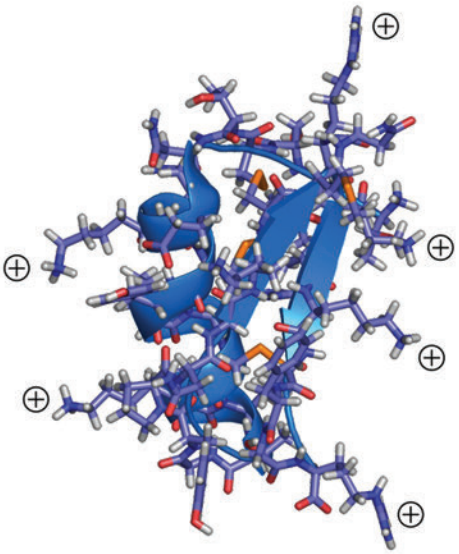
# ION-EXCHANGE CHROMATOGRAPHY FOR BIOMOLECULE ANALYSIS:

## A "How To" Guide

The Measure of Confidence



Agilent Technologies



# INTRODUCTION

Proteins are made up of chains containing numerous amino acids, several of which possess acidic or basic side chain functionalities. This results in an overall charge on the surface of the protein that can be controlled by adjusting the pH of the surrounding solution. The isoelectric point, pI, is the pH at which the net charge of the protein is neutral (the number of positive charges is equal to the number of negative charges). If the pH is below this value, the protein will possess an overall positive charge and can be retained on a negatively charged cation-exchange sorbent; if the pH is above the pI, the protein will be negatively charged overall and can be retained on an anion-exchange sorbent.

In this "how to" guide we discuss ion-exchange (IEX) chromatography, column selection choices, important mobile phase considerations, general rules of thumb for using IEX, instrument considerations, and more.

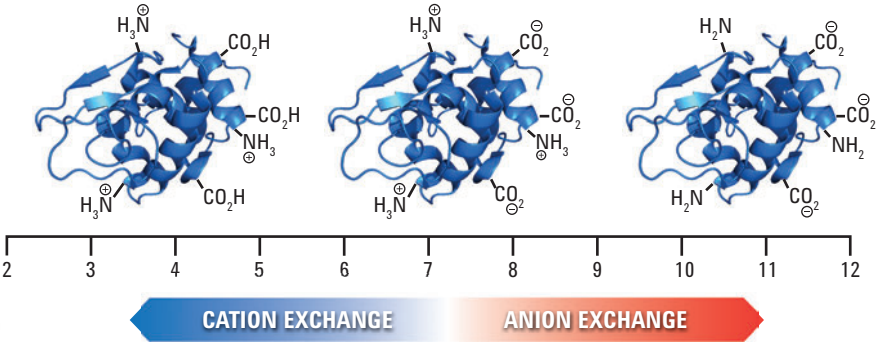



**Figure 1** – Effect of pH on net protein charge

# Separation, based on ionic charge, is typically performed under non-denaturing conditions

Ion-exchange is a widely used method for separating biomolecules based on differences in ionic charge. It is a mild, non-denaturing technique that does not require organic solvents and is therefore frequently used for characterization of proteins in their native or active form, and for purification.

Proteins contain a variety of functionalities that can give rise to differences in charge. Acidic groups include C-terminal carboxylic acids, acidic side chains of aspartic and glutamic acid, and acidic groups arising from sialic acid in glycosylated proteins; basic groups include N-terminal amines and basic side chains of arginine, lysine, and histidine. The overall charge of the molecule is therefore dependent on the pH of the surrounding solution and this in turn will affect the ion-exchange method that can be used. The mobile phase must maintain a controlled pH throughout the course of the separation, and so aqueous buffers are used as eluents.

