An Executive Summary

Native MS for Aggregate and Charge Variant Analysis for Biotherapeutics

Native MS represents a cutting-edge approach to employ MS-compatible mobile phases for non-denaturing SEC and IEX analysis.

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Introduction

Size exclusion chromatography (SEC) and ion exchange chromatography (IEX) are classic techniques employed in the characterization of biotherapeutics under non-denaturing conditions. Standard mobile-phase conditions for these techniques are high in salt concentration, rendering them incompatible with mass spectrometry (MS) detection. Native MS represents a cutting-edge approach to employ MS-compatible mobile phases for non-denaturing SEC and IEX analysis. Learn about aggregate and charge-variant analysis using native-mode MS, including understanding the fundamentals in SEC and IEX for biotherapeutic characterization, the effect of mobile-phase conditions and chromatographic parameters in aggregate analysis by native SEC, and determination of acidic and basic variants of monoclonal antibodies using native weak cation exchange chromatography (WCX).

Increasing Complexity of Biologics

Biologics are a huge field in today's biopharmaceutical market. In 2018, of the top 15 drugs on the market, 10 were biopharmaceuticals in the mAb category.

Additionally, biologics are increasingly complex. As new forms of antibody and new antibody class drugs are developed, new issues are emerging on the analytical side. As well as having bio-originators, biosimilars with very similar or the "same" protein structures are coming to the market. There are issues making sure glycans are the same and/or post-translational modification (PTM)—ensuring these biosimilars have the same use as the originators, but only subtly different in structure.

There are also new mAb formats such as bispecific Fab regions, scFv, BiTE, and new antibody fragment categories that are difficult to analyze. Antibody drug conjugates (ADCs) represent the addition of a small molecule payload to the mAb—these are almost like a "magic-bullet" class of new pharmaceuticals with the added complexity of the drug payload to monitor and control.

All these issues are presenting an analytical challenge to today's researchers, as new tools are needed to look into these species. With analytical challenges, more complicated methods of LC and MS are being examined. More advanced peptide mappings are being used, such as sequence variant analysis, which takes the traditional peptide map a step further into analytical challenges.

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Regarding intact mass and fragment mass, scientists are performing top-down and middle-up experiments, looking at reduced fragments of an antibody or the intact species. Multi-attribute method is an emerging tool where critical quality attributes in the production of biopharmaceuticals are being monitored in a single MS experiment with high-end processing software.

Streamlined Biologics Analysis

Phenomenex developed the bioZen™ series specifically for biopharmaceuticals. At SCIEX's biologics division, it has become the standard for all workflows. The bioZen series has intact C4 and XB-C8 columns, which give good results on reversed-phase analysis of antibodies and antibody fragments, a selection of SEC columns for different-sized aggregate and fragment analysis, a couple of peptide columns, a new cation exchange column, and a glycan column.

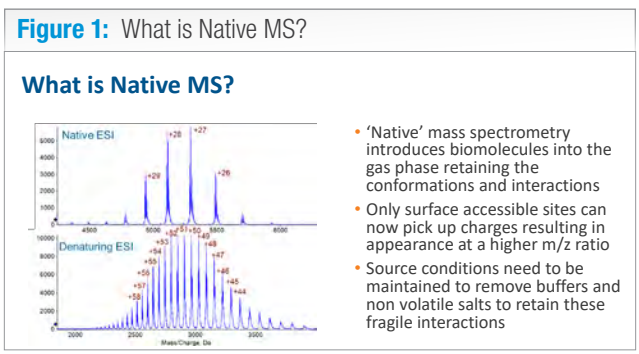
The technologies behind bioZen are the core-shell particles, the modified fully porous particles, and non-porous PS-DVB particles combined with a titanium-based bioinert column. Biopharmaceuticals are known to aggregate and stick to columns heavily, so using bioinert titanium hardware minimizes adsorptive interactions.

What is Native MS?

Native MS or Native-like MS seeks to introduce biomolecules into the gas phase while retaining their solution phase conformations and interactions. Traditional denaturing ESI uses a lot of denaturing solvents, such as methanol and acetonitrile, which unfold the protein. The problem with unfolded proteins is they ionize very well, and they are very producible, but information is lost on how these molecules are interacting with each other in ways such as aggregates, ligand binding, and other known covalent sites.

The bottom spectra on **Figure 1** is over a large number of charged states as all the accessible sites along the molecule become charged. In Native MS, there is a more compact structure, so only the surface accessible sites can pick up charges, resulting in appearance at a higher mass-to-charge ratio in a narrower envelope. In native experiments, the buffers and non-volatile salts have to be balanced to retain fragile interactions, but energy and voltages are used to get these molecules into the gas phase—this balancing act is what's difficult about Native MS.

