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Application Note

Applying Peptide Mapping and Multi-Attribute Method (MAM) Workflow for Biosimilar mAb Drug Products Comparison on the Xevo™ G3 QTof Platform

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Abstract

With the increasing emergence of biosimilar monoclonal antibodies (mAbs), streamlined processes for characterizing these products are highly desirable. Instruments that can provide data to confidently identify and quantify peptide attributes, as well as data analysis pipelines that can expedite and automate analysis while maintaining compliance are essential to acquiring results in a fast-paced, growing market. The new Xevo G3 QTof Platform coupled with the ACQUITY™ Premier UPLC™ System enables robust and reliable analysis of biotherapeutic proteins. The app-based compliance-ready waters_connect™ informatics platform enables streamlined data management from acquisition to analysis. These integrated tools enable efficient biopharmaceutical workflows to be developed, including those for characterization and attribute monitoring.

This study demonstrates how waters_connect can be used with the Xevo G3 QTof for biosimilar mAb characterization and monitoring. The peptide maps of four infliximab samples, including the innovator and three biosimilars, were analyzed for product attributes, including deamidation, oxidation, lysine clipping, and

glycosylation. Differences in their relative abundance between mAbs were quantified using the Peptide MAM App within the compliance-ready waters_connect Software. A stress study of targeted attributes was also carried out for the innovator and one biosimilar to identify critical quality attributes (CQAs) from thermal stress for each mAb. The results showcase how the Xevo G3 QTof Platform with integrated waters_connect data acquisition and processing is a suitable platform for comparative peptide mapping workflows to establish mAb biosimilarity.

Benefits

- High-coverage peptide mapping of innovator and biosimilar mAbs with confident identification and quantification of peptide attributes
- Integrated compliance-ready app-based workflow for streamlined acquiring, processing, and reviewing of peptide mapping data
- Reproducible quantification of low-level peptide attributes for establishing biosimilarity

Introduction

As the patents for monoclonal antibodies (mAbs) expire, an increasing number of biosimilars are being approved by regulatory agencies. With this increasingly competitive market for biosimilar drug products, there is a need to streamline processes to characterize and compare biosimilars to the innovator drug products. Characterizing biosimilars requires a workflow that can confidently identify and quantify various product attributes key to safety, efficacy, and stability. Biosimilar products are highly similar to the active ingredient in the innovator product, but their properties can differ due to differences in production methods as long as those differences generate no clinically meaningful impacts. One of the prominent routes to characterizing and comparing biosimilars is using peptide mapping to analyze post-translational modifications. Enzymatically digesting a mAb and analyzing the peptide fragments with liquid chromatography (LC)-mass spectrometry (MS) enables confirmation of the primary sequence and localization of sites of product variation. The Multi-Attribute Method (MAM) has been gaining increasing popularity to quantify targeted attributes over multiple samples with higher throughput than traditional characterization approaches to data analysis. Rather than using multiple orthogonal analytical techniques for individual attributes, MAM offers the ability to directly monitor numerous attributes across samples using LC-MS.[1]

The Xevo G3 QTof Mass Spectrometer operated under the waters_connect informatics platform (Figure 1) provides a streamlined solution for executing biopharmaceutical workflows. With its updated ion optics to optimize peptide transmission and comprehensive quantification capabilities, the Xevo G3 QTof is fit for purpose for both characterization and monitoring of biotherapeutic attributes. The compliance-ready waters_connect platform handled the entire workflow from sample submission through data analysis and review, and the integrated UNIFI™ App (peptide mapping), scientific library (attribute libraries), and Peptide MAM App (targeted attribute monitoring and new peak detection) enabled a seamless harmonization of characterization and monitoring workflows. This study demonstrates the effectiveness of this combined workflow for peptide map characterization and monitoring of infliximab and three biosimilar drug products (Inflectra®, Avsola®, and Renflexis®). Peptide maps were compared across the four mAbs, and critical quality attributes (CQAs) were identified and reproducibly monitored for the innovator and one of the biosimilars in a stress study.

Figure 1. Xevo G3 QTof platform with integrated waters_connect informatics for compliance-ready app-based data acquisition, processing, review, and reporting.

Experimental

Sample Description

Infliximab samples, including innovator (Remicade®) and biosimilar (Inflectra) were incubated at 37 °C for zero weeks (no stress), one week, or two weeks. All samples, including the two other biosimilars (Avsola and Renflexis), were reduced, alkylated, desalted, tryptic digested, and acidified to 0.1% formic acid. The final concentration was measured to be 0.16 µg/µL.

