# SEC and IEX for Biomolecules

## Column selection and troubleshooting

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LC Columns and Consumables Technical Support
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# Outline

SEC and its mechanism

Column selection – Importance of pore size selection

Mobile phase requirements and considerations

IEX and its mechanism

IEX and isoelectric point (pI)

Considerations for column selection

Mobile phase requirements and considerations

Troubleshooting and common problems

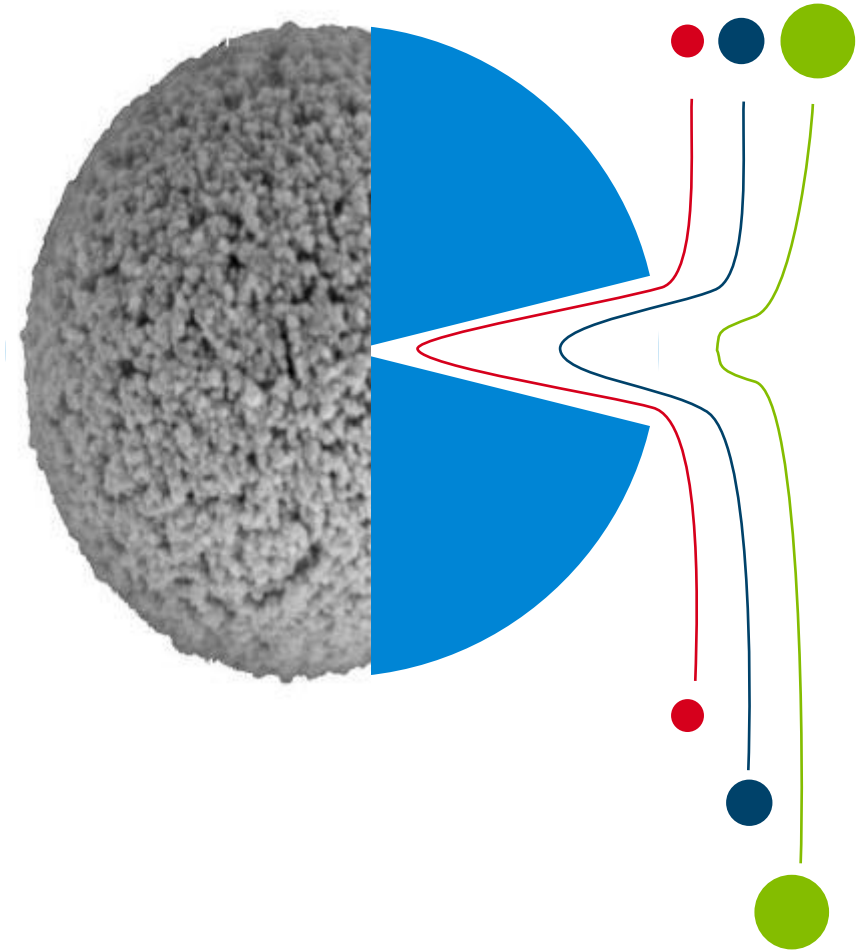
Considerations for column

Considerations for LC system

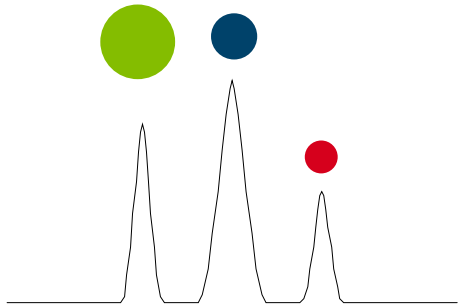
# Size Exclusion Chromatography

# Size Exclusion Chromatography

## Separation by size in solution under nondenaturing conditions



Size in solution is related to retention time:

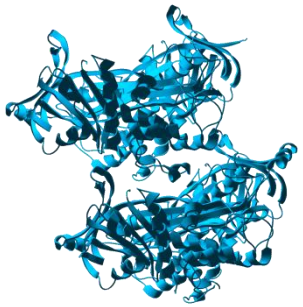


Smaller molecules spend longer in the pores and elute later

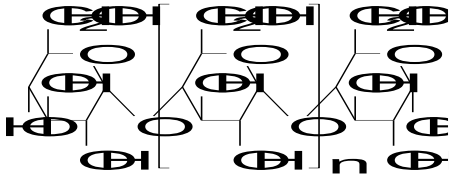
Larger molecules spend less time in the pores and elute sooner

# SEC of Biomolecules and Polymers

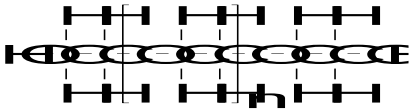
## Size in solution



Ovalbumin
MW 44,330D
385 amino acids



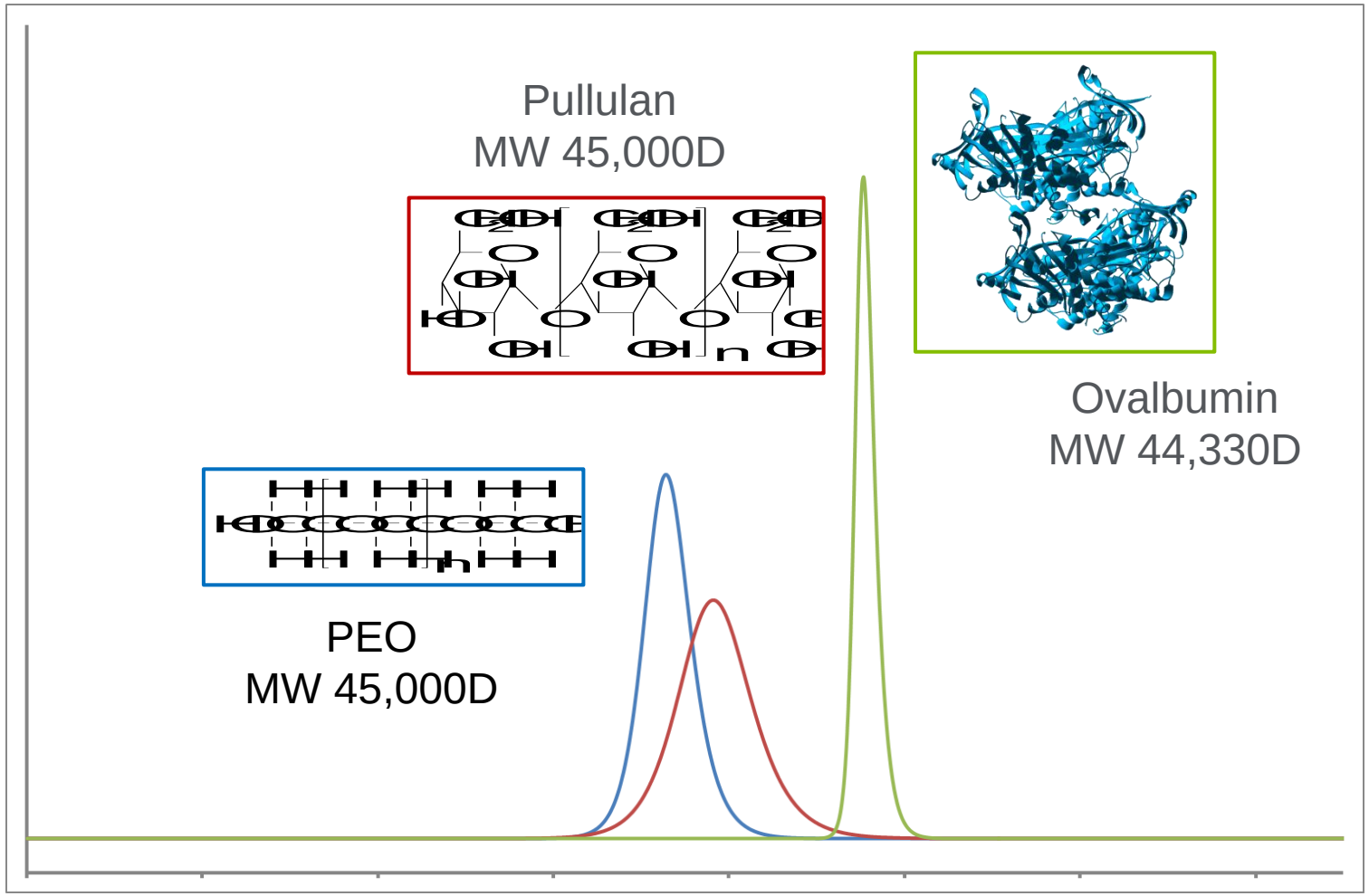
Pullulan
MW 45,000D
n ~ 275



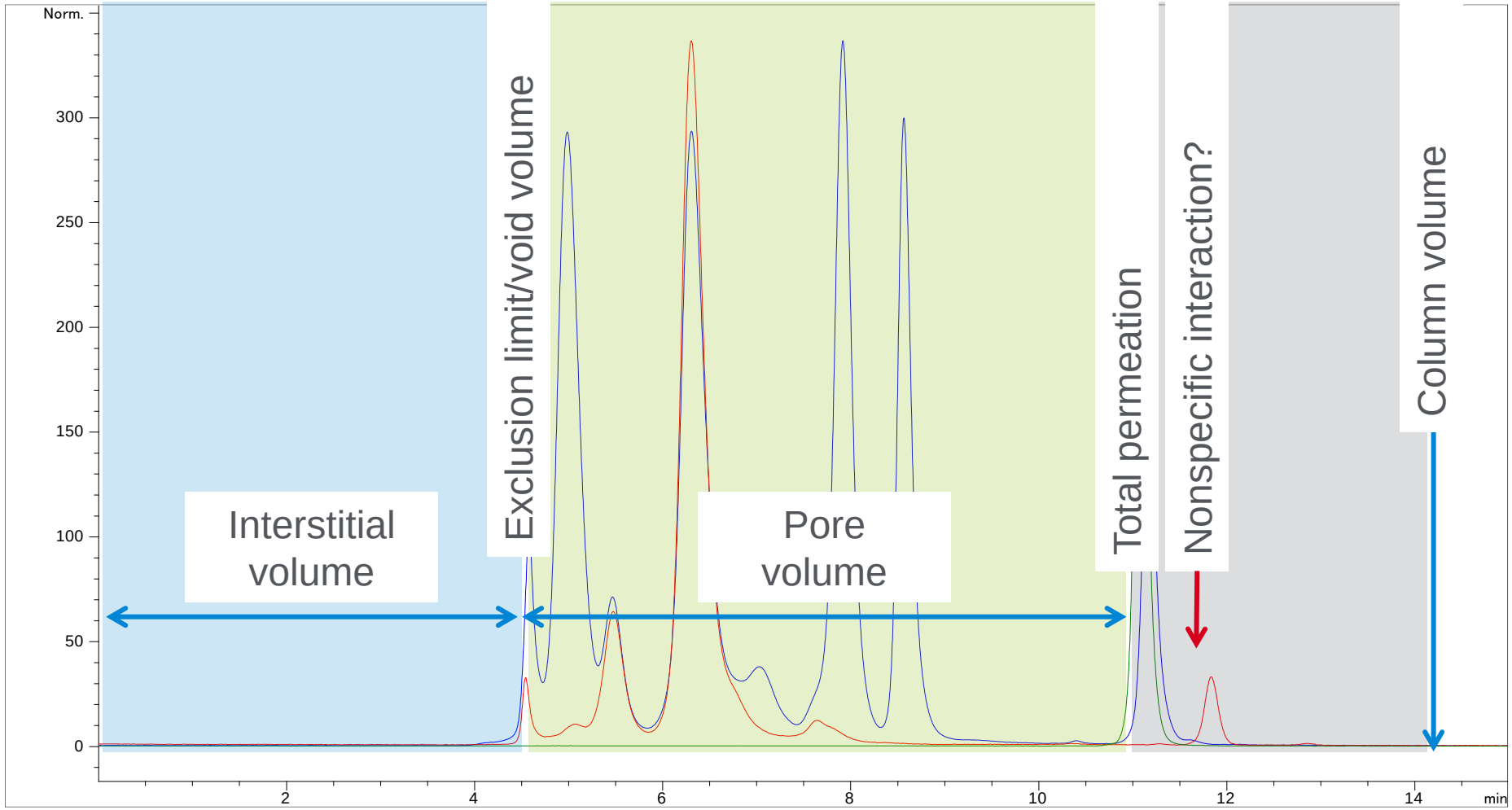
PEO
MW 45,000D
n ~ 1000

# SEC of Biomolecules and Polymers

## Size in solution



# What are These Regions on a Chromatogram?



# Considerations for Column Selection

## Pain points and common challenges for SEC

- Limited resolution – insufficient or incorrect pore sizes can reduce resolution
- Nonspecific interactions contribute to loss of sample and lead to inconsistent results and rework
- Long analysis times mean SEC is typically slow
- Poor pressure stability creates rework and increases costs
- Consistent and reproducible results are desirable
- High salt conditions put excessive wear on the instrument and parts

# What to Look for in an SEC Column

## The right pore size for your sample

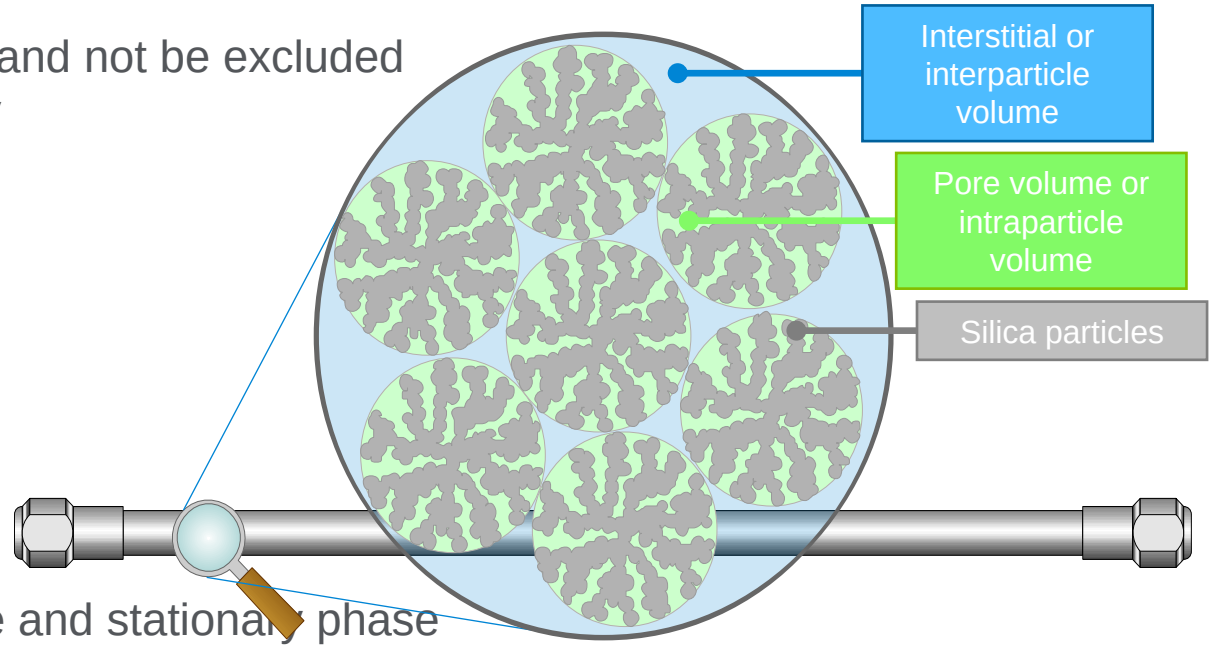
- Pores must be large enough for the sample to permeate and not be excluded
- Pores that are too large will limit the separation capability
- Optimum pore size ~300Å for mAbs

## Well packed column and large pore volume

- Minimize interstitial volume
- Maximize pore volume

## Inert chemistry

- Ideal SEC separations are based only on molecular size
- Best to avoid secondary interactions between the sample and stationary phase




