

SEPARATE

Reverse-Phase Separation of Proteins,
Peptide and Other Biomolecules

John Palmer
August 6, 2008



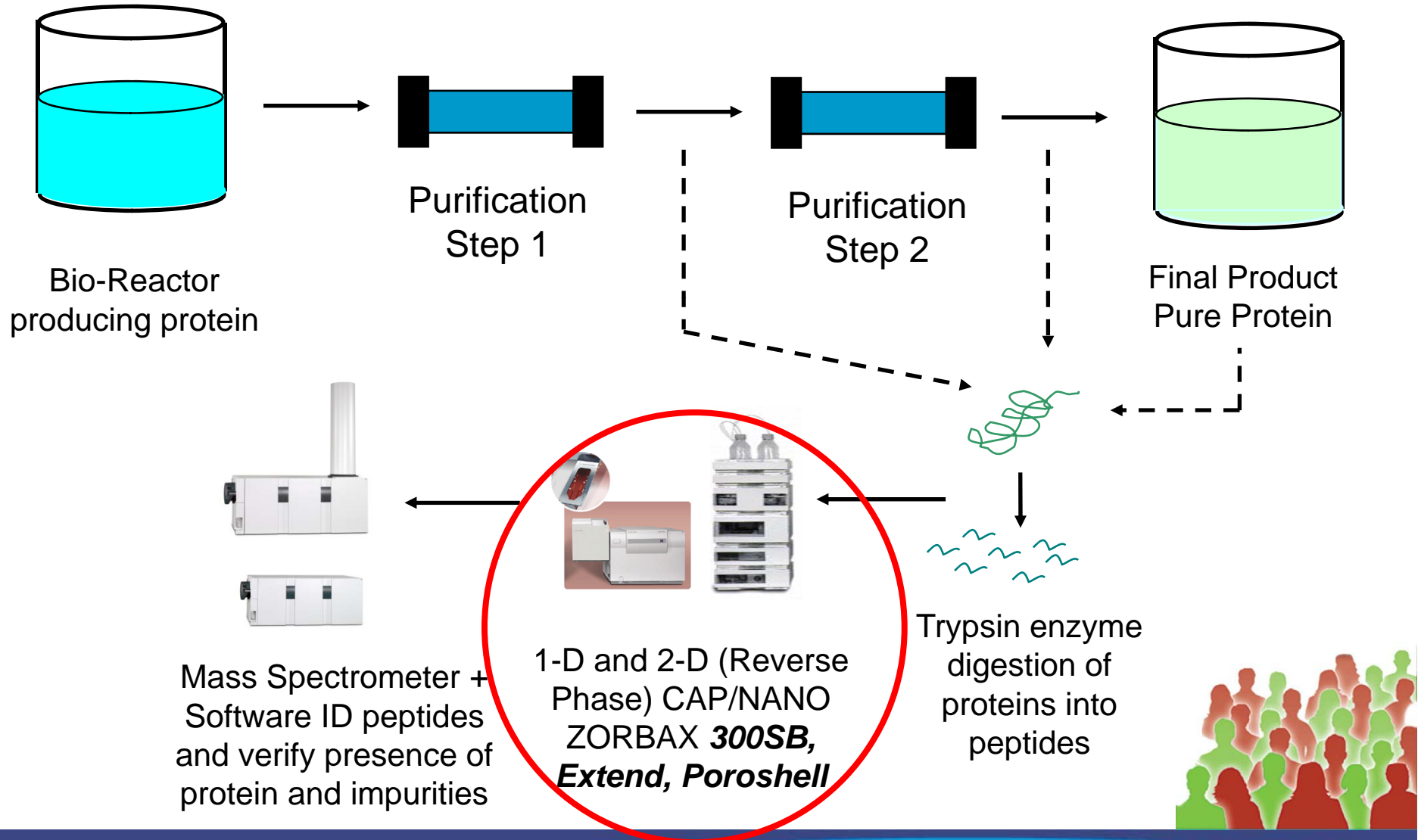
Agilent Technologies

Where is Reversed Phase HPLC Useful in Bio Separations ?

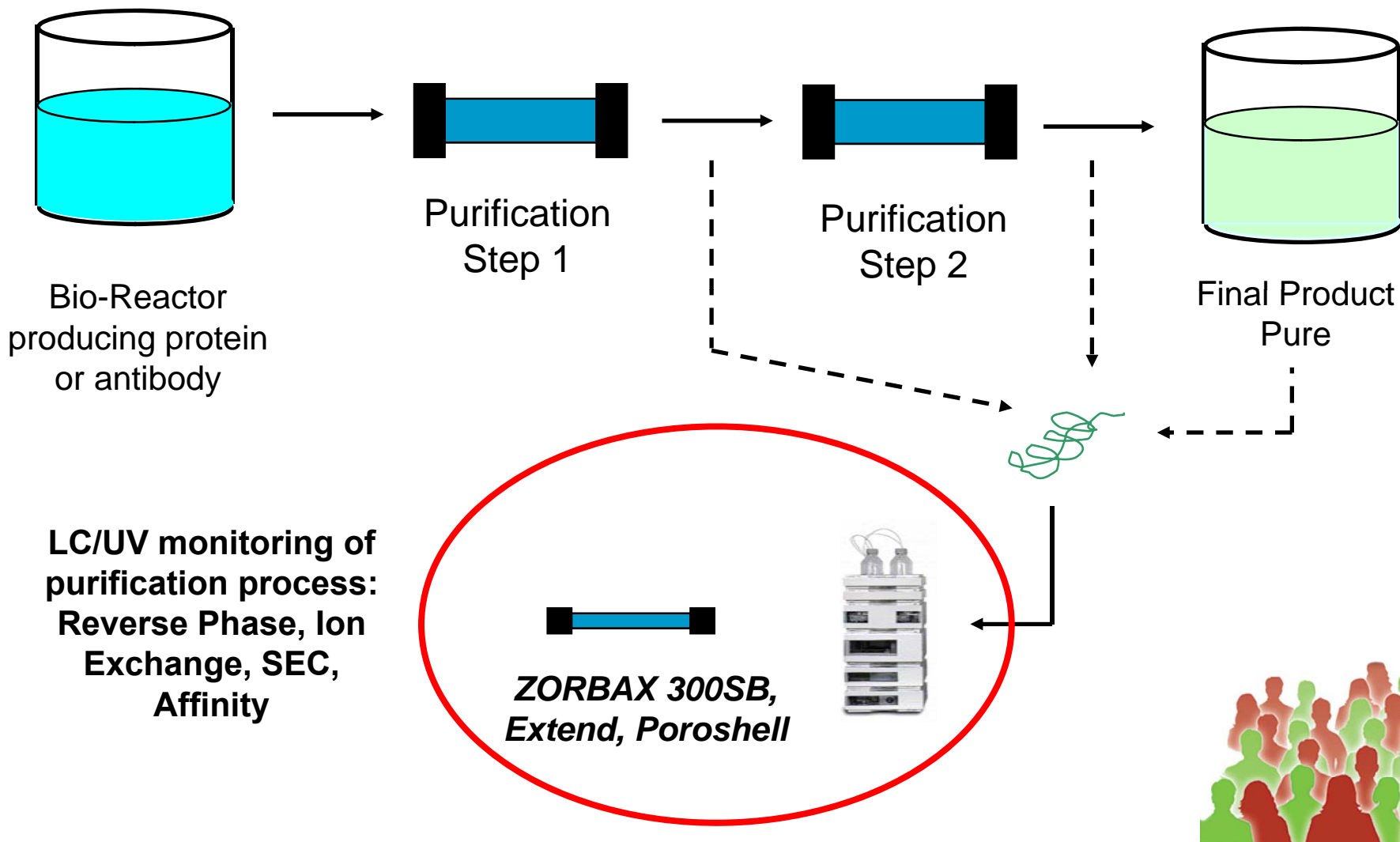
- Proteomics
- New “Bio Pharmaceutical” Development
- Analytical Development
- Stability Testing
- Quality Control



BioPharma Proteomics – Peptide Mapping



BioPharma QA/QC and In-Process Control



Agilent Reversed Phase Columns for Separations of Proteins and Peptides

Requirements

- Wide pore - 300Å for unrestricted access to bonded phase
- LC/MS compatible bonded phases at low and high pH – low bleed, high performance
- Multiple bonded phases for method optimization
- Many configurations for LC/MS compatibility, small sample sizes and 2-D HPLC for proteomics

Columns available

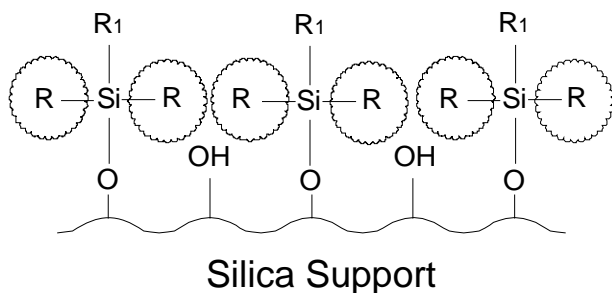
- 300StableBond
- 300Extend
- Poroshell 300SB
- Configurations from nano to prep



ZORBAX 300SB and 300Extend-C18 Columns for the Analysis of Proteins and Peptides

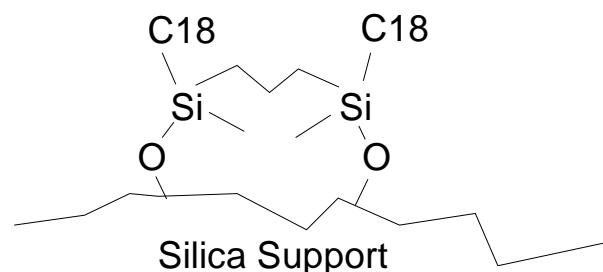
300StableBond

- Four different bonded-phases, 300SB-C18, C8, CN, and C3 for selectivity optimization
- Extremely stable at low pH
- Use with TFA, Formate and Acetate mobile phases
- Stable at high temperature – up to 80 - 90°C



300Extend-C18

- Uses unique bidentate-C18 bonded phase for long lifetime at high pH (up to 11.5)
- Double endcapped
- Can also be used at low pH (2.0)
- Ammonium hydroxide mobile phase good for high pH LC and LC/MS



Typical Conditions for Separations of Peptides and Proteins on 300SB Columns

Column:	4.6 x 150 mm, 5 or 3.5 μm 300SB
Mobile Phase:	A: 95:5, H₂O : ACN with 0.1% TFA B: 5:95, H₂O : ACN with 0.085% TFA
Flow Rate:	1 mL / min.
Temp:	35 - 40°C
Initial Gradient:	0 - 60% B in 60 min.

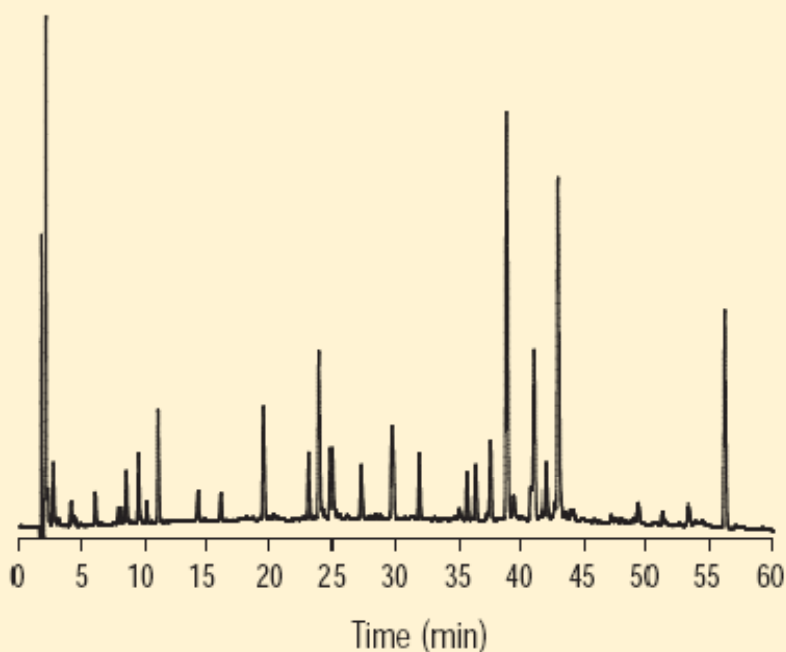


001115P1.PPT



C18 and TFA/CAN Gradients Capable of High Resolution

Microbore ZORBAX 300SB easily resolves this complex mixture



Column: **ZORBAX 300SB-C18**
1.0 x 150 mm, 3.5 μ m
(Agilent Part No. 863630-902)

Mobile Phase: A: 0.1% TFA
B: 0.075% TFA/ 80% ACN

Gradient: 2 - 60% B in 60 min.

Flow Rate: 50 μ L/min

Temperature: 50°C

Detection: UV 215 nm

Sample: tryptic digest of rhGH, 2 μ L

Separate complex peptide mixtures, even at low pH



What Options are Available if the Standard C18/TFA-ACN Gradient Is Not Sufficient?



