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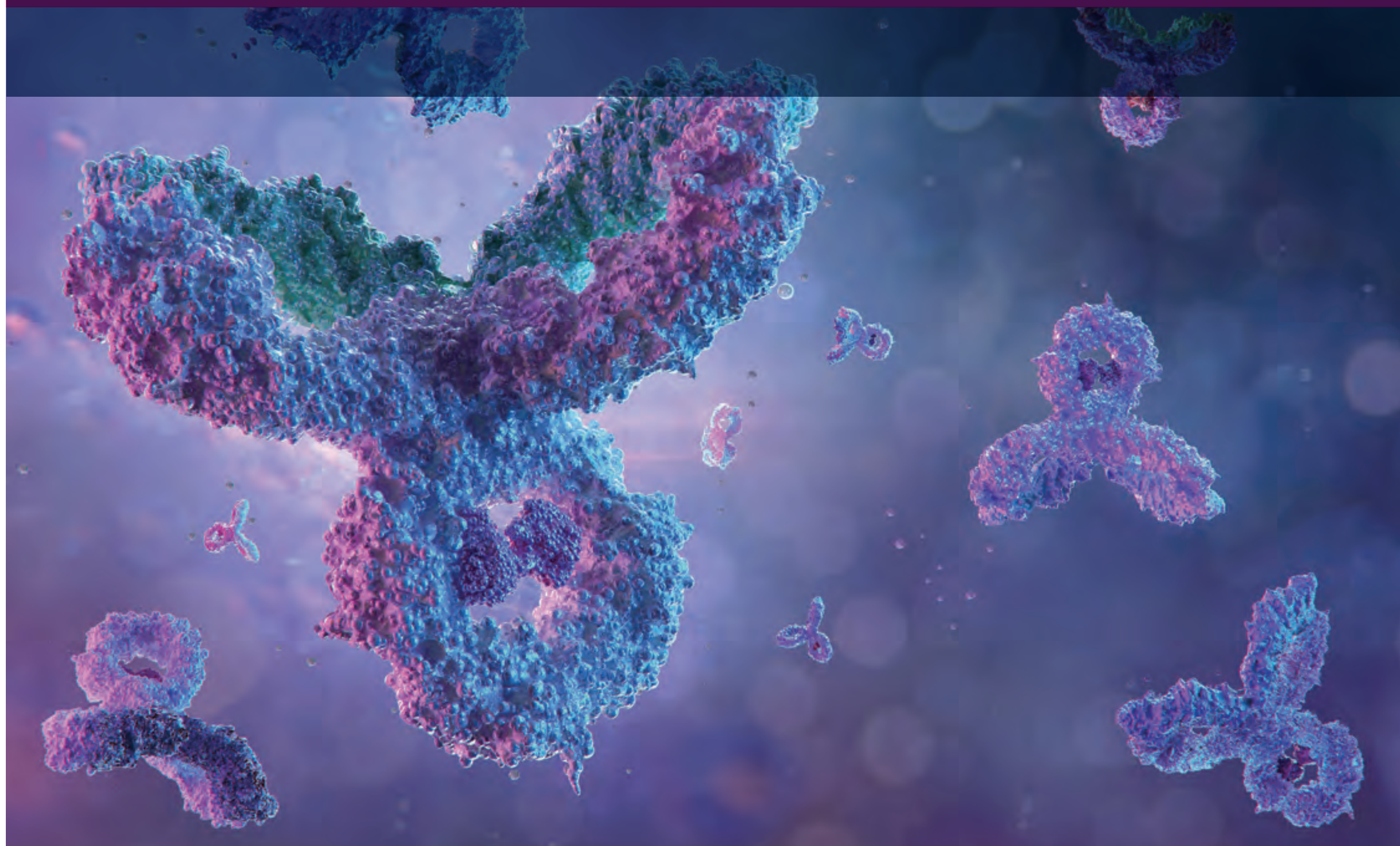
Agilent Biocolumns

Charge Variant Analysis

Application Compendium



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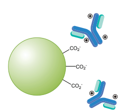
Charge Variant Analysis

Background

The presence of positively charged and negatively charged amino acids and negatively charged glycans (sialic acids) means that large proteins exist as multiple charged species and there are several side reactions that can result in a change in the net charge. Understanding which amino acids or glycans are involved and their specific location within a large biotherapeutic protein is of paramount importance. Variants within the antigen binding region of an antibody are likely to have a more profound effect on function.

Ion exchange chromatography can enable the separation of some charge variants, particularly those positioned on the surface of the protein (rather than hidden within the structure). Nonetheless, separating a molecule may have a net charge of +50 from a variant that is +49 or +51 is still a considerable challenge. Elimination of pore structure and therefore pore diffusion by using nonporous particles goes some way to improving peak shape and gaining resolution. It is often necessary to revert to weak cation exchange columns and to perform extensive method optimization to determine the most appropriate conditions for a particular molecule.



Charge Variant Analysis

Ion exchange chromatography

Enhances the accuracy and speed of biomolecule characterization

Bio MAb

Ideal for monoclonal antibodies

Attribute	Advantage
Rigid, non-porous particles	High-efficiency separations
Hydrophilic, polymeric layer	Eliminates non-specific binding
High density WCX chemistry	High ion exchange capacity ideal for MAbs

Bio IEX

Ideal for proteins and peptides

Attribute	Advantage
Rigid particles with hydrophilic coating	Eliminates non-specific binding
Strong/weak anion, cation chemistries	A column for every separation

Getting Started

Since most proteins contain more basic amino acids than acidic amino acids, most charge variant separations will require cation exchange. However, every protein is different and finding the conditions to deliver the best resolution you require will likely require considerable optimization. Strong cation exchange columns are often easier to work with, however for monoclonal antibodies a weak cation exchange column may be the only way to achieve the desired resolution.

Before beginning method development, it is crucial to determine the isoelectric point, or pI, of the target protein. If the pH of initial mobile phase conditions is too close to the pI of the protein, the protein will not be retained on the column. Depending on how widely the pI of the charge variants differs, the pH may need to be a minimum of 0.5 to 2 pH units away from the isoelectric point of the main species. Proteins may be eluted by either a salt gradient (using high ionic strength to disrupt protein adsorption to the column) or a pH gradient (proteins elute when the pH equals the pI).

It is worthwhile considering an instrument that allows screening of several different columns during method development. It is difficult to predict the outcome of even small changes to method conditions such as ionic strength and pH; both of these factors will influence the net charge on the protein and, in the case of weak ion exchange columns, the net charge on the column too. A rigorous "Quality by Design" approach is recommended. Software to develop a matrix or systematic design of experiments is advisable. Buffer advisor software that can utilize the quaternary HPLC pump capabilities of an Agilent 1260 Infinity II Bio-inert LC can save considerable method development time. Several of the application notes listed in this section, including the “How-To” Guide and the featured application note illustrate how to use buffer advisor to test a range of mobile phase conditions. When the optimum conditions for separation require very low ionic strength buffers at pH levels at the extreme limits of the buffering range then PEEK columns may also be advisable.

Like size exclusion chromatography, ion exchange conditions are typically nondenaturing; the separation is conducted on the intact, native protein. This means that the method is not MS compatible unless combined as the first dimension in a 2D-LC setup. However, quantification can be achieved by UV detection.

Ion-Exchange Chromatography for Biomolecule Analysis: A "How-To" Guide

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.

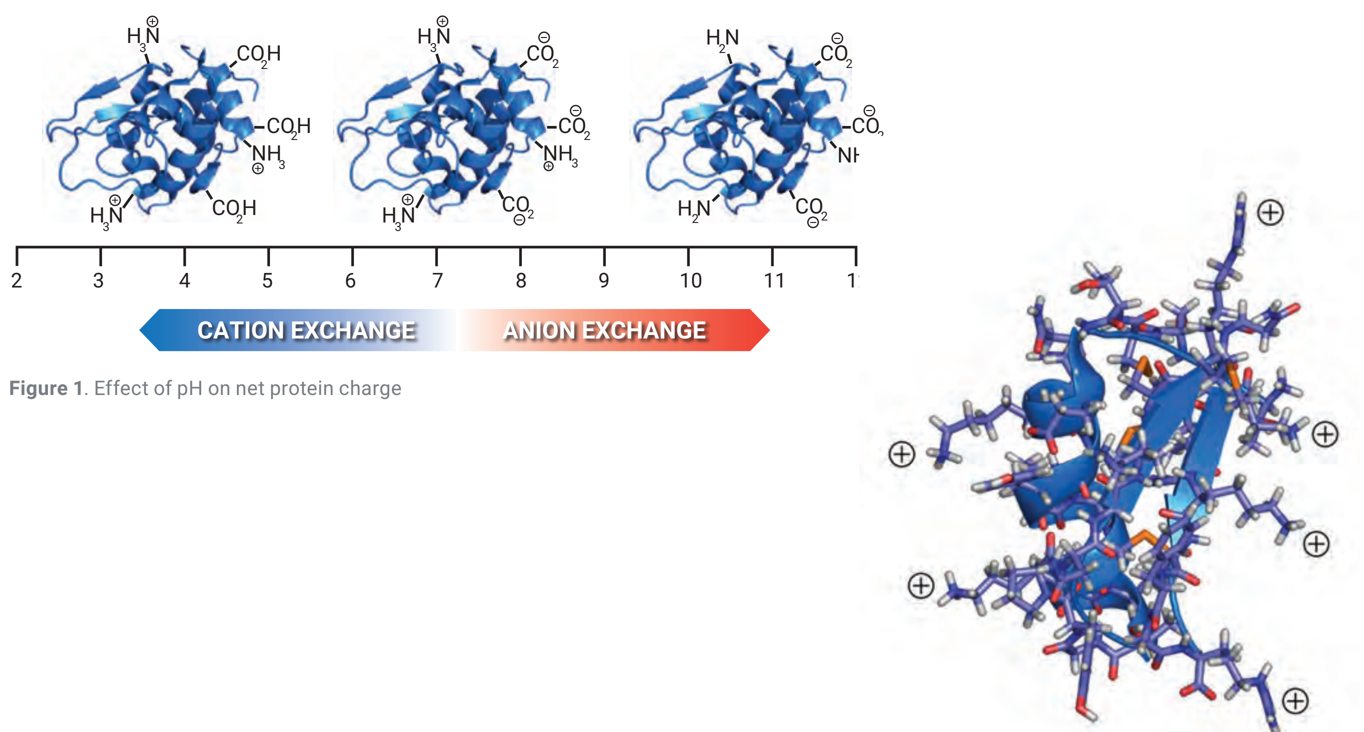


Figure 1. Effect of pH on net protein charge

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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.

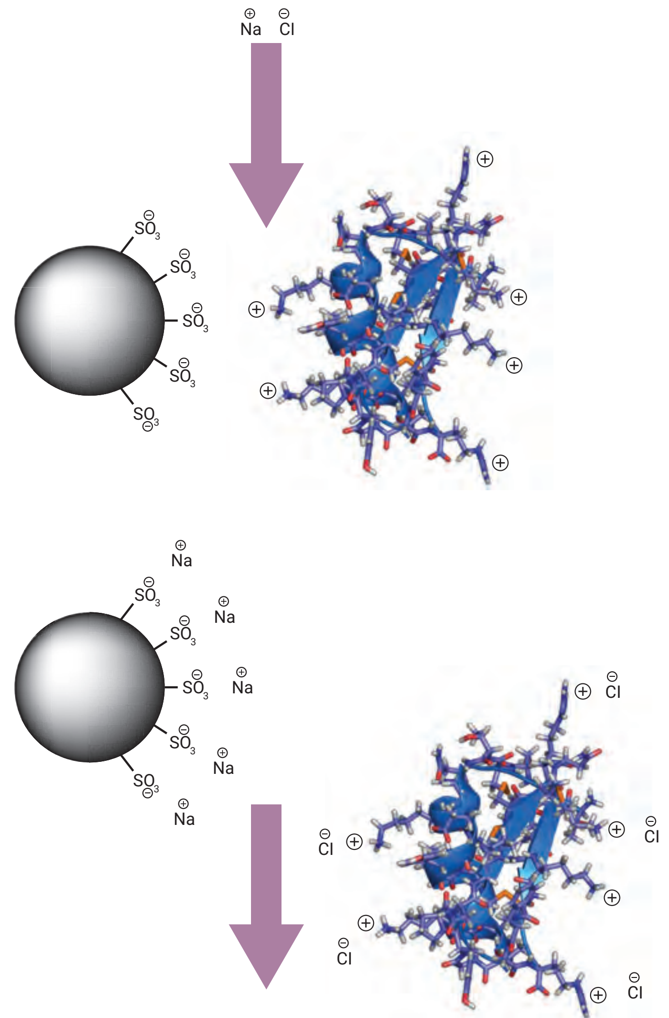


Figure 2. Separation mechanism of ion-exchange

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.

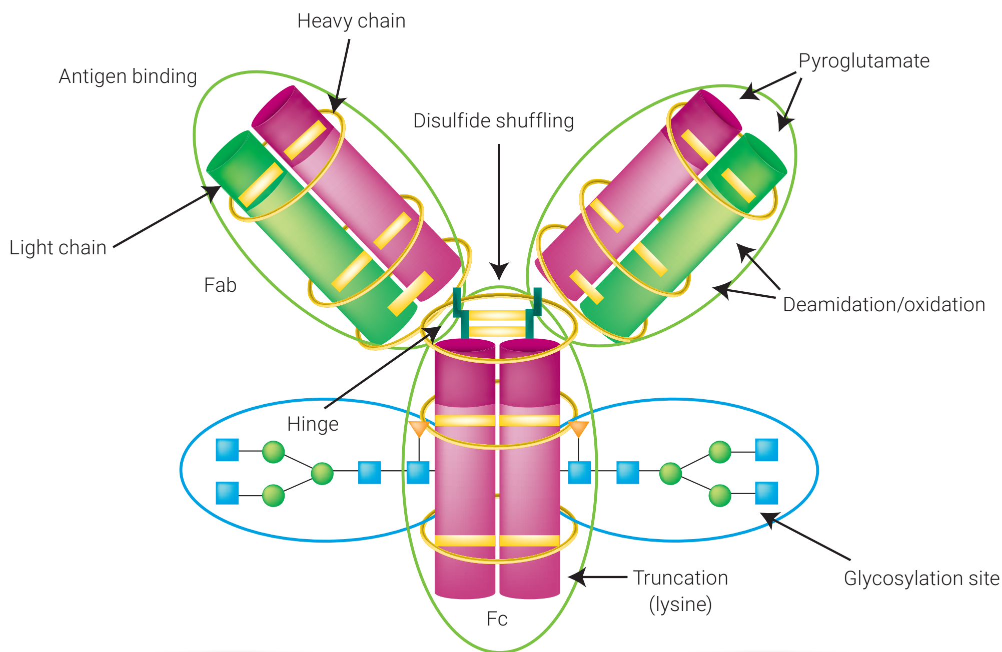
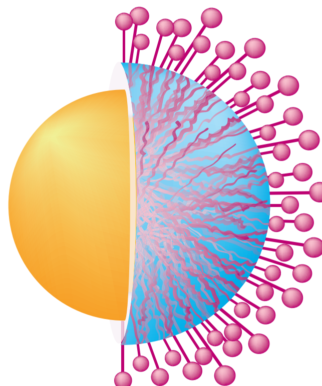


Figure 3. Charged variants of monoclonal antibodies arise through different levels of glycosylation, deamidation, and oxidation of amino acids, and through lysine truncation of heavy chains

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

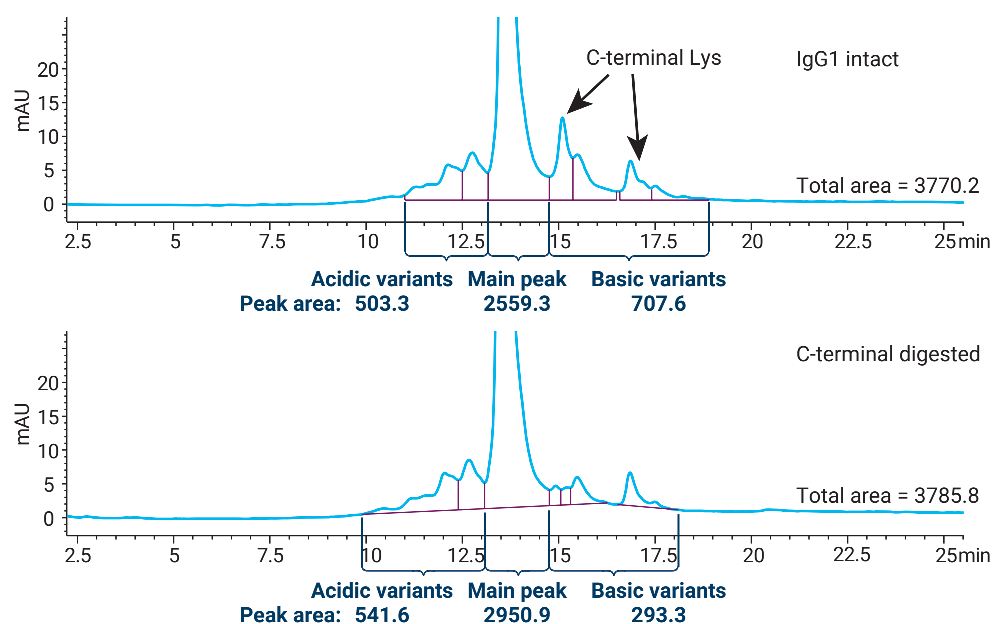


Figure 4. Calculation of C-terminal digested IgG1 using an Agilent Bio MAb 5 µm column on the Agilent 1260 Infinity Bio-inert Quaternary LC. The column delivers high resolution, enabling better peak identification and accurate quantification

Conditions

Parameter	Value
Column:	Bio MAb, PEEK 5190-2407 4.6 x 250 mm, 5 µm
Sample:	5 µL of 1 mg/mL of intact or C-terminal digested IgG1
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 10 mM Na phosphate buffer, pH 5.5 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
Injection:	5 µL

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 Number
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

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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

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

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.

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.

Step 2:

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 Globular proteins and peptides Very large biomolecules/ high speed	PL-SAX 1000 Å PL-SAX 4000 Å PL-SAX 1000 Å 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 Globular proteins Very large biomolecules/ high speed	PL-SCX 1000 Å PL-SCX 4000 Å PL-SCX 1000 Å 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), plasmid DNA, viruses, phages and other macro biomolecules Viruses, DNA, large proteins Plasmid DNS, bacteriophages Proteins, antibodies	Bio-Monolith QA Bio-Monolith DEAE Bio-Monolith SO_3 Bio-Monolith QA Bio-Monolith DEAE 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.

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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.

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 4000 Å 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.

Exceptional separating power

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).