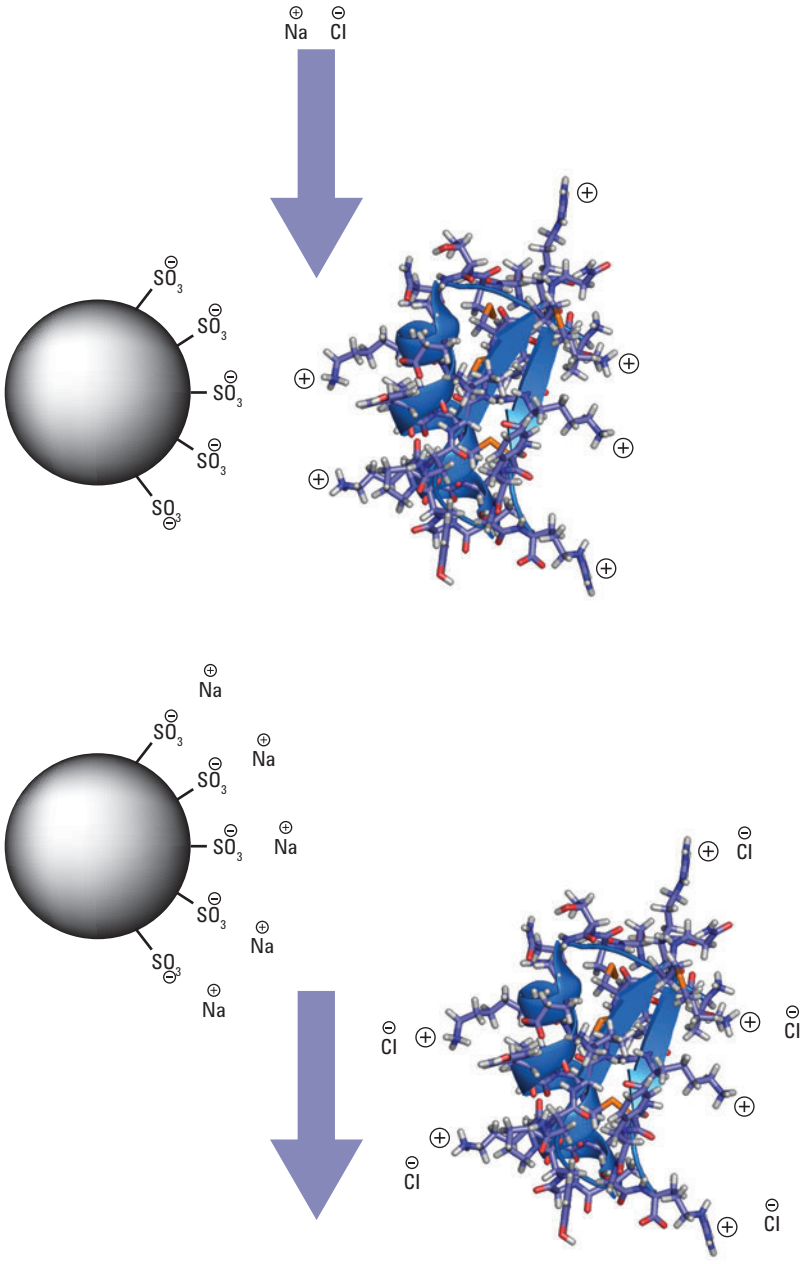



**Figure 2** – Separation mechanism of ion-exchange

The technique of ion-exchange is therefore suitable for separating proteins with differing isoelectric points, but it is equally valuable in separating charged isoforms of a single protein. In the increasingly important field of biopharmaceuticals, where proteins are manufactured through bioengineering and isolated from fermentation reactions, it is important to identify charged isoforms as these indicate a difference in primary structure of the protein. A difference in primary structure could indicate a change in glycosylation, or degradation pathways such as loss of C-terminal residues or amidation/deamidation. They can also result in a change in stability or activity and could potentially lead to immunologically adverse reactions. Ion-exchange is used to separate and quantify charge variants during the development process and also for quality control and quality assurance during manufacture of biotherapeutics. With large molecules such as monoclonal antibodies (mAbs) it is also important to consider the size and structure of the molecule (mAbs are typically 150 kD), particularly as the chromatographic interactions will only occur with surface charges.

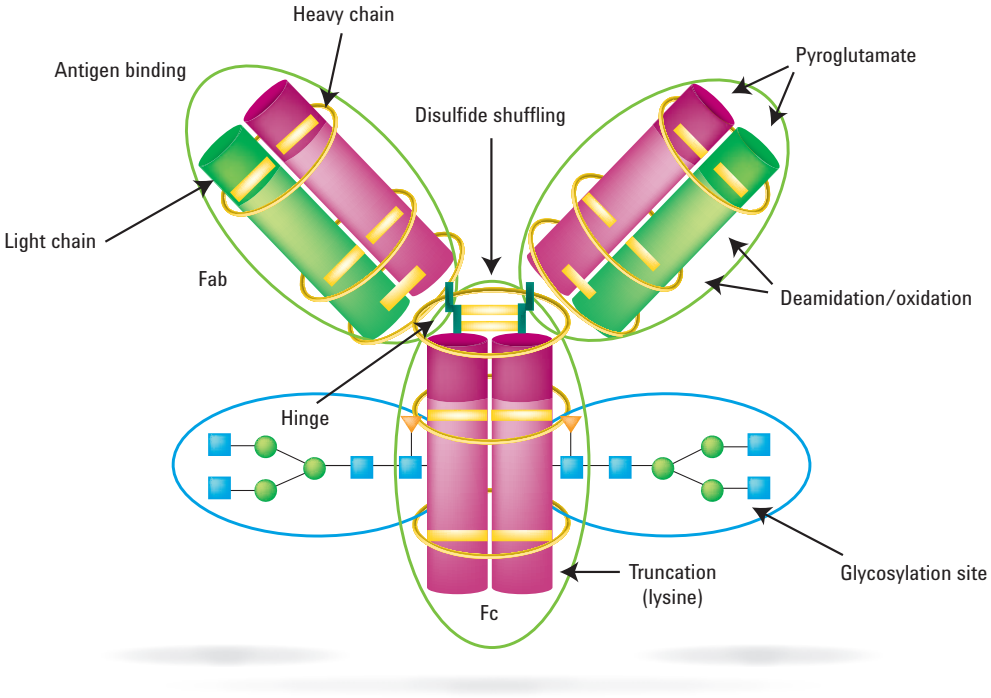
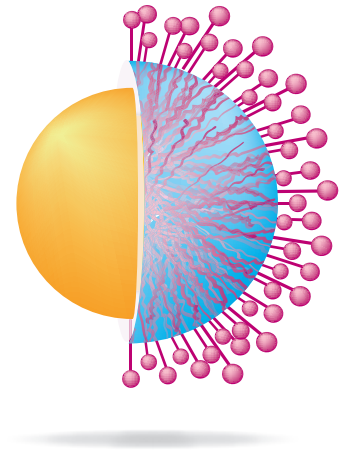



**Figure 3** – Charged variants of monoclonal antibodies arise through different levels of glycosylation, deamidation, and oxidation of amino acids, and through lysine truncation of heavy chains



## Agilent Bio MAb HPLC columns: superior performance from the inside out

- Particles, coating, and bonding are resistant to high pressures, promoting higher resolution and faster separations
- Hydrophilic coating eliminates most nonspecific interactions
- A highly uniform, densely packed, weak cation-exchange (WCX) layer chemically bonded to the hydrophilic, polymeric coating

## Use Bio MAb to identify C-terminal truncation on heavy chains

| | |
|---|---|
| **Column:** | **Bio MAb, PEEK 5190-2407 4.6 x 250 mm, 5 µm** |
| Mobile Phase: | A: 10 mM Na phosphate buffer, pH 5.5<br>B: A + 0.5 M NaCl |
| Flow Rate: | 0.85 mL/min |
| Gradient: | 10 to 35% B from 0-25 min |
| Detector: | UV, 225 nm |
| Sample: | 5 µL of 1 mg/mL of intact or C-terminal digested IgG1 |
| Instrument: | Agilent 1260 Infinity Bio-inert Quaternary LC |

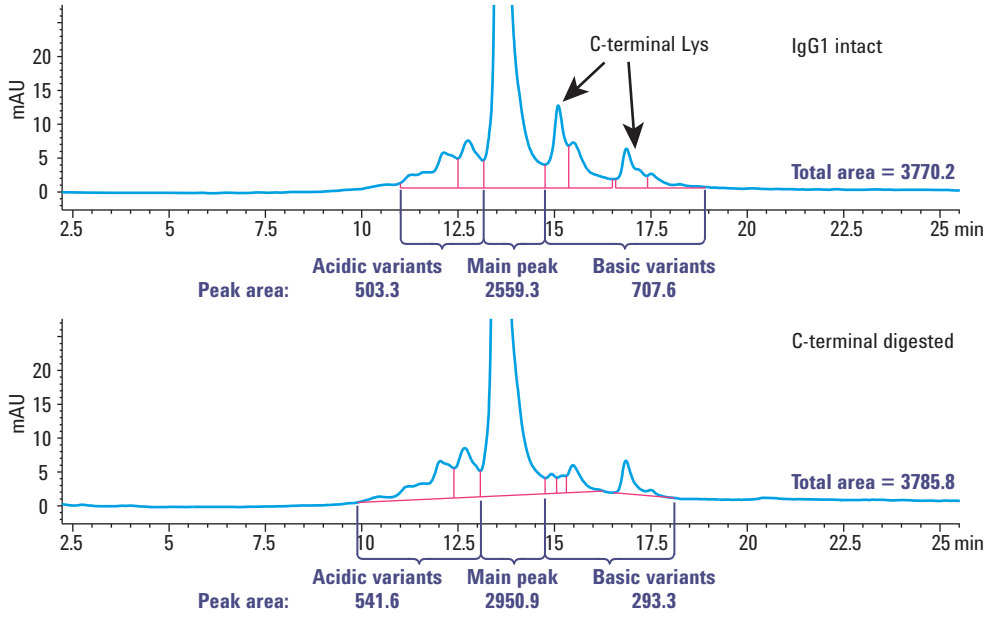



**Figure 4** – Calculation of C-terminal digested IgG1 using an Agilent Bio MAb 5 µm column on the Agilent 1260 Infinity Bio-inert Quaternary LC. The column delivers high resolution, enabling better peak identification and accurate quantification