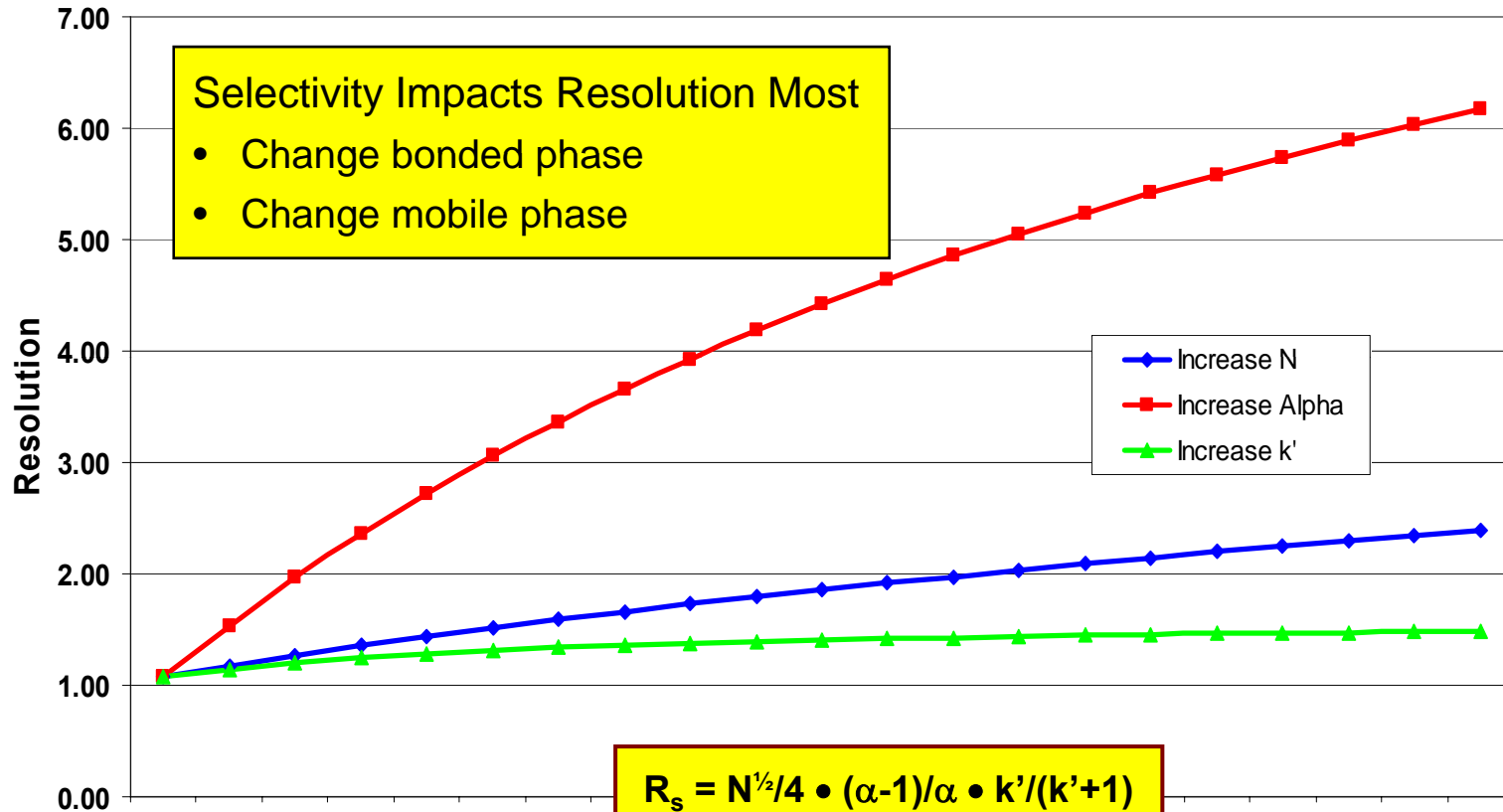
Changes in α Can Be the Key to Improved Separation and Resolution

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

Theoretical Plates Selectivity Retention

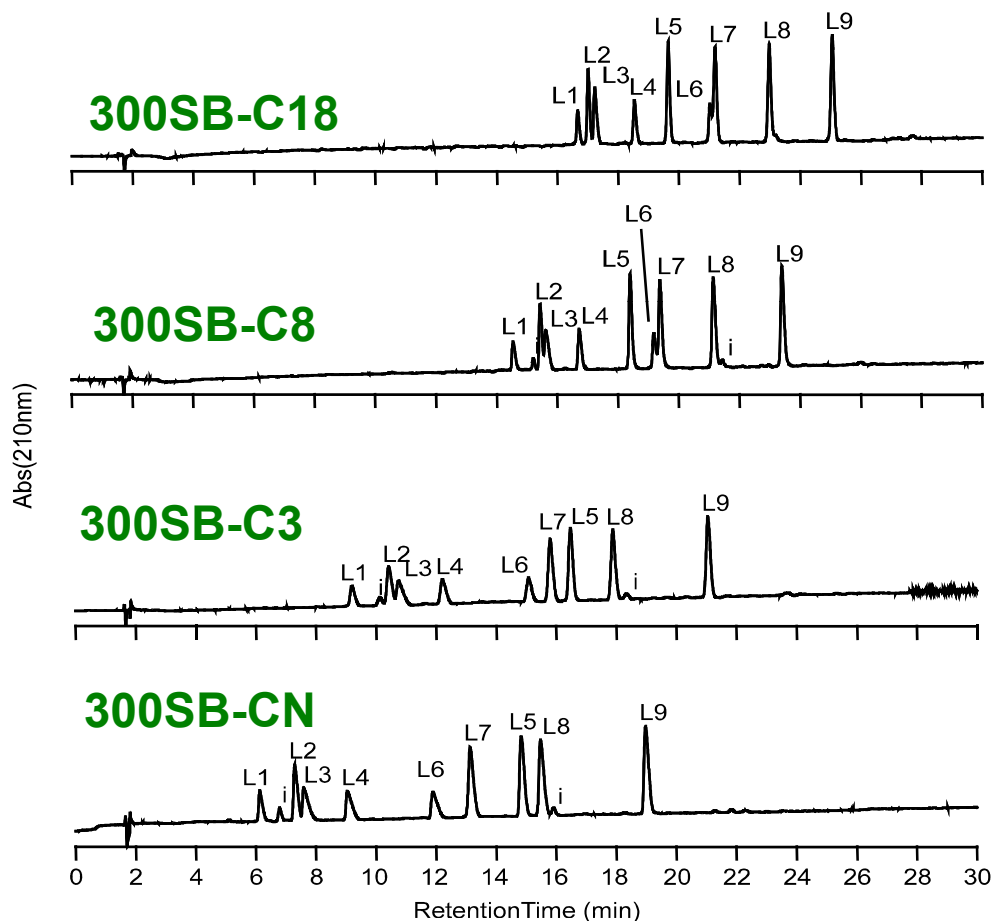


Resolution as a Function of Selectivity, Column Efficiency, or Retention



	5000	10000	15000	20000	25000
Plates:	5000	10000	15000	20000	25000
Alpha:	1.10	1.35	1.60	1.85	2.1
k':	2.0	4.5	7.0	9.5	12.0

Comparison of Small Peptide Selectivity Differences on 300SB Bonded Phases



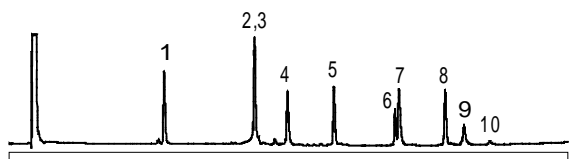
Conditions:

Columns: ZORBAX 300SB, 4.6 x 150 mm, 5 μ m
 Mobile Phase: Gradient, 0 - 26% B in 30min.
 A = 0.1% TFA in Water
 B = 0.1% TFA in Acetonitrile
 Temperature: 40°C
 Sample: 2 μ g of each peptide
 Flow Rate: 1.0 mL / min.
 Detection: UV-210nm

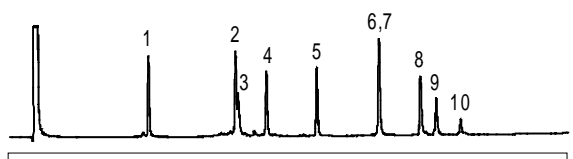


Comparison Separation of Large Polypeptides on 300SB Bonded Phases

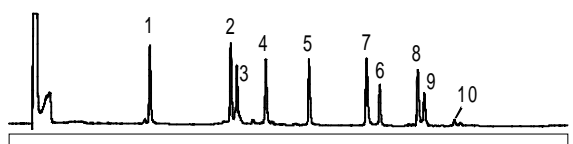
300SB-C18



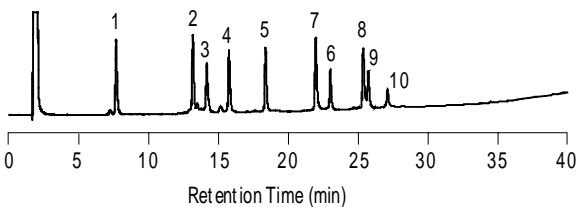
300SB-C8



300SB-C3



300SB-CN



Columns: ZORBAX StableBond 300SB

4.6 x 150 mm, 5 μ m

Mobile Phase: Linear Gradient, 25- 70% B in 40 min

A: 0.1% TFA in Water

B: 0.09% TFA in 80% ACN/20% water

Flow Rate: 1.0 mL/min

Temperature: 60°C

Sample: 3 μ g each protein

1. RNase

2. Insulin

3. Cytochrome C

4. Lysozyme

5. Parvalbumin

6. CDR

7. Myoglobin

8. Carbonic Anhydrase

9. S-100 β

10. S-100 α

- Four different 300SB bonded phases allow selectivity optimization of proteins.

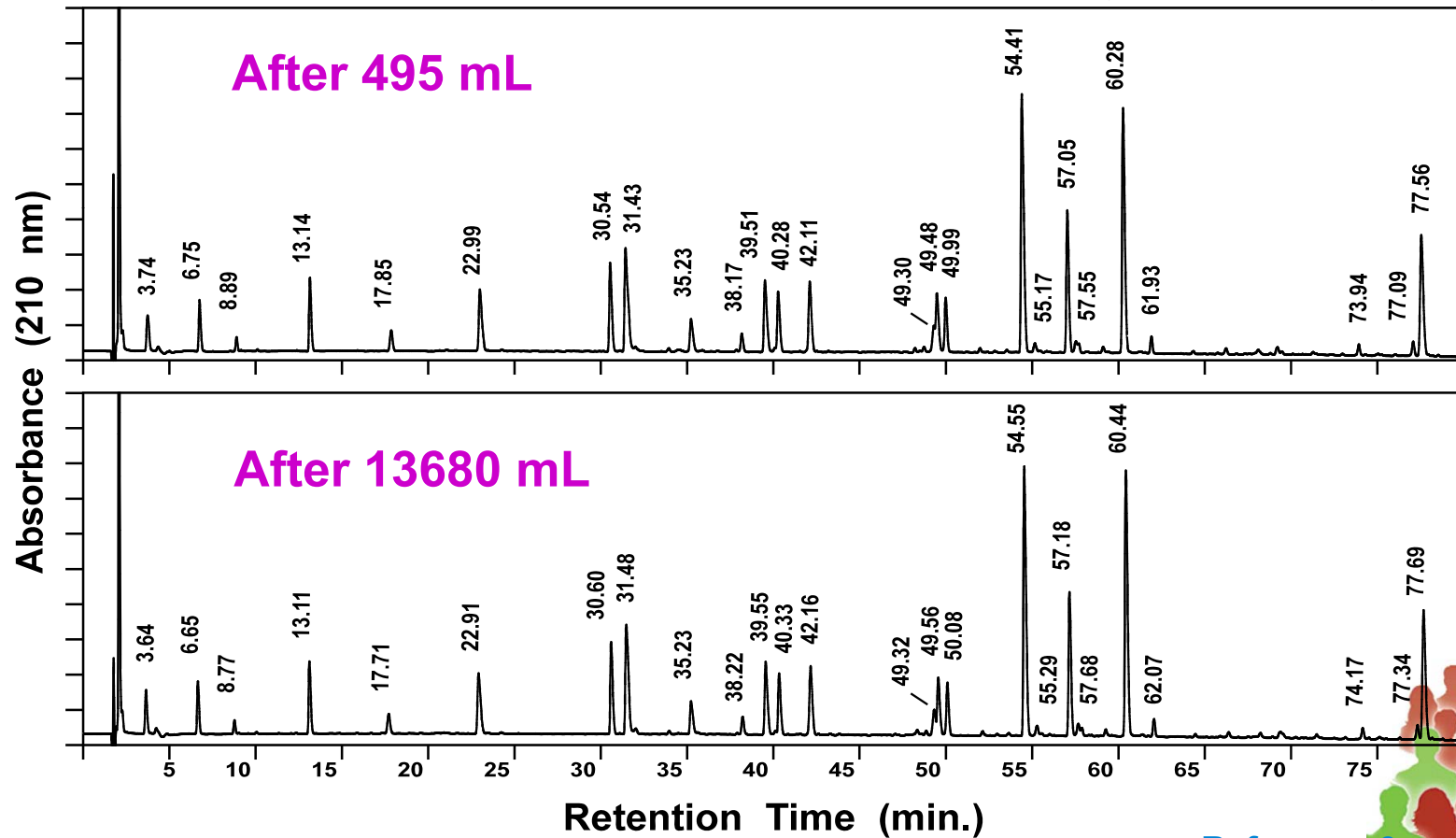


Extended Column Lifetime of ZORBAX 300SB-C8 rhGH Tryptic Digest

Column: ZORBAX 300SB-C8, 4.6 x 150mm

Mobile Phase Gradient 0 - 60% in 120 min. A= 0.1% TFA in Water, B= 0.086% TFA in ACN