Figure 1: What is Native MS?



Antibody Aggregation Pathway

All proteins are presented on paper in a static format, but they are flexing and moving around—there is Brownian motion and certain residues unfolding and flexing on the cells. There is partial unfolding of certain sites that can lead to reversible aggregate formation that will then go back to be the monomer format. But under some conditions, such as pH and temperature, irreversible aggregate formation can happen, which means there are exposed hydrophobic residues encouraging a cascade of aggregation into these large immunogenic particles. This aggregation can cause particle formation, which is insoluble.

> "Biopharmaceuticals are known to aggregate and stick to columns heavily, so using bioinert titanium hardware minimizes adsorptive interactions."

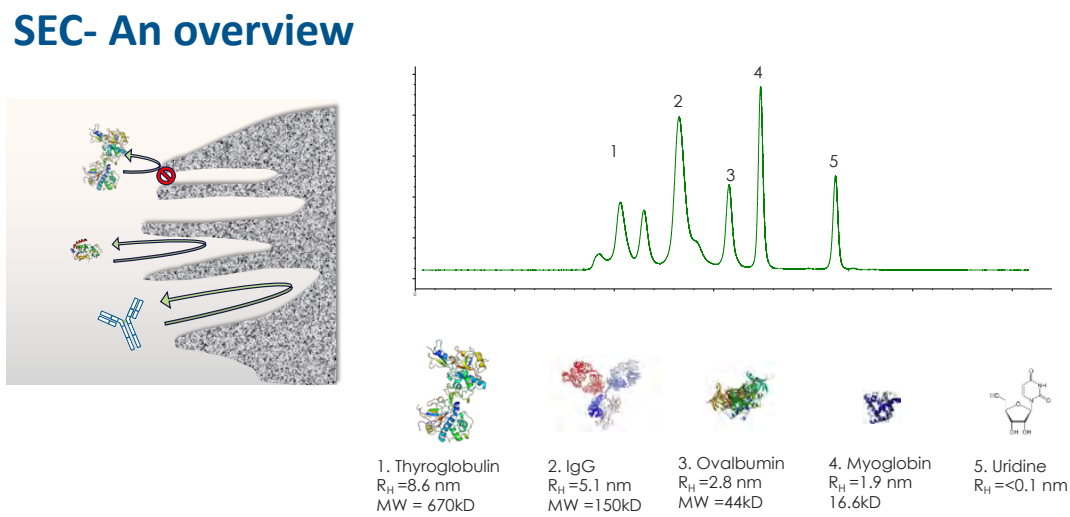
The crux of this aggregation pathway starts with post-translational modifications—disulfide bond formation, deamidation, oxidation, and glycosylation. If there is disulfide unscrambling and two antibodies are close together, the disulfide bonds present can be altered, exposing hydrophobic residues.

Under deamidation, asparagine isomerization happens, reversing the backbone spiral of the protein, again, exposing further hydrophobic residues between proteins that will interact, and therefore, causes aggregation pathways. SEC is the technique utilized to analyze these molecules.

Size Exclusion Chromatography (SEC)

SEC works well with native analysis because it's a non-absorptive technique, and it can work under a variety of native, native-like, or solution-like conditions.

Figure 2: SEC - An Overview



SEC has porous particles that are non-absorptive, and proteins are traveling through the column interacting with these pores. In **Figure 2** is a SEC-standard molecule that contains several proteins of different sizes, from thyroglobulin up at the top, down to uridine, a very small molecule. Thyroglobulin is a very large protein, so it's too large to enter the pores in a SEC-2 column; it cannot interact with the pores of this molecule, and therefore, travel straight through the column rather quickly.

A mAb molecule could interact with some of the pores; however, since these pores are slightly smaller, it might interact with the smaller pores. Therefore, it'll come out after having a slightly longer pathway than thyroglobulin, but it doesn't interact with all the pores. Small molecules like uridine can travel through all the pores; they have a very long pathway going to the column, so they end up coming through last. The larger the molecule is, the quicker it will elute. Small molecules will elute later on down the column.

The hydrodynamic radius of the molecule is what's causing them to interact with these pores, and it correlates very closely with molecular weight. Large molecular weight species have a larger hydrodynamic radius.

Phenomenex has two bioZen SEC columns available: SEC-2 and the SEC-3. The difference between these columns is pore size. SEC-2 is able to separate molecules between 1,000 to 450,000 Daltons, and SEC-3 can separate molecules between 10,000 and 700,000 Daltons. SEC-2 was chosen for the Native MS workflows.