Column dimensions: 7.8 x 300 mm
Column volume = 14.3 mL

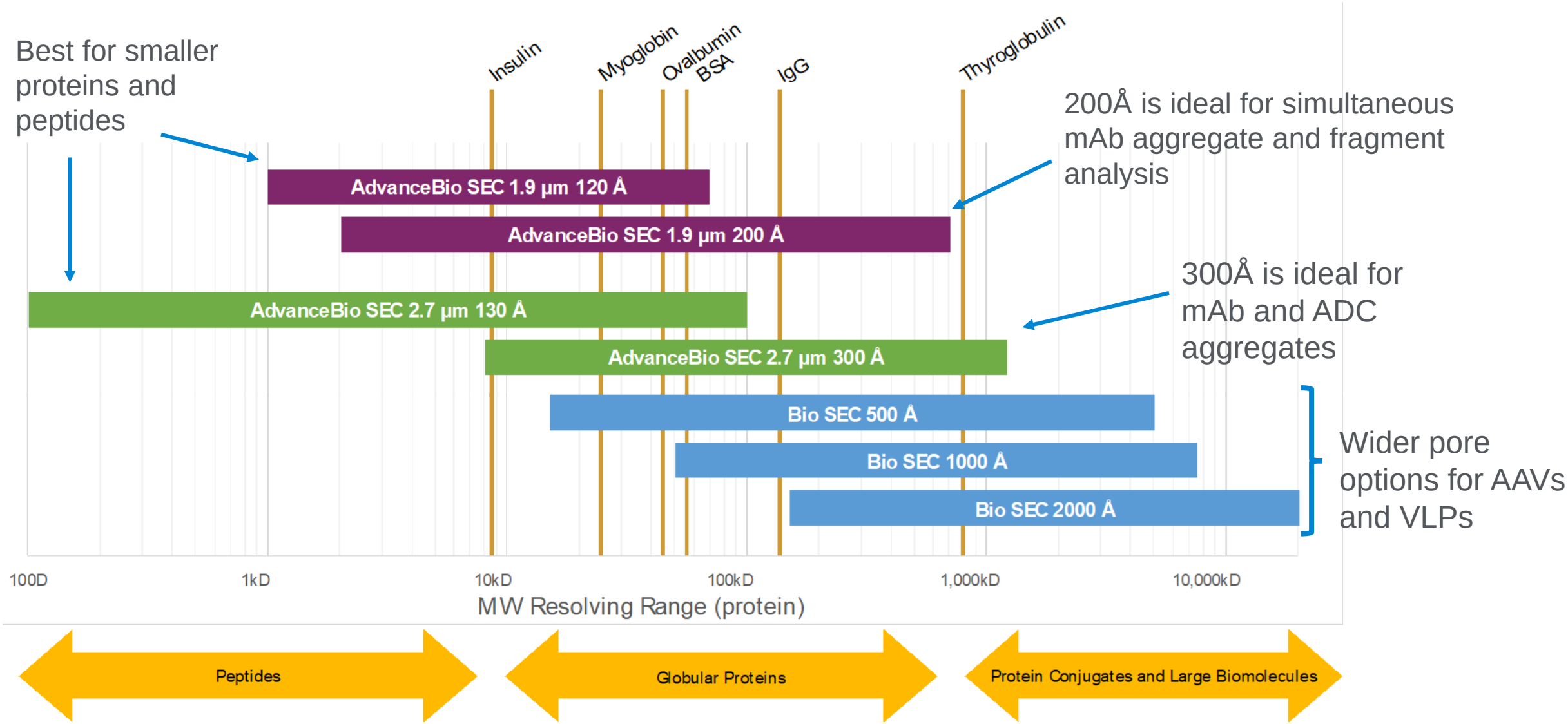
## The right dimensions for your application

4.6 or 7.8 x 150 mm for higher throughput, faster separations

7.8 x 300 mm for higher resolution
4.6 x 300 mm for higher sensitivity

# Choosing an SEC Column for Your Application

Agilent InfinityLab



# Agilent Columns for Aggregate Analysis

Agilent InfinityLab

## AdvanceBio SEC

- Unique hydrophilic coating for minimal secondary interactions
- Large pore volume for maximum separation opportunities
- Excellent peak shape and resolution for challenging samples like ADCs with a simple phosphate buffer
- 130Å and 300Å pore options for 2.7 µm particles
  - 300Å is ideal for mAbs
- 120Å and 200Å pore options for 1.9 µm particles
  - 200Å is ideal for mAbs aggregate and fragment analysis

## Agilent Bio SEC

- MS compatibility
- Pore sizes ranging from 100Å for peptides and small proteins to 2000Å for VLPs

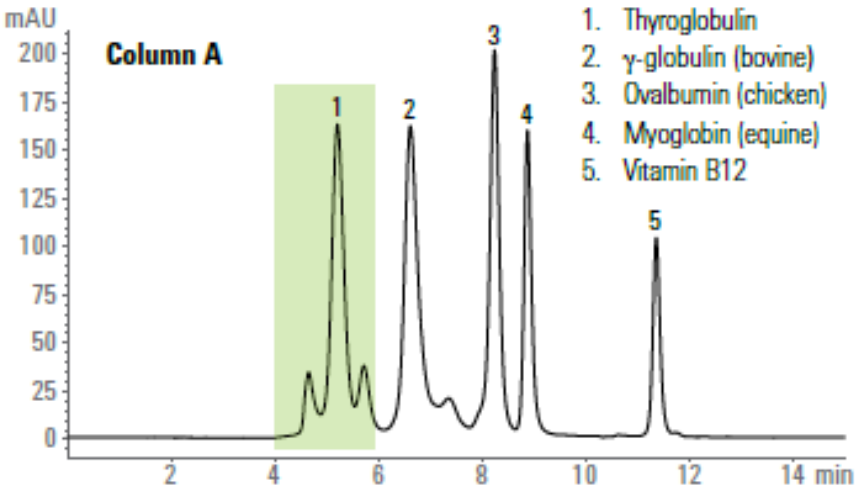
# Importance of Pore Size Selection: Calibrants

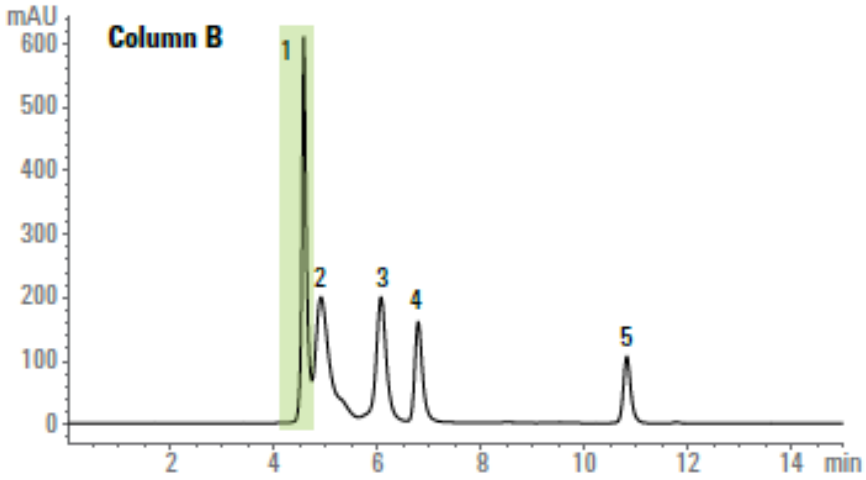
| | |
|---|---|
| Instrument: | Agilent 1260 Infinity Bio-inert Quaternary LC System |
| Mobile phase: | 150 mM phosphate buffer, pH 7.0 |
| Flow rate: | 0.35 mL/min |
| Detector: | UV, 220 nm |
| Sample: | BioRad gel filtration standards mix |

**Column A:** **AdvanceBio SEC 300Å**
**4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)**

**Column B:** **AdvanceBio SEC 130Å**
**4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)**

**BioRad gel filtration standards mix**

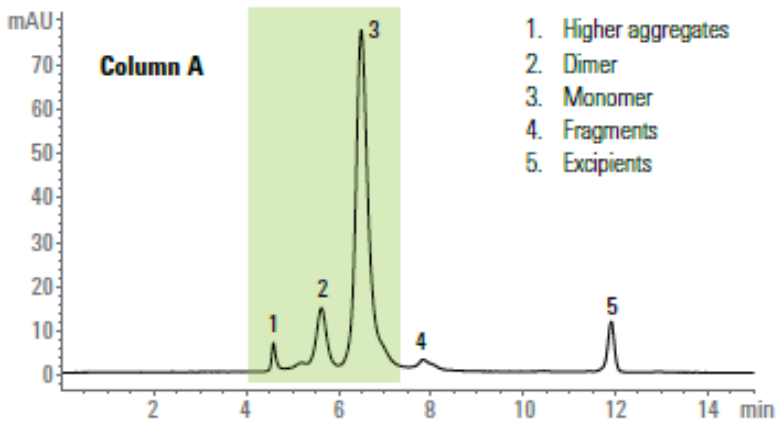


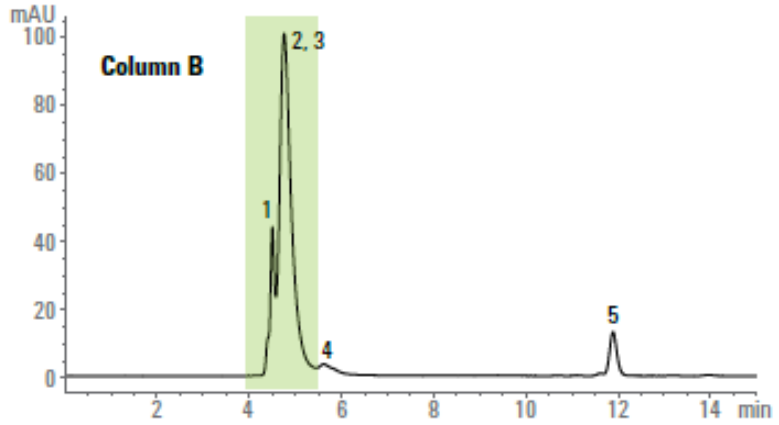



# Importance of Pore Size Selection: Sample

Agilent InfinityLab

**Polyclonal IgG separation**



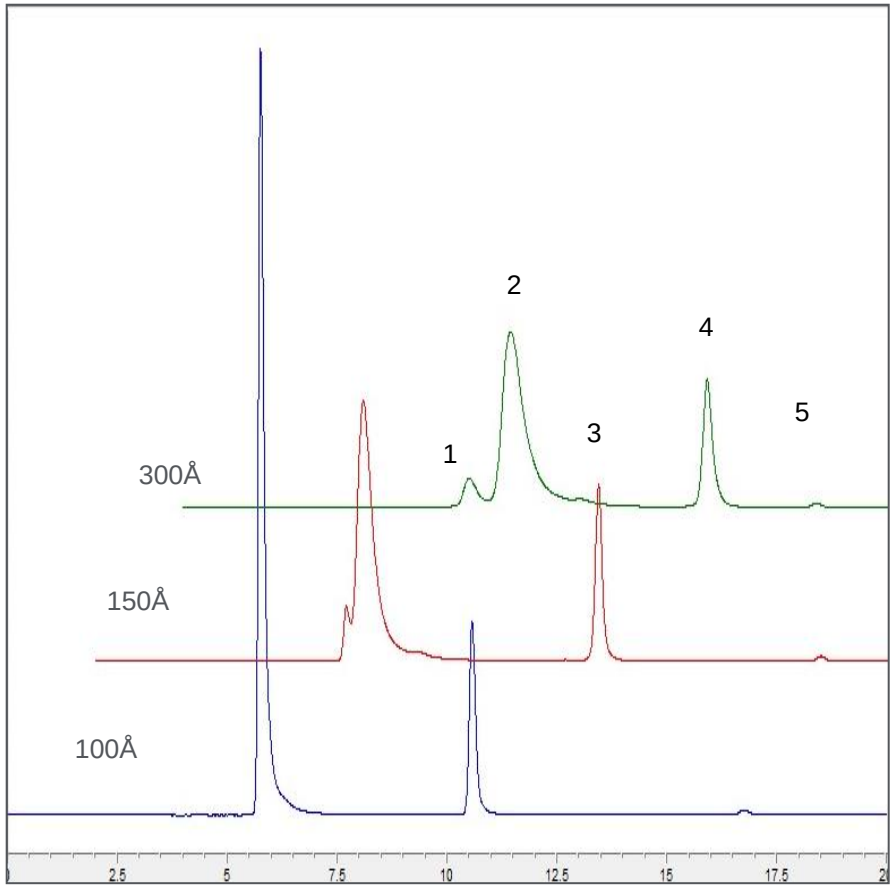




**Column A:** **AdvanceBio SEC 300Å**
**4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)**

**Column B:** **AdvanceBio SEC 130Å**
**4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)**

| | |
|---|---|
| Instrument: | Agilent 1260 Infinity Bio-inert Quaternary LC System |
| Mobile phase: | 150 mM phosphate buffer, pH 7.0 |
| Flow rate: | 0.35 mL/min |
| Detector: | UV, 220 nm |
| Sample: | Polyclonal IgG |

# Pore Size Selection
## Choice for antibody analysis




Eluent: 50 mM $NaH_2PO_4$ + 0.15 M NaCl, pH 6.8
Columns: Bio SEC various pore sizes
Flow: 0.35 mL/min
Detector: UV at 220 nm
System: Agilent 1260 Infinity Bio-Inert LC
Sample: Mouse IgG

1. Dimer
2. Monomer
3. Monomer fragment
4. Azide
5. Retained molecule

# Buffers and SEC: Criteria for Optimal Mobile Phase

- Mobile phase should contain enough buffer/salt (to overcome ionic interactions)
- Mobile phase should not contain too much buffer/salt (to prevent hydrophobic interactions)
- Mobile phase should not alter the analyte (cause degradation/aggregation)
- Mobile phase should be made up fresh and used promptly (bacterial growth is rapid in dilute buffer stored at room temperature).
- Buffer shelf life is <7 days unless refrigerated.
- Mobile phase should be filtered before use. Particulates may be present in water (less likely) or in buffer salts (more likely).

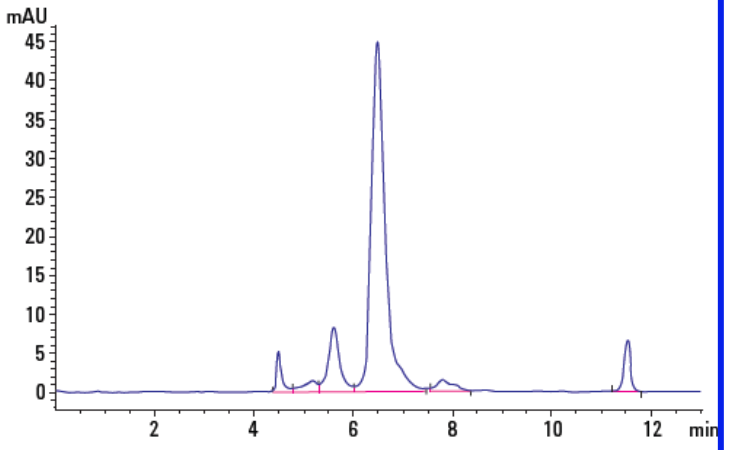
The optimal eluent for the separation should be determined by the characteristics of the column stationary phase and the proteins/polymers to be analyzed so that nonspecific interactions are minimized and the SEC mechanism is maintained.