LC Conditions

LC system:	ACQUITY Premier UPLC System–BSM configuration
Detection:	ACQUITY Premier TUV; 10 mm analytical flow cell; λ = 214 nm
Vials:	QuanRecovery™ with MaxPeak™ HPS Vials (p/n: 186009186)
Column(s):	ACQUITY Premier CSH™ 130 Å C18 1.7 µm, 2.1 × 100 mm
Column temperature:	60 °C
Sample emperature:	8 °C
Injection volume:	2 µL
Flow rate:	0.200 mL/min
Mobile phase A:	0.1% Formic acid in water (LC-MS grade)

Mobile phase B:	0.1% Formic acid in acetonitrile (LC-MS grade)
Gradient:	1–35 %B over 50 min gradient (80 min total run time)

MS Conditions

MS system:	Xevo G3 QTof
Ionization mode:	ESI+
Acquisition range:	100–2000 *m/z*
Capillary voltage:	2.2 kV
Collision energy:	Low energy: 6 V High Energy Ramp: 20–50 V
Cone voltage:	20 V
Source temperature:	120 °C
Desolvation temperature:	350 °C
Cone gas:	35 L/hr
Desolvation gas:	600 L/hr
Intelligent data capture (IDC):	Low (5)

Data Management

Data were acquired and processed using the waters_connect informatics platform (version 2.1.1.13) with UNIFI

App (version 1.9.12.7) and the Peptide MAM App (version 1.0.0.3).

Results and Discussion

Thorough characterization of biosimilars is critical to ensuring the product's comparability to the innovator product. Here, the suitability of the Xevo G3 QTof operating within the integrated waters_connect platform is demonstrated for rigorous comparative analysis of infliximab and approved biosimilars. Although each biosimilar has an identical amino acid sequence to the innovator, differences in the profile of product variants can impact the safety, stability, and efficacy of the drug.

To gain insight into the differences between biosimilars, the peptide maps of each infliximab product were compared. Trypsin-digested samples were analyzed with an ACQUITY Premier UPLC coupled to the Xevo G3 QTof, using MS^E data independent fragmentation to identify peptides and localize sites of modification. Greater than 95% sequence coverage was achieved for each of the four infliximab products with less than 5 ppm mass error in the UNIFI App peptide mapping workflow within waters_connect. Furthermore, the results demonstrated excellent injection-to-injection reproducibility, with the relative standard deviation of peak intensity below 5%. This remarkable reproducibility can be seen in Figure 2A, where overlaid chromatograms from three replicate injections of Remicade show near perfect overlap.

While the chromatograms for each mAb sample look highly similar, as shown in Figure 2B, notable differences were observed in relative abundances of product variants. Across the four products, 47 modifications were localized, including 6 oxidations, 12 deamidations, 28 N-glycosylations, and C-terminal lysine clipping. Example high energy MS^E spectra are shown in Figure 3 for a peptide in its native and oxidized forms. The high coverage of assigned fragment ions enables both confident identification of the peptide and localization of the modification to the methionine amino acid, as evidenced by the y ion series. The differences in relative abundance of modifications were quantified using the Peptide MAM App in waters_connect. This software enables targeted quantification of a list of product quality attributes identified with the UNIFI App Peptide Mapping workflow across samples while remaining within the waters_connect Ecosystem.

