High Resolution and Fast LC and LC/MS of Proteins and Peptides with Poroshell Columns

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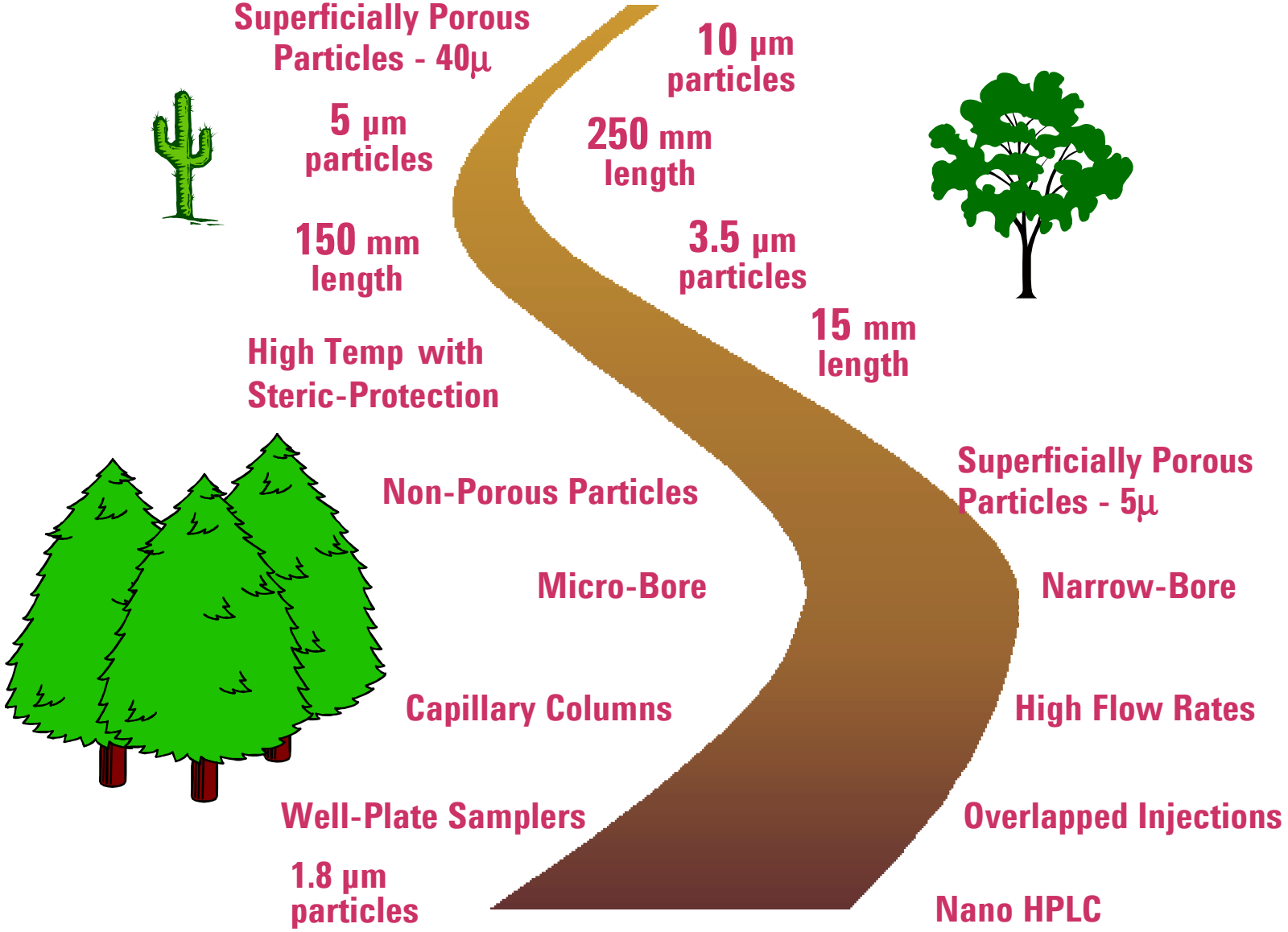
The Path to Ultra-Fast HPLC Separations



Small-Column HPLC Technology

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What Makes High Speed HPLC Possible?

Small particle sizes (< 5 μm)

Short columns (10 cm or less)

Low viscosity mobile phases

Optimized HPLC – minimal extra column volume, low volume flow cell

Detector set to fast response time

High diffusion coefficient of the sample molecules in the mobile phase = rapid diffusion = less band broadening = higher linear velocities with efficient peaks

But large molecules have small diffusion coefficients

Slower Diffusion of Large Molecules Broadens Peaks at High Flow Rates

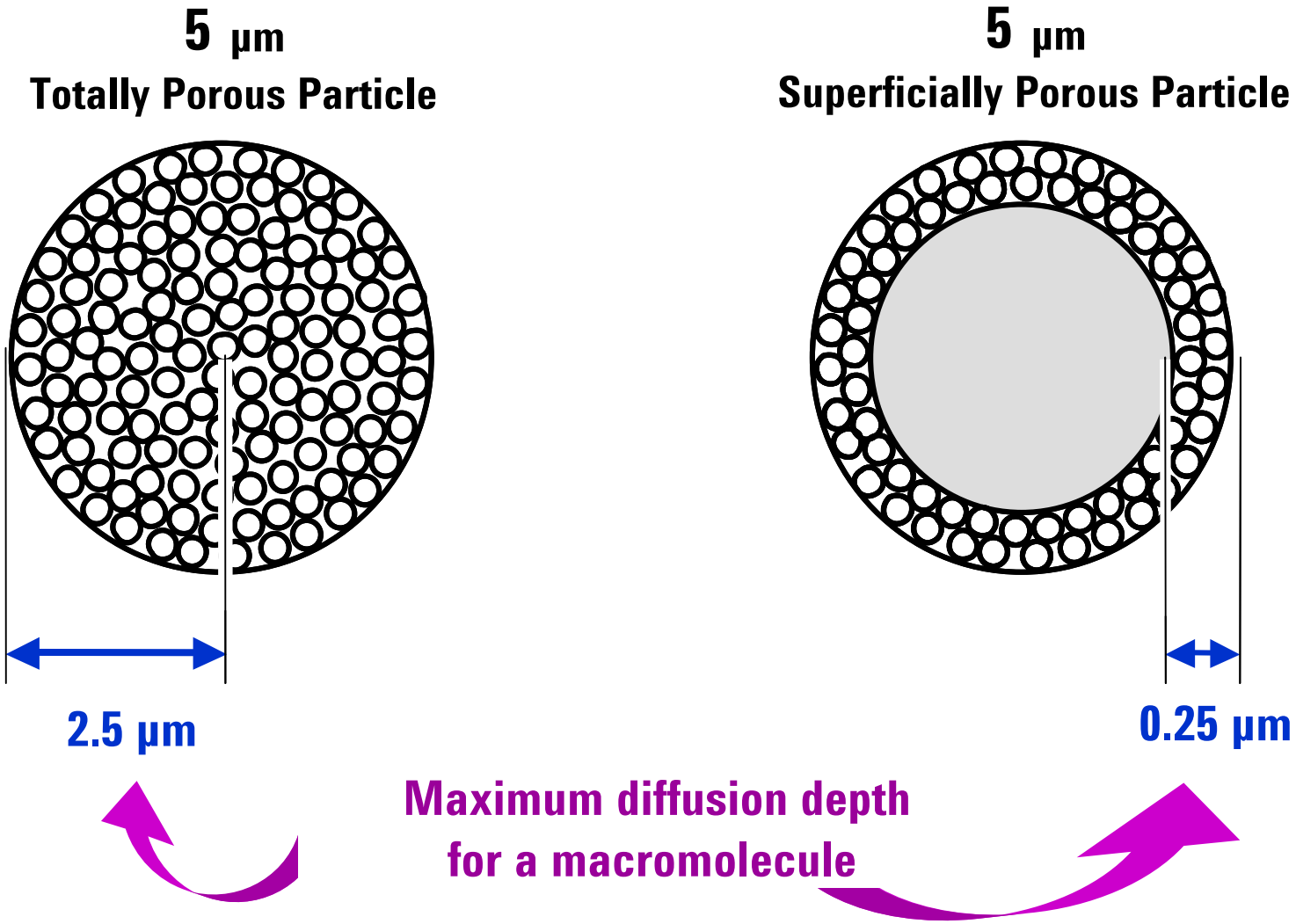
Decrease the diffusion time for macromolecules!

How?

- Increase the Diffusion Rate
  - **Elevate operating temperature**
  - **Decrease solvent viscosity**
- Decrease the Diffusion Distance
  - **Develop very small particles**
  - **Limit diffusion distance into a particle** ← **Zorbax Poroshell Approach**

Comparison of diffusion distance

Totally porous silica versus superficially porous silica



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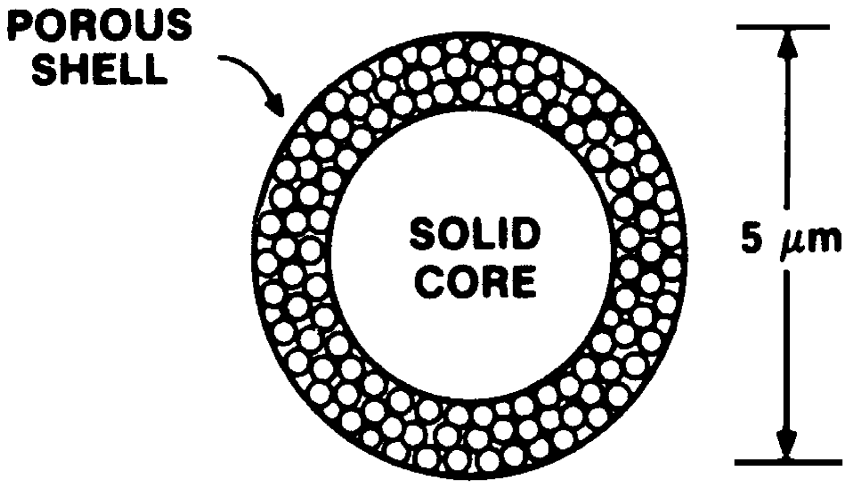
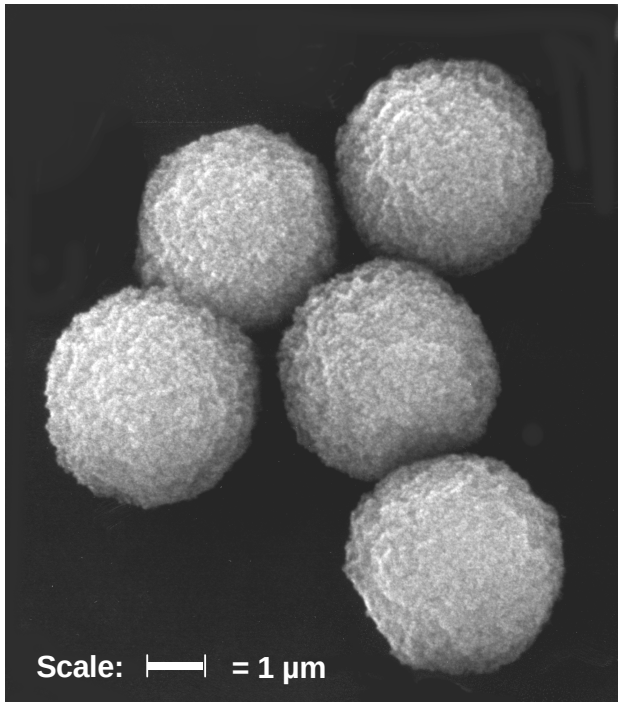
What are Zorbax Poroshell 300SB and 300Extend?

A solid silica core with a superficially porous surface of smaller silica sol particles derivatized with a sterically-protected bonded phase



Schematic of Zorbax Poroshell 300 Superficially-Porous Particle. A RP-HPLC phase such as C18, C8, C3 or Bidentate-C18 are bound to sol particles in the porous shell.



Scanning Electron Micrograph of Poroshell Particles

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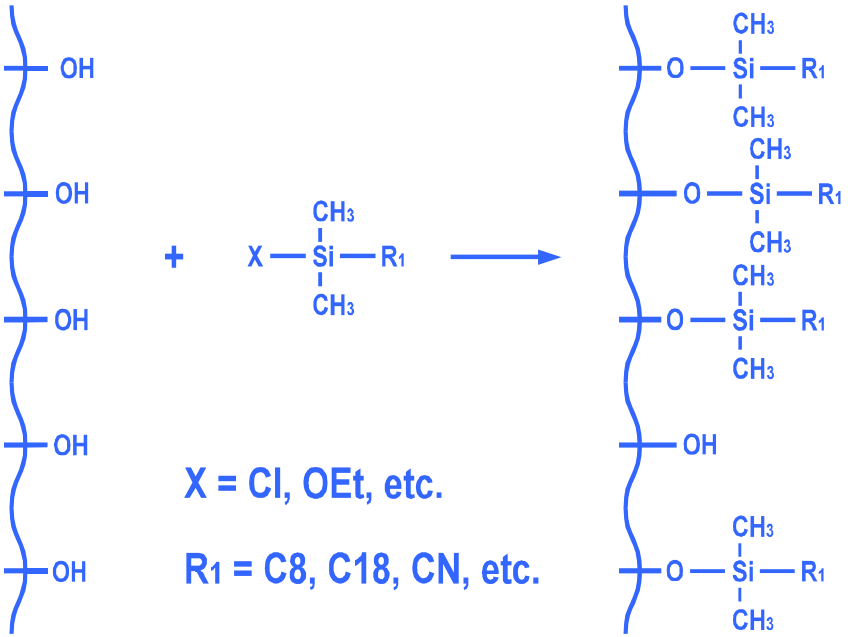
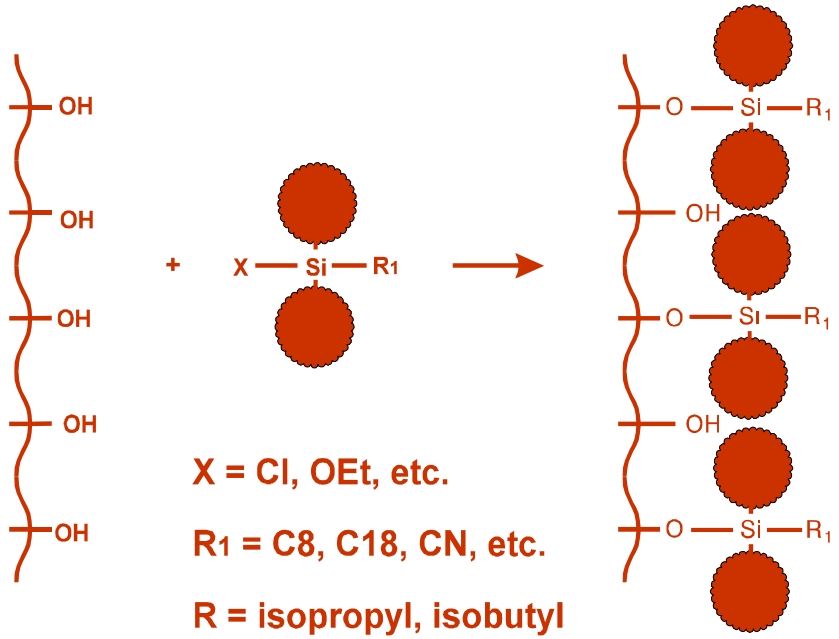
Poroshell is Part of the ZORBAX StableBond Family of Sterically-Protected Silica Phases

HYDROLYTICALLY UNSTABLE CONVENTIONAL, SILOXANE ATTACKED BY HYDRONIUM ION



HYDROLYTICALLY STABLE STERICALLY PROTECTED, SILOXANE RESISTS ATTACK BY HYDRONIUM ION



Advantage of Monolayer Bonding: Single Step, Reproducible Reaction

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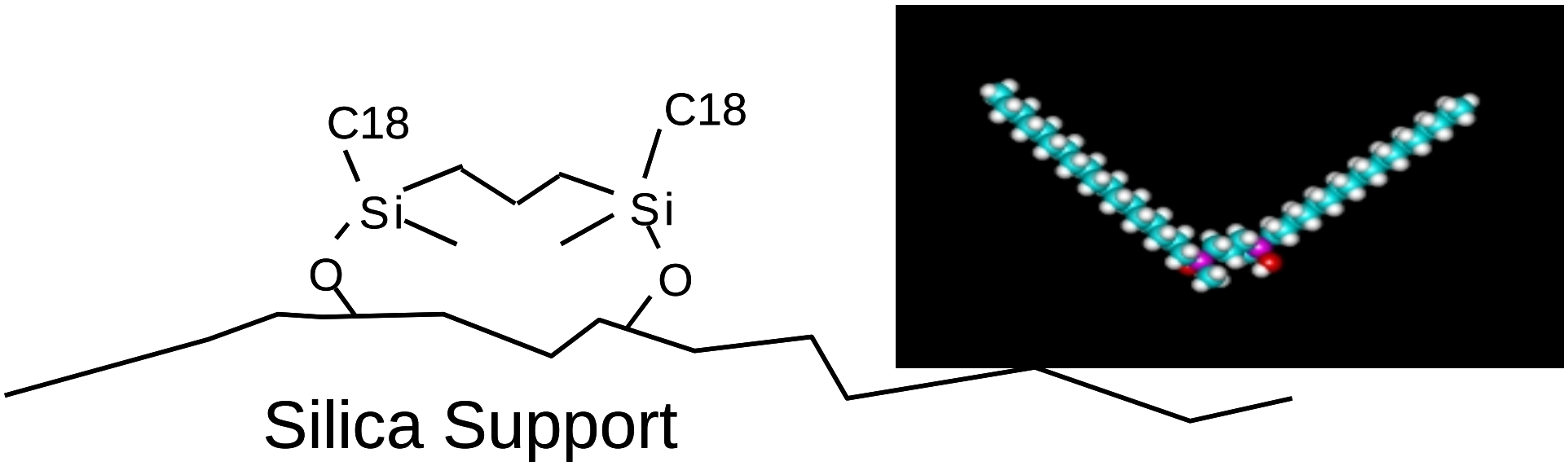
ZORBAX Extend-C18 - A Bidentate C18 Bonded Phase designed for Use at High pH

Now available as Zorbax Poroshell 300Extend-C18



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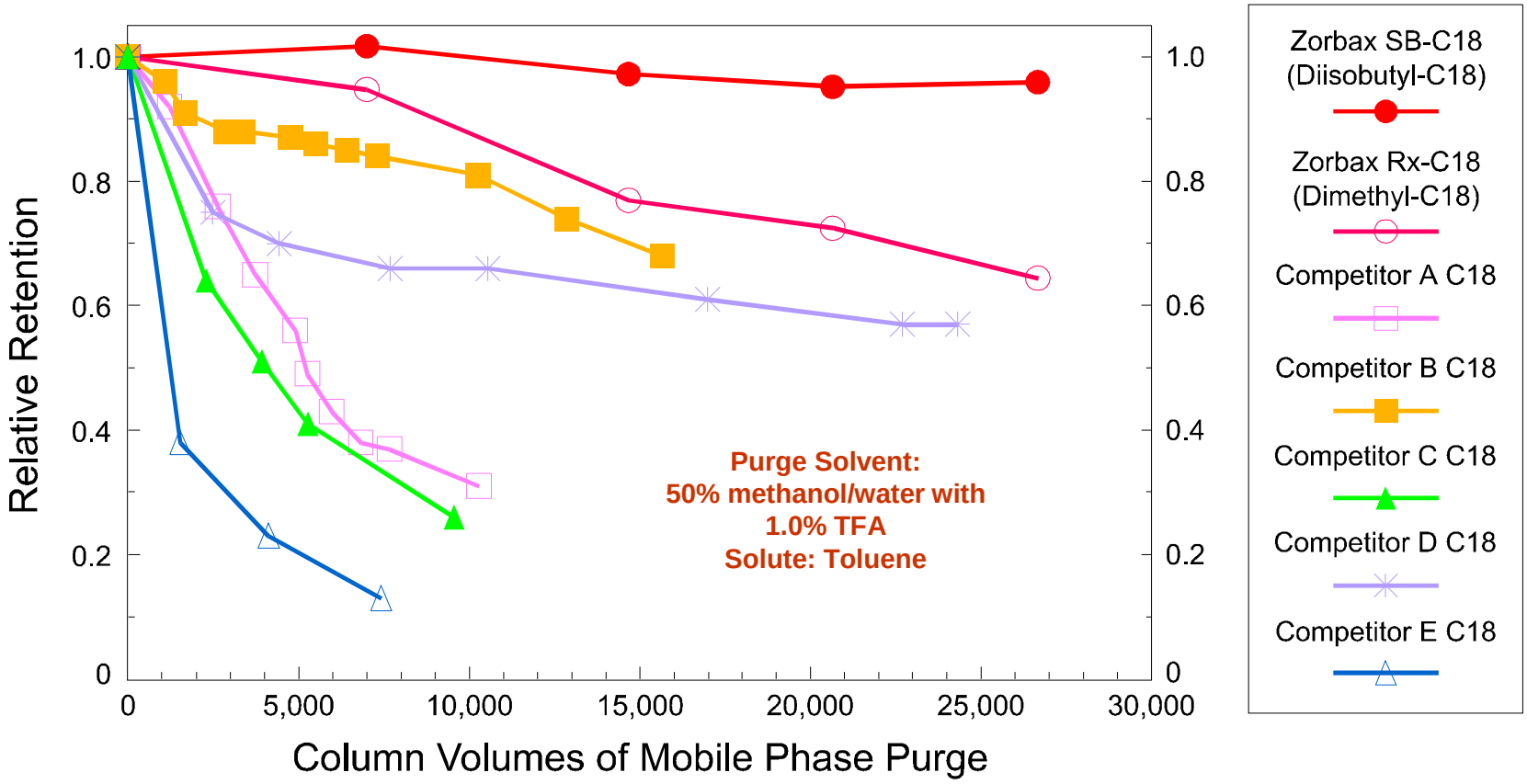
ZORBAX StableBond-C18 shows exceptional stability at low pH and high temperatures (pH 0.8, 90°C)



Kirkland, J.J. and J.W. Henderson, Journal of Chromatographic Science, 32 (1994) 473-480.