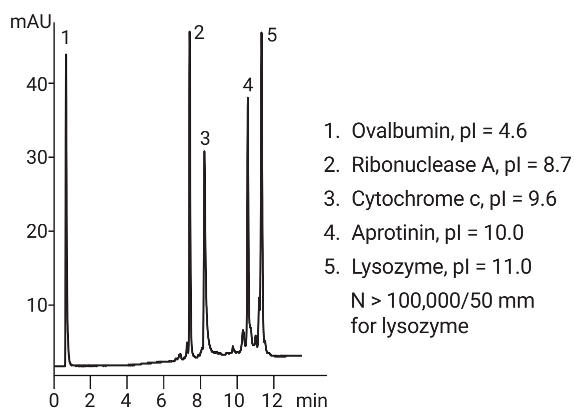

Figure 5. Exceptional separating power of Agilent Bio SCX

Conditions

Parameter	Value
Column:	Bio SCX, stainless steel 5190-2423 4.6 x 50 mm, 3 µm
Buffer: A:	10 mM phosphate, pH 6.0
Flow Rate:	0.5 mL/min
Gradient:	0-1.0 M NaCl, 15 min
Detector:	280 nm

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

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

Conditions

Parameter	Value
Column A:	Bio SCX, stainless steel 5190-2423, 4.6 x 50 mm, 3 µm
Column B:	Bio WCX, stainless steel, 5190-2443 4.6 x 50 mm, 3 µm
Column C:	Bio MAb, stainless steel, 5190-2403 4.6 x 50 mm, 3 µm
Sample:	Ribonuclease A, cytochrome c, lysozyme and protein mix
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 10 mM Sodium phosphate, pH 5.7 B: A + 1 M NaCl
Flow Rate:	0.5 mL/min
Gradient:	0 min - 100 % A : 0 % B 25 min - 0 % A : 100 % B
Temp.:	Ambient
Detector:	Agilent 1260 Infinity Bio-inert Quaternary LC with diode array detector at 220 nm

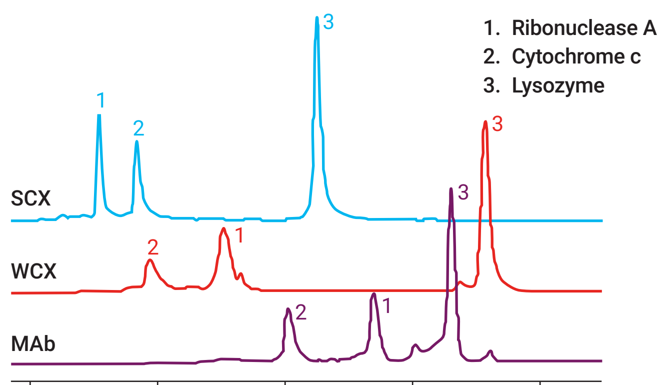


Figure 6. Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

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

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.

Conditions

Parameter	Value
Column A:	Bio WCX, stainless steel 5190-2445 4.6 x 250 mm, 5 µm
Column B:	Bio WCX, stainless steel 5190-2443 4.6 x 50 mm, 3 µm
Sample:	0.5 mg/mL
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 20 mM Sodium phosphate, pH 6.5 B: A + 1.6 M NaClGradient: 0 min - 100 % A : 0 % B
Gradient:	0 to 50 % B
Temp.:	Ambient
Injection:	10 µL
Detector:	UV, 220 nm

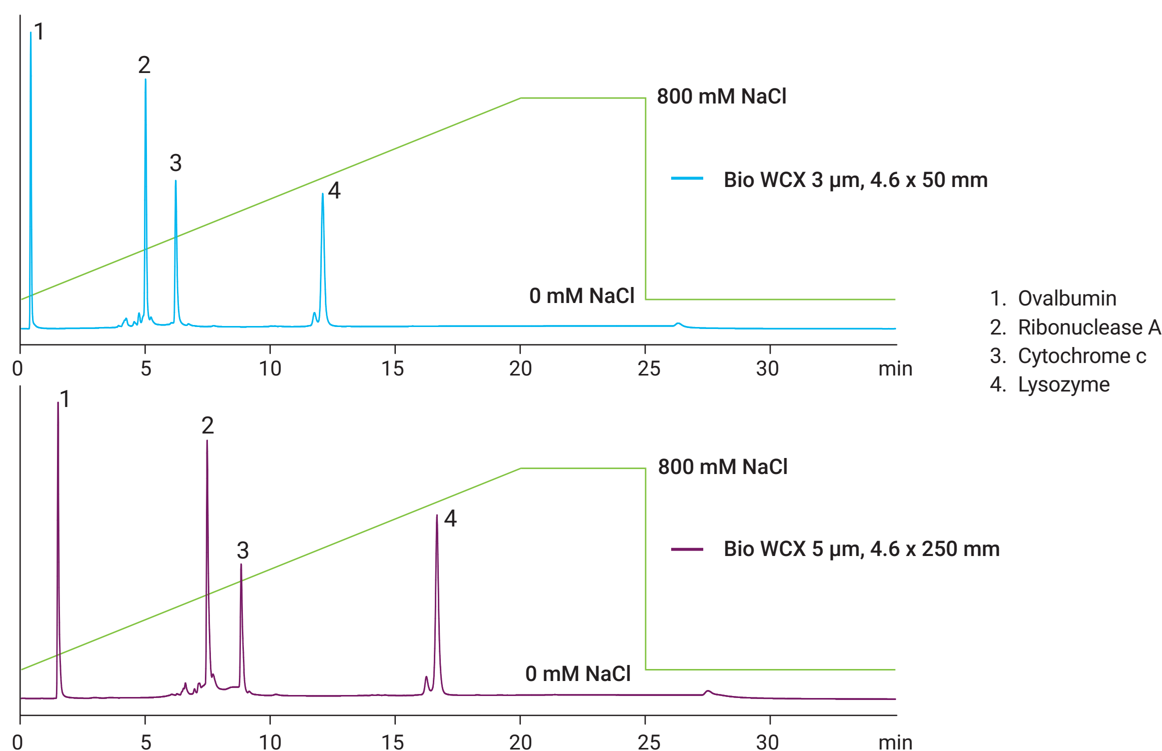


Figure 7. Protein separation on Agilent Bio WCX columns (4.6 x 50 mm, 3 µm and 4.6 x 250 mm, 5 µm) at a flow rate of 1 mL/min. Faster analysis times were achieved with smaller particle size and shorter column length – samples eluted from the longer column in 17 minutes but in only 12 minutes from the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

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.

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**

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

Step 4:

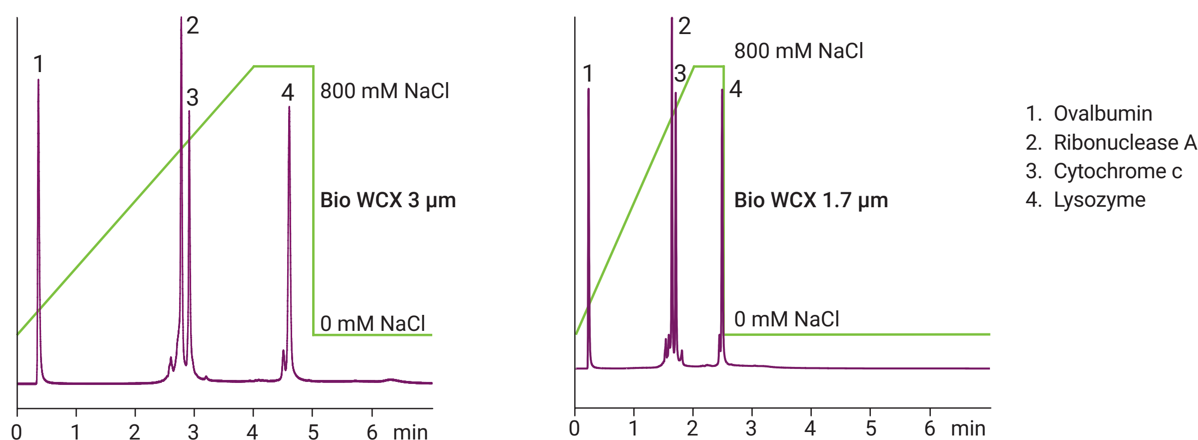
Flow rate

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

Conditions

Parameter	Value
Column A:	Bio WCX, stainless steel 5190-24414.6 x 50 mm, 1.7 µm
Column B:	
Sample:	0.5 mg/mL
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 20 mM sodium phosphate, pH 6.5 B: A + 1.6 M NaCl
Gradient:	0 to 50 % B
Temp.:	Ambient
Injection:	10 µL
Detector:	UV, 220 nm



1. Ovalbumin
2. Ribonuclease A
3. Cytochrome c
4. Lysozyme

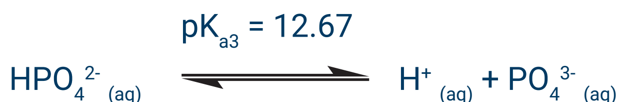
Figure 8. Reduce analysis time – without sacrificing peak shape and resolution – by increasing flow rate

Mobile Phase Selection

Step 5:

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:



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.

Conditions

Parameter	Value
Column A:	Bio MAb, PEEK 5190-2407 4.6 x 250 mm, 5 µm
Sample:	Mix of three proteins, dissolved in PBS (phosphate buffered saline) pH 7.4 Ribonuclease A: 13,700 Da, pl 9.6 Cytochrome c: 12,384 Da, pl 10-10.5 Lysozyme: 14,307 Da, pl 11.35
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
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 mL/min

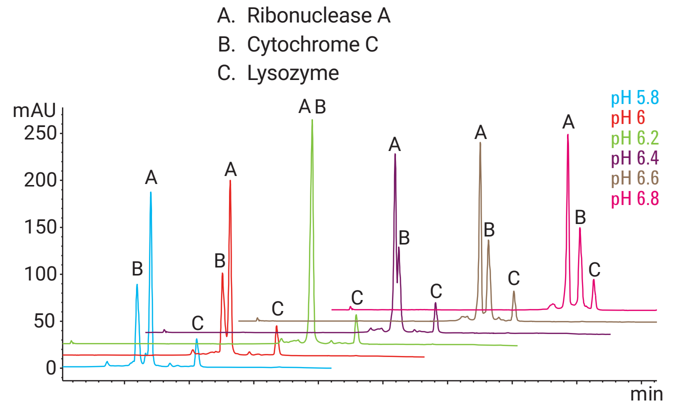


Figure 9. pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients

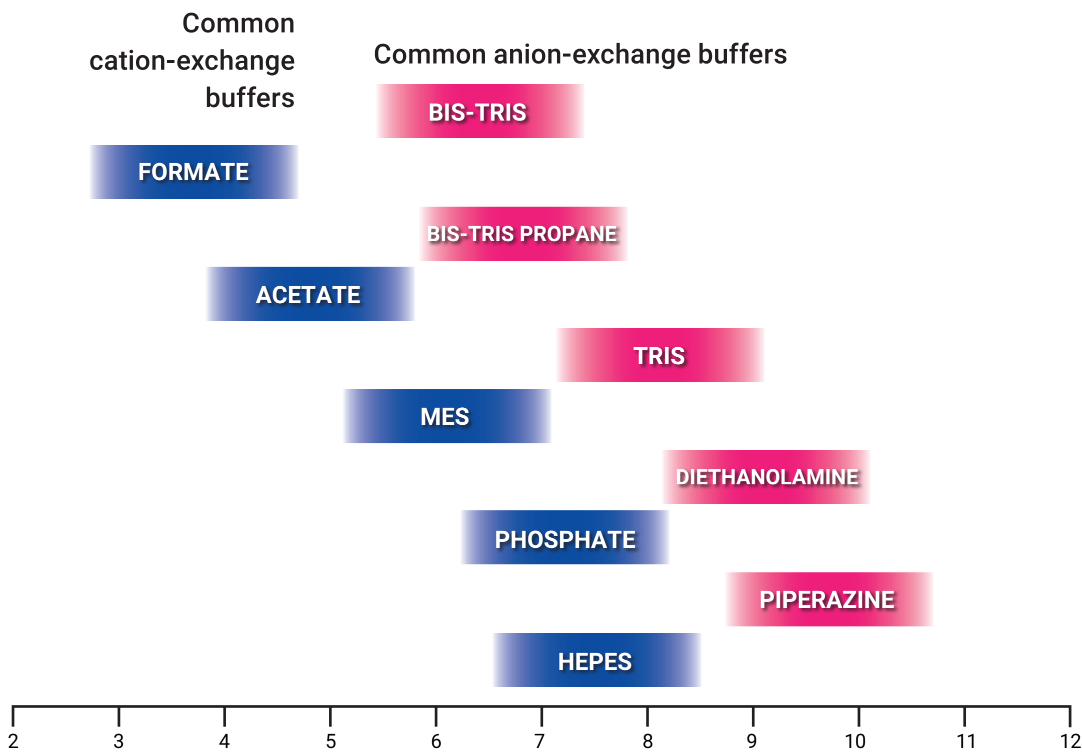


Figure 10. Commonly available buffers for ion-exchange

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).

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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.

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.

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

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.

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.

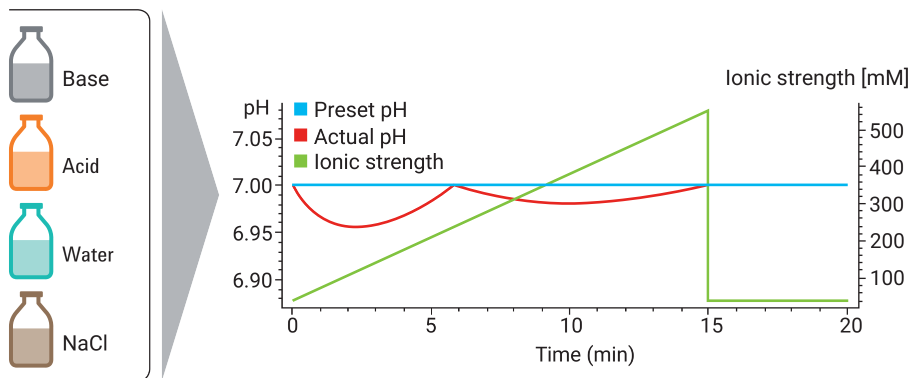

Figure 11. Salt gradients are easily created from stock solutions with Agilent Buffer Advisor Software

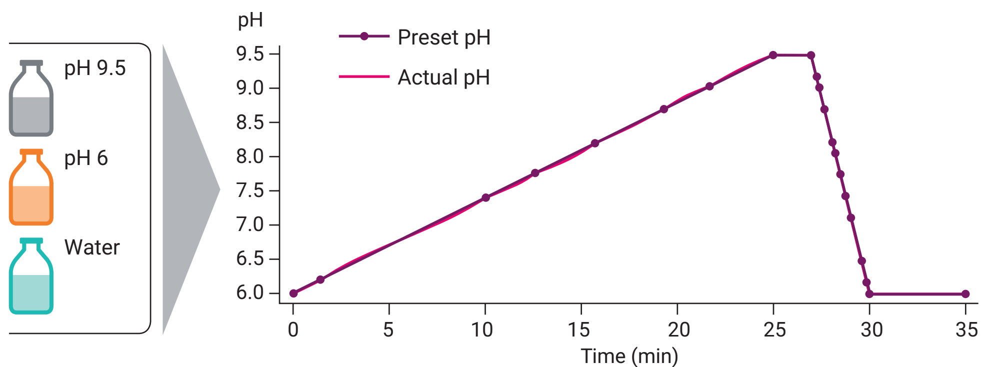

Figure 12. Optimizing buffer strength for a monoclonal antibody separation – pH gradients are easily created from stock solutions

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Conditions

Parameter	Value
Column A:	Bio WCX, stainless steel 5190-2443 4.6 x 50 mm, 3 µm
Column B:	Bio SCX, stainless steel 5190-2423 4.6 x 50 mm, 3 µm
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
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
Temp.:	Ambient
Injection:	5 µL
Detector:	UV, 220 nm

Automated method development for optimized charged-variant separations

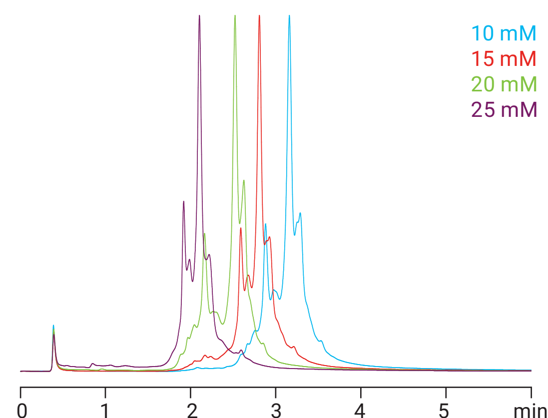

Figure 13. Optimizing buffer strength at pH 6.5 from the screening chromatograms of a monoclonal IgG separation

Conditions

Parameter	Value
Column A:	Bio MAb, PEEK 5190-2407 4.6 x 250 mm, 5 µm
Sample: thermostat	IgG monoclonal antibody
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
Temp.:	30 °C
Data acquisition:	214 and 280 nm
Acquisition rate:	20 Hz
Flow cell:	60 mm path
Injection:	10 µL (needle with wash, flush port active for 7 s)
Detector:	

Bio MAb columns enable precise quantitation, robust methods

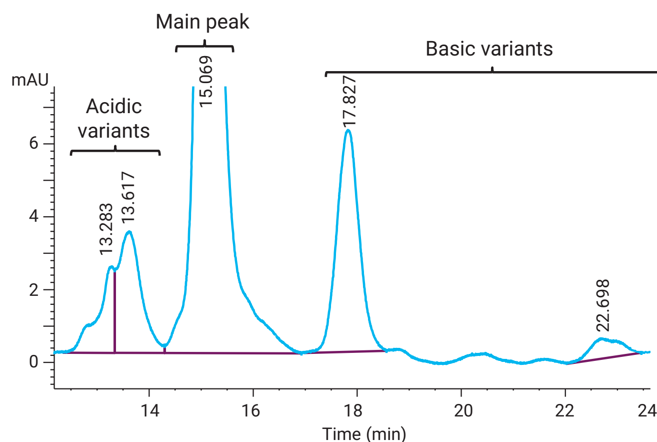

Figure 14. pH gradient-based cation-exchange chromatogram of an IgG1 separation using an Agilent Bio MAb PEEK, 4.6 x 250mm, 5 µm column

Application Note

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Convenient Customization of Your Cation Exchange Analysis

Combining the Agilent 1260 Infinity II Bio Prime LC System, Agilent Buffer Advisor Software, and pH gradients for high-resolving charge variant analysis

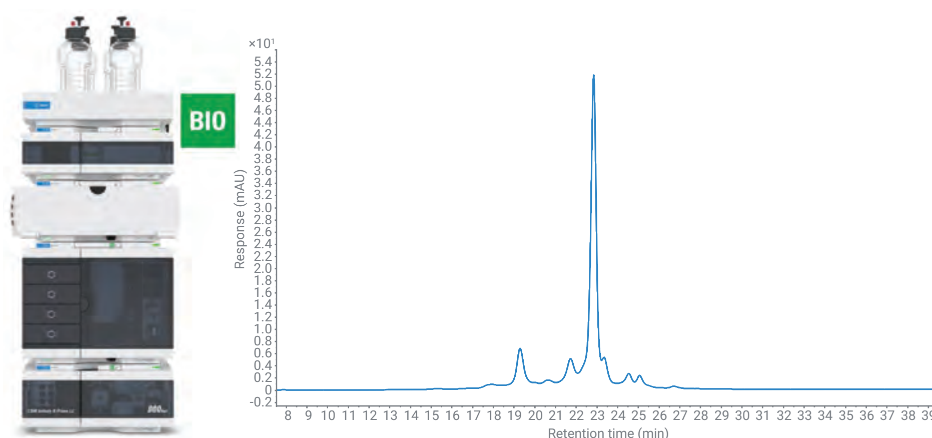
Author

Sonja Schneider
Agilent Technologies, Inc

Abstract

Charge variants separation of monoclonal antibodies can be a challenging task for the chromatographer. Due to the microheterogeneity of the analyzed monoclonal antibody, extensive method development can be necessary to find the optimal desired resolution. Outperforming many traditional salt gradients, the resolving power of pH gradients enables the separation of charge variants in a very efficient way. This application note demonstrates high-resolving and reproducible charge variant analysis of two monoclonal antibodies, trastuzumab and NIST mAb, with different types of pH gradients.

The Agilent 1260 Infinity II Bio Prime LC System, with a completely iron-free flow path and featuring an Agilent 1260 Infinity II Bio Flexible Pump, enables the use of Agilent Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions.



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Introduction

Therapeutic monoclonal antibodies (mAbs) are highly heterogeneous molecules and are composed of a large number of variants. These are naturally occurring in this kind of biopharmaceutical and are not necessarily considered impurities. Proteins in solution have mostly polar and charged amino acids at the protein interface to aqueous media, while the hydrophobic residues tend to self-associate due to hydrophobic interactions. These amino acids on the "outside" of the proteins that are in contact with surrounding liquid are more predisposed to modifications.[1]

Variants, also called protein microheterogeneity, might originate from post-translational modifications during antibody production. In addition, modifications after purification processes, formulation and/or storage can be formed.[2] However, if the variants are present in the pharmaceutical protein, their biological activity might differ and immunogenicity might be enhanced.[2] Hence, the microheterogeneity of the mAbs is subject to extensive analytical characterization to ensure safety and efficacy of the biopharmaceutical.

