IEX and RP Method Development for the Separation of Biomolecules

Tim Rice
Technical Specialist, Bio-Columns
Look Familiar?

Light Chain

Fab

Heavy Chain

Antigen binding

Disulfide shuffling

Pyroglutamate

Deamidation/oxidation

Glycosylation site

Truncation (lysine)

Hinge

Fc

Truncation (lysine)
Where to Begin?

1. Define the separation goals
2. Choose the mode (IEX or RP)
3. Choose the appropriate column
4. Make sure you have the right equipment.
Choose The Right Equipment

Is bio-inertness a concern?
What type of detector is required?
What pressure is required for the column of choice?
The Agilent 1260 Infinity bio-inert quaternary LC

The New Standard in Bioanalysis

100% bio-inert
✓ Precious sample never touch metal surfaces
✓ Extended pH range 1-13 (shortterm 14)
✓ High salt tolerance: 2M salt, 8 M urea
✓ No stainless steel in mobile phase flow path
✓ New capillary technology

UHPLC capability
✓ 600 bar

Superior Ease of Use and Robustness
✓ Active seal wash
✓ Quaternary buffer mixing
✓ Superior Bio-HPLC columns for biotherapeutic characterization
✓ Column compartment for up to 30 cm columns

The choice for both, bioanalytical and biopurification up to 10 ml/min

Agilent Technologies
Ion Exchange
A IEX Bead and a Protein Meet
IEX Mechanism

Negatively Charged Analyte [Anion]
Attracted to Positive Surface

Anion Exchanger
Stationary-phase Particle

Positively Charged Analyte [Cation]
Attracted to Negative Surface

Cation Exchanger
Stationary-phase Particle
IEX Technique

Technique requires gradients for elution.
Proteins interact with the stationary phase due to charge.
Separation based on differences in degree of charge.
Sample is injected in a mobile phase buffer with a low salt concentration – this binds proteins to the column.
Proteins are typically eluted at constant pH with increasing salt gradients (mobile-phase ionic strength) to displace the proteins from the stationary phase.
Higher charge proteins bind more strongly and an increased salt gradient is needed to elute them.
A typical mobile phase will contain NaCl.
Technique does not denature.
Ion Exchange Chromatography of Proteins

- Ion-exchange chromatography (IEC) discriminates between proteins on the basis of accessible surface charges and their corresponding electrostatic interaction with the column’s stationary phase.

- The degree of protein retention is dependent on the strength and number of interactions.

- The 3-D structure of the protein determines which surface residues will be available to contact the column’s stationary phase.
Ion Exchange Chromatography

How it works
Modern method development relies on a thorough evaluation of all possible experimental parameters, for example:

- Buffer/ionic strength
- Buffer pH
- Salt gradient
- Flow rate
- Temperature
Buffer/Ionic Strength

1. Certain ionic strength required to sustain the column function
2. Usually minimal of 10-20mM required
3. Greater than 30mM may prevent adsorption
4. Commonly used salts are NaCl, KCl, and Acetate
5. Elution salt is typically 400-500mM
Buffer and pH Selection

A. Phosphate, Tris, MES, and ACES buffers are commonly used

B. For cation exchange, pH of 4-7

C. For anion exchange, pH of 7-10
Some Guidelines for IEX

1. The General Rule for choosing a Bio IEX column
   – Acidic proteins: SAX or WAX
   – Basic proteins: SCX or WCX

2. Consider the isoelectric point (pI) of your protein when choosing the pH of your mobile phase:
   – If pH > pI, your protein will have a net negative charge
   – If pH < pI, your protein will have a net positive charge

3. The pH of your starting buffer should be 0.5 to 1 pH unit from your pI
   – above pI for Anion Exchange
   – below pI for Cation Exchange
Typical Starting Buffers

**Anion Exchange:**
Buffer A = 20 mM Tris, pH=8.0
Buffer B = 20 mM Tris, 1 M NaCl, pH=8.0

**Cation Exchange:**
Buffer A = 30 mM sodium acetate, pH=4.5
Buffer B = 30 mM sodium acetate, 1 M NaCl, pH=4.5
For Proteins With High pI and Solubility Issues

Buffer A = 30 mM Ethanolamine, 8M urea, pH=10.0
Buffer B = 30 mM Ethanolamine, 8M urea, 1 M NaCl, pH=10.0
Initial Buffer Concentration Is Important!