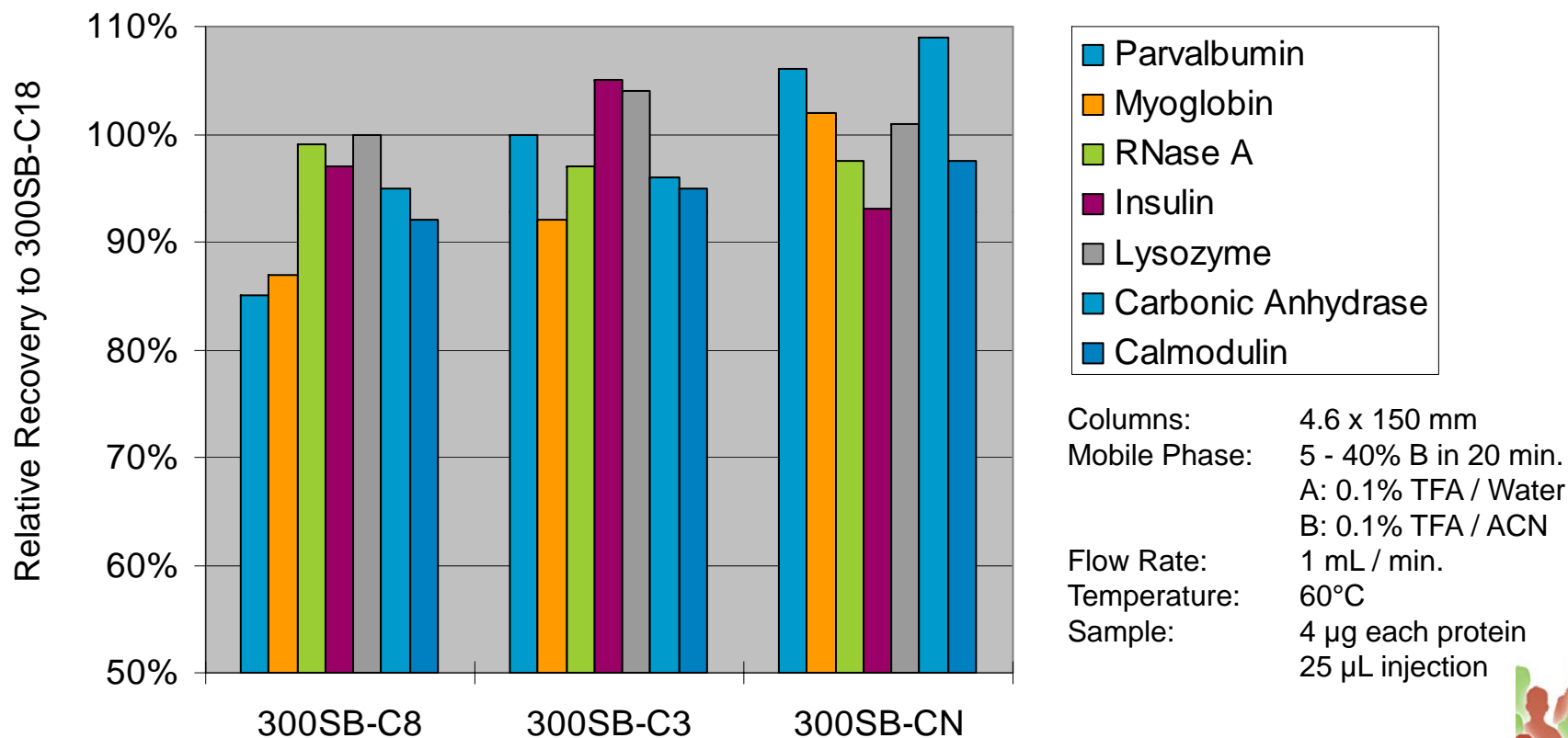
Temp.: 40°C Flow Rate: 1mL/min. Det. UV 210nm Sample: 50 µg of rhGH Tryptic Digest



Reference 3

000336P1.PPT

Recovery of Polypeptides from ZORBAX 300SB Columns

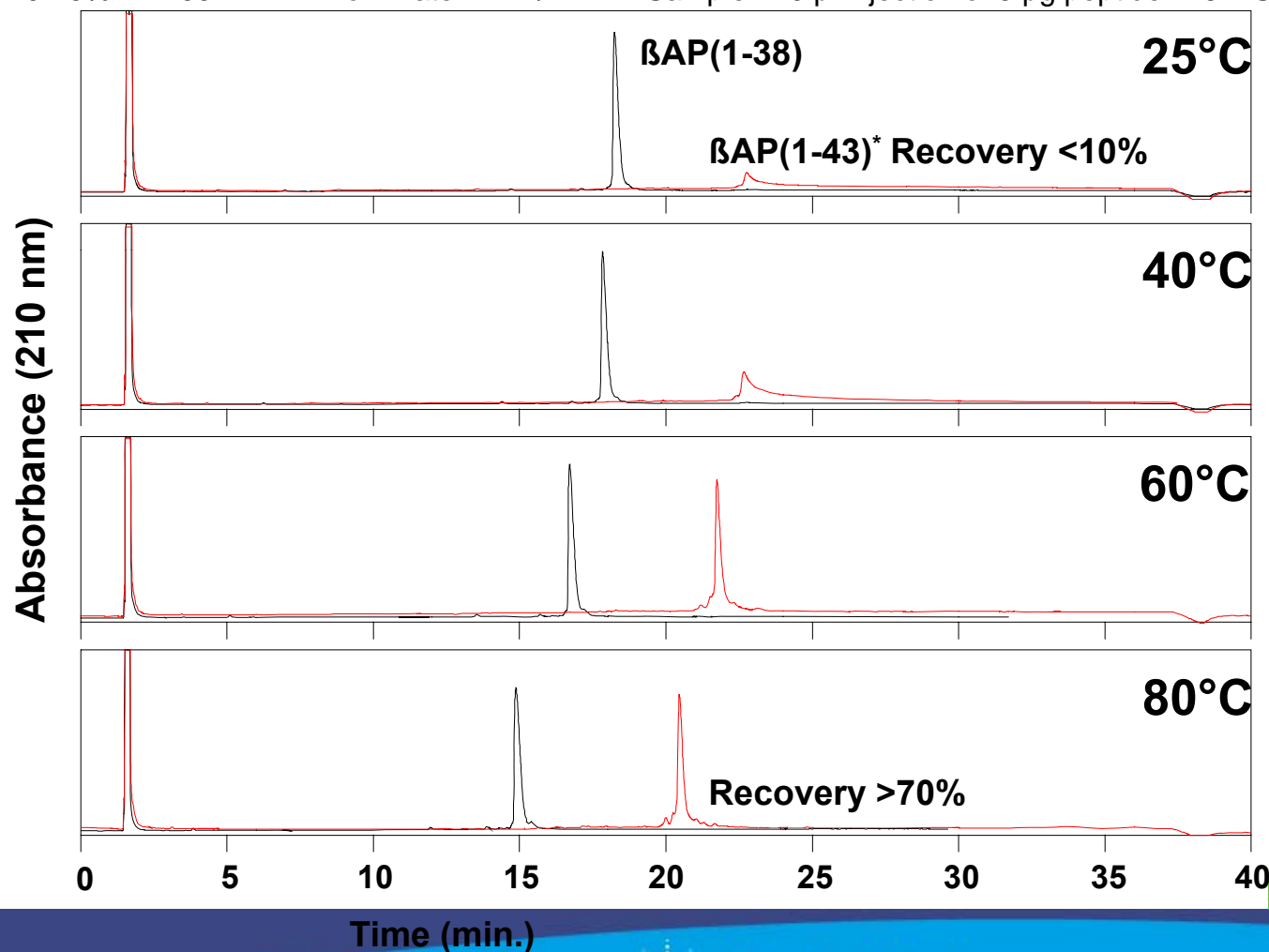


- Recovery may vary depending on bonded phase.
- All 300SB bonded phase generally provide good recovery.

Optimize Recovery by Changing Temperature

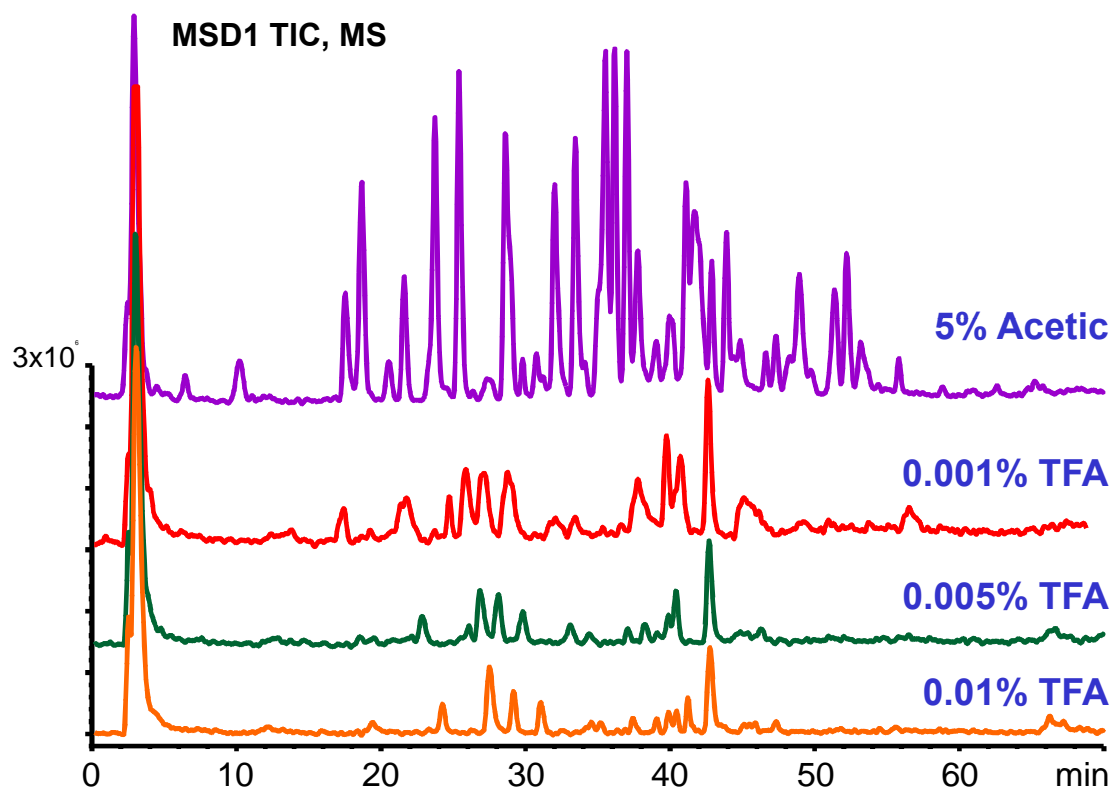
Example: β AP Separation

Column: ZORBAX 300SB-C18, 4.6 x 150 mm, 5 μ m Mobile Phase: A- 0.1%TFA in water, B- 0.09%TFA in acetonitrile
Gradient: 20-45% B in 35 min Flow Rate: 1 mL/min Sample: 10 μ l injection of 5 μ g peptide in 6M Urea/5% HOAc



LC/MS Sensitivity vs. Mobile Phase Modifier

TFA vs. Acetic Acid



Column: **ZORBAX 300SB-C3**
2.1 x 150 mm, 5 μ m

Mobile Phase: Gradient: 0% B hold for 5 min.
0 – 40% B in 55 min.
40 – 100% B in 20 min

A: 5% acetic acid
B: Acetonitrile

Flow Rate: 0.2 mL/min

Instrument: Agilent 1100 MSD

Sample: GluC Digest of BSA

- A significant increase in sensitivity was observed using 5% acetic acid instead of TFA.
- Reducing TFA concentration to 0.001% improved sensitivity very little.

Reference 1



Typical Conditions for Separations of Peptides and Proteins on 300Extend-C18 Columns at High pH

Column: 150 mm Length, 5 or 3.5 μm

300Extend-C18

Mobile Phase: A: 20 mM NH_4OH in water
B: 20 mM NH_4OH in 80% ACN

Flow Rate: 1 mL / min.

Temp: 25 - 30°C

Initial Gradient: 5 - 60% B in 30 min.

- At low pH use the same conditions as on the 300SB columns.



001115P1.PPT

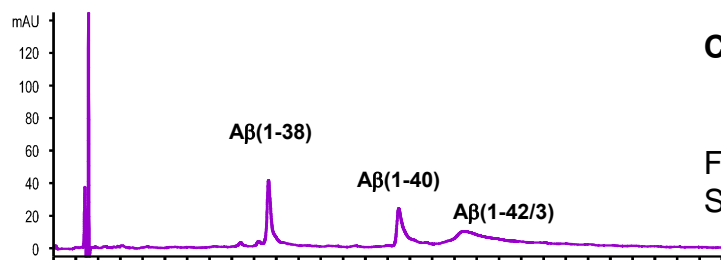


High pH Can be Used for Separating Hydrophobic or Other Low-Solubility Peptides

Comparison of A β Peptide RP-HPLC Separations at Low and High pH

TFA Conditions, 25°C

A- 0.1% TFA in water
B- 0.085% TFA in 80%AcN
33-45%B in 30 min.



Column:

ZORBAX

300Extend C18

2.1 x 150 mm, 5 μ m

Flow Rate:

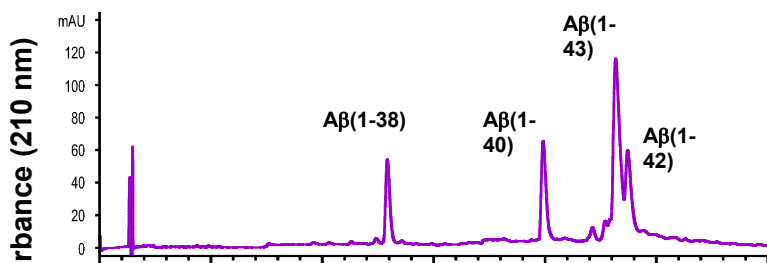
0.25 mL/min

Sample:

5 μ L sample (100 pmol each)

TFA Conditions, 80°C

A- 0.1% TFA in water
B- 0.085% TFA in 80%AcN
29-41%B in 30 min.



Amyloid β Sequences:

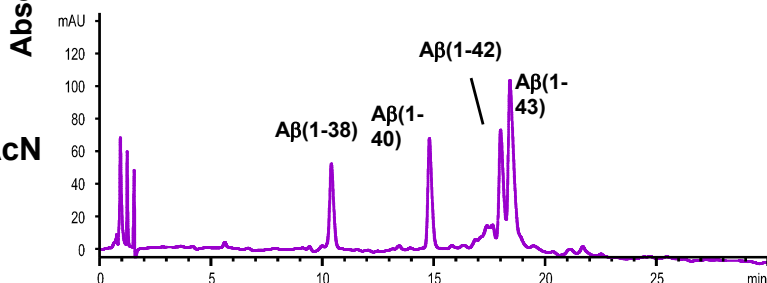
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr
Glu Val His His Gln

Lys Leu¹⁷ Val Phe Phe Ala Glu Asp Val
Gly Ser Asn Lys Gly Ala

Ile Ile Gly Leu Met Val Gly Gly³⁸ Val Val⁴⁰
Ile Ala⁴² Thr⁴³-COOH

NH₄OH Conditions, 25°C

A- 20 mM NH₄OH in water
B- 20 mM NH₄OH in 80%AcN
26-38%B in 30 min.