Learn more about Agilent Bio-IEX columns at **agilent.com/chem/bioHPLC**

# Understanding the requirements for a successful ion-exchange separation


## Step 1: **Sample Preparation**

Sample preparation for ion-exchange chromatography is not unlike that for any protein analysis. The most important aspect is that the sample must be soluble in the eluent and should ideally be dissolved in the mobile phase itself. To protect the column from possible damage we recommend that samples are filtered before use to remove particulates, but filtration should not be used to compensate for poor sample solubility – an alternative eluent may need to be found.

### Captiva Low Protein Binding Filters

Agilent Captiva Premium PES Syringe Filters provide superior and consistent low protein binding for protein-related filtration. The polyethersulfone (PES) filter membranes are a better option than polyvinylidene difluoride (PVDF) membranes for most LC analyses. Agilent PES has similar compatibility to PVDF filters for common LC solvents and is superior in terms of protein binding and cleanliness. Learn more at **www.agilent.com/chem/filtration**

**Captiva Premium PES Syringe Filters**

| Diameter (mm) | Pore Size (µm) | Certification | Housing | Part No. |
|---|---|---|---|---|
| 4 | 0.2 | LC/MS | Polypropylene | 5190-5094 |
| 4 | 0.45 | LC | Polypropylene | 5190-5095 |
| 15 | 0.2 | LC/MS | Polypropylene | 5190-5096 |
| 15 | 0.45 | LC | Polypropylene | 5190-5097 |
| 25 | 0.2 | LC/MS | Polypropylene | 5190-5098 |
| 25 | 0.45 | LC | Polypropylene | 5190-5099 |

For effective sample preparation it is also important to ensure that methods used to dissolve the sample do not change the properties of the sample itself.


## AssayMAP Automated Protein and Peptide Sample Preparation

AssayMAP sample preparation, an automated solution for protein purification, digestion, peptide cleanup, and peptide fractionation, minimizes hands-on time and maximizes workflow reproducibility and efficiency. Standardized user interfaces simplify the workflow while enabling flexible control over key assay parameters. The level of data quality and increased capabilities achievable with AssayMAP technology provide unmatched ability to scale from discovery to validation and production.

- Reproducible results
- Reduced hands-on time
- Simple, user-customizable protocols
- Increased throughput, 8 to 384 samples per day
- Easy method transfer

For an intact protein analysis workflow such as the one presented in this guide, target proteins can be quantitatively purified on the AssayMAP platform using Protein A or Protein G microchromatography cartridges, then fed to HPLC columns to separate and detect intact protein charge variants.

Learn more about AssayMAP technology: **www.agilent.com/lifesciences/assaymap**

# Step 2: **Column Selection**

## Ion-Exchange Column Selection

| 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 | PL-SAX<br>• 1000Å<br>• 4000Å | 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. |
| Globular proteins and peptides | PL-SAX 1000Å | |
| Very large biomolecules/ high speed | PL-SAX 4000Å | |
| Small peptides to large proteins | PL-SCX<br>• 1000Å<br>• 4000Å | 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. |
| Globular proteins | PL-SCX 1000Å | |
| Very large biomolecules/ high speed | PL-SCX 4000Å | |
| Antibodies (IgG, IgM), plasmid DNA, viruses, phages and other macro biomolecules | Bio-Monolith<br>• Bio-Monolith QA<br>• Bio-Monolith DEAE<br>• Bio-Monolith $SO_3$ | Strong cation-exchange, strong and weak anion-exchange phases. Bio-Monolith HPLC columns are compatible with preparative LC systems, including Agilent 1100 and 1200 Infinity Series. |
| Viruses, DNA, large proteins | Bio-Monolith QA | |
| Plasmid DNS, bacteriophages | Bio-Monolith DEAE | |
| Proteins, antibodies | Bio-Monolith $SO_3$ | |

## Column Media Choice

As with most chromatographic techniques, there is a range of columns to choose from. With ion-exchange the first consideration should be "anion or cation-exchange?" There is also the choice of strong or weak ion-exchange. In most circumstances it is best to start with a strong ion-exchange column. Weak ion-exchangers can then be used to provide a difference in selectivity if it is required.

The functional group in a strong cation-exchange column is sulfonic acid, resulting in the stationary phase being negatively charged in all but the strongest acidic mobile phases. Conversely, the functional group in a strong anion-exchange column is a quaternary amine group, which is positively charged in all but the most basic mobile phases. Strong ion-exchange columns, therefore, have the widest operating range. Weak ion-exchange sorbents (carboxylic acids in weak cation-exchangers and amines in weak anion-exchangers) are more strongly affected by the mobile phase conditions. The functionalities are not dissimilar to the charged groups on proteins themselves and the degree of charge can be influenced by ionic strength as well as mobile phase pH.

This can result in a change in resolution that may be subtly controlled and optimized through careful choice of operating conditions. Weak ion-exchangers are therefore an additional tool and can sometimes provide selectivity that is not met by a strong ion-exchange column.

### Exceptional separating power

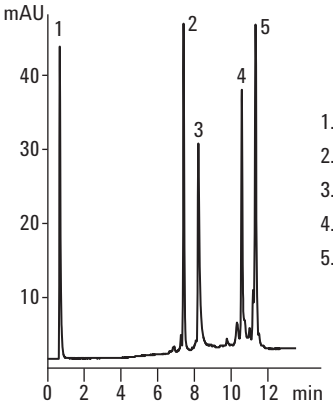
**Column:** **Bio SCX, stainless steel**
**5190-2423**
**4.6 x 50 mm, 3 µm**

Buffer: 10 mM phosphate, pH 6.0

Flow Rate: 0.5 mL/min

Gradient: 0-1.0 M NaCl, 15 min

Detector: 280 nm




1. Ovalbumin, pI = 4.6
2. Ribonuclease A, pI = 8.7
3. Cytochrome c, pI = 9.6
4. Aprotinin, pI = 10.0
5. Lysozyme, pI = 11.0

N > 100,000/50 mm for lysozyme

The hydrophilic, polymeric layer and densely packed ion-exchange functional groups provide extremely sharp peak shapes and high resolution of a mixture of proteins with a broad range of isoelectric points (pI).

