Reverse-Phase Separation of Proteins, Peptide and Other Biomolecules

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Where is Reversed Phase HPLC Useful in Bio Separations?

• Proteomics
• New “Bio Pharmaceutical” Development
• Analytical Development
• Stability Testing
• Quality Control
BioPharma Proteomics – Peptide Mapping

1. Bio-Reactors producing protein
2. Purification Step 1
3. Purification Step 2
4. Final Pure Protein

Mass Spectrometer + Software ID peptides and verify presence of protein and impurities

1-D and 2-D (Reverse Phase) CAP/NANO ZORBAX 300SB, Extend, Poroshell

Trypsin enzyme digestion of proteins into peptides

Agilent Technologies

October 8, 2006
BioPharma QA/QC and In-Process Control

Bio-Reactors producing protein or antibody

Purification Step 1

Purification Step 2

Final Product Pure

LC/UV monitoring of purification process: Reverse Phase, Ion Exchange, SEC, Affinity

ZORBAX 300SB, Extend, Poroshell
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.
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 µm
  (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 µL/min
- **Temperature**: 50°C
- **Detection**: UV 215 nm
- **Sample**: tryptic digest of rhGH, 2 µL

Separate complex peptide mixtures, even at low pH
What Options are Available if the Standard C18/TFA-ACN Gradient Is Not Sufficient?
Changes in $\alpha$ Can Be the Key to Improved Separation and Resolution

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

*Theoretical Plates*  *Selectivity*  *Retention*
Resolution as a Function of Selectivity, Column Efficiency, or Retention

Selectivity Impacts Resolution Most
- Change bonded phase
- Change mobile phase

\[ R_s = \frac{N^{1/2}}{4} \cdot \frac{(\alpha-1)}{\alpha} \cdot \frac{k'}{(k'+1)} \]

<table>
<thead>
<tr>
<th>Plates:</th>
<th>5000</th>
<th>10000</th>
<th>15000</th>
<th>20000</th>
<th>25000</th>
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</thead>
<tbody>
<tr>
<td>Alpha:</td>
<td>1.10</td>
<td>1.35</td>
<td>1.60</td>
<td>1.85</td>
<td>2.10</td>
</tr>
<tr>
<td>( k' ):</td>
<td>2.0</td>
<td>4.5</td>
<td>7.0</td>
<td>9.5</td>
<td>12.0</td>
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</tbody>
</table>
Comparison of Small Peptide Selectivity Differences on 300SB Bonded Phases

Conditions:
- Columns: ZORBAX 300SB, 4.6 x 150 mm, 5 μm
- 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 μg of each peptide
- Flow Rate: 1.0 mL / min.
- Detection: UV-210nm
Comparison Separation of Large Polypeptides on 300SB Bonded Phases

**Columns:** ZORBAX StableBond 300SB
4.6 x 150 mm, 5 μm

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 μg each protein

1. RNase 6. CDR
2. Insulin 7. Myoglobin
3. Cytochrome C 8. Carbonic Anhydrase
4. Lysozyme 9. S-100β
5. Parvalbumin 10. S-100α

• 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

After 495 mL

After 13680 mL
Recovery of Polypeptides from ZORBAX 300SB Columns

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

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

Example: βAP Separation

Column: ZORBAX 300SB-C18, 4.6 x 150 mm, 5 µm
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 µl injection of 5 µg peptide in 6M Urea/5% HOAc

- βAP(1-38)
- βAP(1-43)* Recovery <10%
- Recovery >70%

Temperature: 25°C, 40°C, 60°C, 80°C

Time (min.)
LC/MS Sensitivity vs. Mobile Phase Modifier
TFA vs. Acetic Acid

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

Column: ZORBAX 300SB-C3
2.1 x 150 mm, 5 μm
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

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 NH₄OH in water
B: 20 mM NH₄OH 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.
High pH Can be Used for Separating Hydrophobic or Other Low-Solubility Peptides

Comparison of Aβ 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.