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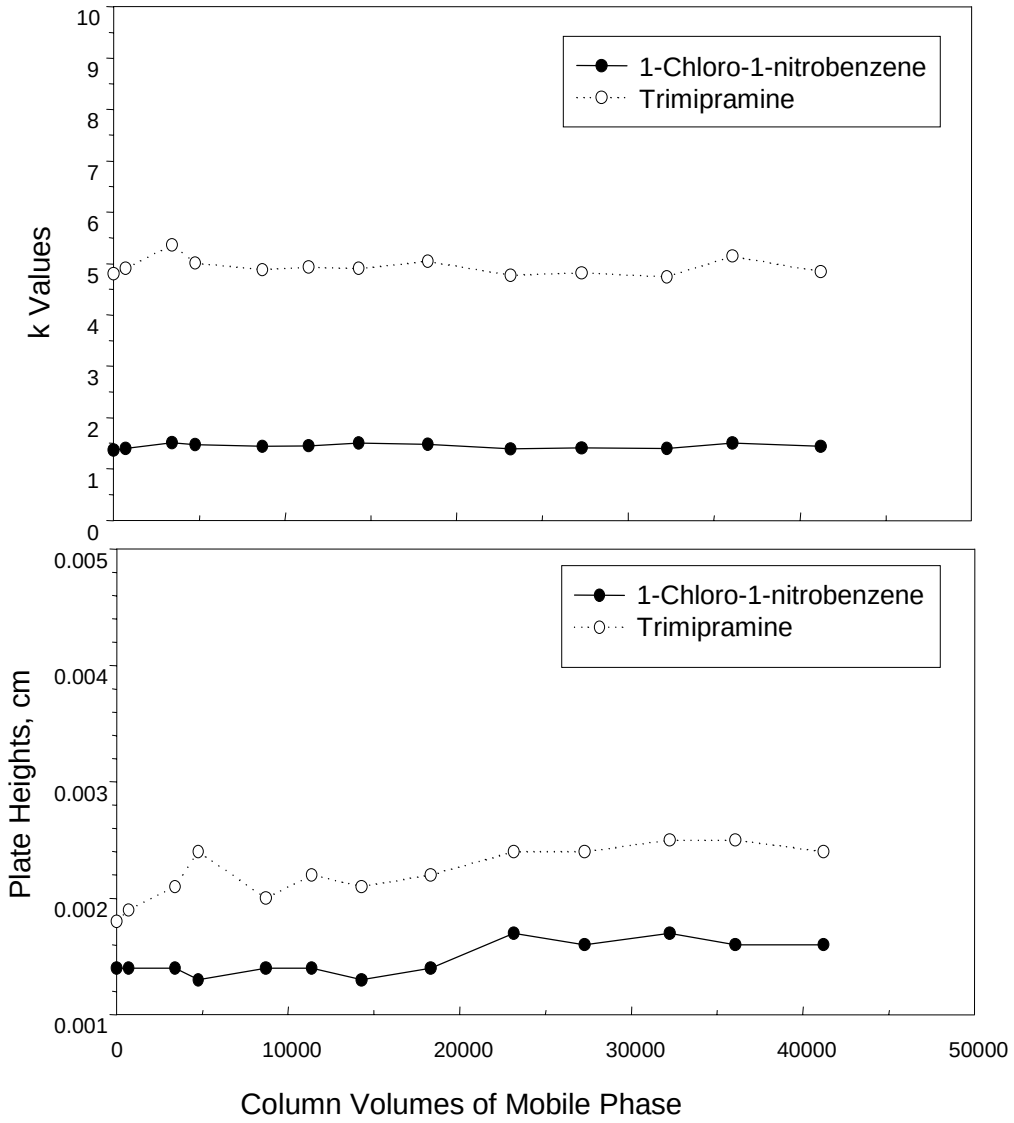
ZORBAX Extend-C18 is Very Stable at High pH

Aging of Extend-C18 column in NH_4OH at pH 10.5



Column: **ZORBAX Extend-C18**
4.6 x 150 mm, 5 µm

Mobile phase: 80% Methanol
20% 20 mM NH_4OH, pH 10.5

Flow Rate: 1.5 mL/min;

Aging at 24°C, tests at 40°C

Consistent retention and plate heights over time indicate stable column at high pH.

Long column lifetime achieved.

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Gradient Equation for Fast HPLC of Proteins

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

Because:

S is a constant for a separation system.

Assuming:

$\Delta\Phi$ is set for maximum resolution.

S and $\Delta\Phi$ drop out of this equation.

$$k^* = \frac{t_G F}{V_m} \sim \frac{V_G}{V_m}$$

$t_G \times F = V_G$ or gradient volume.

k* (relative retention) is dependent upon the ratio of V_G over V_m .

1. If one keeps the same ratio of V_G/V_m, then k* and the peak elution pattern will stay the same.
2. Flow rates of 1 to 3 mL/min on a 2.1 mm i.d. Poroshell column are the equivalent of 5 to 15 mL/min on a 4.6 mm I.d. column !
3. When F is large, t_G should be reduced proportionally; therefore, the very short run times !
4. In comparison of larger columns to the Poroshell configuration, t_G and F are also adjusted .

000994P2.PPT

How Do We Use Poroshell Columns?

Faster separations

- High flow rates for fast analysis with high resolution in comparison to totally porous silica
  - Polypeptides
  - Large proteins
  - Impurities
- High recovery of large proteins
- Elevated temperature

Change selectivity

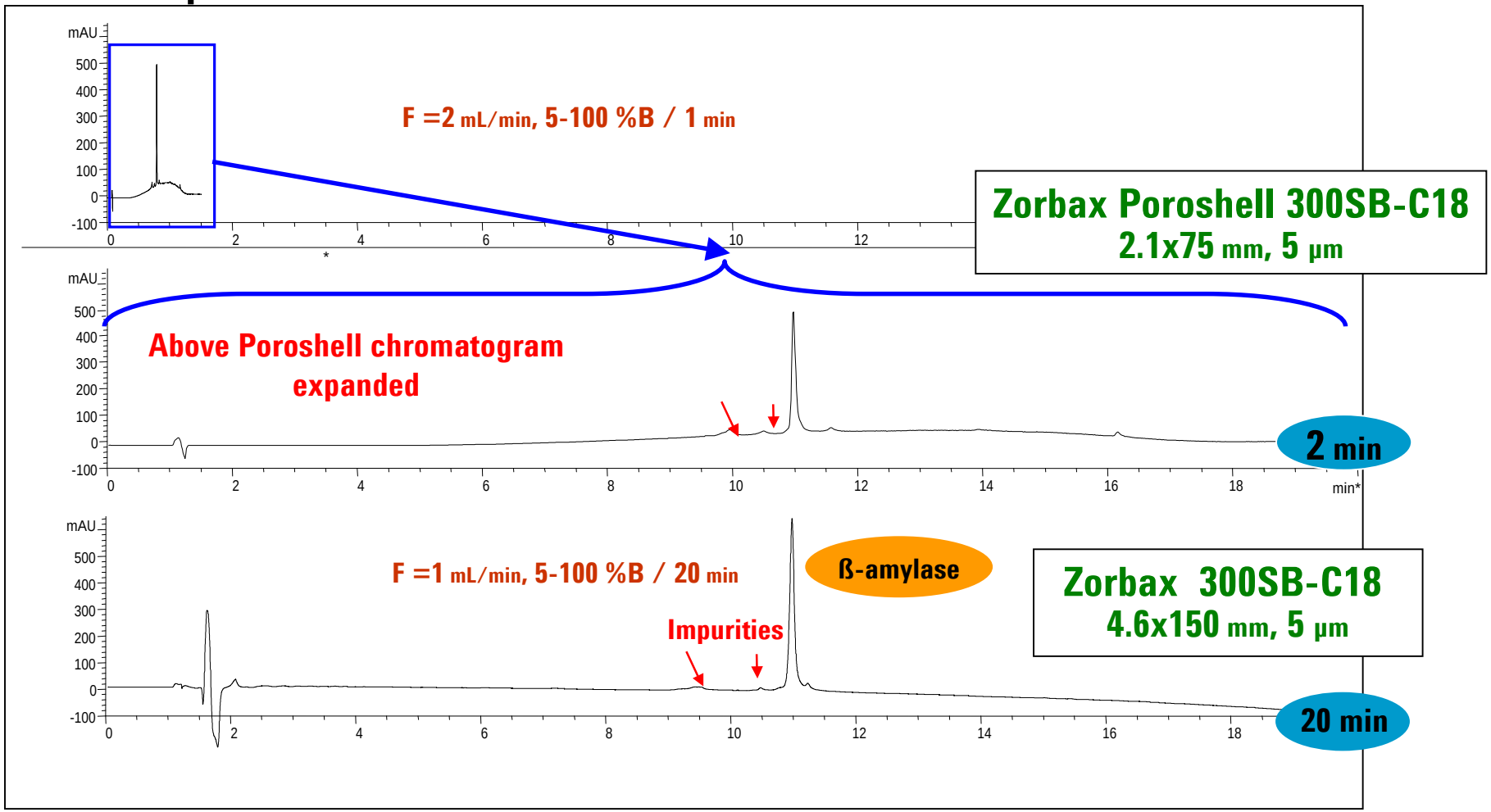
- Different bonded phases - Comparison to totally porous 300SB materials
- High resolution of proteins, heterogeneous proteins, and digests on different bonded phases

LC/MS

- Factors affecting LC/MS of proteins
- High resolution LC/MS of proteins
- Different bonded phases – comparisons to each other
- Peptide mapping at high and low pH

Ultra-Fast Analysis of High-Molecular Weight Protein and Impurities -- ß-Amylase (200KDal)

Comparison of Zorbax Poroshell and Conventional Porous Particle



- Poroshell technology facilitates ultra-fast HPLC analysis of proteins
- Note the similar separation of minor impurities in the 2 and 20 min separations.

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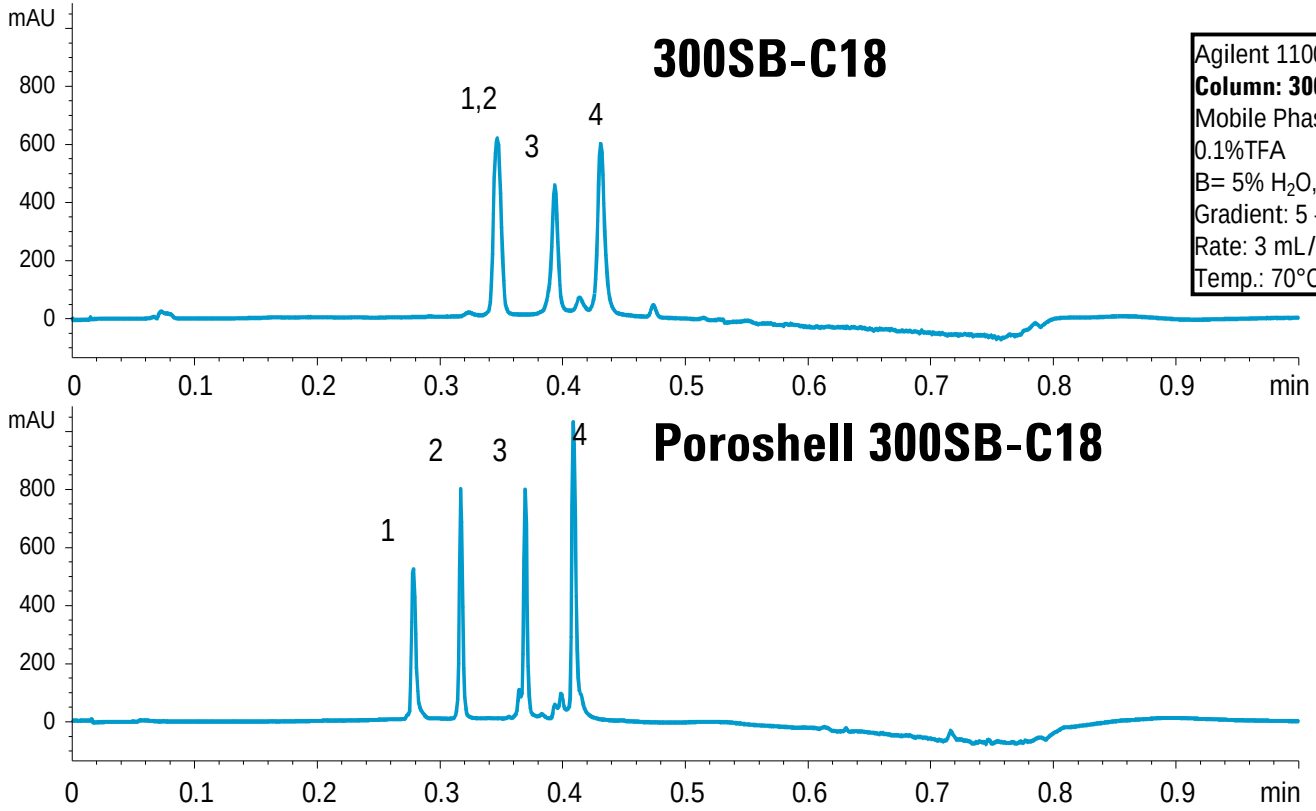
Poroshell Has Different Selectivity from Totally Porous 300SB



Agilent 1100 WPS with AutoBypass
Column: 300SB-C18, 2.1 x 75 mm, 5 µm
Mobile Phase: A= 95% H_2O, 5% ACN with 0.1%TFA
B= 5% H_2O, 95% ACN, with 0.07%TFA
Gradient: 5 –100%B in 0.67 min Flow Rate: 3 mL/min Piston Stroke: 20 µL
Temp.: 70°C Det.: UV 215 nm

- **Poroshell and 300SB-C18 can have different selectivity due to the different ratios of bonded phase on the surface.**

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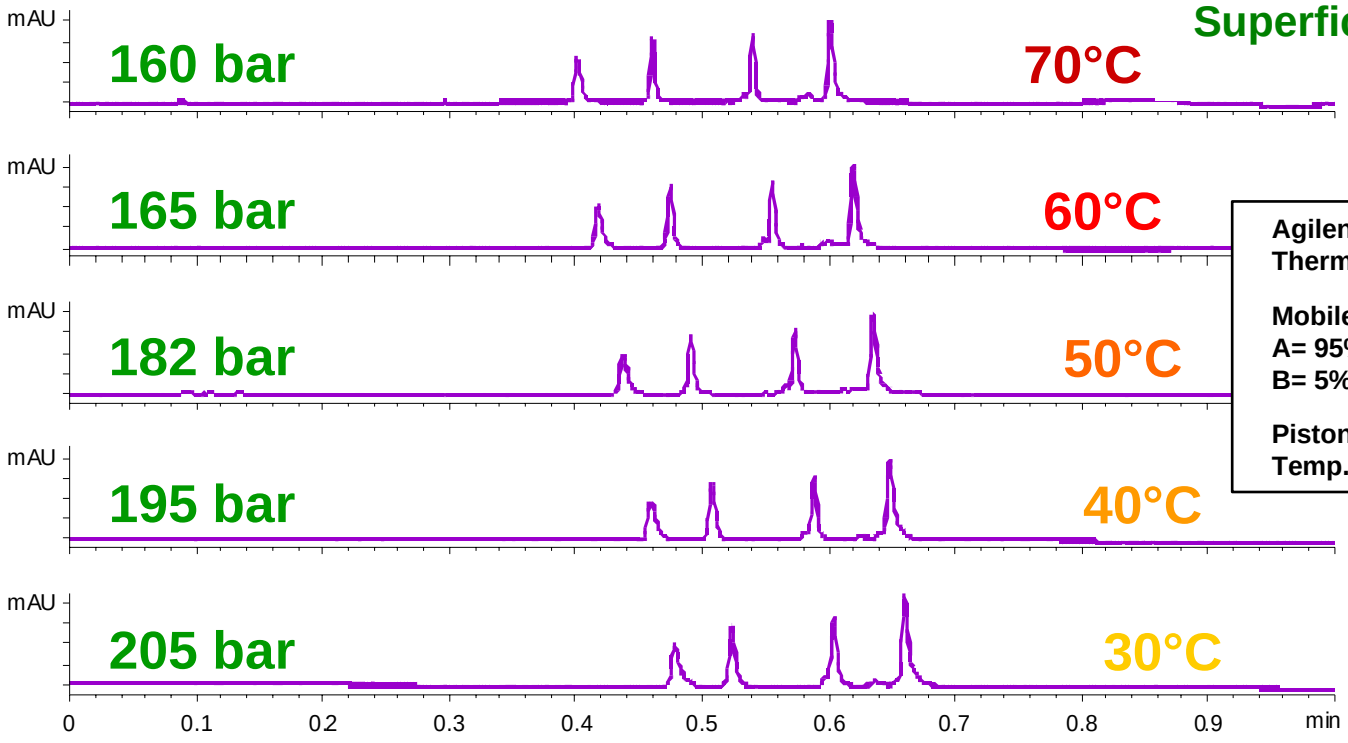
Temperature sharpens peaks and lowers back pressure in ultra-fast protein analysis

Sterically protected C18
Superficially Porous Particle
2.1 x 75mm, 5µm



Agilent 1100 WPS with AutoBypass Thermostatted Column Compartment

Mobile Phase:
A= 95% H_2O, 5% AcN with 0.1%TFA
B= 5% H2O, 95% AcN, with 0.07%TFA

Piston Stroke: 20µL
Temp.: 70°C, Det.: 215 nm

Instrument-Column Synergy in Optimizing Reversed Phase-HPLC Analysis of Proteins and Peptides, ISPPP 2000, Ricker, R.D., and B. E. Boyes, Agilent Technologies, Inc.

- Use of elevated temperature with temperature-resistant bonded phases, reduces viscosity and back pressure, sharpens peaks, increases sample solubility, and reduces retention times.