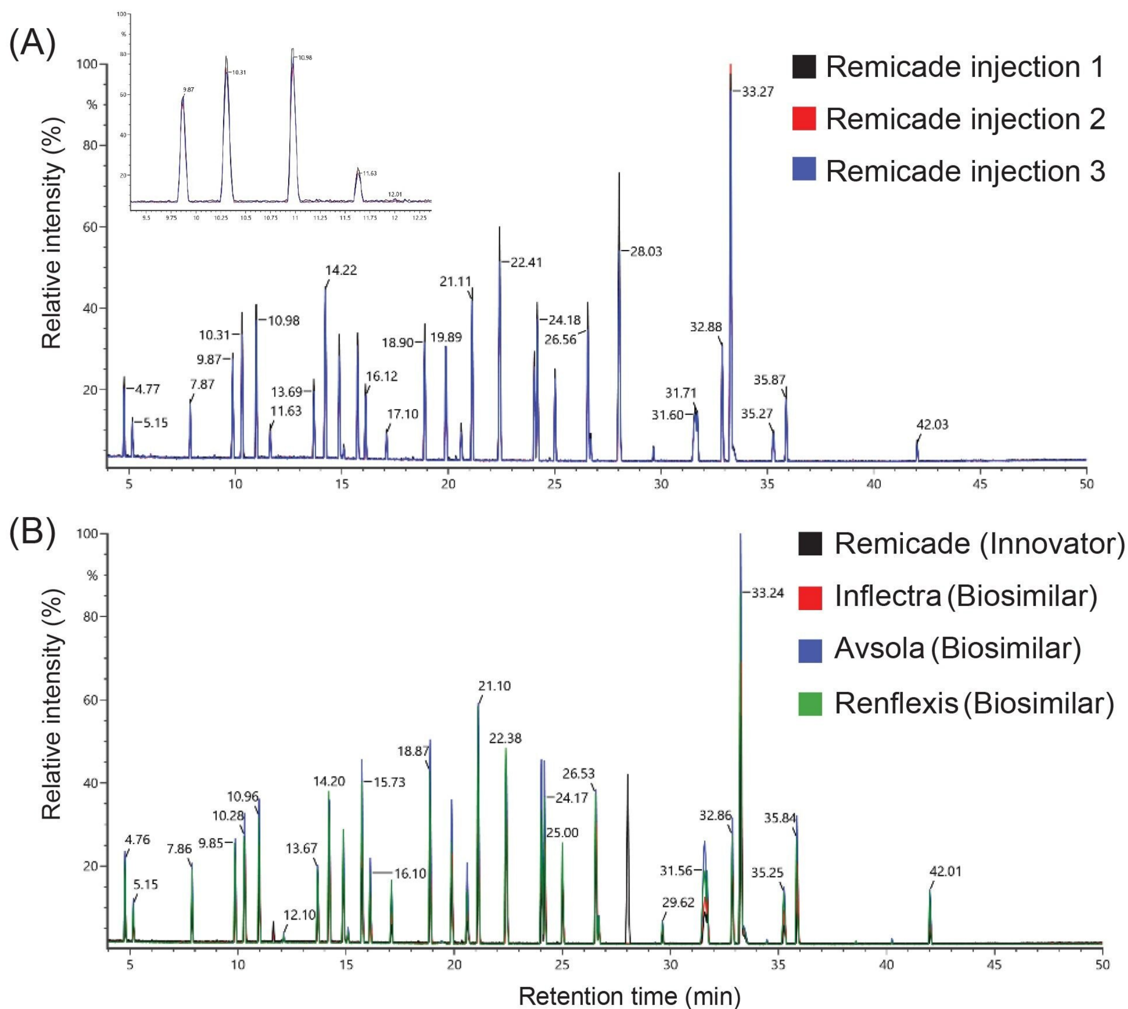


Figure 2. Overlaid base peak ion (BPI) chromatograms of mAb digest, including (A) replicate injections of the innovator (Remicade) sample, showing near perfect overlap of peaks, and (B) injections of the innovator and three biosimilars, indicating similar peptide map profiles.

