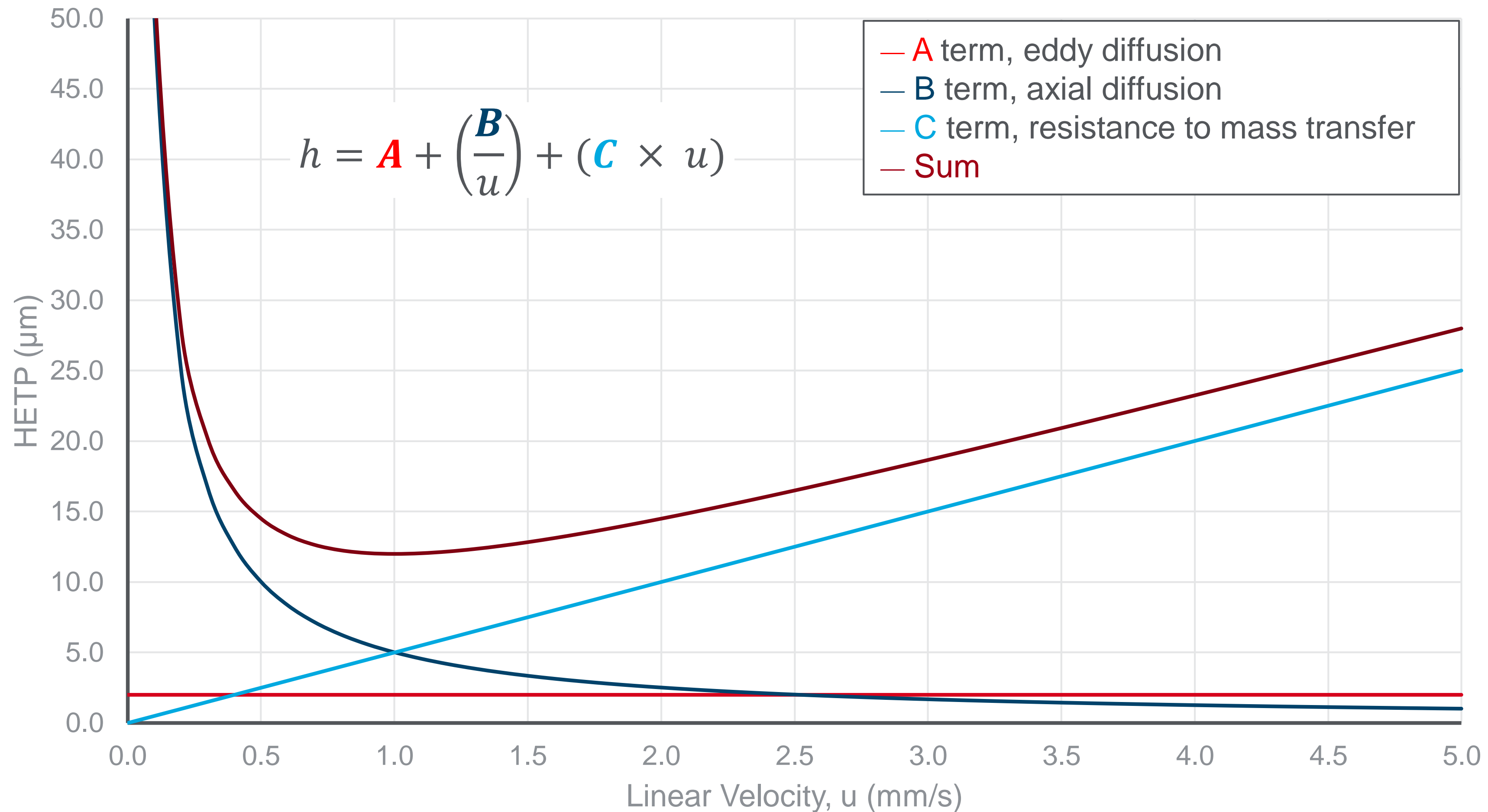
# Column and Instrument Materials

## Nucleotide phosphates on AdvanceBio MS Spent Media (HILIC stationary phase in PEEK lined stainless steel 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

# van Deemter Equation
# Affects on plate count




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



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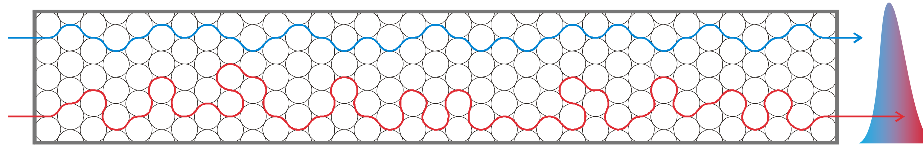
# Factors Affecting Plate Count

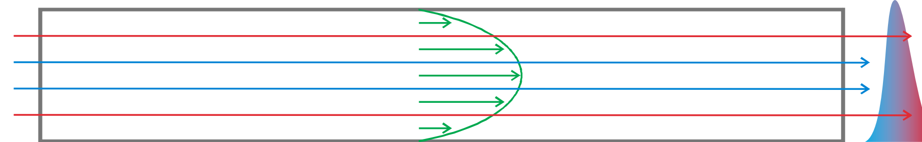
**A** term Eddy diffusion (multiple path effect)



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

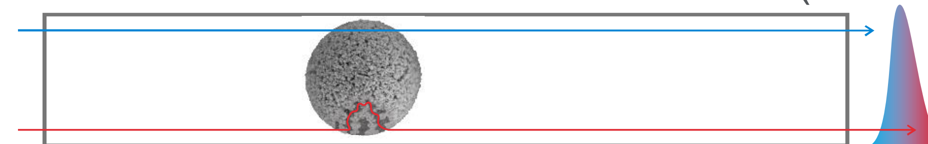
**B** term Molecular diffusion

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



**C** term Mass transfer (within particle)

$w_C \sim d_p^2$





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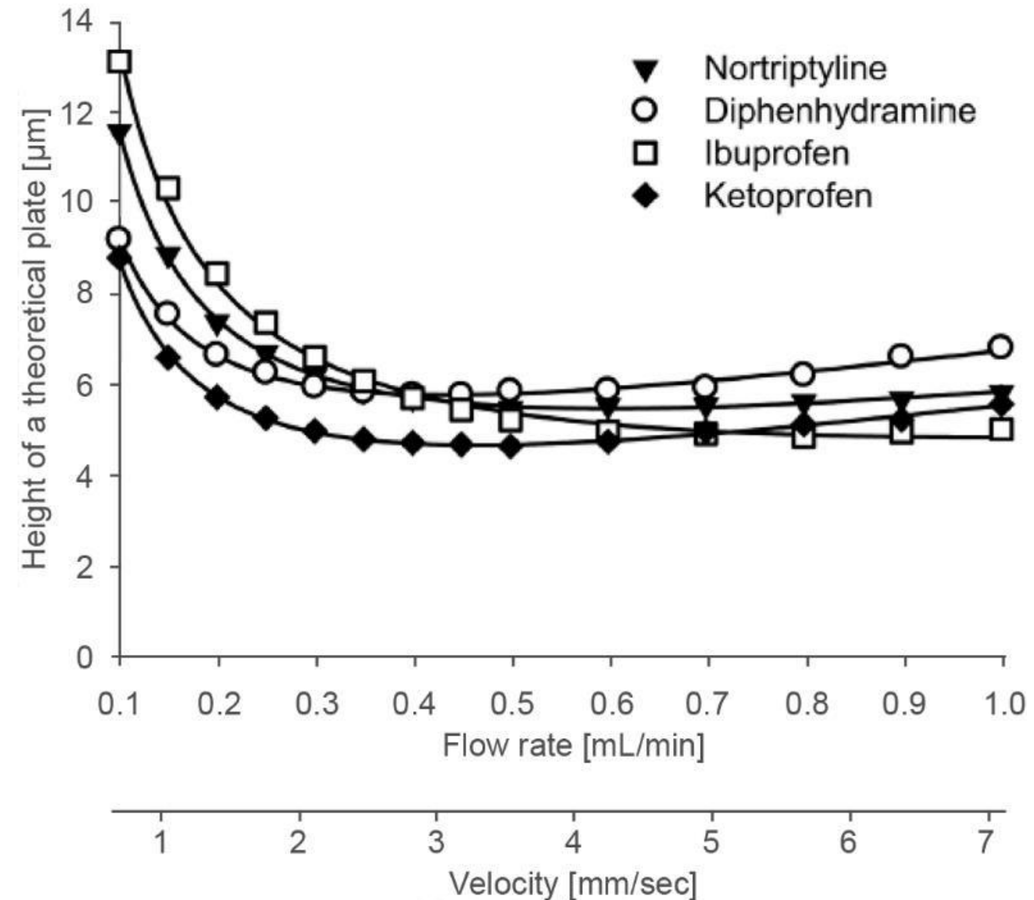
# van Deemter Equation

