Logical Method Development for Peptides and Proteins Using RP-HPLC and SEC
Seminar Outline

1. Gel-filtration separations by SEC

2. Developing methods for protein and peptide separations by RP-HPLC
Mechanism of SEC Separation

- Log (Molecular Weight)
- Linear Separation Range
- Void Volume
- Exclusion Volume
- Elution Volume

FLOW
Advantages of Size-Exclusion Chromatography

• Size-Dependent Separations

• Useable with a Wide Variety of Mobile Phases

• Samples Retain Structure and Activity
Rapid SEC and Effect on Resolution

**Flow mL/min**

- 0.25: Rs = 2.3, Run Time = 60 min
- 0.5: Rs = 1.9, Run Time = 15 min
- 1.0: Rs = 1.4, Run Time = 3.5 min
- 2.0: Rs = , Run Time = 
- 5.0: Rs = , Run Time = 

**Run Time**

- 30 min
- 7.5 min
- 0.25
- 0.5
- 1.0
- 2.0
Practical Applications of SEC

- Desalting and Exchange of Sample Buffer
- Separation of Mono-, Di-, Trimer, Larger Aggregates
- Following Progress of a Reaction
- Separation of Reaction Components and Products, (esp., antibodies, fragments, and conjugates).
- Estimation of Molecular Weight
Separation of Albumin Monomer, Dimer and Aggregate

Conditions:
Column: Zorbax GF-250, 9.4 x 250mm
Mobile Phase: 0.2M Sodium Phosphate, 0.1% Sodium Azide, pH 7.0
Flow Rate: Detection: UV 280nm
Temperature: Sample: 1) Aggregate
2) Albumin dimer

Reprinted with publisher’s permission
Time Course of Antibody Cleavage Monitored by SEC

A. Undigested

Conditions:
- Column: Zorbax GF-250, 9.4 x 250 mm
- Mobile Phase: 100 mM phosphate, pH 7.0
- Flow: 1.0 mL / min.
- Det.: UV 225 nm
- Temp.: 30°C
- Sample: A) Undigested

B. Digested

- B) 5 mg Monoclonal IgG, antibody digested 17 hours with pepsin at pH 3.5
- 25 µL injection volume
Separation of Plasminogen in the Presence and Absence of 6-AHA

Conditions:
- Column: Zorbax GF-250, 9.4 x 250mm
- Mobile Phase: 50mM Sodium Phosphate, 100mM KCl, pH 7.1
- Flow Rate: 0.7 mL / min.
- Detection: UV (microvolts)
- Temperature: 30°C
- Sample: A) Plasminogen
          B) Plasminogen + 10mM 6-AHA (aminohexanoic acid)

Reprinted with publisher’s permission.
Separation of Purified Serum Proteins and Total Serum by Coupled Zorbax GF-250 and GF-450 Columns

Conditions:
- Column: Coupled: Guard Column, Zorbax GF-450 column, 9.4 x 250mm
  Zorbax GF-250 column, 9.4 x 250mm
- Mobile Phase: 0.2M Sodium Sulfate, 0.04M sodium Phosphate, pH 6.8
- Flow Rate: 0.4mL / min.
- Detection: Differential Refractive Index
- Temperature: 20°C
- Sample: Approximately 3mg / ml for each protein
  1) Human IgM (pentameric)
  2) Human α-2 macroglobulin
  3) Human IgA (monomeric)
  4) Human IgG
  5) Human Serum Albumin

Dotted Line: total serum
SEC-Section Conclusions

• SEC can be used for a broad range of size-based analyses

• SEC often used in your samples’ native environment (both material going in and material coming out)

• A rugged SEC material should be used for stability and shorter run times
Break Number 1

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

1. Gel-filtration separations by SEC

2. Developing methods for protein and peptide separations by RP-HPLC
Developing methods for protein and peptide separations by RP-HPLC

- Choosing a Silica (structure and purity)
- Pore-Size Selection (80 and 300Å)
- Consensus Mobile Phase
- Bonded-Phase Selection
- Optimize Separation (N, k’, α, Column Configuration, T)
- Alternative Separation Solutions
Silica Types