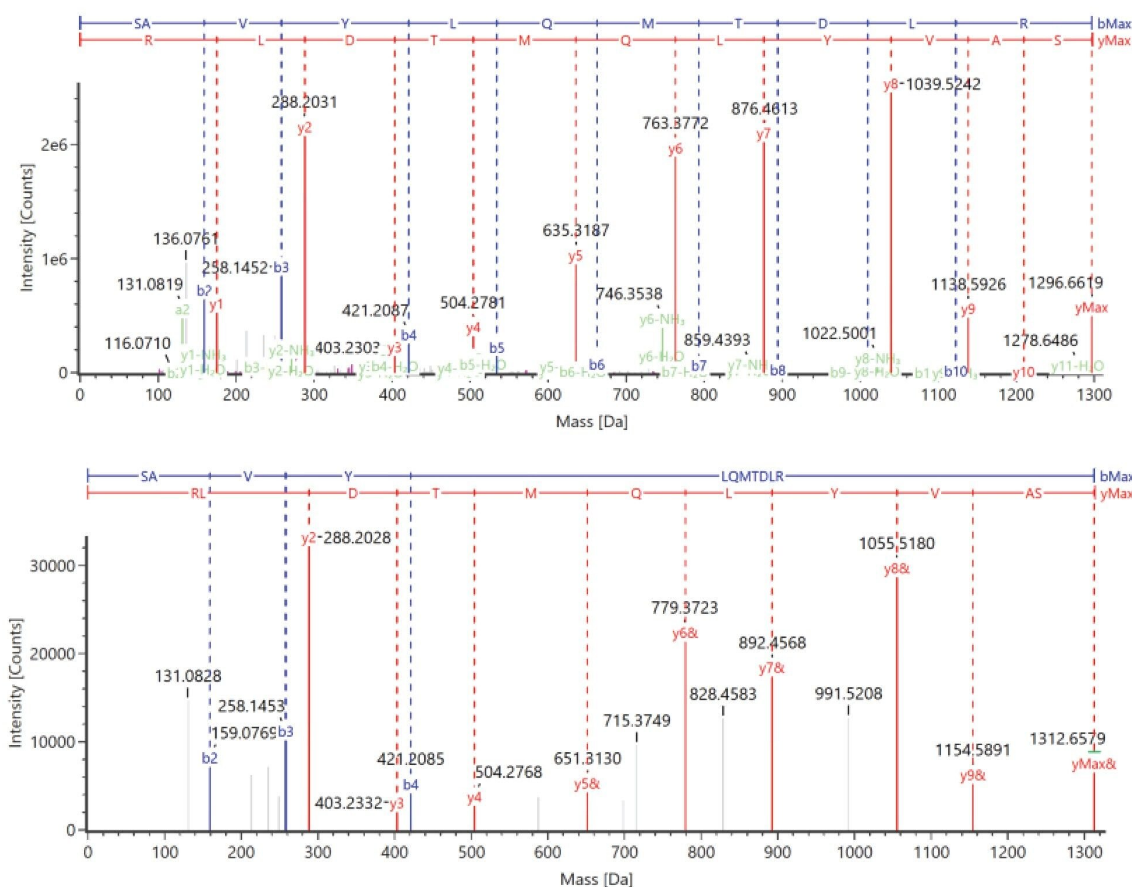
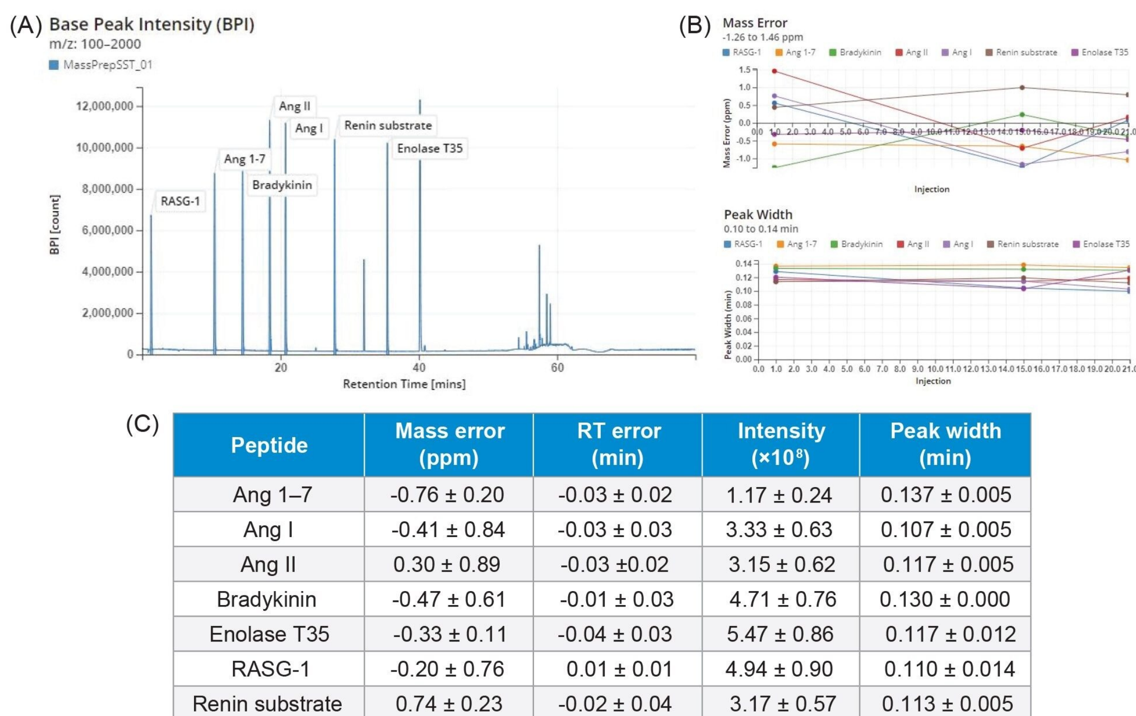


Figure 3. High energy MS^E spectra of HT11 peptide in its (top) unmodified form and (bottom) oxidized form.

Before reviewing quantification results, a system suitability analysis was performed as a part of the Peptide MAM Workflow. By evaluating intermittent injections of a known peptide sample, this step checks to ensure the system is producing acceptable and expected results for the separation and mass detection instrumentation as well as the automated data processing routines. This process is key to instilling confidence in the results, as attribute monitoring requires high analytical rigor. MassPREP™ Peptide Mixture (p/n: 186002337 < https://www.waters.com/nextgen/global/shop/standards--reagents/186002337-massprep-peptide-mixture.html >) was used for system suitability injections. Figure 4B shows two examples of how peptide data are tracked across injections, including mass error and peak width. The visualization of values across replicates enables the user to easily identify anomalies or systemic drifts over time. All four system suitability parameters are shown for each peptide in Figure 4C, reflecting the high mass accuracy (within ±1.5 ppm) and excellent reproducibility of

the platform throughout the duration of the sample sequence. The reproducibility achievable with the Xevo G3 QTof enables subtle trends between samples to be more easily distinguished, while saving time and money by reducing the number of replicates needed to obtain meaningful results for the lowest level product variants.