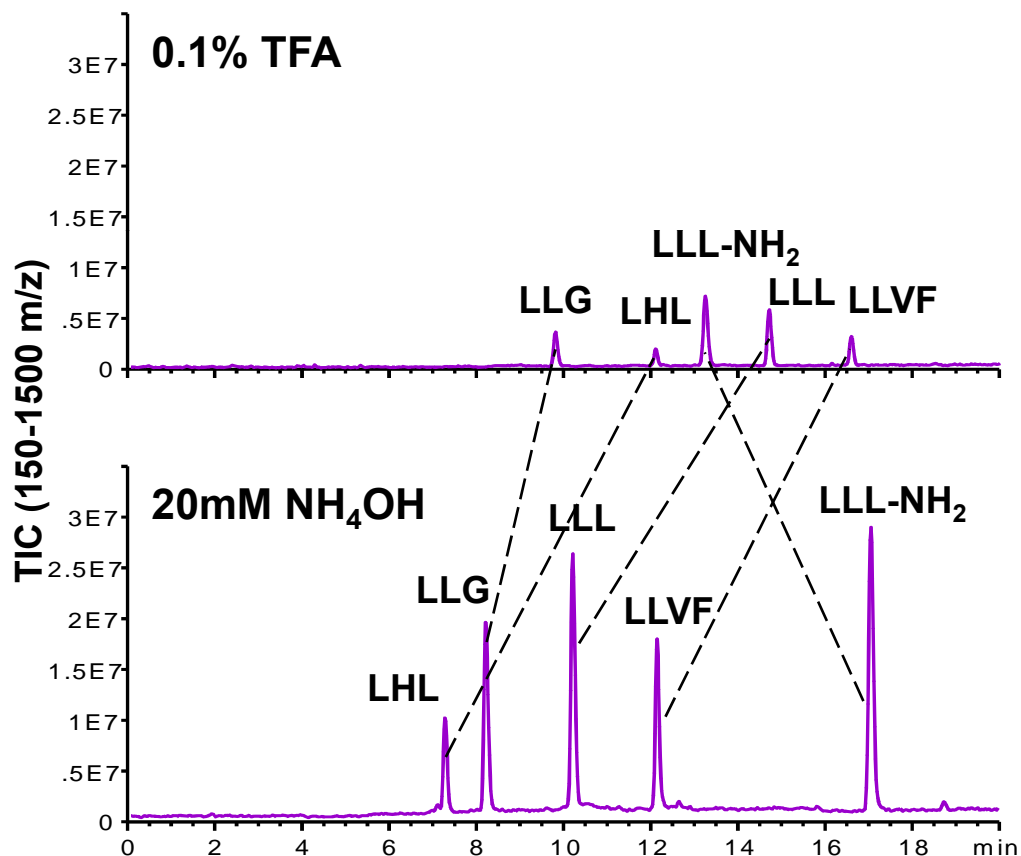
Reference 1

- High pH and room temperature improve peak shape, recovery and change selectivity.



Use Extend-C18 for Different Selectivity at High and Low pH

Comparison of TFA and NH_4OH For Peptide RP-HPLC \ ESI-MS Analysis



Column: ZORBAX Extend C18, 2.1 x 150 mm
 Flow rate: 0.25mL/min
 Temp: 25°C
 Gradient: 5-60% B in 20 min;
 LC/MS: Pos. Ion ESI- Vf 70V, Vcap 4.5 kV,
 N2- 35 psi, 12L/min, 300°C
 4 μL (50 ng each peptide);

TFA Conditions:

A- 0.1% TFA in water
 B- 0.085% TFA in 80% AcN

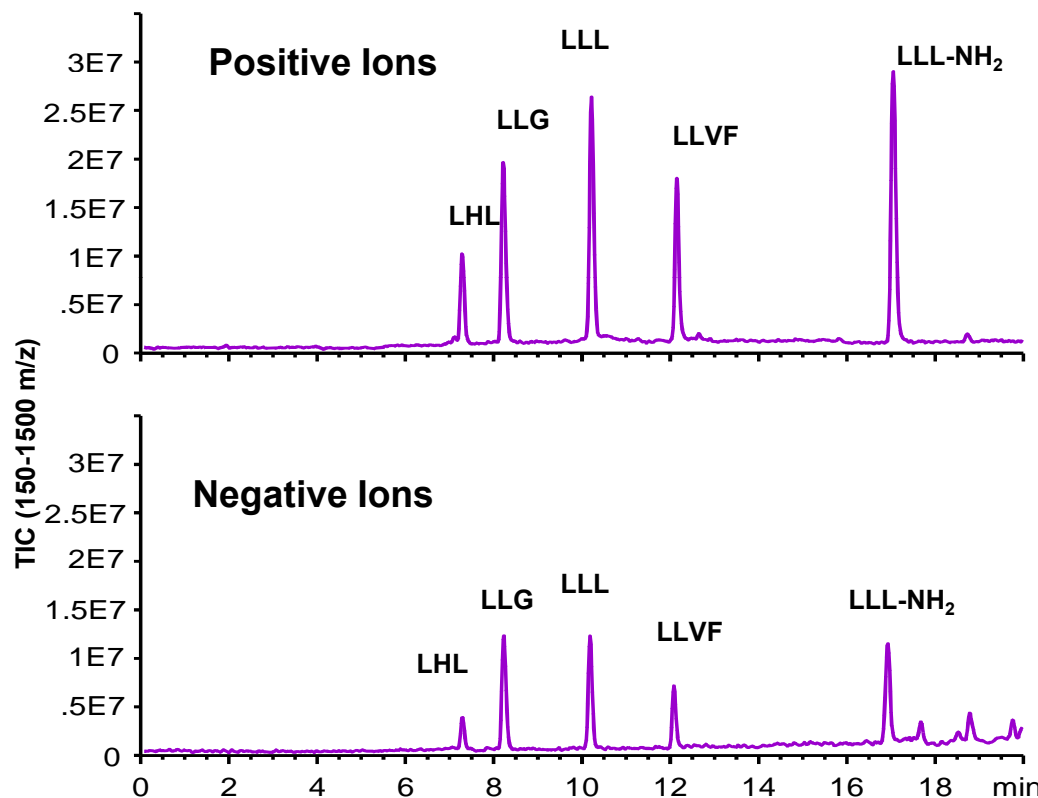
NH_4OH Conditions:

A- 20 mM NH_4OH in water
 B- 20 mM NH_4OH in 80% AcN

Reference 5



Peptide RP-HPLC/ESI-MS Using NH_4OH Mobile Phase Yields Positive and Negative Ion Spectra



Column: ZORBAX Extend C18
 2.1 x 150 mm, 5 μm
Flow rate: 0.25mL/min
Temp: 25°C
Gradient: 5-60% B in 20 min
LC/MS: Pos. Ion ESI- Vf 70V, Vcap 4.5 kV,
 N2- 35 psi, 12L/min, 300°C
Sample: 4 μL (50 ng each peptide)

- The Extend-C18 column is ideal for LC/MS of proteins and peptides at high pH
- Both positive and negative ion MS are possible with NH_4OH mobile phase.



Improved Resolution and Reduced MS Noise at High pH

LC/MS Analysis of Angiotensin on Extend-C18

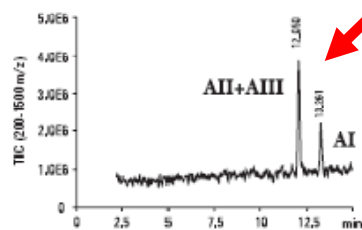
Column: ZORBAX Extend-C18
773700-902
2.1 x 150mm, 5µm

Mobile Phase: Acidic Conditions: A: 0.1% TFA in water B: 0.085% TFA in 80% acetonitrile (ACN)
Basic Conditions: A: 10 mM NH₄OH in water B: 10 mM NH₄OH in 80% ACN

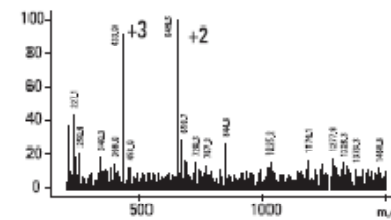
Flow Rate: 0.2 mL/min
Gradient: 15-50% B in 15 min
Temperature: 35°C

MS Conditions: Pos. Ion ESI- Vf 70V, Vcap 4.5 kV, N2- 35 psi, 12 L/min., 325°C
Sample: 2.5 µL sample (50 pmol each)
Angiotensin I, II, III

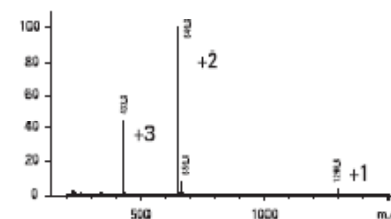
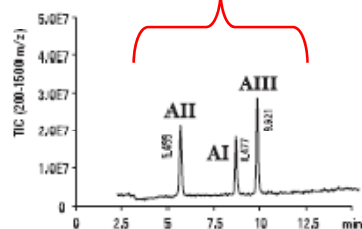
Reference: B.E. Boyes. Separation and Analysis of Peptides at High pH Using RP-HPLC/ESI-MS, 4th WCBP, San Francisco, CA Jan 2000.



AI and AII elute together at lower pH



AI, AII and AIII all resolved at high pH



Both small and large peptides demonstrate selectivity changes at high and low pH. At high pH, due to a change in charge, all three Angiotensins can be resolved. In addition, the spectral clarity of Angiotensin I is dramatically improved at high pH with the ammonium hydroxide mobile phase. The Extend-C18 column can be used for the analysis of small peptides at high pH as well.

High efficiency and good recovery of hydrophobic peptides at high pH.
Ideal for LC/MS with ammonium hydroxide-modified mobile phase.



High Sensitivity, High Resolution and High Speed LC and LC/MS of Proteins and Peptides

- Higher speed separations of proteins and peptides are possible
- High throughput and high efficiency protein applications require reduced analysis times
- Shorter Columns with Smaller Particles Improve Speed for Gradient Separations