Usually minimal of 10-20mM required.

Try several to optimize.
**Effect Of Initial Buffer Concentration**

Columns: Agilent Bio MAb, NP10, 4.6x250mm

Mobile phase: A, Phosphate buffer, pH 7.5  
B, A + 0.1M NaCl

Initial salt:  
A = 20mM phosphate  
B = 10 mM phosphate  
C = 5mM phosphate

Gradient: 15-65%B in 60 min
Flow rate: 0.8 mL/min
Sample: Monoclonal Antibody
Injection: 10 μL (1.5 mg/mL)
Temperature: 25 °C
Detection: UV 214 nm
pH is Very Important To Optimize
Effect of pH on Proteins

Neutral

Basic protein

More arginine, lysine and histidine residues than aspartic acid and glutamic acid residues

Acidic protein

More aspartic acid and glutamic acid residues than arginine, lysine and histidine residues
Effect of pH on Proteins

Reducing pH

Basic protein

Acidic protein

Reducing the pH neutralizes some of the carboxyl groups thereby removing negative charge.
Effect of pH on Proteins

Increasing pH

Basic protein

Acidic protein

Increasing the pH neutralizes some of the basic groups thereby removing positive charge.
### Ion Exchange Sorbents Functionality

<table>
<thead>
<tr>
<th>Ion Exchange Type</th>
<th>Functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong Cation Exchange, SCX</td>
<td>Sulfopropyl, SP</td>
</tr>
<tr>
<td>Weak Cation Exchange, WCX</td>
<td>Carboxyl, CM</td>
</tr>
<tr>
<td>Weak Anion Exchange, WAX</td>
<td>Diethylamino, DEAE</td>
</tr>
<tr>
<td>Strong Anion Exchange, SAX</td>
<td>Quaternary amine, Q</td>
</tr>
</tbody>
</table>

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*Bio-applications Training Program*  
13-17 February 2012
Weak ion-exchange sorbents have a narrower operating pH range than strong ion-exchange sorbents.
Effect of pH on Sorbent

Strong Cation Exchange (SO$_3$H)  Weak Cation Exchange (CO$_2$H)

SCX Titration Curve (unaffected by ionic strength)

WCX Titration Curve (at high ionic strength)

WCX Titration Curve (at low ionic strength)
Effect of pH on Sorbent

Strong Cation Exchange (SO$_3$H)

Weak Cation Exchange (CO$_2$H)

SCX Titration Curve (unaffected by ionic strength)

WCX Titration Curve (at low ionic strength)

WCX Titration Curve (at high ionic strength)
Typical Behaviour of a WCX Sorbent

Uncharged: -CO₂H
No ion exchange capacity

Fully ionised: -CO₂⁻
Maximum ion exchange capacity

High ionic strength eluent (e.g. 100mM)

Weak ionic strength eluent (e.g. 10mM)

In order to ensure the protein is positively charged, eluent pH < pI of protein. This is more difficult with a weak ionic strength buffer.
Cation-exchange: common buffers

- MES (pKa 6.1)
- Phosphate (pKa 7.2)
- HEPES (pKa 7.6)
- Acetate (pKa 4.8)
- Formate (pKa 3.7)
- Citrate (pKa 3.1)

Protein Charge

Positive

Negative

pH

1 2 3

Strong Cation Exchange

Weak Cation Exchange

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Anion-exchange: common buffers

- **Strong Anion Exchange**: DNA, RNA, proteins, peptides, polyanions

- **Weak Anion Exchange**: Saccharides, polyamines, polycarboxylates, polyanions

Buffers:

- TRIS (pKa 8.1)
- Bis-Tris (pKa 6.5)
- Bis-Tris propane (pKa 6.7, 9.1)
- TRIS (pKa 8.1)
- Ethanolamine (pKa 9.5)
- Piperazine (pKa 4.8, 9.7)

Graph showing protein charge and pH range for strong and weak anion exchange.
Typical Behaviour of a WCX Sorbent

Selectivity controlled by pH

- **Retention Time (mins)**: X-axis
- **Salt Content (mM)**: Y-axis
- **pH**: 5.0 to 8.0
- **Salt Conc (mM)**: 0 to 800

**Proteins**
- **Ovalbumin**: Blue line
- **Ribonuclease A**: Red line
- **Cytochrome C**: Green line
- **Lysozyme**: Pink line

- At **pH 5.5**, **Lysozyme did not elute**.