# Operating Parameters

Agilent InfinityLab

## Recommended starting conditions

| | |
|---|---|
| Column: | Agilent AdvanceBio SEC 300Å, 2.7 µm, 7.8 x 300 mm (p/n PL1180-5301) |
| Flow rate: | 1 mL/min |
| Mobile phase: | 150 mM phosphate buffer, pH 7.0 |
| Wavelength: | 220 nm |
| Temperature: | ambient |
| Injection volume: | 5 µL |
| Sample: | IgG |



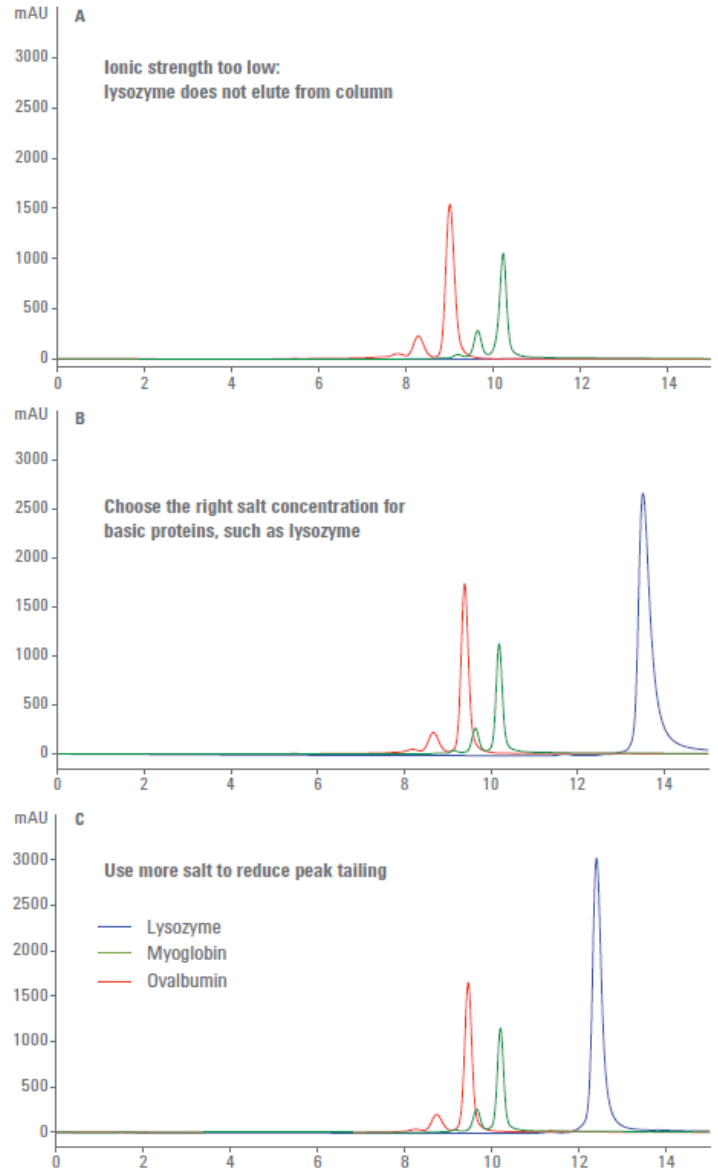

## AdvanceBio 2.7 µm

| Parameter | Conditions |
|---|---|
| Mobile phases | Aqueous buffers<br>150 mM phosphate buffer, pH 7.0<br>Aqueous organic mixes |
| pH | 2 to 8.5 |
| Operating temperature | 20 to 30 ˚C (recommended)<br>80 ˚C (maximum) |
| Operating pressure | <200 bar per column (recommended)<br>400 bar (maximum) |
| Flow rate | 0.1 to 2.0 mL/min for 7.8 mm id<br>0.1 to 0.7 mL/min for 4.6 mm id |
| Protein resolving ranges | 0.1 to 120 kD for 130Å<br>5 to 1250 kD for 300Å |

## AdvanceBio 1.9 µm

| Parameter | Conditions |
|---|---|
| Mobile phases | Compatible with all the SEC mobile phases for UV. Phosphate buffer, pH 7.0, with different salt concentrations, denaturing and native mode SEC-MS mobile phases. |
| pH | 2 to 8.5 |
| Operating temp | 20 to 40 ˚C (recommended)<br>80 ˚C (maximum) |
| Operating pressure | 620 bar (9000 psi) (maximum) |
| Flow rate | For 4.6 × 150 mm, 0.1 to 0.7 mL/min<br>For 4.6 × 300 mm, 0.1 to 0.5 mL/min<br>For 2.1 mm id columns, 0.05 to 0.10 mL/min |
| Protein resolving ranges | 1K to 80 kD for 130Å<br>2K to 700 kD for 300Å |

# Buffer Considerations





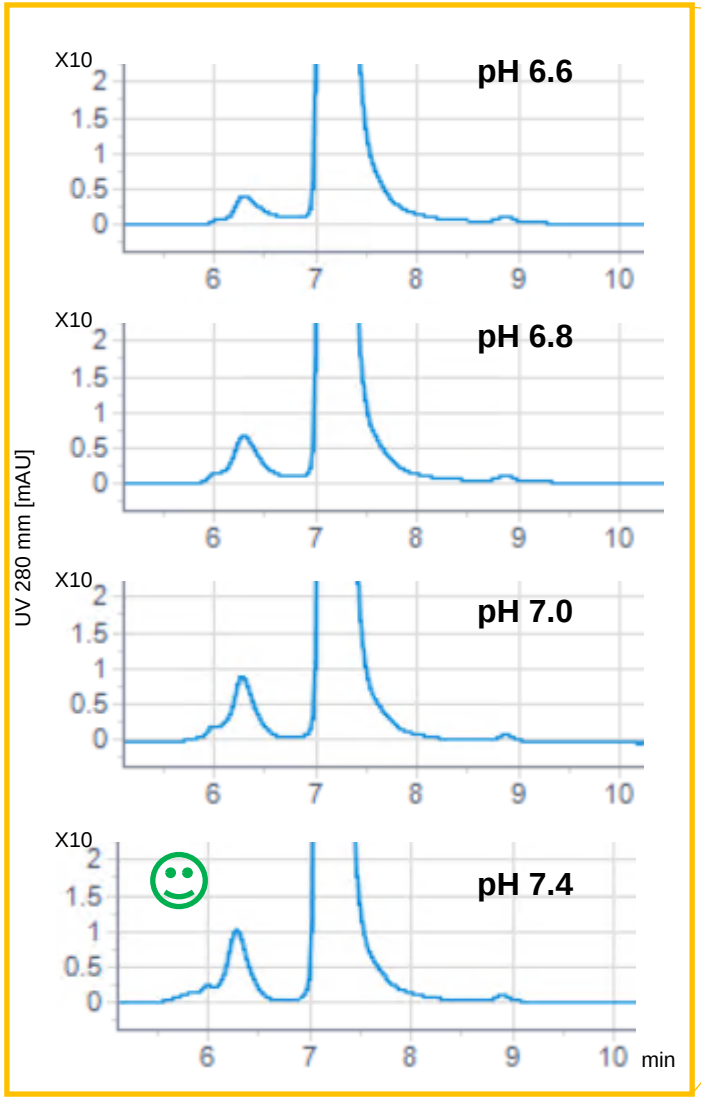
| | |
|---|---|
| **Column:** | **Agilent Bio SEC-3 300Å**<br>**4.6 mm x 300 mm, 3 µm**<br>**(p/n 5190-2513)** |
| Instrument: | Agilent 1260 Infinity Bio-inert Quaternary LC System |
| Flow rate: | 0.35 mL/min |
| Detector: | UV, 220 nm<br>A: Eluent 20 mM phosphate buffer, pH 7 + 50 mM NaCl<br>B: Eluent 20 mM phosphate buffer, pH 7 + 100 mM NaCl<br>C: Eluent 20 mM phosphate buffer, pH 7 + 400 mM NaCl |
| Injection: | 5 µL |
| Sample: | Protein (1 mg/mL 20 mM phosphate buffer, pH 7) |

Pub number: 5991-3651EN

# Mobile Phase Optimization – NISTmAb

Agilent InfinityLab

## 150 mM sodium phosphate




| Buffer (mM) | NaCl (mM) | pH | Asymmetry (As) | Rs (dimer/monomer) |
|---|---|---|---|---|
| 150 | 0 | 6.6 | 1.49 | 2.33 |
| 150 | 0 | 6.8 | 1.43 | 2.35 |
| 150 | 0 | 7 | 1.42 | 2.67 |
| 150 | 0 | 7.4 | 1.41 | 2.78 |
| 200 | 0 | 7.4 | 1.45 | 2.60 |
| 250 | 0 | 7.4 | 1.42 | 2.57 |
| 300 | 0 | 7.4 | 1.40 | 2.45 |
| 350 | 0 | 7.4 | 1.38 | 2.33 |

**Sodium phosphate without NaCl**

**Lower pH, more tailing, less resolution**

**Higher buffer concentration, less tailing but worse resolution**

# Mobile Phase Optimization – NISTmAb

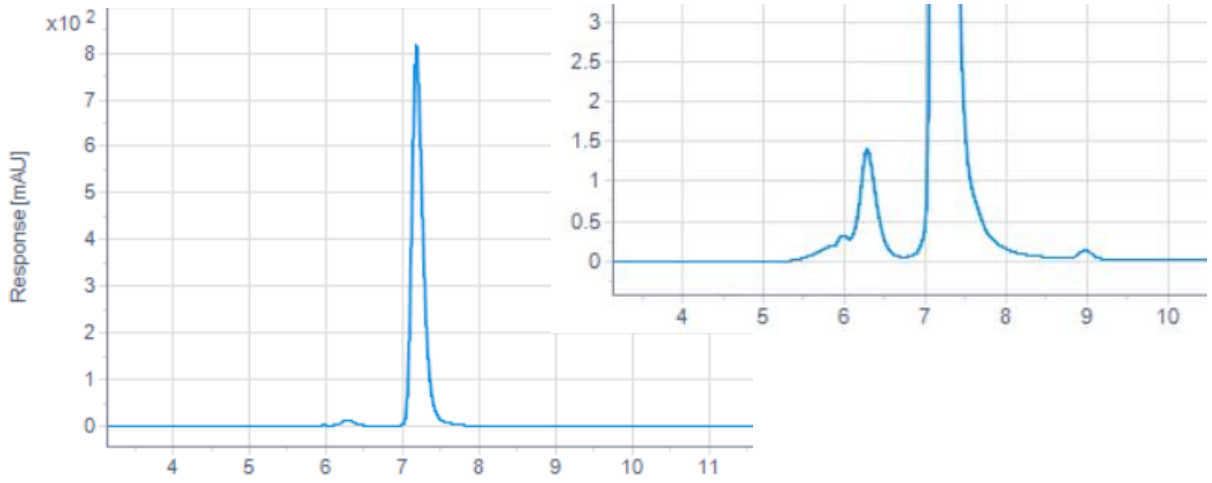
Agilent InfinityLab

## Sodium phosphate concentration and pH screening

| Buffer (mM) | NaCl (mM) | pH | As | Rs (dimer/monomer) |
|---|---|---|---|---|
| 25 | 250 | 6.6 | 1.36 | 2.73 |
| 25 | 250 | 6.8 | 1.36 | 2.86 |
| 25 | 250 | 7 | 1.35 | 2.83 |
| 25 | 250 | 7.4 | 1.37 | 2.86 |

| Buffer (mM) | NaCl (mM) | pH | As | Rs (dimer/monomer) |
|---|---|---|---|---|
| 100 | 250 | 6.6 | 1.36 | 2.87 |
| 100 | 250 | 6.8 | 1.36 | 2.89 |
| 100 | 250 | 7 | 1.35 | 2.83 |
| 100 | 250 | 7.4 | 1.37 | 2.80 |

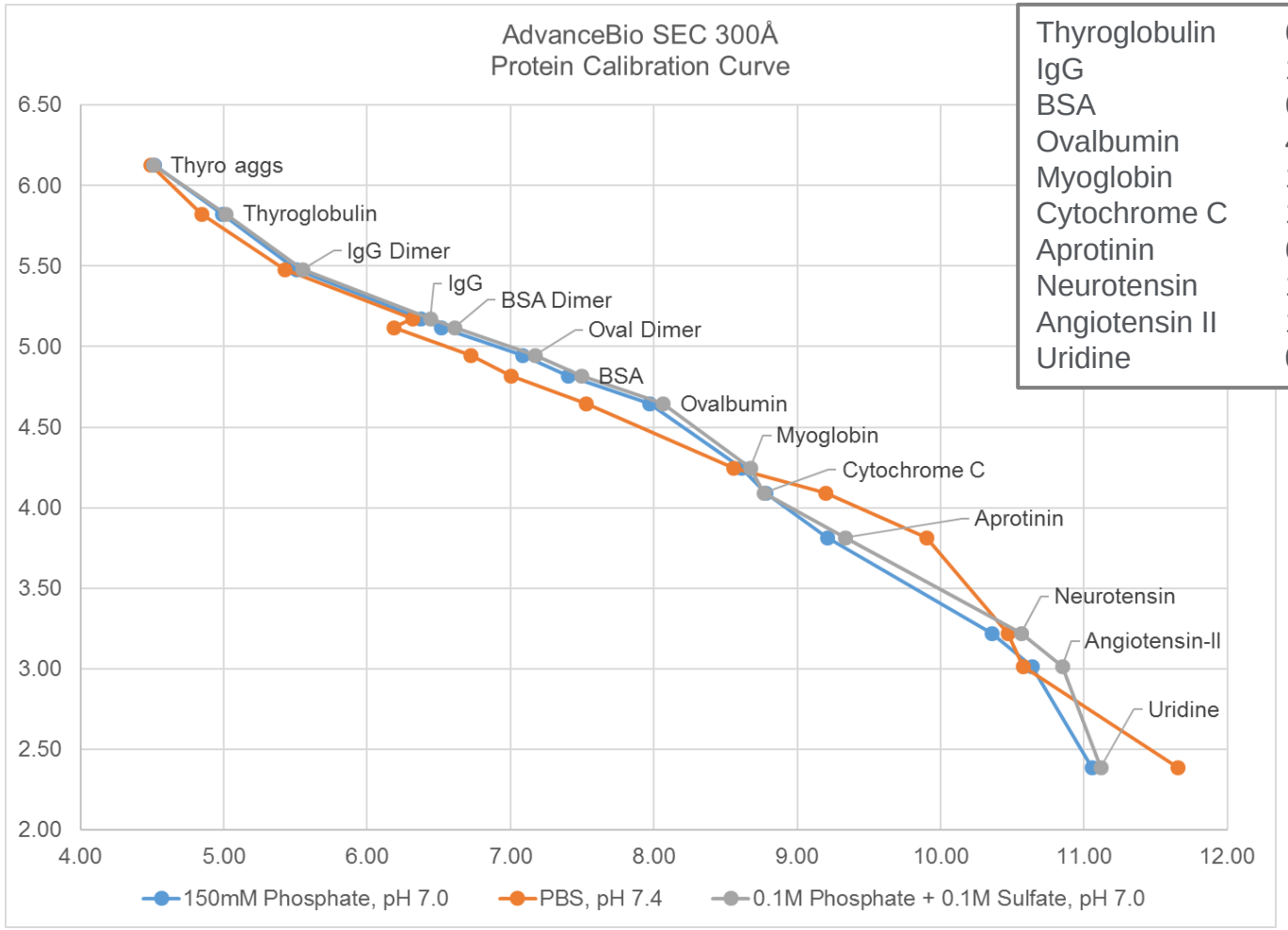
| Buffer (mM) | NaCl (mM) | pH | As | Rs (dimer/monomer) |
|---|---|---|---|---|
| 50 | 250 | 6.6 | 1.35 | 2.87 |
| 50 | 250 | 6.8 | 1.33 | 2.86 |
| 50 | 250 | 7 | 1.36 | 2.85 |
| 50 | 250 | 7.4 | 1.36 | 2.84 |




**For some samples, a certain concentration of NaCl is needed in the mobile phase, with optimized pH for a balance of good peak shape and resolution.**