Cation exchange chromatography (CEX) is considered the gold standard for charge variant analysis of monoclonal antibodies.[3] Classic salt gradients have high resolving power once the method is fully optimized. However, the amount of effort required to develop a high-resolving ion exchange method for protein separation can be very high. Salt concentration, mobile phase pH values, and additives are only a few of the parameters to be optimized. In addition, every molecule and especially biological molecules might show different behavior and the developed methods are not tolerant to large changes in experimental parameters, especially with respect to pH values.[1,4]

pH gradient-based CEX, also known as chromatofocusing, enables high-resolving as well as robust methods for the separation of mAb charge variants.[1,4,5] In typical ion-exchange chromatography (IEX), the molecules are eluted from the column by increasing the ionic strength (mostly with salts like NaCl) of the buffer. In contrast, with pH gradients, the bound molecules are eluted with the changing pH of the buffer. This alters their net surface charge to enable the elution of the bound molecules at their isoelectric point (pI), where the molecule is electrically neutral.

Wide pH gradient methods are more generic and can separate variants from different antibodies within a single buffer system.[1] Also, the method development of pH gradient-based methods is more straightforward and significantly shorter compared to conventional ionic strength-based IEX.

Wide pH gradient methods are more generic and can separate variants from different antibodies within a single buffer system.[1] Also, the method development of pH gradient-based methods is more straightforward and significantly shorter compared to conventional ionic strength-based IEX.

The 1260 Infinity II Bio Prime LC System is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in bio chromatography: The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of ferric ions (e.g. oxidation and protein complex formation) can be avoided.

The 1260 Infinity II Bio Flexible Pump, as a quaternary pump, enables the use of Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. With Buffer Advisor Software, quaternary salt gradients as well as pH gradients can be generated quickly and simply by the calculation of pump timetables for IEX.

This application note presents the analysis of charge variants for trastuzumab and the NIST mAb reference standard with two different pH gradients.

Experimental

Equipment

The Agilent 1260 Infinity II Bio Prime LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS version 2.5 or later versions

Columns

Bio MAb, NP5, 2.1 × 250 mm, PEEK (part number 5190-2411)

Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, tris (tris(hydroxymethyl)aminomethane), imidazole, hydrochloric acid, and piperazine hexahydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

The Agilent 1260 Infinity II Bio Prime LC System comprised the following modules:

- Agilent-NISTmAb (part number 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8

Buffer preparation

The buffers were prepared according to the Stock Solution Recipes from Buffer Advisor Software (see Figure 1).

Quaternary phosphate-based buffer system—calculated by Buffer Advisor

A: Water
B: 1,700 mM sodium chloride
C: 44.5 mM sodium phosphate monobasic
D: 55 mM sodium phosphate dibasic

Note: This setup can be used for salt as well as pH gradient elution—enabling direct comparison and increasing the possibilities for method development.

Binary wide pH gradient buffer system—calculated by Buffer Advisor

With Buffer Advisor, it is also possible to create wide range pH gradients, also termed Composite Buffer. In these cases, only the C and D channels are employed to create the gradient. This experiment used a pH gradient described by Farnan and Moreno[1] and inserted the buffer composition as a User Mixture in the stock solution composition of Buffer Advisor (2.4 mM tris, 1.5 mM imidazole, 11.6 mM piperazine, HCl for pH adjustment to pH 6 & C and 10.5 & D). With this option, the user can construct self-made buffer compositions to enable the desired pH range. Buffer Advisor calculates the ionic strength (IS) as well as the buffering capacity (BC) for both buffer mixtures.

A: Water
B: n/a
C: pH = 6; IS = 22.5 mM; BC = 6.19 mM
D: pH = 10.5; IS = 0.717 mM; BC = 2.31 mM

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. So, for example, for solvent B in the phosphate-buffered gradient with 1,700 mM NaCl, use Sodium Chloride 1.5 M rather than Generic Aqueous or Water in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and hence using the preconfigured solvent tables enables best pump performance.

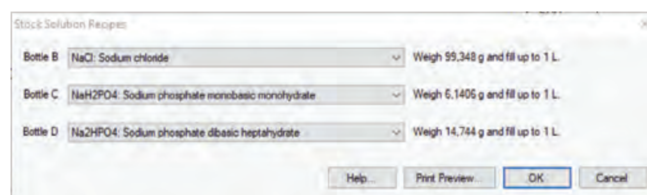

Figure 1. Agilent Buffer Advisor Stock Solution Recipe—quaternary phosphate buffer system.

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Method

Table 1. Quaternary phosphate-based buffer system—salt gradient chromatographic conditions.

Parameter	Value
Solvent	A) Water B) 1,700 mM sodium chloride C) 44.5 mM sodium phosphate monobasic D) 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from 10 to 110 mM NaCl in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
UV Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Table 2. Quaternary phosphate-based buffer system—pH gradient chromatographic conditions.

Parameter	Value
Solvent	A) Water B) 1,700 mM sodium chloride C) 44.5 mM sodium phosphate monobasic D) 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from pH 7 to 8.4 in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
UV Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Table 3. Binary wide pH gradient buffer system/Farnan pH gradients.

Parameter	Value
Solvent	A) n/a B) n/a C) pH = 6; IS = 22,4 mM; BC = 6,14 mM (Farnan Buffer[1]) D) pH = 10,5; IS = 0,717 mM; BC = 2,31 mM (Farnan Buffer[1])
Gradient	Trastuzumab gradient: Gradient from 0 to 50 minutes from pH 8.3 to 10 with a subsequent "wash" step from 51 to 55 minutes at pH 10.5 Stop time: 55 minutes Post time: 20 minutes NISTmAb gradient: Gradient from 0 to 45 minutes from pH 8.9 to 10.5 Stop time: 50 minutes Post time: 20 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
UV Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Results and discussion

With Buffer Advisor, it is possible to calculate both salt as well as pH gradients. With the quaternary phosphate buffer described in the Experimental section of this application note, it is possible to calculate both versions for the separation of trastuzumab charge variants.

Figure 2 displays the overlay of two chromatograms, separating trastuzumab charge variants with a flat salt gradient (blue) and a phosphate-buffered pH gradient from pH 7 to 8.4 (green). The separations by salt and pH gradient are comparable, with slight improvements in resolution when using the pH gradient.

One of the features of Buffer Advisor Software is the improved calculation of linear gradients (salt as well as pH) by adding additional gradient steps within the given gradient to enable perfect linearity without major deviations from the desired/preset pH. To enable this functionality, the Optimize Gradient box in the *4. Create % Timetable* section in the Buffer Advisor user interface (UI) needs to be checked (see red circle in Figures 3A and 3B).

Figures 3A and B showcase the difference between the preset and the actual pH if the box is unchecked (A) and checked (B). With no further optimization from Buffer Advisor, the actual pH can deviate up to 0.4 units from the preset pH, which makes it difficult for the user to rely on the running gradient linearity. By checking the Optimize Gradient box (Figure 3B), additional steps are inserted into the original gradient to ensure linearity of the pH gradient. The Result Pump Gradient Timetable on the bottom left displays the additional inserted steps resulting in the actual gradient being as close as possible to the preset gradient. This gradient timetable can then be exported into the method in OpenLab for an easy transfer without additional time needed for typing.

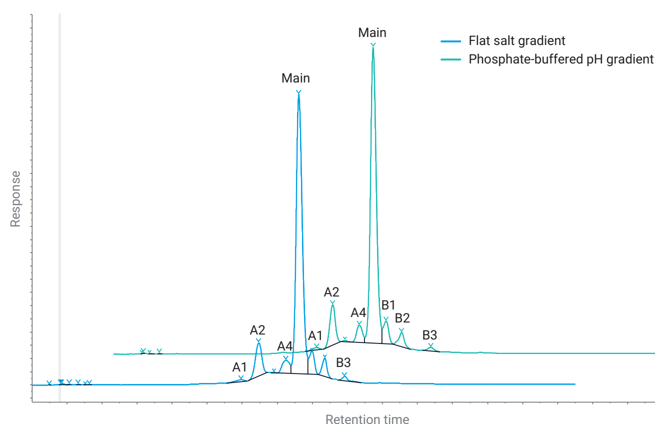


Figure 2. Overlay of two chromatograms for the separation of charge variants with a flat salt gradient (blue) as well as a phosphate-buffered pH gradient from pH 7 to 8.4 (green).

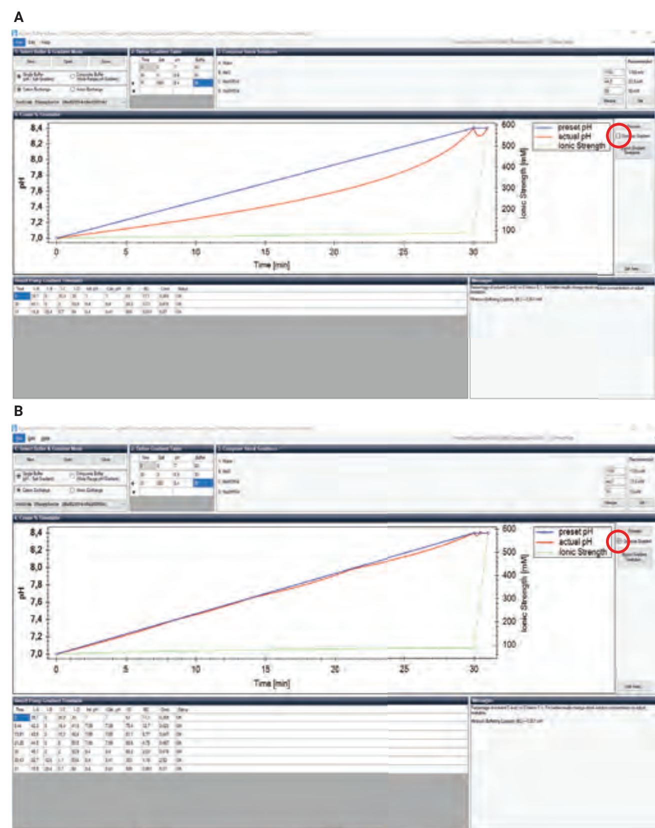


Figure 3. Optimize Gradient function in Agilent Buffer Advisor software to enable highly linear pH gradient. A shows no optimization, B displays the optimized gradient.

Figure 4 shows an overlay of seven subsequent runs of the charge variant analysis of trastuzumab with the phosphate-buffered pH gradient from pH 7 to 8.4. Excellent reproducibility was found for retention time (RT) and area with relative standard deviation (RSD) of less than 0.085% apart from the first two peaks. Due to the minimal area as well as height of the variants A3 and B3, the area reproducibility is higher than 1%.

Within the phosphate-buffered system, method development is limited, especially if the pI of the molecules of interest is not in the pH buffering range between 6 and 8. If the pI of the molecule is too high, elution is not possible using this buffer system. For example, the NIST mAb with a pI of 9.186 needs a different buffer system to enable elution from the CEX column.

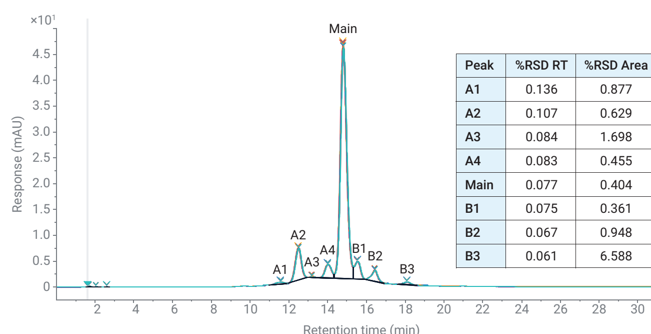
A more generic approach is to use a wide-range pH gradient composed of more than one buffer system. This setup is also incorporated in Buffer Advisor Software under the name Composite Buffer (Wide Range pH Gradient). The pH gradient with pH range 6.0 to 10.5 from Farnan and Moreno (2009)[1] is a suitable buffer system to analyse the charge variants of monoclonal antibodies.

This system was further used and the method optimized for trastuzumab and NIST mAb.

Figure 5 shows the separation of trastuzumab charge variants using the wide-range pH gradient, narrowed from pH 8.3 to 10 to achieve optimal resolution. Compared to the phosphate-buffered pH gradient (see Figure 2), it was possible to resolve two more acidic variants—A1 to A6—eluting before, and one more basic variant eluting after the main peak. Especially the zoomed view in Figure 5B shows the excellent resolution of different charge variants around the main peak, with sharper peaks and enhanced resolving power compared to the shallow salt and pH gradient shown in Figure 2.

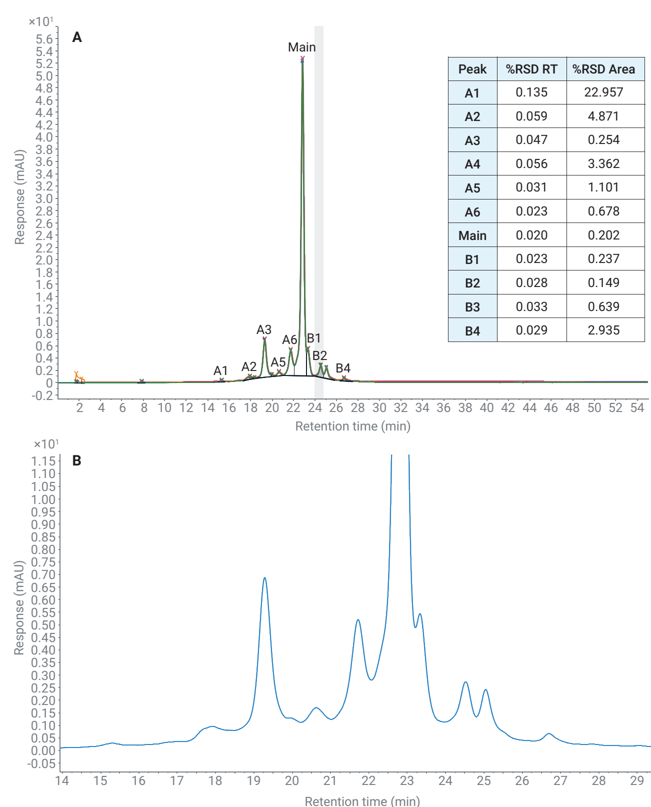
The precision of RT and area was evaluated for all resolved variants (see peak table in Figure 5). Even for extremely small peaks, the precision of RT was excellent, with values below 0.06% RSD except for the first variant A1. The area precision showed excellent values for most of the peaks except for the extremely small ones.

The pH gradient used by Farnan and Moreno[1] has also proven to be ideal for the analysis of the NISTmAb (see Figure 6). For the NISTmAb, the pH gradient was modified to a different pH range due to the different pI of the NISTmAb. With this developed shallow gradient from pH 8.9 to 10.5, it was possible to separate three acidic and two basic variants.



Peak	%RSD RT	%RSD Area
A1	0.136	0.877
A2	0.107	0.629
A3	0.084	1.698
A4	0.083	0.455
Main	0.077	0.404
B1	0.075	0.361
B2	0.067	0.948
B3	0.061	6.588

Figure 4. Overlay of seven consecutive runs of trastuzumab analyzed with phosphate-buffered pH gradient from pH 7 to 8.4 including the precision table for retention time (RT) and area.



Peak	%RSD RT	%RSD Area
A1	0.135	22.957
A2	0.059	4.871
A3	0.047	0.254
A4	0.056	3.362
A5	0.031	1.101
A6	0.023	0.678
Main	0.020	0.202
B1	0.023	0.237
B2	0.028	0.149
B3	0.033	0.639
B4	0.029	2.935

Figure 5. Overlay of seven consecutive runs of trastuzumab using a wide pH gradient from pH 8.3 to 10 including the precision table for retention time (RT) and area A. Zoomed view of a single injection B.

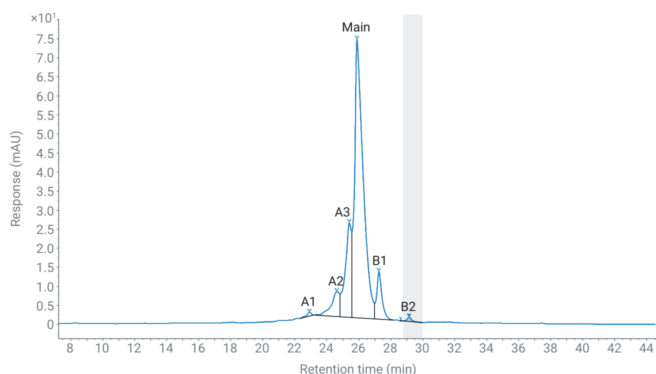


Figure 6. Separation of the NISTmAb with a pH gradient modified according to Farnan and Moreno1 from pH 8.9 to 10.5.

Conclusion

The advantage of pH gradients over salt gradients was demonstrated for the analysis of monoclonal antibodies. pH gradients have been shown to outperform even shallow salt gradients with simplifying method development on the one hand and the generation of high-resolution chromatographic results on the other hand. While the potential of salt gradient method development is rather limited—changes in gradient slope just increase peak width with no further changes in resolution[7]—pH gradients reveal possibilities to further increase resolution and maintain sharp peaks. This was showcased by the analysis of trastuzumab and NIST mAb, especially by the use of the wide range pH gradient, based on Farnan and Moreno.[1] Buffer Advisor Software facilitated dynamic mixing of four stock solutions for the phosphate-buffered systems, preventing time-consuming buffer preparation hands-on time in the lab. In addition, the wide-range pH gradient could be easily calculated by Buffer Advisor. Hence, all methods—developed with Buffer Advisor Software and run on the Agilent 1260 Infinity II Bio Prime LC System with Flexible pump, with its completely iron-free sample flow path—delivered highly reliable and reproducible results.

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How Shallow Can You Go?

Refining charge variant analysis of mAbs with the Agilent 1290 Infinity II Bio LC System

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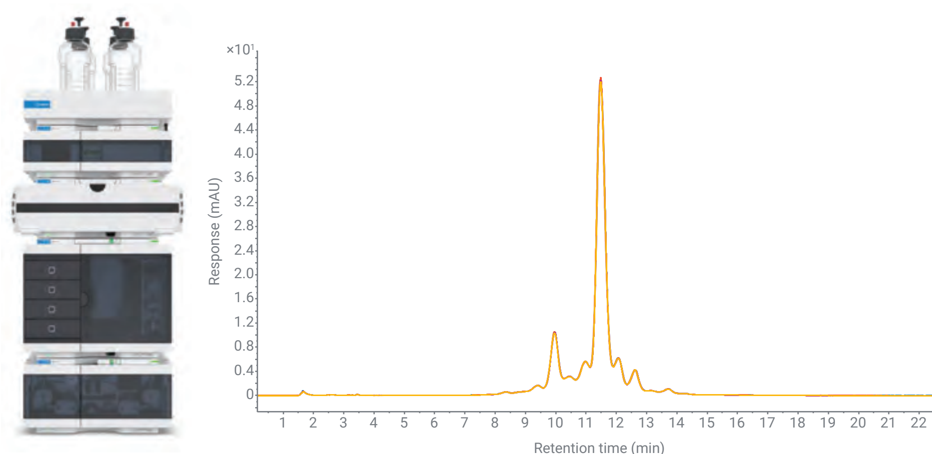
Abstract

Charge variant analysis is a demanding application for applied liquid chromatography systems due to the use of highly corrosive buffer salts in combination with very shallow gradients for optimal separation. The evaluation of different salt gradients was performed on the Agilent 1290 Infinity II Bio LC System and analyzed for resolution as well as reproducibility.

The 1290 Infinity II Bio LC including High-Speed Pump, with its completely iron-free flow path, is optimally suited for the conditions used in biochromatography—avoiding potentially corrosive damage to the system. Excellent reproducibility even for highly challenging shallow gradients was determined, confirming the 1290 Infinity II Bio LC as the next generation of Agilent high-end liquid chromatography systems for high confidence in generated data.