**Figure 5** – Exceptional separating power of Agilent Bio SCX

## Pore Size

Where resolution is more important than capacity, rigid, spherical non-porous particles (with an appropriate surface functionality), as provided by the Agilent Bio IEX product range can be beneficial. For the analysis of exceptionally large biomolecules, or where maximum speed is sought, the Agilent Bio-Monolith column can provide optimum results. Some stationary phases, such as PL-SCX or PL-SAX sorbents, are fully porous with 1000 or 4000Å pores. It is important to ensure the pores are sufficiently large to allow proteins to fully permeate the structure unhindered. This then provides greater surface area and hence greater loading capacity, which is more suited to preparative separations.

Learn more about Agilent Bio-IEX columns at **agilent.com/chem/bioHPLC**

## Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

| | | | | | |
|---|---|---|---|---|---|
| **Column A:** | **Bio SCX, stainless steel**<br>**5190-2423**<br>**4.6 x 50 mm, 3 µm** | **Column B:** | **Bio WCX, stainless steel**<br>**5190-2443**<br>**4.6 x 50 mm, 3 µm** | **Column C:** | **Bio MAb, stainless steel**<br>**5190-2403**<br>**4.6 x 50 mm, 3 µm** |

| | |
|---|---|
| Mobile Phase: | A: 10 mM Sodium phosphate, pH 5.7<br>B: A + 1 M NaCl |
| Flow Rate: | 0.5 mL/min |
| Gradient: | 0 min - 100% A : 0% B<br>25 min - 0% A : 100% B |
| Temperature: | Ambient |
| Detector: | Agilent 1260 Infinity Bio-inert Quaternary LC with diode array detector at 220 nm |
| Sample: | Ribonuclease A, cytochrome c, lysozyme and protein mix |

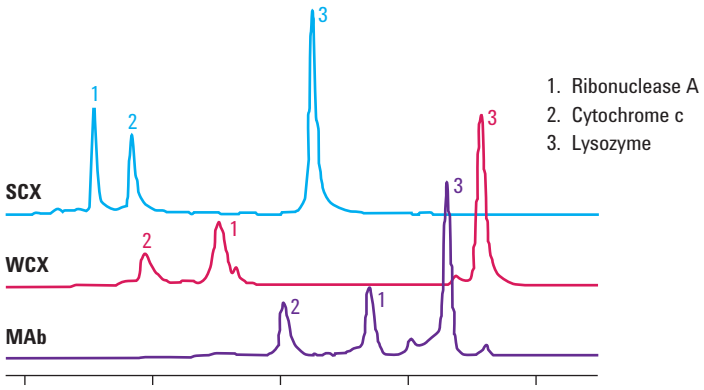



**Figure 6** – Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

## Particle Size

Particle size is an important consideration in column selection. Smaller particle sizes provide more efficient separation, but at the cost of higher operating pressure. Because biomolecules are relatively large and have slower rates of diffusion, smaller particle sizes do not necessarily provide the same level of improvement in resolution that might be seen with small molecules. Furthermore, eluents comprising aqueous buffers are relatively viscous and care must be taken to ensure back pressures are not excessive.

## Column Hardware

Columns are also available in a wide range of dimensions, in PEEK or stainless steel, and packed with a choice of particle sizes. With ion-exchange separation of biomolecules it is not always necessary to employ a 25 cm column. However, there are occasions when the additional column length can improve resolution. Longer columns, nonetheless, also result in higher back pressure, and so larger particle sizes can be required. To improve speed and throughput a shorter column packed with smaller particle sizes will give increased resolution and enable separations to be performed up to 5x faster.

## Achieve faster analysis time with smaller particles and shorter column lengths – speed up your separation by 30%

| | | | | | |
|---|---|---|---|---|---|
| **Column:** | **Bio WCX, stainless steel 5190-2445 4.6 x 250 mm, 5 µm** | Mobile Phase: | A: 20 mM Sodium phosphate, pH 6.5<br>B: A + 1.6 M NaCl | Injection: | 10 µL |
| **Column:** | **Bio WCX, stainless steel 5190-2443 4.6 x 50 mm, 3 µm** | Gradient: | 0 to 50% B | Sample Conc: | 0.5 mg/mL |
| | | Temperature: | Ambient | Detector: | UV, 220 nm |
| | | | | Instrument: | Agilent 1260 Infinity Bio-inert Quaternary LC |

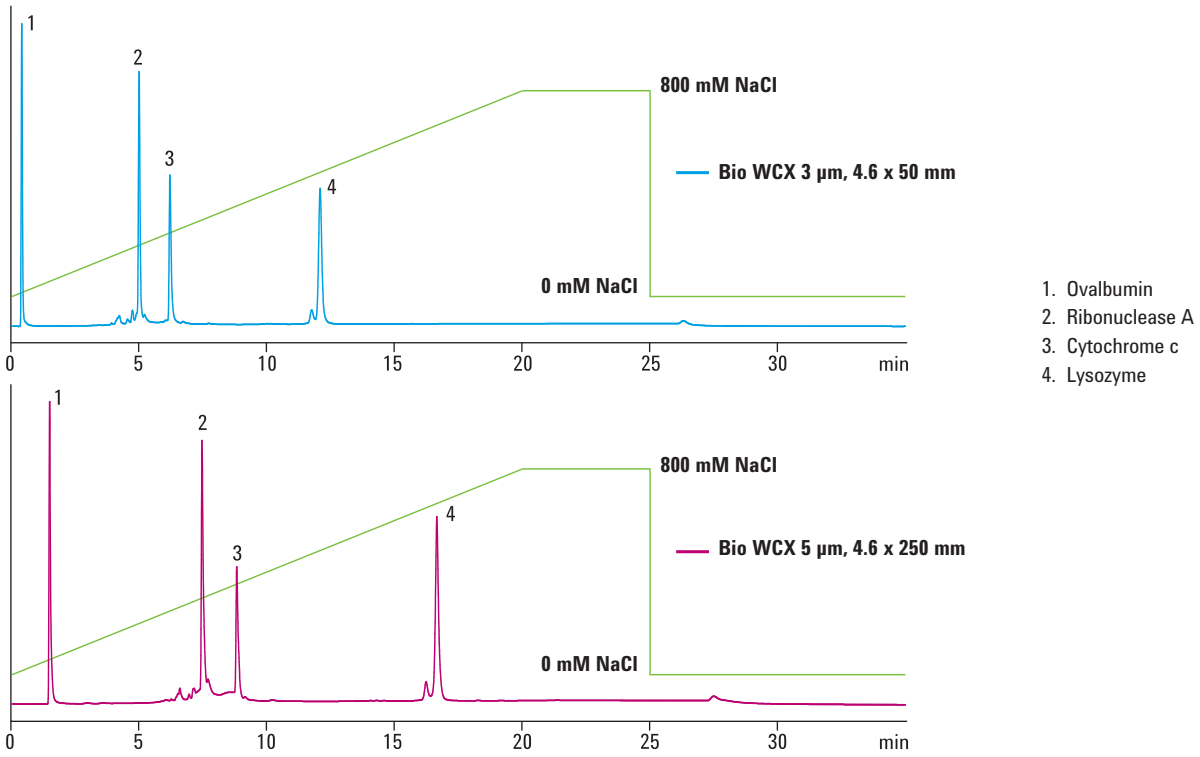



**Figure 7** – Protein separation on Agilent Bio WCX columns (4.6 x 50 mm, 3 µm and 4.6 x 250 mm, 5 µm) at a flow rate of 1 mL/min. Faster analysis times were achieved with smaller particle size and shorter column length – samples eluted from the longer column in 17 minutes but in only 12 minutes from the shorter column