## Curves for different analytes

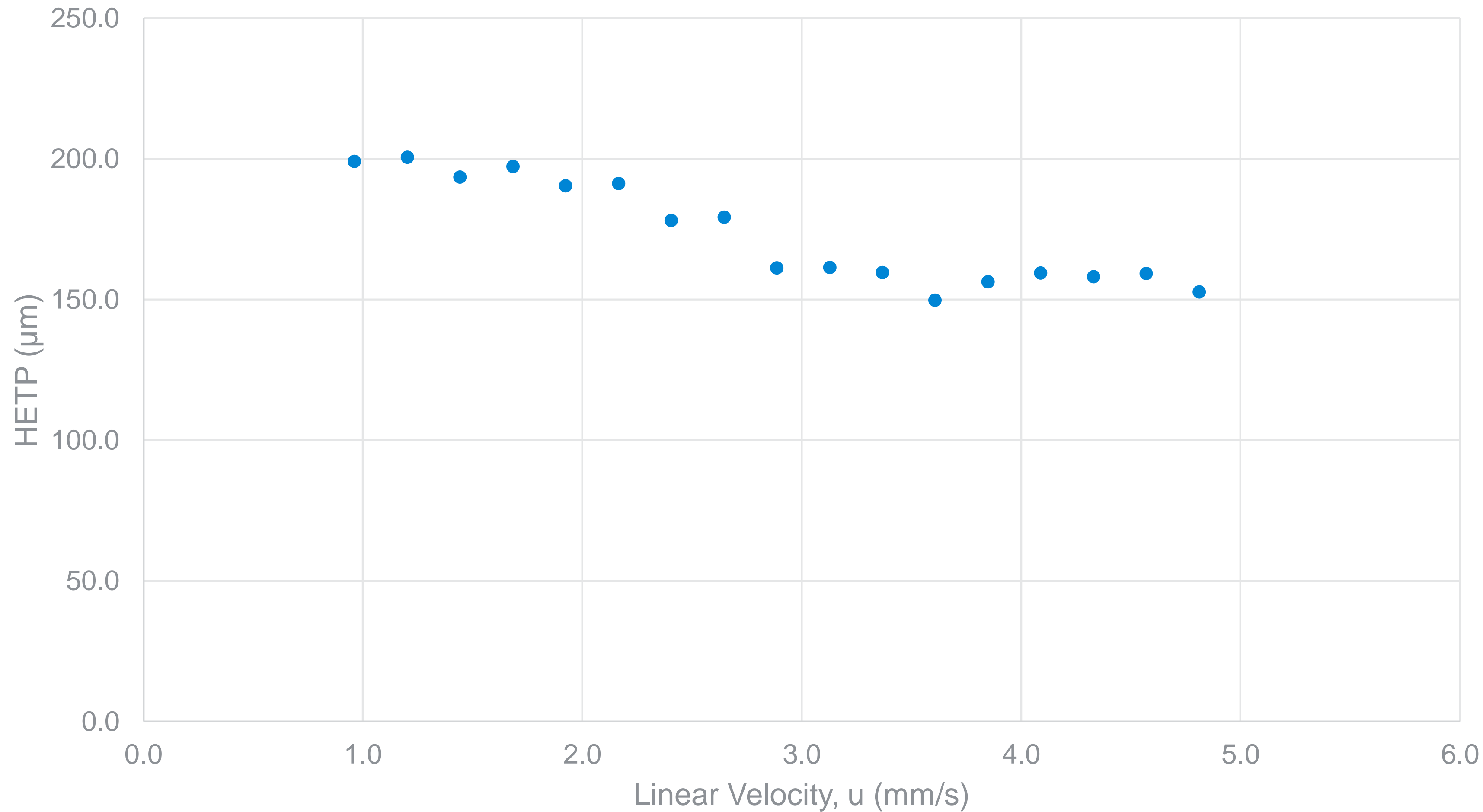
- van Deemter equation for isocratic runs only
- Compound and instrument specific
- Even for sub-2-µm particles not horizontal
- Optimum flow rate depends on compound




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

Are proteins like small molecules?

# van Deemter Plot




Particle morphology:

- Non-porous
- Superficially porous
- Fully porous

Pore size:

- 100Å, 300Å, 450Å, 1000Å

Are proteins like small molecules? The answer is no.



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# van Deemter Equation – C Term – “Resistance to mass transfer”
## Slower diffusion of large molecules broadens peaks at high flow

$w_C \sim d_p^2$

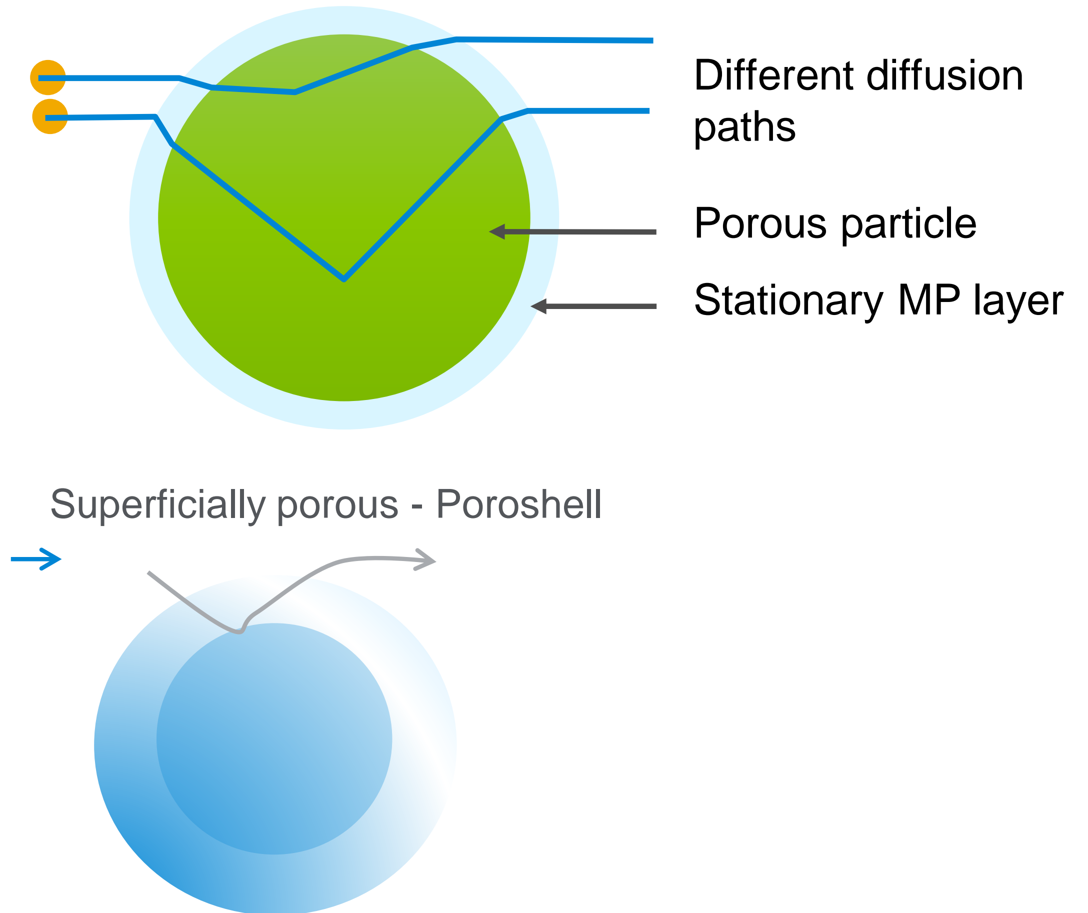
**So… decrease diffusion time for macromolecules!**