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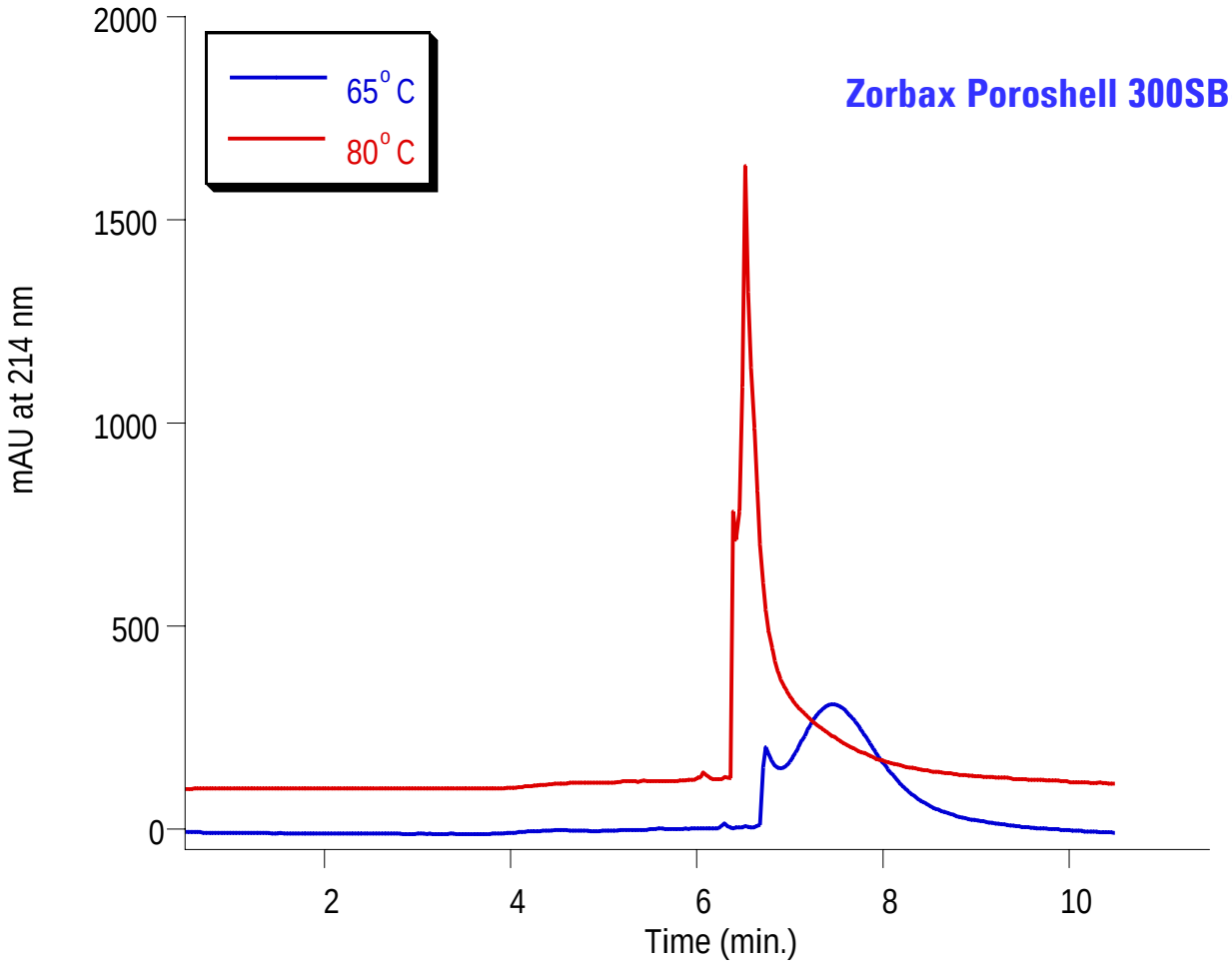
Improved Peak Shape of IgG at Elevated Temperature

Intact IgG (15 ug) at 0.5 ml/min



Zorbax Poroshell 300SB-C18, 5µ, 2.1 x 75 mm

Data courtesy of:
Genentech. Inc
Analytical Chemistry,
Baojen Shyong,
Galahad DePeralta, and
Victor Ling

- **Immunoglobulins have retention characteristics similar to very hydrophobic peptides – peaks are narrower at elevated temperatures and on shorter chain length columns**

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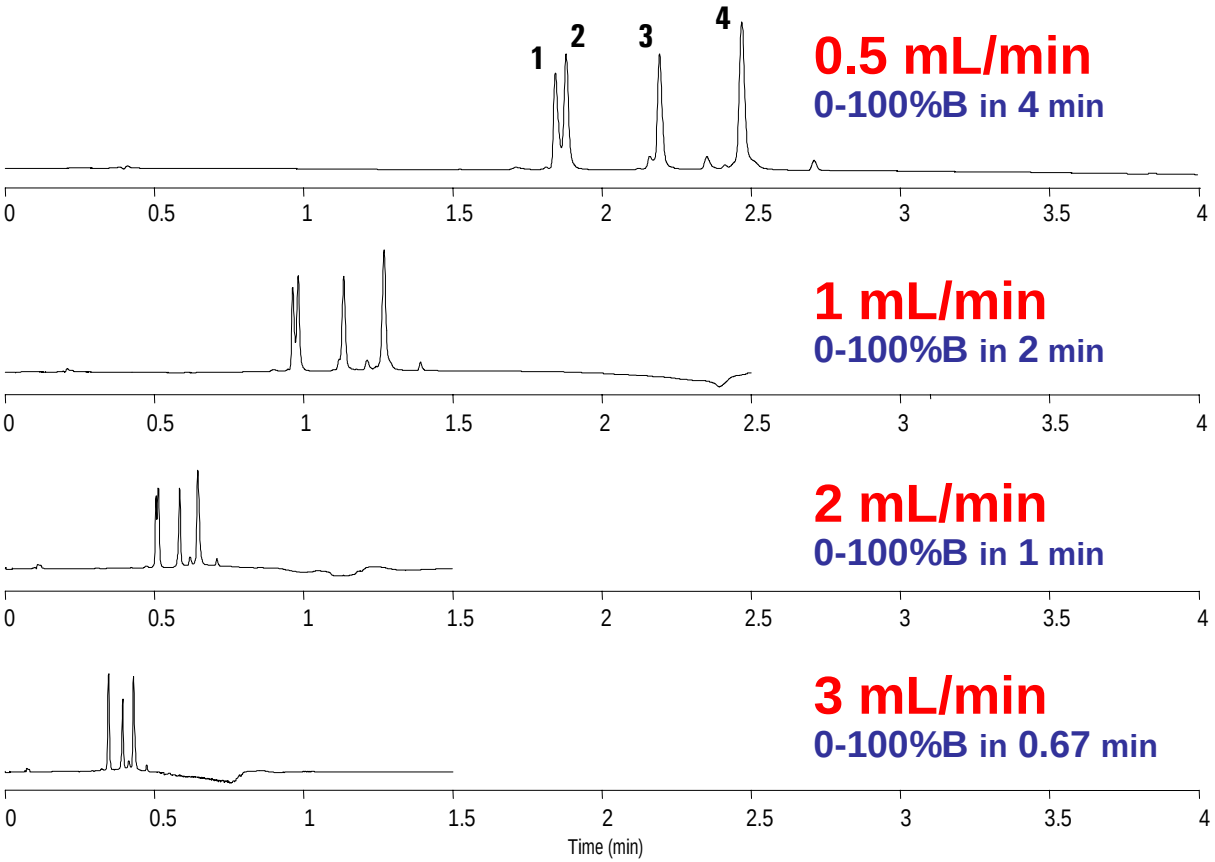
Effect of Increasing Flow Rate in Protein Analysis with use of Totally Porous Silica



Totally Porous
2.1x75mm, 5µm

1. Neurotensin
2. Rnase A
3. Lysozyme
4. Myoglobin

- **Efficiency and resolution are lost at high flow rates on a totally porous 300Å RP column**

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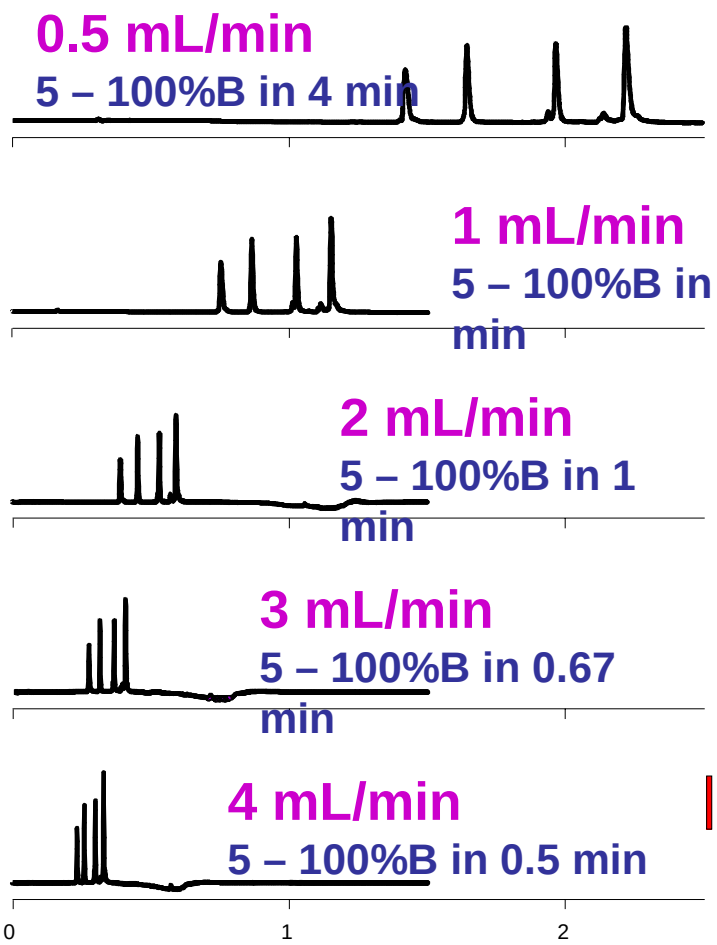
High Efficiency at High Flow Rates for Ultra-Fast Protein Analyses with Poroshell



Agilent 1100 DAD
Agilent 1100 WPS with ADVR

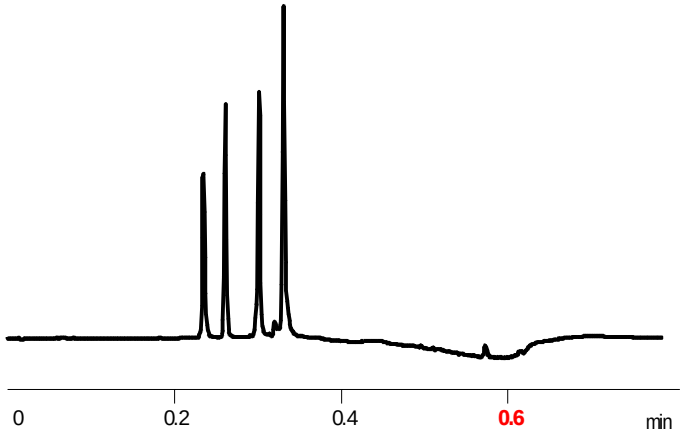
Column: **Poroshell 300SB-C18 2.1 x 75 mm, 5 mm**

Mobile Phase:
A: 95% H_2O, 5% ACN with 0.1% TFA
B: 5% H_2O, 5% ACN with 0.1% TFA

Temperature: 70°C

Detector: UV 215 nm

Sample:
1. Neurotensin
2. RNaseA
3. Lysozyme
4. Myoglobin



- **Resolution is maintained at high flow rates with Poroshell, allowing for efficient peaks and rapid analysis times.**

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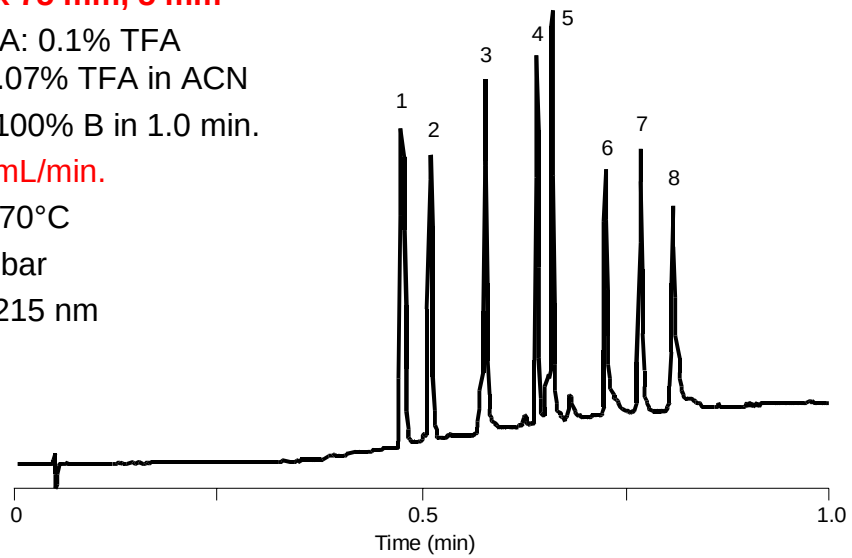
Fast, High Resolution Separation of Peptides and Proteins With Poroshell 300SB-C18 . . . In Seconds

Columns: Poroshell 300SB-C18
2.1 x 75 mm, 5 mm
Mobile Phase: A: 0.1% TFA
B: 0.07% TFA in ACN
Gradient: 5 – 100% B in 1.0 min.
Flow Rate: 3.0 mL/min.
Temperature: 70°C
Pressure: 250 bar
Detection: UV 215 nm



Sample:
1. Angiotensin II
2. Neurotensin
3. RNAse
4. Insulin
5. Lysozyme
6. Myoglobin
7.Carbonic Anhydrase
8.Ovalbumin

- **Only Poroshell can provide high efficiency at higher flow rates for extremely rapid separations of proteins and peptides.**
- **This is due to more rapid mass transfer of the superficially porous particle**

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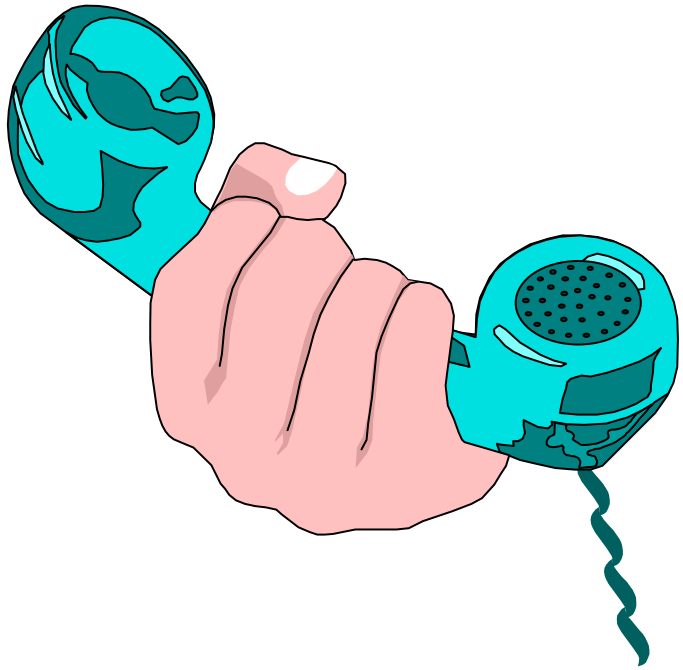
Break Number 1

For Questions and Answers
Press *1 on Your Phone to
Ask a Question



How Do We Use Poroshell Columns?

Faster separations

High flow rates for fast analysis with high resolution in comparison to totally porous silica

- Polypeptides
- Large proteins
- Impurities

High recovery of large proteins

Elevated temperature

Change selectivity

Different bonded phases - Comparison to totally porous 300SB materials

High resolution of proteins, heterogeneous proteins, and digests on different bonded phases

LC/MS

Factors affecting LC/MS of proteins

High resolution LC/MS of proteins

Different bonded phases – comparisons to each other

Peptide mapping at high and low pH

Separation of Seven Monoclonal Antibodies on Zorbax Poroshell 300SB-C8

Column: Zorbax Poroshell 300SB-C8, 2.1x75mm, 5u

Flow: 1.0 ml/min

Detection: 210nm

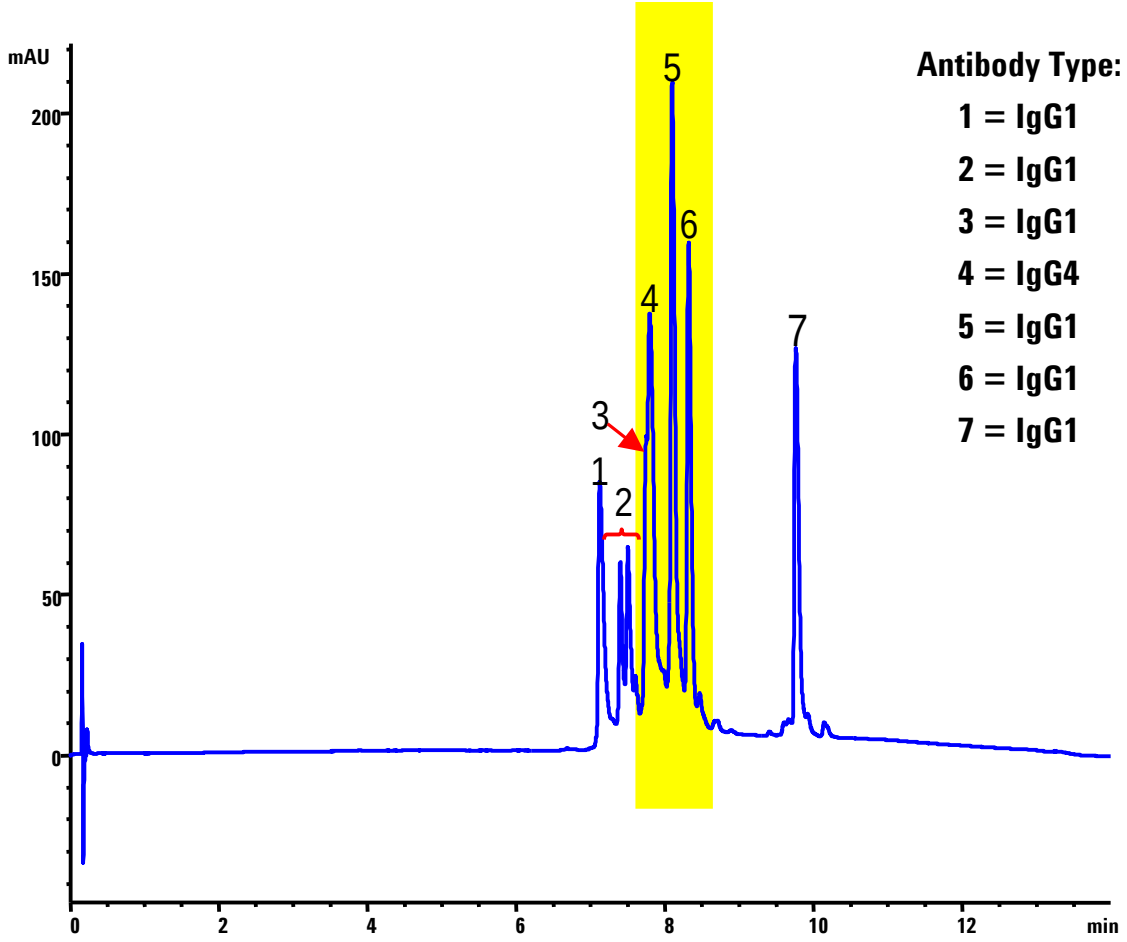
Mobile phase A: H2O-ACN (90:10) + 3 ml/L of MW 300 PEG

Mobile phase B: H2O-ACN (10:90) +3 ml/L of MW 300 PEG

Gradient: 0 min, 19% B; 12 min 41% B;
12.1 min, 19% B; 14 min, 19% B

Temperature: 70° C

Data courtesy of:
Novartis Pharma, Biotechnology, Basel
Dr. Kurt Forrer and Patrik Roethlisberger



- **Zorbax Poroshell 30SB-C8 shows different selectivity for several pairs of monoclonal antibodies.**

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Separation of Seven Monoclonal Antibodies on Zorbax Poroshell 300SB-C3

Column: Zorbax Poroshell 300SB-C3, 2.1x75mm, 5u

Flow: 1.0 ml/min

Detection: 210nm

Mobile phase A: H20-ACN (90:10) + 3ml/L of MW 300 PEG

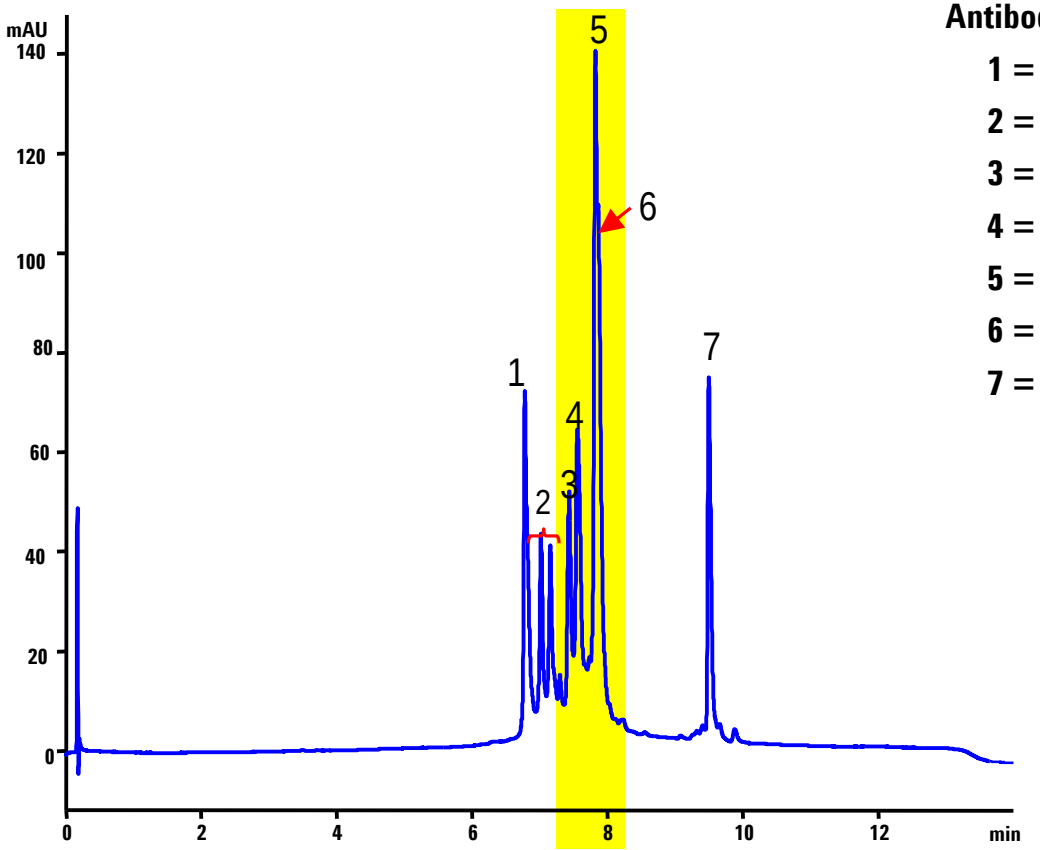
Mobile phase B: H2O-ACN (10:90) + 3ml/L of MW 300 PEG

Gradient: 0 min, 19% B; 12 min 41% B;

12.1 min, 19% B; 14 min, 19% B

Temperature: 70° C

Data courtesy of:
Novartis Pharma, Biotechnology, Basel
Dr. Kurt Forrer and Patrik Roethlisberger



