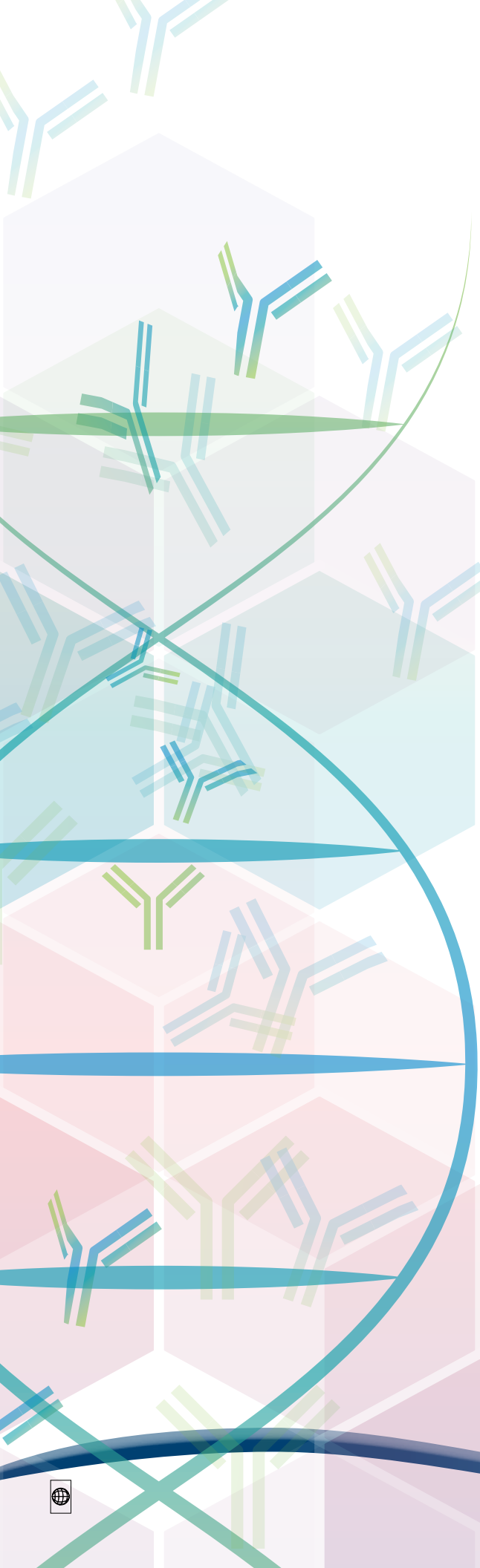


# Biosimilars Chromatographic Characterization

bioZen™
the bio series

**Discover how bioZen columns aid in analyzing infliximab and biosimilars through:**

- **Primary Structure**
- **Higher Order Structure**
- **Post-translational Modifications**

phenomenex®
*. . . breaking with tradition℠*

# Table of Contents

# Introduction

The development of biologic therapeutics is growing at a rapid rate in major pharmaceutical companies with respect to the traditional, small molecule counterpart. Although many different modalities are approved by regulatory agencies, the vast majority of marketed biologics drugs are monoclonal antibodies (mAbs). As with many marketed drugs, competition arises with generic versions when patents expire, which can be a beneficial way to lower drug prices. Biologics, mAbs in particular, also follow this trend and have sparked an increase in biosimilar development in the pharmaceutical industry.

The innovator mAb represents a daunting analytical challenge due to its large size and high complexity. These molecules are produced recombinantly and thus are prone to post-translational modifications (PTMs). Extensive characterization of PTMs is performed to ensure there are no negative implications in safety, efficacy, and immunogenicity. Moreover, during biosimilar research, it is impossible to produce exact replicas of the innovator mAb explaining the terminology of "biosimilar" instead of "generic", which is used in the small molecule arena. Because of this inherent challenge, regulators require evidence of similarity or comparability for biosimilar submissions. Among all the criteria are structural analysis, functional assays, animal studies, and clinical trials.

Regulators provide guidance for the data necessary to achieve similarity or comparability to innovator biologics, but also provide freedom to choose specific assays. The International Congress on Harmonisation (ICH) is a platform for biopharmaceutical companies to coordinate their efforts to unite data requirements. As such, the ICH Q6B[1] and ICH QE5[2] have become the standard quality guidances that connect pharmaceutical companies with regulatory agencies such as the FDA[3] and EMA[4] on biologics characterization and biosimilar acceptance criteria.

Chromatographic analyses are embedded within the ICH Q6B and QE5 as viable options for performing structural analysis. In the ICH Q6B document, liquid chromatography falls under methods for determining physicochemical properties stating that "chromatographic patterns and data on the identity, homogeneity, and purity can be obtained by size exclusion chromatography, reversed phase liquid chromatography, ion-exchange liquid chromatography, affinity chromatography or other suitable procedures." The ICH QE5 document on biosimilarity does not refer to specific chromatographic techniques, but rather defers to the ICH Q6B document as guidance. In addition, the document suggests utilizing more than one analytical technique to confirm characterization.

Given the above criteria, we chose to explore the chromatographic analyses associated with biosimilar characterization. We selected infliximab and two biosimilar products as a case study for these experiments. To frame the results, we chose to follow the structural analysis component in the FDA's "Scientific Considerations in Demonstrating Biosimilarity to a Reference Product" document,[3] which includes: a) primary structures, such as amino acid sequence, b) higher order structures, including secondary, tertiary, and quaternary structure (including aggregation), c) enzymatic post-translational modifications, such as glycosylation and phosphorylation, and d) other potential variations, such as protein deamidation and oxidation.

# II. Primary Structures

The primary structure of a protein can be deduced by many methods, but ultimately are reliant on mass spectrometry (MS) techniques. The identity of all amino acids, the sequence of these amino acids, including sequence variants, and all post-translational modifications can be identified through extensive MS characterization methods. While methods such as the multi-attribute method[5] and 4D-LC[6] for primary structure analysis are still in their infancy, traditional chromatographic methods are the preferred characterization tools of choice for pharmaceutical scientists. This section will cover the reversed phase analysis of biosimilar mAbs through the two most common chromatographic techniques: bottom-up and top-down protein analysis.

## Peptide Mapping – Bottom-Up mAb Analysis

The finest details of a protein's structure are analyzed through peptide mapping, where peptide chains are "mapped" by UV/MS detection to elucidate their quantity and connectivity. For biosimilars, this analysis is perhaps the most critical method for analytical determination of mAb comparability because it can confirm all amino acids present with full coverage, as well as be able to detect any sequence variants. In addition, any post-translational modifications can be identified through peptide mapping, which will be discussed in **Section IV**.

In order to accomplish a peptide mapping analysis, sample preparation through a series of steps including denaturation/reduction, alkylation and enzymatic digestion of the mAb is required. Typical enzymes that achieve efficient digestion are trypsin, LysC, GluC, and pepsin.[7] There are a large number of sample preparation considerations for experimental design, but for the purposes of our studies we selected the most common solution procedure using a tryptic digest. Sample preparation details can be found on **page 17** in the Experimental **Section VI**.

Chromatography plays a critical role in peptide mapping. Traditionally, a C18 column is utilized for peptide maps, but some laboratories prefer a C18 phase with a positive surface. Depending on the protein analyzed and the composition of the digested material, one option may outperform the other. When assessing the infliximab and biosimilars we found that the bioZen™ 2.6 µm Peptide XB-C18 LC column provided excellent separation of the digested material **(Figure 1, p. 5)**.

To assess similarity of the infliximab and its biosimilars, we explored the sequence coverage in terms of all matched peptides and auto-validated peptides **(Table 1, p. 5)**. When assessing the similarity, it is important to compare both heavy chain (HC) and light chain (LC). In these examples we observed high similarity for all matched peptides, but some variation was detected for the auto-validated peptides.

The peptide mapping results for infliximab and biosimilars require further scrutiny in the characterization process but give a starting point for primary structure confirmation. In the next sections we will explore other methods to further assess the similarity of these three proteins.

