Principles and Practices for SEC, IEX for Intact Protein Analysis by UPLC

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Agenda

- Ion-Exchange Chromatography
  - Theory and practice
  - Protein-Pak Hi Res IEX Columns
  - Method Development Strategies

- Size-Exclusion Chromatography
  - ACQUITY UPLC for SEC
    - ACQUITY BEH200 SEC, 1.7 µm Columns
    - ACQUITY BEH125 SEC, 1.7 µm Columns
  - Insulin Analysis
  - Combination of SEC and MS
Ion-Exchange Chromatography

- Separations are based on net surface charge on protein with oppositely charged groups on ion-exchanger.

- Proteins elute from column using either a gradient of increasing salt concentration (most common) or changing pH (less common).
Select buffer pH
Isoelectric Point of a Protein (pI)

Isoelectric point (pI)
Zero net charge at this pH
Select buffer pH
Isoelectric Point of a Protein (pI)

- pH below pI
  Protein has net +ve charge
  pH region **cation** exchange

- pH above pI
  Protein has net -ve charge
  pH range for **anion** exchange
- Select buffer with pKa near to desired pH
- Buffer ions should have same charge as functional groups on packing material (PO$_4^-$ for cation, Tris$^+$ for anion)
Common Customer Concerns

- Reproducibility between columns
- Not getting required resolution from the start
- Recovery and carryover
Protein-Pak Hi Res IEX

<table>
<thead>
<tr>
<th>Description</th>
<th>Protein-Pak Hi Res Q</th>
<th>Protein-Pak Hi Res CM</th>
<th>Protein-Pak Hi Res SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion Exchange</td>
<td>Strong Anion</td>
<td>Weak Cation</td>
<td>Strong Cation</td>
</tr>
<tr>
<td>Functional Group</td>
<td>Quaternary ammonium</td>
<td>Carboxymethyl</td>
<td>Sulfopropyl</td>
</tr>
<tr>
<td>Matrix</td>
<td>Hydrophilic polymer</td>
<td>Hydrophilic polymer</td>
<td>Hydrophilic polymer</td>
</tr>
<tr>
<td>Particle size (μm)</td>
<td>5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Pore size</td>
<td>Non porous</td>
<td>Non porous</td>
<td>Non porous</td>
</tr>
<tr>
<td>i.d. x L (mm)</td>
<td>4.5 x 100</td>
<td>4.5 x 100</td>
<td>4.5 x 100</td>
</tr>
<tr>
<td>Counter ion</td>
<td>Cl⁻</td>
<td>Na⁺</td>
<td>Na⁺</td>
</tr>
<tr>
<td>pH range</td>
<td>3-10</td>
<td>3-10</td>
<td>3-10</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>10-60</td>
<td>10-60</td>
<td>10-60</td>
</tr>
<tr>
<td>pKa</td>
<td>10.5</td>
<td>4.9</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Flow Rates: 0.3-0.6 mL/min 0.5-1.4 mL/min 0.5-1.4 mL/min

\[^{1}\text{Approx Protein Binding Capacity in mgs per column (i.e., BSA for Hi Res Q Column, Lysozyme for Hi Res CM and Hi Res SP Columns)}\]

58 33 25

\[^{1}\text{For optimal resolution of complex samples, do not exceed 20% of the column’s protein binding capacity.}\]
Attributes of Protein-Pak™ Hi Res IEX Columns

- Multi-layered network of ion-exchange groups (SP, CM or Q)
  - Effective diffusion and binding
  - High sample loading and resolution
  - Minimal non-desired interactions

- No MW limitations: non-porous material

- QC tested with protein samples for batch-to-batch reproducibility

- High chemical stability: hydrophilic, polymer-based IEX particles
  - Wide pH range (3-10)
  - High salt concentrations (1M)
  - Standard pressures (up to 1450 psi for CEX and 2175 psi for AEX)
  - Can be cleaned with aggressive washing

- eCord enabled for data tracking
Strategies to Developing an Ion-Exchange Protein Separation

- Selectivity is most conveniently optimized with pH
- Retention is optimized by adjustment of ionic strength
- Changing buffer and counter ion may improve selectivity
- Methods may require adjustment if the temperature is changed
Effect of pH on Selectivity