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*Agilent Technologies*
Impact of mobile phase pH

Columns: Agilent Bio MAb, NP10, 4.6x250 mm

Mobile phase: A, 10 mM phosphate; B, A + 0.1M NaCl
pH: A) pH 7.5
     B) pH 7.0
Gradient: 15-65% B in 60 min
Flow rate: 0.8 mL/min
Sample: Monoclonal Antibody
Injection: 10 µL (1.5 mg/mL)
Temperature: 25 °C
Detection: UV 214 nm
Optimize Resolution

Exploit available particle sizes to increase resolution
Manipulate resolution with gradient slope
The Power Of Particle Size

Column: Bio WCX-NP, 4.6x50mm
Buffer A: 20 mM PBS
Buffer B: A+1.0 M NaCl
Gradient: 0-100%B (20 min)
Flow rate: 1.0 mL/min for NP10, NP5, NP3
0.75 mL/min for NP1.7

Sample
1) Ribonuclease A
2) Cytochrome C
3) Lysozyme
Concentration: 1.0 mg/mL
Detector: 280 nm

Average N~80,000 for WCX-NP1.7
Adjust Gradient Slope to Optimize Resolution

Accomplished by changing:

- gradient time
- % change in ionic strength over time
Monoclonal Antibody Method Development

Optimized Method Conditions

Columns: Agilent Bio MAb, NP10, 4.6x250 mm

Mobile phase: A, 10 mM phosphate, pH 7.5
B, A + 0.1M NaCl

Gradient:
A) 15-75%B in 30 min
B) 15-65%B in 30 min
C) 15-55%B in 30 min
D) 15-47.5%B in 30 min
E) 15-40%B in 30 min

Flow rate: 0.8 mL/min
Sample: Monoclonal Antibody
Injection: 10 µL (1.5 mg/mL)
Temperature: 25 °C
Detection: UV 214 nm
Agilent IEX Columns

Cation exchange
- Agilent Bio WCX
- Agilent Bio MAb
- Agilent Bio SCX
- PL-SCX

Non-porous particles with hydrophilic, ionic coating.

Anion exchange
- Agilent Bio WAX
- Agilent Bio SAX
- Agilent PL-SAX

Porous particles with hydrophilic, ionic coating.

Non-porous particles with hydrophilic, ionic coating.
NEW Ion Exchange Columns

**Agilent Bio IEX**

*High Resolution Ion Exchange Columns*

- Non-porous PS/DVB particles (polystyrene divinylbenzene)
- Uniform polymeric coating with SCX, WCX, SAX, WAX layers, designed for protein and peptide separations
- Available in 10 µm, 5 µm, 3 µm, 1.7 µm particle sizes
- High surface area
- High capacity
Quality by Design

In order to assess the affects of buffer strength and pH, many different eluents will need to be prepared.

Each experiment will involve changing the eluent bottles on the HPLC system and thorough flushing before the next experiment can be undertaken …

… or will it?
The Agilent 1260 Infinity bio-inert quaternary LC
The New Standard in Bioanalysis

100% bio-inert
✓ Precious sample never touch metal surfaces
✓ Extended pH range 1-13 (shortterm 14)
✓ High salt tolerance: 2M salt, 8 M urea
✓ No stainless steel in mobile phase flow path
✓ New capillary technology

UHPLC capability
✓ 600 bar

Superior Ease of Use and Robustness
✓ Active seal wash
✓ Quaternary buffer mixing
✓ Superior Bio-HPLC columns for biotherapeutic characterization
✓ Column compartement for up to 30 cm columns

The choice for both, bioanalytical and biopurification up to 10 ml/min
Buffer Advisor Software
What is *BufferAdvisor*?

- *BufferAdvisor* is an independent utility software to calculate pump time tables for ion-exchange chromatography with 1260 Infinity Bio-inert Quaternary Pump.
- Provides the foundation for automated mixing of acid, base, water and salt in IEX.
- Is capable to calculate the selected buffer concentration and pH and high accuracy.
- Suggests for the most suitable stock solutions and provides recipes.
- Has included more than 50 different buffers and more than 20 were experimentally validated.
- Is capable to correct automatically for pH shifts during salt gradients.
- Provides the option to include user defined buffers can be included.
- Generates an xml file as output which can be imported into the method of the 1260 Infinity Bio-inert Quaternary Pump by a button implemented in the driver.
Reverse Phase
Choose The Initial Bonded Phase (The Right Column):

C18 or C8 or C3 or ........
Buy Them All and Try Them All…..