## Intact Mass - Top-Down mAb Analysis

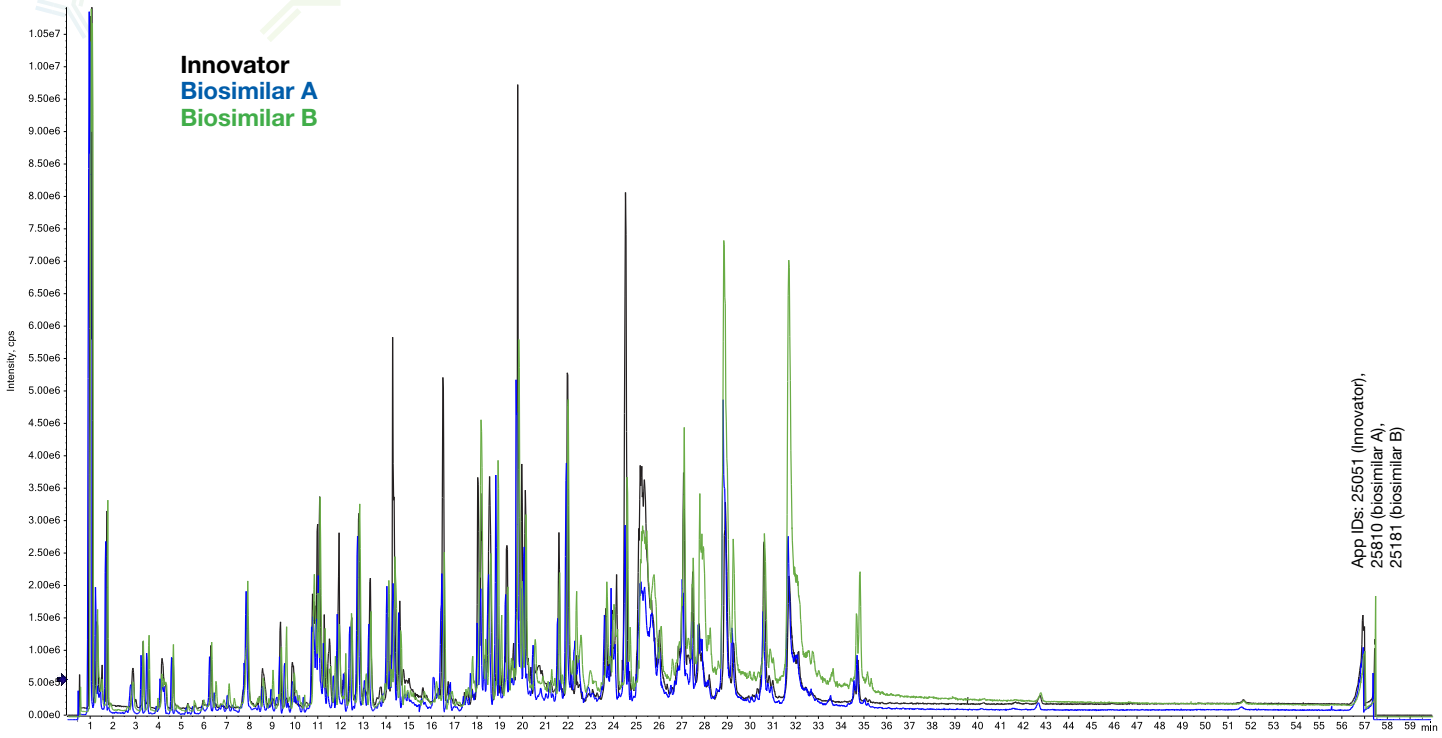
Mass spectrometry is a powerful tool for protein characterization and is often relied upon for primary structure analysis. Performing a reversed phase chromatographic analysis of intact proteins can give detailed information including amino acid sequence and can identify any separated isoforms that may exist. Top-down protein analysis is performed under denaturing mobile phase conditions but does not include any sample preparation (fragmentation or digestion).

The chromatographic portion of an intact mass analysis is critical to obtain good peak shape with minimal carryover. For most mAbs, Phenomenex recommends usage of the bioZen 2.6 µm WidePore C4 LC column for this analysis as it provides good peak shape and excellent MS data. In the case of infliximab, we observed the best MS data with the bioZen 3.6 µm Intact XB-C8. Both of these media are packed in bio-inert titanium (BioTi™) which is designed to mitigate any interactions of the protein with column hardware.

In our analysis of infliximab and biosimilars we generated spectrum at 5,000 resolution with a QTOF instrument. The total ion chromatograms (TICs) are shown in **Figure 2 (p. 6)**. These TICs indicate the subtle differences in chromatography when analyzing infliximab and biosimilars. Comparison of the theoretical mass with the measured mass shows close agreement with infliximab and some variation in measured mass for biosimilars A and B **(Table 2, p. 6)**. Finally, the mass spectra details on the deconvoluted glycoform identification is shown in **Table 3 (p. 7)**. All glycoform data shows reasonable accuracy with under 50 ppm error for each glycoform peak.

Further experimentation can be performed, such as middle-down analyses where the protein is reduced or fragmented. These analyses, typically done with DTT, IdeS, or papain, can provide better details on primary structure. In these cases, a more retentive column is ideal and the bioZen 3.6 µm Intact XB-C8 is the most suitable choice.