Peptide	Mass error (ppm)	RT error (min)	Intensity ($\times 10^8$)	Peak width (min)
Ang 1–7	-0.76 ± 0.20	-0.03 ± 0.02	1.17 ± 0.24	0.137 ± 0.005
Ang I	-0.41 ± 0.84	-0.03 ± 0.03	3.33 ± 0.63	0.107 ± 0.005
Ang II	0.30 ± 0.89	-0.03 ±0.02	3.15 ± 0.62	0.117 ± 0.005
Bradykinin	-0.47 ± 0.61	-0.01 ± 0.03	4.71 ± 0.76	0.130 ± 0.000
Enolase T35	-0.33 ± 0.11	-0.04 ± 0.03	5.47 ± 0.86	0.117 ± 0.012
RASG-1	-0.20 ± 0.76	0.01 ± 0.01	4.94 ± 0.90	0.110 ± 0.014
Renin substrate	0.74 ± 0.23	-0.02 ± 0.04	3.17 ± 0.57	0.113 ± 0.005

Figure 4. System suitability results for MassPREP Peptide Mixture data processed with the Peptide MAM App, including (A) an example chromatogram, (B) examples of values, mass error and peak width, being tracked over injections for each peak, and (C) all suitability parameters for each monitored peak.

The relative quantification of targeted peptide attributes, as measured with the Peptide MAM App, averaged less than 2.0% relative standard deviation for the monitored attributes. An example of how these results are presented within the app is shown in Figure 5, where simplified bar graphs enable rapid evaluation of the data for each targeted attribute.

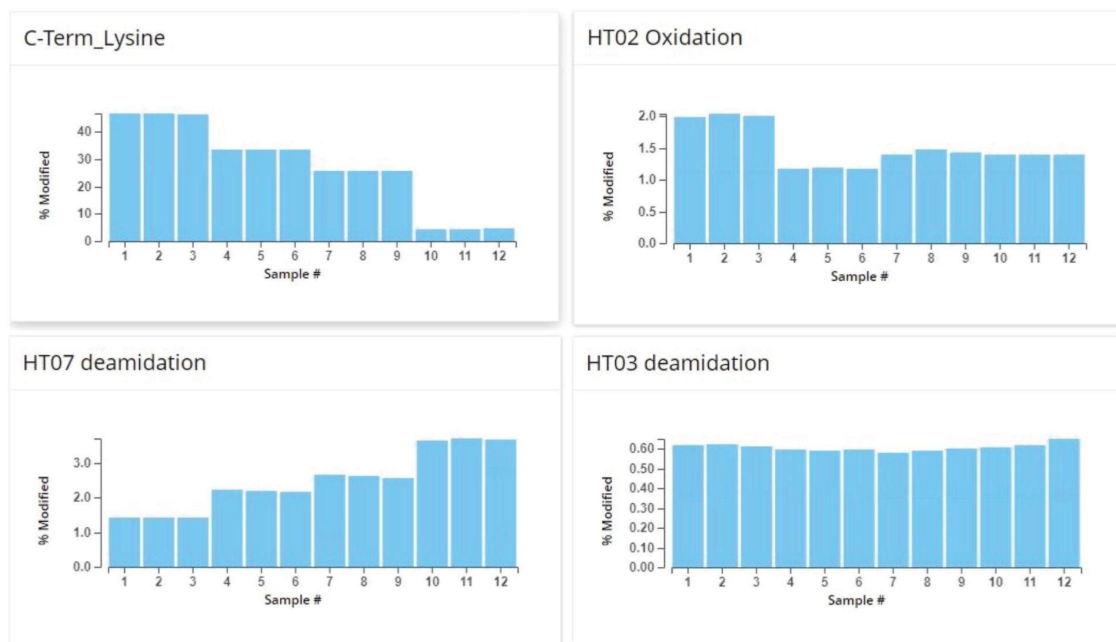


Figure 5. Bar graphs showing results presented in the Peptide MAM App for four monitored attributes, C-terminal Lysine, HT02 oxidation, HT07 oxidation, and HT03 deamidation. Injections are labeled as 1–3 for Remicade, 4–6 for Inflectra, 7–9 for Avsola, and 10–12 for Renflexis.

The quantification results for oxidized and deamidated peptides, as well as C-terminal lysine conjugation, are shown in Figure 6. All six of the oxidation sites monitored showed discernable differences between the infliximab products. For example, heavy chain tryptic peptide (HT) 02, HT11, and one of the sites on HT03 showed greater percentage of oxidation in the innovator, while HT22 showed greater percentage oxidation in two of the biosimilars, Avsola and Renflexis.

Differences in deamidation were less prominent. Of the twelve deamidations monitored, only two showed substantial differences between the biosimilars, including HT07, which had a greater percentage deamidation in the three biosimilars, and one of the sites on HT38, which showed a smaller percentage deamidation in Inflectra and Avsola as compared to Remicade and Renflexis.

The extent of C-terminal lysine clipping was also quantified, as this modification is common during bioproduction and may have an impact on receptor-binding.[4] As shown in Figure 6, the extent of Lysine clipping varies notably between the biosimilars, with Renflexis having the smallest percentage remaining on the peptide.