Increase the diffusion rate

- Elevated operating temperature – Column dependent
- Decreased solvent viscosity – Helps but changes elution

Decrease the diffusion distance

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



# Particle Effect Considerations

| Particle | Diameter | Diffusion Distance | Pore Size | Porosity |
|---|---|---|---|---|
| ZORBAX RRHD 300SB | 1.8µm | 0.9µm | 300Å | 100% (fully porous) |
| AdvanceBio RP-mAb | 3.5µm | 0.25µm (3.0µm solid core) | 450Å | 37% (ultra thin shell) |
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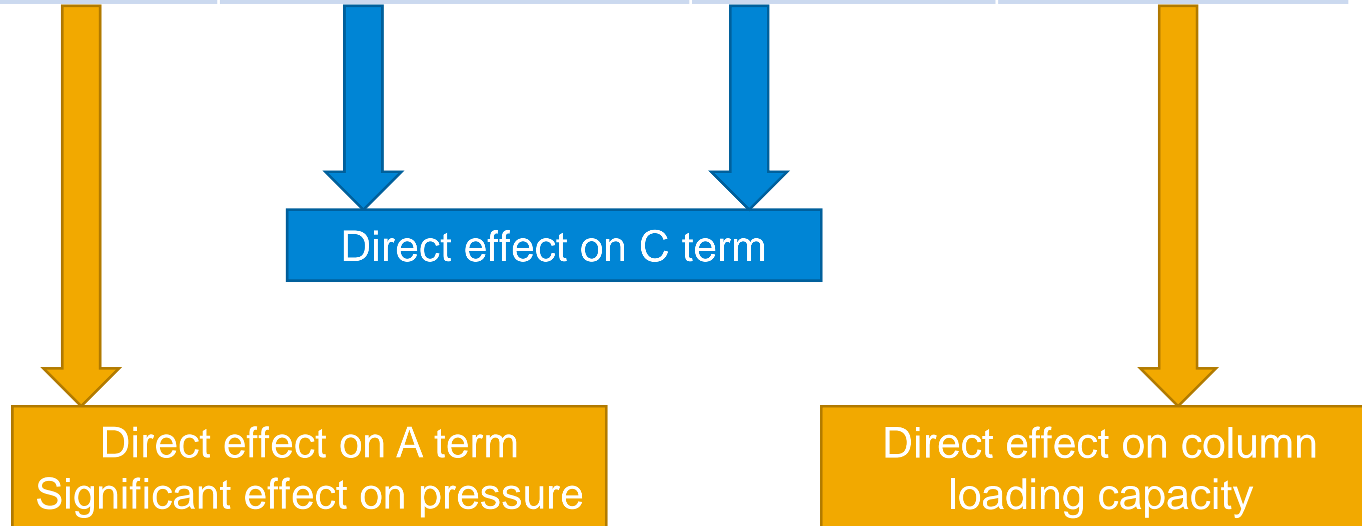
| PLRP-S 1000Å | 5µm | 2.5µm | 1000Å | 100% (fully porous) |





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# Method Conditions

Isocratic or gradient

Mobile phase

- Organic modifier
- Additives

pH

Temperature

Flow rate

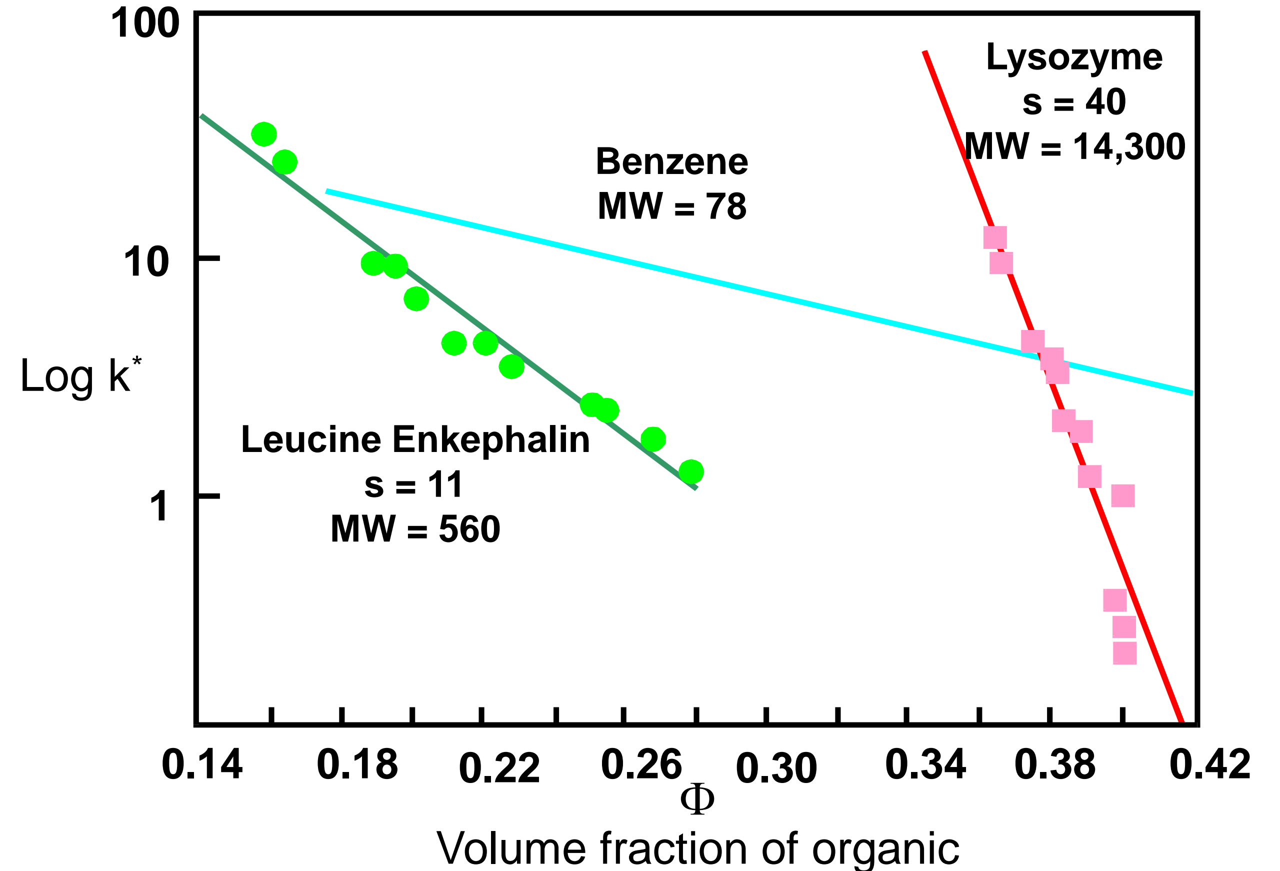
- van Deemter

# Larger Molecules versus Small Molecules

## More sensitive to changes in % organic

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

| | | |
|---|---|---|
| Δ%B | = | Difference between initial and final %B values |
| S | = | Constant that changes with MW:<br>4 for 100 - 500 Da;<br>10 < S < 1000 for peptides and proteins |
| F | = | Flow rate (mL/min) |
| $t_g$ | = | Gradient time (min) |
| $V_m$ | = | Column void volume (mL) |