**Figure 1. TIC Peptide Mapping Traces for Infliximab and Biosimilars**




## Peptide Mapping Method

**Column:** bioZen 2.6 µm Peptide XB-C18

**Dimensions:** 150 x 2.1 mm

**Part No.:** 00F-4768-AN

**Mobile Phase:** A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Acetonitrile

**Gradient:**

| Time (min) | % B |
|---|---|
| 0 | 3 |
| 5 | 3 |
| 63 | 50 |

**Flow Rate:** 0.3 mL/min

**Column Temp:** 40 °C

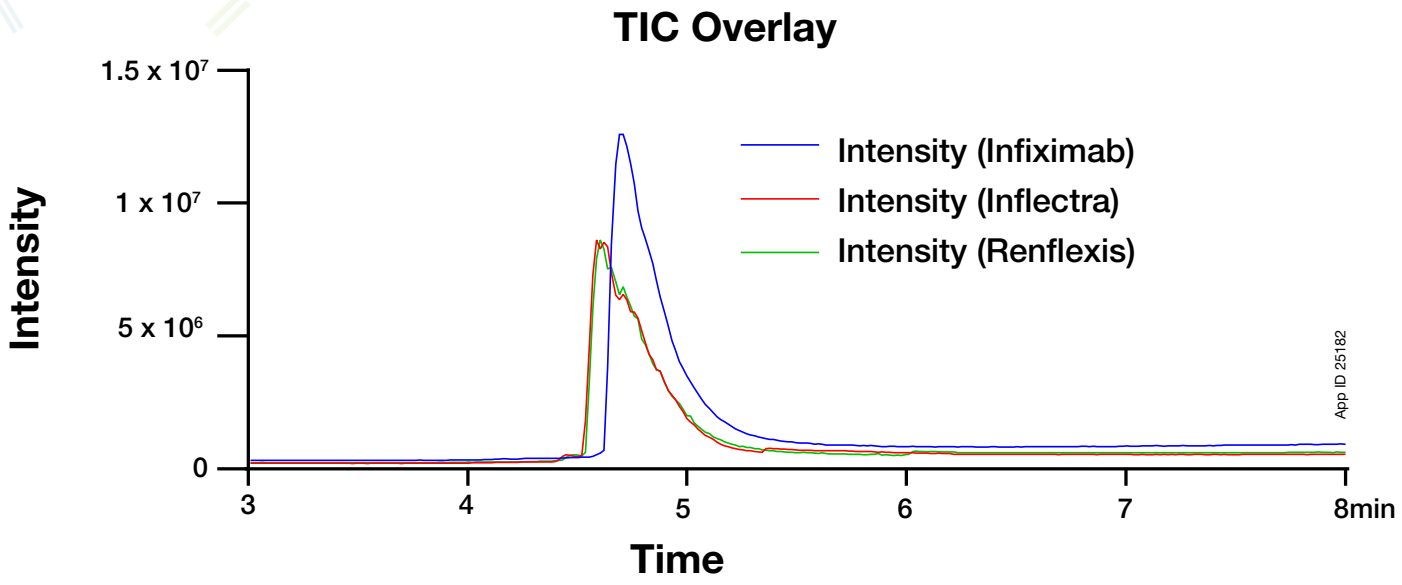
**Detector:** QTOF

**Table 1. Sequence Coverage Results for Peptide Maps**

| | All Matched Peptides | | | | | | Auto Validated Peptides | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | run 1 | | run 2 | | run 3 | | run 1 | | run 2 | | run 3 | |
| | LC | HC | LC | HC | LC | HC | LC | HC | LC | HC | LC | HC |
| Infliximab | 97.7 | 97.3 | 96.3 | 92.2 | 95.8 | 92 | 96.3 | 62.1 | 67.8 | 55.2 | 88.8 | 60.3 |
| Biosimilar A | 97.2 | 93.3 | 93.5 | 98.9 | 97.2 | 87.4 | 69.2 | 68.1 | 44.4 | 46.3 | 71.5 | 62.11 |
| Biosimilar B | 100 | 99.8 | 99.1 | 99.3 | 96.3 | 94 | 72.4 | 82 | 65.9 | 56.5 | 65.4 | 61.2 |

The sequence for the infliximab innovator was used for all peptide mapping identifications.

**Figure 2. Total Ion Chromatograms for Infliximab and Biosimilars**




## Intact Mass Method

**Column:** bioZen 3.6 µm Intact XB-C8
**Dimensions:** 150 x 2.1 mm
**Part No.:** 00F-4766-AN
**Mobile Phase:** A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Acetonitrile/IPA (50:50)

**Gradient:**

| Time (min) | % B |
|---|---|
| 0 | 20 |
| 1 | 20 |
| 10 | 60 |

**Flow Rate:** 0.3 mL/min
**Column Temp:** 90 °C
**Detector:** QTOF

**Table 2. Theoretical versus Measured Mass of Infliximab and Biosimilars**

| | Theoretical | Measured | Measured | Measured | Delta Mass (Da) Average |
|---|---|---|---|---|---|
| Innovator | 148515 | 148516.8 | 148516.7 | 148515.7 | -1.39667 |
| Biosimilar A | 148515 | 148528.4 | 148519.9 | 148519.2 | -7.51167 |
| Biosimilar B | 148515 | 148675.3 | 148676.9 | 148678.2 | -161.797 |

**Table 3. Glycoform Identification after Deconvolution**

| Infliximab | | | | |
|---|---|---|---|---|
| **Glycoform** | **Theoretical Mass** | **Observed Mass** | **Delta m** | **ppm error** |
| G0F/G0F | 148517 | 148515.7 | -1.3 | 8.8 |
| G0F/G1F | 148679 | 148676.3 | -2.7 | 18.2 |
| G0F/G0F +NANA | 148773 | 148773.2 | 0.2 | 1.3 |
| G1F/G2F+NANA | 148935 | 148933.2 | -1.8 | 12.1 |
| G2F/G2F | 149097 | 149093.3 | -3.7 | 24.8 |
| | | | | |
| **Biosimilar A** | | | | |
| Glycoform | **Theoretical Mass** | **Observed Mass** | **Delta m** | **ppm error** |
| G0F/G0F | 148517 | 148516.5 | -0.5 | 3.4 |
| G0F/G1F | 148679 | 148674.6 | -4.4 | 29.6 |
| G0F/G1F+K | 148808 | 148805 | -3 | 20.2 |
| G1F/G1F, G2F/G0F | 148841 | 148834.8 | -6.2 | 41.7 |
| G1F/G1F, G2F/G0F+K | 148970 | 148964.5 | -5.5 | 36.9 |
| | | | | |
| **Biosimilar B** | | | | |
| **Glycoform** | **Theoretical Mass** | **Observed Mass** | **Delta m** | **ppm error** |
| G0F/G0F | 148517 | 148518.9 | 1.9 | 12.8 |
| G0F/G1F | 148679 | 148678.5 | -0.5 | 3.4 |
| G1F/G1F, G2F/G0F | 148841 | 148840 | -1 | 6.7 |
| G1F/G2F | 149003 | 149001.1 | -1.9 | 12.8 |

# III. Higher Order Structure

## Native Protein Analysis

The higher order structure (secondary, tertiary, and quaternary structures) of a protein must be assessed during therapeutic development and various analytical techniques are available to acquire the appropriate data. Most commonly, high resolution techniques including nuclear magnetic resonance (NMR)[8] and X-ray crystallography[9] are deployed as the most powerful tools to acquire the finest of details at the atomic level.

In the above high resolution analyses the protein is not denatured and kept in its native state which provides information on the overall structure of the protein. Chromatographic analyses that are employed for native state protein assessment are size exclusion chromatograph (SEC)[10] and ion-exchange chromatography (IEX),[11] which are used to determine aggregation and charge variants, respectively. This section will cover these two chromatographic methods in the analysis of infliximab and biosimilars.

### Aggregate Analysis by Size Exclusion Chromatography (SEC)

The aggregation of mAbs has therapeutic implications such as safety, efficacy and immunogenicity making it a critical quality attribute to assess during characterization.[12] Determination of high molecular weight (HMW) aggregate and low molecular weight (LMW) fragments and subunits is required throughout therapeutic development. SEC is the most common method to assess aggregation of proteins, but orthogonal methods such as sedimentation velocity analytical ultracentrifugation (svAUC)[13] is also possible.

Monoclonal antibodies are usually immunoglobulin G (IgG) species giving them a molecular weight of 150 kDa. However, when performing SEC, the separation depends on the hydrodynamic volume of the analyte. This means that the radius of the aggregate and monomer needs to be taken into account for proper SEC particle selection to ensure the pore size can host the aggregated species and ultimately provide separation from the monomer mAb. Moreover, modernization of SEC particle technology to sub-2 µm particles allows analysis on ultra-high performance liquid chromatography (UHPLC) instruments.

With the above considerations, we analyzed infliximab and biosimilars on a bioZen™ 1.8 µm SEC-3 column. This column has a 300 Å pore size and the media is packed into BioTi™ column hardware. This titanium hardware is particularly useful for minimization of priming effects of proteins and limits any non-specific secondary interactions of the mAb with the column.[14] Under isocratic conditions, we obtained the chromatograms in **Figure 3, p. 9**. The infliximab innovator provided >99 % monomer, whereas biosimilar A and B provided 95 % and 91 % monomer, respectively **(Table 4, p. 9)**.