Introduction

Monoclonal antibodies (mAbs) are large and highly heterogeneous macromolecules, with a size of around 150 kDa, that are typically generated by recombinant production methods. They are generated in a complex biosynthetic process in which plenty of modifications can occur, leading to hundreds of different variants. Deamidation, oxidation, disulfide bridges, N-glycosylation, N- and C-terminal processing are some of the most common post-translational modifications (PTMs). All these modifications can occur during generation, but also manufacturing and storage contribute to the complexity of these macromolecules. PTMs form a complex isoform profile that needs to be extensively analyzed and monitored, as modifications in the final pharmaceutical might be associated with a loss of biological activity, affected half-life, or immunogenicity.[1] Some of the PTMs result in charge variants of the molecule, which are typically analyzed using ion-exchange chromatography (IEX).[2] Charge variants are considered one of the most important critical quality attributes (CQAs) and therefore strict acceptance criteria and quality controls are to be considered. It is of utmost importance to confirm that the product is correctly manufactured, and to identify and quantify any impurities.

Shallow gradient elution is very common in IEX of proteins. A typical salt gradient in ionic strength mode for the elution of proteins would be approximately 1 to 3 mM/min with a pH value set to a tolerance of ±0.02 pH units.[3]

The 1290 Infinity II Bio LC is equipped with a high-performance High-Speed Pump. The major advantage of binary pumps is that solvent mixing is much more accurate and precise when mixing small proportions of one of the solvent components compared to low-pressure mixing pumps (e.g., quaternary pumps). This type of mixing gives highly precise solvent compositions at the start and the end of a solvent gradient.[4] This is a basis for the generation of reproducible and accurate shallow gradients (below 1%/min from each channel).

The 1290 Infinity II Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in biochromatography: high salt concentrations such as 2 M NaCl, up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl. The complete flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of iron ions (e.g. oxidation, protein complex formation) can be avoided.

This application note presents the analysis of charge variants for trastuzumab and the NISTmAb reference standard. Different salt gradient slopes were tested to find the best resolution possible. The best performing gradient slopes were then evaluated for reproducibility.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS Version 2.5

Columns

Agilent Bio MAb, NP5, 2.1 × 250 mm, PEEK (part number 5190-2411)

Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, hydrochloric acid, sodium hydroxide, tris(hydroxymethyl)-aminomethane, imidazole, and piperazine hexahydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

- Agilent-NISTmAb (p/n 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland)
- The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8

Note: As Adcetris contains many adjuvants, the concentration mentioned here is not the protein concentration only, but the total concentration of all components of the drug.

Buffer preparation

For 2 L of 30 mM phosphate buffer, pH 6.8, 4.45 g of sodium phosphate monobasic monohydrate and 7.44 g of sodium phosphate dibasic heptahydrate was weighed and added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water (› buffer A). 29.22 g of sodium chloride, for a total concentration of 500 mM, was added to an empty, amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer A (› buffer B). The pH values of both prepared buffers were checked and adjusted, if necessary, to pH 6.8 (the addition of high amounts of salt can change the pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

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Method

Table 1. Salt gradient chromatographic conditions.

Parameter	Value
Solvent	A) 30 mM phosphate buffer, pH 6.8, B: 30 mM phosphate buffer, pH 6.8, 500 mM sodium chloride
Gradient	0 or 25 mM—150 mM NaCl in 30 minutes—different shallow gradients for method development 0 mM (trastuzumab) and 25 mM (NIST) to 100 mM NaCl in 30 minutes for reproducibility 25 to 50 mM NaCl in 30 minutes for reproducibility (very shallow gradient) 31 minutes—500 mM NaCl wash Stoptime: 35 minutes Post-time: 15 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm, 10 Hz
Injection	Injection volume: 3 µL for trastuzumab and 2 µL for NIST Sample temperature: 10 °C Needle wash: 3 s in water

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for Solvent B, including 500 mM sodium chloride, use “Sodium Chloride 0.5 M” rather than Generic Aqueous or Water in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

Results and discussion

Method development

To achieve the desired resolution and enable optimal separation, extensive method development is necessary for charge variant analysis. Two parameters are essential to be successful: finding the optimal pH as well as the optimal gradient slope. Both factors can have a major impact on the separation. First, pH scouting is recommended to find the optimal pH for the separation. In earlier experiments, the pH of the used buffers was analyzed from pH 6.4 to 7.4 and was found to be optimal at pH 6.8 for both samples used: trastuzumab and the NISTmAb reference standard (data not shown). The next step is the determination of the ideal gradient slope to enable the efficient separation.

Figure 1 shows an overlay of charge variant analysis of trastuzumab at different gradient slopes ranging from 1% B/min (5 mM/min) down to 0.33% B/min (1.66 mM/min). The shallower the gradient, the higher the requirements to the pump performance. To deliver highly precise solvent compositions during the gradient, the pump needs to work accurately and precisely when mixing small proportions of the solvent components. It has to be considered, though, that for salt gradients, very shallow gradients do not always result in higher resolution, but simply increase peak width (e.g. 0.33% B/min in Figure 1). Therefore, the chosen gradient slope for reproducibility studies was found in the middle of the tested gradients with 0.66% B/min and 3.3 mM/min, which can still be considered shallow.

A similar method development procedure was carried out for the separation of charge variants of the NISTmAb reference standard (see Figure 2). The starting conditions for NIST contained a slighty higher amount of salt due to the higher isoelectric point (pI) of the NIST antibody (pI of ~9.2), compared to trastuzumab with a pI of ~9. For more effective separation of the charge variants of the NISTmAb reference standard, the gradients were slightly shallower compared to trastuzumab, so the most shallow gradient was at 0.17% B/min (0.83 mM/min), which is a challenging task for the pump. For further reproducibility studies, the 0.5% B/min (2.5 mM/min) gradient slope was chosen.

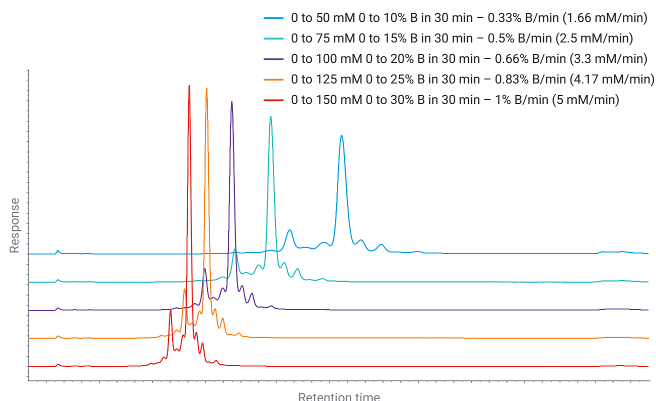


Figure 1. Method development for the separation of trastuzumab with different salt gradient slopes.

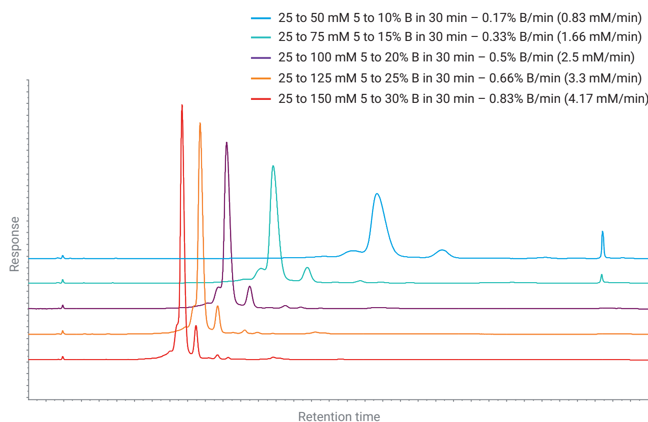


Figure 2. Method development for the separation of the NISTmAb reference standard with different salt gradient slopes.

Reproducibility for trastuzumab charge variant separation

Figure 3 displays reproducibility studies for charge variant separation of trastuzumab (A) with a 0.66% B/min (3.3 mM/min) gradient slope. Figure 3B shows a zoomed view for better visualization of the separated variants. Variants marked with A represent the acidic variants eluting before the main peaks, whereas the basic variants B elute after the main peak. Five acidic variants were resolved before the main peaks and four basic variants eluted after the main peak. All variants and the main peak were evaluated for the precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.052% relative standard deviation (RSD) for RT and below 0.82% RSD for area except for the two very small variant peaks A1 and B3.

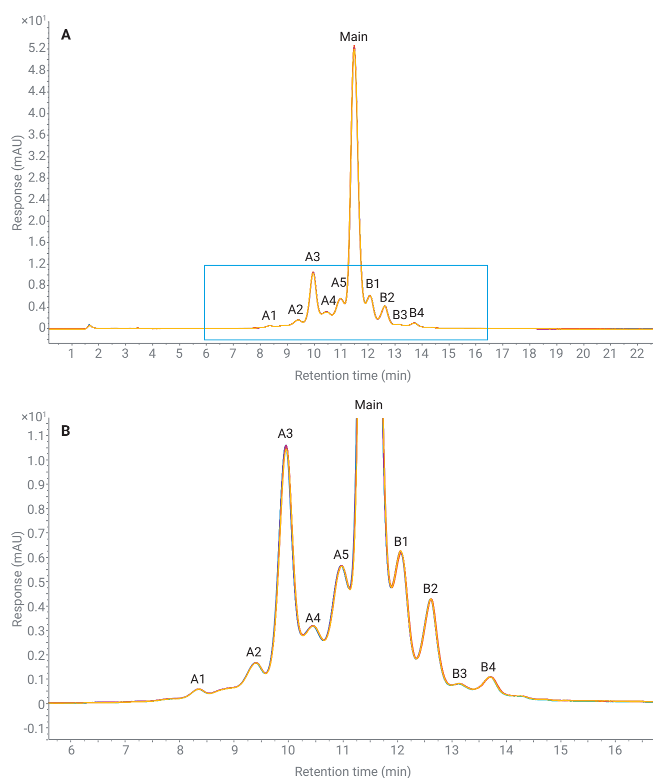


Figure 3. Reproducibility studies with seven subsequent runs for charge variant separation of trastuzumab (A) with 0.66% B/min (3.3 mM/min) gradient slope. (B) Zoomed view.

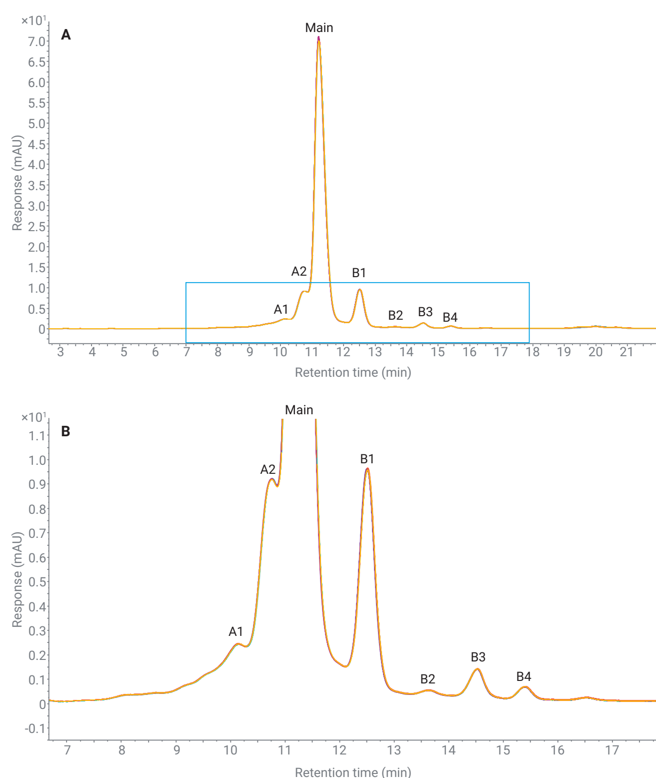
Precision in RSD	RT (%)	Area (%)
A1	0.033	1.793
A2	0.016	0.701
A3	0.026	0.403
A4	0.023	0.813
A5	0.032	0.327
Main	0.033	0.313
B1	0.038	0.329
B2	0.048	0.254
B3	0.046	3.549
B4	0.051	0.812

Reproducibility for NISTmAb charge variant separation

Figure 4 displays reproducibility studies for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope.

Figure 4B displays the zoomed view with two acidic variants and four basic variants. Again, all variants and the main peak were evaluated for precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.06% RSD for RT and below 0.55% RSD for area except for one very small variant peak, B2.

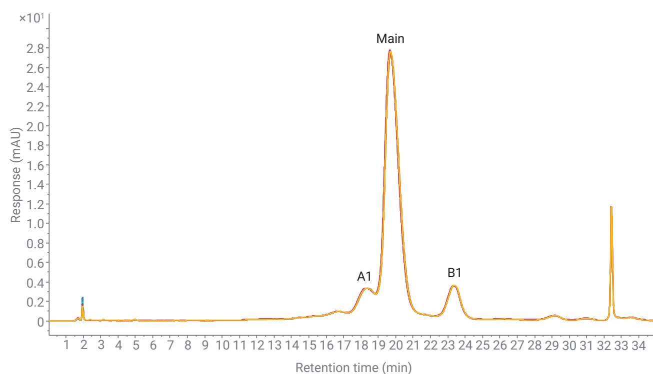
As shown in Figure 2, the shallowest gradient with 0.17% B/min (0.83 mM/min) does not deliver a better resolution compared to gradients such as 0.5% B/min (2.5 mM/min) (still shallow—used in the reproducibility studies).



Precision in RSD	RT (%)	Area (%)
A1	0.058	0.540
A2	0.039	0.374
Main	0.045	0.126
B1	0.042	0.141
B2	0.049	1.469
B3	0.033	0.354
B4	0.033	0.485

Figure 4. Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope. (B) Zoomed view.

However, the 1290 Infinity II Bio High-Speed Pump managed the challenging gradient slope, which is displayed in Figure 5. RT precision over seven subsequent runs was very good (below 0.25% RSD), although the peaks became quite broad with the applied gradient. With increasing peak width, the peak height decreases, which negatively affects the area precision.



Precision in RSD	RT (%)	Area (%)
A1	0.238	6.608
Main	0.102	1.95
B1	0.08	8.157

Figure 5. Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard with 0.17% B/min (0.83 mM/min) gradient slope.

Conclusion

Different salt gradient slopes were evaluated for resolution and reproducibility of the separation of charge variants for trastuzumab and NISTmAb on the 1290 Infinity II Bio LC. At first glance, shallower gradients seemed to improve resolution. However, for both mAbs, the evaluated most shallow gradients did not give the best resolution, as with decreasing slope, the peaks only began to broaden, which led to no further improvement of resolution. The methods with the best combination of high resolution and sharp peak shapes were further evaluated for reproducibility. For a gradient slope of 3.3 mM/min (trastuzumab) and 2.5 mM/min (NISTmAb), excellent reproducibility for RT but also area was documented. The RT precision was below 0.06% RSD for all evaluated peaks. The most shallow gradient tested for NISTmAb was also evaluated for reproducibility, and even for a super shallow gradient with 0.83 mM/min gradient slope, very good RT precision was found (<0.25% RSD). These data show that the 1290 Infinity II Bio LC with its completely iron-free flow path is optimally suited for the conditions used in biochromatography, leading to highly reproducible results.

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Charge Heterogeneity Analysis of Rituximab Innovator and Biosimilar mAbs

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Abstract

This Application Note describes the high-resolution separation of charge variants of innovator and biosimilar rituximab using an Agilent 1260 Infinity Bio-inert Quaternary LC, biocolumns, and an Agilent OpenLAB ChemStation Software tool. An Agilent Bio MAb, 4.6 × 250 mm, 5 µm PEEK ion exchange column features a unique resin designed for the charge-based separation of monoclonal antibodies (mAbs). The optimized salt-gradient showed the differences in acidic and basic charge variant profiles between innovator and biosimilar rituximab. Precision of retention time, height, and area of charge isoforms were well within the acceptable range. C-terminal digestion by Carboxypeptidase B (CPB) revealed the major lysine variant peaks in biosimilar rituximab.

Introduction

Recently, biosimilar products are increasing in popularity in biopharmaceuticals. mAbs can undergo various post-translational modifications (PTMs) including lysine truncation, deamidation, oxidation, glycosylation, and so forth, becoming heterogeneous in their biochemical and biophysical properties. Due to these modifications, charge variants can affect the efficacy, activity, and stability of mAbs as biotherapeutics. Hence, it is very important to characterize the charge heterogeneity in drug development that will serve as a quality control (QC) step in the biopharmaceutical industry. In addition, precise bioanalytical methods are necessary to demonstrate the similarity between a biosimilar and the innovator product.

Cation exchange chromatography (CEX) is the gold standard for charge-sensitive antibody analysis. In CEX, method parameters often need to be optimized for each protein, as ion exchange depends upon the reversible adsorption of charged protein molecules to immobilized ion exchange groups. This Application Note describes the salt-gradient method for separating the charge variants of innovator and biosimilar rituximab using an Agilent 1260 Infinity Bio-inert Quaternary LC and an Agilent Bio MAb NP5, 4.6 × 250 mm, PEEK ion exchange column. The method compares the CEX profiles of innovator and a rituximab biosimilar. Precision of retention time, height, area, and quantification of acidic, basic, and main forms was determined. Carboxypeptidase B (CPB) digestion was performed to study the contribution of C-terminal lysine variants.

Experimental

Instrumentation

An Agilent 1260 Infinity Bio-inert Quaternary LC, operating to a maximum pressure of 600 bar, was used for the experiments. The entire sample flow path was free of any metal components so that the sample did not come in contact with metal surfaces. Solvent delivery was free of any stainless steel or iron components.

Systems

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (TCC) containing bio-inert click-in heating elements (G1316C option 19)
- Agilent 1260 Infinity Diode Array Detector with with 10 mm bio-inert standard flow cell (G1315D)
- Agilent Bio MAb NP5, 4.6 × 250 mm, PEEK (p/n5190-2407)

Software

- Agilent OpenLAB CDS ChemStation Edition, revision C.01.062 Agilent Buffer Advisor, Rev. A.01.01

Table 1. Chromatographic parameters used for IEX chromatography.

Parameter	Conditions				
Mobile phase A	Water				
Mobile phase B	NaCl (850.0 mM)				
Mobile phase C	NaH_2PO_4 (41.0 mM)				
Mobile phase D	Na_2HPO_4 (55.0 mM)				
Gradient	**Time (min)**	**Mobile phase A (%)**	**Mobile phase B (%)**	**Mobile phase C (%)**	**Mobile phase D (%)**
	0	30.3	0	59.6	10.1
	2	26.0	5.0	56.9	12.1
	8	21.5	10.0	54.9	13.6
	20	13.3	19.0	51.9	15.8
	21	30.3	0	59.6	10.1
Injection volume	5 µL				
Flow rate	0.75 mL/min				
Data acquisition	280 nm/4 nm, Ref.: 360 nm/100 nm				
Acquisition rate	5 Hz				
TCC	Room temperature				
Sample thermostat	5 °C				
Post run time	10 minutes				

Reagents, samples, and procedure

Innovator and biosimilar rituximab were purchased from a local pharmacy and stored according to the manufacturer's instructions. Sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, hydrochloric acid (HCl), and sodium hydroxide were purchased from Sigma-Aldrich. All the chemicals and solvents were HPLC grade, and highly purified water was from a Milli Q water purification system (Millipore Elix 10 model, USA). Carboxypeptidase B (C9584) was purchased from Sigma-Aldrich.

Ion exchange chromatography parameters

Table 1 shows the chromatographic parameters for ion exchange chromatography using a 1260 Infinity Bio-inert Quaternary LC. Rituximab (innovator and biosimilar) were diluted to 1 mg/mL in water, and the elution was monitored at 280 nm. Retention time (RT), area, and percent area were used to calculate standard deviation (SD) and relative standard deviation (RSD %) values. Relative percent area was used to quantify the charge variants of mAbs.

Carboxypeptidase B digestion

Biosimilar and innovator rituximab were diluted to 1 mg/mL using 10 mM sodium phosphate buffer, pH 7.5. To these, 0.25 units of CPB was added and incubated at 37 °C. At various time points, the reaction mixture was aliquoted and quenched with acetic acid before analysis.