Antibody Type:

1 = IgG1

2 = IgG1

3 = IgG1

4 = IgG4

5 = IgG1

6 = IgG1

7 = IgG1

- **Zorbax Poroshell 300SB-C3 shows different selectivity for several pairs of monoclonal antibodies.**

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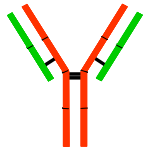
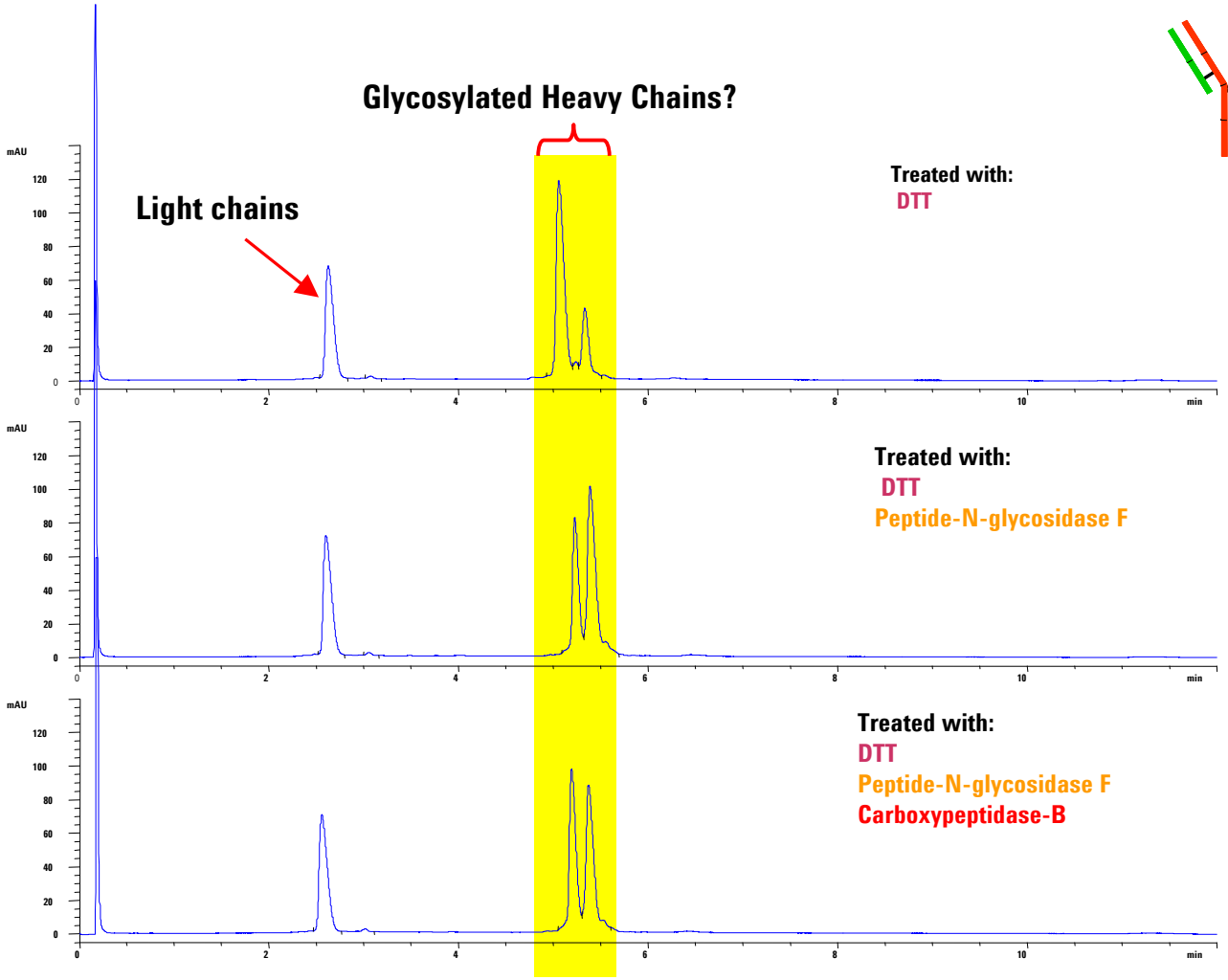
Monoclonal IgG1 Chains on ZORBAX Poroshell 300SB-C8





Column: Zorbax Poroshell 300SB-C8, 2.1x75mm, 5u

Flow: 1.0 ml/min

Detection: 210nm

Mobile phase A: H2O-ACN (90:10) +3 ml/L of MW 300 PEG

Mobile phase B: H2O-ACN (10:90) + 3 ml/L of MW 300 PEG

Gradient: 0 min, 25% B; 10 min 40% B;
10.1 min, 25% B; 12 min, 25% B

Temperature: 70° C

Data courtesy of:
Novartis Pharma, Biotechnology, Basel
Dr. Kurt Forrer and Patrik Roethlisberger

It is known that the heavy chain is glycosylated. What is not yet known for certain is whether one or both peaks above are glycosylated initially and what the difference is between the two peaks after the glycosylation is removed. Current thought is that they are likely to be conformers but there is no real proof of this yet.

Monoclonal Antibody Chains on ZORBAX Poroshell 300SB-C8

Column: Zorbax Poroshell 300SB-C8, 2.1x75mm, 5um

Flow: 1.0 ml/min

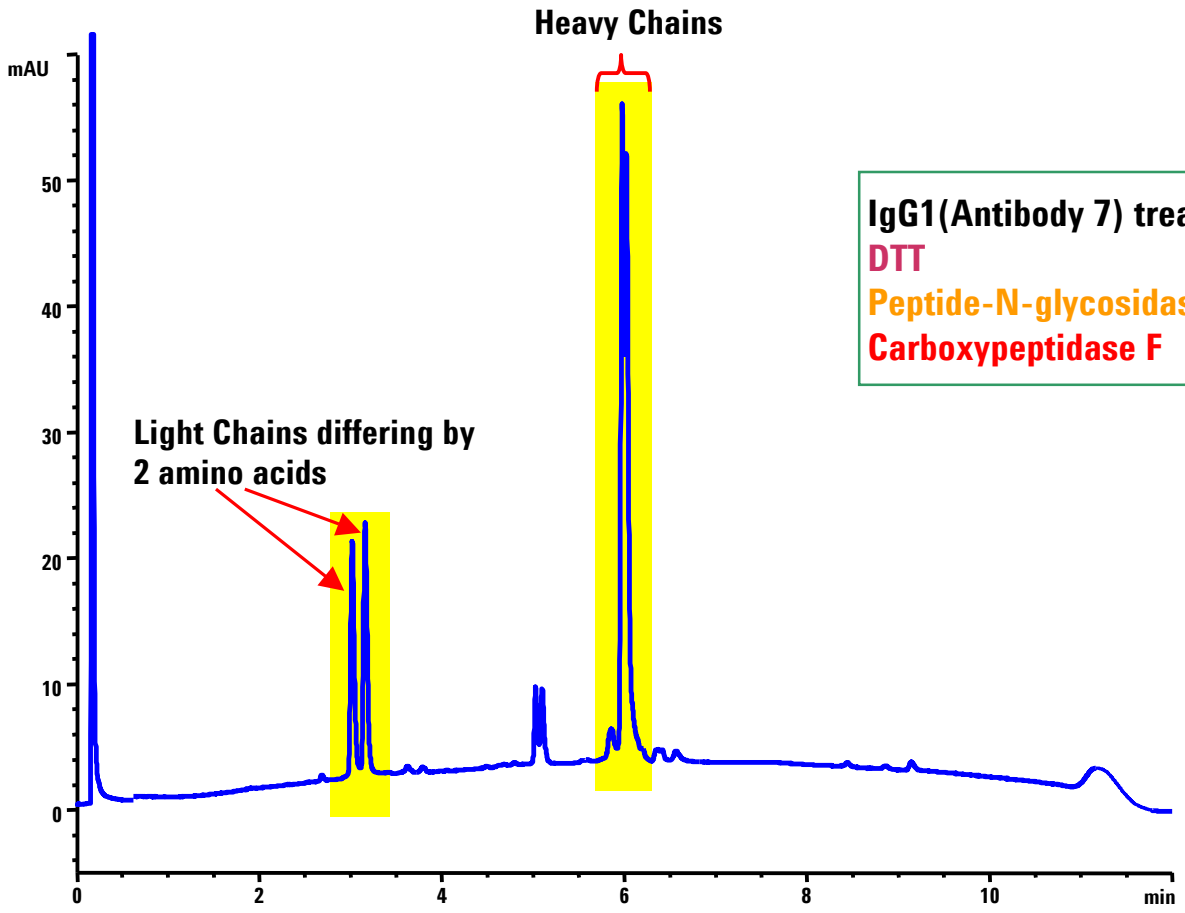
Detection: 210nm

Mobile phase A: H2O-ACN (90:10) + 3 ml/L of MW 300 PEG

Mobile phase B: H2O-ACN (10:90) + 3 ml/L of MW 300 PEG

Gradient: 0 min, 20% B; 10 min 50% B; 10.1 min, 20% B; 14 min, 20% B

Temperature: 70° C



IgG1(Antibody 7) treated with:
DTT
Peptide-N-glycosidase F
Carboxypeptidase F

Data courtesy of:
Novartis Pharma, Biotechnology, Basel
Dr. Kurt Forrer and Patrik Roethlisberger

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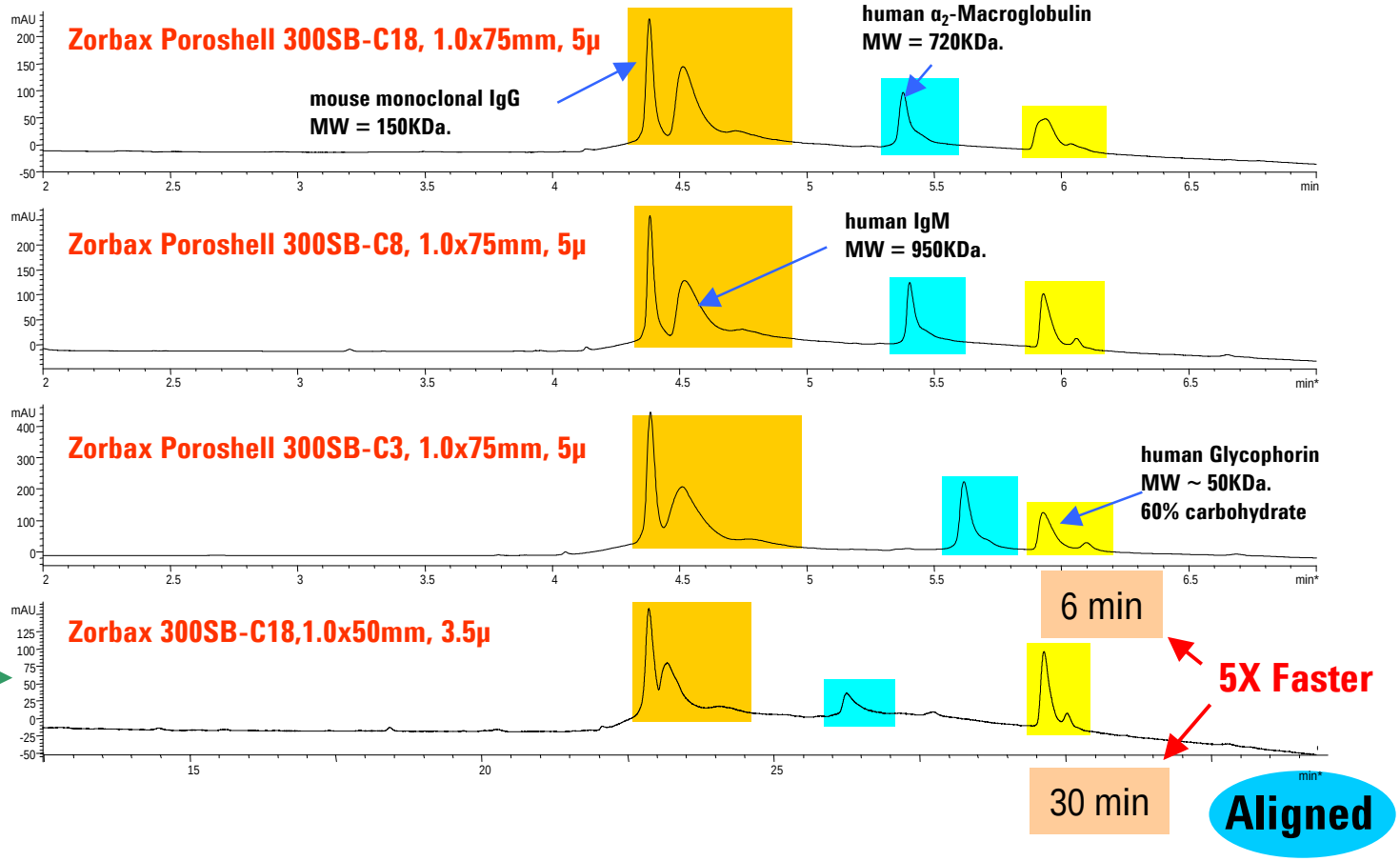
Separation of Large and Glycosylated Proteins on ZORBAX Poroshell 300SB-C18, C8, and C3

Column: Zorbax Poroshell 300SB-C18, C8 or C3, 1.0 x 75mm, 5µ
Flow: 0.454ml/min
Mobile phase A: 0.1% TFA in H_2O
Mobile phase B: 0.07% TFA in ACN
Gradient: 5% B —> 100% B in 10 min
Detector: DAD, 212nm, 1.7 µl flow cell
<0.01min peak width
Temperature: 70° C
Bypass mode, Binary Pump, no mixer

Column: Zorbax 300SB-C18, 1.0x50mm, 3.5µ
Flow: 0.071ml/min
Mobile phase A: 0.1% TFA in H_2O
Mobile phase B: 0.07% TFA in ACN
Gradient: 5%B —> 100%B in 50 min
Detector: DAD, 212nm, 1.7µl flow cell
<0.01 min peak width
Temperature: 70° C
Bypass mode, Binary Pump, no mixer



- **Peak widths, resolution, and analysis times are superior on Poroshell columns**

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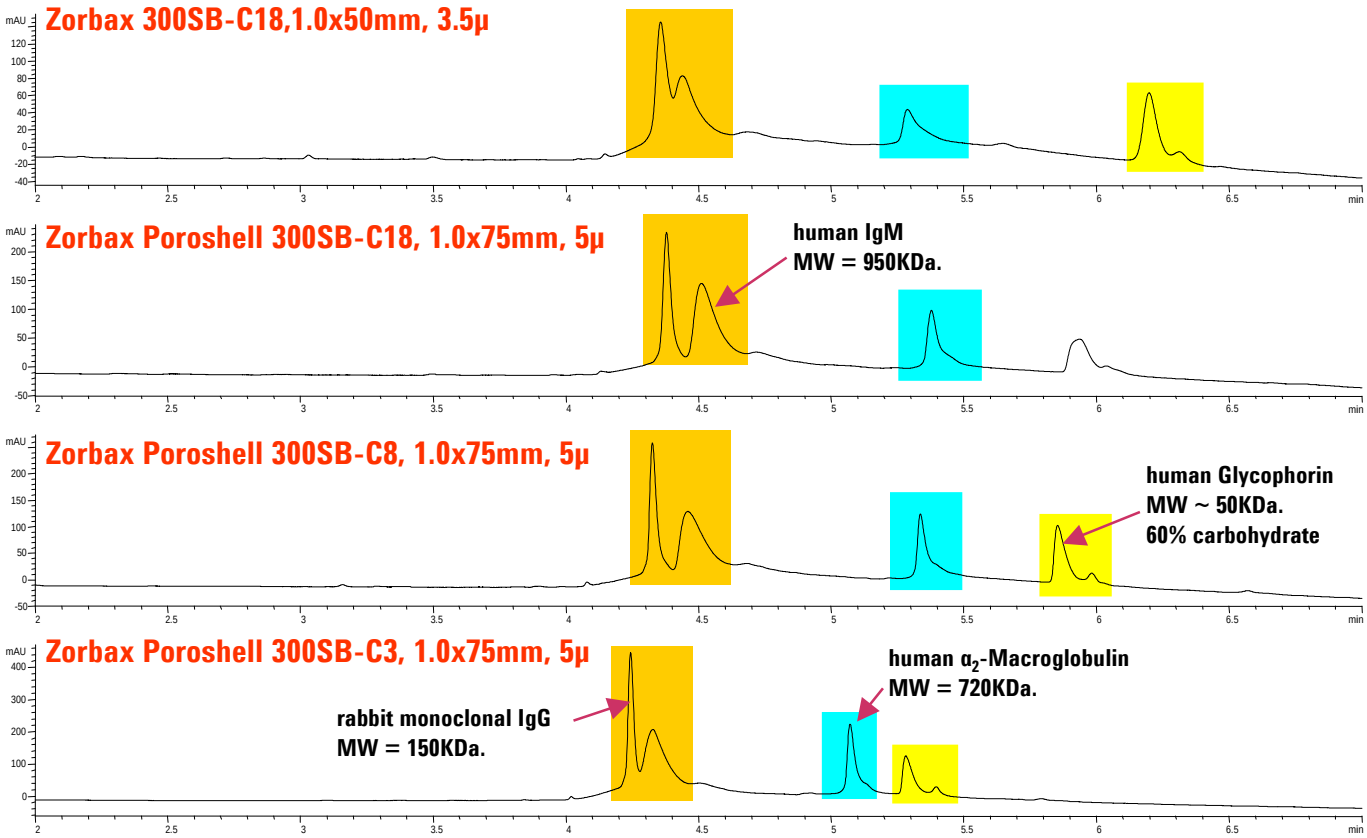
Separation of Large and Glycosylated Proteins on ZORBAX Poroshell 300SB-C18, C8, and C3

Column: Zorbax Poroshell 300SB-C18, C8 or C3, 1.0x75mm, 5µ and Zorbax 300SB-C18, 1.0x50mm, 3.5µ
Flow: 0.454ml/min
Mobile phase A: 0.1% TFA in H_2O
Mobile phase B: 0.07% TFA in ACN
Gradient: 5% B —> 100% B in 10 min
Detector: DAD, 212nm, 1.7 µl flow cell <0.01min peak width
Temperature: 70° C
Bypass mode, Binary Pump, no mixer



- **Zorbax Poroshell C8 and C3 offer advantages over Zorbax 300SB-C18 for large and heavily glycosylated proteins**
- **Zorbax Poroshell C18 offers advantages for all but the most heavily glycosylated proteins**

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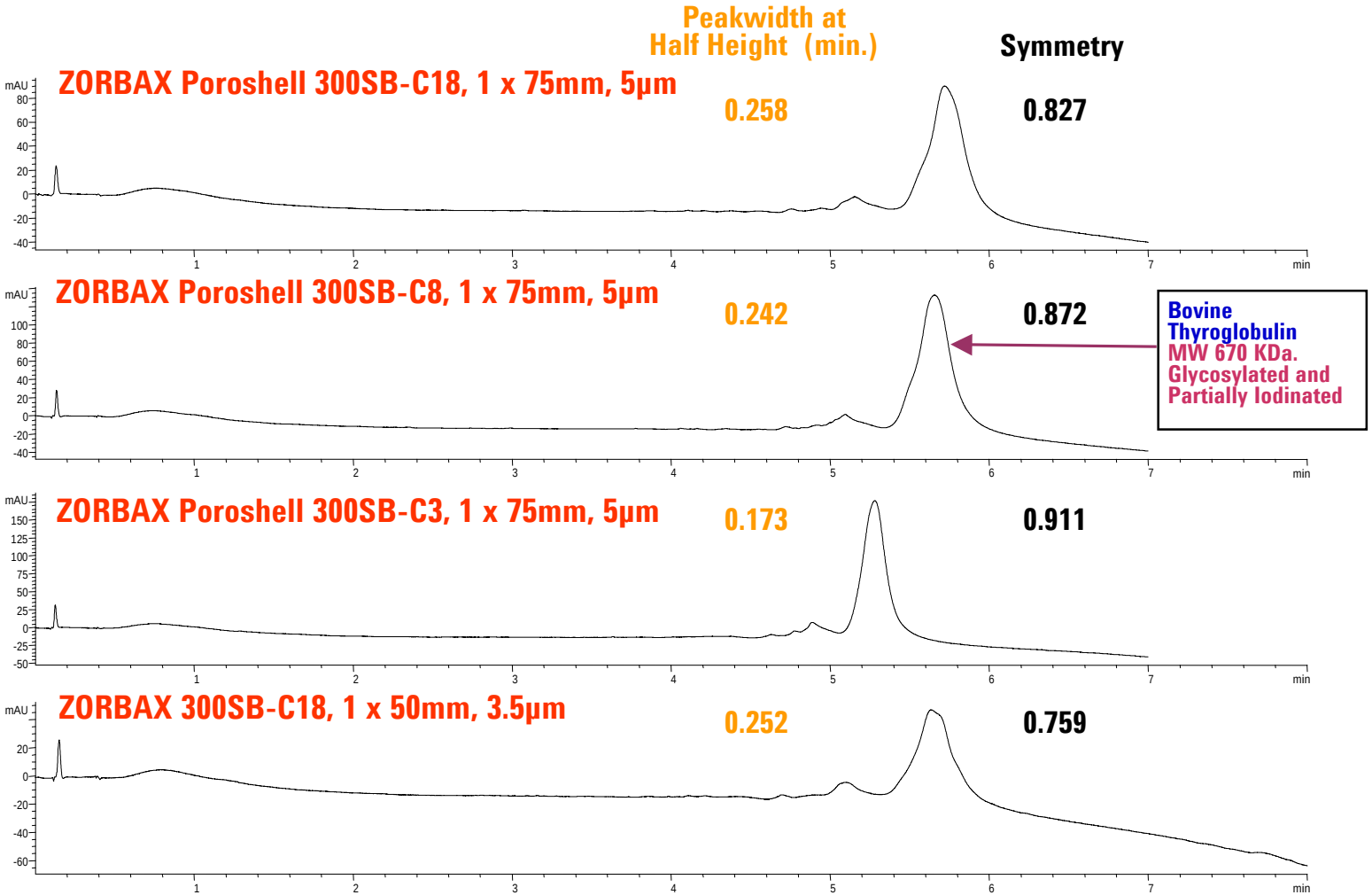
Chromatography of Thyroglobulin - a Doubly Heterogeneous Protein – on ZORBAX Poroshell C3, C8, and C18