**Rx-SIL**
( Silica Sol )

**Xerogel**

<table>
<thead>
<tr>
<th>STRUCTURE:</th>
<th>UNIFORM SUB PARTICLES</th>
<th>“SPONGE-LIKE,” POLYMERIC NETWORK</th>
</tr>
</thead>
<tbody>
<tr>
<td>POROSITY (%):</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>SURFACE AREA (M²/G):</td>
<td>100 Å / 150</td>
<td>100 Å / 300</td>
</tr>
<tr>
<td>STRENGTH:</td>
<td>HIGH</td>
<td>WEAK</td>
</tr>
<tr>
<td>HIGH pH RESISTANCE:</td>
<td>GOOD</td>
<td>POOR</td>
</tr>
<tr>
<td>PURITY:</td>
<td>HIGH</td>
<td>LOW - HIGH</td>
</tr>
<tr>
<td>PORE SIZE DISTRIBUTION:</td>
<td>NARROW</td>
<td>BROAD</td>
</tr>
</tbody>
</table>
Chromatographic Improvement Using Highly Purified Zorbax Rx-Sil

Original ZORBAX

Conditions: Flow Rate: 2.0 mL / min.
Mobile Phase: 5% 2-Propanol in Heptane

ZORBAX Rx-Sil (1987)
Developing methods for protein and peptide separations by RP-HPLC

- Choosing a Silica (structure and purity)
- Pore-Size Selection (80 and 300Å)
- Consensus Mobile Phase
- Bonded-Phase Selection
- Optimize Separation \((N, k', \alpha, \text{Column Configuration}, T)\)
- Alternative Separation Solutions
Molecules Must Enter Pores

(A,B) Enter Pores

(C) Will be Excluded
Effect of Pore Size and Molecular Size on Peak Width
Gradient Separations

- Leu Enkephalin (M.W. = 556)
- Angiotensin II (M.W. = 1046)
- Insulin B (M.W. = 3,496)
- Cyt C (M.W. = 12,327)
- RNase (M.W. = 13,684)
- Lysozyme (M.W. = 13,900)
Effect of Pore Size on Sample Load
Gradient Separation

Load (µg injected)

PW 1/2

Angiotensin II (300Å)
Angiotensin II (80Å)
Pore Size Recommendation

\[ \leq 4000 \text{ MW} \rightarrow \text{Use 80Å pore columns to maximize loading capacity and retention} \]

\[ 4000 \text{ to } 500,000 \text{ MW} \rightarrow \text{Use 300Å pore columns to maintain high efficiency. Increase column diameter to increase loading capacity} \]
Break Number 2

- For Questions and Answers
- Press *1 on Your Phone to
- Ask a Question
Developing methods for protein and peptide separations by RP-HPLC

- Choosing a Silica (structure and purity)
- Pore-Size Selection (80 and 300Å)
- Consensus Mobile Phase
- Bonded-Phase Selection
- Optimize Separation ($N$, $k'$, $\alpha$, Column Configuration T)
- Alternative Separation Solutions
Reasons for Consensus Conditions for Protein / Peptide Mobile Phases

**Acetonitrile**
- Low UV Cutoff, 190 nm
- Volatile
- Good solubilization, denaturant

**TFA**
- Low UV Absorbance
- Volatile
- Good solubilization, denaturant
- Acidic - improves peak shape
- Mild anionic ion pair - more retention of lys, other free amines
- Compatible with MS

**TFA / Water : TFA / ACN mobile phase**
- Low pH (0.1% TFA, pH ≈ 1.9) suppresses silanol interactions
- Relatively non-viscous - high efficiency, low pressure

---

Column: 4.6 x 150 mm 300SB-C8
Mobile
Phase: A: 95:5, H₂O : ACN, 0.1% TFA
       B: 5:95, H₂O : ACN, 0.085% TFA
Gradient: 0 - 60% B in 60 min.
Flow: 1 mL / min.
Temp: 35 - 40°C
Developing methods for protein and peptide separations by RP-HPLC