SEC and Native MS is different than reversed phase in how the flow rate impacts resolution. On **Figure 3,** there are two UV spectra done under LC conditions, not LC-MS conditions, where there is a 0.2 and a 0.3 mL flow rate. There is one preceding species (aggregate), one large species (monomer), and some smaller species afterward (fragments). On a lower flow rate, more fine features of aggregates can be observed in the first eluting peak. With SEC, a lower flow rate typically improves the chromatography. This feature is compatible with MS, and these low flow rates of 0.2 mL/min can work well with SCIEX's mass spectrometers.

SEC is a non-adsorptive technique, so under these conditions, there should be no interaction between the molecules of interest and the column stationary phase. Analytes will only elute by molecular weight if separation is without any interaction in the secondary phase. Optimizing the buffer conditions ensures there is as little interaction between the column and the molecules as possible.

Figure 3: Effect of Flow-rate on SEC

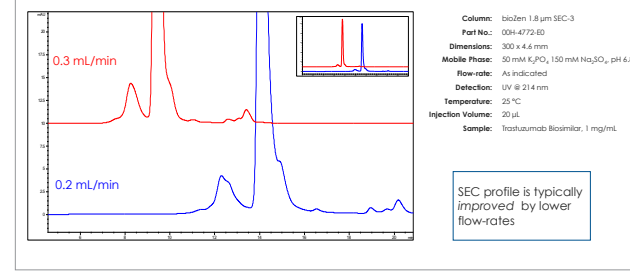
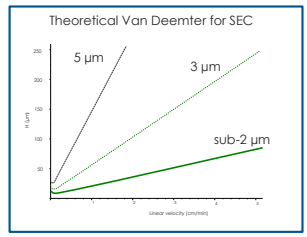


Figure 4: Non-Adsorptive Chromatography

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = RT\ln k$$

Under ideal size exclusion conditions, enthalpy (ΔH^0) should be zero, therefore:

$$\ln K_D = -\Delta S^0/R$$

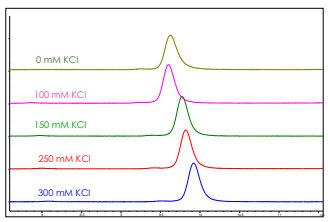


Under LC conditions, with LC-UV, very high potassium and phosphate salts are used to mask the secondary interactions to the column. However, under MS conditions, these have to be replaced with more MS-friendly buffers such as ammonium acetate and ammonium bicarbonate.

The equation on **Figure 4** shows that separation under traditional separation techniques is driven by the Gibbs-free energy. However, the separation equation is for a non-adsorptive technique, in which case actual enthalpy is zero and separation is driven by entropy.

The graph on the slide is the theoretical plot of the Van Deemter equation. It shows with smaller particle sizes using SEC, better separation at lower linear velocities is obtained. On **Figure 5,** the difference between no potassium chloride and various high-level potassium chloride is observed. In general, the peak shape and peak intensity improve with higher salt buffer conditions.

Figure 5: Effect of Salt of Aggregate Recovery



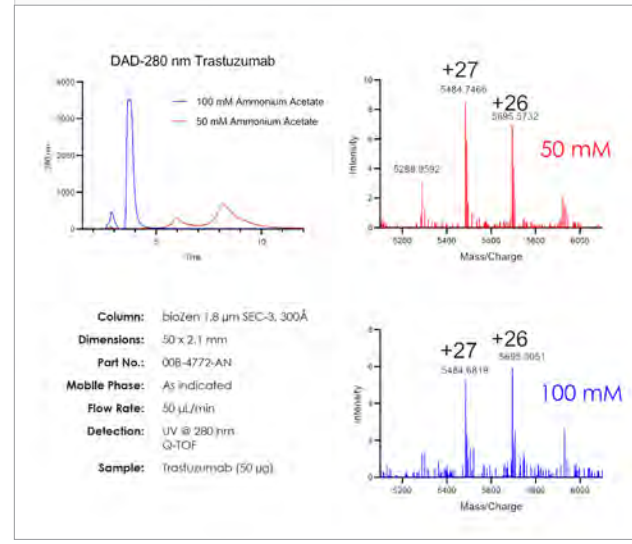
Column: 1.8 µm SEC-2
Dimensions: 300 X 4.6 mm
Mobile Phase: 50mM Potassium Phosphate + KCl, as noted
Flow Rate: 0.3 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Sample: Biosimilar (mAb 2)

Salt Concentration (mM KCl)	Retention Time (t)	Peak Width	Peak Symmetry
0	9.63	0.1472	0.661
100	9.60	0.1718	0.78
150	9.77	0.1753	0.785
250	9.82	0.1767	0.788
300	9.91	0.1822	0.778

Is MS-friendly Mobile Phase Capable of Replicating High-Salt Conditions of Traditional SEC?