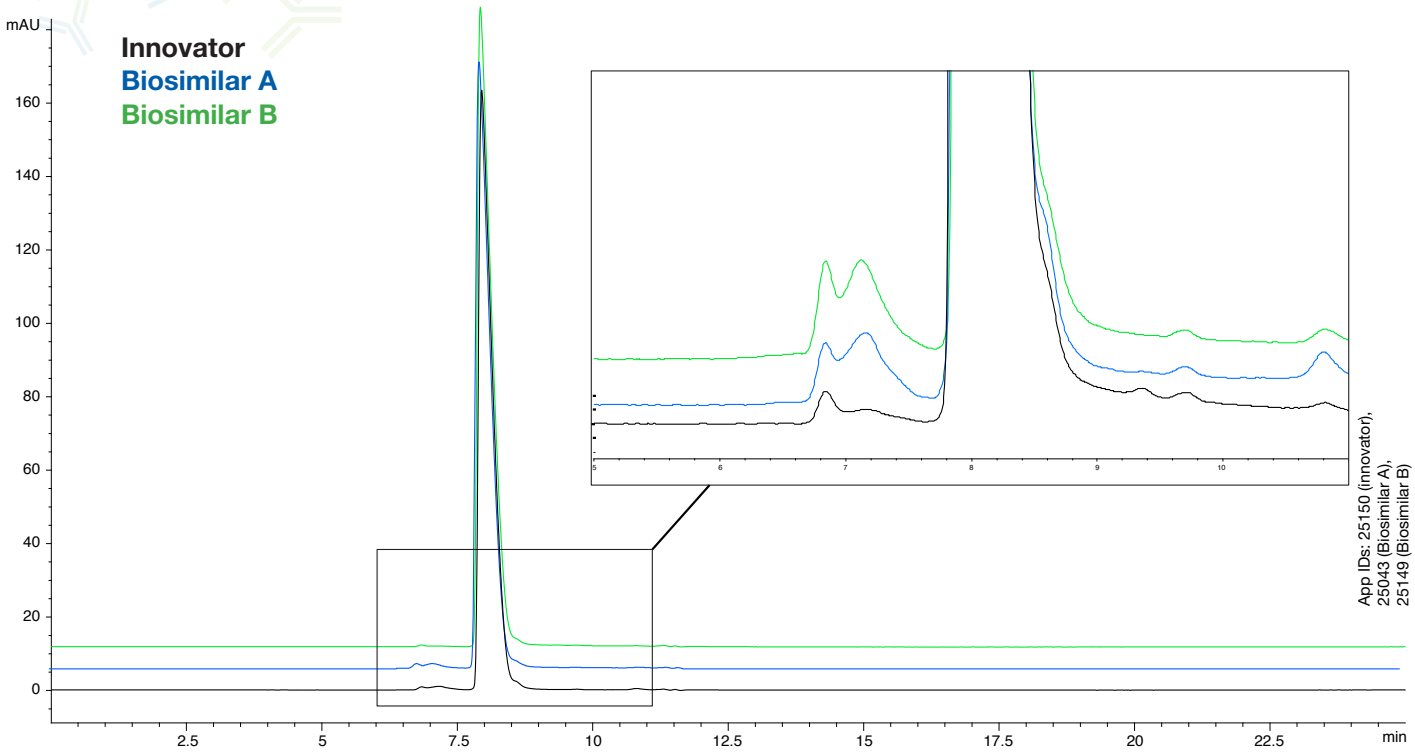
### Charge Variant Analysis by Ion-Exchange Chromatography (IEX)

All monoclonal antibodies have accessible amino acid side chains that create an overall surface charge on the molecule. Changes in the surface charge influence the isoelectric point (pI) of the protein which can impact its therapeutic effects.[11] Therefore, it is a critical quality attribute that is mandated by regulators to assess during development. While not commonly grouped under higher order structure determination, charge variant analysis (CVA) provides quaternary structure information and, chromatographically, is performed under native conditions.

The recombinant production of monoclonal antibodies generates chemical modifications to amino acids including deamidation, oxidation, and C-terminal lysine truncation. As a result, the acidic and basic variants of the protein are produced. Two common methods to analyze charge heterogeneity are ion-exchange chromatography (IEX)[11] and imaging capillary electrophoresis (iCE).[15] These complementary techniques are used throughout development of biotherapeutics with both providing valuable information on the acidic and basic charge variants.

The chromatographic profiling of charge variant isoforms by IEX can be performed with either anionic or cationic media. Cation-exchange chromatography (CEX) utilizes anionic media; weak cation-exchange (WCX) employs a carboxylate phase while strong cation-exchange (SCX) uses a sulfonate phase. Alternatively, anion-exchange chromatography (AEX) utilizes cationic media weak anion-exchange (WAX) contains an amino phase and strong anion-exchange (SAX) has a quaternary ammonium phase. The pI of the protein analyte will determine which type of IEX to perform. In general, more basic mAbs with a pI greater than 6, will utilize CEX because the surface charge of the protein is appropriately cationic at a lower pH. Weak cation-exchange is utilized more than strong cation-exchange as it can offer similar resolution with lower backpressure. Finally, it should be noted that for CVA by IEX, it is critical that column hardware is bio-inert. Most column vendors employ a bio-inert polyether ether ketone (PEEK) hardware, which provides excellent recovery

**Figure 3. Aggregate Analysis of Infliximab and Biosimilars**




but is less amenable to UHPLC conditions due to backpressure limitations.

With the above considerations in mind, Phenomenex built the bioZen™ 6 µm WCX column, which is packed in BioTi™ bio-inert titanium hardware. This non-porous polymeric particle is specially grafted with polycarboxylate stationary phase that provides excellent resolution of charge variants. Moreover, the titanium hardware does not have the pressure limitations of PEEK and can be run at much higher flow rates as a result. We performed CVA on infliximab and biosimilars with this column at optimal reduced linear velocity of 1 mL/min. Following the lead of Genentech, a pH gradient was employed to generate the results in **Figure 4 (p. 10, pH Gradient).** Notably, these mobile phase conditions provided 14 % acidic and 47 % basic variants for the innovator biologic and biosimilar A having much different results with 33 % acidic and 9 % basic variants. Modifying the mobile phase to a salt gradient provided the data in **Figure 4 (p. 10, Salt Gradient**. Consistent results to the pH gradient mobile phase were observed, demonstrating the versatility in mobile phases for these analyses **(Table 5, p. 10).**

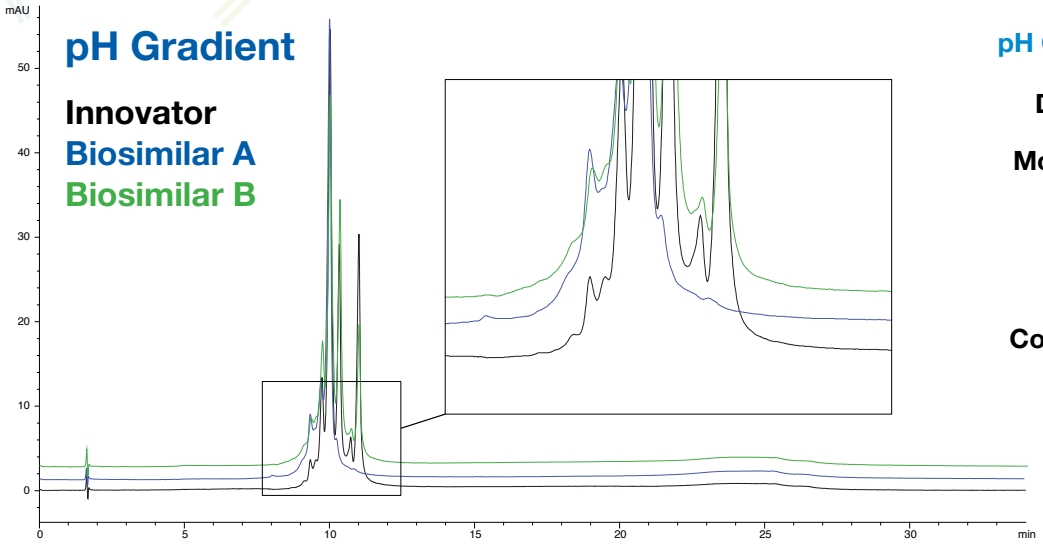
## Size Exclusion (SEC) Method

**Column:** bioZen 1.8 µm SEC-3
**Dimensions:** 300 x 4.6 mm
**Part No.:** 00H-4772-E0
**Mobile Phase:** 50 mM Potassium phosphate, 250 mM Potassium chloride pH 6.8
**Gradient:** Isocratic
**Run time:** 30 min
**Flow Rate:** 0.3 mL/min
**Column Temp:** Ambient
**Detector:** Agilent® 1260