Column: as shown Flow: 0.454mL/min Mobile phase A: 0.1% TFA in H_2O Mobile phase B: 0.07% TFA in ACN Gradient: 5% B —> 100% B in 10 min Detector: DAD, 212nm, 1.7 µl flow cell <0.01min peak width Temperature: 70° C Bypass mode, Binary Pump, no mixer



- **Best peak width and symmetry on Zorbax Poroshell 300SB-C3**

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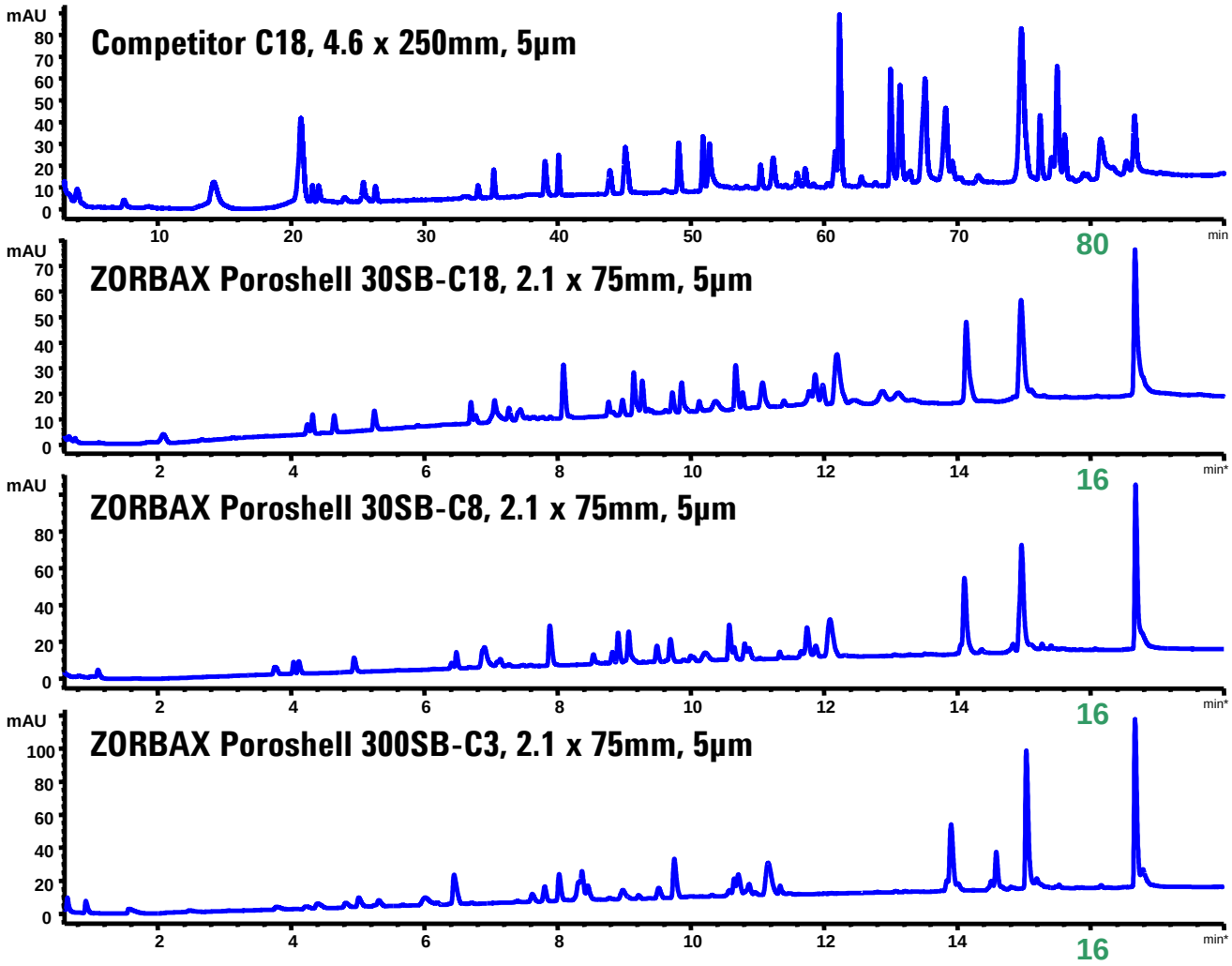
High Speed HPLC Peptide Maps of a Monoclonal Antibody on Several ZORBAX Poroshell Phases



Conditions: Mobile phase A = 0.1% TFA in water Mobile phase B = 0.1% TFA in ACN;
Gradient: 1min, 0% B; 10min, 0% B; 110min, 50% b; 115 min, 70% B;120 min, 70% B; 125min, 0% B; 135 min, 0% B
Temperature: ambient;
Detection: VWD, 210nm; Injection: 50 µl Lys-C digest of Human Monoclonal Antibody;
Flow: 0.3 ml/min;

Conditions:
Mobile phase A = 0.1% TFA in water
Mobile phase B = 0.1% TFA in ACN;
Gradient: 0 min, 0% B; 20 min, 50% B; 20.5 min, 100% B; 21.5 min, 100% B
Temperature: 70°C;
Detection: VWD, 210nm;
Injection: 10 µl Lys-C digest of Human Monoclonal Antibody;
Flow: 1.0 ml/min

Aligned

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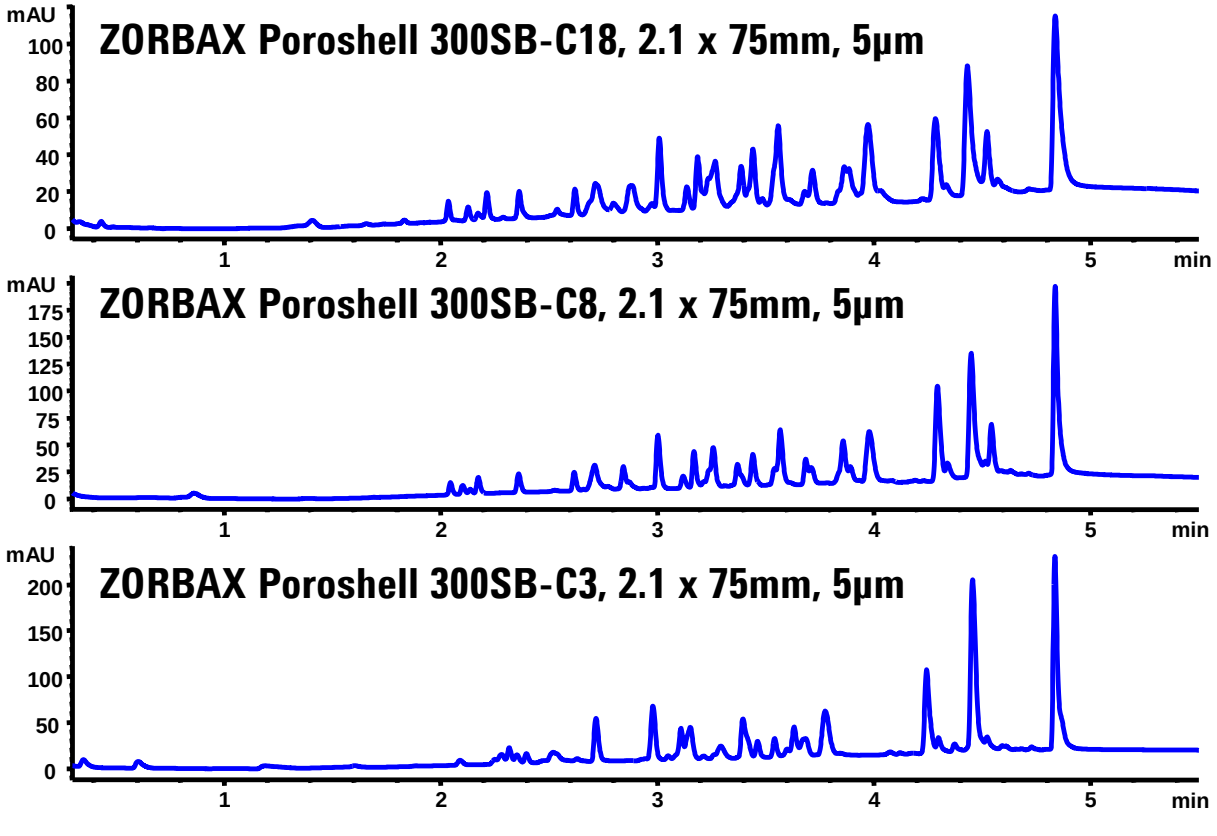
Ultra High Speed HPLC Peptide Maps of a Monoclonal Antibody on Several ZORBAX Poroshell Phases



Conditions:
Mobile phase A = 0.1% TFA in water
Mobile phase B = 0.1% TFA in ACN;
Gradient: 0 min, 0% B; 5.5 min, 55% B; 5.6 min, 55% B; 7.0 min, 0% B
Temperature: 70°C;
Detection: VWD, 210nm;
Injection: 10 µl Lys-C digest of Human Monoclonal Antibody;
Flow: 1.0 ml/min;

- **ZORBAX Poroshell technology facilitates *ultra*-fast HPLC analysis of peptides**

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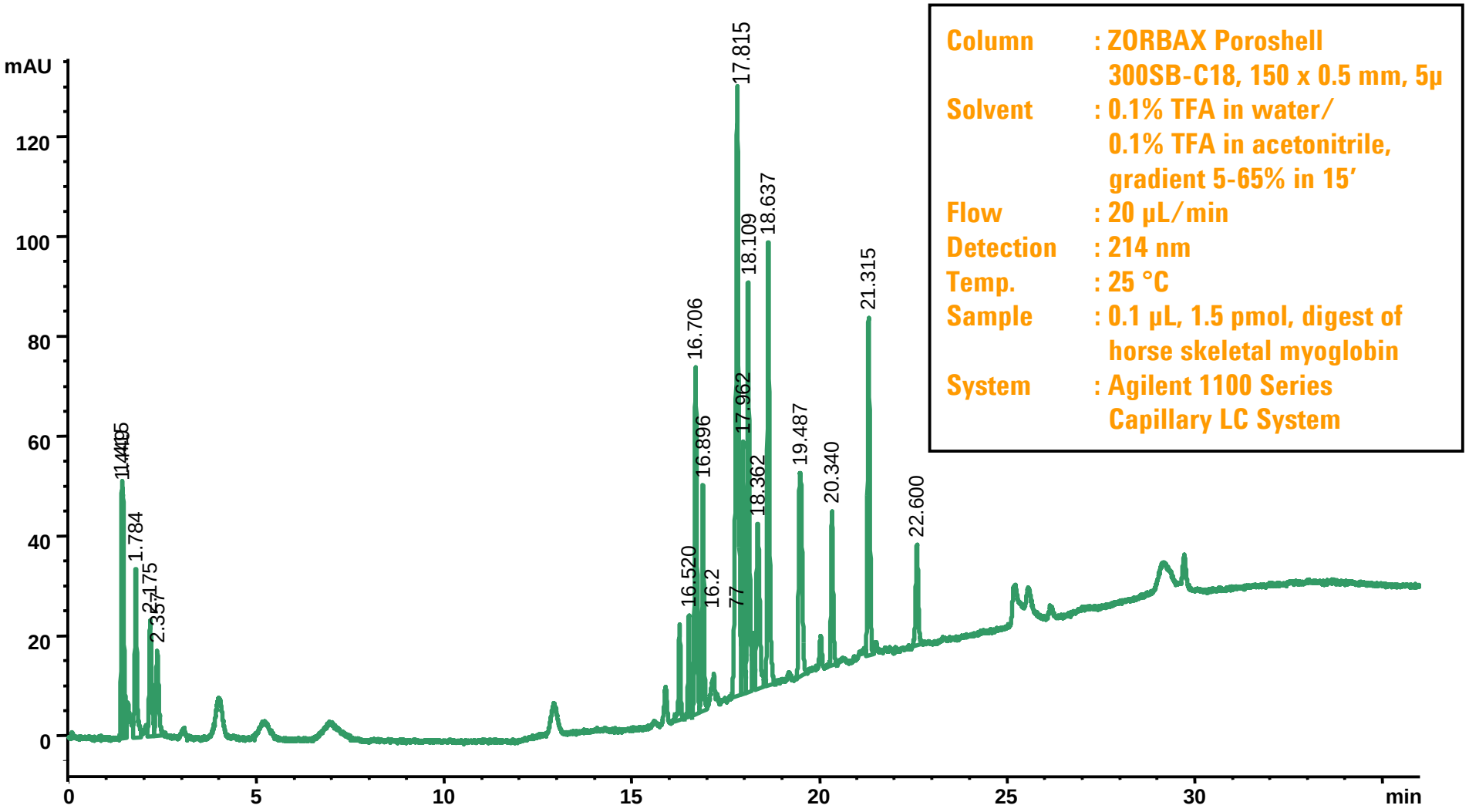
Peaks detected in peptide maps of a Lys-C digest of a monoclonal antibody

Column	# of Peaks recognized (120 min. run)	# of Peaks recognized (20.5 min. runs)	# of Peaks recognized (5.6 min. runs)
Competitor V C18	57		
Poroshell-C18		55	46
Poroshell-C8		58	48
Poroshell-C3		54	47

Separation of a Tryptic Digest on Poroshell Capillary HPLC Column



Column	: ZORBAX Poroshell 300SB-C18, 150 x 0.5 mm, 5µ
Solvent	: 0.1% TFA in water/ 0.1% TFA in acetonitrile, gradient 5-65% in 15’
Flow	: 20 µL/min
Detection	: 214 nm
Temp.	: 25 °C
Sample	: 0.1 µL, 1.5 pmol, digest of horse skeletal myoglobin
System	: Agilent 1100 Series Capillary LC System

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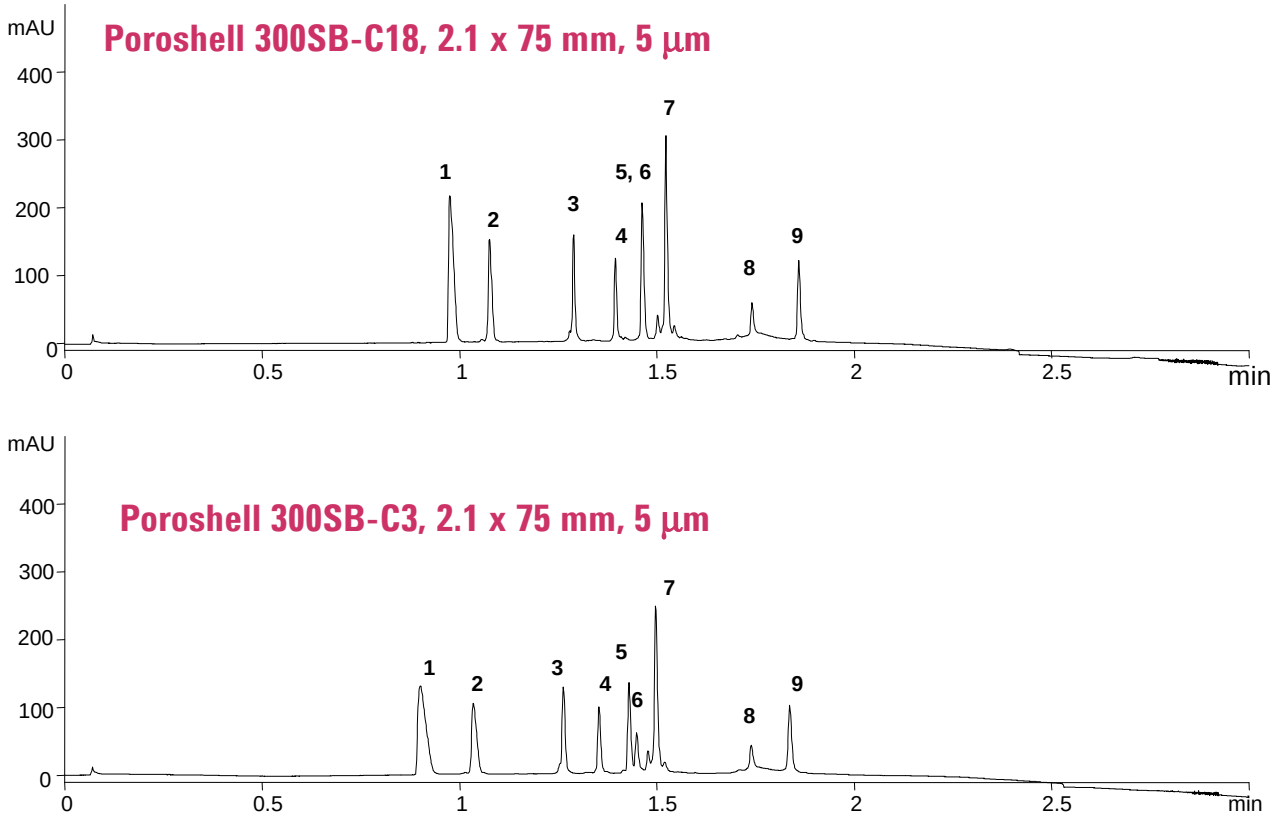
More Poroshell Bonded Phases Provide Selectivity Options to Enhance Resolution

Conditions: columns – as listed Mobile Phase: A 0.1% TFA/H_2O, B 0.07% TFA/MeCN; Gradient: 5-100% B in 3.0 min Temperature: 70 ^{0}C; Flow rate: 0.5 ml/min; Detector: UV 215 nm; Samples: 1. Angiotensin II 2. Neurotensin 3. RNase A 4. Insulin B Chain 5. Insulin 6. Cytochrome C 7. Lysozyme 8. Myoglobin 9. Carbonic Anhydrase



- Poroshell 300SB-C3 provides complete resolution of Insulin and Cytochrome C, where as the 300SB-C18 does not.

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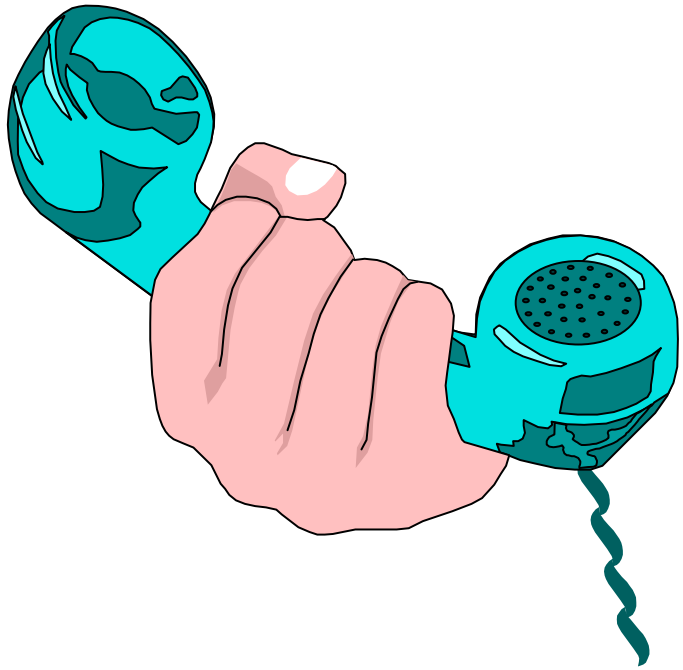
Break Number 2

For Questions and Answers
Press *1 on Your Phone to
Ask a Question



How Do We Use Poroshell Columns?