On **Figure 6,** a comparison of 50 mM of ammonium acetate and 100 mM of ammonium acetate. Looking at the chromatographic traces, the difference in peak shape is much higher than the different concentrations of potassium chloride. So, the ammonium acetate is not doing as good a job of buffering in these conditions; therefore, a lot of work has been done trying to optimize how the MS conditions, the LC conditions, and the flow rate can interact to give the best conditions.

Figure 6: Mobile Phase, Effect on Sensitivity



SCIEX X500B QTOF

SCIEX X500B QTOF was designed specifically for biologic molecules. It works well for routine analysis such as peptide mapping and intact samples such as the aforementioned Native MS experiments. SCIEX OS was specifically designed to be a more new-age MS software with easy-to-use menus that doesn't require as much in-depth MS usage to get the best out of the data.

On **Figure 7** is a SEC standard under Native MS conditions. On the left-hand side of the slide, there is a UV trace of four molecules leeching off a bioZen SEC-2 column with 100 mM ammonium acetate. The IgG elutes first, then BSA, carbonic anhydrate (CA), and myoglobin.

Figure 7: Native MS Seperation of SEC Standards

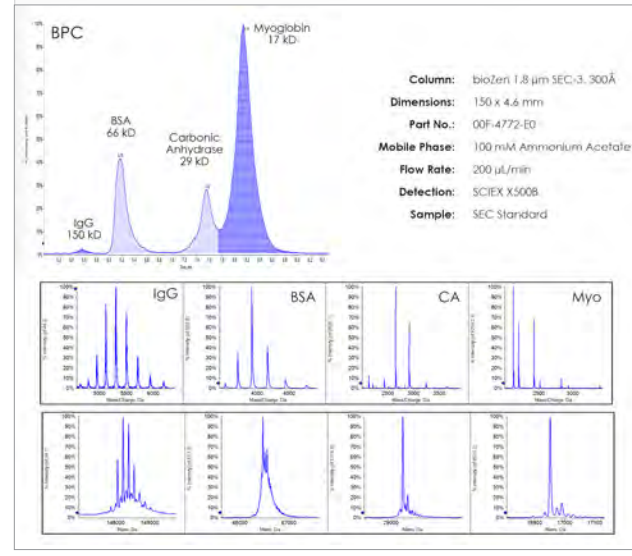


Figure 8: Aggregate Analysis of NIST mAb with bioZen SEC-2

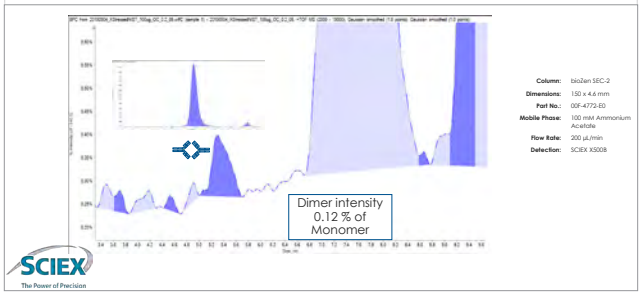
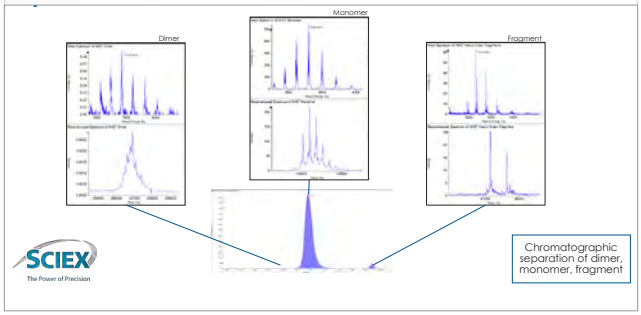


Figure 9: Spectra for NIST mAb



The LC-UV shows good separation, but the MS adds an extra level of information, so now the raw MS spectra is observed for these molecules. Not only is the peak eluting off an LC-UV, but specific information on the nature of these molecules is available—the aggregation, the accurate molecular weight—with the same sort of separation as you would get under high-salt conditions. Separation power going into these MS conditions isn't lost, but rather a lot of information on the molecules involved is gained.

Figure 8 shows the aggregate analysis of the NIST monoclonal antibody on the bioZen SEC-2 column. The top inset is the total ion chromatogram for this sample. It's mainly dominated by one major species with a slightly smaller species eluting later. There is a peak eluting beforehand, however, there's a lot noise in the spectra. Without any MS added, the nature of the small species and the late-eluting peaks would be unknown only using LC-UV analysis.