# Importance of Buffer Selection

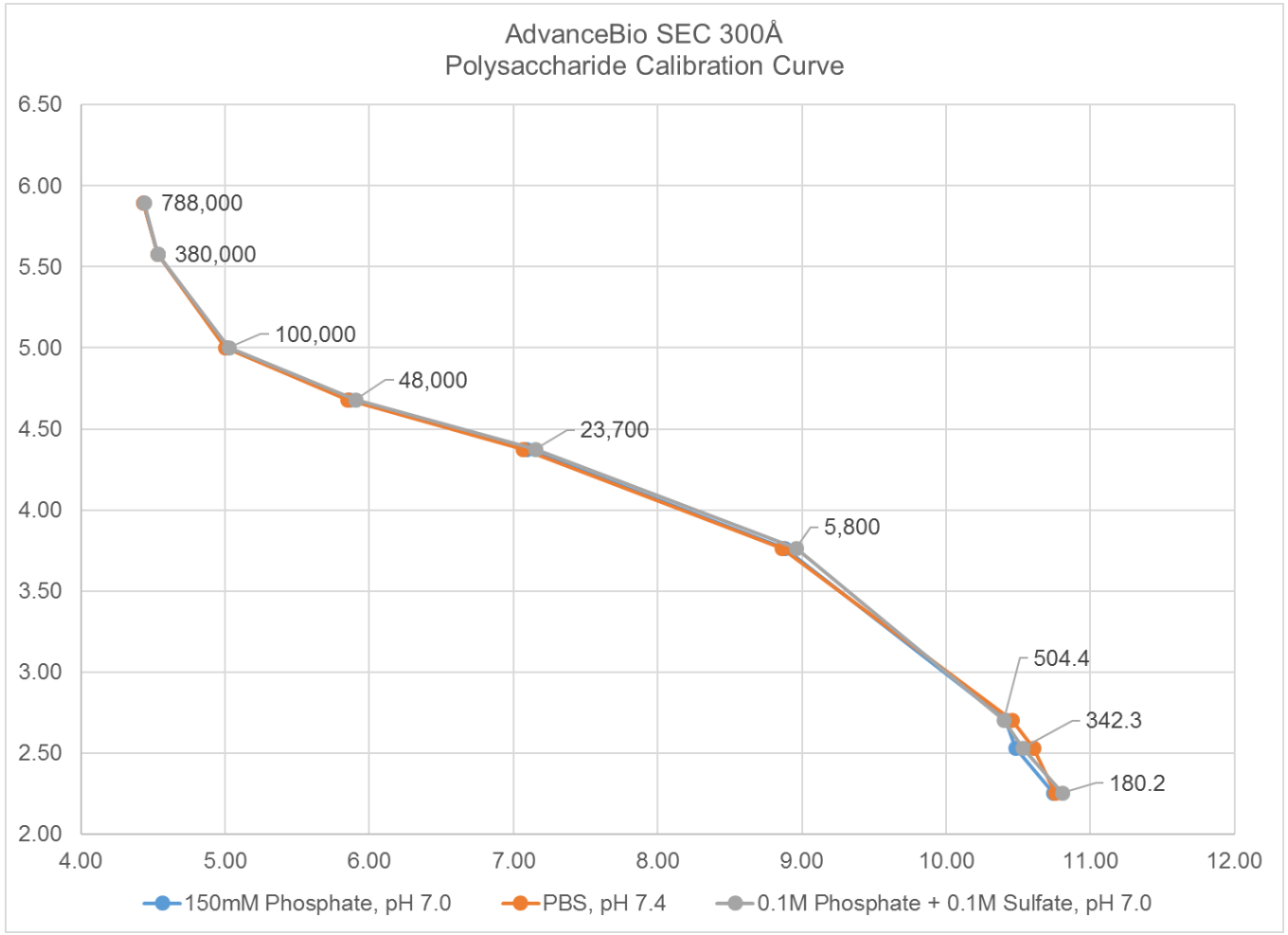
## Peptide and protein calibration




| | |
|---|---|
| Thyroglobulin | 670kD |
| IgG | 150kD |
| BSA | 66kD |
| Ovalbumin | 44kD |
| Myoglobin | 17kD |
| Cytochrome C | 12kD |
| Aprotinin | 6.5kD |
| Neurotensin | 1.7kD |
| Angiotensin II | 1.0kD |
| Uridine | 0.24kD |

# Importance of Buffer Selection

## Polysaccharide calibration



# Ion Exchange Chromatography

# Ion Exchange Chromatography
## Common terminology and acronyms

SAX/WAX – Strong anion exchange and weak anion exchange

SCX/WCX – Strong cation exchange and weak cation exchange

| Resin Type | Cation Exchange | Anion Exchange |
|---|---|---|
| Net charge of molecule of interest | + | – |
| Charge of resin | – | + |

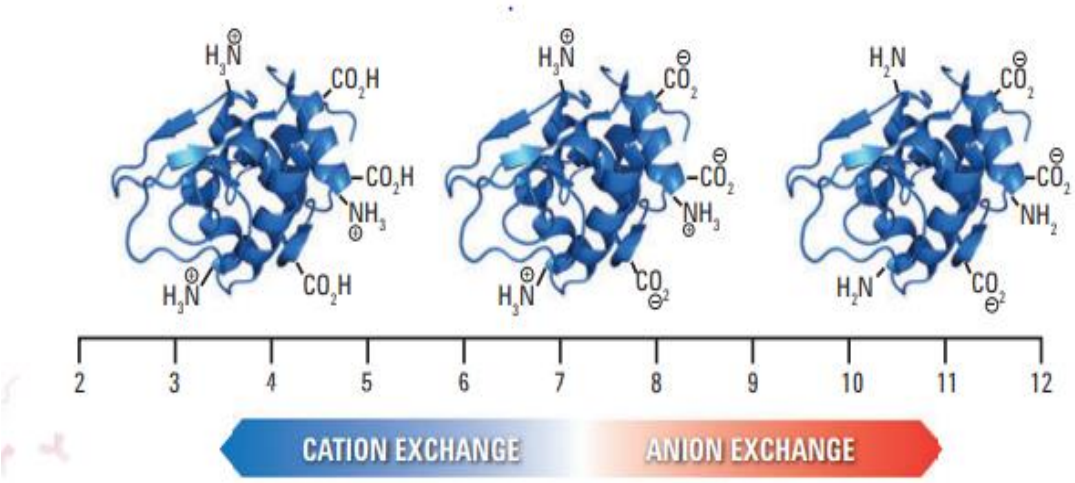



**Figure 1** – Effect of pH on net protein charge

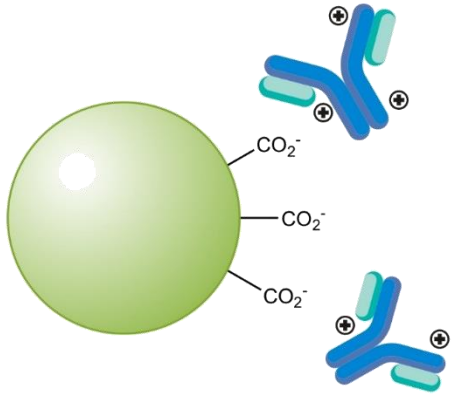
# IEX and Charge Variants

## How are charge variants monitored?

Charge variant analysis is often done by ion exchange (IEX) chromatography followed by UV detection.

**For this technique, the *isoelectric point* (pI) of the protein is critical.**

**This is the pH where the net charge on the protein is zero.**




# IEX Technique for Proteins

The technique requires a gradient for elution.

Protein interaction is based on accessible surface charges and corresponding electrostatic interaction with the column stationary phase.

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

Separation is based on differences in degree of charge

The sample is injected into a mobile phase buffer with a low salt concentration

- Binds proteins to the column

Typically eluted at constant pH with increasing salt gradients (mobile-phase ionic strength)

- Displaces proteins from stationary phase

A higher charge means proteins bind more strongly

- Increased salt gradient needed to elute

Typical mobile phase will contain NaCl

Technique does not denature

# IEX Mechanism Example

## Basic protein on strong cation exchange packing

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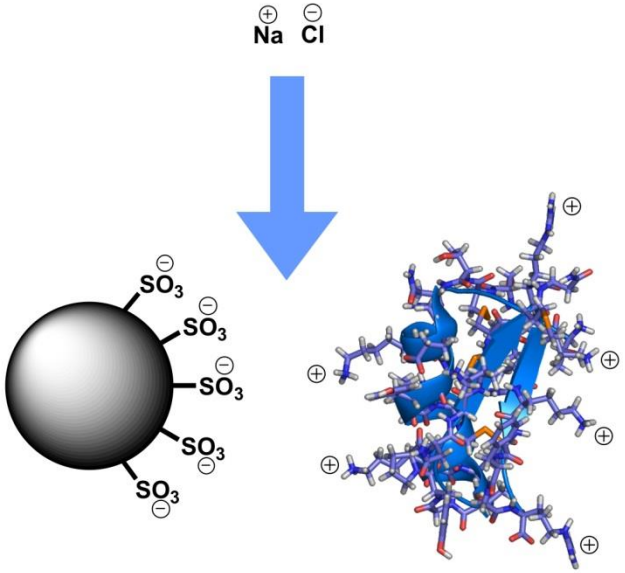
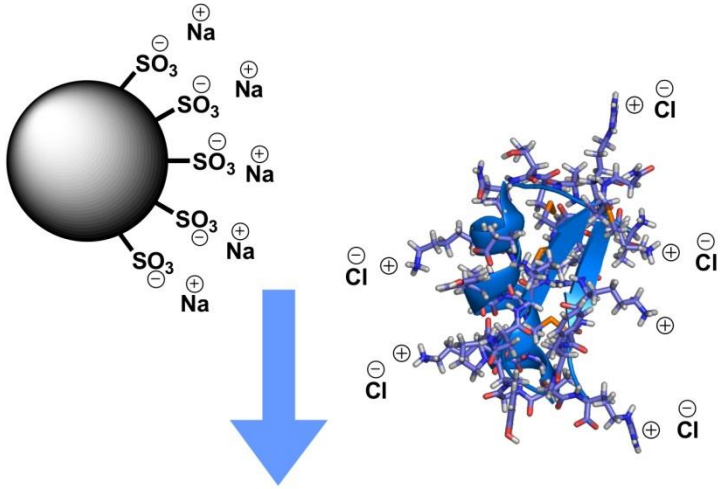
### Low salt to bind




### High salt to elute

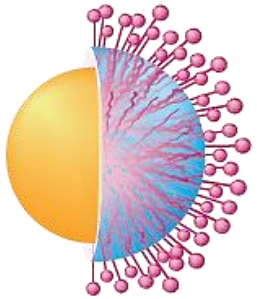



Elution order will correlate with the number of positive charges

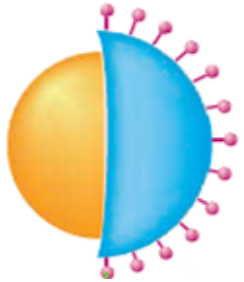


Agilent

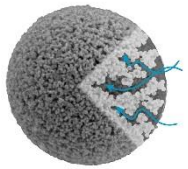
# Agilent Columns for Ion Exchange



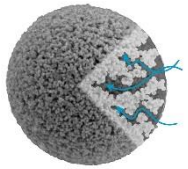
**Agilent Bio mAb**
- Polymeric weak cation exchange (WCX)
- Nonporous for high efficiency
- High density WCX coating optimized for mAb separations



**Agilent Bio IEX**
- Polymeric WCX, WAX, SCX, SAX
- Nonporous for high efficiency
- Peptides and proteins



**PL-SAX**
- Totally porous particles for scale up to purification
- Oligos, peptides, and proteins



**PL-SCX**
- Totally porous particles for scale up to purification
- Peptides and proteins

**Bio-Monolith IEX**
- Monolith format for high speed separations of macromolecules
- QA (SAX), DEAE (WAX), $SO_3$ (SCX)

# Ion Exchange Product Families

| | Particle | Porosity | Functionalities | Particle Sizes | Pore Size | Application |
|---|---|---|---|---|---|---|
| Agilent Bio-IEX | Polymer | Nonporous | SAX, WAX, SCX, WCX | 1.7 µm, 3 µm, 5 µm 10 µm | N/A | Peptides proteins |
| Agilent Bio MAb | Polymer | Nonporous | WCX | 1.7 µm, 3 µm, 5 µm 10 µm | N/A | IgG |
| PL-SAX | PS/DVB | Fully porous | SAX | 5 µm, 8 µm, 10 µm 30 µm | 1000A, 4000A | Peptides, oligos, proteins |
| PL-SCX | PS/DVB | Fully porous | WCX | 5 µm, 8 µm, 10 µm 30 µm | 1000A, 4000A | Peptides, proteins |
| Bio-Monolith IEX | Polymer | Monolith | QA, DEAE, $SO_3$ | N/A | N/A | Biomacromoleucles |

1. Nonporous particles for high efficiency analytical separations
2. Porous particles for scale up to purification
3. Monoliths for high speed separations

# General Guidelines

The general rule for choosing an IEX column for proteins.

- Acidic proteins: SAX or WAX
- Basic proteins: SCX or WCX

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

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

If your pI is unknown:

- Start with pH 6.0 for cation-exchange
- Start with pH 8.0 for anion-exchange

# Column Selection – Ion Exchange

| Application | Agilent Columns | Notes |
|---|---|---|
| Monoclonal antibodies | Bio MAb | Thorough characterization of monoclonal antibodies includes the identification and monitoring of acidic and basic isoforms. Agilent Bio MAb HPLC columns feature a unique resin specifically designed for high-resolution charge-based separations of monoclonal antibodies. |
| Peptides and proteins | Bio IEX | Agilent Bio IEX columns are packed with polymeric, nonporous, ion-exchange particles. The Bio IEX columns are designed for high resolution, high recovery and highly efficient separations. |
| Proteins, peptides and deprotected synthetic oligonucleotides<br><br>Globular proteins and peptides<br>Very large biomolecules/<br>high speed | PL-SAX 1000 Å<br>PL-SAX 4000 Å<br><br>PL-SAX 1000 Å<br>PL-SAX 4000 Å | The strong anion exchange functionality, covalently linked to a fully porous chemically stable polymer, extends the operating pH range. In addition, the anion-exchange capacity is independent of pH. For synthetic oligonucleotides, separations using denaturing conditions of temperature, organic solvent, and high pH are all possible. The 5 µm media delivers separations at high resolution with the 30 µm media used for medium pressure liquid chromatography. |
| Small peptides to large proteins<br><br>Globular proteins<br>Very large biomolecules/<br>high speed | PL-SCX 1000 Å<br>PL-SCX 4000 Å<br><br>PL-SCX 1000 Å<br>PL-SCX 4000 Å | PL-SCX is a macroporous PS/DVB matrix with a very hydrophilic coating and strong cation-exchange functionality. This process is controlled to provide the optimum density of strong cation-exchange moieties for the analysis, separation and purification of a wide range of biomolecules. The 5 µm media delivers separations at higher resolution with the 30 µm media used for medium pressure liquid chromatography. |
| Antibodies (IgG, IgM),<br>plasmid DNA, viruses,<br>phages and other macro biomolecules<br><br>Viruses, DNA, large proteins<br>Plasmid DNS, bacteriophages<br>Proteins, antibodies | Bio-Monolith QA<br>Bio-Monolith DEAE<br>Bio-Monolith $SO_3$<br><br>Bio-Monolith QA<br>Bio-Monolith DEAE<br>Bio-Monolith $SO_3$ | Strong cation-exchange, strong and weak anion-exchange phases.<br>Bio-Monolith HPLC columns are compatible with preparative LC systems, including Agilent 1100 and 1200 Infinity Series. |

Table from: Agilent BioColumns: Charge Variant Analysis
Publication number: 5994-0034EN