**pH 6.6**

1. α-Chymotrypsinogen
2. Ribonuclease A
3. cytochrome c

**pH 5.0**

1. α-Chymotrypsinogen
2. Ribonuclease A
3. cytochrome c

- Column: Protein-Pak Hi Res CM 4.6 x 100 mm column
**Fine tune pH**

**pH Effect on mAb Separation**

- **Column:** Protein-Pak Hi Res CM 4.6 x 100 mm column
- **Gradient:** 0.0 - 0.10 M NaCl, 20mM Sodium Phosphate in 40 min
- **Flow:** 0.5 mL/min

**Graphs:**
- **pH 6.4**
- **pH 6.6**
- **pH 6.8**
Effect of Salt Gradient Slope

- Higher salt gradients result in earlier elution of bound proteins
- High salt wash may be needed in shallower gradients to elute tightly bound proteins
- Column: Protein-Pak Hi Res CM 4.6 mm x 100mm

<table>
<thead>
<tr>
<th>Protein</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>1</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>2</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>3</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>4</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5</td>
</tr>
</tbody>
</table>

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Effect of Salt Gradient Slope: Ovalbumin Variants

- Longer gradient: increased resolution, lower sensitivity
- Column: Protein-Pak HI Res Q, 4.6 x 100 mm
Buffer can alter selectivity and retention of proteins at same pH (6)

- Column: Protein-Pak Hi Res CM 4.6 x 100 mm column
Counter ion may change selectivity and retention of proteins

- Effects tend to be minimal
Temperature Effects

- Changes may be similar to those observed with pH change
- Column: ProteinP-ak Hi Res CM 4.6 x 100 mm column
IEX Summary

- Protein-Pak Hi Res IEX column benefits
  - Consistent batch-to-batch performance (tested with protein standards)
  - Minimal column related carryover
  - Stable over a wide pH range

- Method Parameters to optimize are
  - Selectivity is most conveniently optimized with pH
  - Retention is optimized by adjustment of ionic strength
  - Changing buffer and counter ion may improve selectivity
  - Methods may require adjustment if the temperature is changed
Agenda

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  - Theory and practice
  - Protein-Pak Hi Res IEX Columns
  - Method Development Strategies

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    - ACQUITY BEH125 SEC, 1.7 µm Columns
  - Insulin Analysis
  - Combination of SEC and MS
Common Customer Concerns

- Column-to-column reproducibility
  - Changes in retention time
  - Changes in spacing between peaks
  - Changes in resolution
- Column lifetime
  - Peak shape deteriorates over time
  - Increased pressure
  - Changes in resolution
- Tailing of specific proteins
- Resolution
- Throughput
UPLC-SEC vs HPLC-SEC of mAb monomer and aggregates

ACQUITY BEH200 SEC, 1.7 µm 4.6 x 300 mm

HPLC 100% Silica-Diol SEC 250Å 5µm 7.8 x 300 mm

2.26 % Aggregate

2.24 % Aggregate

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Larger system dispersion decreases component resolution

**HPLC System**

- BEH200 SEC 1.7um Column (4.6 x 300mm)
- USP Res= 1.37

**Waters ACQUITY UPLC System**

- BEH200 SEC 1.7um Column (4.6 x 300mm)
- USP Res= 2.37
Application Areas

- Determination of protein molecular weight
- Molecular weight range of 10,000 to 450,000 Daltons
- Determination of size heterogeneity in a protein sample
- Quantitation of protein aggregates primarily in therapeutic monoclonal antibodies.
The packing material is based on our patented Bridged Ethyl Hybrid base particle and effective diol bonding, which provide a stable chemistry with minimal secondary interactions.

- Significant reduction of undesired secondary interactions respect 100% Silica-Based Diol coated Columns
BEH200 SEC, 1.7um Column Batch Test

<table>
<thead>
<tr>
<th>Analyte</th>
<th>pI</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Thyroglobulin, 3 mg/mL</td>
<td>4.6</td>
<td>669,000</td>
</tr>
<tr>
<td>2. IgG, 2 mg/mL (Vicam)</td>
<td>6.7</td>
<td>150,000</td>
</tr>
<tr>
<td>3. BSA, 5 mg/mL</td>
<td>4.6</td>
<td>66,400</td>
</tr>
<tr>
<td>4. Myoglobin, 2 mg/mL</td>
<td>6.8, 7.2</td>
<td>17,000</td>
</tr>
<tr>
<td>5. Uracil, 0.1 mg/mL</td>
<td>N/A</td>
<td>112</td>
</tr>
</tbody>
</table>
BEH200 SEC, 1.7um
Batch-to-Batch Reproducibility

Batch 1, Column 1

Batch 1, Column 2

Batch 1, Column 3

Batch 2, Column 1

Batch 2, Column 2
What’s new?