Faster separations

- High flow rates for fast analysis with high resolution in comparison to totally porous silica
  - Polypeptides
  - Large proteins
  - Impurities
- High recovery of large proteins
- Elevated temperature

Change selectivity

- Different bonded phases - Comparison to totally porous 300SB materials
- High resolution of proteins, heterogeneous proteins, and digests on different bonded phases

LC/MS

- Factors affecting LC/MS of proteins
- High resolution LC/MS of proteins
- Different bonded phases – comparisons to each other
- Peptide mapping at high and low pH

Poroshell Columns for LC/MS of Proteins

High resolution separations of proteins

Formic or acetic acid containing mobile phases for better LC/MS sensitivity

Optimize and balance LC and MS parameters for high sensitivity

MS Parameters	LC Parameters
Flow rate	Flow Rate
Scan speed	Resolution
Peak width	Peak width
Step size	

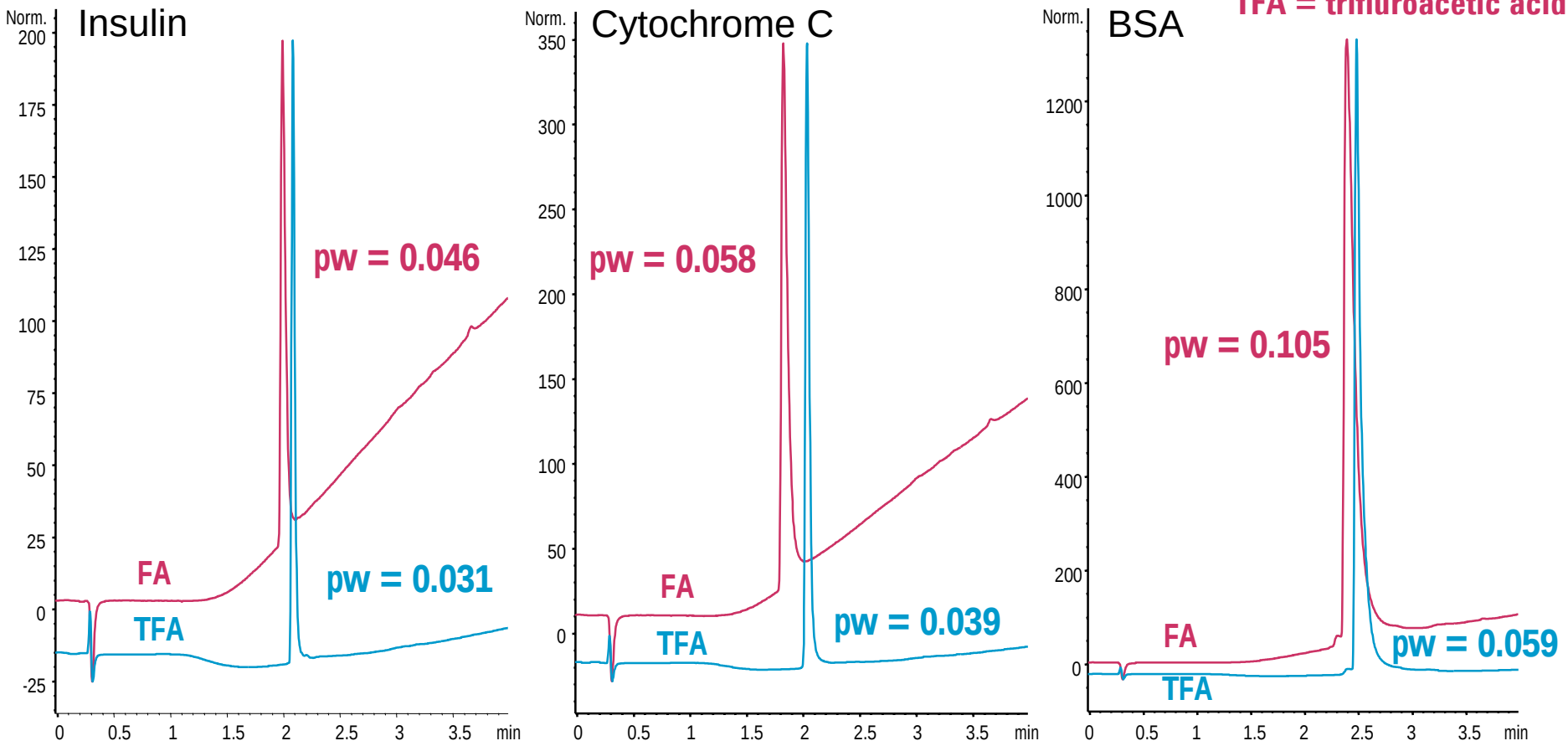
Excellent elution and recovery of proteins improves LC/MS

Effect of LC/MS Acid Modifier on Peak Width using Poroshell

Column: Poroshell 300SB-C18, 2.1 x 75 mm, 5 µm Mobile Phase Gradient: 20-100% B in 5.5 min. A: water + 0.1% TFA or FA B: ACN + 0.1% FA or TFA Flow Rate: 500 µL/min Temperature: 60°C Injector: 1 µL DAD: 220/4 nm

DAD 220, 4 nm

FA = formic acid
TFA = trifluroacetic acid



- **Formic acid gives high efficiency with only slightly larger peak widths on Poroshell**

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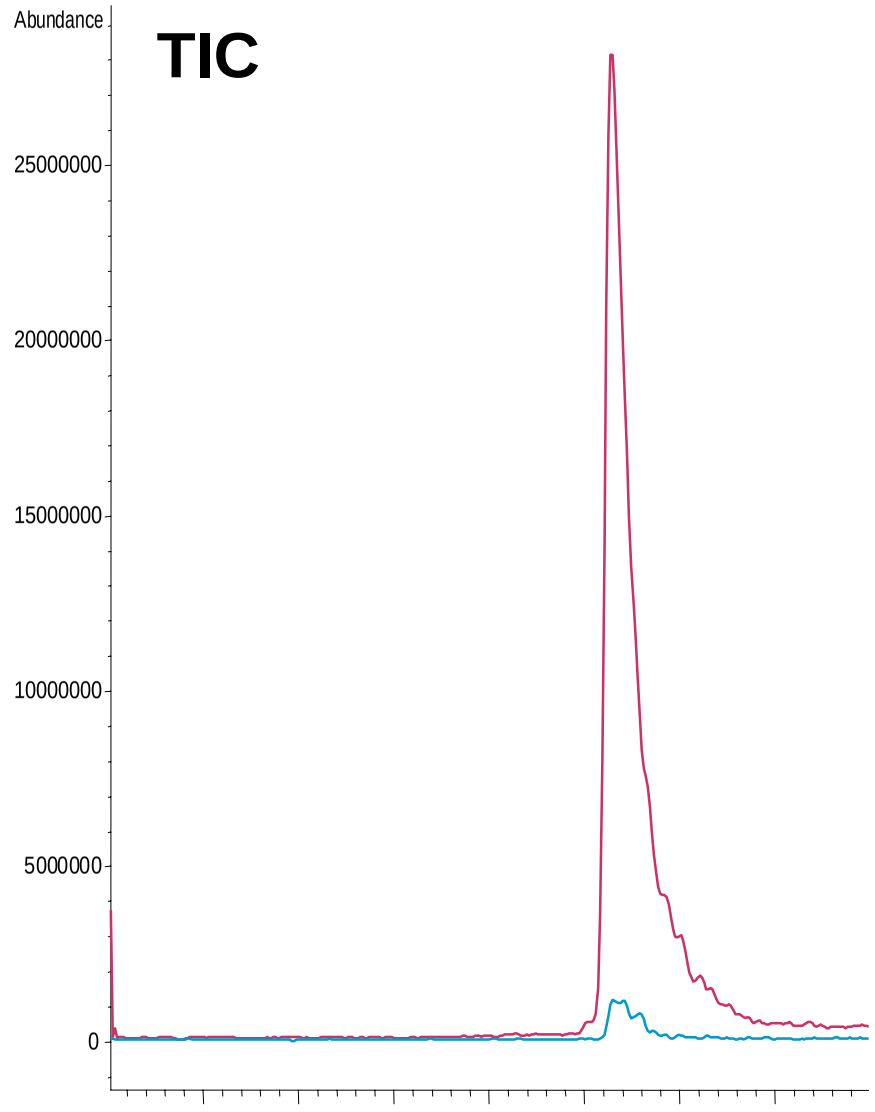
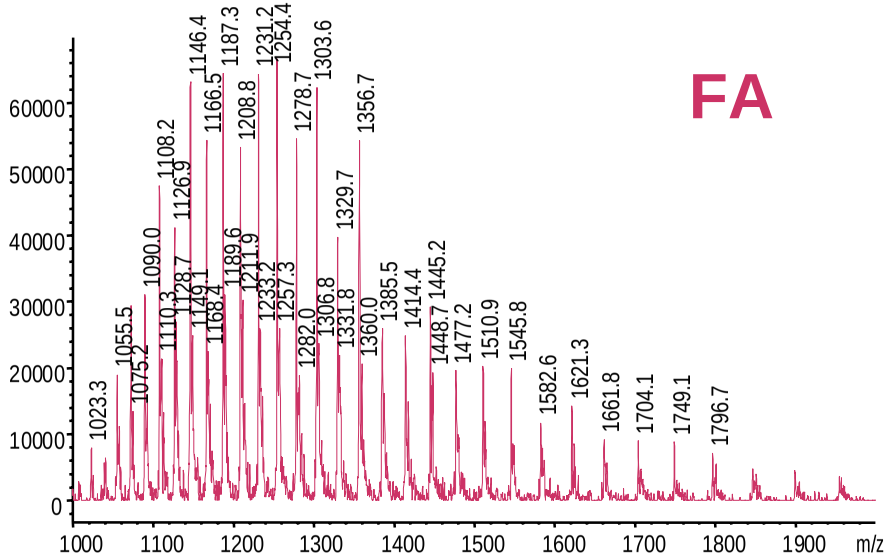
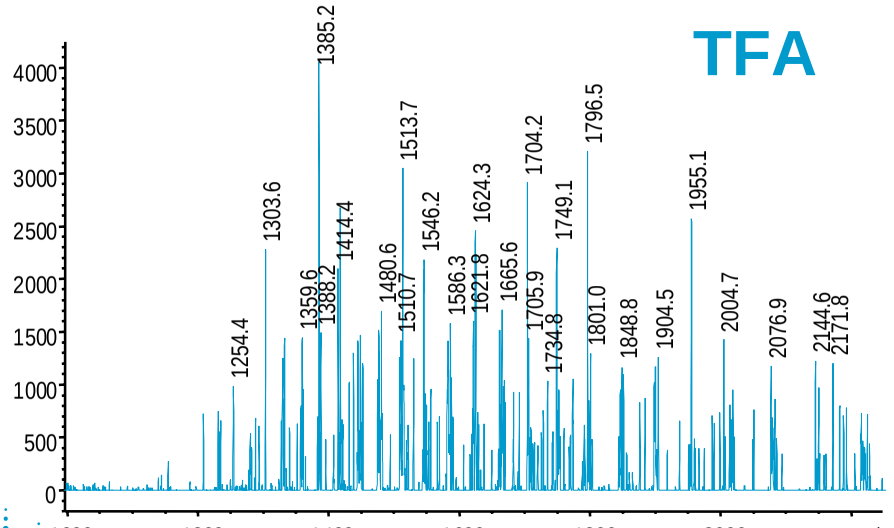
Effect of Modifier on MS Response: 10 pmol BSA

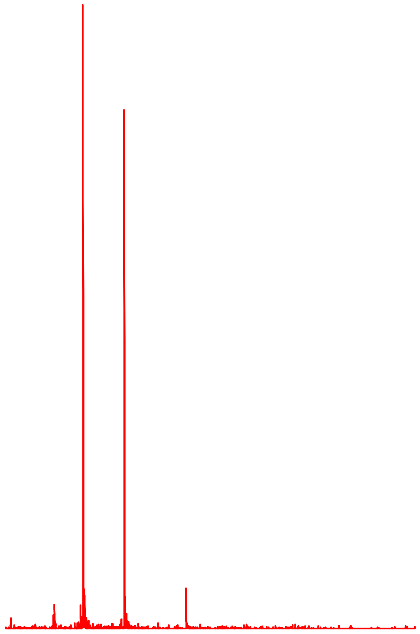




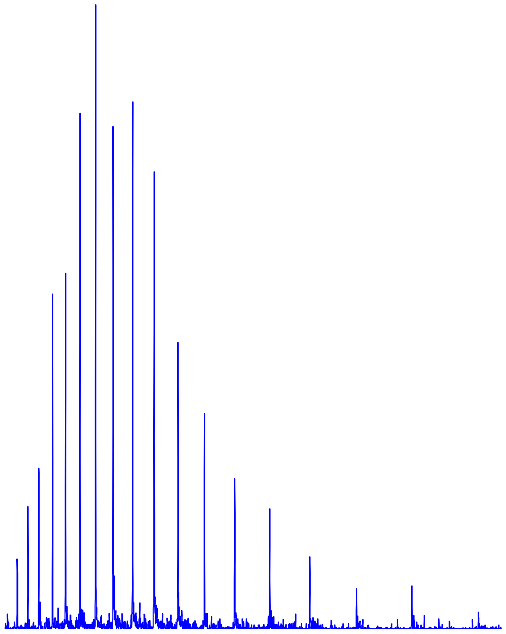


Good Sensitivity with Formic Acid
Mass Spectra of Several Proteins

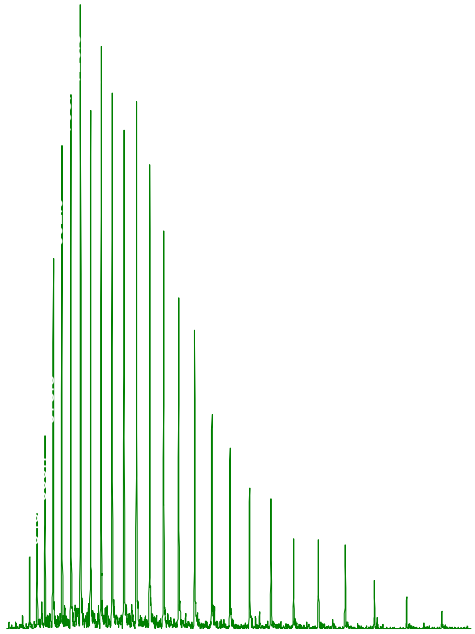
5 pmol Insulin
MW 5733



5 pmol Myoglobin
MW 16950



10 pmol Carbonic anhydrase
MW 29022

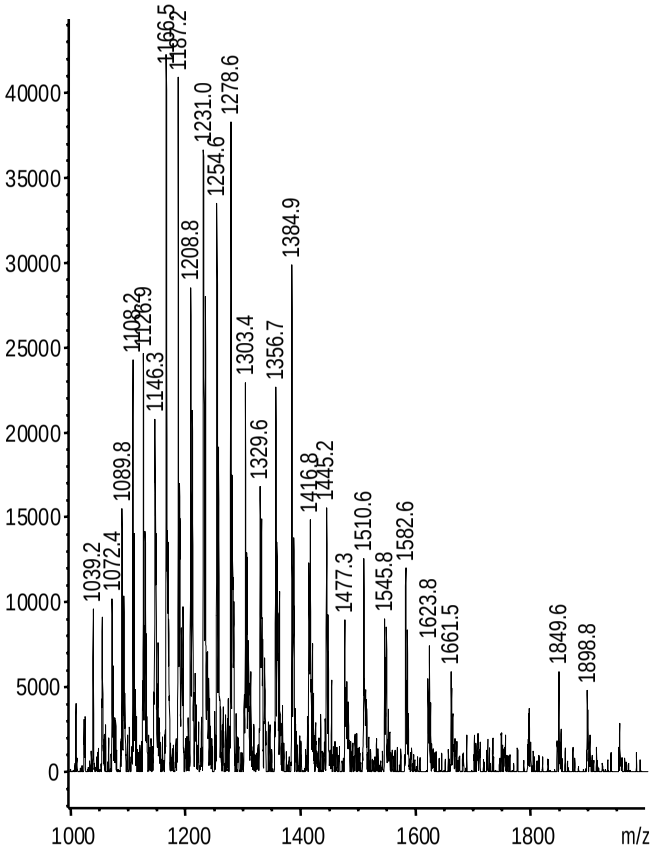


Column: Poroshell 300SB-C18, 2.1 x 75 mm, 5 µm Mobile Phase Gradient: 20-100% B in 5.5 min. A: water + 0.1% formic acid B: ACN + 0.1% formic acid Flow Rate: 500 µL/min Temperature: 60°C Injector: 1 µL DAD: 220/4 nm LC/MS: Pos. Ion ESI –, Vcap 6000 V, Drying gas flow: 12 l/min Drying gas temperature: 350°C Nebulizer: 45 psi, Fragmentor volatage: 140 V Scan: 600 – 2500 Stepsize: 0.15 Peakwidth: 0.06 min

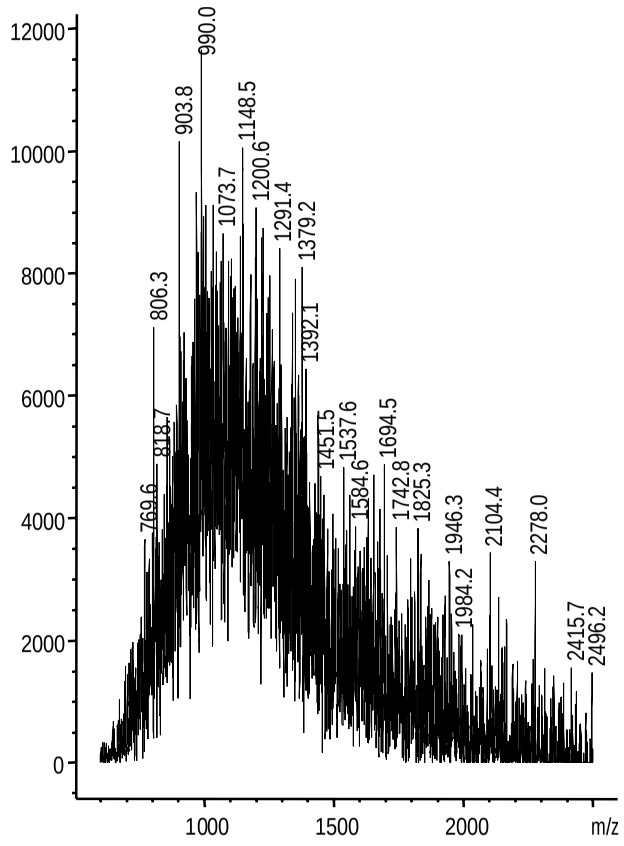
- **Both small and large proteins generate clear mass spectra using Poroshell with formic acid.**

Impact of Protein Heterogeneity on Mass Spectra

5 pmol BSA



100 pmol phosphorylase B



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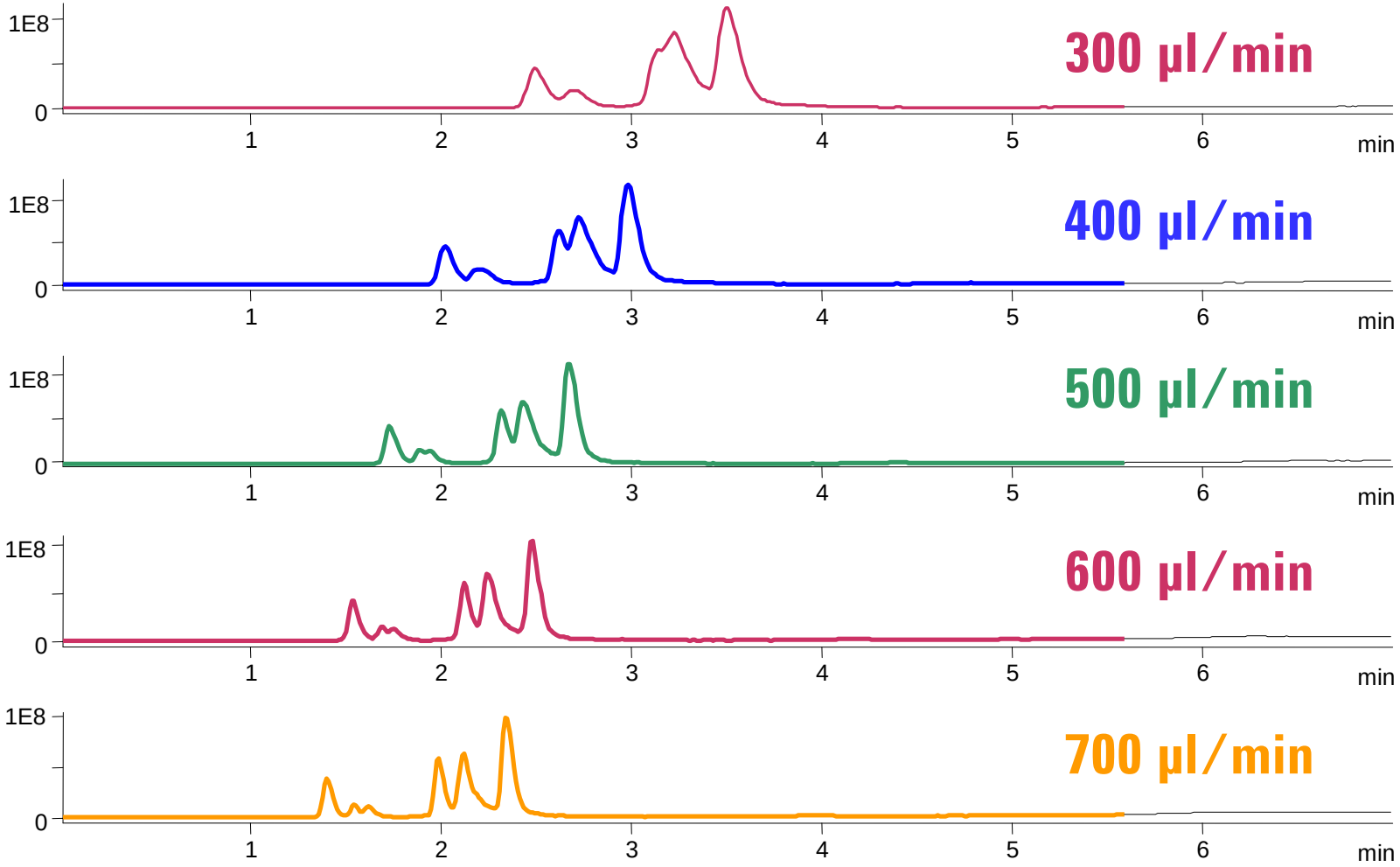
Optimize LC/MS Separation: Effect of Flow Rate