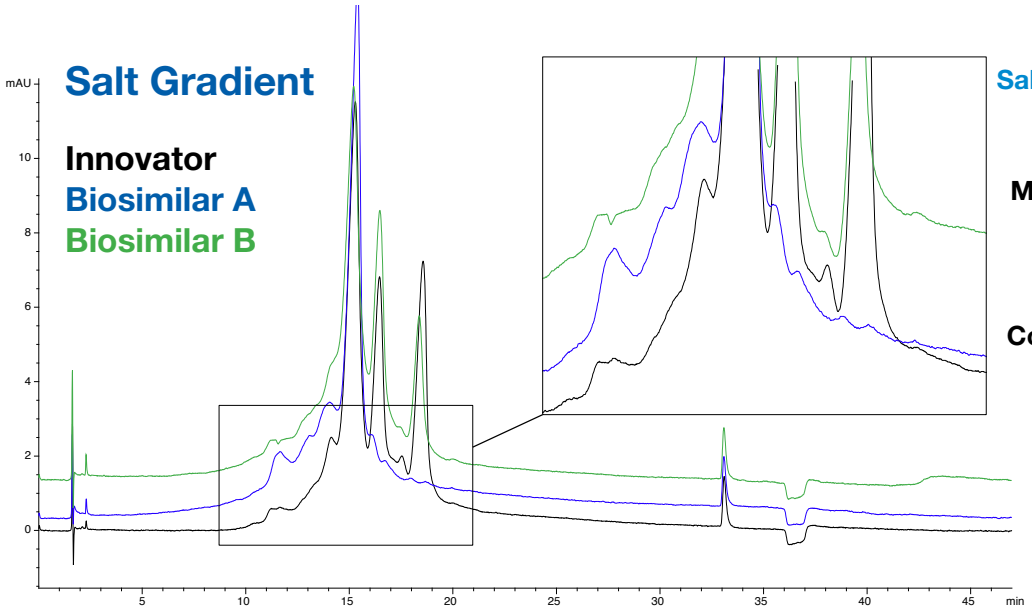
**Table 4. Percent Monomer of Infliximab and Biosimilars from SEC Analysis**

| | % Monomer |
|---|---|
| Infliximab | 100 |
| Biosimilar A | 99 |
| Biosimilar B | 91 |

**Figure 4. Charge Variant Analysis of Infliximab and Biosimilars using pH and Salt Gradient**




**pH Gradient**
**Column:** bioZen™ 6 µm WCX
**Dimensions:** 250 x 4.6 mm
**Part No.:** 00G-4777-E0
**Mobile Phase:** A: CX-1 Gradient A Buffer (pH 5.6)
B: CX-1 Gradient B Buffer (pH 10.2)
**Gradient:** 0-100 % B in 20 min
**Flow Rate:** 1mL/min
**Column Temp:** 30 °C
**Detector:** UV @ 280 nm




**Salt Gradient**
**Column:** bioZen 6 µm WCX
**Dimensions:** 250 x 4.6 mm
**Part No.:** 00G-4777-E0
**Mobile Phase:** A: 20 mM MES (pH 5.6)
B: 20 mM MES, 0.3 M NaCl (pH 5.6)
**Gradient:** 20-50 % B in 30 min
**Flow Rate:** 1mL/min
**Column Temp:** 30 °C
**Detector:** UV @ 280 nm

**Table 5. Analysis of Acidic and Basic Charge Variants from WCX Experiments**

## pH Gradient

| | % Acidic | Neutral | % Basic |
|---|---|---|---|
| Innovator | 14 | 39 | 47 |
| Biosimilar A | 33 | 58 | 9 |
| Biosimilar B | 23 | 35 | 42 |

## Salt Gradient

| | % Acidic | Neutral | % Basic |
|---|---|---|---|
| Innovator | 14 | 37 | 49 |
| Biosimilar A | 31 | 60 | 9 |
| Biosimilar B | 21 | 40 | 39 |

# IV. Enzymatic Post-translational Modifications (PTMs)

Monoclonal antibodies are recombinantly generated through cellular production and as a result, minor changes in conditions and cell function influence the final mAb product structure.[16] These PTMs come in a variety of forms including glycosylation, deamidation, oxidation, lysine clipping, and phosphorylation. The PTMs can influence the efficacy of the mAb and have safety implications, which is why individual PTMs are found as CQAs in biotherapeutic development. Many chromatographic methods are capable of monitoring PTMs, some of which were included in previous sections. In this section we take a closer look at glycosylation and the details that can be observed in peptide mapping.

## Glycosylation

Monoclonal antibody function is dramatically influenced by glycosylation and therefore is a critical quality attribute that must be assessed in therapeutic development.[17] Specifically, some key glycosylation profiles that impact mAb function are:

1. ADCC (Antibody Dependent Cellular Cytotoxicity) - where the effector cell lyses the target cell, is heavily influenced by core fucosylation. Removal of fucose will increase ADCC activity.
2. Presence of galactose (i.e. “galactosylated”) glycans affect both cell dependent cytotoxicity or CDC and increase clearance
3. High mannose glycans increase clearance considerably
4. The presence of the terminal sialic acid n-glycoylneuraminic acid increases immunogenicity
5. Gallili antigen or the gal alpha gal, is an epitope present in the carbohydrate region in many non-primate mammals and elicits an immunogenic response. Anti-gal is the most abundant antibody in humans, accounting for 1 % of immunoglobulins.

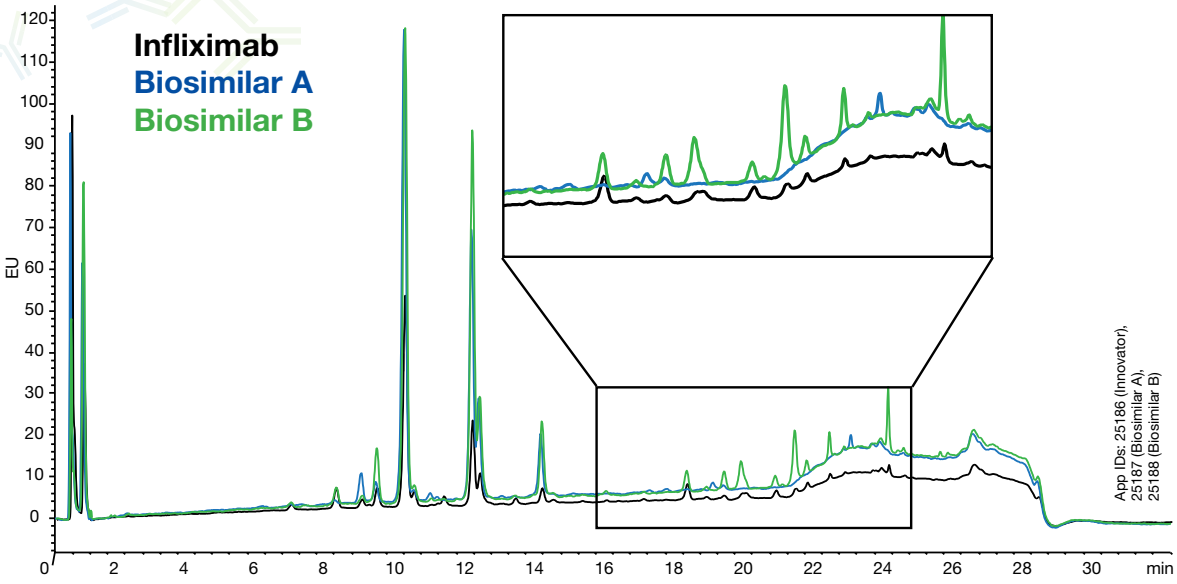
Because of these implications, the characterization of glycosylation on the innovator mAb along with the follow-on biologic is critical.