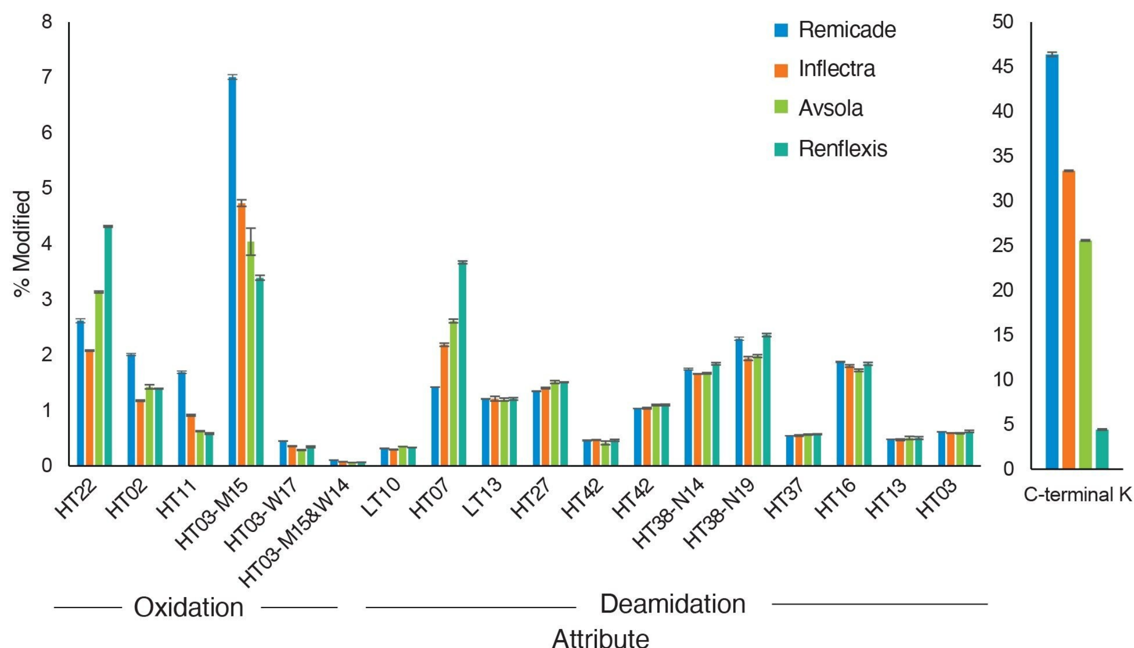


Figure 6. Relative abundances of peptide attributes (oxidation, deamidation, and C-terminal Lysine conjugation) between an innovator and three biosimilars. Error bars indicate standard deviation based on three replicate injections. For peptides with multiple modification sites, the modified amino acid is indicated. H heavy chain; L light chain; T tryptic peptide.

The percentage of various N-glycovariants showed variation between the infliximab products, likely due to manufacturing and cell-line differences.[5] Figure 7 shows the relative abundance of 28 N-glycoforms of the HT26 peptide, categorized into three groups: high-abundance (above approximately 2% relative abundance), low-abundance (below approximately 2% relative abundance), and immunogenic (glycans containing either N-glycolylneuraminic acid (NeuGc) or galactose-alpha-1,3-galactose (alpha-gal)).

In all four mAbs, FA2 and FA2G1 were the most prominent glycoforms, comprising approximately 50% and 30%, respectively, of the total abundance of all forms of the peptide. However, the exact relative amount of each varied between the mAbs, with Avsola having the highest abundance of FA2 and the lowest abundance of FA2G1. Conversely, Inflectra had the lowest abundance of FA2 and the greatest abundance of FA2G2.

The immunogenic glycoforms were in greater abundance in Remicade and Inflectra compared to Avsola and Renflexis, as is expected due to the difference in cell lines, with the former originating from murine cell lines and

the latter from Chinese hamster ovary cell lines.

The low-abundance glycoforms showed largely contrasting relative abundances between the four mAbs. For example, A1, M5A1G1, FM5A1, and A1G1 were in the highest relative abundance in Remicade, while A2G1 and M6 showed the highest relative abundance in Renflexis, and A2G1 was highest in Inflectra. Differences in glycosylation are important to note, as they may impact the ability of the drug to provoke a desired or undesirable immune response.

In addition to a comparison of peptide maps, the innovator and one biosimilar (Inflectra) were also subjected to a stress study. Stress testing experiments are important in biotherapeutic development to obtain information pertinent to developing analytical methods, determining dosage forms, identifying impurities, calculating shelf life, and comparing degradation pathways for biosimilar products.[6,7] The stress, either thermal, chemical, or mechanical, is intended to accelerate protein degradation and elevate impurity levels, providing insight into how proteins' efficacy and immunogenicity may be affected. These studies enable identification of CQAs, which are peptide attributes that are critical to the product's potency, efficacy, and safety.