The MS data on **Figure 9** is the dimer for the NIST mAb, showing the raw and reconstructed data for the dimer. A very good spectra of a NIST mAb monomer is in the center, both in the raw and reconstructed data. In addition, the specific nature of the fragments is available. A couple of fragments elute together—a heavy chain Fab region, and some of the glycoforms on the monomer and fragment can be observed. Although there are high-salt conditions with less intensity than the standard reverse phase, there is good quality spectra that's able to identify key details.

On **Figure 10** are four commercially available antibodies with difference profiles that were analyzed by SEC UV-MS (the UV cell is in line before the MS). Bevacuzimab elutes in approximately six minutes; there is a dimer visible, followed by a mAb fragment and the monomer, and then more fragments. Cetuximab elutes with no dimer visible, only a monomer and a series of fragments. Rituximab has a heavy chain, light-chain fragment with small fragments. Trastuzumab elutes mainly as a monomer but under two peaks.

Looking at the LC-UV data of bevacuzimab, the nature of these species involved is unknown, but when the extra dimension of the MS is added in, there is a very clear dimer spectra and eluting with a very high mass-to-charge ratio. When reconstructed into a form where specific glycoforms can be identified, there is also clear monomer spectra, almost as good as you would get with any reversed-phase experiment.

Adding in the UV data, the relative level of the dimer versus the monomer can be quantitated. The dimer has about 2.6 percent relative area, and the monomer about 88 percent of the area, and some fragments lifting at 9.1 percent of the area. So, these were quantified in the UV data, but there is the added level of specific information from the MS.

Ion Exchange-Native HRMS for Analysis of mAb Charge Variants

Figure 11 offers an overview of common PTMs that occur on monoclonal antibodies along with its typical Y depiction. The yellow dots indicate common post-

Figure 10: Aggregate Analysis of mAb with bioZen SEC-2

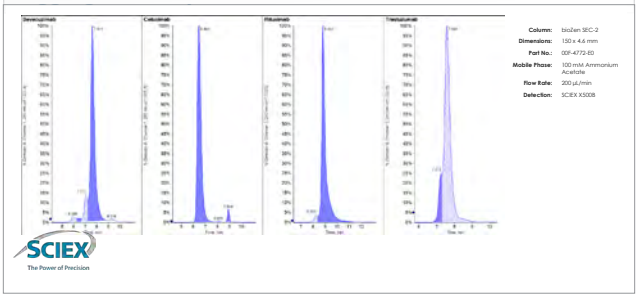
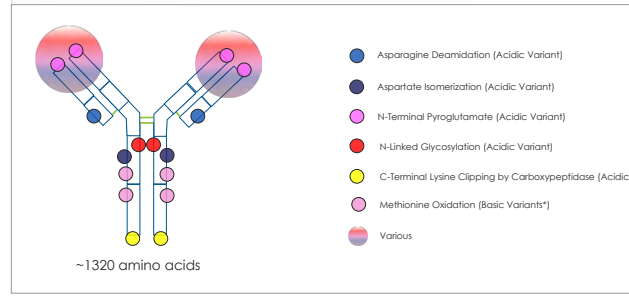


Figure 11: Common PTMs with mAbs



transitional modifications that occur, including C terminal lysine clipping. Many drug products do not have any C-terminal lysine, so one thing to look for is whether there is any residual lysine at the C terminus, which would result in charge variants with an additional one or two lysines. On the N terminus, there's a variety of PTMs that are possible, including the pyroglutamate formation, which leads to an acidic variant. In general, there's a good number of variants that can occur with these monoclonal antibodies.

PTMs can occur during manufacturing, purification, and storage, and they're considered critical quality attributes because they can affect efficacy, activity, and stability. Routinely in drug manufacturing, PTMs are monitored during manufacturing and formulation with ion exchange chromatography with UV detection using a salt gradient or a pH gradient. However, monitoring PTMs with ion exchange coupled online with Native high-resolution MS allows for rapid identification of these modifications. Native MS gives simpler spectra, i.e., there's less spectral overlap, which facilitates identification.