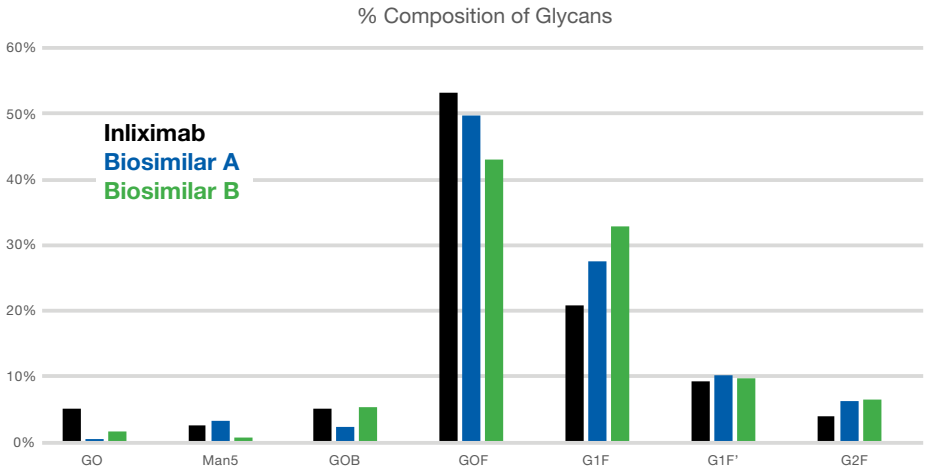
Methods to analyze N-glycans include analysis of released glycans and glycopeptides[18] or the analysis of intact or fragmented proteins.[19] The latter option is capable of determining glycan content along with site occupancy of the glycans, however, it requires much more rigorous data interpretation and there are no universal methods to perform this analysis. Therefore, most scientists opt for the released N-glycan process because it offers generality and can be performed in high throughput environments.

Performing released N-glycan analysis requires an enzyme to cleave the glycans from the glycoprotein followed by labeling with a fluorophore and subsequent analysis by a fluorescence detector (FLD) or MS instrument. Traditionally, the labeling reagent for this workflow is incorporated through a reductive amination of the released N-glycan with an activated aniline, such as 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA) or procainamide. More recent advances to accelerate the labeling step proceed through reaction with an activated carbamate (InstantPC™, for example) to generate a more stable urea. In addition, these newer reagents contain a tertiary amine rendering them capable of MS analysis. Hydrophilic interaction chromatography (HILIC) is then performed to separate the labeled N-glycans.

During our analysis of infliximab and biosimilars we opted for labeling with InstantPC followed by HILIC analysis on a bioZen™ 2.6 µm Glycan column. To cleave the glycans from the protein, PNGase F was employed followed by a clean-up step. Subsequent labeling with InstantPC and label clean up with a HILIC solid phase extraction tube provided the appropriate material for analysis. Subjection to the bioZen Glycan column provided the chromatograms in **Figure 5 (p. 12)**.

**Figure 5. Released N-Glycan Analysis by HILIC**




The major species for each of the infliximab antibodies are G0F, G1F and G2F

## Glycan Method

**Column:** bioZen™ 2.6 µm Glycan
**Dimensions:** 100 x 2.1 mm
**Part No.:** 00D-4773-AN
**Mobile Phase:** A: 100 mM Ammonium formate pH 4.5
B: Acetonitrile

**Gradient:**

| Time (min) | % B |
|---|---|
| 0 | 78 |
| 20 | 65 |
| 26 | 40 |

**Flow Rate:** 0.5 mL/min
**Column Temp:** 50 °C
**Detector:** ACQUITY® H-Class

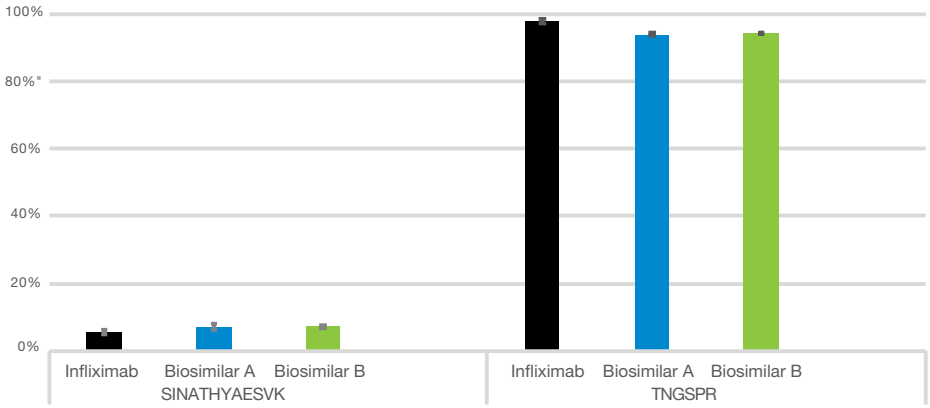
## Deamidation and C-Terminal Lysine Clipping

The deamidation of asparagine (Asn) residues on a mAb is a post-translational modification that can lead to less effective binding and altered pharmacokinetic (PK) data.[20] Typically occurring in the complementary determining regions (CDRs), this PTM generates a negatively charged aspartate (Asp) or isoaspartate (isoAsp) and can be monitored by IEX as described previously because it is an acidic variant. Confirmation of the deamidation can be performed through peptide mapping analysis of specific peptide chains. In the case of infliximab and biosimilars, we observed minimal variation (less than 5 %) for deamidation across the innovator and follow-on products **(Figure 6, top)**.

C-terminal lysines play an important role in mAb function and their cleavage is another PTM that is closely monitored in biotherapeutic development. Most often analyzed by IEX, confirmation of structure can be determined through peptide mapping. In the case of infliximab and biosimilars, we observed nearly 100 % lysine cleavage on biosimilar A with infliximab and biosimilar B giving less than 70 % cleavage **(Figure 6, bottom)**.

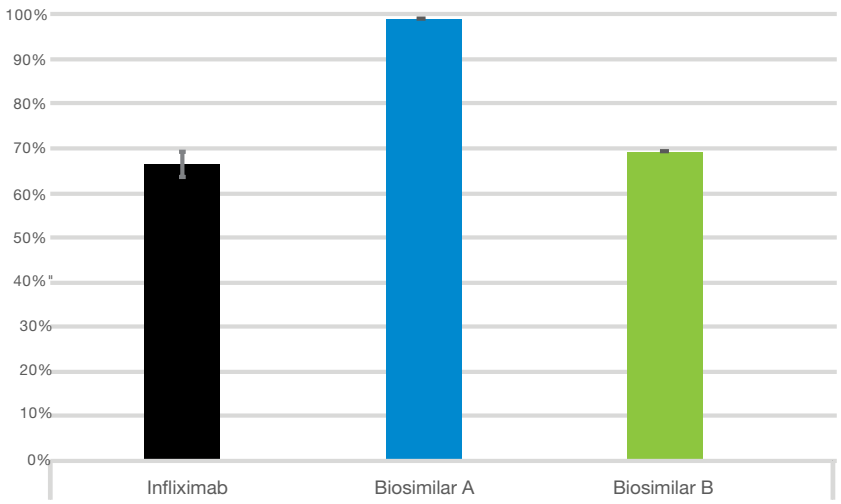
**Figure 6. Deamidation and Lysine Cleavage Results for Infliximab and Biosimilars**

### Asparagine Deamidation




Deamidation levels did not have more than 5% variation.

### Lysine Cleavage



## Methionine Oxidation

One critical PTM that influences factors such as binding and serum half-life is methionine oxidation.[21] UHPLC-MS/MS is capable of determining the extent of methionine oxidation at low levels through peptide mapping. In our analysis of infliximab and biosimilars we monitored three peptide chains containing methionine. In each of these analyses, variation between the innovator and biosimilars did not exceed 15 % **(Figure 7)**.