- Choosing a Silica (structure and purity)
- Pore-Size Selection (80 and 300Å)
- Consensus Mobile Phase
- **Bonded-Phase Selection**
- Optimize Separation \( (N, k', \alpha, \text{Column Configuration}, T) \)
- Alternative Separation Solutions
Column-Technology Improvement for Low-pH Separation

HYDROLYTICALLY UNSTABLE
CONVENTIONAL

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{CH}_3 \\
\text{OH} & \quad X - \text{Si} - R_1 \\
\text{OH} & \quad \text{CH}_3 \\
\end{align*}
\]

\[X = \text{Cl, OEt, etc.} \]

\[R_1 = \text{C8, C18, CN, etc.} \]

HYDROLYTICALLY STABLE
STERICALLY PROTECTED
Used in Zorbax StableBond columns

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{CH}_3 \\
\text{OH} & \quad X - \text{Si} - R_1 \\
\text{OH} & \quad \text{CH}_3 \\
\end{align*}
\]

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{CH}_3 \\
\text{OH} & \quad \text{CH}_3 \\
\end{align*}
\]

\[X = \text{Cl, OEt, etc.} \]

\[R_1 = \text{C8, C18, CN, etc.} \]

\[R = \text{isopropyl, isobutyl} \]

End-capping groups are labile at low pH

End-capping groups are not used

Monofunctional Silanes Yield Single Step, Reproducible Reaction
ZORBAX SB-C18 Shows Exceptional Stability At Low pH - High Temperature (pH 0.8, 90°C)

Purge Solvent: 50% methanol/water with 1.0% TFA
Solute: Toluene

Stability of ZORBAX SB-CN at pH 2.0, 50°C

ZORBAX StableBond® Family of Selectivities

Pore-Size Availability

300Å / 80Å

\[
\begin{array}{c}
\text{SB-CN} \\
\text{SB-C3} \\
\text{SB-phenyl} \\
\text{SB-C8} \\
\text{SB-C18}
\end{array}
\]

Bulky diisobutyl (with C18) or diisopropyl (with C8, C3, CN, phenyl) side-chains result in stable long and short-chain monofunctional ligands.
Small-Peptide Selectivity Differences on Different Bonded Phases

**Conditions:**
- **Columns:** ZORBAX 300SB, 4.6 x 150mm
- **Mobile Phase:** Gradient, 0 - 26% B in 30min.
  - A = 0.1% TFA in Water
  - B = 0.1% TFA in Acetonitrile
- **Temperature:** 40°C
- **Sample:** 2µg of each peptide
- **Flow Rate:** 1.0 mL / min.
- **Detection:** UV-210nm
Recovery of Polypeptides from ZORBAX 300SB Columns: Effect of Bonded Phase

- 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

Relative Recovery to 300SB-C18

- Parvalbumin
- Myoglobin
- RNase A
- Insulin
- Lysozyme
- Carbonic Anhydrase
- Calmodulin
Effect of Bonded-Phase Ligand on Recovery of a Synthetic Lipopeptide: Effect of Bonded Phase

Conditions:
15 µL (15 µg) of peptide in 0.1% TFA/DMSO
4.6 mm ID x 150 mm Zorbax columns
1 mL/min, 60°C, 10 - 90% B in 40 min
A – 0.1% TFA/water, B – 0.1% TFA/AcN

- 300SB-CN, Recovery 59%
- 300SB-C8, Recovery 75%
- 300SB-C18, Recovery 89%
Break Number 3

- For Questions and Answers
- Press *1 on Your Phone to
- Ask a Question
Developing methods for protein and peptide separations by RP-HPLC

- Choosing a Silica (structure and purity)
- Pore-Size Selection (80 and 300Å)
- Consensus Mobile Phase
- Bonded-Phase Selection
  - Optimize Separation ($N$, $k'$, $\alpha$, Column Configuration, $T$)
- Alternative Separation Solutions
How Resolution Varies with $k'$, $N$, and $\alpha$
Improving Resolution Using $k^*$

Resolution Relationship for Gradient Elution

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

$\Delta \Phi = \text{change in volume fraction of organic}$

$S = \text{constant}$

$F = \text{flow rate}$

$t_G = \text{gradient time (min.)}$

$V_m = \text{column void volume}$

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

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

Thus, all of these increase $k^*$:

1. Longer gradient time $t_G$
2. Shorter column $V_m$
3. Higher flow rate $F$
4. Shorter organic range $\Delta \Phi$
Resolution Can Often Be Improved Using a Shorter Column Length (Decreasing Vm)

Gradient: 2-75% B in 30 min.
Mobile Phase: A= 5:95, ACN : H₂O 0.1% TFA  
B= 95:5, ACN : H₂O 0.085% TFA
Flow Rate: 1 mL / min.
Injection: 10 µL, 2.6 µg each
Temp: 35°C
UV: 215 nm

1. GLY-TYR
2. VAL-TYR-VAL
3. [GLU]-ß PROTEIN AMYLOID FRAGM 1-16
4. [TYR₈] BRADYKININ
5. MET-ENK
6. LEU-ENK
7. ANGIOTENSIN II
8. KINETENSIN
9. RNASE
10. INS (EQUINE)
Gradient Steepness Effects Retention ($k^*$) and Resolution

$t_G = 5$

$t_G = 10$

$t_G = 20$

$t_G = 40$

0% B

100% B

0 10 20 30 40
Rapid Resolution with Reduced Particle Size

Mobile Phase: A: 95:5, H₂O:ACN with 0.1% TFA, B: 5:95, H₂O:ACN with 0.085% TFA F=1 mL/min, Det.: 215nm, Sample: 1-10µg protein (10µL inj.) in 6M Gu-HCl, pH7.0

**ZORBAX 300SB-C8**

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

**Rapid Resolution**

10-60% B in 50 min.

10-60% B in 30 min.

10-60% B in 10 min.

**Agilent Technologies**

Slide 42

000976P1.PPT
Rapid, Reproducible Analysis of a Protein Sample -- Fast Re-equilibration

1. Met Enkephalin
2. Leu Enkephalin
3. Angiotensin II
4. Neurotensin
5. RNase
6. Insulin (BOV)
7. Cytochrome C
8. Lysozyme
9. Calmodulin
10. Myoglobin
11. Carbonic Anhydrase

ZORBAX 300-SB-C8
Properties - 3.5 µm vs. 5 µm Particle Size

- Surface area \((m^2/g)\)
- Pore size \((80\text{Å} \text{ or } 300\text{Å})\)
- Surface coverage \((\mu\text{M} / m^2)\)
- Selectivity \((\alpha)\)
- Retentivity \((k')\)

Increased Resolution \((R_s)\)
Effect of Particle Size on Peak Width
Calmodulin Tryptic Digest

A = 0.1% TFA in H₂O,  B = 0.095% TFA in 80% ACN/ H₂O; 5-60%B / 90 min
Flow=0.75 mL/min; 45 µL inj. (75 µg); 210 nm

5.0 µm, 35°C
P = 110 bar

3.5 µm, 35°C
P = 170 bar

Agilent Technologies
Effect of Temperature on Peak Width

Calmodulin Tryptic Digest

A= 0.1% TFA in H₂O,  B= 0.095% TFA in 80% ACN/ H₂O; 5-60%B / 90 min
Flow=0.75 mL/min; 45 µL inj. (75 µg); 210 nm

3.5 µm, 35°C  
P = 170 bar

3.5 µm, 85°C  
P = 88 bar

Zorbax  
80Å SB-C18

Agilent Technologies
Using Higher Temperatures

Reduces Analysis Time
May Change Selectivity
May Increase Recovery
New Selectivities Using Elevated Temperature and Stable, Short-Chain Bonded Phases
Zorbax 300SB-C3

Ambient

35° C

45° C

60° C

1. Leucine Enkephalin
2. Angiotensin II
3. RNase A
4. Insulin (BOV)
5. Cytochrome C
6. Lysozyme
7. Myoglobin
8. Carbonic Anhydrase

Mobile Phase:
A: 5:95 ACN : Water with 0.10 % TFA (v/v%);
B: 95:5 ACN : Water with 0.085% TFA (v/v%)