Stainless steel columns are used, but salt gradients can prove aggressive and cause corrosion if left in contact with the column. PEEK columns do not suffer from this problem and can be beneficial for molecules that are metal-sensitive, though they operate at lower back pressures. For a metal-free sample flow path, a PEEK column run with a bio-inert instrument such as the Agilent 1260 Infinity Bio-inert Quaternary LC should be used.

## Column Diameter

Column diameter can also be important, depending on the amount of sample being analyzed. If only limited amounts of material are available, 2.1 mm id columns (operated at 0.35 mL/min) are useful. But it is important to minimize system volumes between the column and detector when using smaller id columns to prevent excessive dispersion and loss of resolution.


The Agilent 1260 Infinity Bio-inert Quaternary LC is an ideal HPLC instrument for ion-exchange chromatography

# Step 3:
# HPLC System Considerations

An ideal choice for this type of analysis is the Agilent 1260 Infinity Bio-inert Quaternary LC. It handles challenging solvent conditions with ease, such as extreme pH values of pH 1 to pH 13, and buffers with high salt concentrations.

Corrosion-resistant titanium in the solvent delivery system and metal-free materials in the sample flow path create an extremely robust instrument.

## Detection

For biomolecules such as proteins that consist of multiple amino acids linked via amide bonds, UV detection at 210 nm or 220 nm will give the best signal strength and sensitivity. However, some of the eluents commonly employed in ion-exchange have a strong background absorbance at low wavelengths, and so it may be necessary to use 254 nm or 280 nm instead. These wavelengths are only sensitive to amino acids with aromatic or more conjugated side chains, which will result in much lower sensitivity.

### Optimize interaction-free chromatography

Agilent Bio-inert LC supplies provide robust, interaction-free results to ensure increased system efficiency – while improving chromatographic reliability with sharper peaks and more reproducible analysis.

Learn more:
**www.agilent.com/chem/biosupplies**

# Step 4: **Flow Rate**

Typical flow rate for use with 4.6 mm id columns is 0.5 to 1.0 mL/min. For some applications the speed of analysis is crucial. Shorter columns can be used to reduce the analysis time – 50 mm instead of the conventional 150 mm or 250 mm – or flow rates can be increased, or both (taking care not to exceed column pressure limitations).

## Smaller particle sizes provide increased resolution

**Column:** **Bio WCX, stainless steel**
**5190-2441**
**4.6 x 50 mm, 1.7 µm**

Mobile Phase: A: 20 mM sodium phosphate, pH 6.5
B: A + 1.6 M NaCl

Gradient: 0 to 50% B

Temperature: Ambient

Injection: 10 µL

Sample Conc: 0.5 mg/mL

Detector: UV, 220 nm

Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC

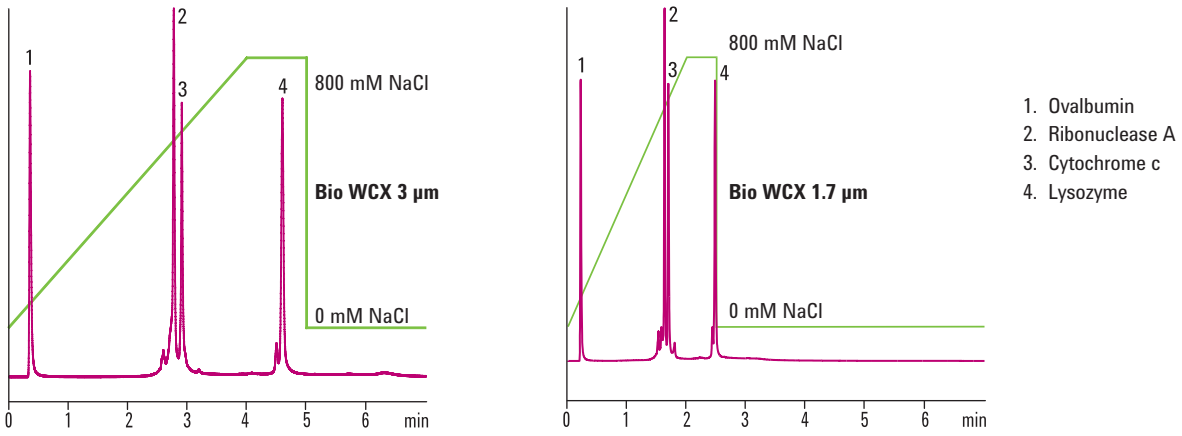



**Figure 8** – Reduce analysis time – without sacrificing peak shape and resolution – by increasing flow rate

Learn more about Agilent Bio-IEX columns at **agilent.com/chem/bioHPLC**

# Step 5: **Mobile Phase Selection**

Initial mobile phase selection will be dictated by the pI of the protein and the method of analysis, i. e. cation- or anion-exchange. **Figure 10** shows the range of buffers commonly available.

The role of the buffer is to control the change in pH during the separation and therefore maintain a consistent charge on the compounds being analyzed. It is important to remember that a buffer will only satisfactorily perform this role if it is within one pH unit of its dissociation constant, pKa. Phosphoric acid or phosphates possess three dissociation constants:

$$H_3PO_{4\,(aq)} \xrightleftharpoons{pK_{a1} = 2.12} H^+_{(aq)} + H_2PO_4^-{}_{(aq)}$$