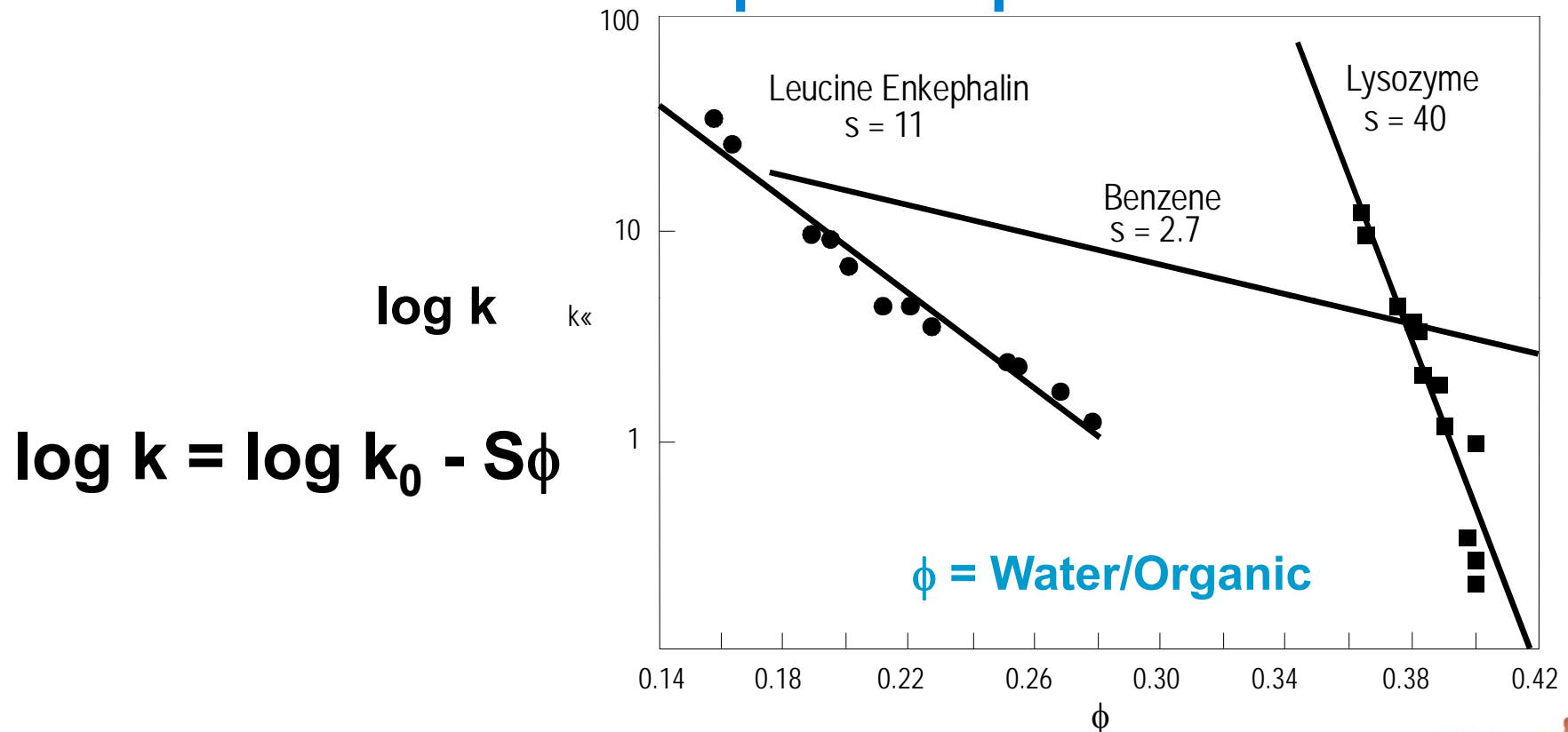




GRADIENT ELUTION



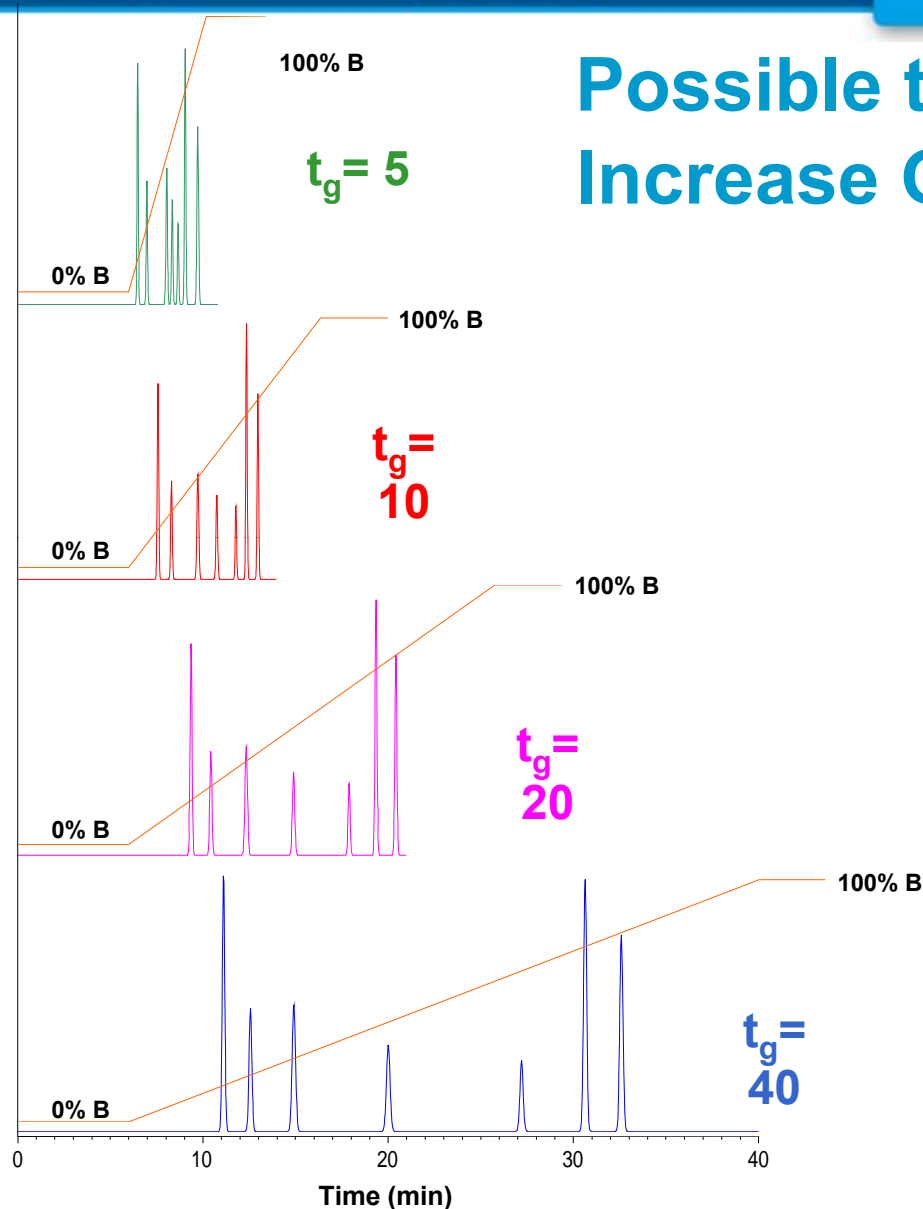
Why Gradients Are Preferred for Protein and Peptide Separations



Large proteins like Lysozyme (14,000 MW) are more sensitive to changes in mobile phase conc of organic modifier (15X) than small molecules like benzene (78 MW) and 4X more sensitive than leucine enkephalin (600 MW).



Possible to Manipulate Terms to Increase Gradient Retention (k^*)



$$k^* \propto \frac{t_g F}{S \Delta\%B V_m}$$

$1/k^* = \text{gradient steepness} = b$

F = flow rate (mL/min.)

t_g = gradient time (min.)

V_m = column void volume (mL)

$\Delta\Phi$ = change in % B solvent

S = constant



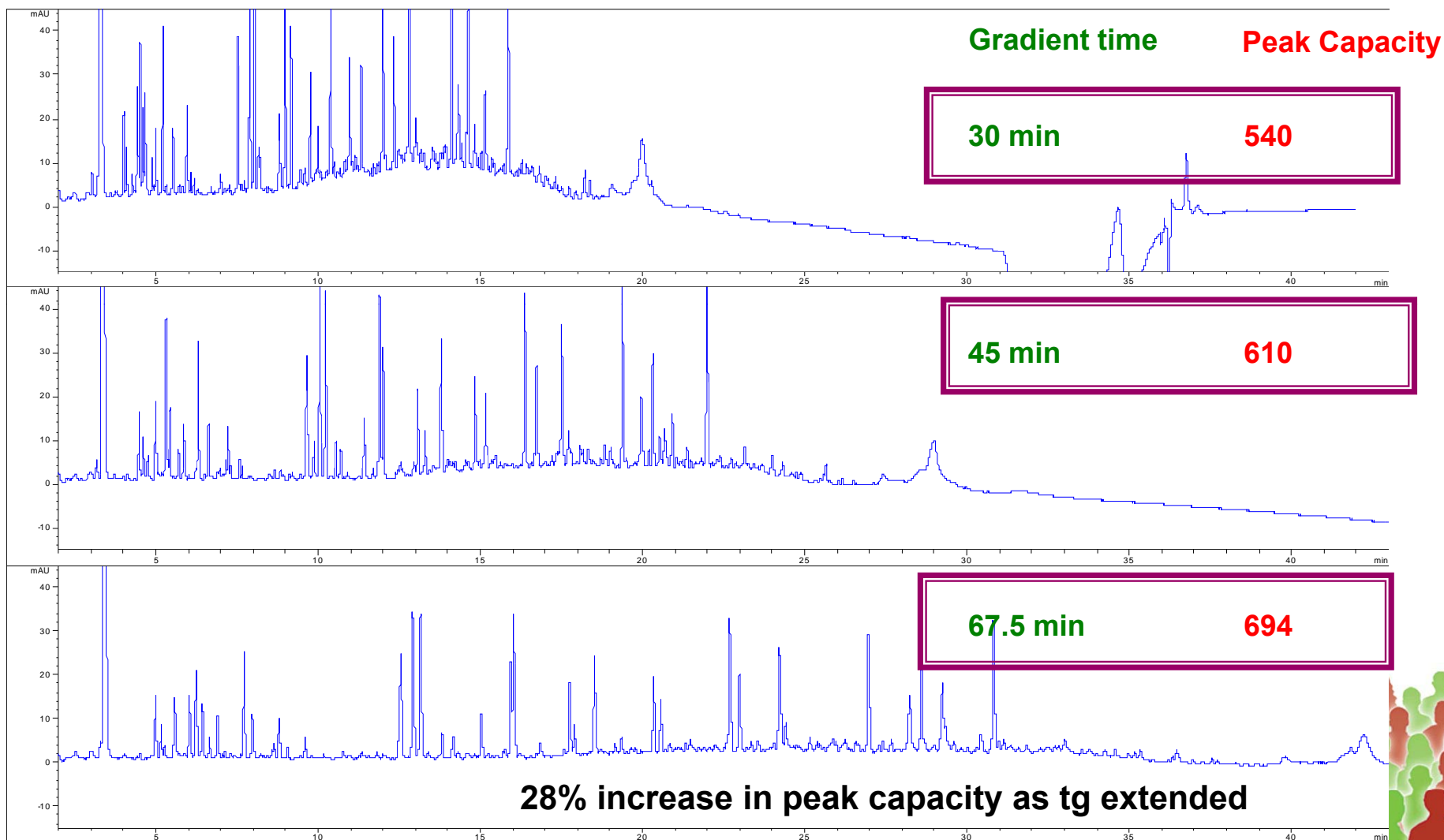
000995P1.PPT



Agilent Technologies

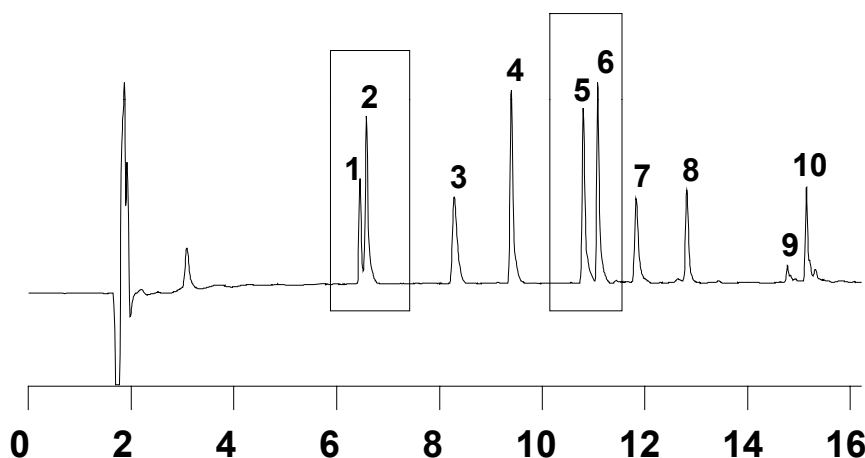
BioColumn Separations
October 8, 2006

Increase in t_G Improves k^* and Peak Capacity

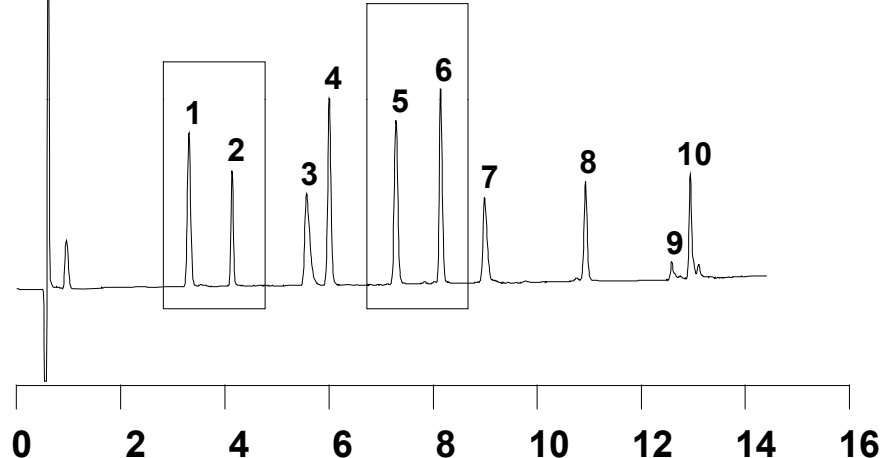


Improving R_s Using Short Column Length (V_m), Smaller Particle Size (N), Constant Time (t_G)

300SB-C8, 4.6 x 150 mm, 5 μ m



300SB-C8, 4.6 x 50 mm, 3.5 μ m



Mobile Phase: A: 95% Water : 5 % ACN, 0.1% TFA
B: 5% Water : 95% ACN, 0.085% TFA
Gradient: 10-60% B in 30 min.

Flow Rate: 1.0 mL / min.

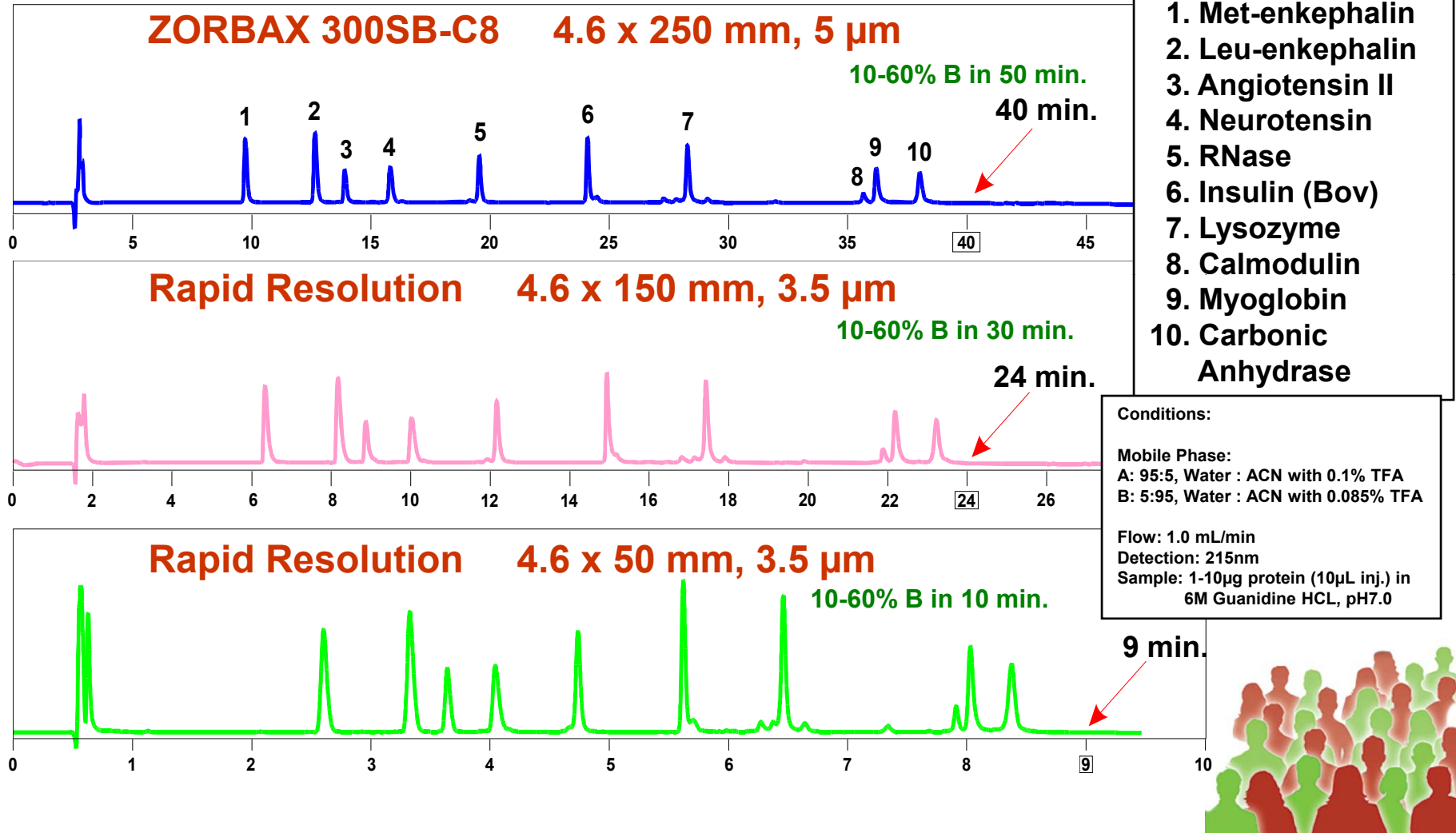
Temperature: Ambient

Sample: 1. Gly-Tyr
2. Val-Tyr-Val
3. [Gln¹¹] Amyloid- β -
Protein Fragm 1-16
4. (TYR8) Bradykinin
5. Met-Enk

6. Leu-Enk
7. Angiotensin II
8. Kinetensin
9. RNase
10. Insulin (Eq.)

000997P1.PPT

Maintain Resolution and Reduce Time by Keeping t_R / V_m Relationship Constant



What Makes Very High Speed HPLC Possible?