- Lysozyme is 15 times more sensitive to changes in organic modifier than benzene
- Four times more sensitive than leucine enkephalin

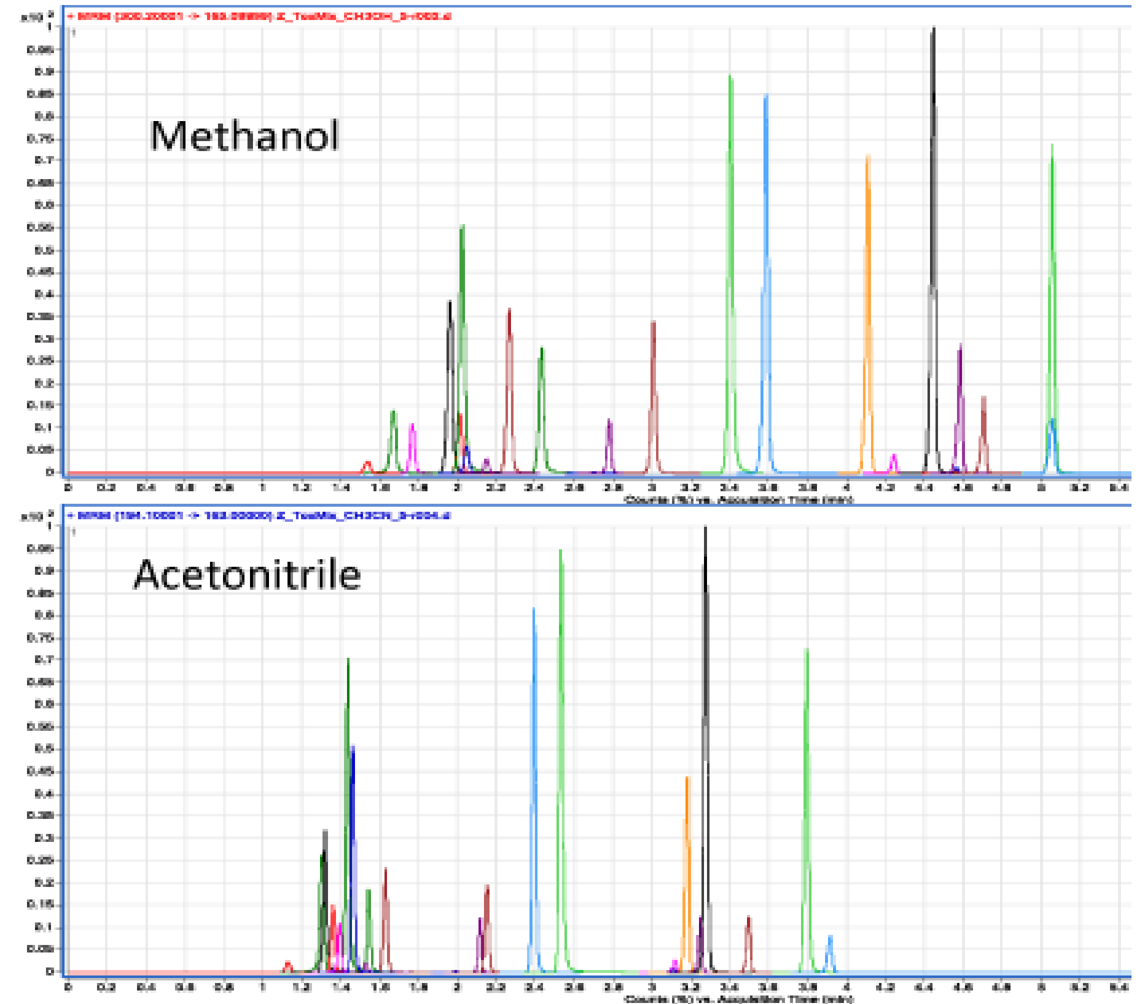
# Exploring Organic Modifiers – Small Molecules

Why?

- It's easy – ACN and 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, Agilent pub. No. 5990-6345EN.



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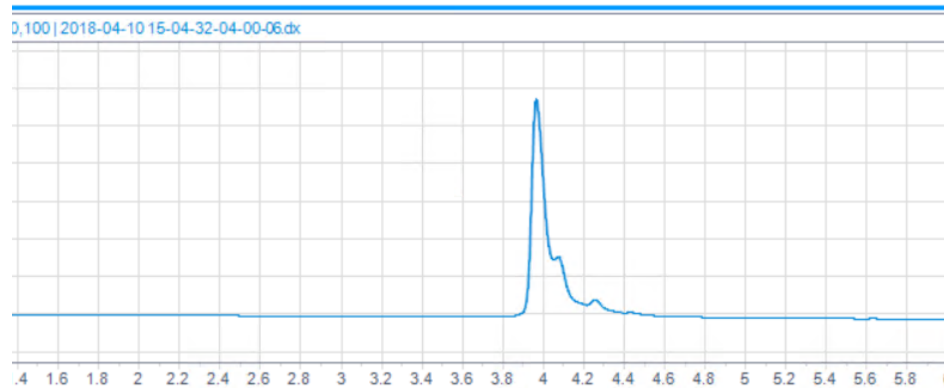
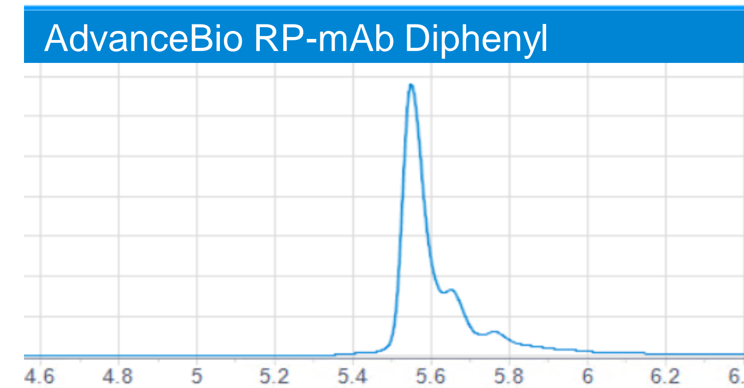
# Mobile Phase: Organic Modifier

| Columns | 2.1 x 50mm |
|---|---|
| Mobile phase | A: water:IPA 98:2 + 0.1% TFA<br>B: IPA:ACN:MPA 70:20:10 |
| Temperature | 80 °C |
| Flow rate | 0.5 mL/min |
| Gradient | 25 – 45% B in 10 min |

| Columns | 2.1 x 50mm |
|---|---|
| Mobile phase | A: 0.1% TFA in water<br>B: 0.08% TFA in ACN |
| Temperature | 80 °C |
| Flow rate | 0.5 mL/min |
| Gradient | 25 – 45% B in 10 min |