Results and Discussion

The Agilent Buffer Advisor Software is an ideal tool to generate pH or ionic strength gradients for protein charge variant separation. It reduces the time required for method development. In this study, a series of method development scouting runs were carried out using the Buffer Advisor Software for optimal mAb charge variant separation. Figure 1 shows the charge variant profiles of innovator and biosimilar rituximab on a Bio MAb PEEK column, demonstrating high-resolution separation of charge variants in 20 minutes with three distinct peaks in biosimilar (Buffer: 30 mM, pH: 6.3, and NaCl: 0–161.5 mM). The Agilent Bio MAb columns contained a highly uniform, densely packed, weak cation exchange resin. Early and late-eluting peaks were called acidic and basic variants, respectively. The peak at 11.4 minutes was designated as the main peak. The overlay of five replicates of innovator and biosimilar rituximab shows excellent separation reproducibility (Figure 2). The average RTs and area RSDs for main peak are shown in the figure. The RSDs are within the acceptable range, which demonstrates the precision of the system.

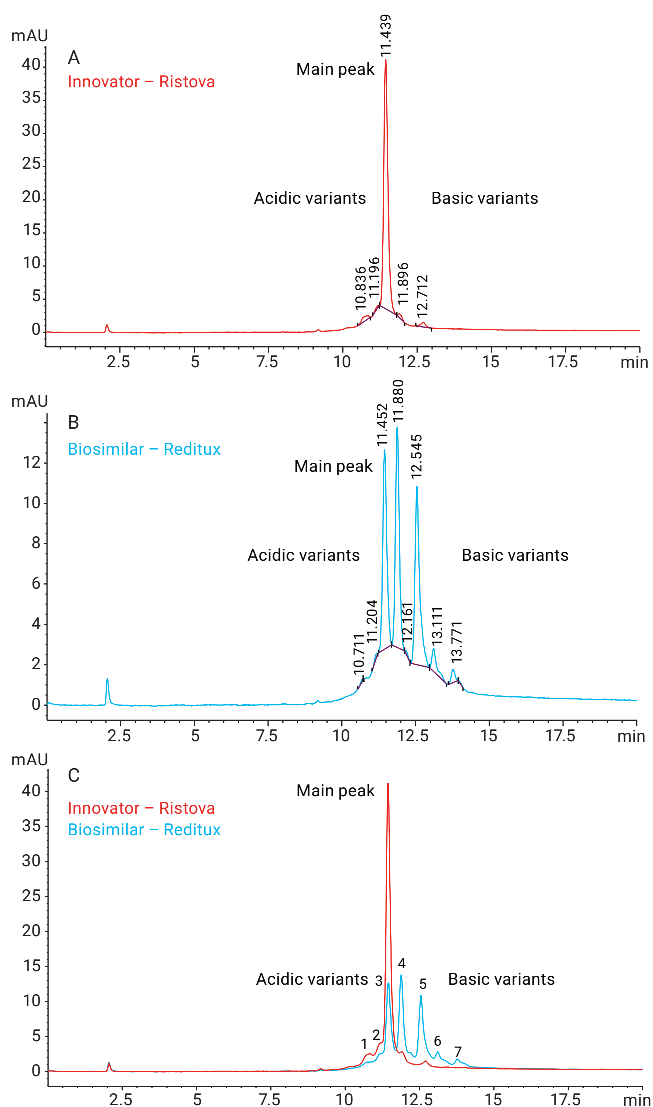

Figure 1. Charge variant profiles of innovator (A) and biosimilar (B) rituximab using an Agilent Bio MAb 5 µm column. C) Overlay of innovator and biosimilar rituximab. Peaks 1 and 2: acidic variants; 3: main form; 4, 5, 6 and 7: basic variants.

The high-resolution separation of mAbs facilitated the quantification of charge variants using peak areas. Table 2 summarizes the area percent of charge variants of five consecutive analyses. There was a significant difference in the area percent of the charge variants between two mAbs. The main form in the innovator rituximab was found to be 93.21 % and 29.78 % in biosimilar rituximab. The major charge variant in biosimilar rituximab was 69.46 % basic variants as compared to the innovator product (3.22 %).

Table 2. Charge variants quantification by area %, n = 5.

Innovator – Ristova	RT (min)	Area %
Acidic variant	10.84, 11.21	3.56
Main peak	11.44	93.21
Basic variant	11.9, 12.7	3.22
Biosimilar – Ristova	**RT (min)**	**Area %**
Acidic variant	10.73, 11.22	0.76
Main peak	11.45	29.78
Basic variant	11.87, 12.15, 12.59, 13.1, and 13.77	69.46

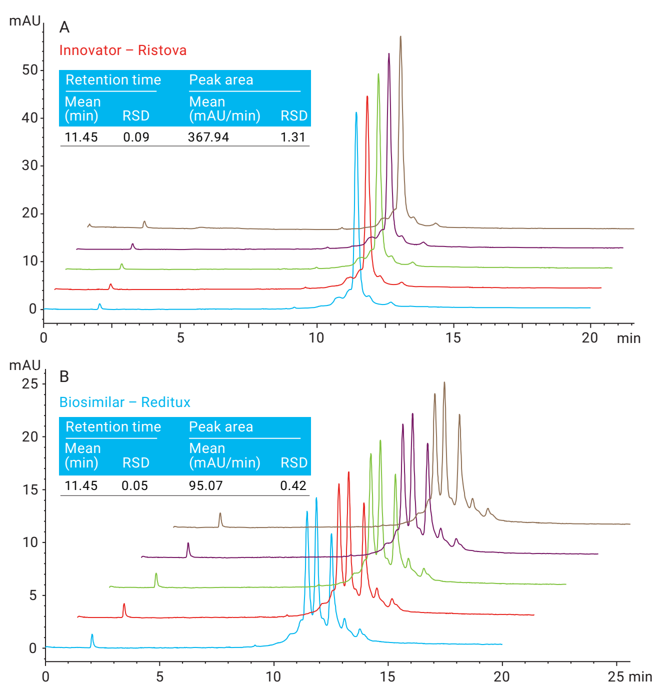

Figure 2. Overlay of five replicates of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column. Insert table shows the precision of retention time and area for main peak, n = 5.

To further characterize the basic variant mAU peaks, both mAbs were subjected to carboxypeptidase B digestion. Figures 3A and 3B show the overlay 50 of the IEX profiles before and after C-terminal cleavage of innovator and biosimilar rituximab, respectively. The disappearance of basic variant peaks after carboxypeptidase B treatment confirmed that the peaks correspond to lysine 20 variants. Figure 4 shows the overlay of the IEX profiles of biosimilar rituximab after CPB treatment and innovator rituximab without CPB treatment, 0 revealing the charge variant similarity between the mAbs.

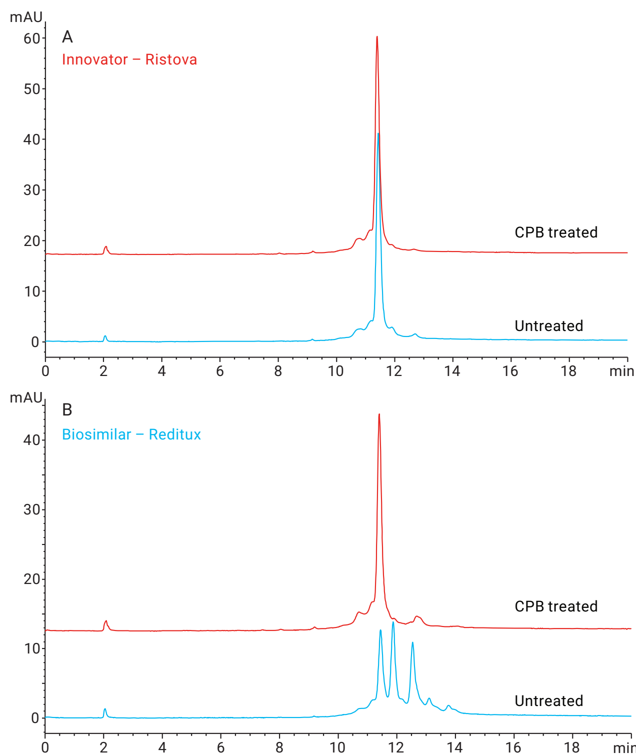

Figure 3. Characterization of basic charge variants. Separation of CPB treated (overnight) and untreated of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column.

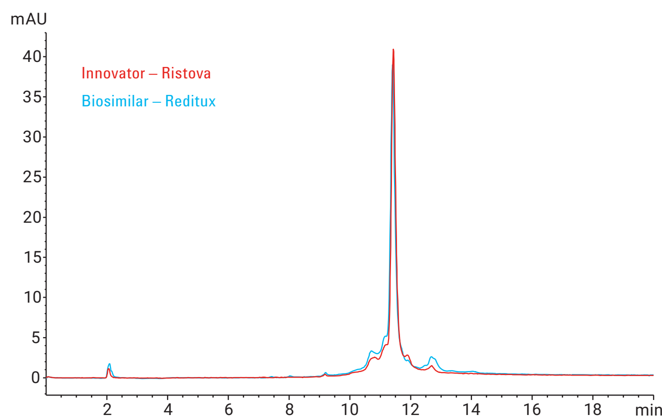

Figure 4. Overlay of innovator rituximab without CPB treatment (red) and biosimilar rituximab after CPB treatment (blue).

Conclusion

The salt-gradient method described in this Application Note demonstrates the high-resolution separation of charge variant profiles of mAbs on an Agilent Bio MAb, 4.6 × 250 mm, 5 µm PEEK column. The innovator and biosimilar rituximab had different separation profiles with different degrees of acidic and basic variants. Carboxypeptidase B digestion confirmed that the major basic variant peaks in biosimilar correspond to lysine variants. The Agilent 1260 Infinity Bio-inert Quaternary LC with Bio MAb PEEK columns and reproducible method make this solution particularly suitable for the QA/QC analysis of mAbs for the biopharmaceutical industry.

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Agilent publication number 5991-0895EN

Agilent publication number 5990-6844EN

Agilent publication number 5991-0565EN

Application Note

Charge Variant Analysis

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Faster Separations Using Agilent Weak Cation Exchange Columns

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Abstract

Ion exchange is a commonly used technique for the separation of complex protein mixtures. Traditionally, such separations are performed using shallow gradients of increasing salt concentration with long column lengths providing the necessary resolution. The columns have often been packed using large diameter particles to minimize backpressure. This Application Note demonstrates how analysis times can be significantly reduced, increasing throughput without compromising analytical performance, by exploiting the benefits of small particle size, non-porous ion exchange sorbents.

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Introduction

Proteins, polypeptides and oligonucleotides are often analyzed by ion exchange chromatography because they are complex molecules with multiple charges on their surfaces. The technique is ideally suited to the separation of charged biomolecules as it is nondenaturing and can provide good performance and resolution.

Traditionally, this has meant using highly porous particles to enable such large molecules to permeate the particles. In turn, columns of 15 cm or 25 cm in length, packed with 5 µm or 10 µm particles are commonly used.

The advent of non-porous sorbents such as Agilent's Bio IEX range, comprising a rigid polymeric core particle with a grafted hydrophilic layer containing the ion-exchange functionality, can improve resolution. This is because the diffusion-limited band broadening associated with a molecule penetrating the core of a large particle is eliminated. In turn, this means smaller particles and shorter column lengths can be used to significantly improve throughput, greatly reducing analysis times. The benefits for improved productivity for tasks such as fraction analysis are immediately evident.

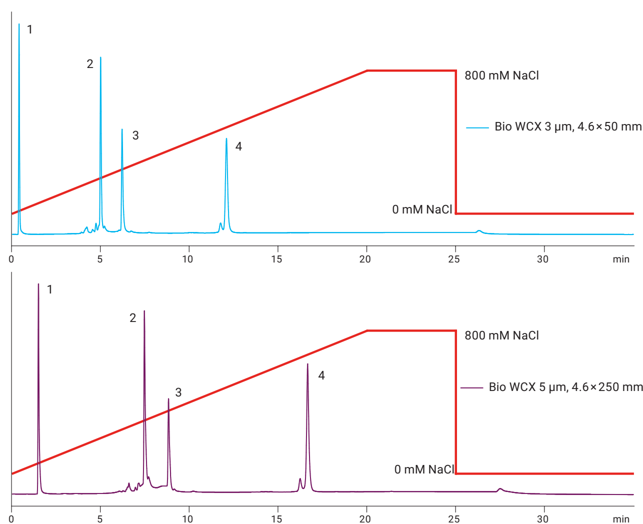

Figure 1. Protein separation on Agilent Bio WCX 5 µm 4.6 × 250 mm versus Agilent Bio WCX 3 µm, 4.6 × 50 mm (flow rate 1.0 mL/min).

Materials and methods

Agilent Bio IEX columns are packed with rigid polymeric, nonporous particles grafted with a functionalized hydrophilic polymer layer. The resultant 1.7, 3, and 5 µm rigid particles provide high resolution and high separation efficiency by reducing the band broadening effects resulting from diffusion limitations with totally porous particles. The chemically bonded hydrophilic coating significantly reduces the effects of nonspecific binding and results in greater levels of recovery.

Conditions, Bio-Monolith column

Parameter	Value
Columns:	Agilent Bio WCX 5 µm, 4.6 × 250 mm SS (p/n5190-2445) Agilent Bio WCX 3 µm, 4.6 × 50 mm SS (p/n5190-2443) Agilent Bio WCX 1.7 µm, 4.6 × 50 mm SS (p/n5190-2441)
Sample:	Ovalbumin (1), Ribonuclease A (2), Cytochrome c (3), Lysozyme (4)
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 20 mM sodium phosphate, pH 6.5 B: A + 1.6 M NaCl
Gradient:	0 to 50 % B
Temp.:	Ambient
Injection volume:	10 µL
Conc.:	0.5 mg/mL
Detection:	UV, 220 nm

Results and Discussion

The performance of a column, as measured by plate count, is dependent on particle size and column length. From this it may be inferred that a shorter column packed with smaller particles can be used to achieve the same level of performance when compared to a longer column packed with larger particles (Figure 1). This is commonly found in practice. However, for gradient elution, further modifications to the method need to be employed to provide the additional benefits of shorter run times and greater productivity.

Converting gradient times into column volumes is a useful way of calculating the shorter gradient program and can provide the desired outcome in terms of higher speed separations (Table 1). However, smaller particle sizes may require higher flow rates to attain maximum performance. This is illustrated by the van Deemter curves shown in Figure 2.

To maximize the separation efficiency using the Agilent Bio WCX 3 µm, 4.6 × 50 mm column, the 4 minute gradient separation was carried out at 1.0, 1.5, 2.0, and 2.5 mL/min (Figure 3). As expected, the higher linear velocity created from higher flow rates improved the peak shape.

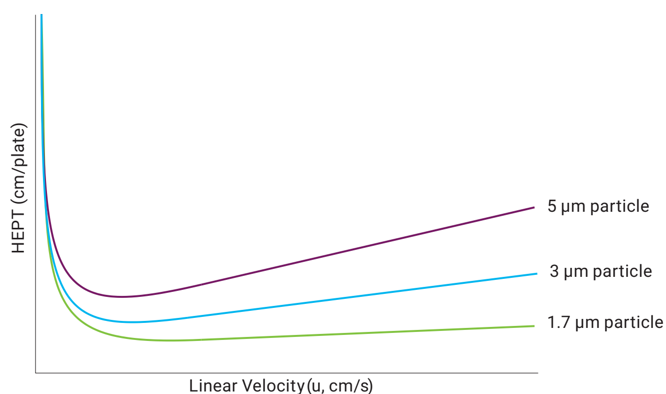

Figure 2. Typical van Deemter curves.

Table 1. Gradient Time to Column Volume Conversion

Time (minutes)	mM NaC	#CV
0	0	0.0
20	800	4.8
25	800	6.0
25.01	0	6.0
35	0	8.4

#CV = number of column volumes at 1.0 mL/min (4.6 Å~ 250 mm column)

Time (minutes)	mM NaCl	#CV
0	0	0.0
4	800	4.8
5	800	6.0
5.01	0	6.0
7	0	8.4

#CV = number of column volumes at 1.0 mL/min (4.6 Å~ 50 mm column)

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In comparison, the Agilent Bio WCX 1.7 µm, 4.6 × 50 mm column provided sharper peaks under identical conditions (Figure 4).

Increasing the flow rate should mean that it is possible to further reduce the gradient time. This was investigated using the Bio WCX 1.7 µm, 4.6 × 50 mm column. The 0 to 800 mM NaCl gradient was reduced from 4 to 2 minutes.

It was found that at a flow rate of 1.7 mL/min the backpressure remained below 400 bar and still provided exceptional peak shape and resolution (Figure 5).

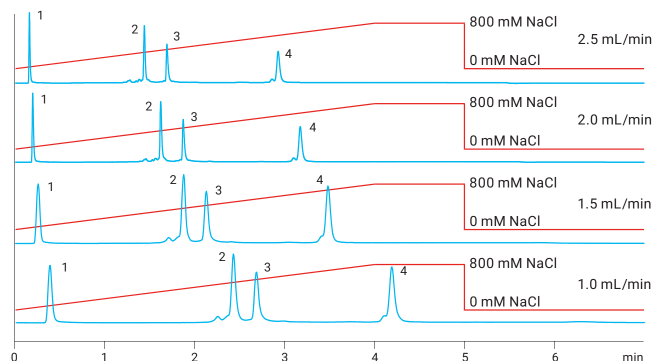

Figure 3. Effect of flow rate on chromatographic performance (Agilent Bio WCX 3 µm, 4.6 × 50 mm).

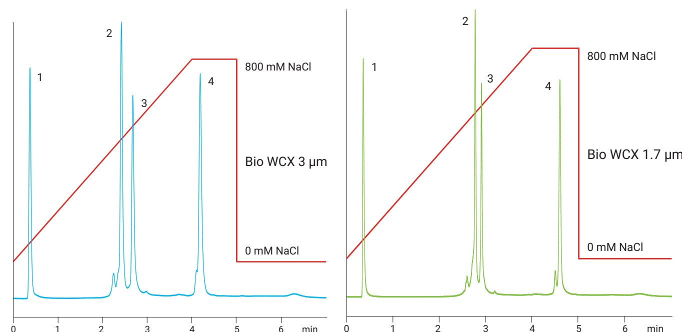

Figure 4. Comparison of Agilent Bio WCX 3 µm, 4.6 × 50 mm versus Agilent Bio WCX 1.7 µm

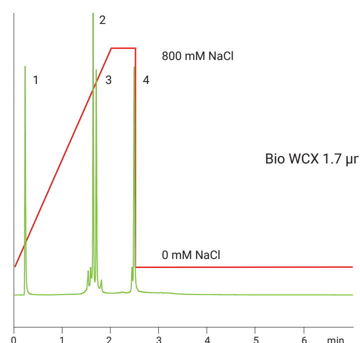

Figure 5. Agilent Bio WCX 1.7 µm, 4.6 × 50 mm for protein separations under 3 minutes (flow rate 1.7 mL/min)

Conclusions

We have shown that by using shorter 5 cm columns packed with smaller particle size (3 µm and 1.7 µm), Agilent Bio WCX products can lead to significant reductions in run times from 20 or 30 minutes down to less than 3 minutes, and still retain excellent peak resolution. This enables much higher throughput in time-critical applications.

The backpressure of 400 bar shows that, by reducing the analysis time dramatically from over 30 minutes to less than four minutes for the entire gradient, a 600 bar system such as the Agilent 1260 Infinity Bio-inert LC is still sufficient.