The Experiment

The general experimental overview and CEX conditions are laid out in **Figures 12 and 13.** The bioZen 6 µm cation exchange material used in **Figure 14** is based on a monodisperse non-porous polystyrene divinylbenzene polymer bead. The non-porous polymeric bead is grafted with a hydrophilic layer to minimize any hydrophobic interaction of the protein with the hydrophobic polystyrene divinylbenzene bead. On top of the hydrophilic layer, a linear polymer carboxylic acid is grafted, which forms the ion exchange functionality. Particle size distributions are shown in **Figure 15,** on the X axis is the particle diameter, and on the Y axis is the relative intensity or the count of particles. The bioZen 6 µm has a very narrow particle size distribution when compared to some standard commercial products—approximately 5 µm material and a 10 µm material. This narrow particle size distribution improves efficiency and reduces back pressure.

Optimized Cation Exchange Buffers

After a fair amount of experimentation, the buffers that work best for charge variant Native MS are shown in **Figure 16.** The A buffer is 20 mM ammonium acetate adjusted to pH 5.2 with acetic acid. The mobile phase B is 5 mM ammonium acetate, pH 10.2 adjusted with ammonium hydroxide. The buffer composition was optimized with separation robustness in mind, meaning the same reproducible results day to day.

One way separation robustness is ensured is by using the highest buffer capacity while using the lowest practical ionic strength. This explains the difference in

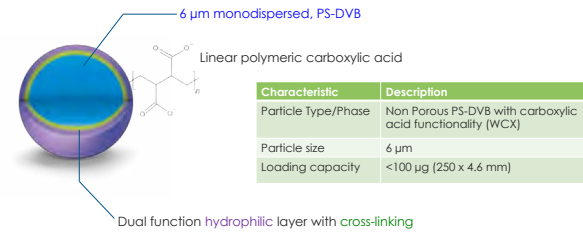
Figure 12: Experimental Overview – CEX-native HRMS

- **Buffer composition and pHs optimized for CV analysis**
- **Gradient range and slope optimized for each mAb**
- **MS conditions optimized to maximize sensitivity, minimize adducts**

Figure 13: EXperimental – CEX Conditions

- **All columns 2.1mm ID**
  - Different lengths used: 250mm and 50mm (150mm)
- **Column Type**
  - Weak cation exchange (WCX) – bioZen™ WCX 6µm
- **Flow rate: 0.2 mL/min (0.3mL/min)**
- **Temperature: 30° C**
- **Injected mass: 50 -100 µg on-column**

Figure 14: bioZen µ6 WCX



Characteristic	Description
Particle Type/Phase	Non Porous PS-DVB with carboxylic acid functionality (WCX)
Particle size	6 µm
Loading capacity	<100 µg (250 x 4.6 mm)

Figure 15: Comparison to 5 & 10 µm WCX

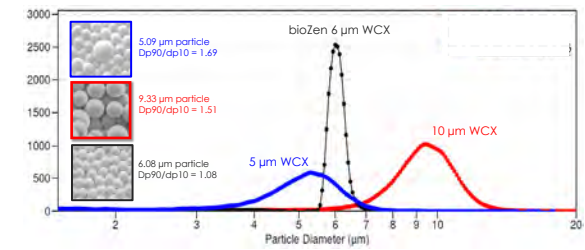


Figure 16: Experimental – Optimized CEX Buffers

- **Buffers:**
  - A = 20mM NH_4Ac pH 5.2 (HAc)
  - B = 5mM NH_4Ac pH 10.2 (NH_4OH)
- **Unique, optimized buffer composition:**
  - Separation robustness
  - buffer capacity (highest) vs ionic strength (lowest practical)
  - pH linearity – lower concentration B buffer gives finer pH control
  - Speed column equilibration - higher concentration A buffer
  - pH stability – 2 weeks at room temperature (inlet air filter, SecurityCAP™)
  - Metal free salt, acid, base used (≥99.99% trace metals basis) – reduce adducts

compositions between A and B in **Figure 16**—the lower concentration of B buffer allows larger changes to be made in the percent B, which gives finer control of pH and helps to improve pH linearity. While conversely, having a higher concentration of the A buffer allows speed column equilibration.

Many different buffer compositions and components have been mentioned in the literature, but after experimenting, they didn't offer optimal pH stability. But with this particular combination, the pH was stable for up to two weeks at room temperature, i.e., the same buffer preparation could be used for up to two weeks.

An important note is an inlet air filter must be used on the mobile phase reservoirs in order to get this lifetime. Phenomenex has a commercial product, SecurityCAP™, that provides an inlet air filter preventing bacteria and dust from contaminating the mobile phase, and thus, extends buffer lifetime.

First Results

Figure 17 shows a separation of trastuzumab on a 250 mm bioZen WCX column. There is a very detailed separation of the different isoforms present. The gradient is from 20% B to 50% B, over 25 minutes. The back pressure is about 80 bar, and at this flow rate, 0.2 mL/min, there is additional room to speed things up by increasing the flow rate.