- Acetonitrile - organic solvent for reversed-phase separation of Proteins just like with small molecules.
- Trifluoroacetic acid is the preferred mobile-phase additive.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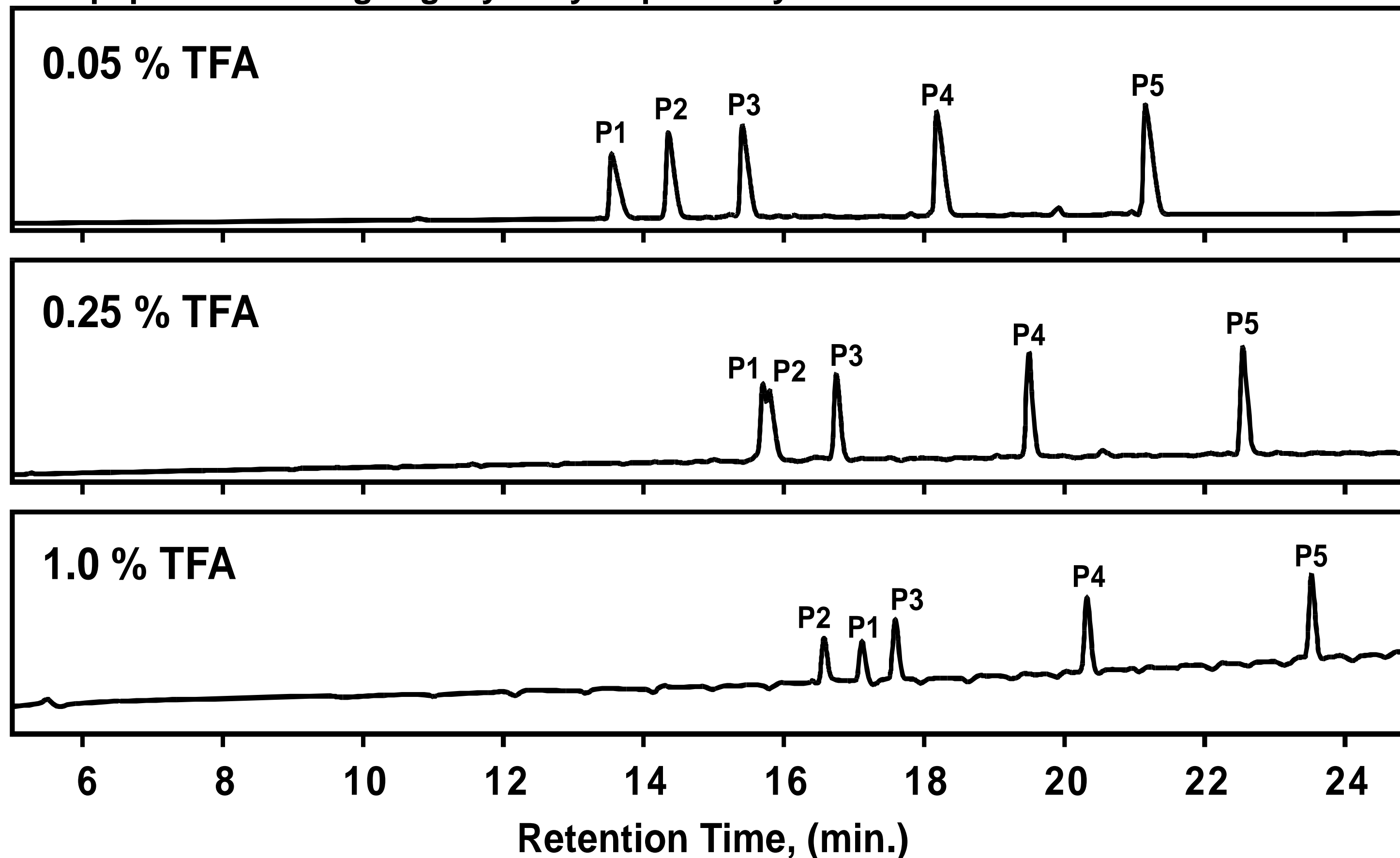
# TFA Concentration

## Affect on hydrophilic 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**



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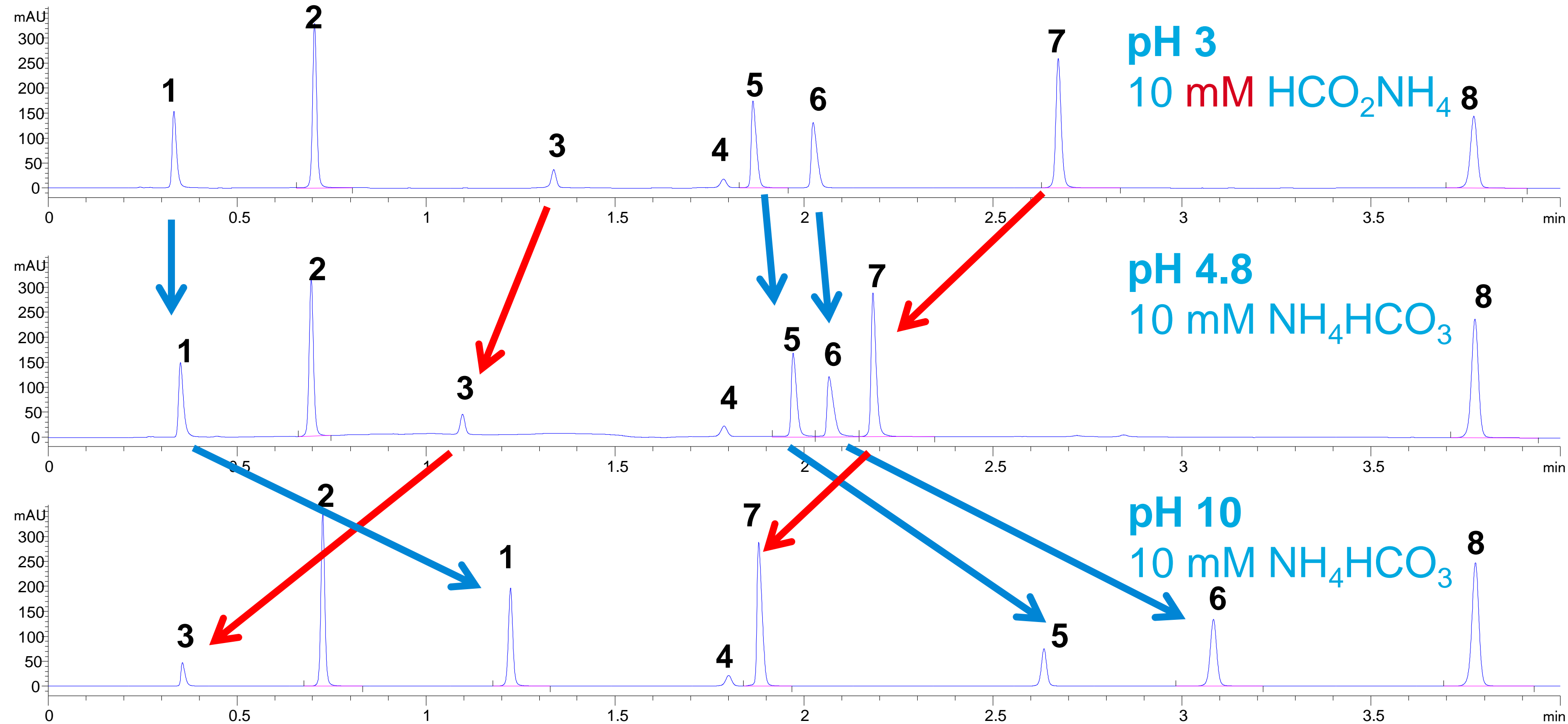
# Selectivity Can be Controlled by Changing pH