Not really feasible, right?
How do you narrow it down?
Consider Molecular Weight

- Larger molecular weights separate better with smaller ligands
  - Upper Mw of C18 = 70kDa
- Not much difference between C8, C3 & diphenyl
  - Upper Mw of C3/C8/DP = 180kDa
Pore Size

Generally speaking, the “go to pore” size for most protein applications using RP is 300 Å.

Smaller pore sizes are available for smaller proteins and peptides.

Larger pore sizes are available for larger proteins and synthetic molecules.
About Particle Size

Smaller particle sizes will increase resolution power

Coupled with smaller column lengths, decrease time and increase throughput

Agilent offers many options down to 1.8 um for RP
As Column Volume Decreases, Decrease Gradient Time (tg) to Keep Gradient Retention ($k^*$) and Resolution Constant

- **ZORBAX 300SB-C8** 4.6 x 250 mm, 5 µm
  - 10-60% B in 50 min.
  - 40 min.

- **Rapid Resolution** 4.6 x 150 mm, 3.5 µm
  - 10-60% B in 30 min.
  - 24 min.

- **Rapid Resolution** 4.6 x 50 mm, 3.5 µm
  - 10-60% B in 10 min.
  - 9 min.
Initial Separation Conditions

Mobile Phase:  
A: 95% H2O/5% ACN, 0.1% TFA  
B: 5% H2O/95% ACN, 0.1% TFA  

Gradient: 0-60% B in 60 min  

Temp: 35-40 °C  
Flow Rate: 1ml/min
Optimize Organic Modifier

In order of increasing elutropic force and decreasing polarity:
Water
Methanol
Acetonitrile
N-Propanol
THF
About Ion Pair Agents

- Typically TFA is used
- If LC/MS is used, can substitute Formic or Acetic acid
Optimize Temperature

- Higher column temperature can dramatically improve resolution and recovery
- Check manufacturer specs for compatibility
- Agilent Zorbax Stablebond columns are rated to 80 C
Effect of Column Temperature on Retention $k$ and Selectivity $\alpha$

1. Column temperature can be used to fine tune a separation by affecting both the retention $k$ and selectivity $\alpha$.
2. About a 1% increase in $T$ leads to a 1 to 2% decrease in $k$.
3. Increase in $T$ leads to decrease in Pressure due to decrease in mobile phase viscosity.
4. Increase in $T$ also leads to decrease in peak widths.
5. Also, use of a thermostat column compartment improves retention time precision.
Optimize Mobile Phase pH

- Start with acidic pH first
- If not ideal, move to mid or high pH, check manufacturers specs for limits
- Selectivity will change because acidic amino acids will become negatively charged and basics may lose their charge
- Ammonium hydroxide is an excellent mobile phase for high pH separations using LC/MS
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.
Adjust Gradient Slope to Optimize Resolution

Accomplished by changing:

- gradient time
- % change in organic modifier over time
Effect of Gradient Time

Steep

\( t_G = 5 \)

\( t_G = 10 \)

Shallow

\( t_G = 20 \)

\( t_G = 40 \)
Agilent RP Column Choices for Biomolecules
Zorbax Silica

Zorbax Stablebond 300

300 Å pore size
available in C-3, C-8, C-18, and CN
non-endcapped
particle sizes: 3.5, 5, and 7
NEW!
Zorbax 300 SB RRHD for Proteins and Peptides!

• Stablebond 300 silica
• C-18, C-8, C-3, and unique diphenyl bonded phase
• 1.8 um particle size
• 1200 Bar pressure limit for uHPLC
Poroshell 300

300 Å pore size
Stablebond chemistry
available in C-3, C-8,C-18, and C-18 Extend
5 um particle size
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!
PLRP-S

Polystyrene divinylbenzene bead
Available in 100, 300, 1000, and 4000Å pore sizes
Particle sizes 3, 5, 8, 10, and higher
Various geometries from Nano, Capillary to preparative
In Closing

Method Development requires a substantial time investment, but the practical approaches presented today will help you develop robust methods that give you the results you need.

Thank you for your attention!
Questions?