The main peak raw mass spectrum along with the charge state is shown on **Figure 18.** The center is + 27 and the far right is + 25—this is an example of the raw mass spectrum in a Native MS separation. The masses are in the range of 5,500 m/z, so most of the intensity in the desired range, and it tails off toward each end. The raw mass spectrum has been deconvoluted using software, so the average molecular weight of the species can be obtained.

Figure 19 shows all the different glycoforms that are co-eluting because there are no charges associated with them. Again, cation exchange is separating these different isoforms based on charge. Since these glycoforms do not have any differing charges, they all co-elute, which is one reason why there is a number of peaks in the mass spectrum for one chromatographic peak.

The Basic Variant

In the case of trastuzumab, all of the C-terminal lysine in the primary drug products are removed. So, the basic variant is where one of the C terminal lysine is still attached to the molecule, which gives a basic variant. In the reconstructed spectrum, there are some shoulders, which is a little unusual.

On the top panel of **Figure 20** is the reconstructed mass spectrum of the main isoform, or the main peak,

Figure 17: Results–Trastuzumab–biozen WCX 6 µm

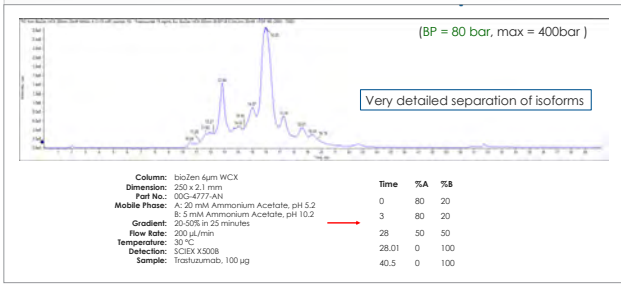


Figure 18: Trastuzumab Mass Spectra

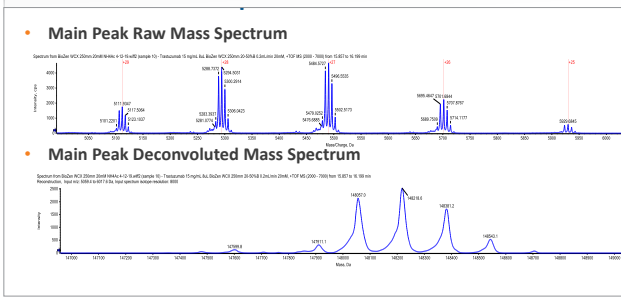
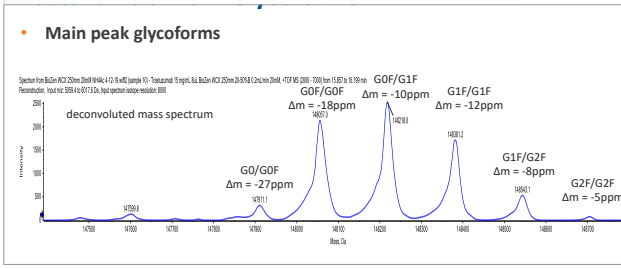


Figure 19: Trastuzumab Main Glycoforms



along with the glycoforms indicated. The mass change from one glycoform to the next is about 162, while a lysine variant adds 128 to the mass of the molecule. The shoulders are in the reconstructed spectrum of the lysine variant, e.g., on the lower panel, the G0F/G0F+lysine is indicated with the arrow, and it also runs into the mass of G0F/G1F, which differs by 162. So, there is some spectral overlap with a co-eluting isoform. The same pattern repeats itself throughout the mass spectrum because each sugar adds 162 to the mass, while the lysine adds 128, which explains the shouldering.

To further confirm that's the lysine variant, the mAb is deglycosylated with PNGase F. The chromatogram looks very similar to the chromatogram shown in **Figure 17**. PNGase F removes all of the sugar molecules from the mAb, and since sugars typically dont carry any charge, the separation is essentially unaffected. Looking at the lysine variant again, there are only two peaks in the raw mass spectrum. Once deconvoluted, one of those masses turns out to be the mass of the main peak, while the second mass is that of the lysine variant. This is a very common way of confirming lysine variants with mAbs.