# Buffers in IEX Chromatography

- Well controlled, precise buffers enhance reproducibility and method robustness
- Make buffers fresh, and filter before use
- Confirm composition of buffers, especially for commercial products – is there added NaCl when you don't expect it?
- It is better to adjust the pH with a mixture of buffers than by adding acid or base, especially for more than a small change
- For IEX, the mobile phase pH must be at least 1 pH unit away from the pI of the protein (isoelectric point, where the protein is charge neutral)
  - Neutral protein will not be retained on the column, operating pH must be far enough from pI that protein has a net charge
  - Guidelines range from 0.5 to 2 pH units as a minimum separation

# IEX Conditions

## Buffer/ionic strength

Certain ionic strength is required to sustain the column function

Usually minimum of 10–20 mM required

Greater than 30 mM may prevent adsorption

Commonly used salts are NaCl, KCl, and acetate

Elution salt is typically 400–500 mM – 1M

## Buffer and pH selection

Phosphate, tris, MES, and ACES buffers are commonly used

For cation exchange, pH of 4 to 7

For anion exchange, pH of 7 to 10

## Anion exchange

Buffer A =
20 mM Tris, pH = 8.0

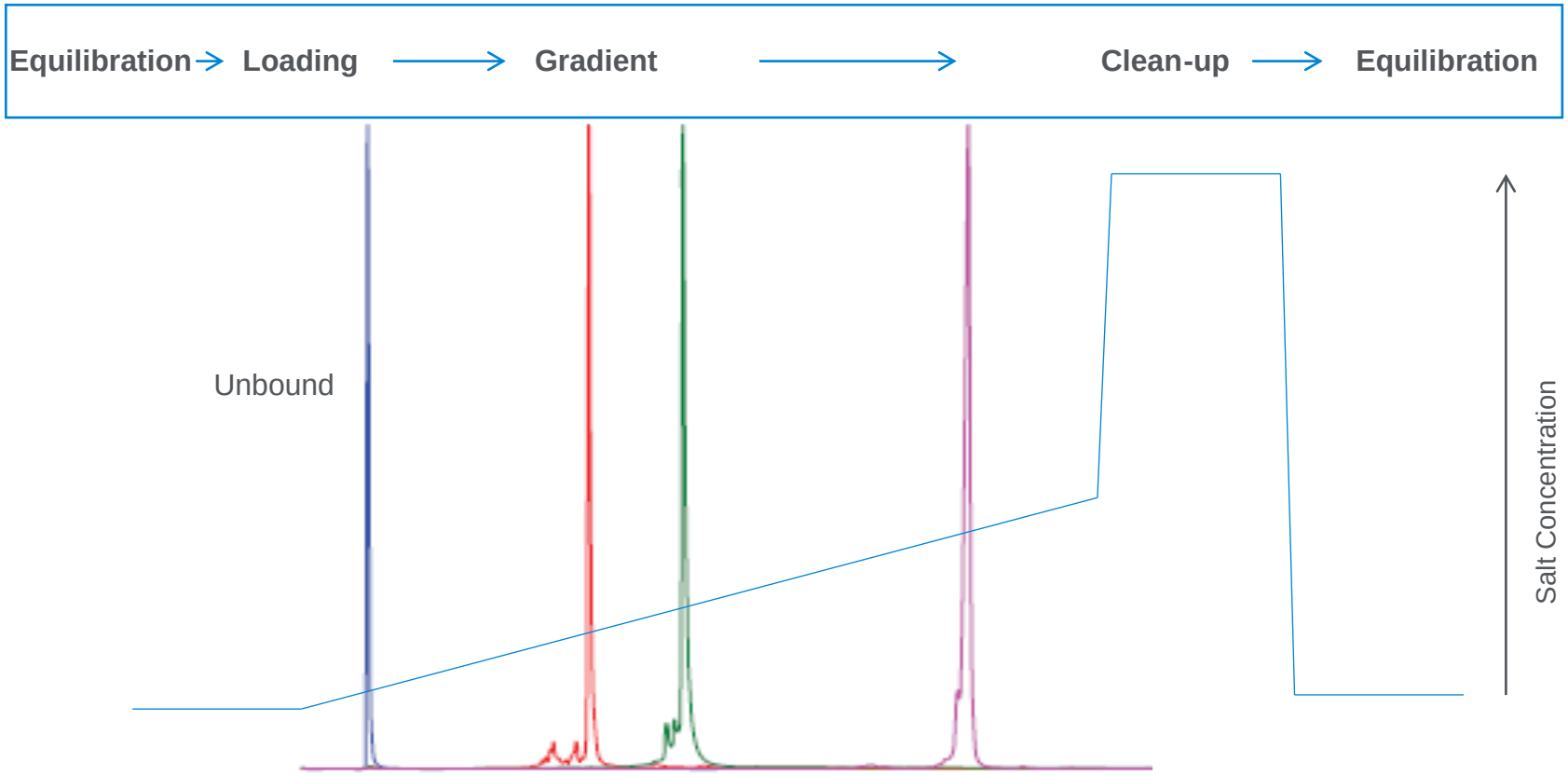
Buffer B =
20 mM Tris, 1 M NaCl, pH = 8.0

## Cation exchange

Buffer A =
30 mM sodium acetate, pH = 4.5

Buffer B =
30 mM sodium acetate, 1 M NaCl, pH = 4.5

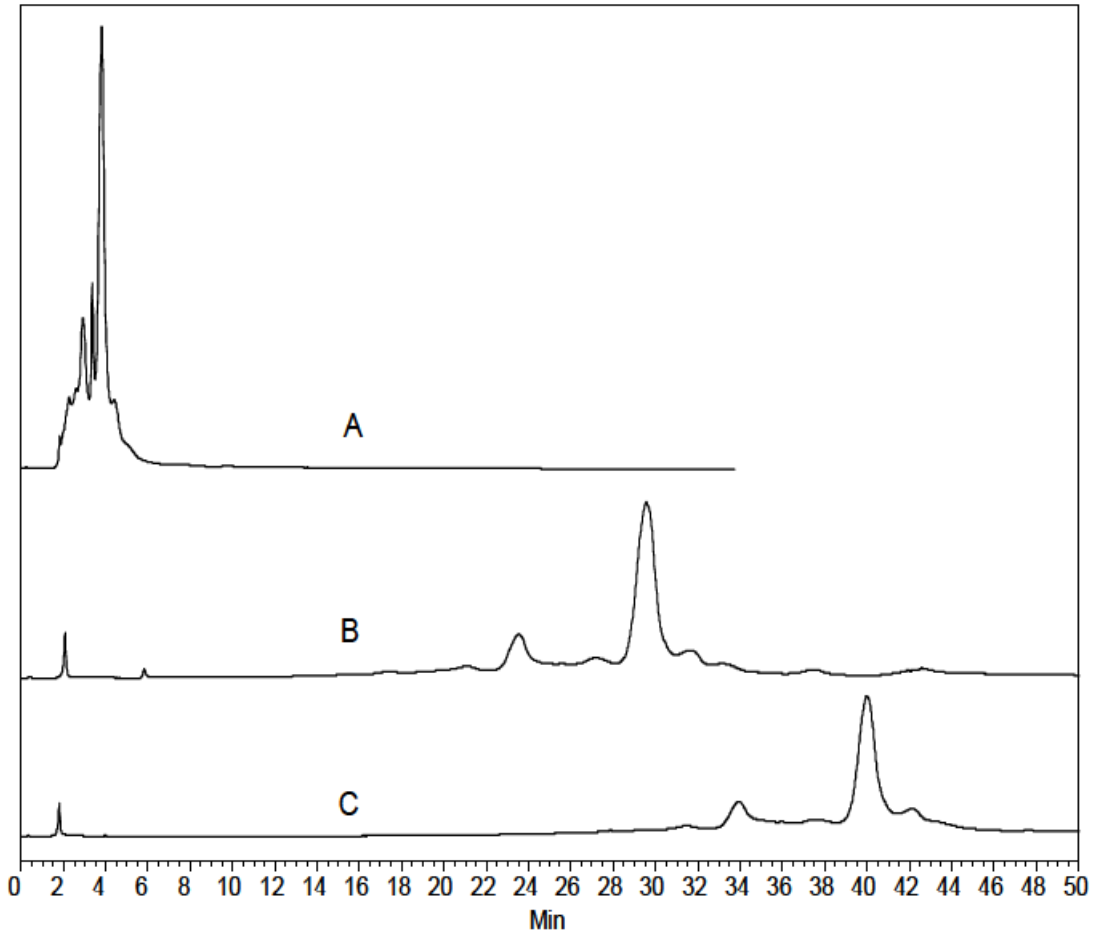
# Ion Exchange Separation Sequence




Equilibration/clean-up is typically 5 to 10 column volumes – essential for reproducibility

[Ion-exchange chromatography for biomolecule analysis: A “how-to” guide]

# Impact of Buffer Concentration




Column: Agilent Bio MAb, NP10, 4.6 x 250 mm

Mobile phase:

A: Phosphate buffer, pH 7.5

B: A + 0.1M NaCl

Initial buffer concentration:

A = 20 mM phosphate

B = 10 mM phosphate

C = 5 mM phosphate

Gradient: 15-65%B in 60 min

Flow rate: 0.8 mL/min

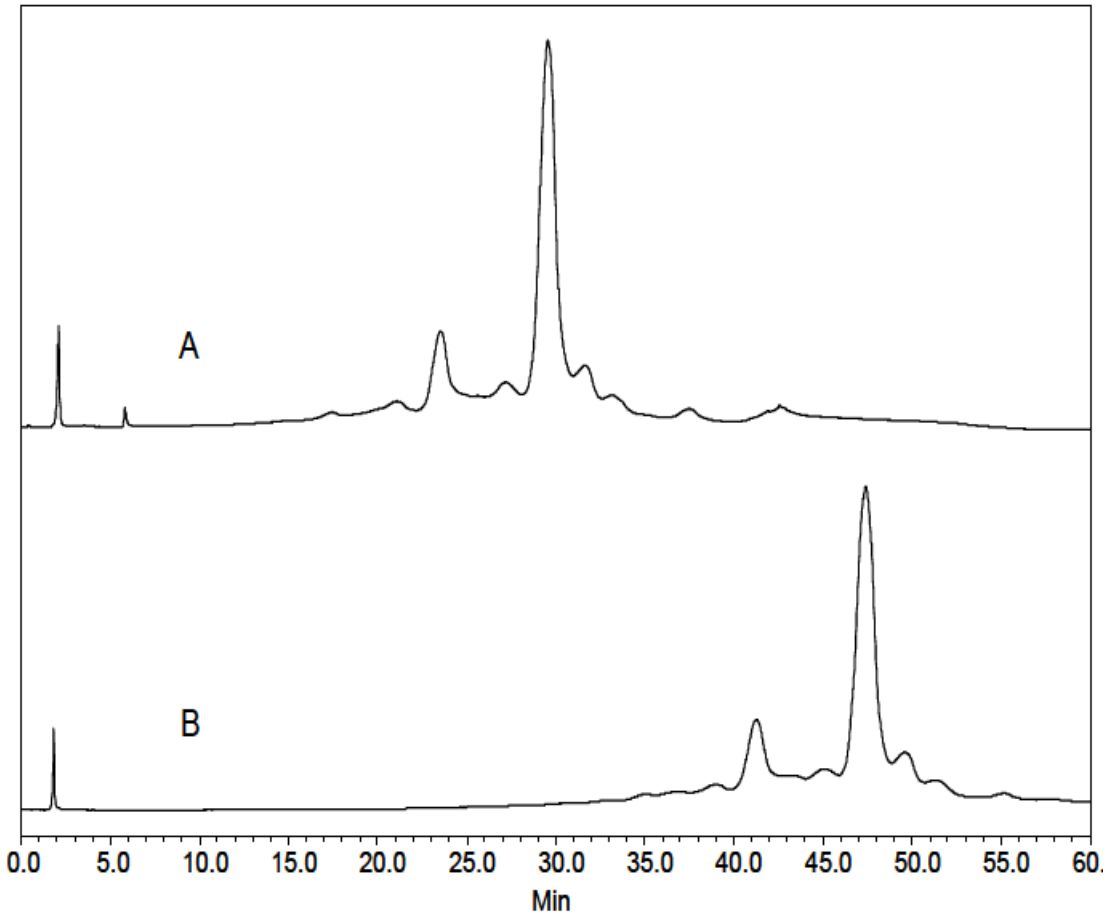
Sample: Monoclonal antibody

Injection: 10 µL (1.5 mg/mL)

Temperature: 25 °C

Detection: UV 214 nm

# Effect of pH




Column: Agilent Bio MAb, NP10, 4.6 x 250 mm

Mobile phase:

A: 10 mM phosphate;

B: A + 0.1 M NaCl

pH:

A = pH 7.5

B = pH 7.0

Gradient: 15 to 65%B in 60 min

Flow rate: 0.8 mL/min

Sample: Monoclonal antibody

Injection: 10 µL (1.5 mg/mL)