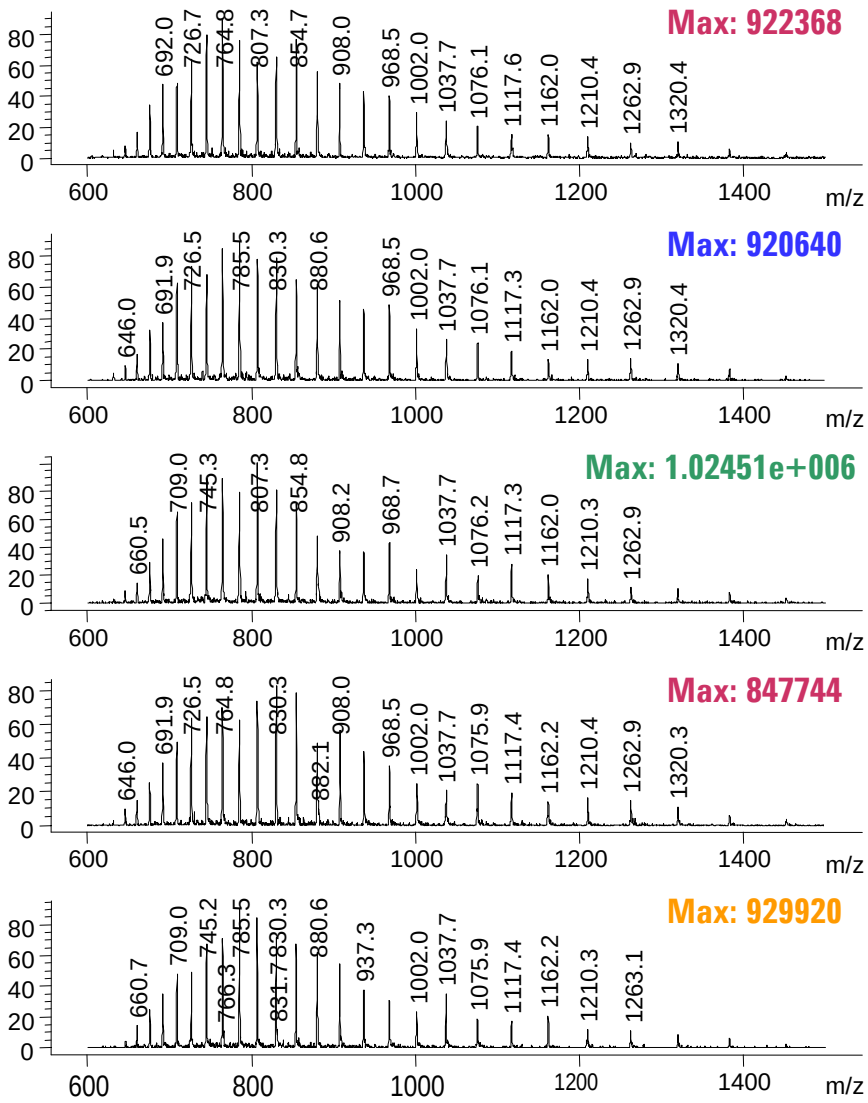
Column: Poroshell 300SB-C18, 1.0 x 75 mm, 5 µm Mobile Phase Gradient: 20-100% B in 5.5 min. A: water + 0.1% formic acid B: ACN + 0.1% formic acid Flow Rate: as shown Temperature: 80°C Injection volume: 1 µL Sample: insulin, lysozyme, cytochrome C, myoglobin, BSA, carbonic anhydrase LC/MS: Pos. Ion ESI –, Vcap 6000 V, Drying gas flow: 12 l/min Drying gas temperature: 350°C Nebulizer: 45 psi, Fragmentor volatage: 140 V Scan: 600 – 2500 Stepsize: 0.15 amu Peakwidth: 0.06 min



- **The flow rate is more critical to the chromatographic separation than the MS.**

Effect of Flow Rate on Mass Spectra

- **Flow rate changes have almost no impact on the mass spectra From 300 – 600 µL/min**



300 µl/min

400 µl/min

500 µl/min

600 µl/min

700 µl/min

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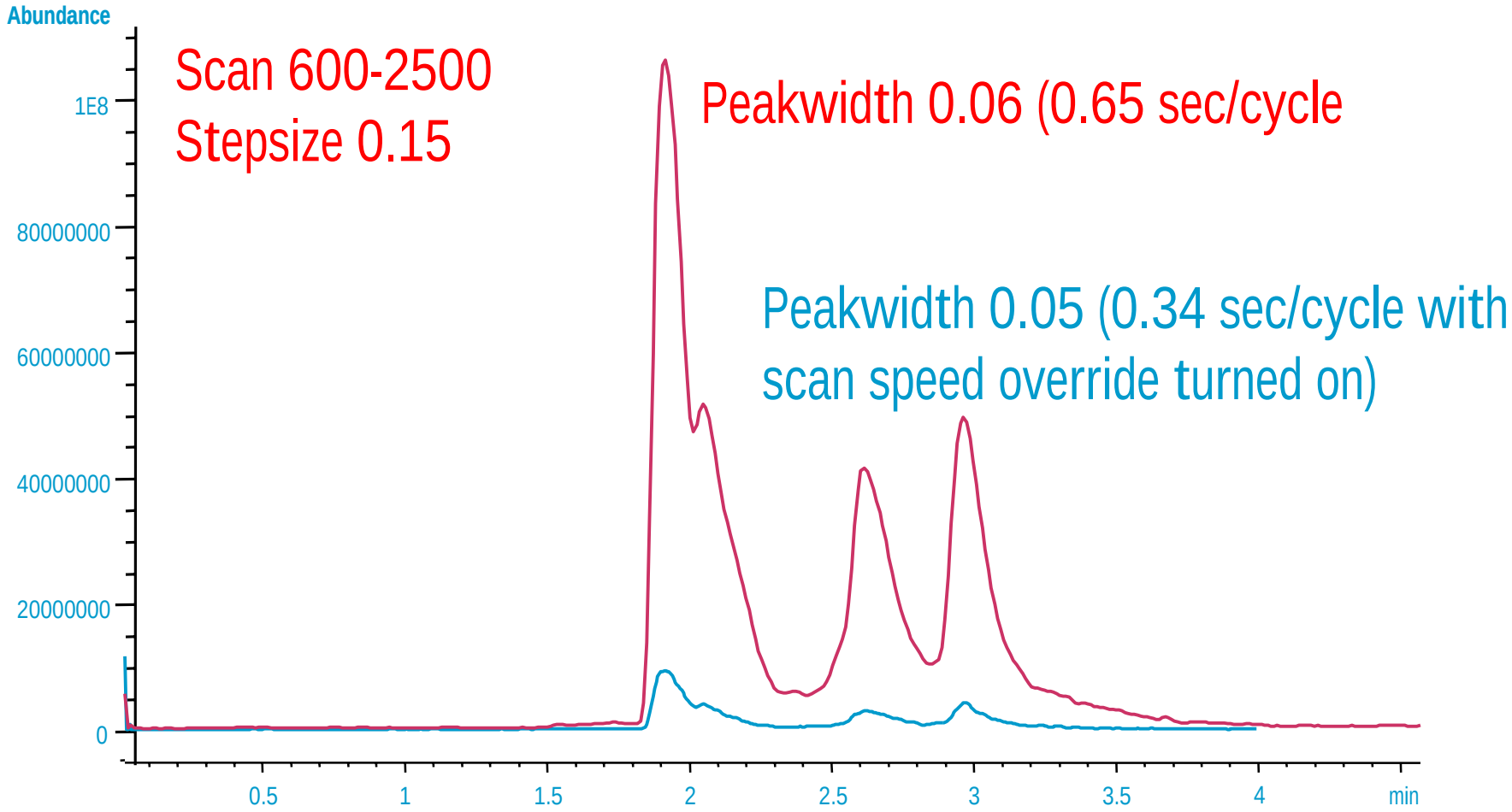
Impact of Scan Speed on Sensitivity



- **A slower scan speed as defined by MS settings, allows for better peak definition for better sensitivity**

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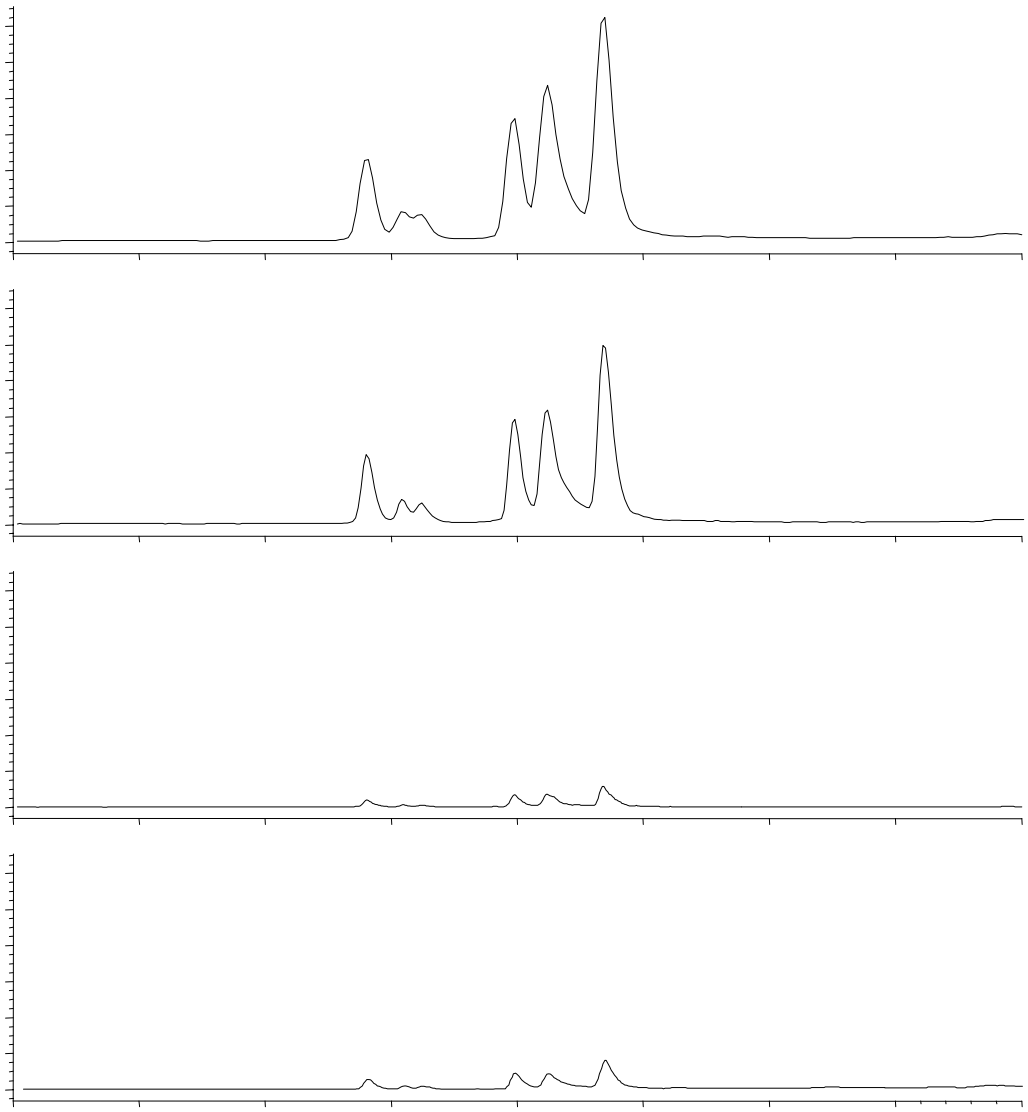
Effect of MS Settings on TIC Signal



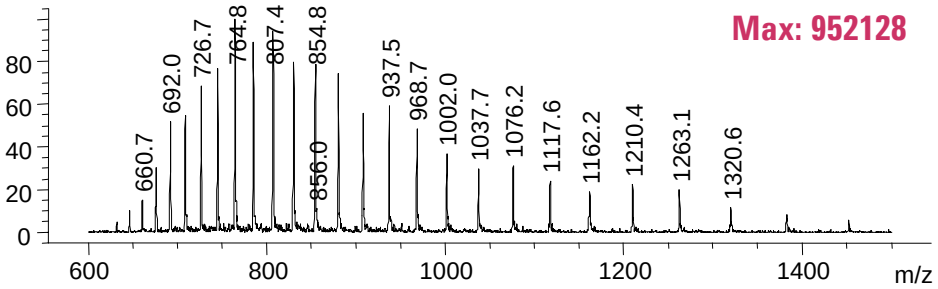
Pw=0.1
Stepsize = 0.15
Loose chromatographic resolution

Pw=0.06
Stepsize = 0.15
Best compromise of speed, chromato-graphic behavior and MS results

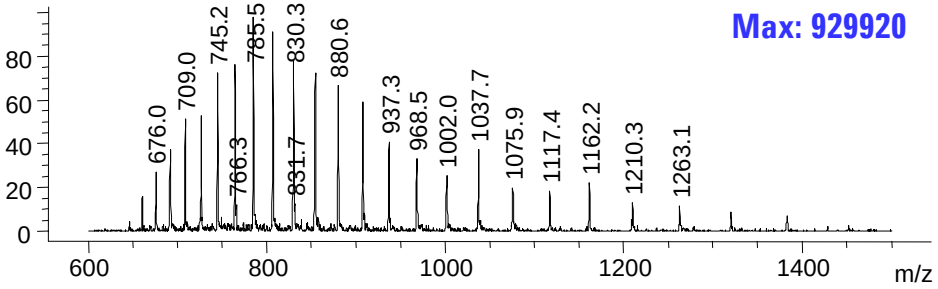
Pw=0.04
Stepsize = 0.15
Scanspeed override ON
Sacrifices MS signal (ion transmission)

Pw=0.04
Stepsize = 0.25
Better chromatographic resolution but loss of MS signal

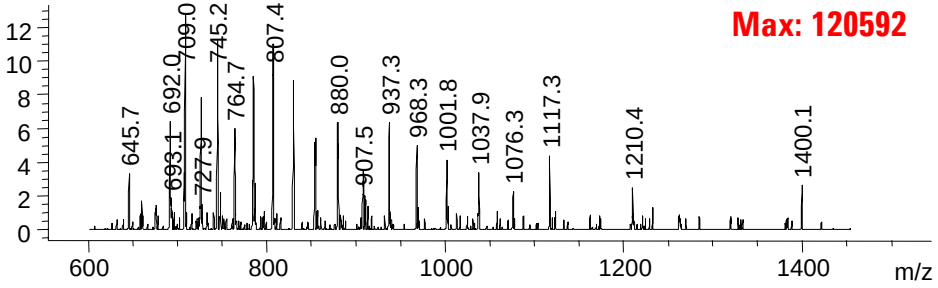
Effect of MS Settings on Spectral Quality



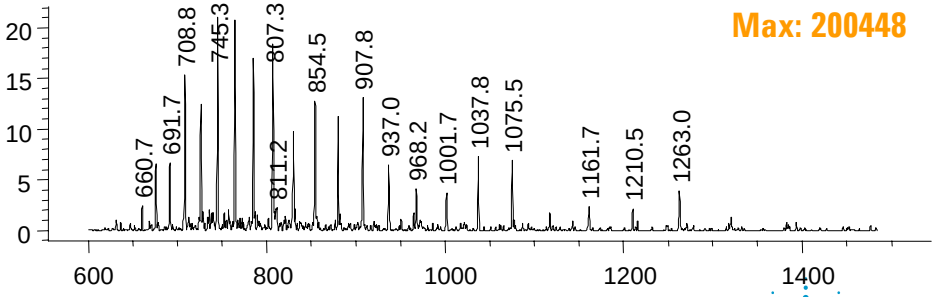
Pw=0.1
Stepsize = 0.15
Lose chromatographic resolution



Pw=0.06
Stepsize = 0.15
Best compromise of speed, chromatographic behavior and MS results

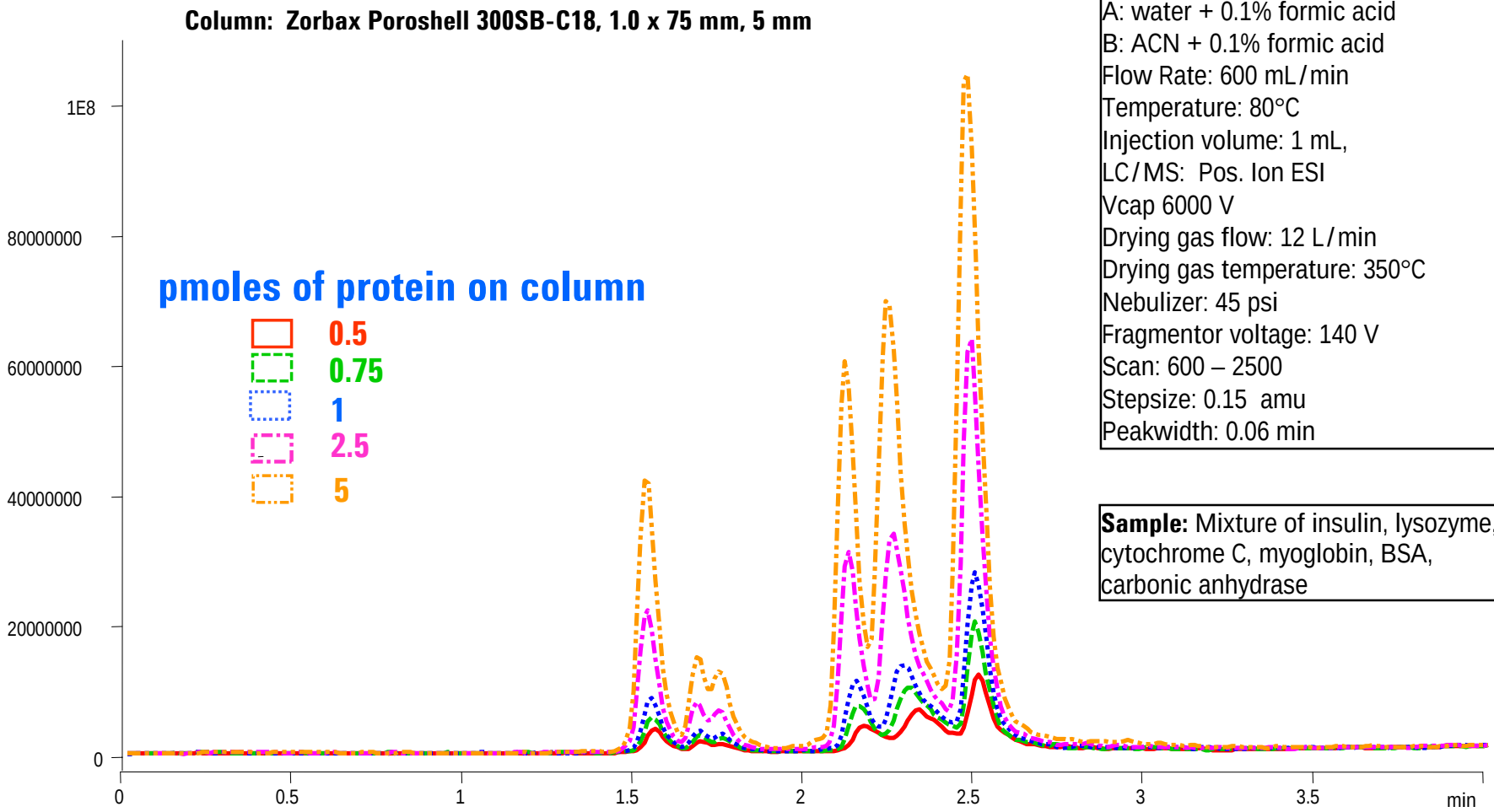


Pw=0.04
Stepsize = 0.15
Scanspeed override ON
Sacrifices MS signal (ion transmission)



Pw=0.04
Stepsize = 0.25
Better chromatographic resolution but loss of MS signal

High Flow Rates and High Sensitivity LC/MS Using 1.0 mm ID Zorbax Poroshell



Mobile Phase Gradient: 20 -100% B in 5.5 min.
A: water + 0.1% formic acid
B: ACN + 0.1% formic acid
Flow Rate: 600 mL/min
Temperature: 80°C
Injection volume: 1 mL,
LC/MS: Pos. Ion ESI
Vcap 6000 V
Drying gas flow: 12 L/min
Drying gas temperature: 350°C
Nebulizer: 45 psi
Fragmentor voltage: 140 V
Scan: 600 – 2500
Stepsize: 0.15 amu
Peakwidth: 0.06 min

Sample: Mixture of insulin, lysozyme, cytochrome C, myoglobin, BSA, carbonic anhydrase

- These TIC's show good sensitivity with only 0.5 pmoles on column.