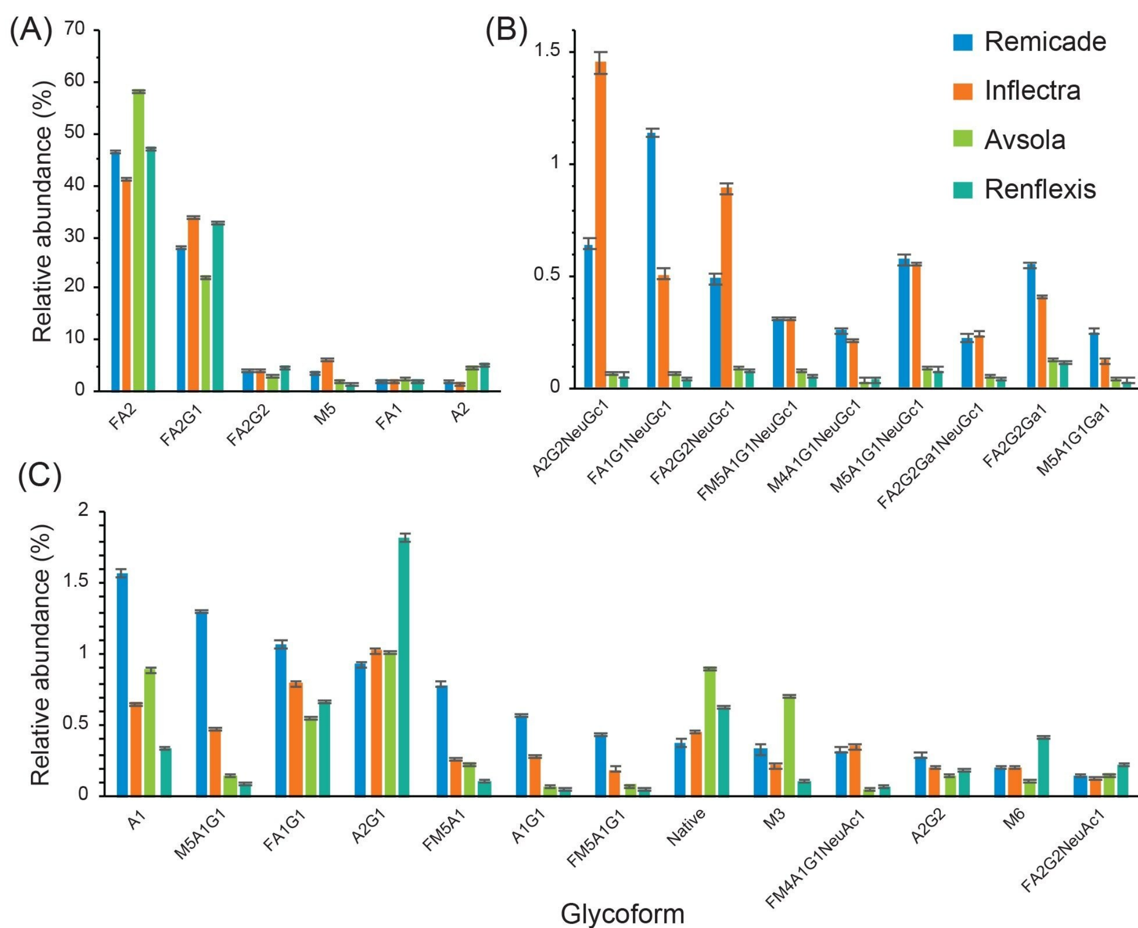


Figure 7. Relative abundances of glycosylated heavy chain peptide HT26 across innovator and three biosimilars, categorized by (A) high-abundance glycoforms, (B) immunogenic glycoforms, and (C) lower-abundance glycoforms. Glycoforms are labeled based on Oxford nomenclature. Error bars indicate standard deviation based on three replicate injections.

Here, the stress experiment was employed to monitor changes in CQAs in Remicade and Inflectra using the Peptide MAM App. Samples were subjected to temperature stress for one or two weeks prior to analysis and compared to an unstressed sample. Figure 8 shows a mirror plot comparing the unstressed (control) and two-week stressed samples of Inflectra vs Remicade. While the chromatograms look similar in the mirror plots with no obvious differences in major peaks, minor changes in several CQAs were identified with the MAM App, as shown in Figure 9. Many of the CQAs showed elevated responses in the stressed samples, though not all showed consistent increases between the innovator and biosimilar. For example, oxidation of HT22 and HT02

decreased in the two-week sample of Remicade but increased in the two-week sample of Inflectra. Others showed consistent results between the two, such as deamidation of HT07 and HT38, which both progressively increased in one- and two-week samples of both mAbs. No notable differences were observed in the relative abundances of glycoforms in the stressed samples or the relative amounts of Lysine clipping (data not shown). Biosimilars are not expected to have identical patterns of product variation, and any differences would be risk assessed to determine potential impacts to the functional and safety profiles of the molecules.