**Figure 7. Methionine Oxidation MS/MS Data and Comparative Results**

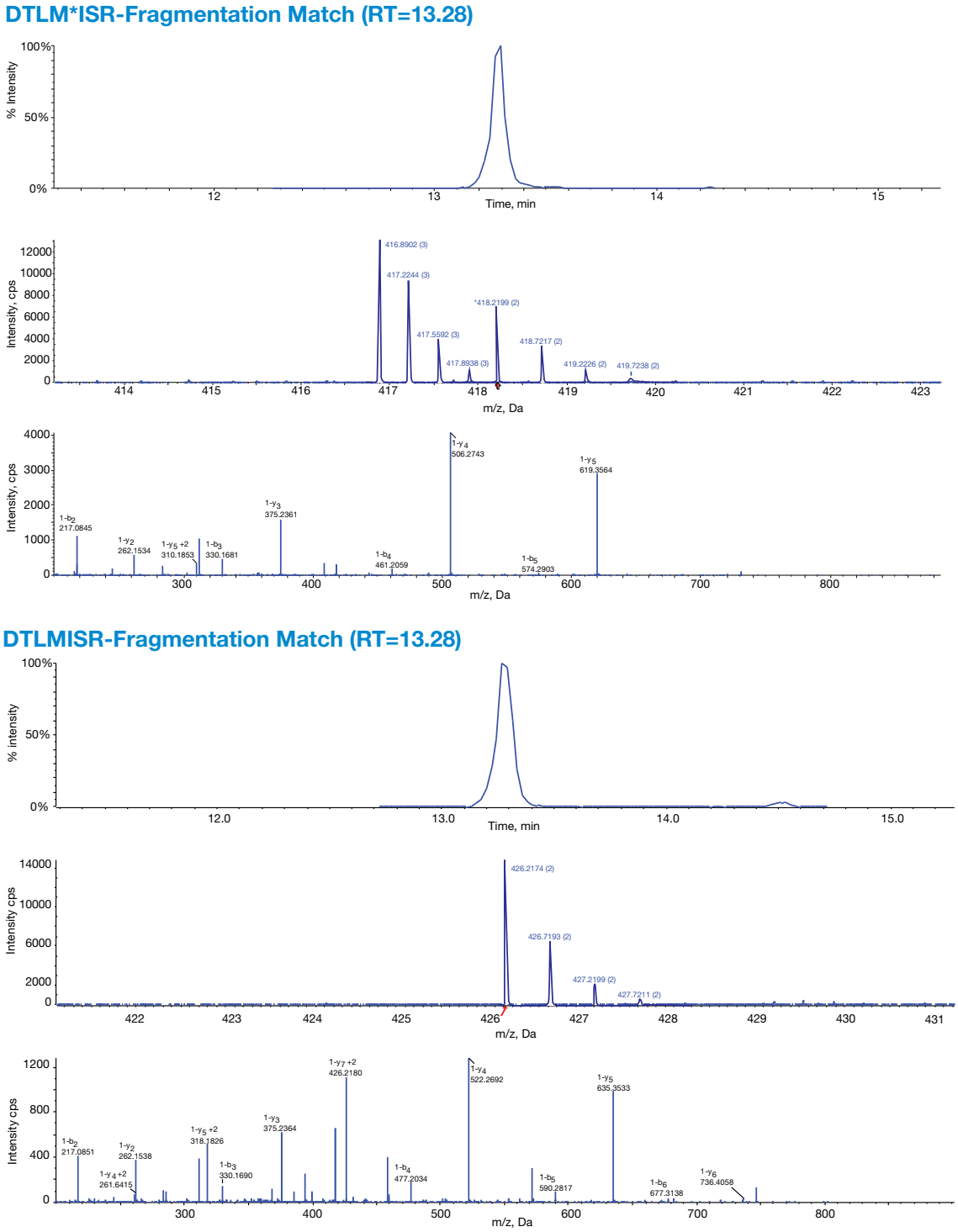
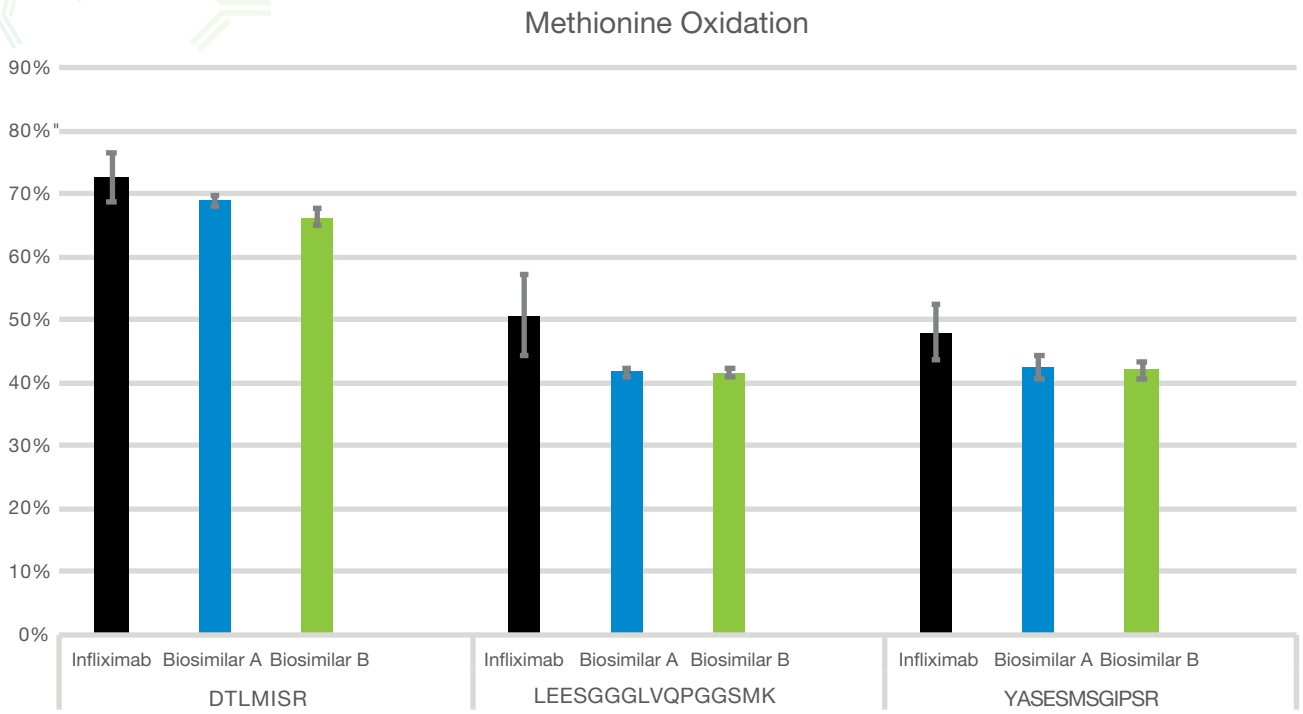


**Figure 7. Methionine Oxidation MS/MS Data and Comparative Results (*continued*)**




Oxidation did not vary more than 15 %

# V. Conclusion

Biosimilars will continue to dominate biopharmaceutical pipelines for the foreseeable future. As new modalities emerge as therapeutic agents, the analytical demand for characterization may increase. Moreover, regulations will evolve as more evidence is gathered and presented at forums such as the ICH conferences. Therapeutic comparability is critical when proposing a new drug application and harmonization on how to best demonstrate comparability is essential.

Chromatography is an indispensable tool for analytical scientists to probe the structural features of biological molecules. As methods progress toward more sophisticated techniques, solutions providers will adapt to create novel technologies to support the industry. Phenomenex bioZen™ columns are only one example of the new advances available to bolster analytical development methods. Their utility in the characterization of infliximab and biosimilars shows the broad applicability to structural analysis of biosimilars. Choosing the right technologies to generate appropriate data for therapeutic development is a must. Opting for chromatography columns designed specifically for these modalities is a good starting point.

# VI. Experimental Section

## General

Dithiothreitol (DTT), iodoacetamide (IAA), MES hydrate, sodium phosphate monobasic, sodium phosphate dibasic, sodium sulfate, guanidine, ammonium bicarbonate were purchased from Sigma-Aldrich® (St. Louis, MO). Trypsin Gold was purchased from Promega® (Madison, WI). Sodium Chloride was purchased from VWR® (Radnor, PA). Sodium Hydroxide, dPBS, Optima LC-MS grade water, Optima LC-MS grade acetonitrile, Optima LC-MS grade isopropanol were purchased from Thermo Fisher® (Hampton, NH). pH gradient buffers (CX-1 & CX-2) were purchase from Thermo Fisher (Hampton, NH). Infliximab and biosimilars were purchased from Myoderm (Norristown, PA). Peptide mapping and intact mass was done on an Agilent® 1290 II coupled to a QTOF. Glycan mapping was done on a Waters® ACQUITY® H-Class.