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**



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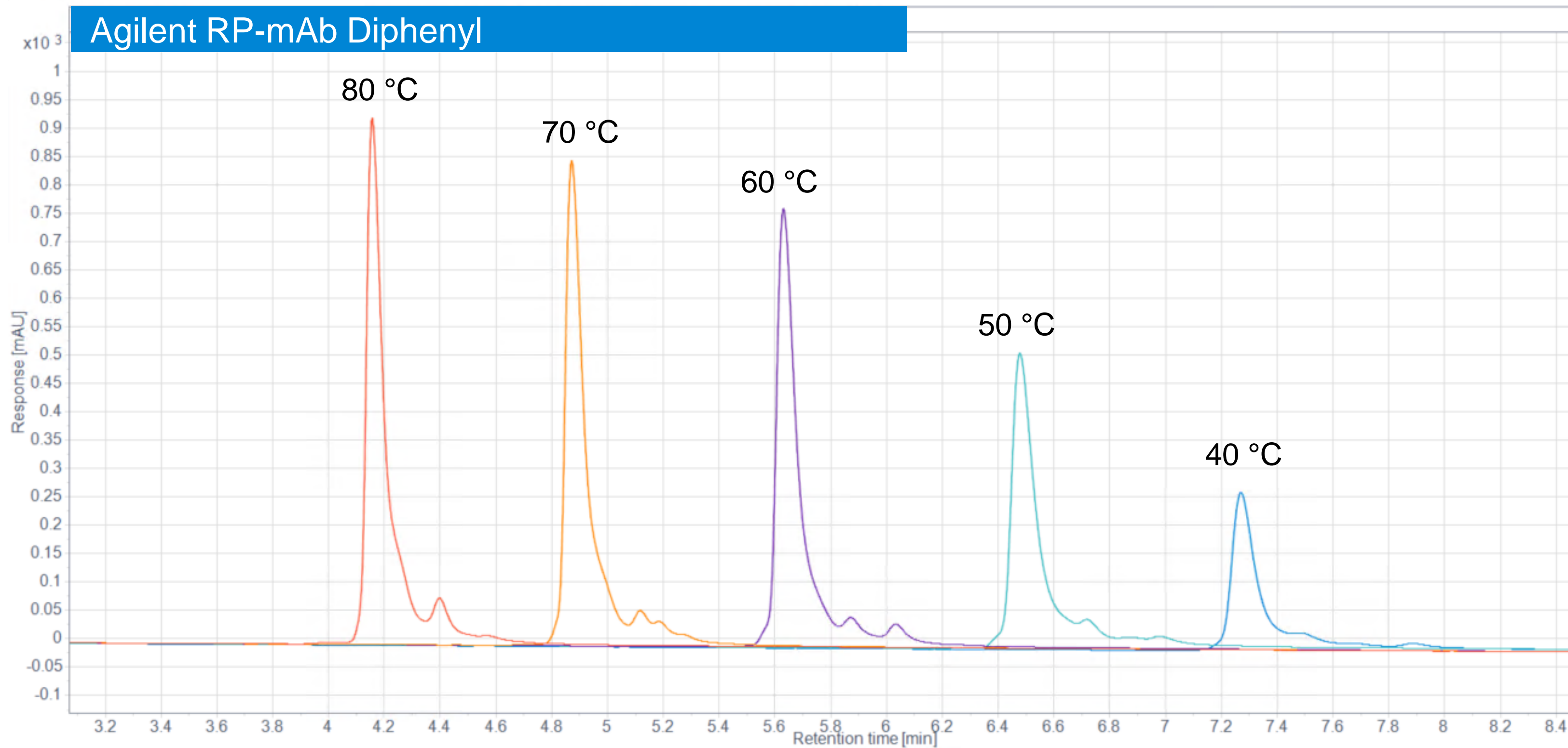
# Effect of column temperature




Increasing temperature:

- Reduces mobile phase viscosity
- Improves mass transfer (for sharper peaks)
- Leads to shorter retention times
- Reduces operating pressure
- Can cause degradation of some proteins



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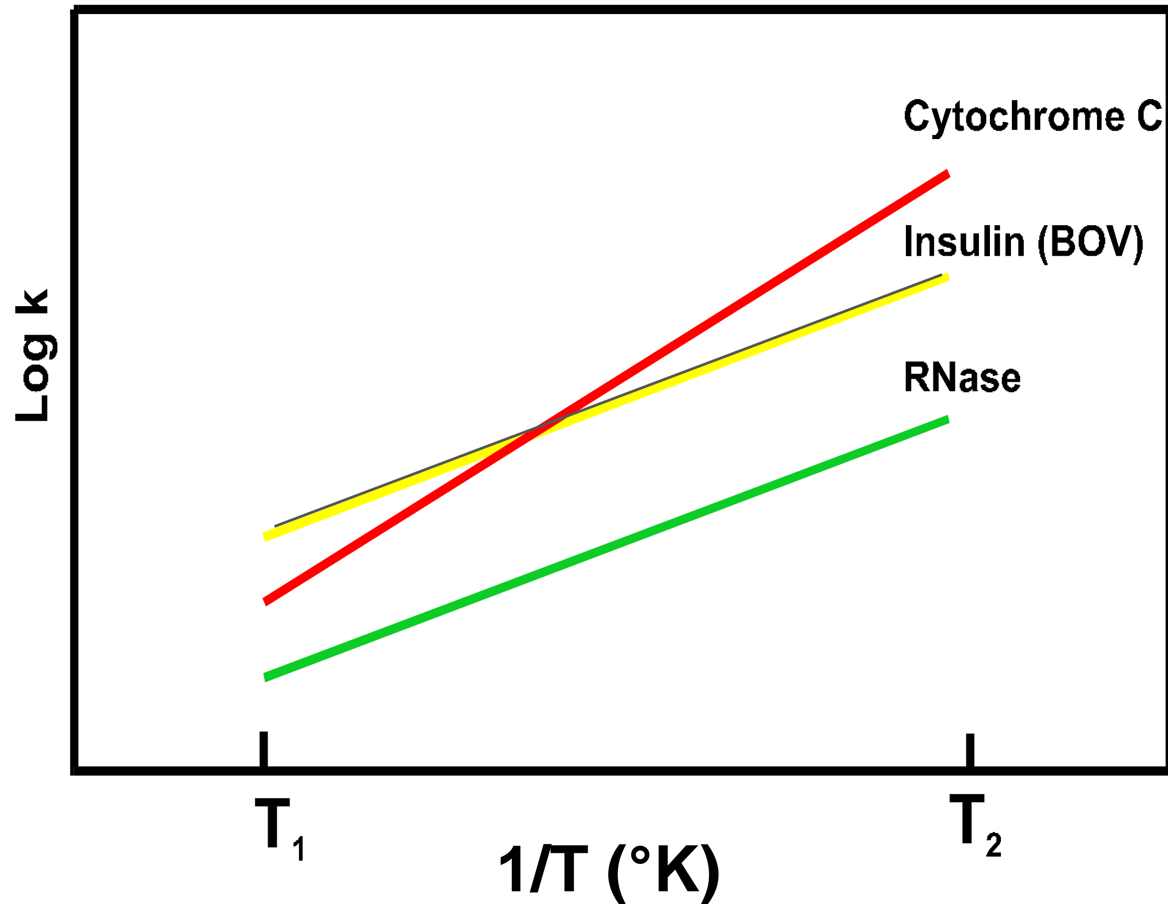
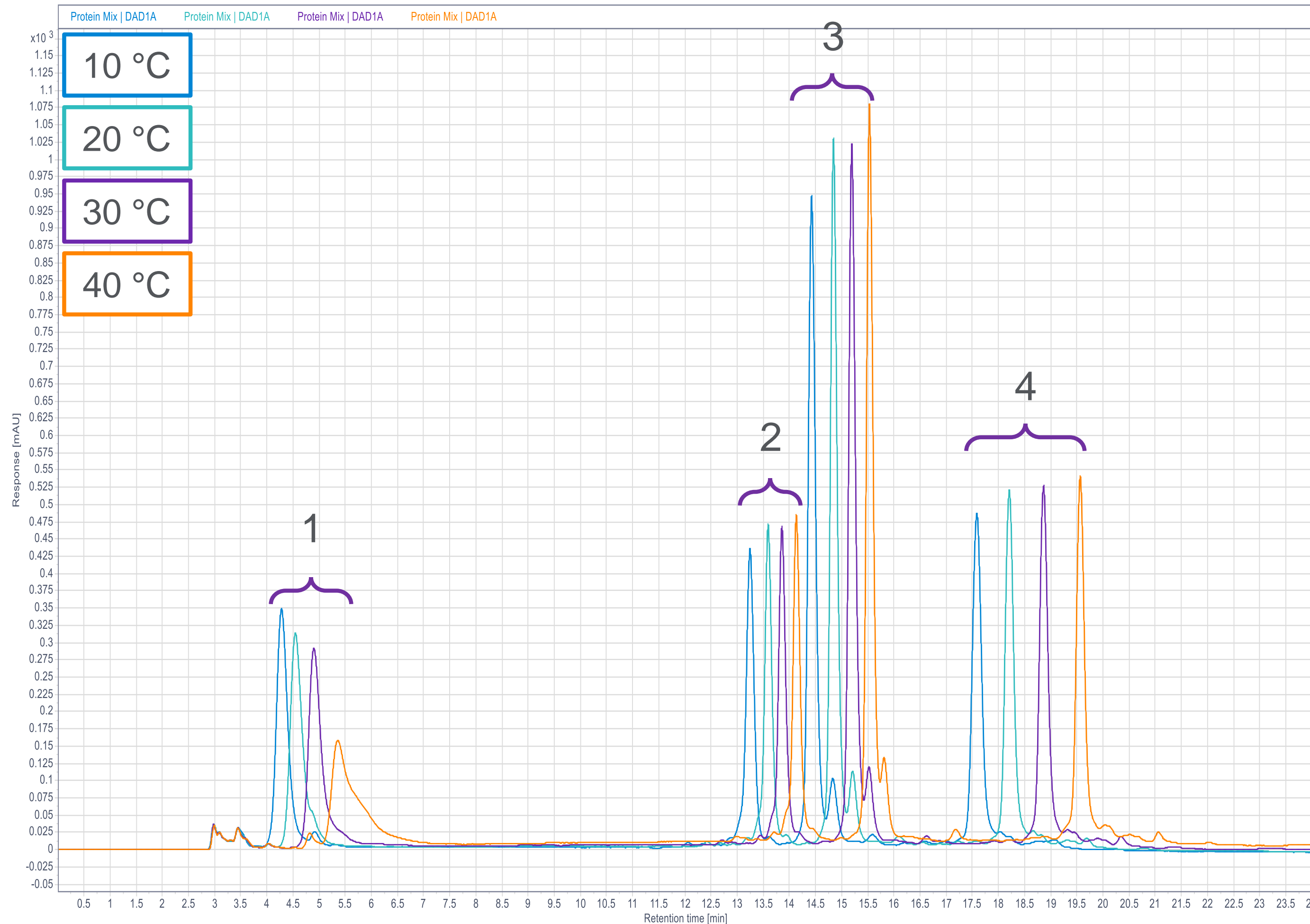
# Increasing Temperature