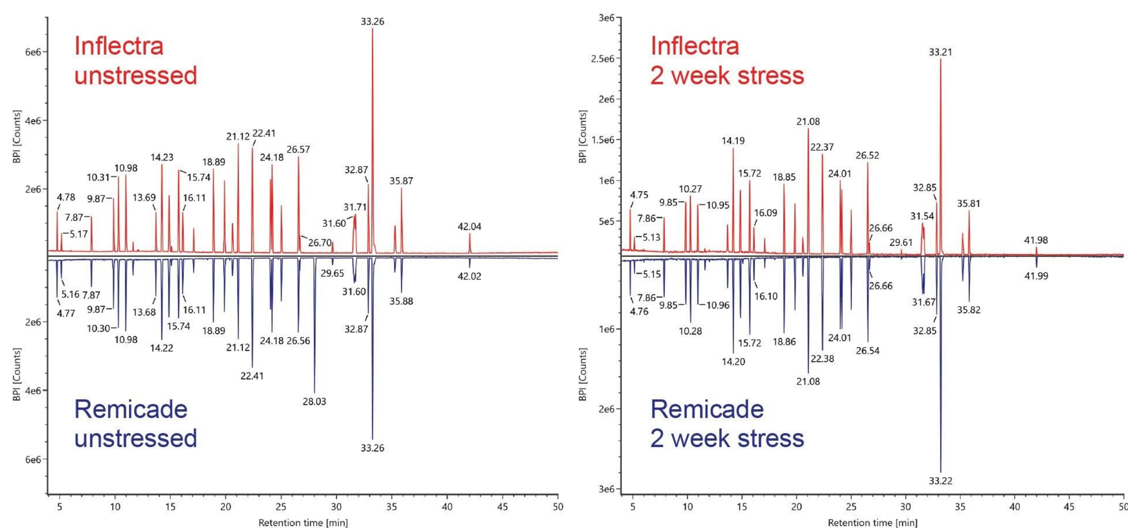


Figure 8. Mirror plots showing the comparison of Inflectra to Remicade for both (left) unstressed samples and (right) samples subjected to two weeks of elevated temperature stress.

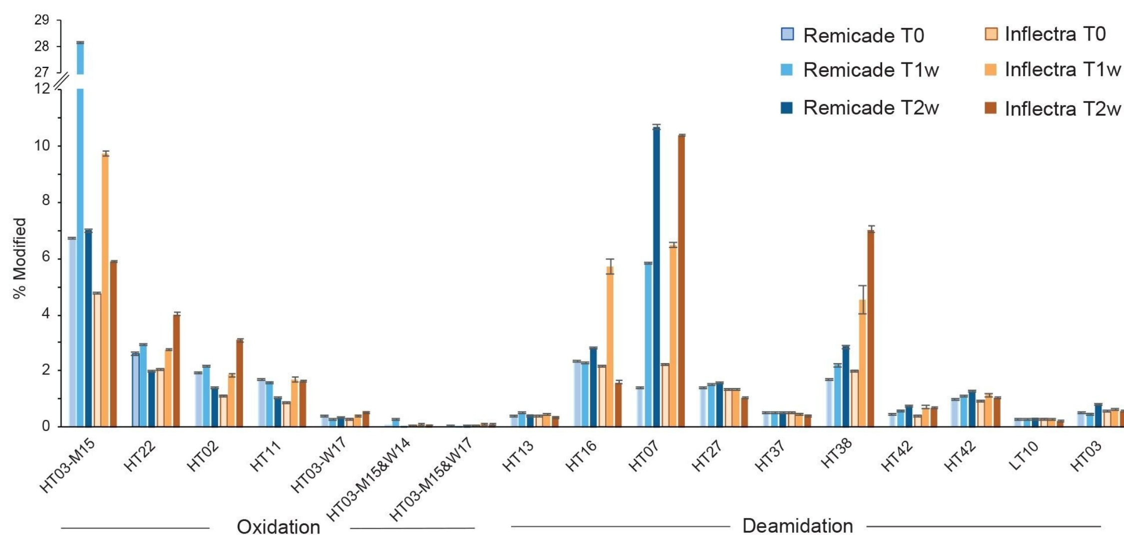


Figure 9. Relative abundance of oxidized and deamidated peptides in Remicade and Inflectra across each stress condition, including unstressed (T0), one week stress (T1w), and two weeks stress (T2w). Error bars indicate standard deviation based on three replicate injections. For peptides with multiple modification sites, the modified amino acid is indicated. H heavy chain; L light chain; T tryptic peptide.

Conclusion

Thorough characterization of biosimilar mAbs is critical to ensuring the safety and efficacy of the products and to relying on the innovator's experience to reduce clinical burdens for the follow-on product. The Xevo G3 QTof Platform with compliance-ready waters_connect Informatics enables robust and efficient analysis of product attributes through streamlined peptide mapping and peptide MAM workflows. Using this platform for primary structure characterization, this study achieved high sequence coverage of innovator and three biosimilar infliximab samples. With the integrated UNIFI App and Peptide MAM App, peptide attributes were identified, relatively quantified, and compared between the four mAb products. Additionally, potential CQAs were identified and quantified in a stress study. These results demonstrate how the Xevo G3 QTof can be used seamlessly with waters_connect data acquisition and processing for biosimilar peptide map characterization and attribute

monitoring workflows.

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