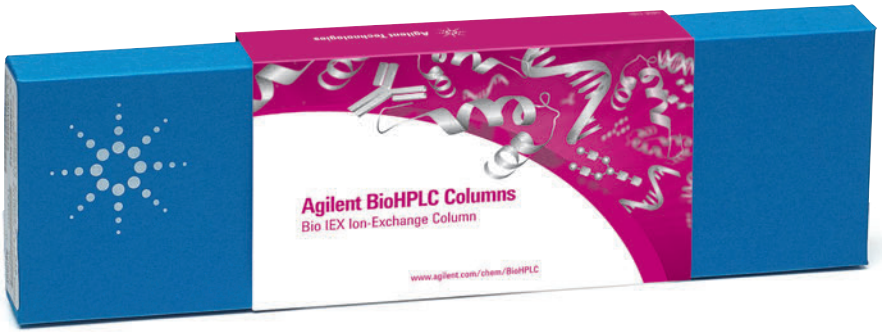
$$H_2PO_4^-{}_{(aq)} \xrightleftharpoons{pK_{a2} = 7.21} H^+_{(aq)} + HPO_4^{2-}{}_{(aq)}$$

$$HPO_4^{2-}{}_{(aq)} \xrightleftharpoons{pK_{a3} = 12.67} H^+_{(aq)} + PO_4^{3-}{}_{(aq)}$$

Phosphate buffers in the range pH 6 to 7 are therefore suitable for cation-exchange chromatography, typically in concentrations of 20 to 30 mM, and have the advantage of low background absorbance at 210 nm. It is important to make up buffers systematically and accurately, as even minor differences in ionic strength or pH can affect the retention time of proteins to different extents, and could result in poor resolution and variability in the chromatographic profile.

Unlike strong ion-exchange columns that are fully ionized under normal operating conditions, it is important to realize that the buffer pH and ionic strength can affect the degree to which a weak ion-exchange column is ionized. This is one of the tools available to alter selectivity, to achieve a desired separation.

However, to elute biomolecules from the column, a competing ion must be introduced. Typically, this will be accomplished by a linear sodium chloride gradient. Eluent A will comprise the buffer adjusted to the appropriate pH. Eluent B will contain the same concentration of buffer with a higher concentration of sodium chloride, perhaps 0.5 M, with the pH then adjusted to the same value.



| | |
|---|---|
| **Column:** | **Bio MAb, PEEK**<br>**5190-2407**<br>**4.6 x 250 mm, 5 µm** |
| Flow Rate: | 1 mL/min |
| Mobile Phase: | A: Water<br>B: 1.5 M NaCl<br>C: 40 mM $NaH_2P0_4$<br>D: 40 mM $Na_2HP0_4$<br>By combining predetermined proportions of C and D as determined by the Buffer Advisor Software, buffer solutions at the desired pH range and strength were created. |
| Sample: | Mix of three proteins, dissolved in PBS (phosphate buffered saline) pH 7.4<br>Ribonuclease A: 13,700 Da, pI 9.6<br>Cytochrome c: 12,384 Da, pI 10-10.5<br>Lysozyme: 14,307 Da, pI 11.35 |
| Instrument: | Agilent 1260 Infinity Bio-inert Quaternary LC System |

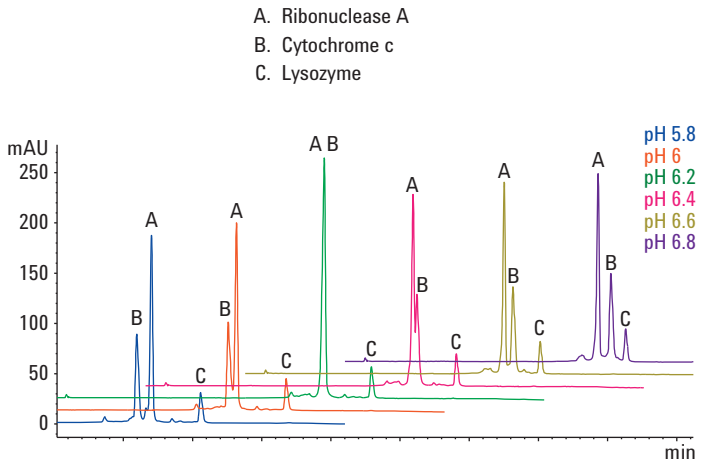



**Figure 9** – pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients

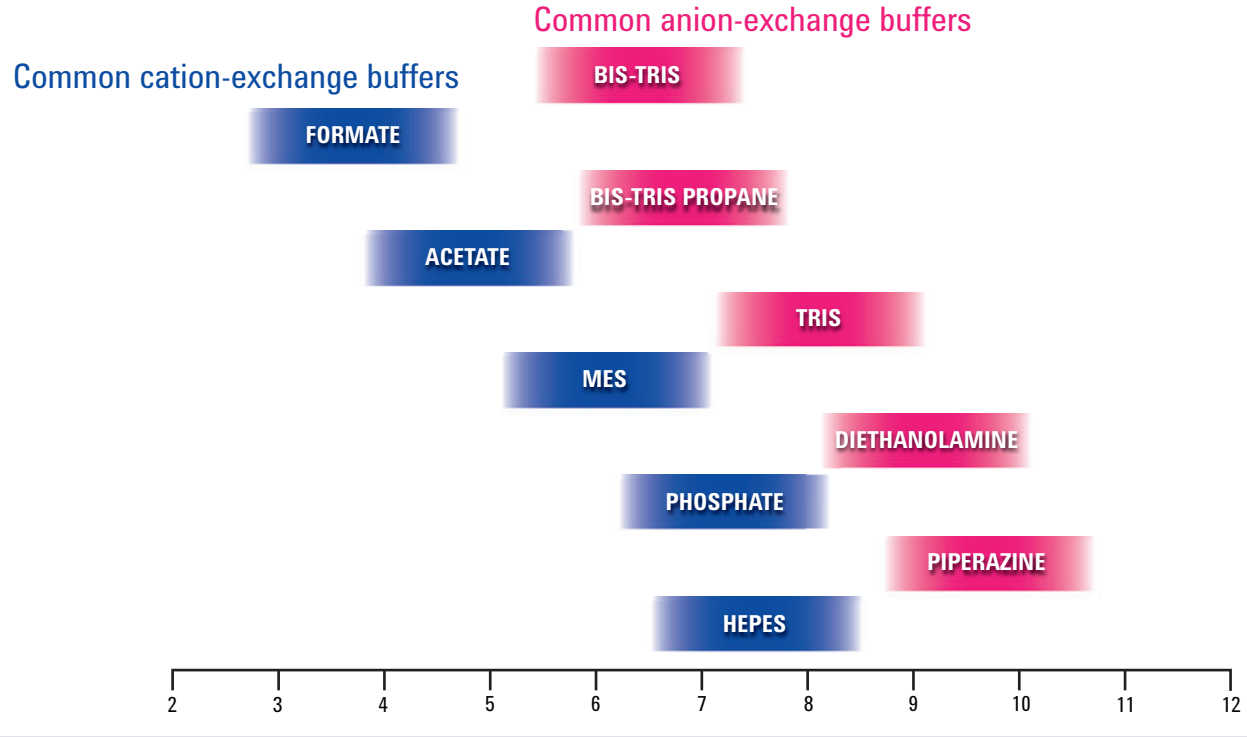



**Figure 10** – Commonly available buffers for ion-exchange

Learn more about Agilent Bio-IEX columns at **agilent.com/chem/bioHPLC**

# Developing an Effective Ion-Exchange Method


It must be remembered that biomolecules such as monoclonal antibodies are incredibly complex. A typical mAb comprises over 1,300 individual amino acids. Of these, perhaps 130 have acidic side chains and 180 have basic residues. The likelihood is that a monoclonal antibody will have a net positive charge at neutral pH and therefore should be separated using a cation-exchange column. However, it is difficult to predict the actual isoelectric point, pI, of such a molecule, and so some method development or optimization should be anticipated.