- Reduces analysis time
- May change selectivity




Caution: Excessive heat can cause faster sample and/or column degradation!

# Effect of column temperature in HIC




Increasing temperature:

- Reduces mobile phase viscosity
- May lead to loss in resolution
- Leads to longer retention times
- Reduces operating pressure
- Can cause degradation of some proteins

1 Cytochrome C
2 Ribonuclease A
3 Lysozyme
4 α-Chymotrypsinogen A



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# Troubleshooting

Recovery
- Molecule size
- Metals
- Is method optimized?

Detection
- Chromophore
- MS

Column cleaning

# What Affects Recovery?

Chain length

Hydrophobicity/aggregation

Mobile phase

Solubility of sample

Size

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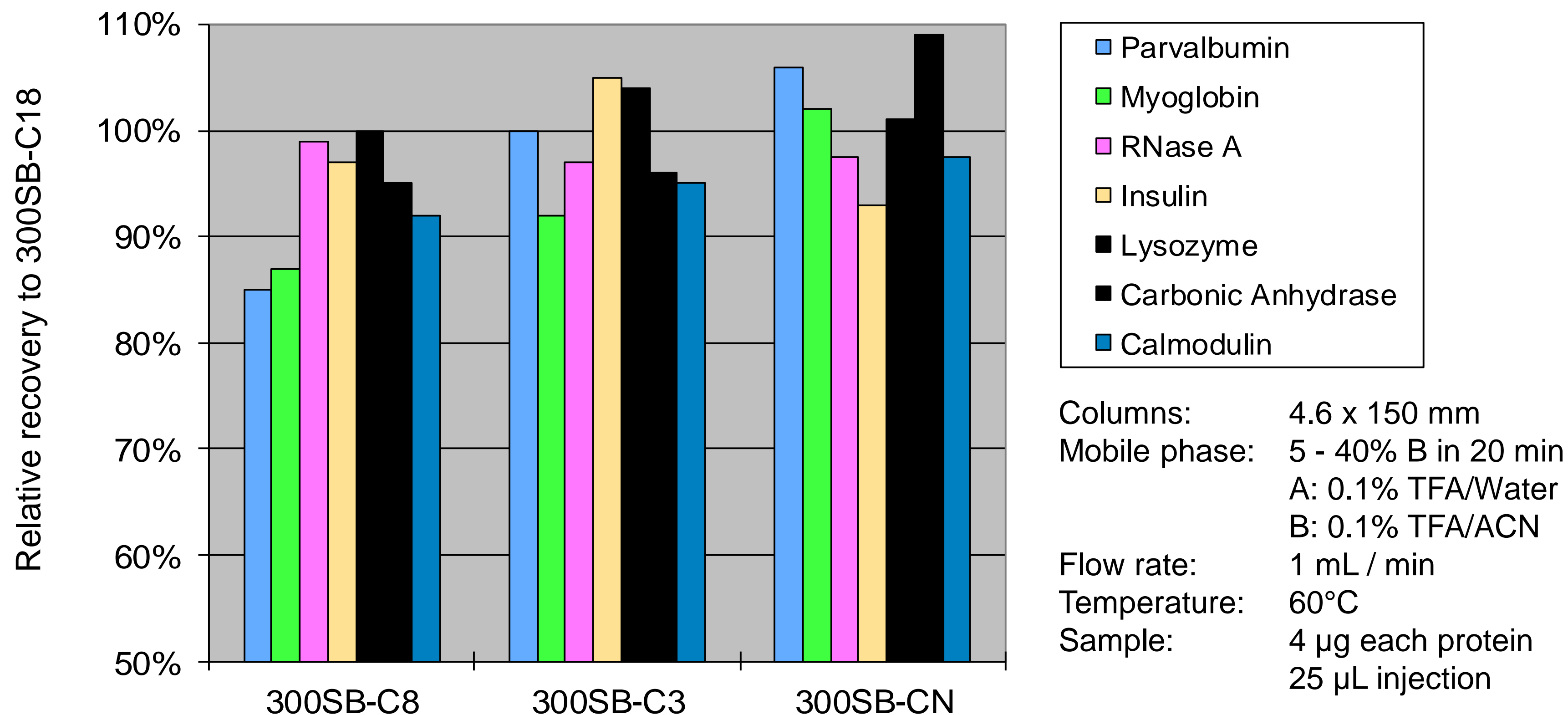
# Recovery of Polypeptides from ZORBAX 300SB Columns