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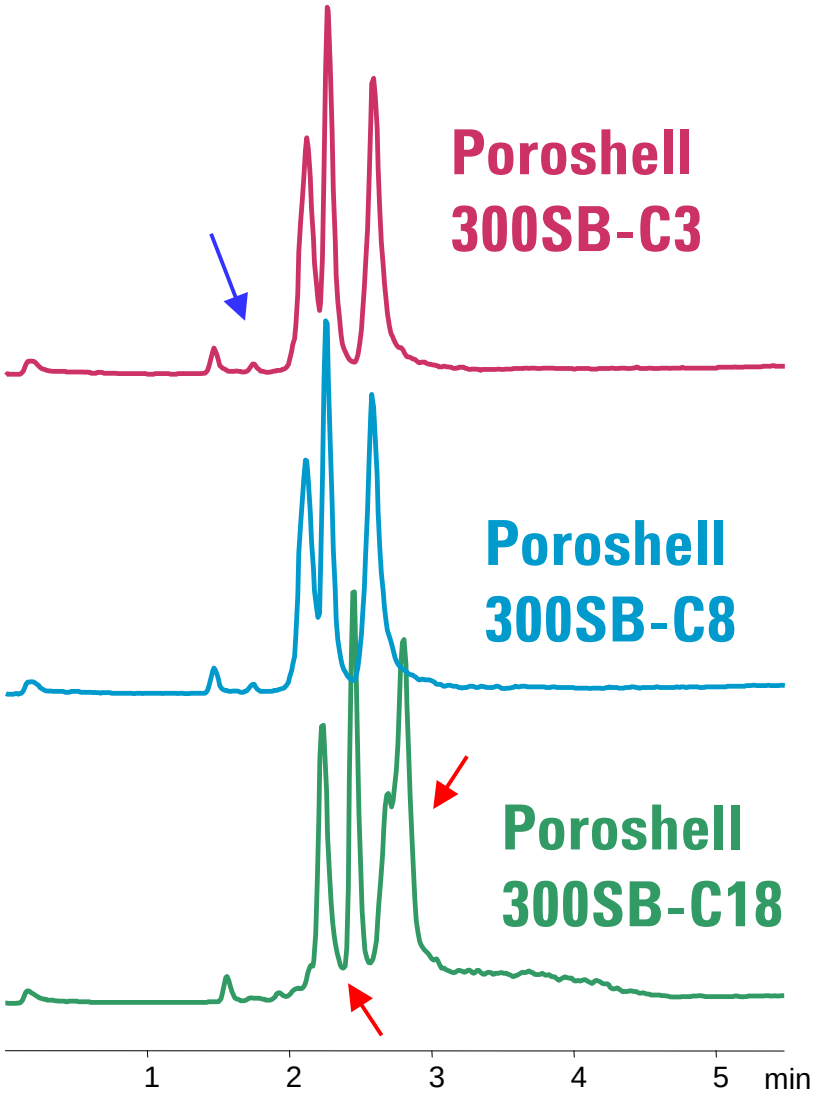
Selectivity, Resolution Options with Multiple Poroshell Bonded Phases



Column: Poroshell 300SB, 2.1 x 75 mm, 5 µm
Mobile Phase Gradient: 20-100% B in 5.5 min.
Mobile Phase: A: water + 0.1% Formic Acid B: ACN + 0.1% Formic acid
Flow Rate: 500 µL/min
Temperature: 60°C
Injection: 1 mL
Sample: Phosphorylase B, BSA, Ovalbumin, Carbonic anhydrase, Soyben trypsin inhibitor, alpha-lactalbumin and sucrose
Detection: Electrospary ionization: positive ion
Vcap: 6000V
Drying Gas: 12L/min 350ºC
Nebulizer: 45 psi
Scan: 600-2500 amu
Step size:0.15 amu
Peak width:0.06 min

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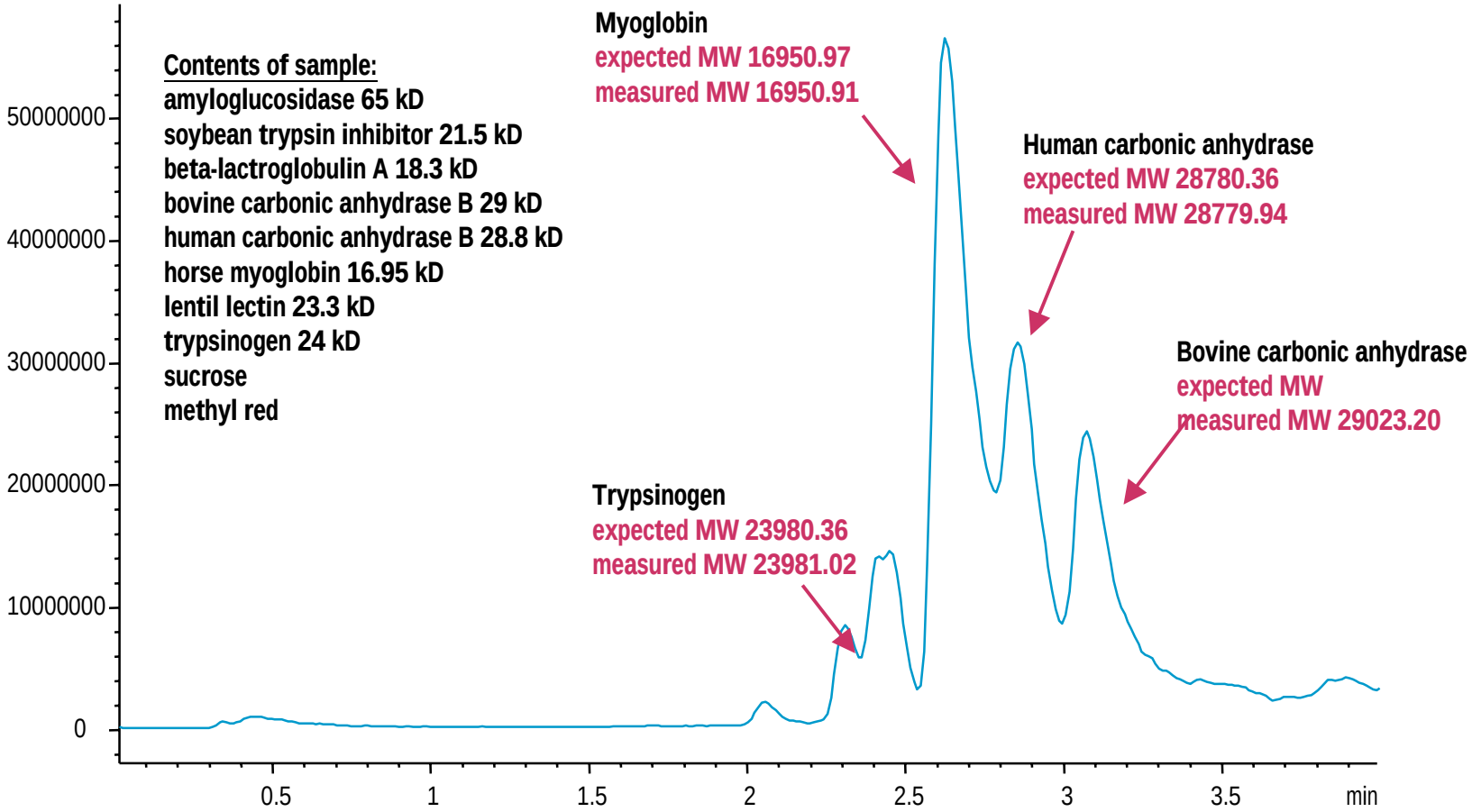
Pharmacia pI 3-10 Standard run on Poroshell 300SB-C18



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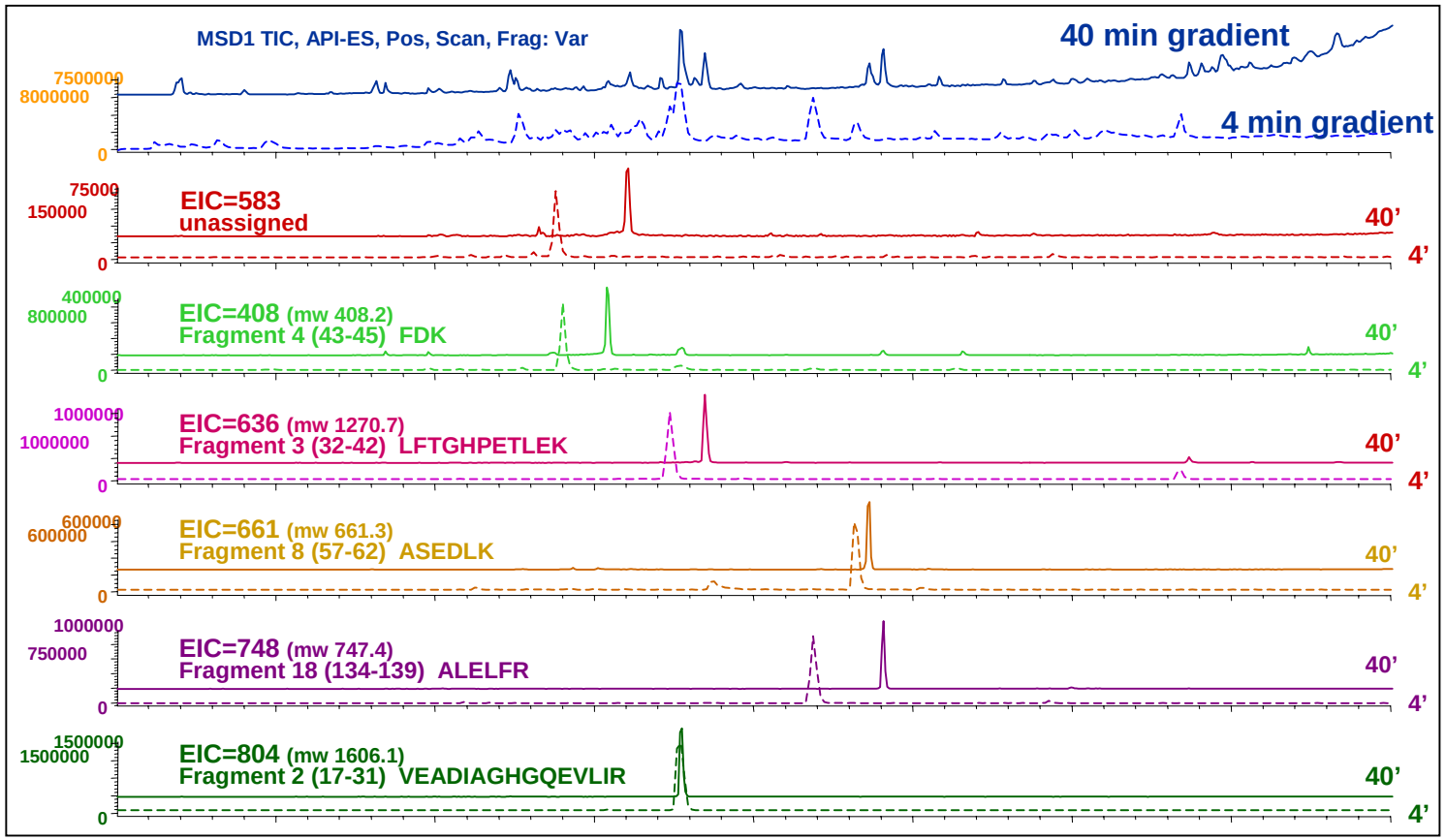
Comparison of Speed in Peptide Mapping by LC-MS using ZORBAX Extend-C18 and Poroshell Extend-C18



ZORBAX Extend-C18 (2.1 x 150mm, 3.5µm)
Flow= 0.2mL/min, Gradient 0 – 100%B in 40 min
Mobile Phase A= 10mM NH_4OH, H_2O; B= 10mM NH_4OH, MeOH
Agilent 1100 LC-MSD, Scan 200-1500m/z,
Drying Gas= 10L/min; 35psig; Temp= 350°C
POS ESI mode, 4500V, Fragmentor Ramp 70 @ 50m/z to 120 @ 1500m/z
Sample: myoglobin tryptic digest 50 pmol in 5µL

ZORBAX Poroshell Extend-C18 (2.1 x 75mm, 5µm)
Flow= 1.0mL/min, Gradient 0 – 100%B in 4 min
Mobile Phase A= 10mM NH_4OH, H_2O; B= 10mM NH_4OH, MeOH
Agilent 1100 LC-MSD, Scan 200-1500m/z,
Drying Gas= 13L/min; 60psig; Temp= 350°C
POS ESI mode, 4500V, Fragmentor Ramp 70 @ 50m/z to 120 @ 1500m/z
Sample: myoglobin tryptic digest 50 pmol in 5µL

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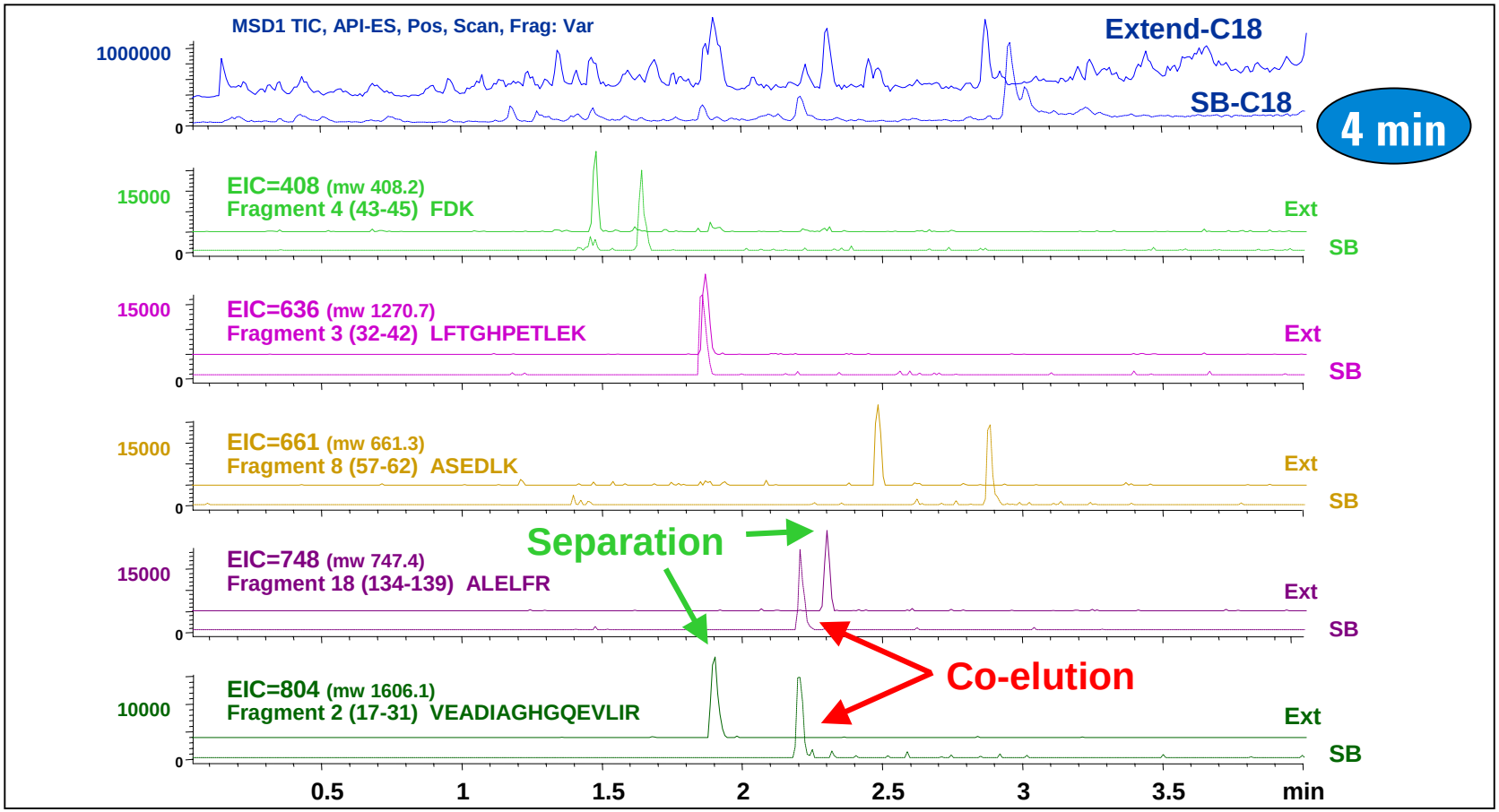
Comparison of Peptide Mapping by LC-MS using Poroshell 300Extend-C18 and Poroshell 300SB-C18 at high and low pH



ZORBAX Poroshell 300Extend-C18 (2.1 x 75mm, 5µm)
Flow= 1.0mL/min, Gradient 0 – 100%B in 4 min
Mobile Phase A= 10mM NH_4OH, H_2O; B= 10mM NH_4OH, MeOH
Agilent 1100 LC-MSD, Scan 200-1500m/z
Drying Gas= 13L/min; 60psig; Temp= 350°C, POS ESI mode, 4500V
Fragmentor Ramp 70 @ 50m/z to 120 @ 1500m/z, 0.52 sec/cycle
Sample: myoglobin tryptic digest: 50 pmol in 5µL

ZORBAX Poroshell 300SB-C18 (2.1 x 75mm, 5µm)
Flow= 1.0mL/min, Gradient 0 – 100%B in 4 min
Mobile Phase A= 0.1% Formic Acid, H_2O; B= 0.1% Formic Acid, MeOH
Agilent 1100 LC-MSD, Scan 200-1500m/z
Drying Gas= 13L/min; 60psig; Temp= 350°C, POS ESI mode, 4500V
Fragmentor Ramp 70 @ 50m/z to 120 @ 1500m/z, 0.52sec/cycle
Sample: myoglobin tryptic digest: 50 pmol in 5µL

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Summary

- Poroshell columns have a porous shell and solid core to reduce the diffusion distance and time for proteins.
- This allows for fast analysis of proteins – including very large proteins with high recovery.
- It also allows for high efficiency and good resolution of protein impurities.
- Use of sterically protected 300SB and 300 Extend phases further allows these columns to be operated at very high temperature and low or high pH.
- These columns are ideal for LC/MS of proteins and peptides because they give high resolution of complex samples.

Bibliography

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[2]. R.D. Ricker, B.J. Permar, and B.A. Bidlingmeyer, HPLC Analysis of Proteins in the Fast-Lane, PittCon2002, March 18-21, 2002 New Orleans

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[4] Cliff Woodward, Ron Majors, Bernard J. Permar, and Robert D. Ricker, The Use of a Family of Superficially Porous Columns compatible with Standard and Capillary HPLCs to achieve fast separations of very Large, Heterogeneous Proteins, ISPPP 2003, Orlando

Acknowledgements

1) Genentech. Inc, Analytical Chemistry (Baojen Shyong, Galahad DePeralta, and Victor Ling) for the use of their data on the effects of temperature on antibody separations

2) Novartis Pharma, Biotechnology, Basel (Dr. Kurt Forrer and Patrik Roethlisberger) for their data and discussions on the analysis of monoclonal antibodies and their fragments.

HPLC Column Technical Support

800-227-9770 (phone: US & Canada)*

302-993-5304 (phone)*

* *Select option 4, then option 2.*

916-608-1964 (fax)

www.agilent.com/chem

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Q & A for breaks 1 & 2

Break 1

Q1: When you show the speed capabilities of Poroshell you have an implied assumption that everything in the LC is optimized for it.What happens if the system is set up for standard columns?

A1: What happens is not necessarily pretty. You will see less of an advantage for high speed columns. To optimize means to install narrow bore tubing throughout, eliminate as much extra column volume as possible, choose the best flow cell for your column diameter, and optimize the speed of data collection of your detectors. Once this is done, the advantages of Poroshell are obvious.

Q2: When you show data for column lifetime, you do it in terms of column volumes and run standards at regular intervals. Why don't you do it in terms of number of injections?

A2: With the number of columns to be checked and samples to be run, it simply takes too long; so, we run it the other way. The examples shown are really only for comparing one column to another not for running against an absolute standard. Every manufacturer wants to know how well they do relative to their competition. This helps improve products.

Break 2

Q1: I noticed that PEG 300 was included in the mobile phases during the monoclonal antibody chromatograms. Why?

A1: It turns out that antibodies are very 'sticky' on reversed phase columns and always leave some molecules bound to the column. After several runs an increase in backpressure becomes very noticeable. If PEG 300 is included in the mobile phase, this buildup does not happen. I should note, however, that you must not use PEG 300 when running into your LC/MS; you will see PEG forever as a background contaminant. The buildup can be washed off by running at 100% ACN until the pressure returns to initial conditions.

Q2: C3 seems to be better than C18 on the biggest molecules. I do not understand why.

A2: C3 was better on the very heterogeneous large molecules studied. We think that this is because C3 has the lowest retention; i.e., it has fewer ways to interact with a large complicated molecule and thus will show less broadening due to interaction than the long chain, C18. It is not absolutely certain that it will be best on all large molecules.