- Small particle sizes
 - Short columns (<100mm)
 - Low viscosity mobile phases
 - Optimized HPLC – minimal extra column volume, low volume flow cell, minimal delay volume for gradients
 - Detector set to fast response time
 - High diffusion coefficient of the sample in the mobile phase
- But large molecules have small diffusion coefficients!!**



Slower Diffusion of Large Molecules Limits Speed and Resolution

So, decrease the diffusion time for macromolecules!

Increase the Diffusion Rate

- Elevate operating temperature -- Need stable bonded phase
- Decrease solvent viscosity -- Helps but changes elution

Decrease the Diffusion Distance

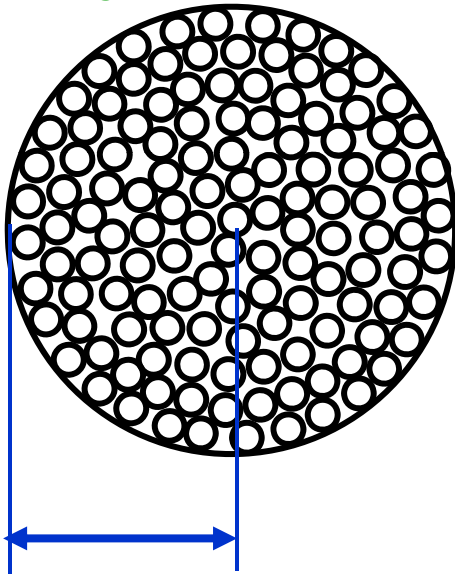
- Develop very small particles (<2-um) -- High back pressure and plugging
- Limit diffusion distance into a particle!



Comparison of Diffusion Distance

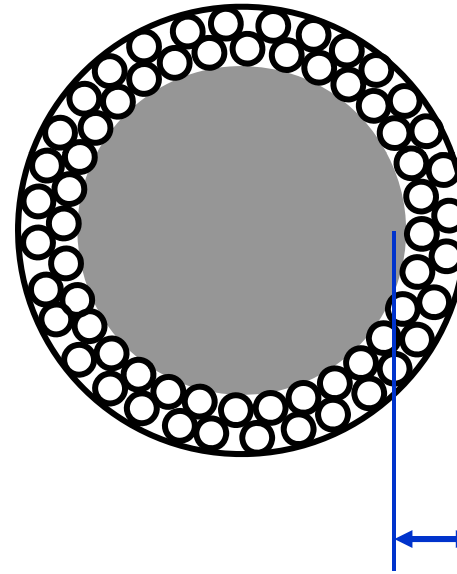
Totally porous silica vs. superficially porous silica

5 μm
Totally Porous Particle



2.5 μm

5 μm
Superficially Porous Particle



0.25 μm

**Required diffusion distance
for a macromolecule
reduced 10 fold !**



Poroshell is Used for Fast Separations at High Flow Rates

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



Flow Rates for Poroshell Columns

Column Internal Diameter	Porous Particle Flow Rate Range	Poroshell Flow Rate Range
2.1 mm	0.1 – 0.3 mL/min	0.3 – 3 mL/min
1.0 mm	30 – 60 μ L/min	0.08 - 0.75 mL/min

- **Very high flow rates can be used effectively with Poroshell columns**



High Flow Rates with 2.1 mm ID Poroshell for High Resolution and Fast Separations

Columns: Poroshell 300SB-C18
2.1 x 75 mm, 5 μ m

Mobile Phase:A: 0.1% TFA

B: 0.07% TFA in ACN

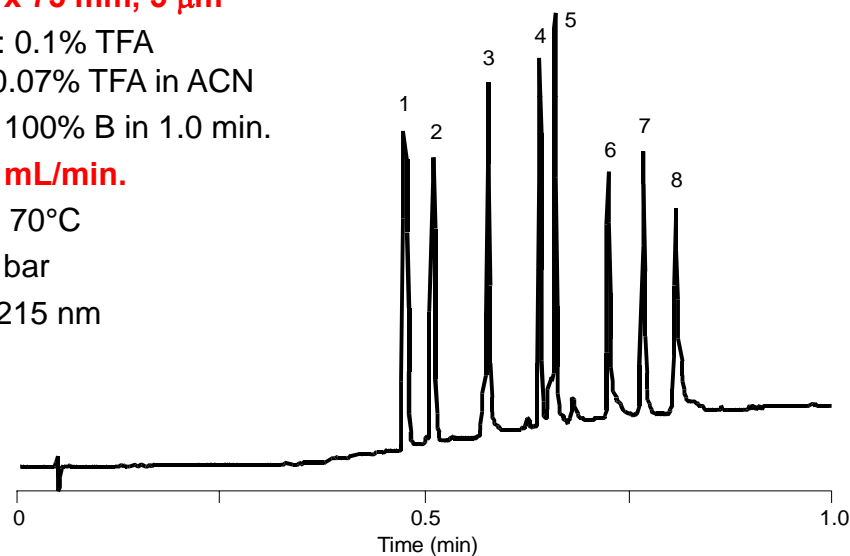
Gradient: 5 – 100% B in 1.0 min.

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



Poroshell Has Different Selectivity from Totally Porous 300SB

- Different selectivity due to the different ratios of bonded phase on the surface.

Agilent 1100 WPS with AutoBypass
B= 5% H₂O, 95% ACN, with 0.07%TFA
Det.: UV 215 nm

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

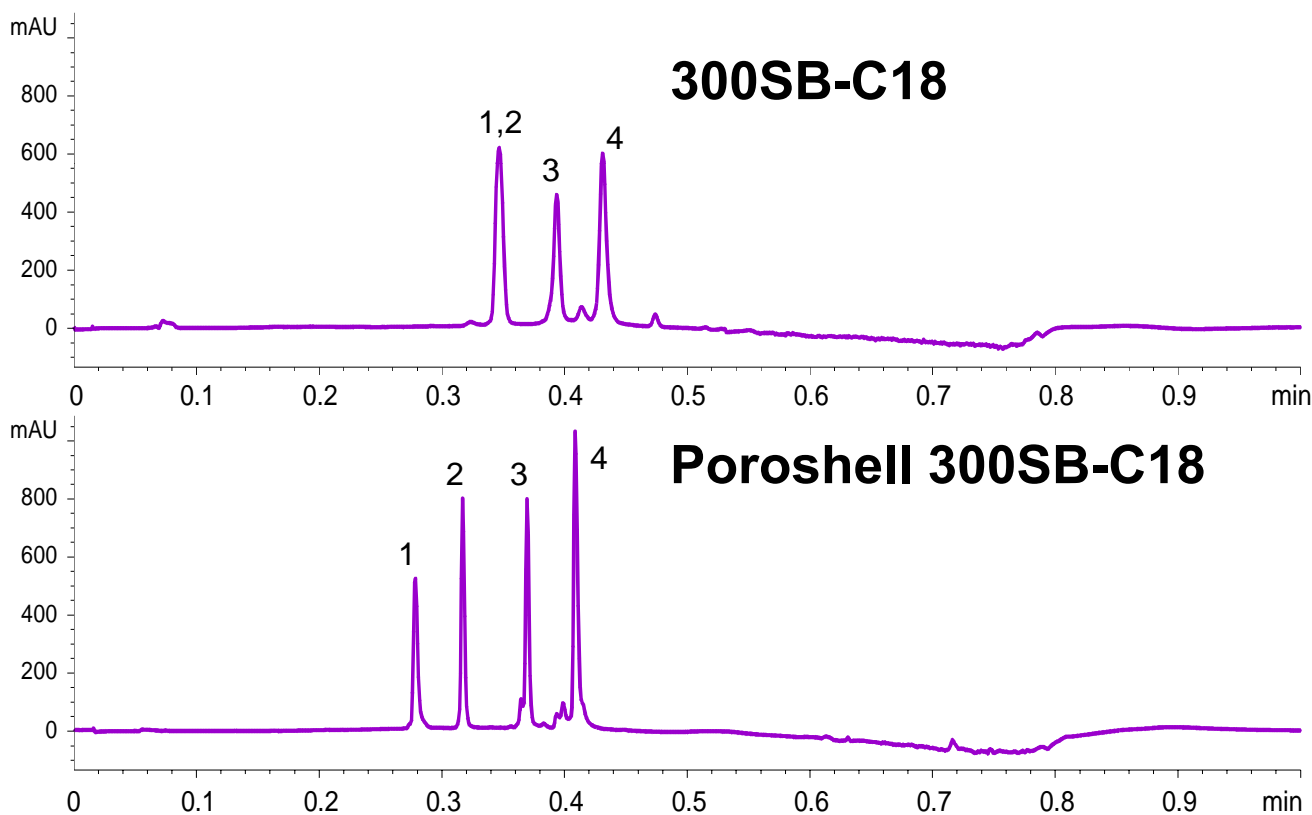
Mobile Phase: A= 95% H₂O, 5% ACN with 0.1%TFA