**001007P1.PPT**

# Proteins and Mass Spectrometry

## 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

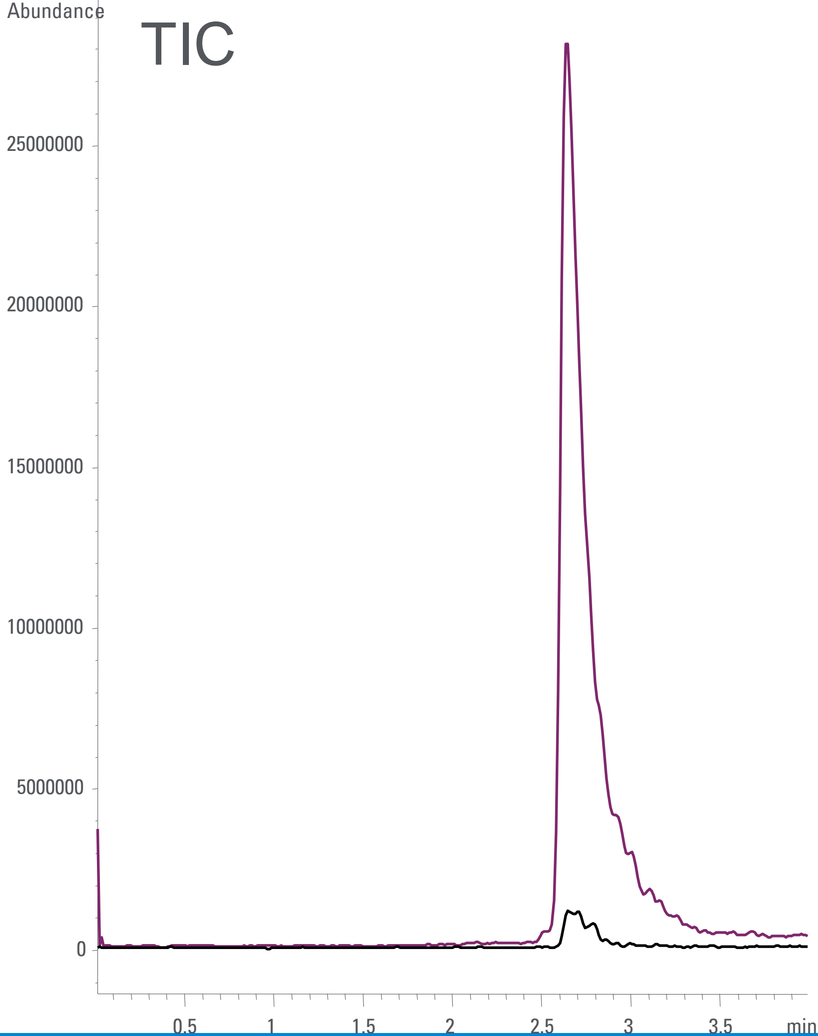
Denature

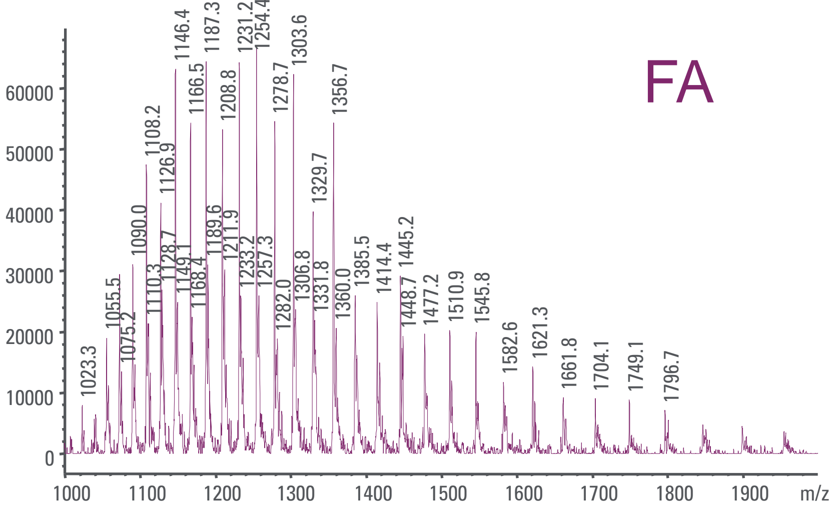
- Chaotropes: Guanidine, Urea
- Reductants: DTT, BME, Ascorbic acid
- Temperature

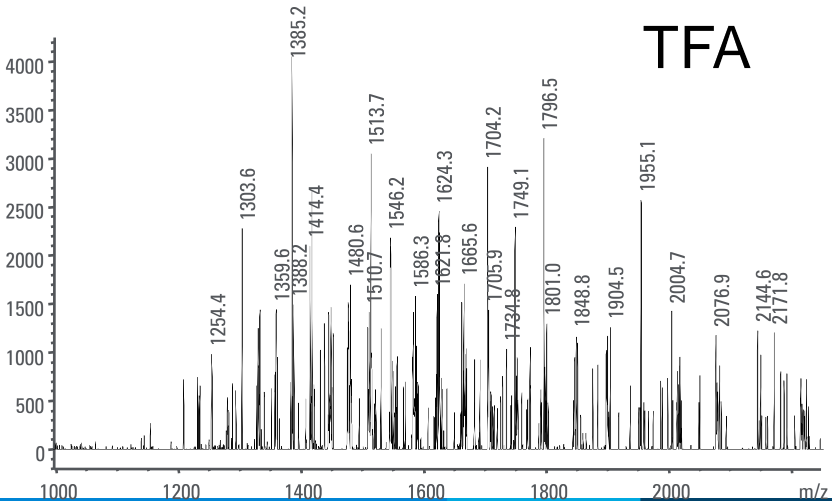
Digestion

Derivatize

# Effect of Modifier on MS Response: 10 pmol BSA





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# 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 3 M guanidine in 50% IPA

***Silica based RP columns only**

Before using either hexane or methylene chloride the column must be flushed with isopropanol and again before returning to your reversed phase mobile phase.

# Caring for your columns …

1. Column installation
2. Column use
3. Column storage
4. Column clean-up
5. Common problems
   - Pressure
   - Retention time
   - Resolution
   - Peak shape

**REFER TO THE USER GUIDE / CARE GUIDE !**

# 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 or GC/MS Columns and Supplies

Option 2 for LC or LC/MS 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

# Eliminating Sticking with Wash Step

1. LC Disconnected from MS and going directly to waste
2. IPA at 5 mL/min for 5 min
3. Water at 5 mL/min for 5 min
   - Or flow at 0.5 mL/min for 1 hour
4. 0.5% Phosphoric Acid in 90% Acetonitrile / 10% Water
   - Flow at 0.1 mL/min overnight
5. Water at 5 mL/min for 5 min
   - Or flow at 0.5 mL/min for 1 hour
6. Mobile Phase at 5 mL/min for 5 min
   - Or flow at 0.25 mL/min for 1 hour
7. Reconnect LC to MS and proceed with analysis

# InfinityLab Deactivator Mobile Phase Additive

- Add to mobile phase after passivating system with phosphoric acid
- Safe for TOF
- No system dedication, like ion pairing
- Compatible with all Poroshell columns, not just HILIC-Z

p/n: 5191-4506