Temperature: 25 °C

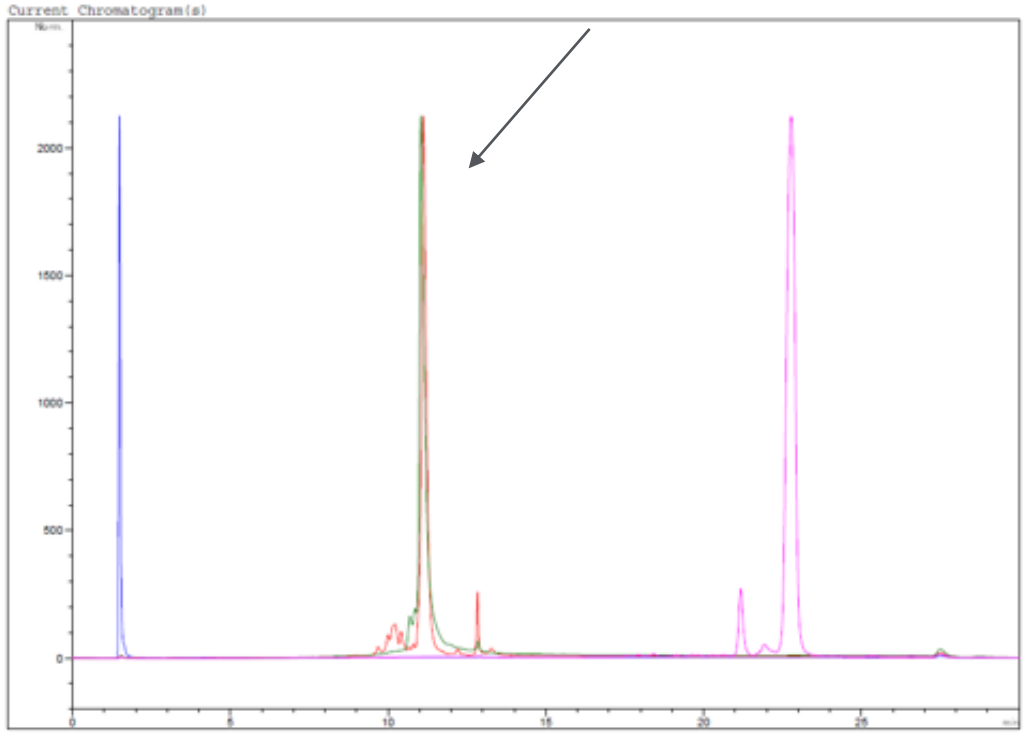
Detection: UV 214 nm

# Weak Cation Exchange

## pH as a tool to modify selectivity

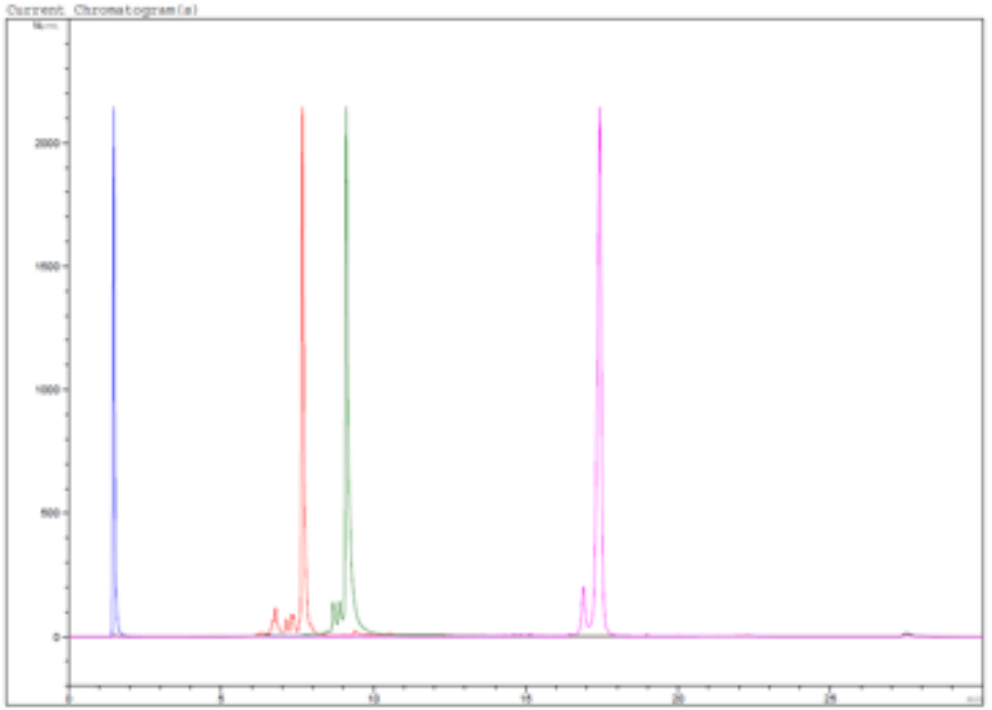
pH 6.0

pH 6.0: Ribonuclease A and Cytochrome C co-elute




1. Ovalbumin pI 4.5
2. RNase A pI 9.4
3. Cytochrome C pI 9.8
4. Lysozyme pI 11

pH 6.5



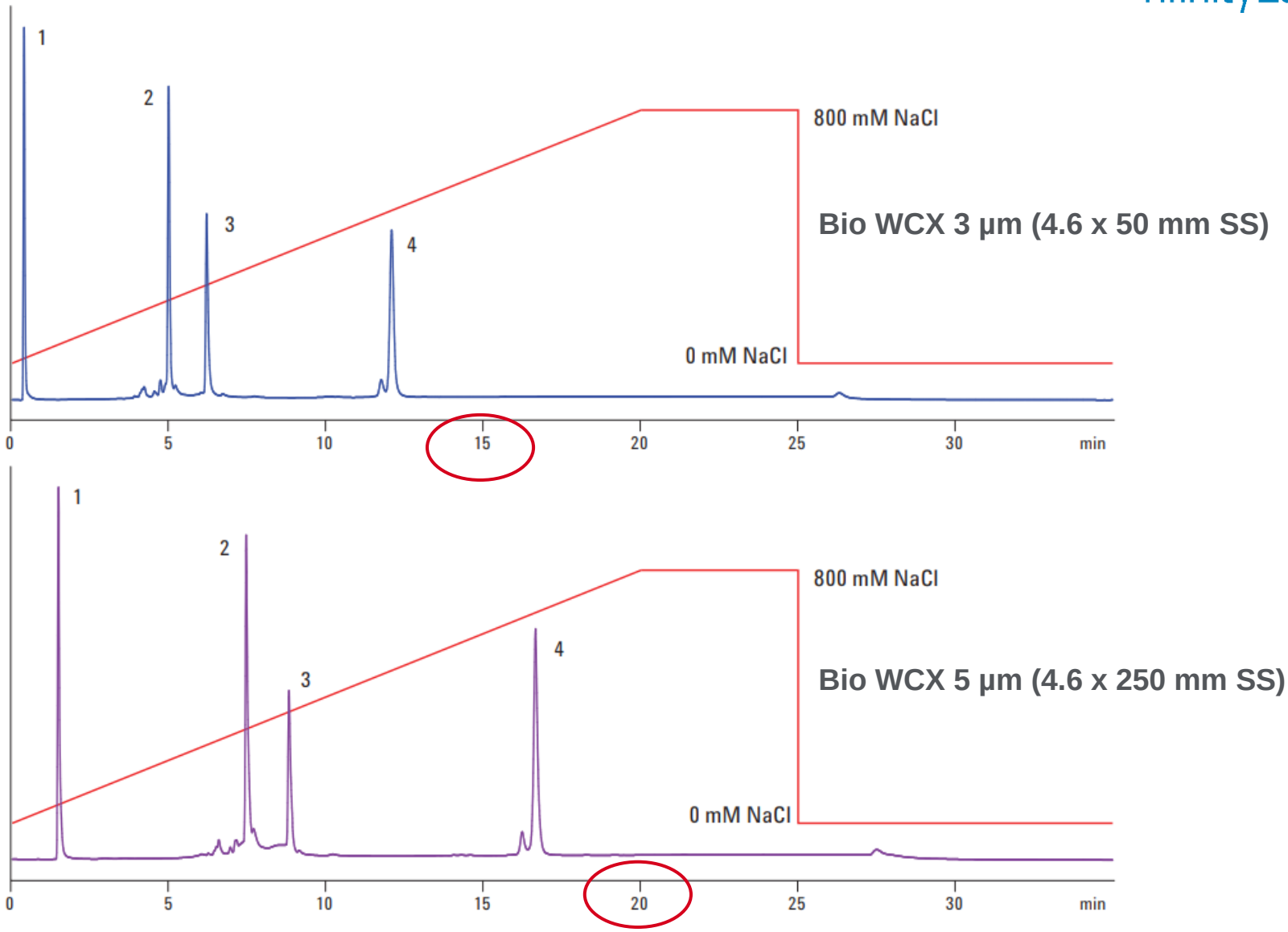

Column: Bio WCX NP5 (250 x 4.6 mm SS)
Gradient: 20 mM sodium phosphate buffer
0-750 mM NaCl (0-20 mins)
Flow rate: 1.0 mL/min
Sample: 5 µL
Detection: UV, 220 nm

# Achieve Faster Analysis Time

Agilent InfinityLab

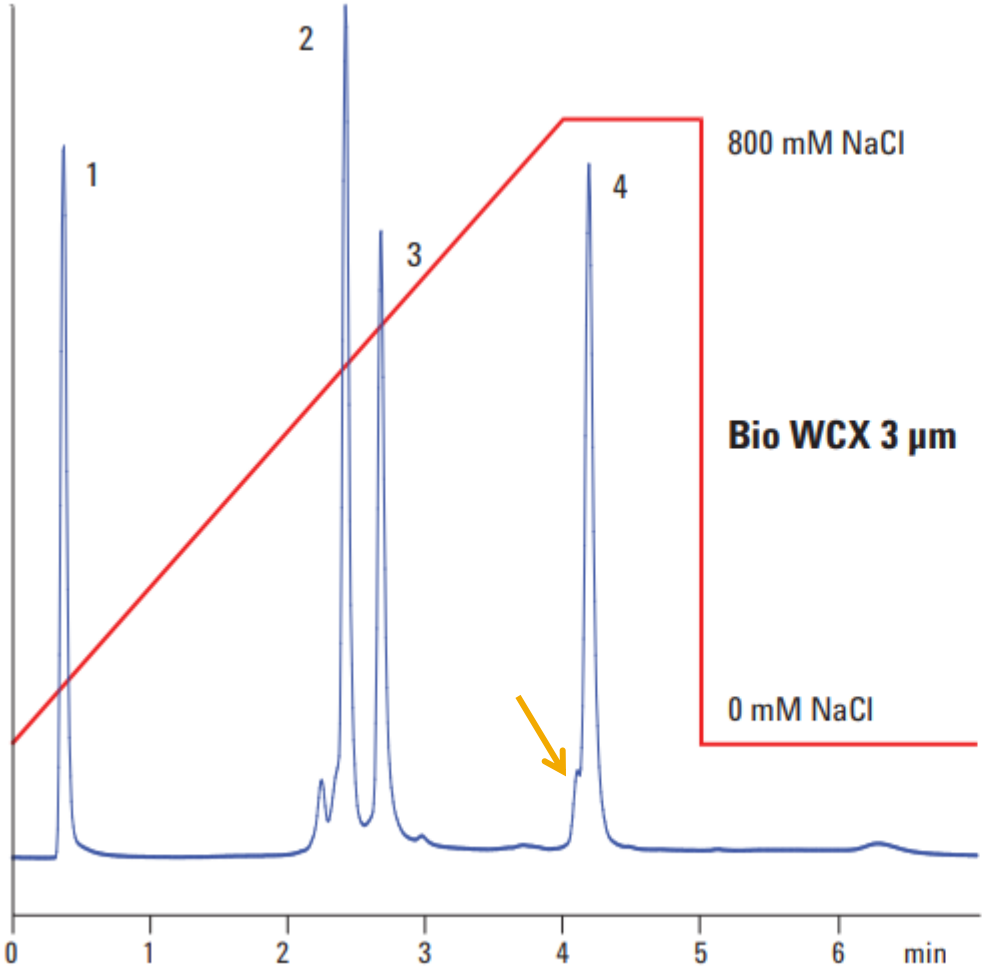
Smaller particles and shorter column lengths

| | | |
|---|---|---|
| Gradient | 20 mM sodium phosphate buffer, pH 6.5<br>0-800 mM NaCl (0-20 mins) | |
| Flow rate | 1.0 mL/min | |
| Sample | 10 µL inj. | |
| Detection | UV, 220 nm | |
| Samples: | | |
| 1. | Ovalbumin | pI 4.5 |
| 2. | RNase A | pI 9.4 |
| 3. | Cytochrome C | pI 9.8 |
| 4. | Lysozyme | pI 11 |

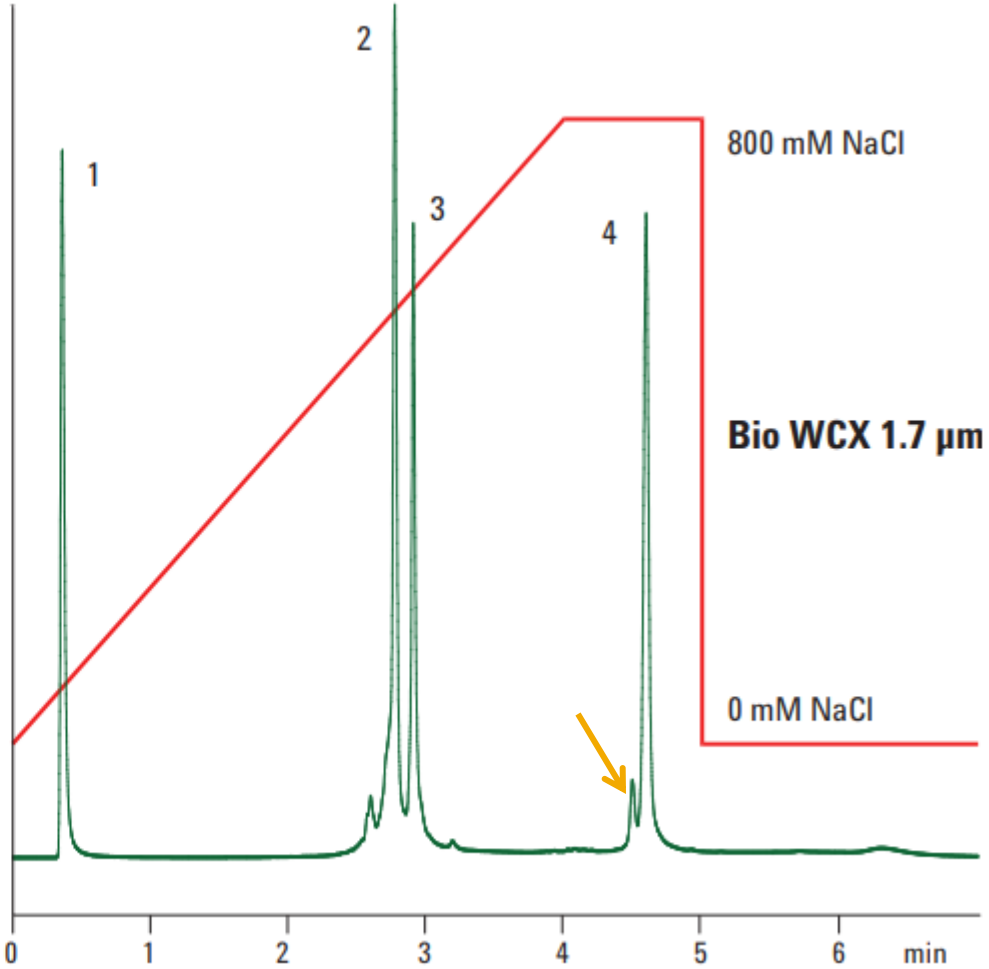



Reference technical note publication number: [5990-9931EN]

# Higher Resolution Separations with1.7 µm IEX columns




Bio WCX 3 µm, 4.6 x 50 mm
0.5 mL/min



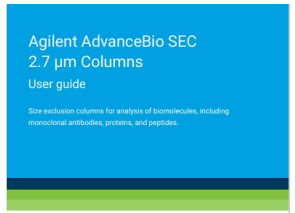

Bio WCX 1.7 µm, 4.6 x 50 mm
0.5 mL/min ← Not UHPLC

Reference technical note publication number: 5990-9931EN

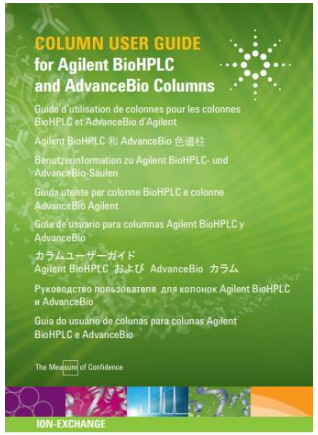
# Troubleshooting and Column Care

# Know Your Column

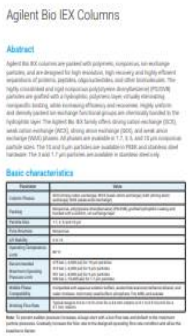
## See the column user guide










The user guide provides information for:

- Shipping solvent
- Technical specifications
- Column conditions
- Mobile phases guidelines
- Recommended starting gradients
- Column care
- Tips for getting the best chromatography
- Troubleshooting
- Column cleaning and storage

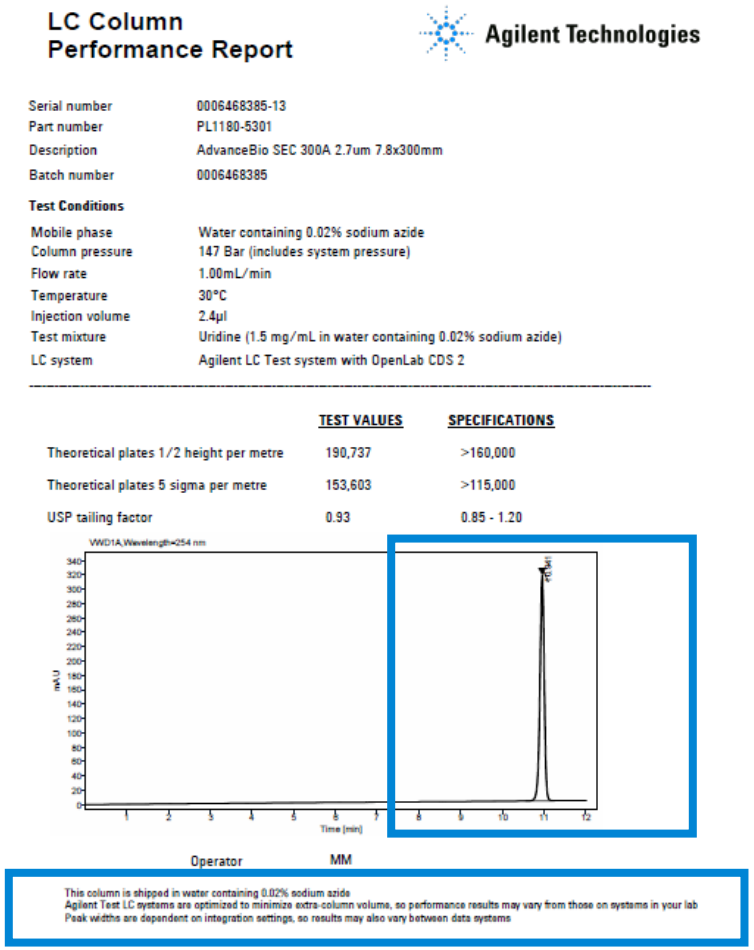
From Agilent.com, the link for SEC and IEX columns user guides is:

Bio LC Column User Guides | Agilent

# Benchmark Your Column

Agilent InfinityLab

## Every new column should be “QC” tested on your instrument



LC Column Performance Report

Agilent Technologies

| | |
|---|---|
| Serial number | 0006468385-13 |
| Part number | PL1180-5301 |
| Description | AdvanceBio SEC 300A 2.7um 7.8x300mm |
| Batch number | 0006468385 |

**Test Conditions**

| | |
|---|---|
| Mobile phase | Water containing 0.02% sodium azide |
| Column pressure | 147 Bar (includes system pressure) |
| Flow rate | 1.00mL/min |
| Temperature | 30°C |
| Injection volume | 2.4µl |
| Test mixture | Uridine (1.5 mg/mL in water containing 0.02% sodium azide) |
| LC system | Agilent LC Test system with OpenLab CDS 2 |

| | TEST VALUES | SPECIFICATIONS |
|---|---|---|
| Theoretical plates 1/2 height per metre | 190,737 | >160,000 |
| Theoretical plates 5 sigma per metre | 153,603 | >115,000 |
| USP tailing factor | 0.93 | 0.85 - 1.20 |



Operator MM

This column is shipped in water containing 0.02% sodium azide
Agilent Test LC systems are optimized to minimize extra-column volume, so performance results may vary from those on systems in your lab
Peak widths are dependent on integration settings, so results may also vary between data systems

### Performance verification based on Agilent checkout

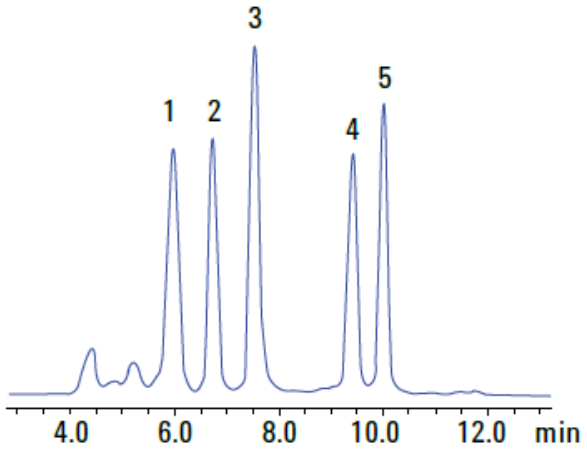
- Run Agilent checkout before use
  - Record the difference between your instrument and the performance report (use as a base value)
- Run again if the column seems to lose performance
  - Compare with results from the first run

### Performance verification based on in-house checkout

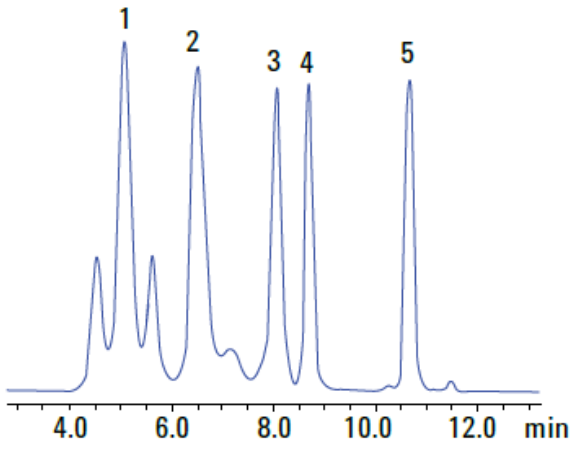
- Run in-house checkout before use
  - Record key specifications, such as tailing factor, plates, backpressure
- Run again if the column seems to lose performance
  - Compare with results from the first run

# What to Use for Your SEC Column?

## AdvanceBio SEC protein standards




AdvanceBio SEC 130Å Protein Standard separation on AdvanceBio SEC 130Å column




AdvanceBio SEC 300Å Protein Standard separation on AdvanceBio SEC 300Å column


| **AdvanceBio SEC 130Å Protein Standard** p/n 5190-9416, 1.5 mL vial) | |
|---|---|
| **Analyte** | **MW** |
| 1. Ovalbumin | 45,000 |
| 2. Myoglobin | 17,000 |
| 3. Aprotinin | 6,700 |
| 4. Neurotensin | 1,700 |
| 5. Angiotensin II | 1,000 |

| **AdvanceBio SEC 300Å Protein Standard** (p/n 5190-9417, 1.5 mL vial) | |
|---|---|
| **Analyte** | **MW** |
| 1. Thyroglobulin | 670,000 |
| 2. γ-globulin | 150,000 |
| 3. Ovalbumin | 45,000 |
| 4. Myoglobin | 17,000 |
| 5. Angiotensin II | 1,000 |

# Take Care Installing Your Column

- Set the maximum back pressure for your system which will be appropriate for your column
- Check the solvent that the column was shipped in
- Know which mobile phase is currently running in your LC system
- Care should be taken not to pass any mobile phase through the column that may cause a precipitate to form
- If mobile phase additives are used (such as buffers or organics), it is advisable to do an intermediate flush with a mobile phase of the correct composition, but without these additions
- Flush the LC system with mobile phase through to the column inlet connection
- Note the correct flow direction on column when connecting it to the LC system
- Introducing the mobile phase to the column at a reduced flow rate while monitoring back pressure is recommended
- Gradually increase the flow rate of your column to the working flow rate and allow for column equilibration

# Transferring of Solvents for SEC Columns

## Special considerations for SEC columns

Care must be taken to avoid overpressuring your column.
This can be a problem when transferring to solvents of higher viscosity.

Column flushing must be done at low flow rates:

- For 2.7 µm particle columns:
  - At no more than 0.1 mL/min for 4.6 mm columns
  - At no more than 0.2 mL/ min for 7.8 mm columns
  - Keep column pressure under 200 bar

- For 1.9 µm particle columns:
  - At no more than 0.1 mL/min for 4.6 mm columns
  - At no more than 0.05 mL/min for 2.1 mm columns
  - Keep column pressure under 400 bar

- Take care to avoid precipitation

# Cleaning for SEC Columns

## Example column cleaning instructions, AdvanceBio SEC 2.7 µm

Agilent InfinityLab

### Suggested cleaning solutions

- For strongly adsorbed contaminants:
  - High salt concentration at low pH (for example, 0.5 M Na2SO4, pH 3) or 0.5 M guanidine hydrochloride
- Organic solvent for hydrophobic materials:
  - Up to 50% methanol, ethanol, or isopropanol
- Acidic reagents for basic contaminants:
  - 0.1% TFA, formic acid, or acetic acid in 15% acetonitrile

For column specific instructions, see column user guide

- Flush the column in the direction of the flow arrow
- Reduce the flow rate to keep the back pressure below 200 bar
- Rinse the column with at least five column volumes of ultrapure water before and after flushing with cleaning solution.
- Use at least 20 column volumes of cleaning solution

Note: It is **not** recommended to use the three cleaning solutions sequentially. Choose the most appropriate for your possible contaminant.

Take care to avoid precipitation of buffer salts and overpressuring the column due to mobile phase viscosity differences.

# Cleaning for IEX Columns

For column specific instructions,
see column user guide

- Mild contamination
  - Columns can be efficiently cleaned with 1.0 M NaCl in mobile phase A solution (equilibration buffer).
- Moderate contamination
  - The recommended cleaning buffer for the SCX and WCX columns is 50 mM phosphate buffer in 1.0 M NaCl, pH 10.
  - The recommended cleaning buffer for the SAX and WAX columns is 150 mM potassium nitrate, pH 4.0.
- Severe contamination
  - Columns can be washed in reversed direction with 25 mM NaOH for 15 CV at 0.25 mL/min.
- Remove hydrophobic contaminants
  - If the columns are contaminated with hydrophobic proteins, these can be removed using 50 to 75% ethanol or acetonitrile for at least 15 CV.

# Cleaning and Solvent Transfer for IEX Columns

- As a first option:
  - To clean the column, flush the column in the reverse direction for at least 15 CV at no more than 50% of the maximum operating pressure limit of the particle size.

- Care must be taken to avoid overpressuring your column.
  - Reduce the flow rate to keep column back pressures low

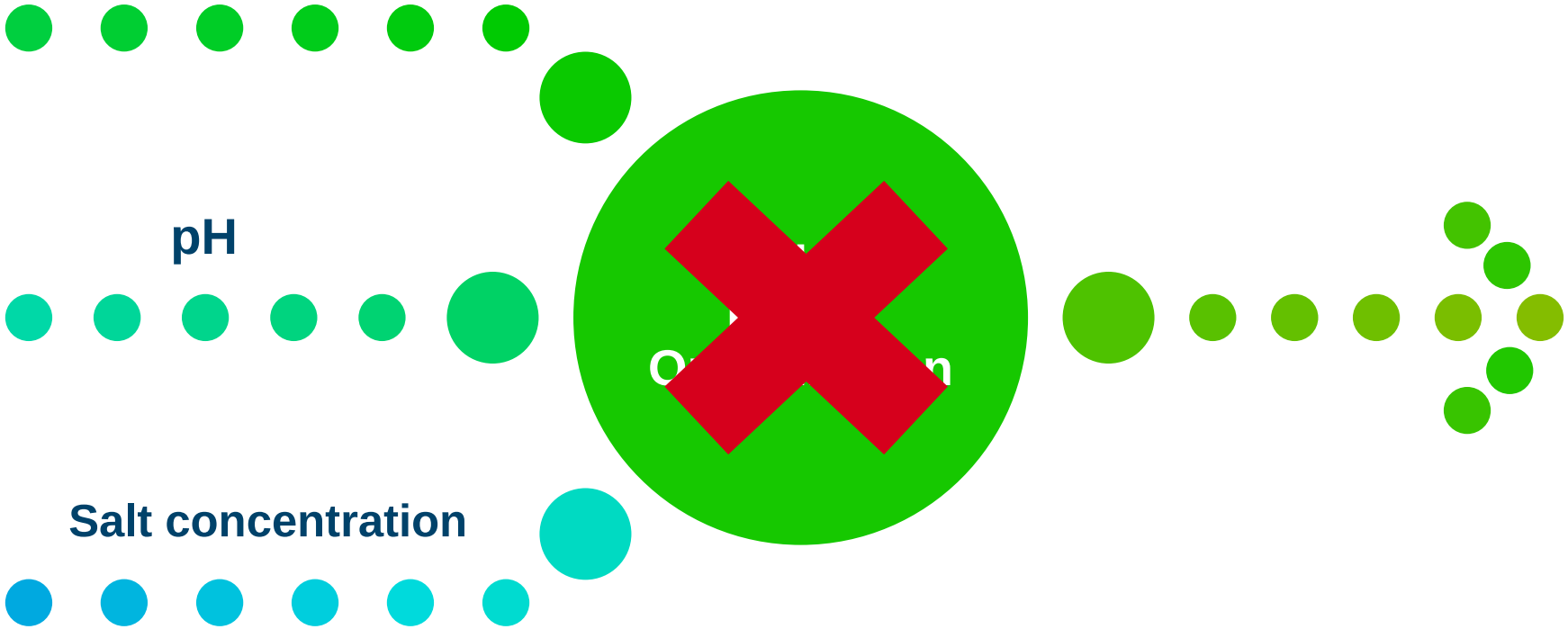
- After rinsing with **any** cleaning solution:
  - Rinse the columns with at least 10 CV of deionized water
  - Next, rinse the columns with mobile phase B for 10 CV
  - Equilibrate the columns with equilibration buffer (such as mobile phase A or a mixture of mobile A and B) for 10 CV

- If organics were used for cleaning:
  - Rinse the column with 100% deionized water for 10 CV to remove organic solvents

**Note**: Before using organics to clean the columns, ensure that the columns are flushed with water to remove salt. This will prevent salt precipitation, which could increase column backpressure significantly and damage the columns.

# Importance of Consistent Buffer Preparation

Agilent InfinityLab

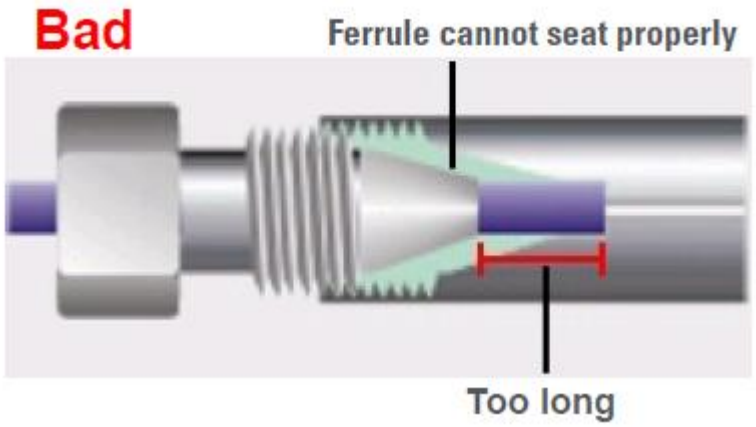


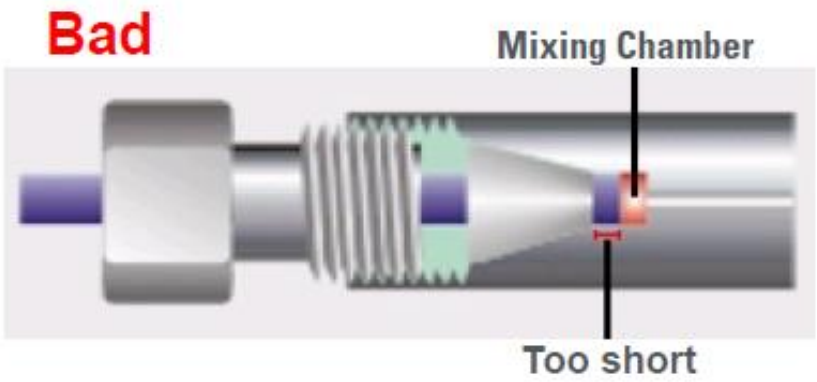

Poor mobile phase preparation can result in problems with:

- Peak resolution
- Peak symmetry
- Peak retention time

# Considerations for Your HPLC System

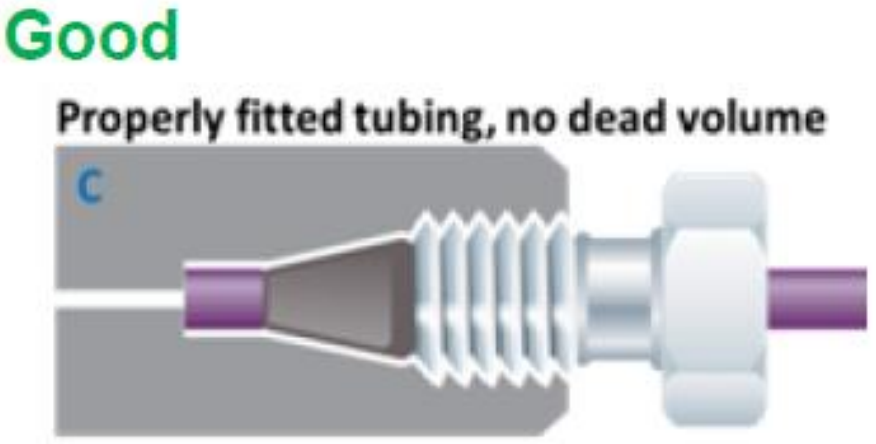
## Make correct connections



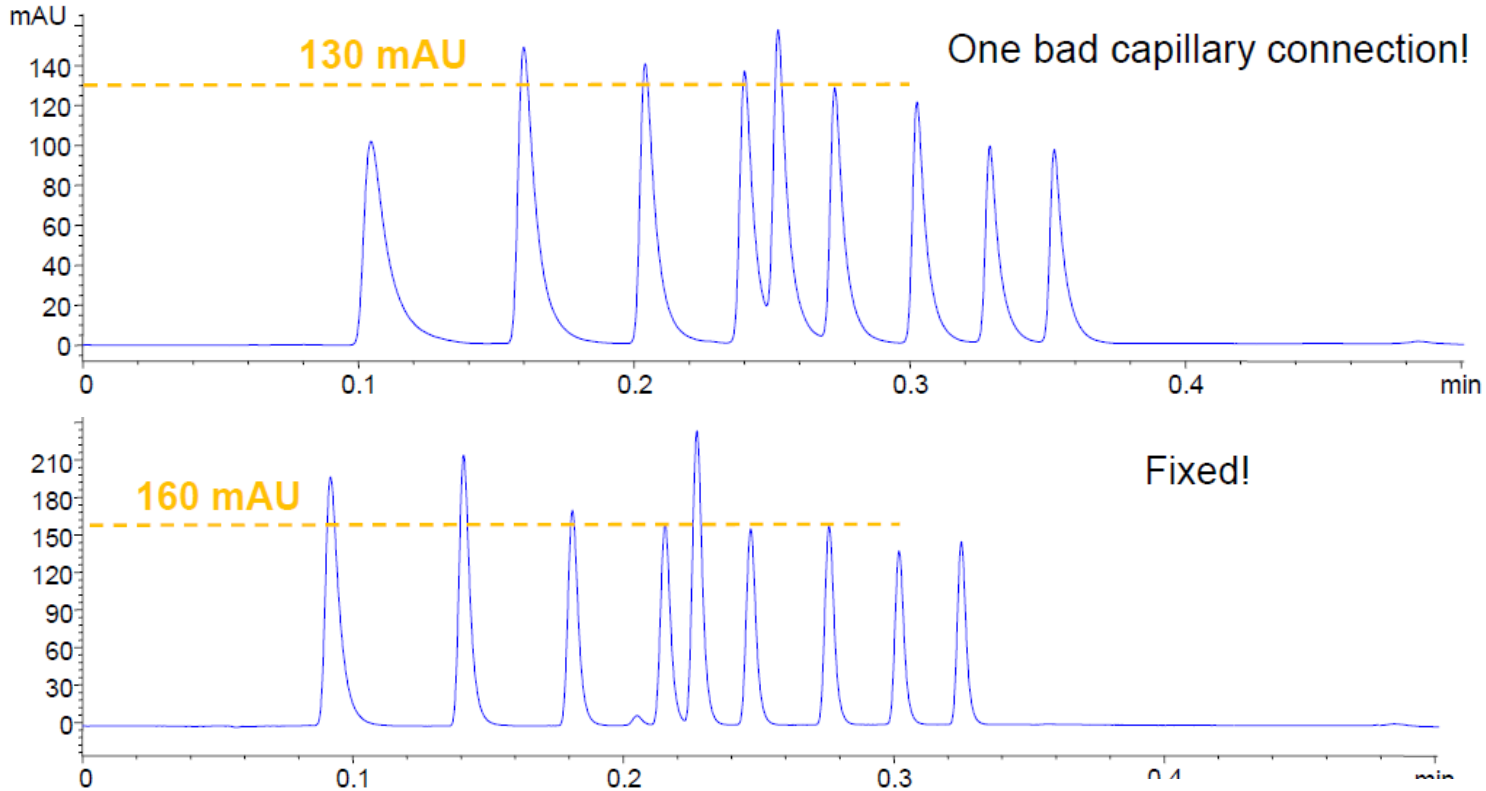




**Poor fitting connections**

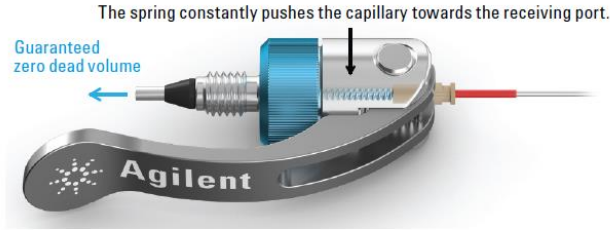
- Will broaden or split peaks, or cause tailing
- Will typically affect all peaks, but especially early eluting peaks
- Can cause carryover



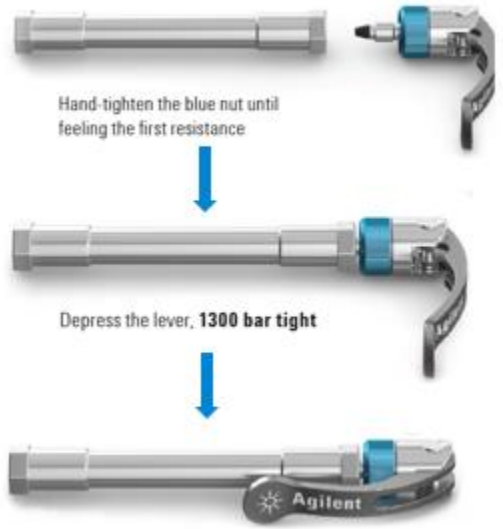
# Importance of Correct Connections




Quick Turn




Quick Connect



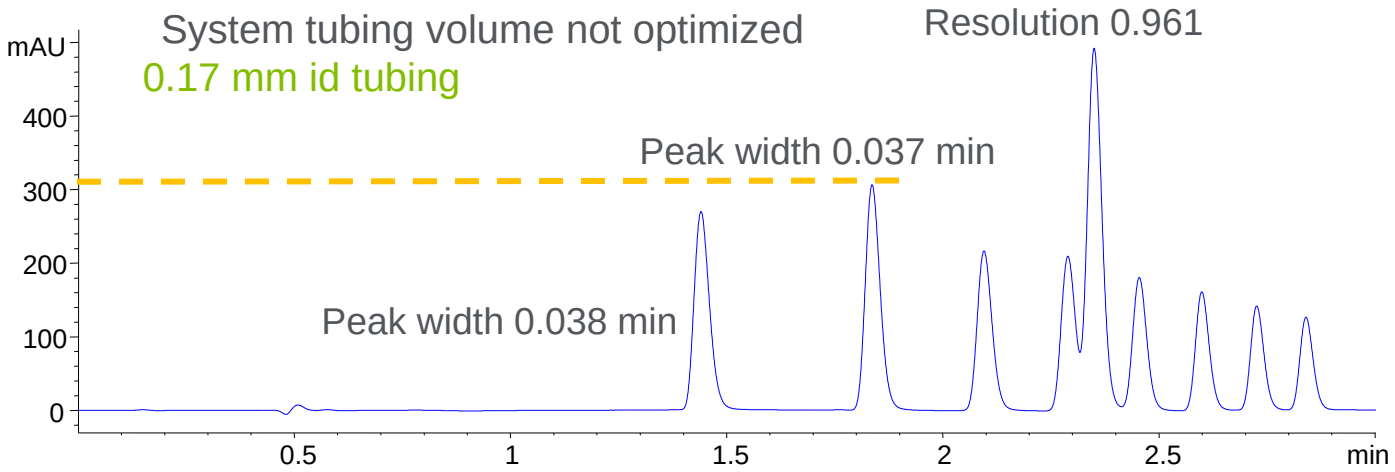

Correct connection every time

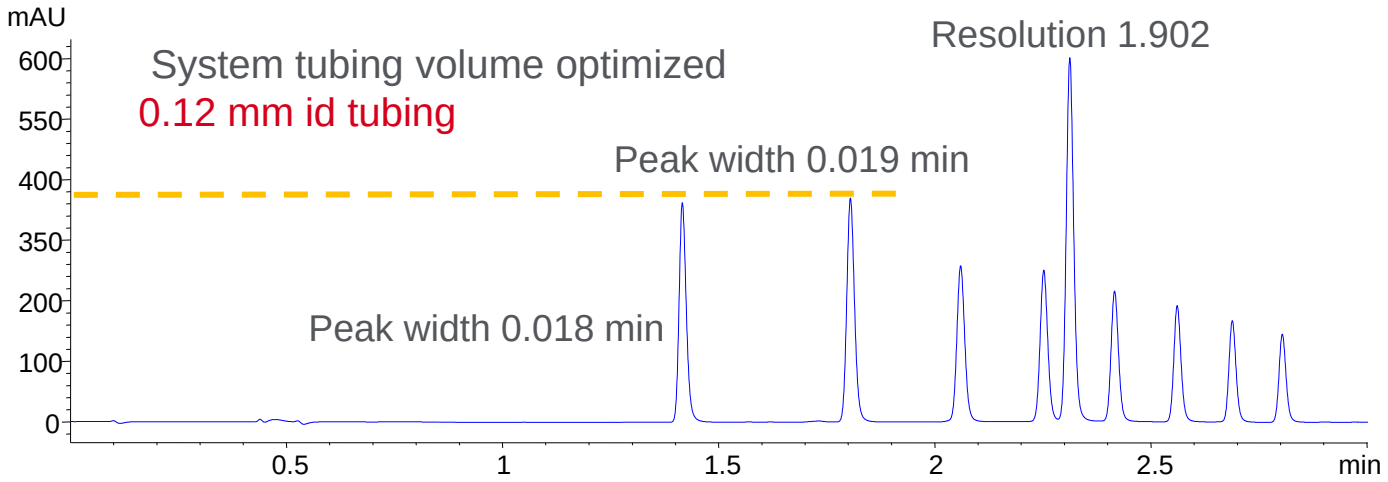
Compatible to 1300 bar

Agilent technical note: Agilent InfinityLab UHPLC Fittings

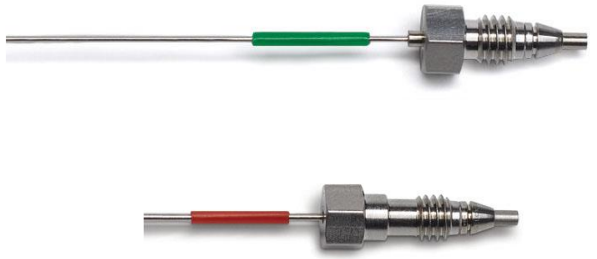
Publication number: 5991-5525EN

# Optimizing Connecting Tubing Volume for UHPLC Columns




| Length | 10 mm | 50 mm | 100 mm | 150 mm |
|---|---|---|---|---|
| Tubing id | Volume | Volume | Volume | Volume |
| 0.17 mm (green) | 0.227 µL | 1.1 µL | 2.27 µL | 3.3 µL |
| 0.12 mm (red) | 0.113 µL | 0.55 µL | 1.13 µL | 1.65 µL |



# Agilent Biomolecule Columns Portfolio

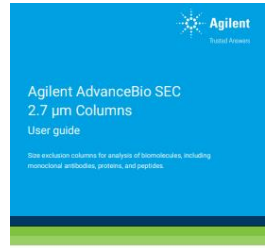
Agilent InfinityLab

| Titer Determination | Aggregate Analysis | Intact Purity and PTM Analysis | | Peptide Mapping and PTM Analysis | Charge Variant Analysis | Glycan Analysis | Amino Acid/Cell Culture Media Analysis | |
|---|---|---|---|---|---|---|---|---|
| Affinity | Size Exclusion | Reversed Phase >150 Å | Hydrophobic Interaction | Reversed Phase <150 Å | Ion Exchange | Hydrophilic Interaction | Reversed Phase <150 Å | Hydrophilic Interaction |
| Bio-Monolith rProtein A | AdvanceBio SEC 1.9 µm PEEK-lined | PLRP-S 1000Å 5 µm PEEK-lined | AdvanceBio HIC | AdvanceBio EC-C18 PEEK-lined | Bio mAb/Bio IEX 5 µm PEEK | AdvanceBio Glycan Mapping | AdvanceBio Amino Acid Analysis | AdvanceBio MS Spent Media PEEK-lined |
| Bio-Monolith Protein A | AdvanceBio SEC 1.9 µm | PLRP-S | | AdvanceBio Peptide Mapping | Bio mAb (WCX) | | ZORBAX Eclipse AAA 3.5 µm | |
| Bio-Monolith Protein G | AdvanceBio SEC 2.7 µm | AdvanceBio RP mAb 450Å | | AdvanceBio Peptide Plus | Bio IEX (SAX, WAX, SCX, WCX) | | | |
| | Bio SEC-3 | ZORBAX RRHD 300 Å, 1.8 µm | | ZORBAX RRHD 300 Å, 1.8 µm | PL SCX, SAX | | | |
| | Bio SEC-5 | ZORBAX 300SB 3.5, 5 and 7 µm | | | Bio-Monolith (QA, DEAE, SO3) | | | |
| | ZORBAX GF250 and GF450 | Poroshell 300 5 µm | | | | | | |
| | | | | Stainless steel column hardware | Solid PEEK or PEEK lined bioinert column hardware | | | |

| Oligonucleotide Impurity Analysis | | Capsid Analysis | |
|---|---|---|---|
| Reversed Phase | Ion Exchange | Aggregation – SEC | Empty/Full - SAX |
| AdvanceBio Oligonucleotide | PL-SAX | Bio SEC-5 | Bio SAX |
| PLRP-S | Bio SAX | | Bio SAX |
| | Bio SAX | | |

# LC Columns and Supplies Resources

- BioHPLC column catalog: 5994-0974en-agilent.pdf
- BioLC column user guides: Bio LC Column user guides | Agilent
- IEX for Biomolecules “How to” guide: 5991-3775EN (agilent.com)
- Buffer Advisor: Agilent Buffer Advisor Technical Overview
- SEC for Biomolecules “How to” guide: 5991-3651EN_LR.pdf (agilent.com)
- InfinityLab supplies catalog: InfinityLab LC Supplies (agilent.com)
- LC Handbook: LC-Handbook-Complete-2.pdf (Agilent.com)
- LC troubleshooting poster: LC Troubleshooting Guide (Agilent.com)
- Agilent Community: Agilent Community
- Consumables Community: Agilent Collection of Columns, Supplies, and Standards Resources - Consumables - Agilent Community
- App finder: Application Finder | Agilent
- Agilent Peak Tales podcasts: peaktales.libsyn.com
- Webinars, upcoming and recorded: Webinars | Agilent

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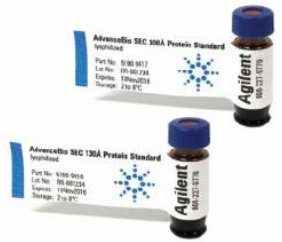


# Contact Agilent Chemistries and Supplies Technical Support

Available in the U.S. and Canada, 8-5 all time zones

1-800-227-9770 option 3, option 3:

Option 1 for GC and GC/MS columns and supplies

Option 2 for LC and LC/MS columns and supplies

Option 3 for sample preparation, filtration, and QuEChERS

Option 4 for spectroscopy supplies

Option 5 for chemical standards

Option 6 for Prozyme products

gc-column-support@agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

chem-standards-support@agilent.com

pzi.info@agilent.com

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# Thank you for attending

**Any questions?**

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