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Application Note

Charge Variant Analysis

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Optimizing Protein Separations with Cation Exchange Chromatography Using Agilent Buffer Advisor

Protein separation with the Agilent 1260 Infinity Bio-inert Quaternary LC System

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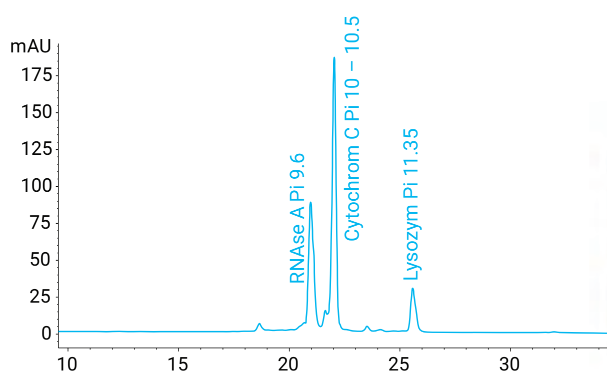
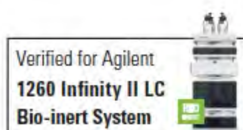
Abstract

This Application Note shows that the Agilent Buffer Advisor software in combination with the Agilent 1260 Infinity Bio-inert Quaternary LC System is an ideal solution for automated protein separation by ionic strength gradients. Usually, pH scouting using premixed two-component gradients is time-consuming and work-intensive. Dynamically mixed four-component gradients calculated by the Buffer Advisor software shorten and simplify the workflow for pH scouting. In addition, excellent retention time precision and pH consistency were gained using the gradients calculated by the Buffer Advisor software.





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Introduction

Proteins consist of many different amino acids comprising weak acidic (carboxylic) and basic (amine) groups. Therefore, proteins are amphoteric molecules that exist mostly as zwitterions in a certain pH range. The pH where the protein has no net charge and does not interact with a charged medium is the isoelectric point (pI). In ion exchange chromatography (IEX), the unique relationship between net surface and pH can be used for optimal protein separation. The pH defines the number of charges on the protein and also helps to stabilize the native structure of the protein in the buffer used during analysis.

To ensure optimal binding and elution characteristics of proteins of interest to the IEX column, pH and ionic strength of the deployed buffer are important factors. Even small changes in these two parameters can affect the separation. As a consequence, pH scouting is an important method to find the optimal separating conditions when working with ionic strength gradients. In contrast to pH-gradients, the pH is kept constant in ionic strength gradients. By increasing the ionic strength (salt concentration) of the mobile phase, the less strongly bound proteins are eluted earlier than the stronger bound proteins.

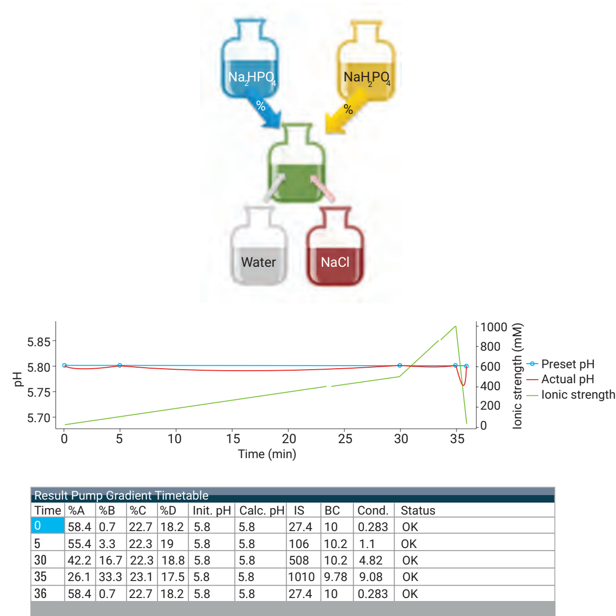
In general, a premixed two-component gradient is prepared for analysis with a starting buffer of low ionic strength and an elution buffer containing high ionic strength. This includes the following preparation steps:

- Dissolving the appropriate buffering compounds at defined concentration
- Titrating the pH with acid/base to the desired pH of the mobile phase
- Splitting the buffer and adding salt to one portion (elution buffer)
- Titrating the pH of the elution buffer with acid/base to the desired pH, if necessary

To perform pH scouting using premixed two-component gradients, prepare different bottles of buffer. To test, for example, six different pH values, it is necessary to prepare 12 bottles of premixed buffer. In contrast, with dynamically mixed four-component gradients, it is necessary to prepare only four bottles to generate various pH values. Further, dynamical mixing of a buffer eliminates the necessity to titrate the buffer solutions manually, which is typically time-consuming and errors prone. The application of dynamically mixed four-component gradients simplifies method development and reduces the time needed for buffer preparation to a large extent by just providing four bottles with stock solutions:

- Line A: Water
- Line B: Salt solution
- Line C: Acidic buffer component
- Line D: Basic buffer component

Using the four stock solutions, different buffers at different pH and salt concentration can be prepared. The Buffer Advisor software is a helpful tool to calculate the percentages of the stock solutions in order to achieve the desired pH, buffer concentration, and ionic strength (Figure 1).



Result Pump Gradient Timetable

Time	%A	%B	%C	%D	Init. pH	Calc. pH	IS	BC	Cond.	Status
0	58.4	0.7	22.7	18.2	5.8	5.8	27.4	10	0.283	OK
5	55.4	3.3	22.3	19	5.8	5.8	106	10.2	1.1	OK
30	42.2	16.7	22.3	18.8	5.8	5.8	508	10.2	4.82	OK
35	26.1	33.3	23.1	17.5	5.8	5.8	1010	9.78	9.08	OK
36	58.4	0.7	22.7	18.2	5.8	5.8	27.4	10	0.283	OK

Figure 1. Quaternary mixing to create a salt gradient with constant pH.

The Buffer Advisor software generates a timetable, which can be imported into the method of the 1260 Infinity Bio-inert Quaternary LC Pump using the Import Solvent Blending File function of the Agilent OpenLAB CDS ChemStation Edition software (Figure 2).

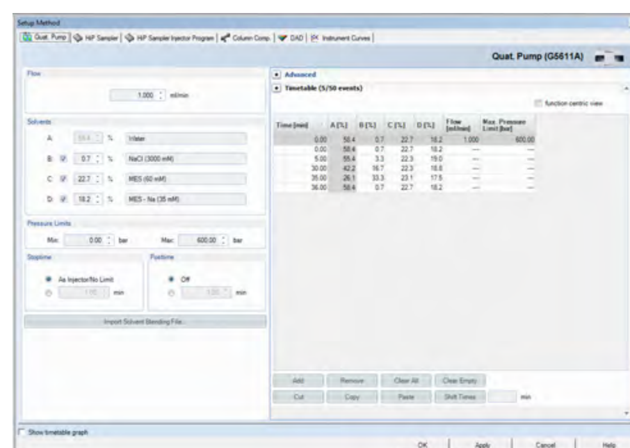


Figure 2. The generated Timetable can be imported into the method of the Agilent 1260 Infinity Bio-inert LC System through the Agilent OpenLAB CDS ChemStation Edition software.

The Buffer Advisor software can be applied for ionic strength or pH gradients in anion or cation exchange chromatography. The software provides a wide choice of different selectable buffers for single buffer (ionic strength gradients) or for composite buffer (pH gradients) applications. Depending on the proteins of interest and the used columns, the user can select buffers either for anion or cation exchange (Figure 3). To ensure optimal buffering capacity, recommended pH ranges and concentrations in which the buffers should be used are displayed.

A common issue in ion exchange chromatography with ionic strength gradients is the decrease in pH as an effect of added neutral salt like NaCl1,2. The Buffer Advisor software counteracts this issue by recalculation of the overall mobile phase composition considering the concentration of acidic and basic buffer (Line C and D) to maintain the desired constant pH. In addition, if the pH deviation gets too large, the Buffer Advisor software automatically inserts additional time points into the pump timetable.

Figure 3. Buffer list for cation exchange chromatography, sorted by recommended pH range.

Experimental

Instrumentation

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) with bio-inert solvent heat exchangers
- Agilent 1260 Infinity Diode Array Detector VL (G1315D with bio-inert standard flow cell, 10 mm)
- Agilent 1260 Infinity Bio-inert Analytical-scale Fraction Collector (G5664A)

Column

- Agilent Bio MAb Column, PEEK, 4.6 × 250 mm, 5 µm

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.03 [32]
- Agilent Buffer Advisor, Rev. A.01.01

Solvents

Buffer A: H_2O

Buffer B: NaCl 3 M

Buffer C: MES (2-(N-morpholino) ethanesulfonic acid monohydrate) 60 mM

Buffer D: MES-Na (2-(N-morpholino) ethanesulfonic acid sodium salt) 35 mM

Sample

Mix of three proteins, solved in PBS (phosphate buffered saline), pH 7.4

Ribonuclease A: 13,700 Da pI 9.6

Cytochrom C: 1 2,384 Da pI 10–10.5

Lysozyme: 14,307 Da pI 11.35

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). MES (2-(N-morpholino)ethanesulfonic acid monohydrate) and MES-Na (2(N-morpholino)ethanesulfonic acid sodium salt) were purchased from Merck, Darmstadt, Germany. NaCl was purchased from VWR, Radnor, PA, USA.

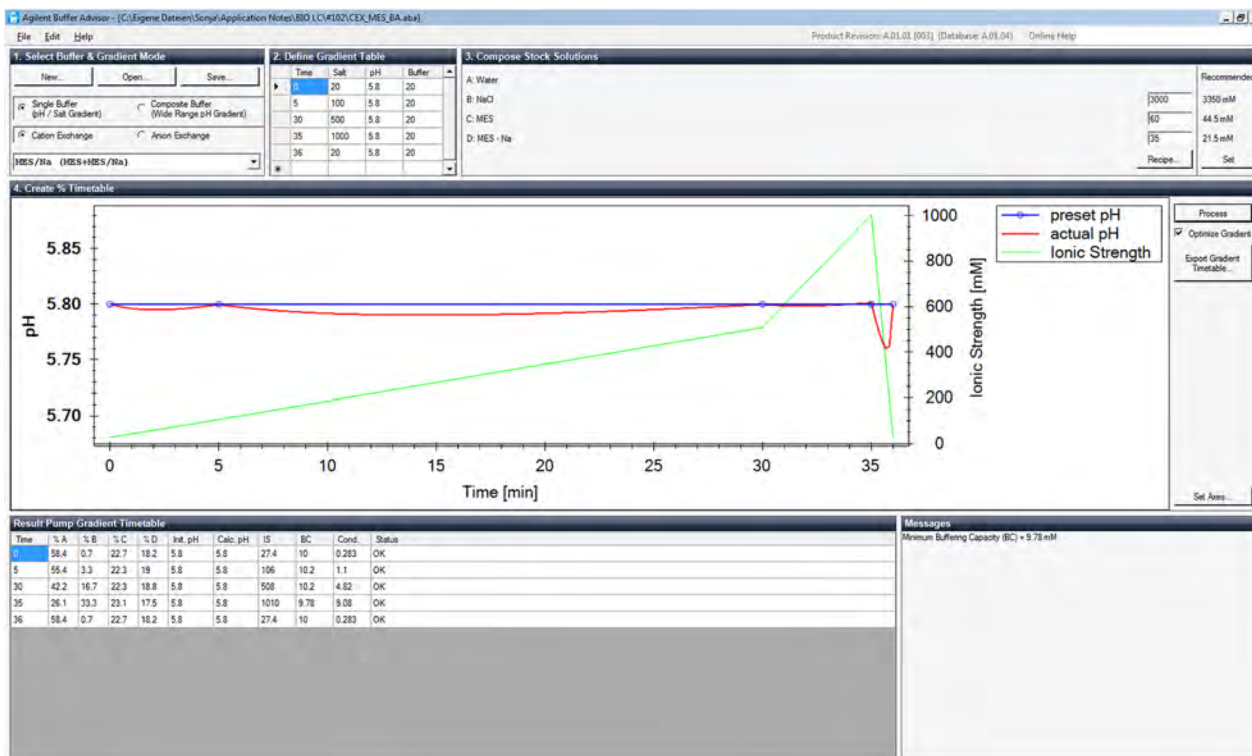


Figure 4. Agilent Buffer Advisor software, showing the steps described in "Results and discussion".

Conditions

Parameter	Value
Flow rate:	1 mL/min
Gradient:	0 min – 20 mM NaCl 5 min – 20 mM NaCl 30 min – 500 mM NaCl 35 min – 1000 mM NaCl 36 min – 20 mM NaCl
Injection volume:	10 µL
Thermostat:	4 °C
Temperature TCC:	25 °C
DAD:	280 nm/4 nm
Ref.:	Off
Peak width:	> 0.05 min (1.0 s response time)(5 Hz)

Stock Solution Recipes

Bottle B	NaCl: Sodium chloride	Weight 175.32 g and fill up to 1 L
Bottle C	MES: MES	Weight 11.714 g and fill up to 1 L
Bottle D	MES - Na: MES sodium salt	Weight 7.6027 g and fill up to 1 L

Help... Print Preview... OK Cancel

Figure 5. Stock solution recipes

Results and discussion

pH scouting was performed using calculations from the Agilent Buffer Advisor software for pH values from 5.8 to 6.8. A mix of three proteins (ribonuclease A, cytochrome C and lysozyme) was separated using a four-component salt gradient at six different pH values. Dynamically mixed four-component gradients were generated using the calculations from the software. The Buffer Advisor software simplifies the generation of different four-component gradients by calculating the percentage of the individual stock solutions in the mobile phase at defined time points (Figure 4).

1. After definition of the gradient parameters, such as time, maximum salt concentration, pH, and buffer concentration
2. The Buffer Advisor software calculates the needed stock concentrations. The Recipe button displays the absolute amount of needed chemicals for the preparation of the stock solutions (Figure 5). These proposed stock concentrations can be adjusted by the user.
3. Select the Process tab.
4. The Buffer Advisor software calculates the needed amount of each channel to maintain the correct pH during the complete chromatographic run. Furthermore, it calculates whether the pH, salt concentration and buffer concentration entered is suitable for the buffer system that was selected. The timetable displays also additional data, such as buffering capacity of the mobile phase.

The pH scouting for the three-protein mix of ribonuclease A (A), cytochrome C (B) and lysozyme (C) demonstrates the benefits of the Agilent Buffer Advisor software (Figure 6). Even small pH changes of 0.2 have a strong influence on the retention of the proteins on the weak cation exchange (WCX) column. Changes in the elution order become obvious when the pH is changed from 5.8 to 6.8.

Manual preparation of corresponding buffers for premixed two-component gradients includes several steps. For each pH and for each prepared bottle (one with low and one with high ionic strength), a manual titration of the buffers is necessary. pH scouting for six different pH values in order to achieve the optimal resolution results in preparation of 12 solvent bottles (including weighing chemicals, pH adjustment). This is a very time-consuming procedure and highly prone to error and variation.

In contrast, the Buffer Advisor software is capable of automatically and reproducibly mixing all six separation conditions out of four stock solutions without any manual interference. The optimal resolution was achieved at pH 5.8 (Figure 6).

Based on the results, the user has various options on how to proceed:

1. Fine-tuning of the resulting pH values and gradients
2. Transfer of the dynamically mixed four-component gradient to other instruments through the OpenLAB CDS ChemStation timetable
3. Implementation of pH scouting results into two-component gradients using premixed buffers

In the last case, however, deviations from correct pH are expected, due to the pH optimization procedure of the Buffer Advisor software.

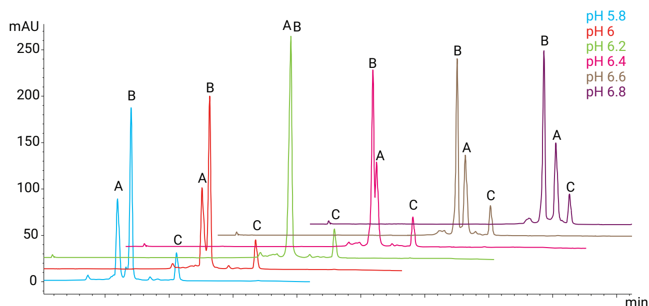


Figure 6. pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients.

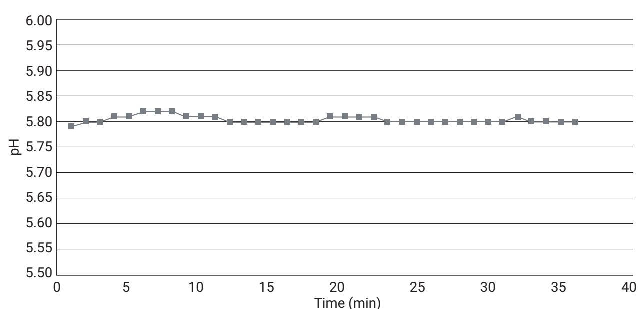


Figure 7. Off line pH measurement

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Conclusions

Using dynamically mixed four-component gradients, calculated by the Buffer Advisor software, shortens and simplifies the workflow for pH scouting. The employment of dynamically mixed gradients calculated with the Buffer Advisor software results in a significant decrease in buffer preparation time, particularly when compared to manual preparation of buffers for premixed two-component gradients. The Buffer Advisor software provides a wide range of prevalidated, user-selectable buffer systems for anion and cation exchange chromatography and delivers recipes for preparation of the most suitable stock solutions. Due to pH optimization of the software, resulting pH values are more accurate and precise than those resulting from premixed gradients formed out of manually prepared buffer solutions. The Buffer Advisor software counteracts this issue by the recalculation of the four-component gradient regarding the concentration of acidic and basic buffer to maintain the desired constant pH.

The Buffer Advisor software in combination with the Agilent 1260 Infinity Bio-inert Quaternary LC System is excellent for generating four-component gradients. The calculations of Buffer Advisor software lead to exact and reproducible protein analysis while providing an excellent tool for automated pH scouting and accurate ion exchange chromatography. The Buffer Advisor software is, therefore, an ideal tool for automatic development of analytical methods in ion-exchange chromatography, which can be seamlessly transferred to the corresponding QA/QC departments.

References

1. R. J. C. Brown & M. J. T. Milton. Observation of a combined dilution and salting effect in buffers under conditions of high dilution and high ionic strength, *Accred Qual Assur* 8(11): 505-510, **2003**.
2. A. E. Voinescu et al. Similarity of Salt Infl uences on the pH of Buffers, Polyelectrolytes, and Proteins, *J. Phys. Chem.* B 110: 8870-8876, **2006**.

Application Note

Charge Variant Analysis

Agilent
Trusted Answers

Fully Automated Characterization of Monoclonal Antibody Charge Variants Using 4D-LC/MS

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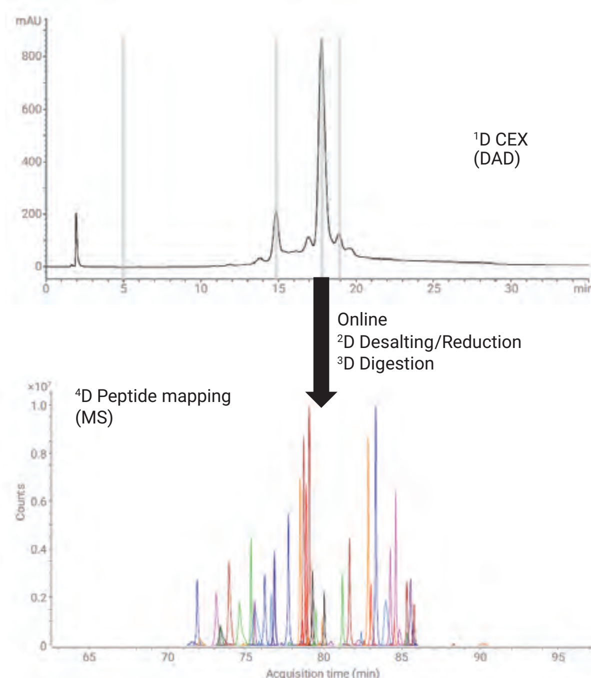
Abstract

This application note describes the fully automated and in-depth characterization of monoclonal antibody (mAb) charge variants by four-dimensional liquid chromatography/mass spectrometry (4D-LC/MS) using the Agilent InfinityLab 2D-LC Solution and the Agilent 6545 LC/Q-TOF system. Charge variants resolved by cation-exchange chromatography (CEX) are collected in loops installed on a multiple heart-cutting valve and consecutively subjected to online desalting, denaturation, reduction, and tryptic digestion prior to LC/MS-based peptide mapping.