Figure 20: Trastuzumab Lysine Variant

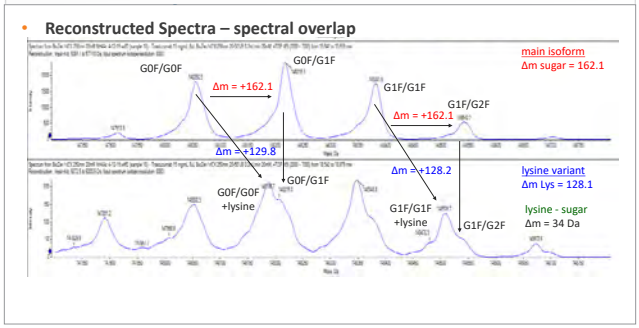


Figure 21: Results–Trastuzumab–biozen WCX 6 µm

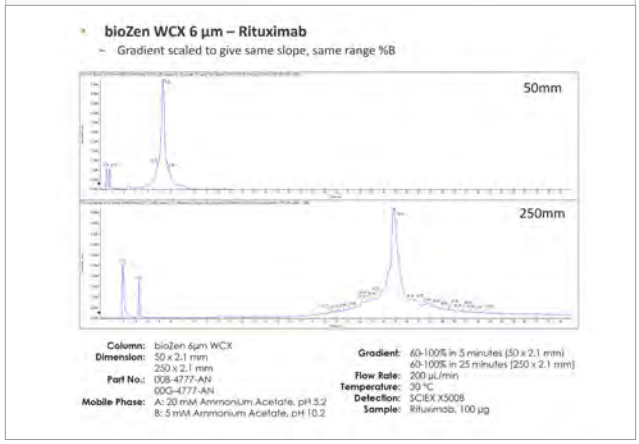


Figure 22: Column Length – 150mm vs 50mm

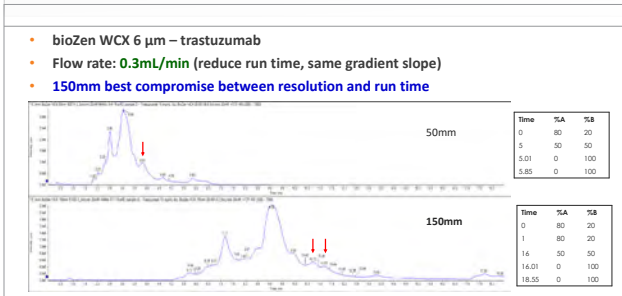
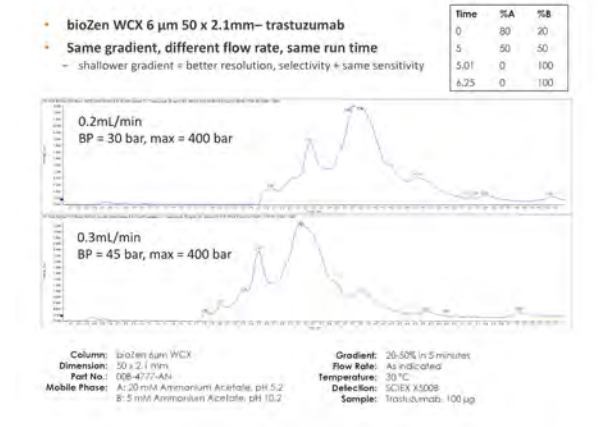


Figure 23: Flow Rate–0.2 vs 0.3 mL/min



The Effect of Column Length

Figure 21 shows the comparison of the separation for a 50 mm column versus the 250 mm column. The gradient is scaled to give the same slope on both columns.

The separation on the 50 mm column is five times as fast. The 250 mm column offers a significant improvement in the chromatographic resolution throughout the chromatogram, which makes it much simpler to identify any new PTMs that may result from a change in the process.

As shown in **Figure 21**, even though there is significantly better chromatographic resolution of the different components, there is a very similar mass spectrum for the +1 lysine variant—whether it's the 50 mm column or the 250 mm column. This shows shorter columns can be used as long as the user knows what to look for.

Figure 22 compares a 150 mm column to a 50 mm column with an increased flow rate to 0.3 mL/min to reduce the run time. On the 50 mm column, the single peak indicated by the red arrows is resolved into two peaks on the 150 mm column, showing significant improvements in resolution with an increase in column length.

The Effect of Flow Rate

Figure 23 shows the effect of using 0.2 versus the 0.3 mL flow rate with the same column, the bioZen 6 µm WCX in 50 x 2.1 mm format, and the same gradient. If the gradient table is not changed then, the higher flow rate gives a shallower gradient and thus, better resolution and selectivity.

Conclusion

The novel, robust, MS-compatible buffer system described in this work gave very reproducible mAb charge variant analysis results with a bioZen 6 µm WCX column with approximately two weeks shelf life at room temperature. For MS, a 150 x 2.1 mm column at 0.3 mL/min offers the optimum compromise between chromatographic resolution and separation speed, providing the best chance of identifying new charge variants. For UV, a 250 x 2.1 mm column operated at 0.4 mL/min is optimal.