Gradient: 5 –100%B in 0.67 min

Flow Rate: 3 mL/min

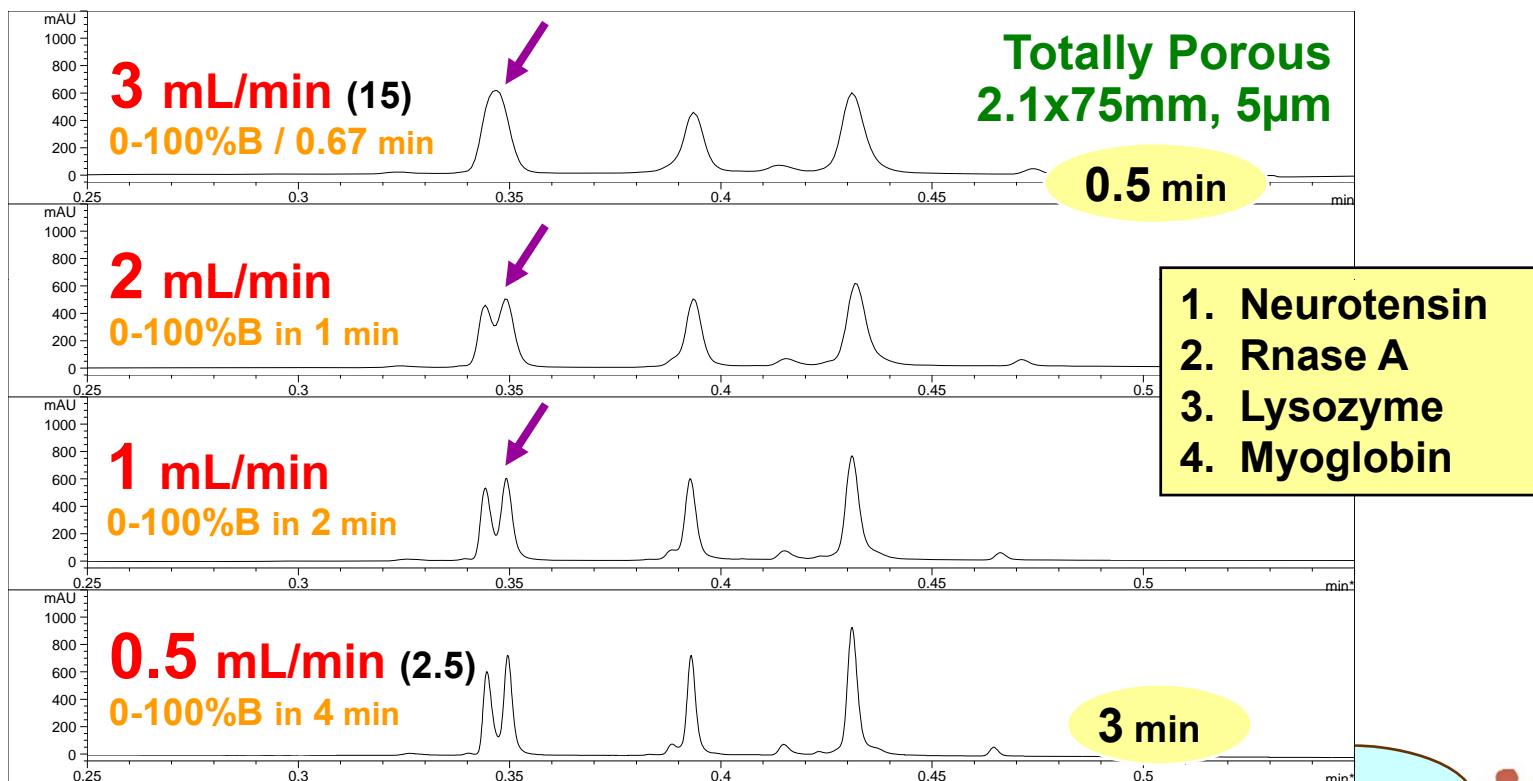
Piston Stroke: 20 μL

Temp.: 70°C



Effect of Increasing Flow Rate in Protein Analysis Using Totally Porous Silica

Resolution Loss



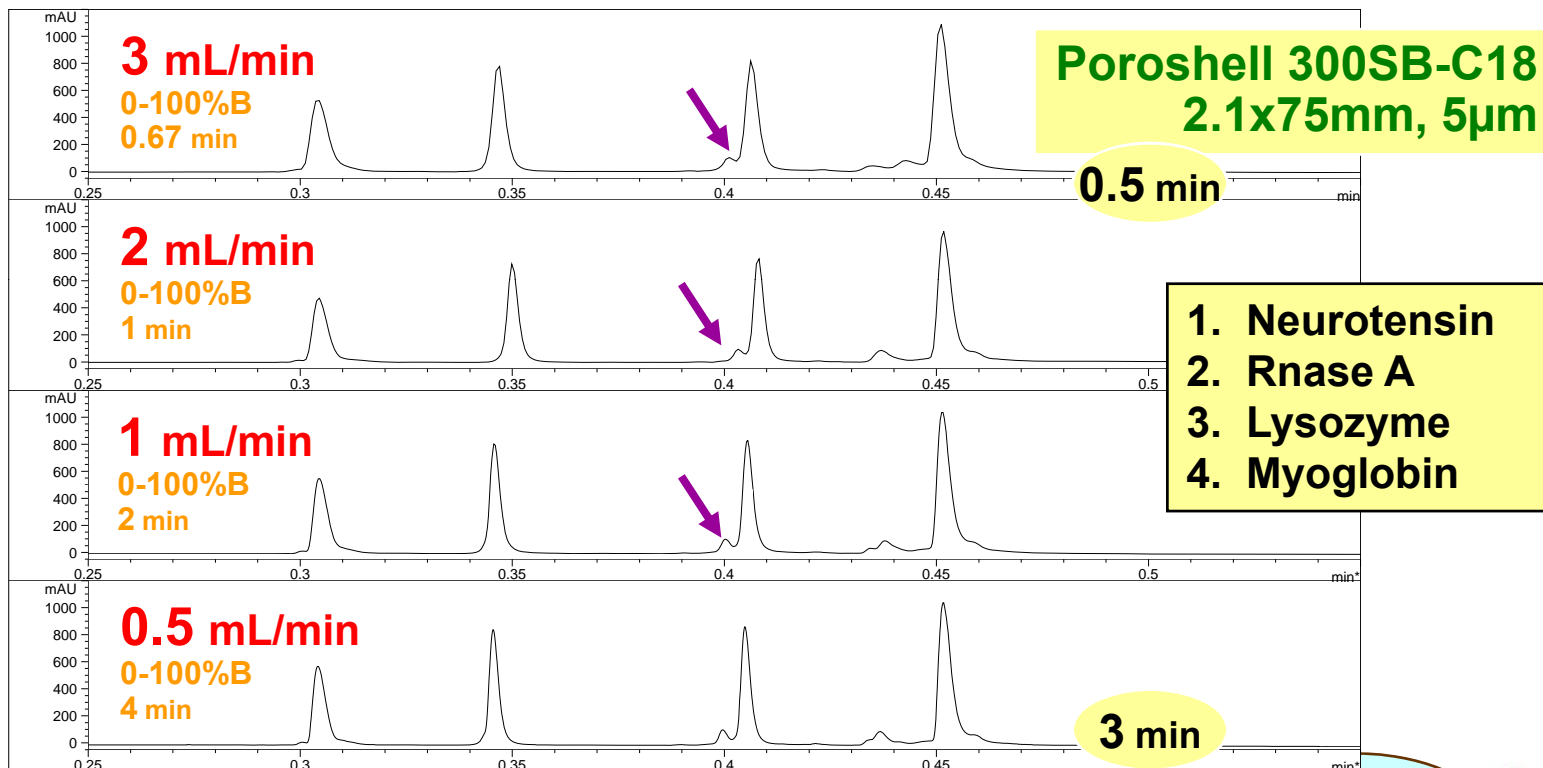
Agilent 1100 WPS with AutoBypass; Piston Stroke: 20 μ L, Temp.: 70°C, Det.: 215 nm
Mobile Phase: A= 95% H₂O, 5% AcN with 0.1%TFA; B= 5% H₂O, 95% AcN, with 0.07%TFA

Aligned

- As flow rate increases, peak width increases and resolution is lost in protein analysis when using totally porous particles!

Effect of Increasing Flow Rate in Protein Analysis with Poroshell

Sustained Efficiency and Resolution



Agilent 1100 WPS with AutoBypass; Piston Stroke: 20 μ L, Temp.: 70°C, Det.: 215 nm
Mobile Phase: A= 95% H₂O, 5% AcN with 0.1%TFA; B= 5% H₂O, 95% AcN, with 0.07%TFA

- As flow rate increases, peak width and resolution are maintained when using superficially porous particles!

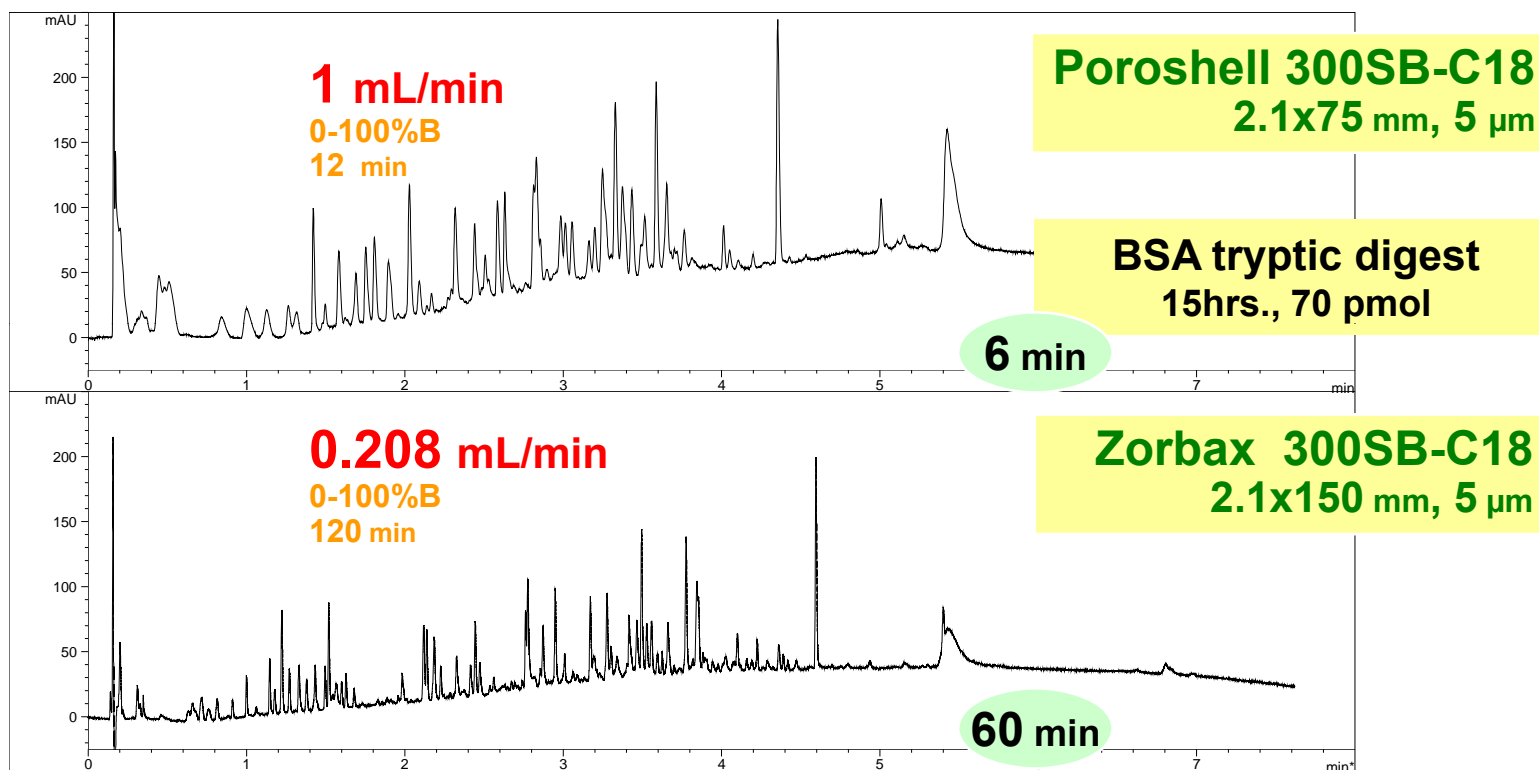
Poroshell is Used for High Resolution

High resolution and fast analysis

- High resolution of protein digests
- High resolution of protein impurities
- High resolution for LC/MS of proteins



High Resolution and Fast Analysis – Separation of a Protein Digest on Poroshell 300SB-C18



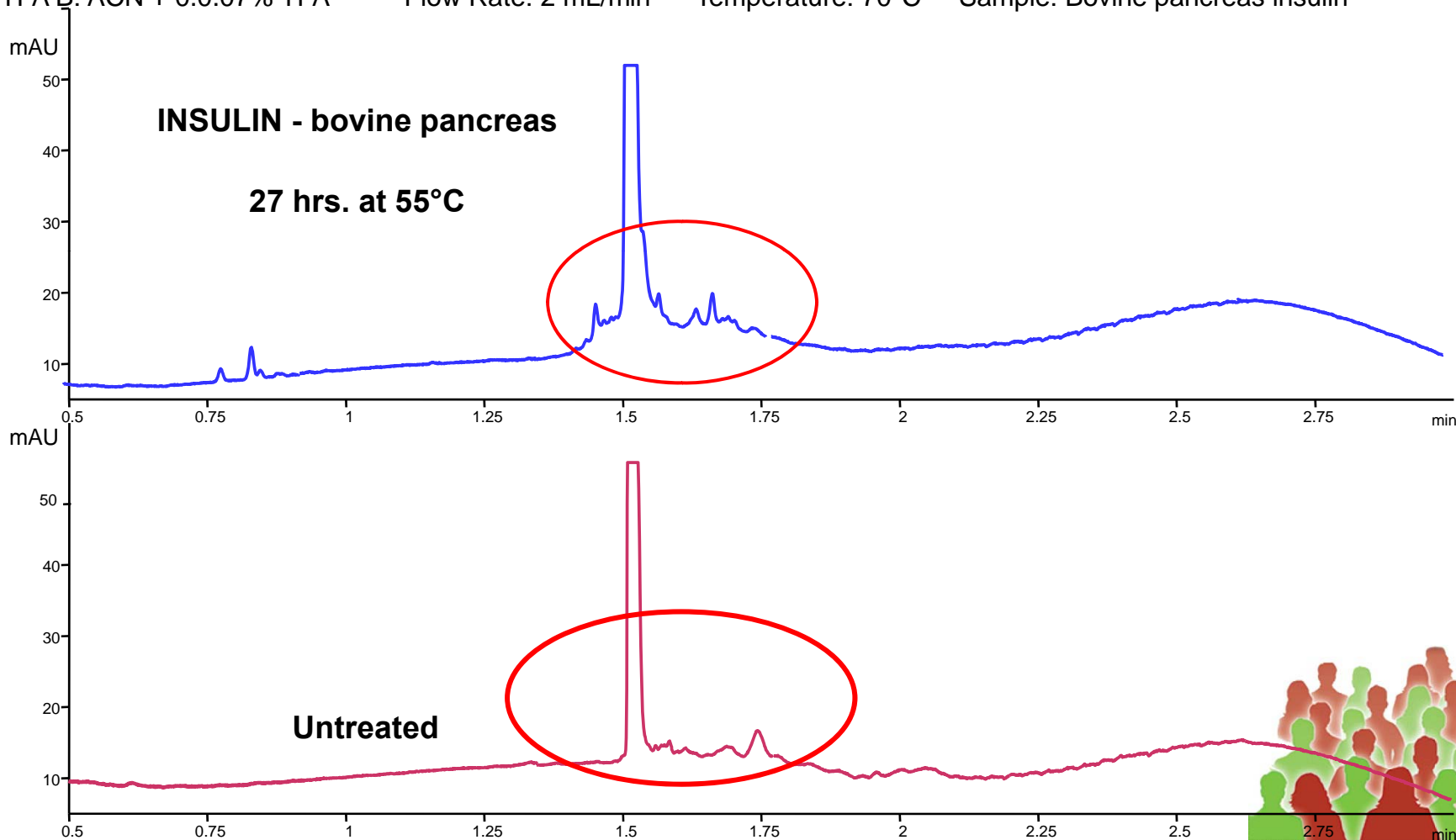
Agilent 1100 WPS with AutoBypass; Piston Stroke: 20 μ L, Temp.: 70°C, Det.: 215 nm
Mobile Phase: A= 95% H₂O, 5% AcN with 0.1%TFA; B= 5% H₂O, 95% AcN, with 0.07%TFA

- **Extremely fast (6 min), high, resolution of BSA tryptic digest, using Poroshell 300SB-C18.**
- **Under typical conditions, a comparable run takes 60min on a totally porous particle.**



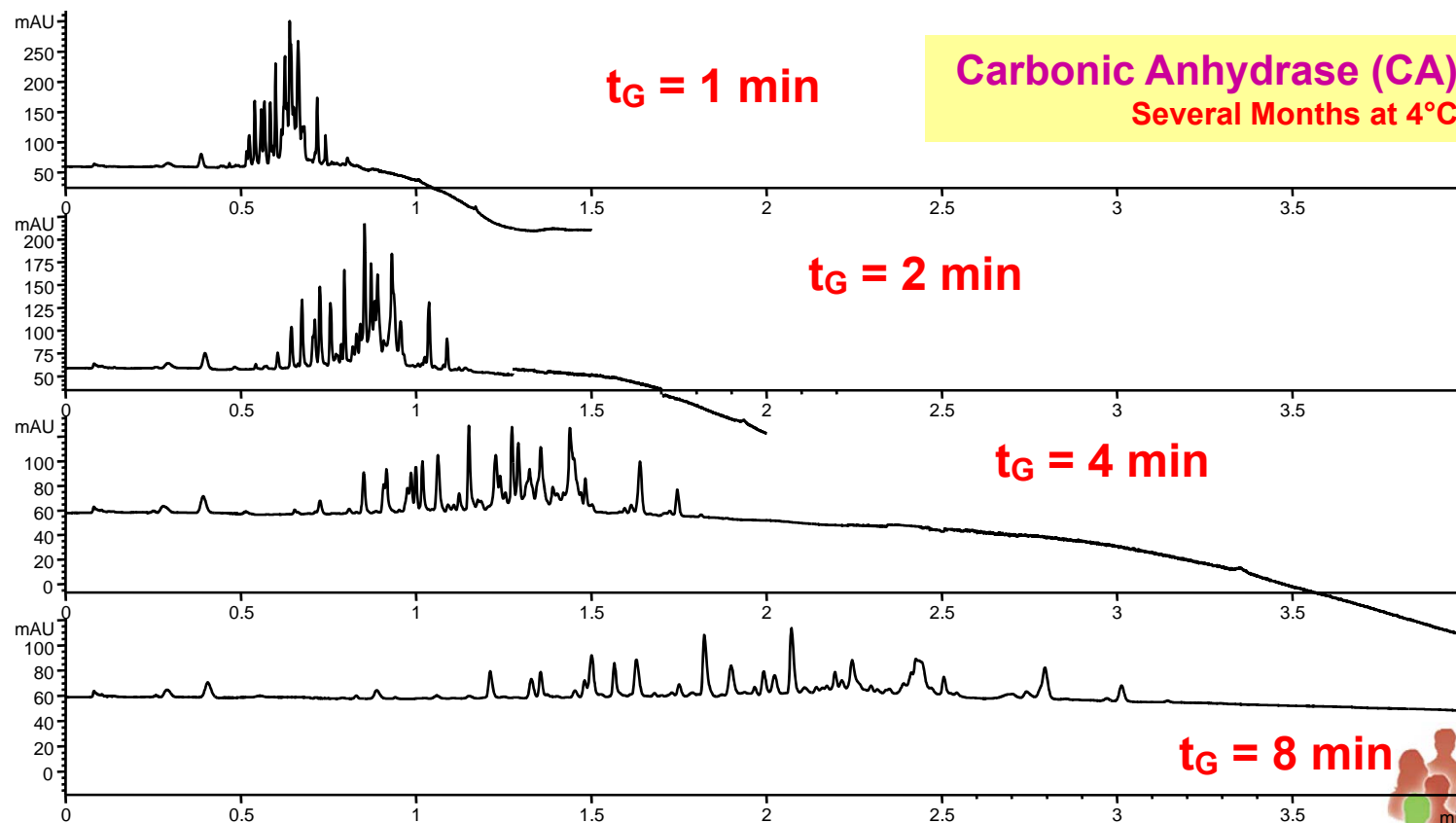
Fast Analysis of Insulin (5.7 kDa) Impurities Using Poroshell 300SB-C18