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Introduction

Protein biopharmaceuticals have emerged as important therapeutics for the treatment of various diseases including cancer, cardiovascular diseases, diabetes, infection, inflammatory, and autoimmune disorders.[1-3] Protein biopharmaceuticals come in many flavors and include monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), fusion proteins, hormones, growth factors, cytokines, therapeutic enzymes, blood factors, vaccines, and anticoagulants. Given their obvious benefits in terms of safety and efficacy, these molecules have substantially reshaped the pharmaceutical market, and today, over 350 products have been approved for human use in the United States and the European Union.[1-3] This represents approximately one quarter of the total pharmaceutical market, with mAbs being the fastest growing class of pharmaceuticals.

Together with a huge therapeutic potential, these molecules come with an enormous, analytically demanding structural complexity.[1,2] In contrast to small molecule drugs, biopharmaceuticals are large (mAbs have an MW of approximately 150 kDa) and heterogeneous. They are the product of one or a couple of genes. However, hundreds of possible variants that differ in post-translational modifications (PTMs), amino acid sequence, higher-order structure, etc. may coexist, all making up the profile, safety, and efficacy of the product.[1-3] Consequently, their in-depth structural characterization involves a significant number of analytical tools, with chromatography (LC) and mass spectrometry (MS) at the forefront.

A key technology to study charge variants that might arise from PTMs such as asparagine deamidation, C-terminal lysine truncation, N-terminal cyclization (pyroglutamate formation), sialylation, etc. is CEX. In CEX, electrostatic interaction between the anionic groups of the stationary phase and cationic groups on the protein surface form the basis of the separation. The protein is loaded on the column at a mobile phase pH below its isoelectric point (pI), and elution is achieved using a salt or pH gradient. CEX buffers are typically composed of nonvolatile constituents, making these methods incompatible with MS. Peak identification is a laborious task involving peak collection and desalting prior to MS analysis.[4] With the recent introduction of commercial and robust ^{2}D-LC instrumentation, this series of events is now commonly performed in an online automated manner.[5-9] Peaks eluting from the CEX column are stored in loops and subjected to online desalting using reversed-phase (RPLC) or size exclusion chromatography (SEC) prior to MS measurement. Both comprehensive (LC×LC) and (multiple) heart-cutting ^{2}D-LC (LC-LC) have been used.[5-9]

To unambiguously identify CEX peaks, however, peptide mapping is required. While protein measurement is indicative of identity and highlights dominant modifications with mass differences beyond the mass accuracy of the MS instrument, it typically does not provide the actual amino acid sequence, nor does it allow us to localize modifications. Addressing the latter, and inspired by previous work,[10-12] the current application note describes a fully automated online ^{4}D-LC/MS setup incorporating first dimension (^{1}D) CEX, peak collection, ^{2}D desalting, denaturation, reduction, ^{3}D trypsin digestion, and ^{4}D RPLC/MS-based peptide mapping for the in-depth characterization of mAb charge variants.

Experimental

Materials

Acetonitrile (HPLC-S), water (ULC/MS), and formic acid (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands). NaH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$, NaCl, NH_4HCO_3, Tris base, and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). UltraPure Tris-HCl pH 7.5 was purchased from ThermoFisher Scientific (Waltham, MA, USA). Type I water was produced from tap water by an arium pro Ultrapure Lab Water System from Sartorius (Göttingen, Germany). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland).

Sample preparation

Trastuzumab was diluted to 7 mg/mL in ^{1}D CEX mobile phase A (MPA: 10 mM sodium phosphate pH 7.65). Deamidation was induced by incubating trastuzumab at 37 °C for 3 days in high pH conditions (100 mM Tris pH 9.0) and subsequently buffer-exchanged to 7 mg/mL in ^{1}D CEX mobile phase A.

Instrumentation

An Agilent 1290 Infinity II 2D-LC system equipped with the multiple heart-cutting option, an additional Agilent 1260 Infinity II quaternary pump and Agilent 1260 Infinity II isocratic pump, two additional 2-position/6-port valves, and a zero dead volume T-piece were used. Stainless steel tubing with an internal diameter of 0.12 mm was applied. The configuration is schematically represented in Figure 1 and summarized in this application note. Diode array detection (DAD) was used in the first (CEX) and fourth dimension (RPLC). Additionally, an Agilent 6545 LC/Q-TOF with a Jet Stream ESI source was used for detection after the fourth and final dimension.

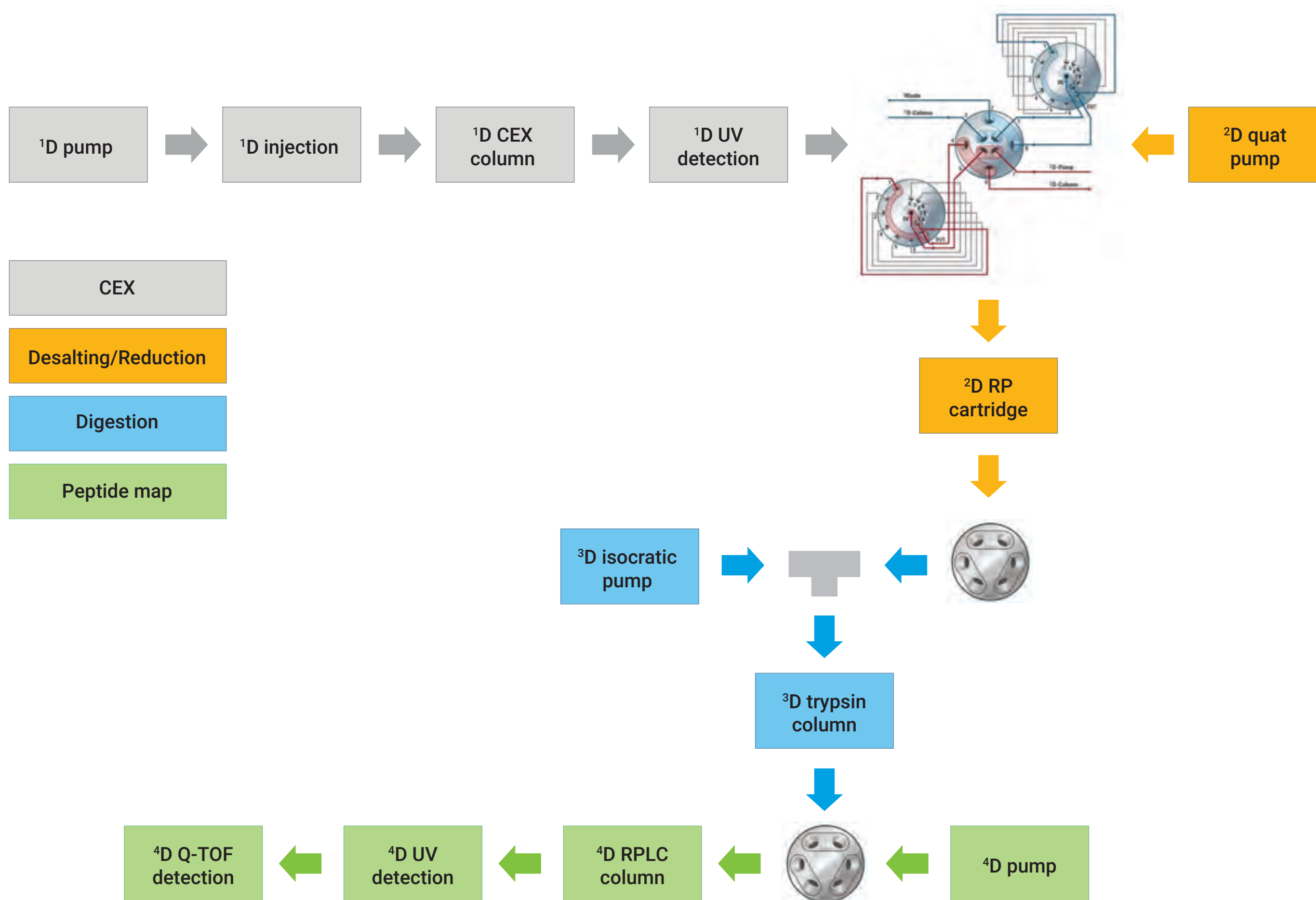

Figure 1. 4D-LC/MS configuration incorporating 1D CEX separation and charge variant peak collection using multiple heart-cutting, 2D RPLC-based desalting, denaturation, reduction, 3D trypsin digestion, and 4D RPLC-MS based peptide mapping.

Configuration

^{1}D: Cation-exchange chromatography:

- G7120A Agilent 1290 Infinity II high-speed pump
- G7167B Agilent 1290 Infinity II multisampler with sample thermostat (option 101)
- G7116B Agilent 1290 Infinity II multicolumn thermostat (MCT) with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)
- G7117B Agilent 1290 Infinity II diode array detector with a 3.7 mm HDR InfinityLab Max-Light cartridge cell (G4212-60032)

Note: A short 3.7 mm detector flow cell was installed to reduce the signal intensity and prevent saturation of the UV signal

2D-LC with multiple heart-cutting

- Agilent 1290 Infinity valve drive (G1170A) with 2D-LC valve (G4236A)
- Two Agilent 1290 Infinity valve drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops

^{2}D: Reversed-phase chromatography for desalting, denaturation, and reduction:

G7111B Agilent 1260 Infinity II quaternary pump with active inlet valve (AIV) (option 032)

^{3}D: Trypsin digestion

- G7111B Agilent 1260 Infinity II quaternary pump with active inlet valve (AIV) (option 032)

^{4}D: Reversed-phase chromatography for peptide mapping:

- G7120A Agilent 1290 Infinity II high-speed pump
- G7116B Agilent 1290 Infinity II MCT with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)
- G7117B Agilent 1290 Infinity II DAD with a 10 mm InfinityLab Max-Light cartridge cell (G4212-60008)
- G6545A Agilent 6545 LC/Q-TOF with Jet Stream ESI source

Note: Orachrom StyrosZyme TPCK-Trypsin and Agilent AdvanceBio peptide mapping columns are both contained in different zones of one single column compartment and maintained at 40 and 60 °C, respectively.

Software

- Agilent OpenLab CDS ChemStation revision C.01.07 SR4 [505]
- 2D-LC add-on software revision A.01.04 [017]
- Agilent MassHunter for instrument control (B.09.00)
- Agilent MassHunter with BioConfirm add-on for data analysis (B.07.00)

Method

^{1}D and ^{4}D were configured in the 2D-LC software, while ^{2}D and ^{3}D were controlled in a regular method setup and were programmed as repetitive events. The cycle time of these events was 110 minutes, identical to the ^{4}D cycle time programmed in the 2D-LC software. MassHunter acquisition was triggered by a remote start from the 2D-LC system.

Four heart-cuts were taken across the CEX analysis. The first heart-cut at 4.8 minutes is a blank cut, which enables preconditioning of all dimensions before the analysis of the actual CEX heart-cuts of interest.

^{1}D: Cation-Exchange Chromatography			
Column	Agilent Bio MAb, nonporous (2.1 mm × 250 mm, 5 µm) (p/n 5190-2411)		
Temperature	30 °C		
Mobile phase A	10 mM sodium phosphate, pH 7.65		
Mobile phase B	10 mM sodium phosphate, pH 7.65 + 100 mM NaCl		
Flow rate	0.2 mL/min		
Gradient	**Time (min)**	**B (%)**	
	0	5	**CEX analysis**
	36	70	
	36.5	100	
	46	100	
	46.5	5	
	60	5	
Injection	100 µg		
Detection	220 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		

Multiple Heart-Cutting ^{1}D > ^{2}D	
Sampling Timetable Trastuzumab	
Cut	**Time (min)**
1 – Blank	4.80
2 – Pre-peak	14.93
3 – Main Peak	17.55
4 – Post-peak	18.99

^{2}D: Reversed-Phase Chromatography for Desalting, Denaturation, and Reduction (Manually Entered Repetitive Event)						
Column	Polymer-based desalting cartridge, 2.1 × 10 mm					
Temperature	23 °C					
Mobile Phase A	0.1% (v/v) formic acid in water					
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile					
Mobile Phase C	20 mM DTT in 100 mM Tris-HCl, pH 7.5					
Gradient	**Time (min)**	**A (%)**	**B (%)**	**C (%)**	**Flow (mL/min)**	
	10	99	1	0	0.5	**Desalting and focusing**
	10.01	0	0	100	0.2	**Reduction**
	20	0	0	100	0.2	
	20.01	99	1	0	0.5	
	25	99	1	0	---	**Desalting and elution**
	25.01	40	60	0	---	
	27	40	60	0	0.5	
	27.01	40	60	0	0.015	**Elution and digestion**
	68	40	60	0	0.015	
	68.01	0	100	0	0.5	
	85	0	100	0	---	
	95	99	1	0	---	
	120	99	1	0	---	
Valve	27 min: Pos 1 & Pos 2 (start trypsin digestion) 67 min: Pos 2 & Pos 1 (start peptide mapping)					

[3]D: Trypsin Digestion (Manually Entered Repetitive Event)			
Column	Orachrom StyrosZyme TPCK-Trypsin PEEK (2.1 × 150 mm)		
Temperature	40 °C		
Mobile Phase	50 mM NH_4HCO_3, pH 8		
Gradient	**Time (min)**	**Flow (mL/min)**	
	25	0.06	
	25.01	0.135	**Digestion**
	67	0.135	
	67.01	0.06	
	135	0.06	
Valve	27 min: Pos 1 -> Pos 2 (start trypsin digestion) 67 min: Pos 2 -> Pos 1 (start peptide mapping)		

Data processing

Measured signals were matched onto the trastuzumab light- and heavy-chain sequences using the BioConfirm algorithm incorporated in the MassHunter software. Mass tolerance for matching experimental data onto the sequence was set at 8 ppm. Extracted ion chromatograms (EICs) obtained at 20 ppm mass accuracy were used to monitor PTMs such as deamidation.

Results and Discussion

A scheme of the fully automated online 4D-LC/MS protein analyzer, incorporating CEX, peak collection, desalting, denaturation, reduction, trypsin digestion, and peptide mapping, is shown in Figure 1. CEX peaks are collected in 40 µL loops installed on a multiple heart-cutting valve and transferred one by one to a polymeric RP cartridge where desalting, denaturation, and reduction take place. The reduced mAb, trapped on the cartridge, is subsequently eluted into the trypsin column by raising the acetonitrile concentration. Using a T-piece, trypsin digestion buffer is mixed with the reversed-phase mobile phase to have optimal digestion conditions and to reduce the acetonitrile concentration.

[4]D: Reversed-Phase Chromatography for Peptide Mapping (Repetitive Event Controlled by 2D-LC Software)			
Column	Agilent AdvanceBio peptide mapping (2.1 × 150 mm × 2.7 µm) (p/n 651750-902)		
Temperature	60 °C		
Mobile Phase A	0.1% (v/v) formic acid in water		
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile		
Flow Rate	0.4 mL/min		
Gradient	**Time (min)**	**B (%)**	
	0	1	
	8.5	1	
	9	100	
	15	100	
	16	1	
	20	1	**Load digest on peptide mapping column**
	64	1	
	64	1	**Peptide mapping**
	97	45	
	98	100	
	103	100	
	104	1	
	110	1	
DAD Detection	214 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		

MS Detection	
Parameter	**Source**
Positive Ionization	
Drying Gas Temperature	300 °C
Drying Gas Flow	8 L/min
Nebulizer Pressure	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	8 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	1,000 V
Fragmentor	175 V

Parameter	Acquisition
Mode	Extended dynamic range (2 GHz)
Data Acquisition Range	*m/z* 100 to 3,200
	1 spectrum/s
	Centroid acquisition
Switch diverter valve to MS after 67 minutes	

During the digestion, the trypsin column is in-line with the peptide mapping column, and generated peptides are focused at the head of the RPLC column. After 20 minutes, a valve switch initiates the elution of the digest into the MS.

The CEX chromatogram of the mAb trastuzumab is shown in Figure 2. Trastuzumab, commercialized as Herceptin, is a humanized IgG1 binding the HER2 receptor, thereby finding use in the treatment of HER2 positive metastatic breast cancer. With a pI of 8.45, the mAb is positively charged at the CEX mobile phase pH, thereby governing interaction with the negatively charged chromatographic resin. Upon eluting the mAb using a NaCl salt gradient, various charge variants were revealed, which were subsequently subjected to online peptide mapping. Figure 3A schematically presents the 4D-LC/MS experiment involving the analysis of three CEX peaks (pre-, main-, and post-peaks) and a CEX blank as shown in Figure 2. Figure 3B zooms in on the pressure and DAD profiles of one cycle and shows the desalting, denaturation, reduction, digestion, and peptide mapping of the main CEX peak. Over 90% sequence coverage could be obtained. Peptides identified are shown in Table 1, and an overlay of the LC/MS compound chromatograms is provided in Figure 4. Peptides not covered are typically small and/or hydrophilic and are not focused at the head of the peptide mapping column during the digestion. For this reason, they are diverted to waste.

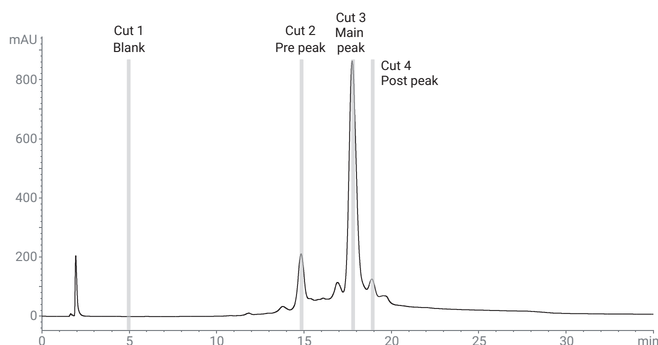

Figure 2. CEX chromatogram of the monoclonal antibody trastuzumab. Conditions according to reference 13. Heart-cuts taken are indicated in gray.

Next to sequence information, peptide mapping also reveals modifications and modification sites. Figure 5 shows the online peptide mapping of the CEX pre-, main-, and post-peak, and in particular the EICs of two peptides (i.e., light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42) containing a potential deamidation site at position 30, and heavy-chain peptide WGGDGFYAMDYWGQGTLVTVSSASTK (HC 99–124) containing a potential isomerization site at position 102). From the data, it could be deduced that the pre-peak corresponds to a deamidated variant, with asparagine converted to aspartate at position 30 on one of the light chains. It could also be demonstrated that the post-peak carries an isoaspartate at position 102 on one of the heavy chains. This is clearly visualized by the peak doublets corresponding to the modified and nonmodified variants. These results are in accordance with those reported by Harris et al., who performed offline fraction collection and peptide mapping on Herceptin acidic and basic variants.4

The same experiment was performed on a high-pH stressed Herceptin sample (Figure 6). Such conditions are known to induce deamidation, thereby rendering the mAb more acidic. The CEX profile presented in Figure 6 shows an acidic shift, and the peptide map data of CEX peaks 1 and 2 show a double deamidated variant, with both light chains deamidated at position 30. CEX peaks 3 and 4 correspond to a single deamidation event, with one light chain deamidated at position 30. The difference between peaks 1 and 2, and 3 and 4 originate from another deamidation, this time at position 387 in the heavy chain. This deamidation site appears in two peptides (one fully cleaved and one miscleaved) that are apparently digested differently when a deamidation exists.

Figure 3. (A) Schematic representation of the different stages of the 4D-LC/MS experiment focusing on the pre-, main-, and post-peak as well as a CEX blank region as shown in Figure 2. (B) Focus on desalting, denaturation, reduction, digestion, and peptide mapping of the main CEX peak.

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Table 1. Peptides identified in the CEX main peak following online RPLC/MS-based peptide mapping.

RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
79.1	1880.9972	66635668	6.61	EVQLVESGGGLVQPGGSLR	HC(001-019)	1880.9956	0.8	0
76.8	1109.5539	25727274	2.55	LSCAASGFNIK	HC(020-030)	1109.5539	0.0	0
81.2	2180.0864	16055279	1.59	LSCAASGFNIKDTYIHWVR	HC(020-038)	2180.0837	1.2	1
76.6	1088.5410	13618352	1.35	DTYIHWVR	HC(031-038)	1088.5403	0.6	0
76.2	829.4442	24354704	2.42	GLEWVAR	HC(044-050)	829.4446	-0.5	0
71.9	1083.5360	19879112	1.97	IYPTNGYTR	HC(051-059)	1083.5349	1.0	0
72.1	1181.6059	3708836	0.37	GRFTISADTSK	HC(066-076)	1181.6041	1.6	1
73.1	968.4819	26423682	2.62	FTISADTSK	HC(068-076)	968.4815	0.4	0
79.3	2260.1184	317550	0.03	FTISADTSKNTAYLQMNSLR	HC(068-087)	2260.1158	1.1	1
81.3	3518.6474	320893	0.03	FTISADTSKNTAYLQMNSLRAEDTAVYYCSR	HC(068-098)	3518.6446	0.8	2
76.8	1309.6451	24858112	2.47	NTAYLQMNSLR	HC(077-087)	1309.6449	0.1	0
79.9	2568.1769	5903991	0.59	NTAYLQMNSLRAEDTAVYYCSR	HC(077-098)	2568.1737	1.2	1
71.6	1276.5392	2994056	0.30	AEDTAVYYCSR	HC(088-098)	1276.5394	-0.1	0
85.3	2783.2545	16863744	1.67	WGGDGFYAMDYWGQGTLVTVSSASTK	HC(099-124)	2783.2537	0.3	0
78.7	1185.6398	68405792	6.79	GPSVFPLAPSSK	HC(125-136)	1185.6394	0.4	0
77.7	1263.6494	36588096	3.63	STSGGTAALGCLVK	HC(137-150)	1263.6493	0.1	0
88.3	6655.2898	221285	0.02	DYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK	HC(151-213)	6655.2857	0.6	0
79.8	1374.7171	214690	0.02	VDKKVEPKSCDK	HC(214-225)	1374.7177	-0.4	3
84.9	2729.4093	7103687	0.71	THTCPPCPAPELLGGPSVFLFPPKPK	HC(226-251)	2729.4073	0.7	0
73.4	834.4277	13166738	1.31	DTLMISR	HC(252-258)	834.4269	1.0	0
82.0	2897.4175	220680	0.02	DTLMISRTPEVTCVVVDVSHEDPEVK	HC(252-277)	2897.4151	0.9	1
84.3	4556.2041	547132	0.05	DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(252-291)	4556.1992	1.1	2
79.3	2081.0013	17858876	1.77	TPEVTCVVVDVSHEDPEVK	HC(259-277)	2080.9987	1.2	0
83.0	3739.7881	18901388	1.88	TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(259-291)	3739.7828	1.4	1
78.6	1676.7966	5991668	0.59	FNWYVDGVEVHNAK	HC(278-291)	1676.7947	1.1	0
84.6	1807.0008	38113724	3.78	VVSVLTVLHQDWLNGK	HC(305-320)	1806.9992	0.9	0
83.3	2227.2022	63210428	6.27	VVSVLTVLHQDWLNGKEYK	HC(305-323)	2227.2001	0.9	1
82.4	2458.3080	2959244	0.29	VVSVLTVLHQDWLNGKEYKCK	HC(305-325)	2458.3043	1.5	2
81.6	2886.5495	183447	0.02	VVSVLTVLHQDWLNGKEYKCKVSNK	HC(305-329)	2886.5426	2.4	3
73.9	837.4964	38694668	3.84	ALPAPIEK	HC(330-337)	837.4960	0.5	0
75.4	1285.6662	875645	0.09	EPQVYTLPPSR	HC(348-358)	1285.6667	-0.4	0
75.6	1903.9366	36378636	3.61	EPQVYTLPPSREEMTK	HC(348-363)	1903.9350	0.8	1
80.5	2989.5263	1849832	0.18	EPQVYTLPPSREEMTKNQVSLTCLVK	HC(348-373)	2989.5253	0.3	2
78.4	1721.8701	179925	0.02	EEMTKNQVSLTCLVK	HC(359-373)	1721.8692	0.6	1
78.9	1103.6013	45037560	4.47	NQVSLTCLVK	HC(364-373)	1103.6009	0.4	0
81.6	2543.1245	29221288	2.90	GFYPSDIAVEWESNGQPENNYK	HC(374-395)	2543.1241	0.2	0
85.7	4398.0307	5032842	0.50	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	HC(374-412)	4398.0281	0.6	1
85.9	4954.3531	225838	0.02	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK	HC(374-417)	4954.3502	0.6	2
82.8	1872.9144	57538216	5.71	TTPPVLDSDGSFFLYSK	HC(396-412)	1872.9146	-0.1	0
82.6	2429.2370	970108	0.10	TTPPVLDSDGSFFLYSKLTVDK	HC(396-417)	2429.2366	0.2	1
77.8	2986.3744	1042270	0.10	SRWQQGNVFSCSVMHEALHNHYTQK	HC(418-442)	2986.3715	1.0	1
78.5	2743.2427	46302832	4.60	WQQGNVFSCSVMHEALHNHYTQK	HC(420-442)	2743.2384	1.6	0
74.6	659.3487	26985572	2.68	SLSLSPG	HC(443-449)	659.3490	-0.5	0
76.7	1877.8787	2655889	0.26	DIQMTQSPSSLSASVGDR	LC(001-018)	1877.8789	-0.1	0
79.5	2551.2398	9011163	0.89	DIQMTQSPSSLSASVGDRVTITCR	LC(001-024)	2551.2371	1.1	1

Table 1. Continued.

RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
75.6	1989.9932	10501849	1.04	ASQDVNTAVAWYQQKPGK	LC(025-042)	1989.9908	1.2	0
74.8	2286.1771	370620	0.04	ASQDVNTAVAWYQQKPGKAPK	LC(025-045)	2286.1757	0.6	1
84.3	1771.9519	27274394	2.71	LLIYSASFLYSGVPSR	LC(046-061)	1771.9509	0.6	0
85.8	4129.8936	10160642	1.01	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	LC(067-103)	4129.8892	1.1	0
85.3	4599.1803	2980494	0.30	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK	LC(067-107)	4599.1792	0.2	1
84.5	4755.2888	6157193	0.61	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKR	LC(067-108)	4755.2803	1.8	2
82.2	2101.1217	3894068	0.39	RTVAAPSVFIFPPSDEQLK	LC(108-126)	2101.1208	0.4	1
84.0	1945.0220	32666390	3.24	TVAAPSVFIFPPSDEQLK	LC(109-126)	1945.0197	1.2	0
90.2	3666.8789	1457746	0.14	TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR	LC(109-142)	3666.8756	0.9	1
85.5	1739.8676	23403588	2.32	SGTASVVCLLNNFYPR	LC(127-142)	1739.8665	0.6	0
75.9	2676.2628	852142	0.08	VQWKVDNALQSGNSQESVTEQDSK	LC(146-169)	2676.2627	0.0	1
80.0	4160.0087	11663837	1.16	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	LC(146-183)	4160.0033	1.3	2
79.7	4766.2746	403170	0.04	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK	LC(146-188)	4766.2683	1.3	3
78.8	3618.7073	16391463	1.63	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	LC(150-183)	3618.7021	1.5	1
78.6	4224.9705	725845	0.07	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK	LC(150-188)	4224.9670	0.8	2
77.3	4490.1265	478190	0.05	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK	LC(150-190)	4490.1209	1.3	3
78.9	6290.0188	328711	0.03	VDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTK	LC(150-207)	6290.0085	1.6	4
79.0	1501.7515	2071466	0.21	DSTYSLSSTLTLSK	LC(170-183)	1501.7512	0.2	0
74.0	2689.3218	547667	0.05	ADYEKHKVYACEVTHQGLSSPVTK	LC(184-207)	2689.3170	1.8	2
73.4	2083.0562	6116953	0.61	HKVYACEVTHQGLSSPVTK	LC(189-207)	2083.0521	2.0	1
75.3	1817.8988	25691042	2.55	VYACEVTHQGLSSPVTK	LC(191-207)	1817.8982	0.3	0

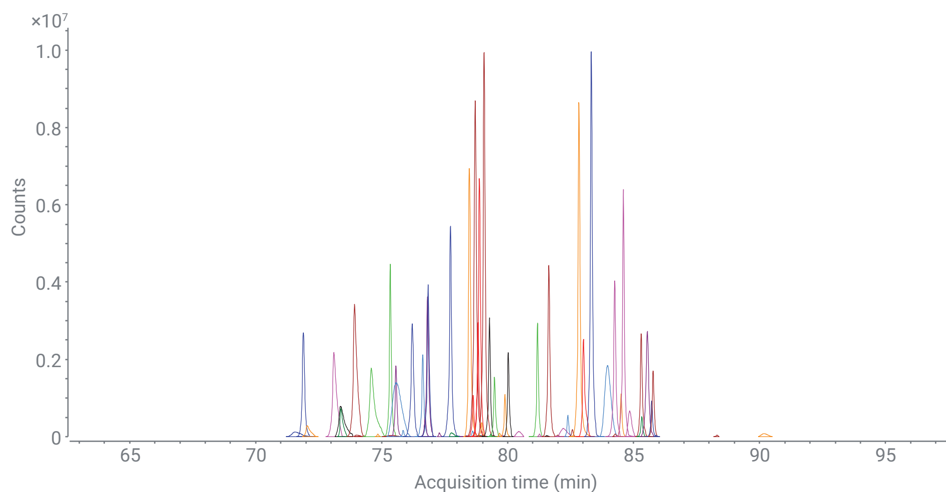

Figure 4. Overlaid RPLC/MS compound chromatograms of MS-identified peptides in the CEX main peak following online peptide mapping.

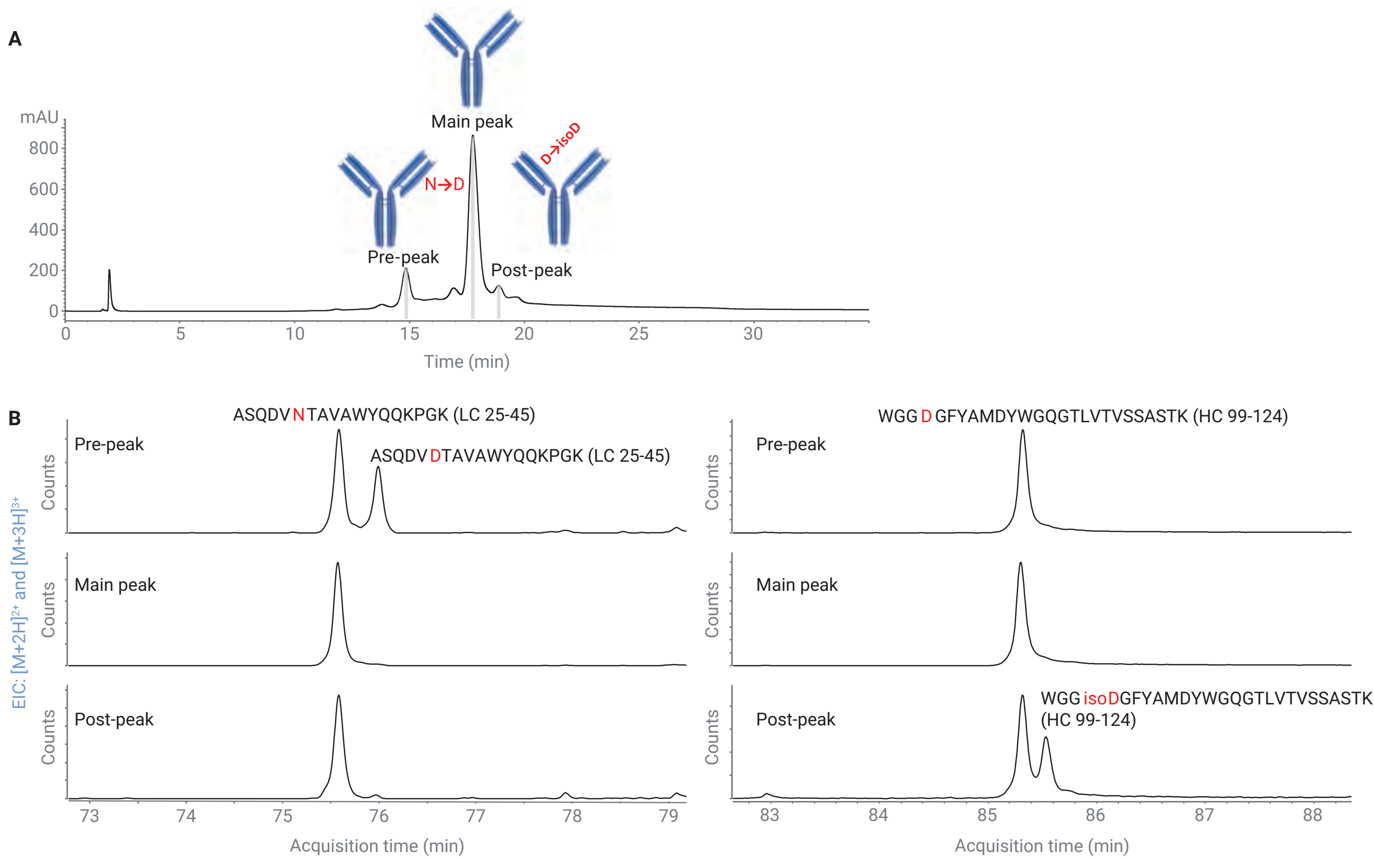

Figure 5. Online peptide mapping of trastuzumab CEX pre-, main-, and post-peaks. (A) the CEX chromatogram and (B) the extracted ion chromatograms of light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQDVDTAVAWYQQKPGK (LC 25–42), heavy-chain peptide WGGDGFYAMDYWGQGTLVTVSSASTK (HC 99–124) and isomerized heavy-chain peptide WGGisoDGFYAMDYWGQGTLVTVSSASTK (HC 99–124).

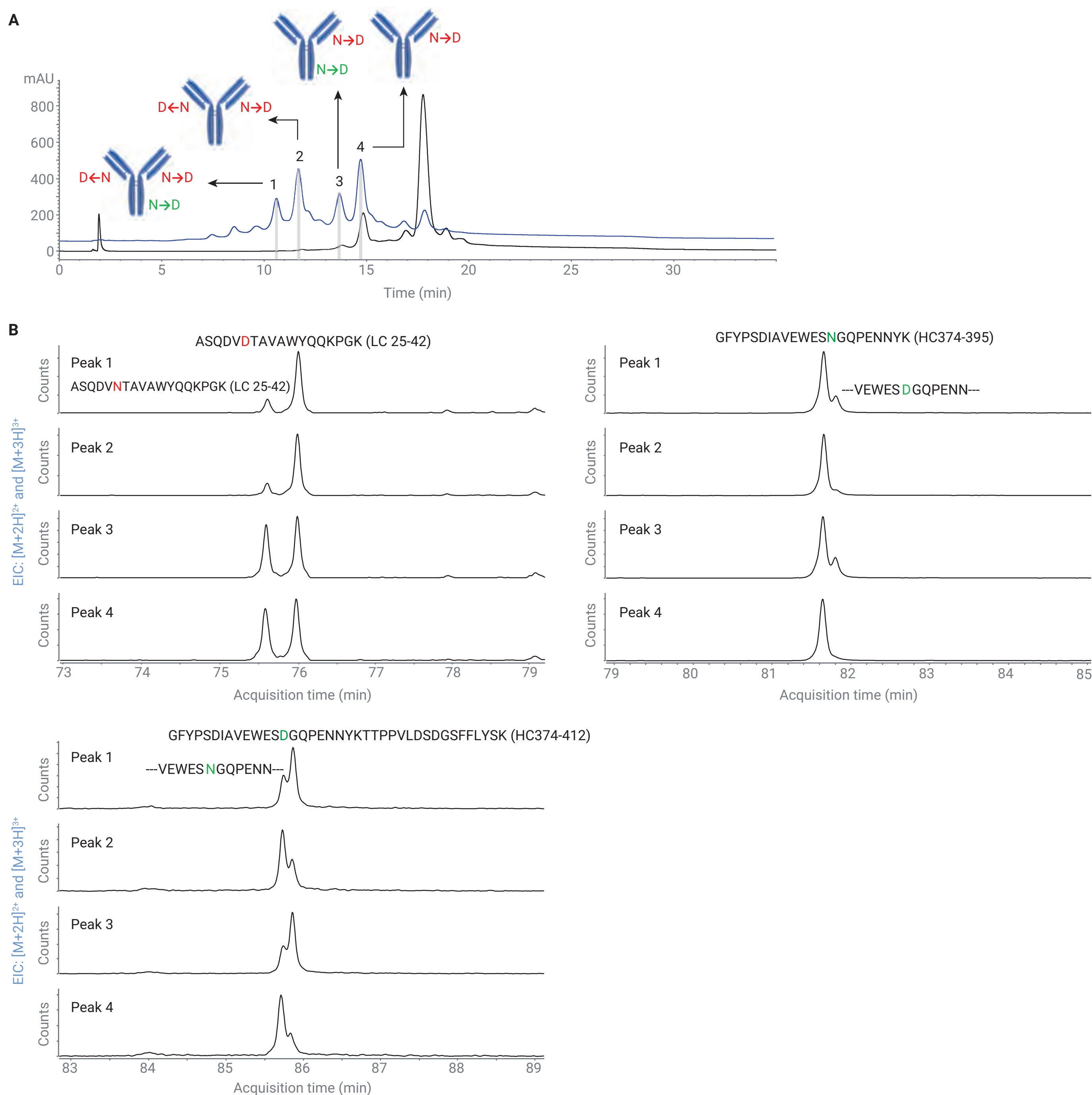

Figure 6. Online peptide mapping of high-pH stressed trastuzumab CEX peaks 1, 2, 3, and 4. (A) the overlaid CEX chromatograms of nonstressed and high-pH stressed trastuzumab and (B) the extracted ion chromatograms of light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQDVDTAVAWYQQKPGK (LC 25–42), heavy-chain peptide GFYPSDIAVEWESNGQPENNYK (HC 374–395), deamidated heavy-chain peptide GFYPSDIAVEWESDGQPENNYK (HC 374–395), heavy-chain peptide GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK (HC374–412), and deamidated heavy-chain peptide GFYPSDIAVEWESDGQPENNYKTTPPVLDSDGSFFLYSK (HC 374–412).

Conclusion

A fully automated 4D-LC/MS protein analyzer incorporating 1D CEX separation and charge-variant peak collection using multiple heart-cutting, 2D RPLC-based desalting, denaturation, reduction, 3D trypsin digestion, and 4D RPLC/MS-based peptide mapping was described and successfully applied to characterize acidic and basic variants observed in the CEX profile of nonstressed and high-pH stressed trastuzumab. This multidimensional system is based on the InfinityLab 2D-LC Solution and the 6545 LC/Q-TOF system. A variant of this 4D-LC/MS design can readily be configured by replacing CEX in the first dimension by Protein A affinity chromatography, size exclusion chromatography, hydrophobic interaction chromatography, etc.

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Additional Application Notes

Publication Number	Title
5991-7442EN	Seamless Method Transfer from an Agilent 1260 Infinity Bio-inert LC to an Agilent 1260 Infinity II Bio-inert LC
5991-5273EN	Characterize mAb Charged Variants by Cation-exchange Chromatography
5991-5274EN	Characterize Fab and Fc Fragments by Cation-exchange Chromatography
5991-0895EN	Analysis of Intact and C-terminal Digested IgG1 on an Agilent Bio MAb 5 µm Column
5990-9629EN	pH Gradient Elution for Improved Separation of Monoclonal Antibody Charge Variants
5991-1407EN	High-resolution Analysis of Charge Heterogeneity in Monoclonal Antibodies Using pH-gradient Cation Exchange Chromatography
5991-1408EN	Protein Separation with pH Gradients Using Composite Buffer Systems Calculated by the Agilent Buffer Advisor Software
5991-4722EN	Reducing Cycle Time for Charge Variant Analysis of Monoclonal Antibodies
5991-3365EN	Simple Method Optimization in mAb Charge Variant Analysis using pH Gradients Generated from Buffer Advisor with Online pH and Conductivity Monitoring
5990-9270EN	Separation of Protein Standards on Agilent 3 µm Ion-Exchange Columns by Cation Exchange Chromatography
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