Gradient: 15-53 % B in 20 minutes, postime: 12 min.
Flow: 1.0ml/min, Protein concentration: 2-6µg, Inj. vol.: 10µL, UV/VIS=215 nm, Temp.: 35 °C
Effect of Column Temperature on βAP Peptide Separation

ZORBAX 300 SB-C18 (4.6 x 150 mm)
1 ml/min.; 20-45% B / 35 min.; A=0.1% TFA in H2O, B=0.09% TFA in ACN

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Absorbance (210 nm)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>βAP(1-38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>βAP(1-43)*</td>
<td>Recovery &lt;10%</td>
</tr>
<tr>
<td>40°C</td>
<td>10 µl injection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µg peptide in</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6M Urea/5% HOAc</td>
<td></td>
</tr>
<tr>
<td>60°C</td>
<td>10 µl injection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µg peptide in</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6M Urea/5% HOAc</td>
<td></td>
</tr>
<tr>
<td>80°C</td>
<td>10 µl injection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µg peptide in</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6M Urea/5% HOAc</td>
<td></td>
</tr>
</tbody>
</table>

Recovery >70%
Developing Methods for Protein and Peptide Separations by RP-HPLC

- Choosing a Silica (structure and purity)
- Pore-Size Selection (80 and 300Å)
- Consensus Mobile Phase
- Bonded-Phase Selection
- Optimize Separation ($N$, $k'$, $\alpha$, Column Configuration, $T$)
- Alternative Separation Solutions
Family of Bonded Phases For Best Stability, Peak Shape Across the pH Range

**StableBond**
- Designed specifically for stability at low pH
- Use appropriate buffers (e.g., Tris, Bis-Tris-Propane)
- Non-endcapped

**Eclipse XDB**
- Designed specifically for stability at neutral pH
- Dimethyl-alkyl (C8, C18)
- Proprietary double endcapped

**Bonus-RP**
- Designed for stability at acidic and neutral pH
- Proprietary triple endcapped
- Polar alkyl phase

**Extend**
- Designed specifically for high pH
- Patented bidendate bonded phase
- Endcapped
Reasons for Separating Basic Solutes at Intermediate pH (4-8) or Higher

- Sample components are unstable at low pH
- Basic compounds elute too quickly
- Required band spacings are not found at low pH
- Near the pK_i, greatest band spacing changes occur; moving out of this region makes method rugged
- At high pH, many basic compounds are not ionized and separate without ionic interaction with silanols
Comparison of Angiotensins Separation with TFA and NH4OH

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

Basic Conditions
A- 10 mM NH₄OH in water
B- 10 mM NH₄OH in 80% AcN

Zorbax Extend C18
(2.1 x 150 mm)

HP 1100
MSD: Pos. Ion ESI
Vf 70V, Vcap 4.5 Kv
N₂=35psi, 12L/min.
325°C
Gradient: 15-50%B / 15 min.
0.2 mL/min
Temp: 35°C
Sample: 2.5 µL
(50 pmol each)
Separate Peptides/Proteins at High pH with Low-Noise LC/MS
Comparison of Angiotensin I Mass Spectra With TFA and NH4OH

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

Conditions: 2.5 μL sample (50 pmol); Extend-C18, 2.1 x 150 mm; 0.2 mL/min; 35°C; 15-50% B in 15 min.; Pos. Ion ESI-Vf 70V, Vcap 4.5 kV, N₂ - 35 psi, 12 L/min., 325°C

Basic Conditions
A- 10 mM NH₄OH in water
B- 10 mM NH₄OH in 80%AcN

Note that the +1 ion is visible with the high-pH mobile phase, but buried in the noise with the low-pH mobile phase.
Method Development Summary

- Method development is a logical sequence
  - Choose silica
  - Choose pore size
  - Use consensus mobile phase
  - Choose solubilization solution
  - Choose column chemistry for pH range
  - Optimize separation ($k^*$, $a^*$, N, column configuration, T)
  - Study alternative solutions

- Stable reversed-phase media offer flexibility to use temperature and short-chain bonded phases as selectivity tools

- Use appropriate instrumentation
Break Number 4

• For Questions and Answers
• Press *1 on Your Phone to
• Ask a Question
Wrap-up E-Seminar Questions