- BEH 125 SEC UPLC Column
  - 15 cm/30 cm/Guard
  - Launched on January 10th
  - Linear range from 1.000 to 80.000 Dalton

<table>
<thead>
<tr>
<th>Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>186006504</td>
<td>ACQUITY UPLC BEH125 SEC 1.7µm 4.6x30 Gd</td>
</tr>
<tr>
<td>186006505</td>
<td>ACQUITY UPLC BEH125 SEC 1.7µm 4.6x150mm</td>
</tr>
<tr>
<td>186006506</td>
<td>ACQUITY UPLC BEH125 SEC 1.7µm 4.6x300mm</td>
</tr>
</tbody>
</table>
Calibration Curves for ACQUITY UPLC BEH200 and BEH125, SEC, 1.7 μm Columns

BEH200, SEC, 1.7μm

Thyroglobulin (~ 669,000 Da)
IgG (~ 150,000 Da)
Conalbumin (~ 75,000 Da)
Rnase A (~ 13,700 Da)
Ovalbumin dimer (~ 88,000 Da)
Ovalbumin (~ 44,000 Da)
Aprotinin (~ 6,500 Da)
Angiotensin II (~ 1,045 Da)

BEH125, SEC, 1.7μm

Uracil (~ 112 Da)
Resolution of Proteins and Peptides

- Conditions: 25mM Sodium Phosphate, 150mM Sodium Chloride, pH 6.8, 0.4 mL/min
- BEH125 column provides increased resolution throughout the lower end of the peptide mass range (132 29,000).
Insulin Analyses by Traditional HPLC-SEC vs UPLC-SEC

Waters Alliance HPLC System
Insulin HMWP SEC 10 µm (7.8 x 300mm)

Waters ACQUITY UPLC System
BEH125, SEC, 1.7 µm (4.6 x 300mm)

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Over 800 injections the retention time of the insulin monomer peak and the resolution between insulin monomer and dimer peaks are maintained.
What about SEC – MS?
MS: Xevo G2 Q Tof
- Conditions: 100mM Ammonium Formate, Flow rate: 0.15 mL/min on BEH200 15 cm
- Post UV detection additive: ACN, 0.8% Formic acid
Extracted Spectrum

Intact IgG
MW 148,221
Peak 1

Clip
MW 100,764
Peak 2

Low MW Species
Peak 3

- Deconvoluted molecular weight determined using MaxEnt1
Summary: Waters ACQUITY UPLC SEC System Solution

- New SEC column chemistries based on BEH particles
- True UPLC separation
- Application benefits
  - Reduced secondary interaction
  - Improved physical and chemical column lifetime
  - Improved column-to-column reproducibility
  - Improved resolution
  - Improved throughput
- Synergistic combination of UPLC system and column
- Higher throughput compared to traditional HPLC
Multi-Mode Chromatography
Automated with ACQUITY UPLC H-Class Bio
Mouse Ascites Fluid

SEC
ACQUITY UPLC BEH200
4.6x150mm
0.5mL/min
20mM Na phosphate,
pH 6.8 150mM NaCl

Cation Exchange
Protein-Pak Hi Res SP
4.6x100mm
0.5mL/min
20mM phosphate
pH 6
0-250mM NaCl
20mins

Anion Exchange
Protein-Pak Hi Q
4.6x100mm
0.5mL/min
20mM Tris
pH 7.5
0-250mM NaCl
20mins

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Interesting Application Notes/posters on IEX and SEC

- IEX Method Development of a Monoclonal Antibody and Its Charge Variants [720003836en](www.waters.com) on www.waters.com
- Multi-Mode Analytical Separations of Proteins [720003854en](www.waters.com) on www.waters.com
- Improving the Lifetime of UPLC Size-Exclusion Chromatography Columns Using Short Guard Columns [720004034en](www.waters.com) on www.waters.com
- Technology Brief with SEC and IEX guidelines [720004182en](www.waters.com) on www.waters.com
- Technology Brief on SEC with MS [720004018en](www.waters.com) on www.waters.com
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2010-2011

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