Column: Poroshell 300SB-C18, 2.1 x 75 mm, 5 μ m Mobile Phase Gradient: 5-100% B in 5 min. A: water + 0.1% TFA B: ACN + 0.007% TFA Flow Rate: 2 mL/min Temperature: 70°C Sample: Bovine pancreas insulin



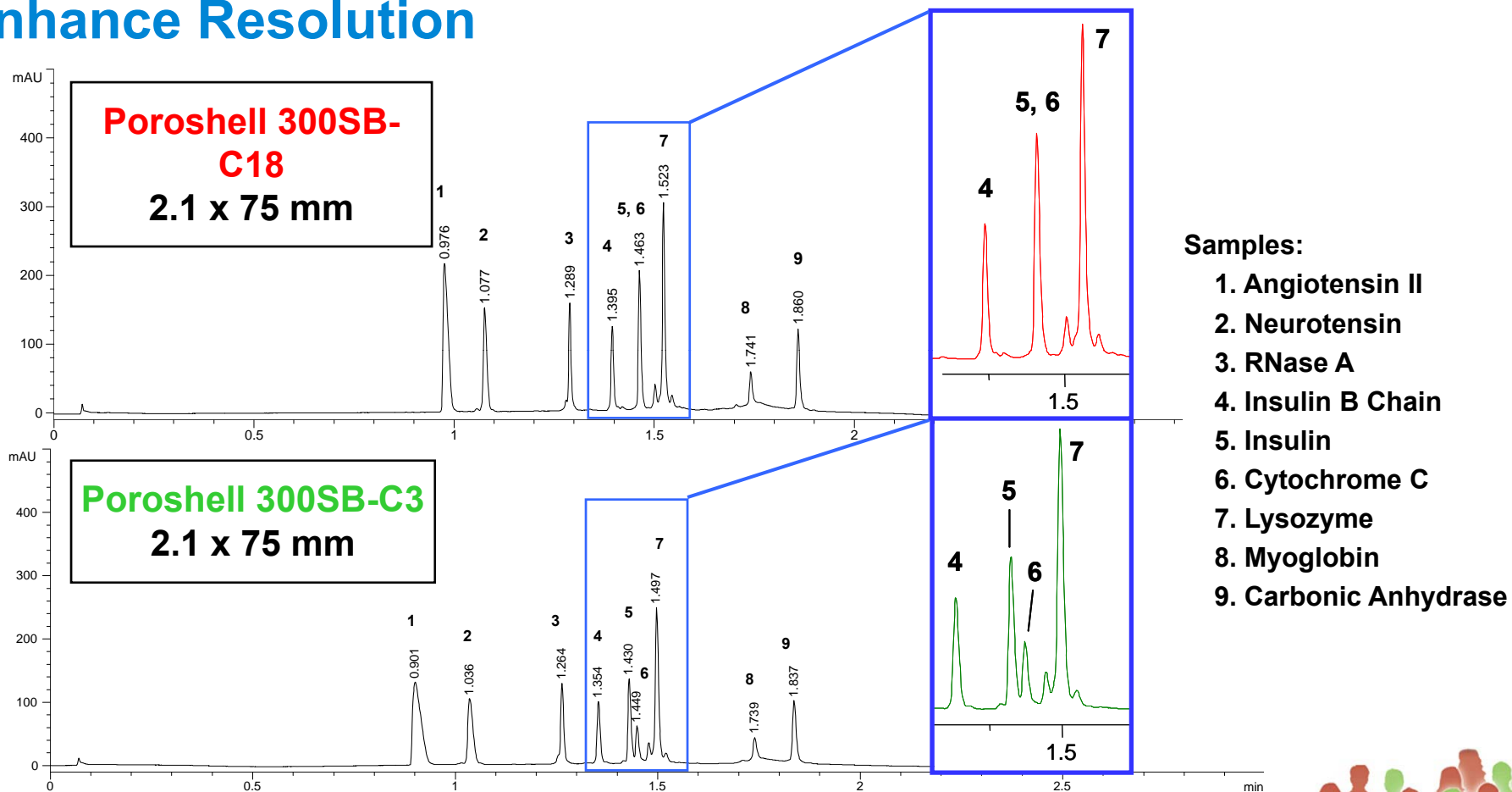
Ultra High Speed Resolution Optimization Using RP-HPLC on Poroshell 300SB-C18

Column: Poroshell 300SB-C18, 2.1 x 75 mm, 5 μ m Mobile Phase Gradient: 5-100% B in t_G min. A: water + 0.1% TFA B: ACN + 0.007% TFA Flow Rate: 2 mL/min Temperature: 70°C Sample: Carbonic Anhydrase 1 mg/mL Injection Volume: 2 μ L



- High resolution is obtained in 2 or 3 min.

Poroshell Bonded Phases Provide Selectivity Options to Enhance Resolution

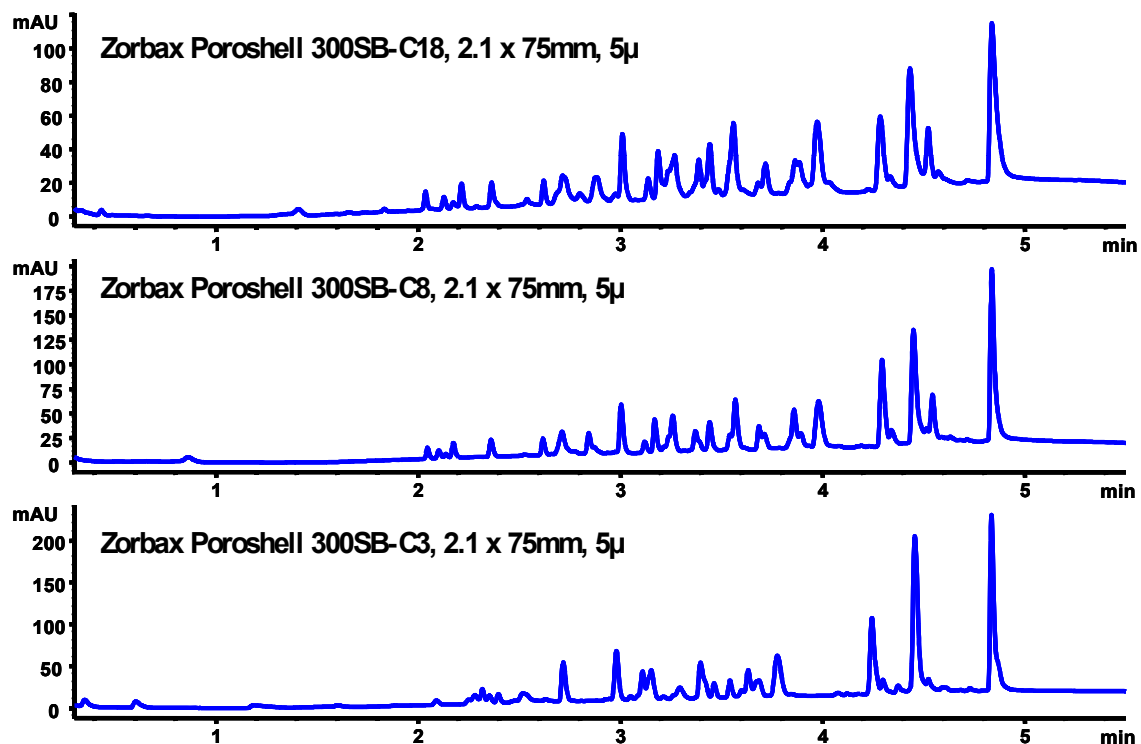


Column: Agilent Poroshell (2.1 x 75 mm); Temp.: 70 °C; Flow: 0.5 mL/min; Det: UV 215 nm
Mobile Phase: A= 0.1% TFA/H₂O, B= 0.07% TFA/ACN; Gradient: 5-100% B in 3.0 min

- Changing from SB-C18 to SB-C3, within the Poroshell family results in resolution of peaks 5 and 6, still in 3 min!

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

Original method – 120 min t_G using a C18 4.6 x 250 mm – 57 peaks detected



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

46 to 48 peaks, 1/20
analysis time

Aligned

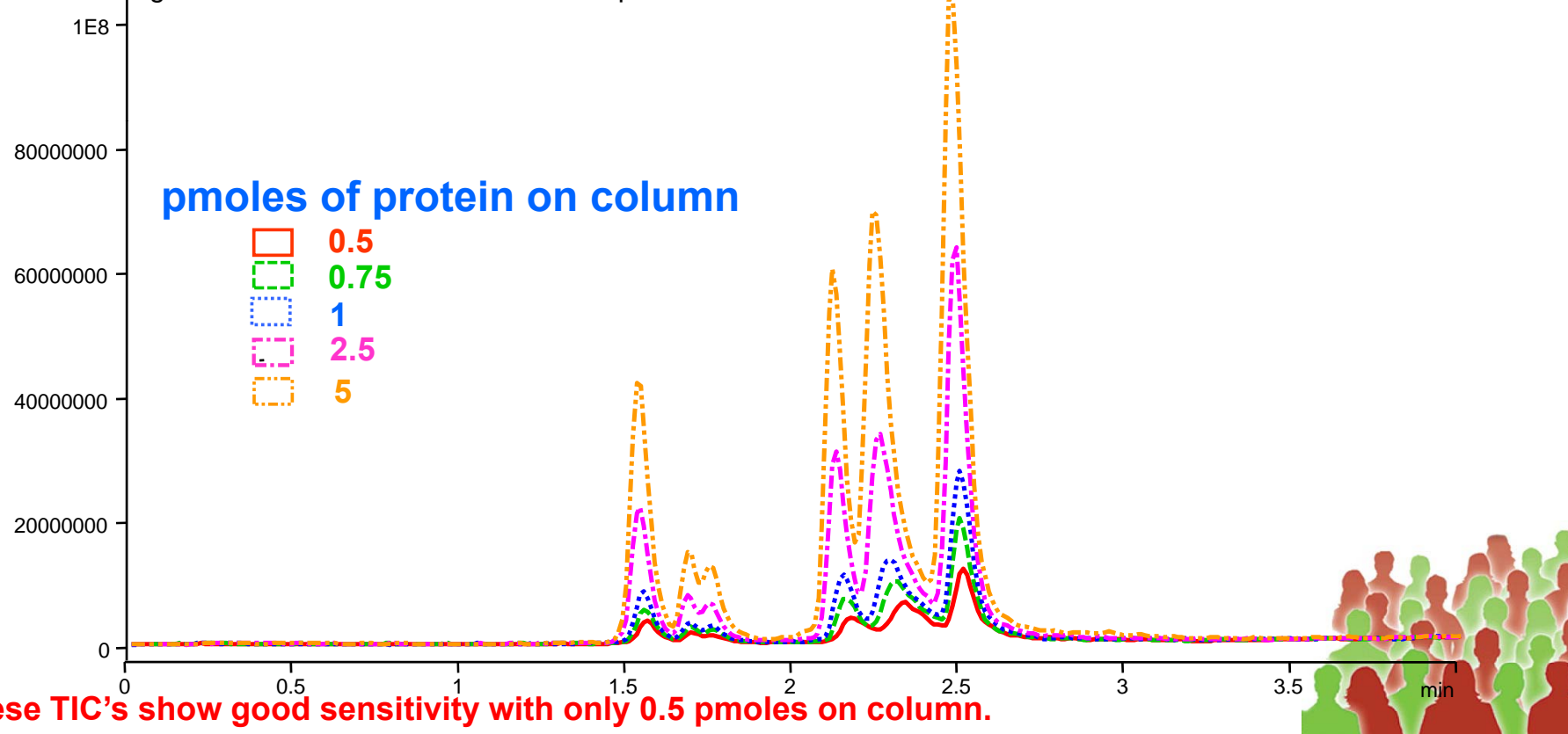
Data courtesy of:
Novartis Pharma,
Biotechnology, Basel
Dr. Kurt Forrer
Patrik Roethlisberger

For more details see
5989-0590EN

• Zorbax Poroshell technology facilitates ultra-fast HPLC analysis of peptides

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

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:** 600 $\mu\text{L}/\text{min}$ **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 voltage:** 140 V **Scan:** 600 – 2500 **Stepsize:** 0.15 amu **Peakwidth:** 0.06 min



Summary



- **Variety of ZORBAX 300 Columns for RP HPLC Separation of Biomolecules**
 - 300SB for Low pH and High Temperature
 - 300 Extend for pH 2 to 11.5 for Enhanced Resolution and Reduced LC/MS Noise
 - 300 Poroshell for High Speed, High Resolution RP HPLC
- **Variety of Bonded Phases, Particle Sizes and Column Sizes**
 - 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.
- **All Columns Compatible with LC/MS “Friendly” Mobile Phases**



Thank you for attending.

To learn more about the sample simplification with the Multiple Affinity Removal System, visit **Proteomics Solution Source** at www.agilent.com/chem/separate1

To reserve your space for the next e-Seminar, register today:

IDENTIFY - Identify, Characterize and Measure Bio-molecules in a Variety of Sample Sources

October 22, 2008 at 1 p.m. EDT