**TFA Conditions, 80°C**
- A- 0.1% TFA in water
- B- 0.085% TFA in 80%AcN
- 29-41%B in 30 min.

**NH₄OH Conditions, 25°C**
- A- 20 mM NH₄OH in water
- B- 20 mM NH₄OH in 80%AcN
- 26-38%B in 30 min.

**Column:**
- ZORBAX
  - 300Extend C18
  - 2.1 x 150 mm, 5 μm

**Flow Rate:**
- 0.25 mL/min

**Sample:**
- 5 μL sample (100 pmol each)

Amyloid β Sequences:
- Asp Ala Glu Phe Arg His Asp Ser Gly Tyr
- Glu Val His His Gln Lys Leu 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

- 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 NH₄OH 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

NH₄OH Conditions:
A- 20 mM NH₄OH in water
B- 20 mM NH₄OH in 80% AcN

Reference 5
Peptide RP-HPLC/ESI-MS Using NH₄OH 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:** 4μL (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 NH₄OH 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- V=70V, Vcap=4.5 kV, N2: 35 psi, 12 L/min, 325°C
- **Sample:** 2.5 µL sample (50 pmol each)


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
why gradients are preferred for protein and peptide separations

\[
\log k = \log k_0 - S \phi
\]

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 ($k^*$)

$$\frac{tgF}{S \Delta%B V_m}$$

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

- $F = \text{flow rate (mL/min.)}$
- $t_g = \text{gradient time (min.)}$
- $V_m = \text{column void volume (mL)}$
- $\Delta\Phi = \text{change in % B solvent}$
- $S = \text{constant}$
Increase in $t_G$ Improves $k^*$ and Peak Capacity

28% increase in peak capacity as $t_G$ extended
Improving $R_s$ Using Short Column Length ($Vm$), Smaller Particle Size ($N$), Constant Time ($t_G$)

300SB-C8, 4.6 x 150 mm, 5µm

300SB-C8, 4.6 x 50 mm, 3.5µm

Sample: 1. Gly-Tyr
2. Val-Tyr-Val
3. $[\text{Gln}^{11}]$ 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.)

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
Maintain Resolution and Reduce Time by Keeping $t_G / V_m$ Relationship Constant

**ZORBAX 300SB-C8** 4.6 x 250 mm, 5 µm

1. Met-enkephalin
2. Leu-enkephalin
3. Angiotensin II
4. Neurotensin
5. RNase
6. Insulin (Bov)
7. Lysozyme
8. Calmodulin
9. Myoglobin
10. Carbonic Anhydrase

- Mobile Phase: A: 95:5, Water : ACN with 0.1% TFA B: 5:95, Water : ACN with 0.085% TFA
- Flow: 1.0 mL/min
- Detection: 215nm
- Sample: 1-10µg protein (10µL inj.) in 6M Guanidine HCL, pH7.0

**Rapid Resolution** 4.6 x 150 mm, 3.5 µm

10-60% B in 30 min.

12 min.

**Rapid Resolution** 4.6 x 50 mm, 3.5 µm

10-60% B in 10 min.

9 min.
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-µm) -- 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

<table>
<thead>
<tr>
<th>Column Internal Diameter</th>
<th>Porous Particle Flow Rate Range</th>
<th>Poroshell Flow Rate Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 mm</td>
<td>0.1 – 0.3 mL/min</td>
<td>0.3 – 3 mL/min</td>
</tr>
<tr>
<td>1.0 mm</td>
<td>30 – 60 μL/min</td>
<td>0.08 - 0.75 mL/min</td>
</tr>
</tbody>
</table>

- 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 μm
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

![300SB-C₁₈ Chart]

![Poroshell 300SB-C₁₈ Chart]
**Effect of Increasing Flow Rate in Protein Analysis Using Totally Porous Silica**

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

- 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

- Extremely fast (6 min), high, resolution of BSA tryptic digest, using Poroshell 300SB-C18.
- Under typical conditions, a comparable run takes 60 min 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 μm  
**Mobile Phase Gradient:** 5-100% B in 5 min. A: water + 0.1% TFA  
B: ACN + 0.0.07% TFA  
**Flow Rate:** 2 mL/min  
**Temperature:** 70°C  
**Sample:** Bovine pancreas insulin

**INSULIN - bovine pancreas**

27 hrs. at 55°C

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

Column: Poroshell 300SB-C18, 2.1 x 75 mm, 5 μm  Mobile Phase Gradient: 5-100% B in tG min. A: water + 0.1% TFA B: ACN + 0.0.07% TFA  Flow Rate: 2 mL/min  Temperature: 70°C  Sample: Carbonic Anhydrase 1 mg/mL  Injection Volume: 2 μL

- High resolution is obtained in 2 or 3 min.
Poroshell Bonded Phases Provide Selectivity Options to Enhance Resolution

• 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: 0°C; Detection: VWD, 210 nm; Injection: 10 μl 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

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

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

For more details see 5989-0590EN
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 μL/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 volatage: 140 V  
Scan: 600 – 2500  
Stepsize: 0.15 amu  
Peakwidth: 0.06 min

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

[Graph showing pmoles of protein on column]
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