## Sample Preparation

- Samples should ideally be dissolved in the mobile phase (eluent A).
- If the sample is cloudy, it may be necessary to change the mobile phase conditions.
- Filtration or centrifugation can be used to clarify samples, but these processes could alter the composition of the sample.
- Samples should be made up fresh and analyzed as soon as possible. Refrigeration can increase the "shelf life" of samples.
- Bacterial growth can develop quickly in buffer solutions.

## Column Media Choice

- The choice between anion- and cation-exchange depends on the isoelectric point of the protein(s) of interest.
- Strong ion-exchangers are a good first choice, with weak ion-exchange offering a difference in selectivity if it is required.

## Column Selection

- **Pore size** – proteins of interest must be able to freely permeate the particles. Non-porous spherical particles provide highest resolution for analytical separations, where column loading capacity is not a major concern.
- **Particle size** – use smaller particles for higher resolution (which results in higher back pressure).
- **Column length** – shorter 50 mm columns can be used for more rapid separations, particularly with smaller particles, and longer 250 mm columns where additional resolution may be required.
- **Column id** – use smaller columns for reduced solvent consumption and smaller injection volumes (beneficial if sample is limited).


## Mobile Phase

- The mobile phase should contain buffer to maintain the desired operating pH, typically 20 mM. The pH and ionic strength of the buffer can affect resolution on weak ion-exchange products and so the optimum conditions should be found experimentally.
- Addition of sodium chloride to the mobile phase will alter the pH. Re-adjust as necessary.
- Make up fresh mobile phase and use promptly because bacterial growth is rapid in dilute buffer stored at room temperature.
- Buffer shelf life is less than seven days unless refrigerated.
- Filter before use. Particulates can be present in water (less likely) or in buffer salts (more likely).

## Column Conditioning and Equilibration

For reproducible ion-exchange separation, the column equilibration and cleanup phases of the gradient are critical. Protein elution is achieved by increasing the ionic strength or changing the eluent pH, or both, and so at the end of each analysis the column must be equilibrated back to the starting conditions, ionic strength, and pH. If this is not done, the next column run will have a different profile as the protein will interact differently with the column.


Fast buffer scouting with Agilent Buffer Advisor software. Watch video: **agilent.com/chem/bufferadvisor-video**

## Software

One additional tool that can be used to simplify your workflow is the Agilent Buffer Advisor Software.

Agilent Buffer Advisor Software eliminates the tedious and error-prone method development steps of buffer preparation, buffer blending and pH scouting, by providing a fast and simple way to create salt gradients (**Figure 11**) and pH gradients (**Figure 12**). Using the mixing principle of the 1260 Infinity Bio-inert Quaternary pump, the Buffer Advisor Software facilitates dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. In addition, buffers are prepared more accurately, which makes for more robust method transfer to other laboratories.

To create a salt gradient, an increasing amount of salt solution from channel D is mixed with the acidic and basic buffer components from channels A and B, and with water for dilution from channel C.

Learn more about Agilent Bio-IEX columns at **agilent.com/chem/bioHPLC**

Initial screening of twenty experiments was achieved from just four mobile phase eluents instead of needing forty different solutions. The software automatically blends the buffers to create the desired pH and buffer strengths. The gradient timetable can then be programmed in the quaternary, as shown in **Figure 13**.

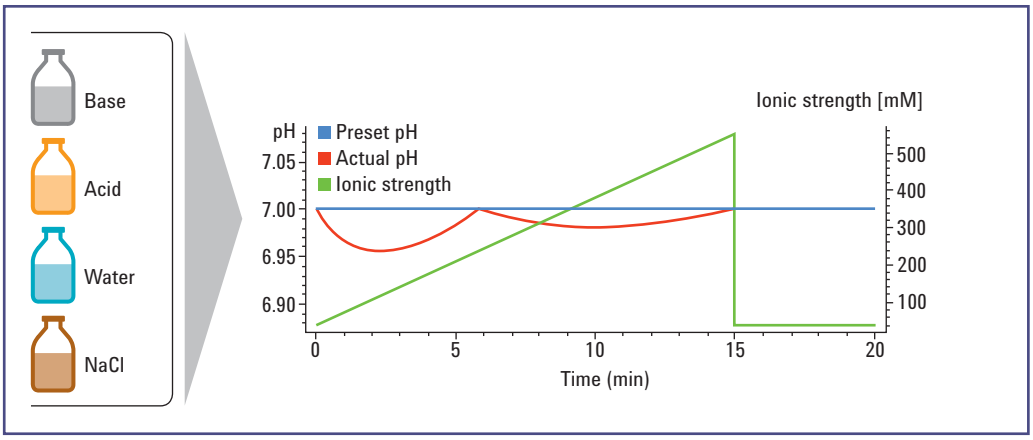



**Figure 11** – Salt gradients are easily created from stock solutions with Agilent Buffer Advisor Software

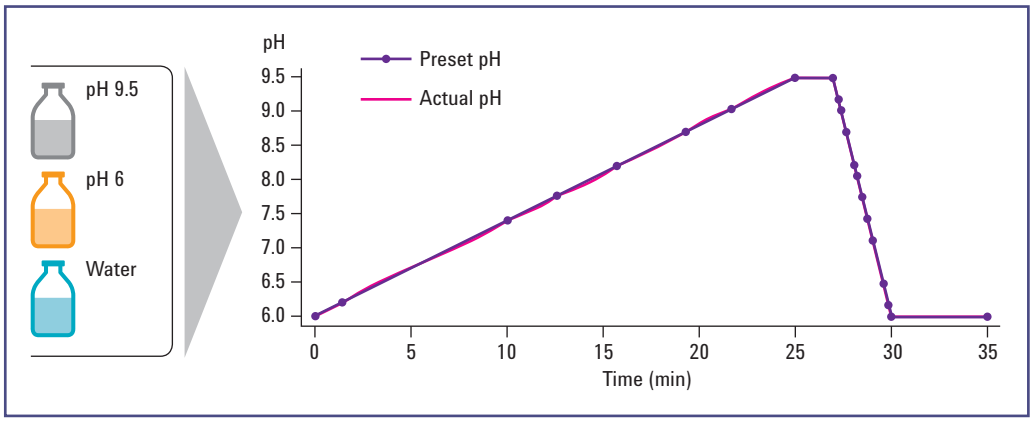



**Figure 12** – Optimizing buffer strength for a monoclonal antibody separation – pH gradients are easily created from stock solutions

## Automated method development for optimized charged-variant separations

**Column:** **Bio WCX, stainless steel**
**5190-2443**
**4.6 x 50 mm, 3 µm**

**Column:** **Bio SCX, stainless steel**
**5190-2423**
**4.6 x 50 mm, 3 µm**

Mobile Phase: A: Water
B: 1.5 M NaCl
C: 40 mM $NaH_2P0_4$
D: 40 mM $Na_2HP0_4$
By combining predetermined proportions of C and D as determined by the Buffer Advisor Software, buffer solutions at the desired pH range and strength were created.