For all applications, bioZen™ LC columns were used from Phenomenex® (Torrance, CA).

# Ordering Information bioZen

| bioZen Columns (mm) | | | | | | Biocompatible Guard Cartridges | | |
|---|---|---|---|---|---|---|---|---|
| | 50 x 2.1 | 100 x 2.1 | 150 x 2.1 | 50 x 4.6 | 150 x 4.6 | for 2.1 mm | for 4.6 mm | Holder |
| | | | | | | /3pk | | ea |
| bioZen 2.6 µm Glycan | 00B-4773-AN | 00D-4773-AN | 00F-4773-AN | — | — | AJ0-9800 | — | AJ0-9000 |
| | | | | | | /3pk | | ea |
| bioZen 1.6 µm Peptide PS-C18 | 00B-4770-AN | 00D-4770-AN | 00F-4770-AN | — | — | AJ0-9803 | — | AJ0-9000 |
| | | | | | | /10pk | /10pk | ea |
| bioZen 3 µm Peptide PS-C18 | 00B-4771-AN | — | 00F-4771-AN | 00B-4771-E0 | 00F-4771-E0 | AJ0-7605 | AJ0-7606 | KJ0-4282 |
| | | | | | | /3pk | | ea |
| bioZen 1.7 µm Peptide XB-C18 | 00B-4774-AN | 00D-4774-AN | 00F-4774-AN | — | — | AJ0-9806 | — | AJ0-9000 |
| | | | | | | /3pk | /3pk | ea |
| bioZen 2.6 µm Peptide XB-C18 | 00B-4768-AN | 00D-4768-AN | 00F-4768-AN | 00B-4768-E0 | 00F-4768-E0 | AJ0-9806 | AJ0-9808 | AJ0-9000 |
| | | | | | | /3pk | /3pk | ea |
| bioZen 2.6 µm WidePore C4 | 00B-4786-AN | 00D-4786-AN | 00F-4786-AN | 00B-4786-E0 | 00F-4786-E0 | AJ0-9809 | AJ0-9811 | AJ0-9000 |
| bioZen 3.6 µm Intact XB-C8 | 00B-4766-AN | 00D-4766-AN | 00F-4766-AN | 00B-4766-E0 | 00F-4766-E0 | AJ0-9812 | AJ0-9814 | AJ0-9000 |

| | 50 x 2.1 | 100 x 2.1 | 150 x 2.1 | 250 x 2.1 | 50 x 4.6 | 100 x 4.6 | 150 x 4.6 | 250 x 4.6 | 300 x 4.6 | for 4.6 mm | Holder |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | /3pk | ea |
| bioZen 1.8 µm SEC-2 | 00B-4769-AN | — | 00F-4769-AN | — | — | — | 00F-4769-E0 | — | 00H-4769-E0 | AJ0-9850 | AJ0-9000 |
| bioZen 1.8 µm SEC-3 | 00B-4772-AN | — | 00F-4772-AN | — | — | 00D-4772-E0 | 00F-4772-E0 | — | 00H-4772-E0 | AJ0-9851 | AJ0-9000 |
| | | | | | | | | | | for 4.6 mm | Holder |
| | | | | | | | | | | /10pk | ea |
| bioZen 6 µm WCX | 00B-4777-AN | 00D-4777-AN | 00F-4777-AN | 00G-4777-AN | 00B-4777-E0 | 00D-4777-E0 | 00F-4777-E0 | 00G-4777-E0 | — | AJ0-9400 | KJ0-4282 |

### Sample Preparation

| bioZen Solid Phase Extraction | Format | Sorbent Mass | Part No. | Unit |
|---|---|---|---|---|
| bioZen N-Glycan Clean-Up | Microelution 96-Well Plate | 5 mg/well | 8M-S009-NGA | 1/box |


### bioZen MagBeads

| Coating | Formats | Part No. |
|---|---|---|
| Streptavidin | 25 mg (1.25 mL) | KS0-9531 |
| | 50 mg (2.50 mL) | KS0-9532 |
| | 500 mg (25 mL) | KS0-9533 |

## Peptide Mapping

**Sample Preparation:**

100 µg of antibody was buffer exchanged with PBS. It was then diluted 1:1 with 5 M Guanidine. To that, stock, DTT was added (1:10) and incubated at 56 °C for 30 min. Then stock IAM was added (2x the amount of DTT) and incubated in the dark for 45 min. The reduced and alkylated mixture was then buffer exchanged into PBS. Trypsin was then added (1:20 trypsin/antibody) and incubated at 37 °C overnight. The tryptically digested samples were then placed in a speedvac until dry. The pellet was resuspended with mobile phase A and a total of 2 µg was injected onto a bioZen™ 2.6 µm Peptide XB-C18 column.

## Intact Mass

**Sample Preparation:**

Antibodies were diluted to 1 mg/mL with dPBS.

Antibody (3 µg) was injected directly on a bioZen 2.6 µm WidePore C4 column.

## Size Exclusion Chromatography (SEC)

**Buffer Preparation:**

Make stock solutions of 0.5 M sodium phosphate monobasic, 0.5 M sodium phosphate dibasic and 1 M sodium sulfate. Filter each of the stock solutions with a 0.2 µm membrane filter to remove any particulates. In a 1 L graduated cylinder, add 250 mL of 1 M sodium sulfate, then add 600 mL HPLC water or Milli-Q® water. Add a total of 100 mL of both sodium phosphate stock solutions to a pH of 6.8. Then add 50 mL HPLC or Milli-Q water. The final mobile phase was filtered using a 0.2 µm membrane filter to remove any particulates.

Antibody (20 µg) was injected directly on a bioZen 1.8 µm SEC-3 column.

## Weak Cation-Exchange Chromatography

**Sample Preparation:**

Salt Buffer Preparation: (20 mM MES pH 6.0) - 7.81 ± 0.01 g MES hydrate (MW 195.24) was added to a clean 2 L volumetric flask. To this 1.6 L HPLC grade water was also added. Using a magnetic stir bar, the solution was stirred until all solids were dissolved. Using a pH meter, the pH of the solution was measured and adjusted to pH 6.0 ± 0.1 with sodium hydroxide (approximately 16 mL). A final volume of 2.0 L was made up by adding HPLC grade water. The final mobile phase was filtered using a 0.2 µm membrane filter to remove any particulates.

(20 mM MES + 300 mM NaCl pH 6.0) - 7.81 ± 0.01 g MES hydrate (MW 195.24) and 35.06 ± 0.01g NaCl (MW 58.44) was added to a clean 2 L volumetric flask. To this, 1.6 L HPLC grade water was also added. Using a magnetic stir bar, the solution was stirred until all solids were dissolved. The final mobile phase was filtered using a 0.2 µm membrane filter to remove any particulates.

Antibody (30 µg) was injected directly on a bioZen 6 µm WCX column.

## Glycan Analysis

**Sample Preparation:**

To prepare infliximab and biosimilars for chromatographic analysis, the Gly-X™ N-Glycan Rapid Release and Labeling with InstantPC™ kit from ProZyme® was utilized. The protein was first denatured with the denaturation reagent provided and incubated at 90 °C for 3 min. Subsequent enzymatic digestion with N-Glycanase® (PNGase F) at 50 °C for 5 min provided the released glycans. Immediate labeling with InstantPC dye for 1 min at 50 °C followed by clean-up through a Gly-X clean-up plate provided the labeled N-glycans. The bioZen N-Glycan Clean-Up plate (Phenomenex, Part No.: 8M-S009-NGA) can be used as an alternative 96-well clean-up plate within this method. 1 µL of the elution from the glycan clean-up plate was injected on a bioZen 2.6 µm Glycan column.

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# Biosimilars Chromatographic Characterization

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