Flow Rate: 1.0 mL/min

Gradient: Conditions for chromatograms shown:
pH 5.0 to 7.0, 10 to 25 mM buffer strength
0 to 500 mM NaCl, 0 to 15 min
500 mM NaCl, 15 to 20 min

DOE experiments
pH 5.0 to 7.0
0 to 200 mM, 0 to 250 mM, and 0 to 300 mM

Temperature: Ambient

Detector: UV, 220 nm

Injection: 5 µL

Sample: IgG monoclonal antibody

Sample Conc: 2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)

Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC

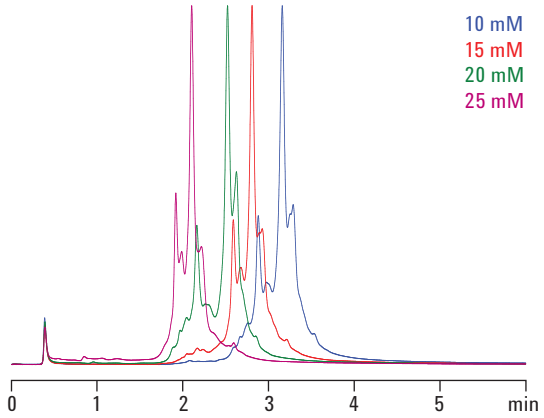



**Figure 13** – Optimizing buffer strength at pH 6.5 from the screening chromatograms of a monoclonal IgG separation

## Bio MAb columns enable precise quantitation, robust methods

**Column:** **Bio MAb, PEEK**
**5190-2407**
**4.6 x 250 mm, 5 µm**

Mobile Phase: A: 10 mM sodium phosphate buffer, pH 6.0
B: 10 mM sodium bicarbonate buffer, pH 9.5

Flow Rate: 1.0 mL/min

Gradient:

| Time (min) | Mobile phase (% B) |
|---|---|
| 0 | 0 |
| 25 | 100 |
| 27 | 100 |
| 30 | 0 |

Post time: 5 min

Injection: 10 µL (needle with wash, flush port active for 7 s)

Temperature: 30 °C

Detector: Data acquisition: 214 and 280 nm
Acquisition rate: 20 Hz
Flow cell: 60 mm path
Sample thermostat: 5 °C

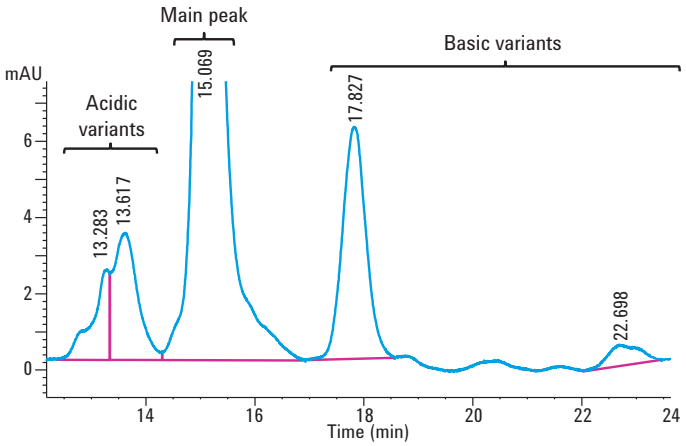
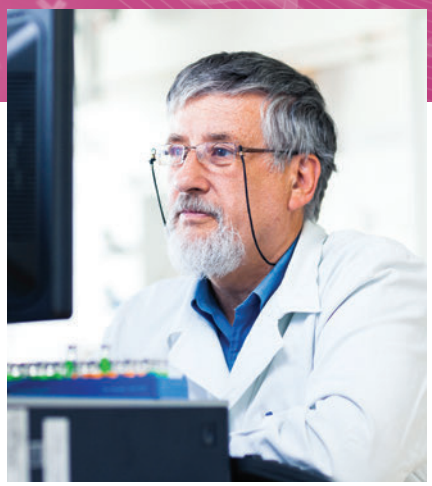


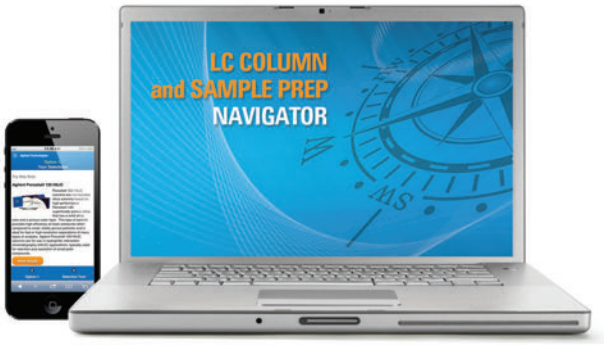

**Figure 14** – pH gradient-based cation-exchange chromatogram of an IgG1 separation using an Agilent Bio MAb PEEK, 4.6 x 250mm, 5 µm column

# Partnering with you to get great results



Increasing challenges require better answers. Our solutions enable biopharmaceutical scientists to innovate in disease research, accelerate drug discovery, and have greater confidence throughout development and manufacturing. A broad range of Agilent solutions in genomics research, automation, separation, and detection technologies – along with workflow-driven software solutions – helps deliver the answers required to bring effective therapeutics to market.

Learn about the Agilent solutions for biopharma
agilent.com/chem/togetherbiopharma




## Navigate your way to a successful result

agilent.com/chem/navigator

With a wealth of biocolumns and small columns available, Agilent has introduced the LC columns and Sample Prep NAVIGATOR to help you choose the right column for your application.

The NAVIGATOR presents four easy search options:

- By part number – cross-reference for LC columns and sample prep products to find the best Agilent replacement
- By column – recommendations based on method
- By compound – drop down list
- By USP method

In addition, the tool offers column support to optimize chromatography, sample prep product recommendations, and quick access to technical support resources and other tools.


Printed in the USA May 2, 2014
5991-3775EN

Agilent Technologies