Application Note

Biotherapeutics and Biosimilars

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LC/MS/MS Peptide Mapping Comparison of Innovator and Biosimilars of Rituximab

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Abstract

The development and manufacturing of biosimilars require comparability studies with innovator biotherapeutic products. This Application Note presents a peptide mapping analysis of monoclonal antibody (mAb) biologics to assess the similarities and differences between innovator and two biosimilars of rituximab using an Agilent 6545XT AdvanceBio LC/Q-TOF system. The sequence coverage map of tryptic digested mAbs was >97%. Liquid chromatography/mass spectrometry (LC/MS) peptide separation profiles show differences in peptide abundances between innovator and biosimilar mAbs, revealing different extents of post-translational modifications (PTMs) such as deamidation and oxidation.

Introduction

The production of mAb-based therapeutics is growing rapidly.[1] Patents covering innovator mAbs that entered the market approximately two decades ago are expiring, opening opportunities for the development of cost-effective biosimilars. Biosimilars are close copies of their reference products, but are not identical due to inevitable differences in their manufacturing processes. These processes are highly complex, and slight deviations may introduce variability and intrinsic molecular heterogeneity to the product. Regulatory agencies require thorough characterization of biosimilars to prove they are comparable to innovator products in terms of safety, purity, and efficacy. The goal of biosimilar product development is to elucidate the similarities and differences between innovator and biosimilar drugs. Analytical comparability is a fundamental requirement to show biosimilarity.

Critical quality attributes (CQAs) of biologics define their efficacy and safety. PTMs such as glycosylation, oxidation, and deamidation variants are amongst the CQAs that can significantly impact biological function. In the biopharmaceutical industry, LC/MS-based peptide mapping serves as a primary tool for protein identification as well as monitoring PTMs. High resolution, reliable chromatographic separations, and accurate mass measurements are essential for the success of peptide mapping experiments.

In this study, peptide mapping of an innovator and two biosimilar mAbs of rituximab was performed using an integrated workflow consisting of an Agilent AssayMAP Bravo liquid-handling platform, an Agilent 1290 Infinity II LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF, and data analysis through Agilent MassHunter BioConfirm software (Figure 1). The percentage sequence similarity of the heavy chain, light chain, and intact mAbs were compared to determine the amino acid coverage. The results of the study demonstrate the suitability of Agilent's peptide mapping workflow for identifying and monitoring important PTMs such as deamidation and oxidation.

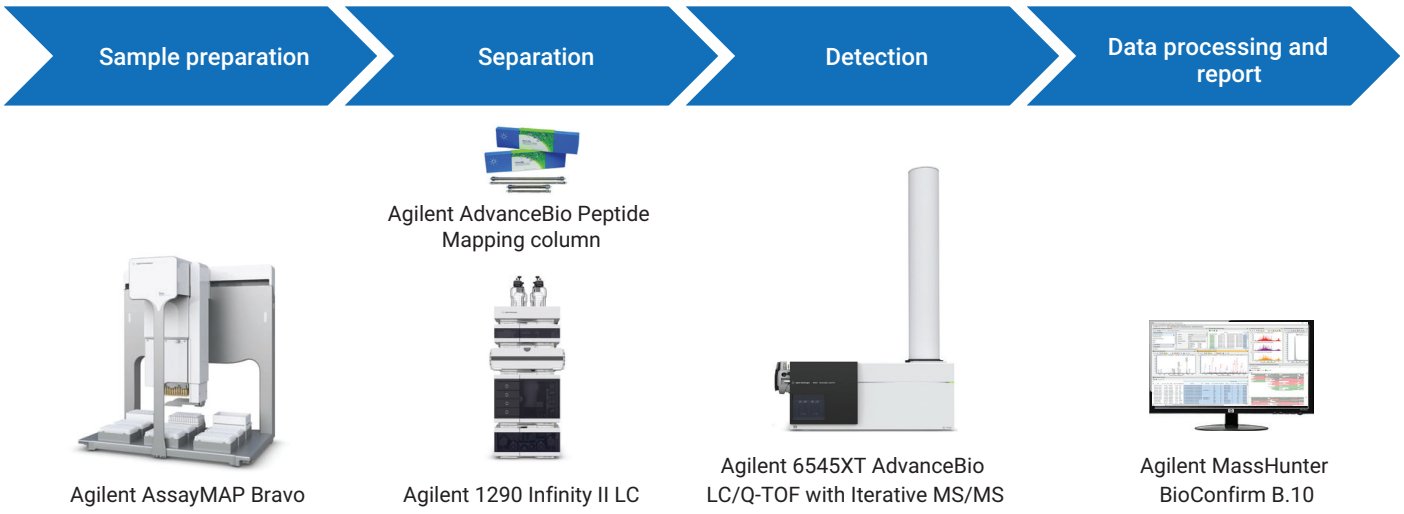

Figure 1. Agilent peptide mapping workflow.

Experimental

Materials

Innovator and biosimilar monoclonal antibodies were purchased from a local distributor (Singapore), and stored according to the manufacturers' instructions. Trizma base, guanidine-hydrochloride, *tris*(2-carboxyethyl)phosphine (TCEP), iodoacetamide (IAA), formic acid, trifluoroacetic acid, and LC/MS grade solvents were purchased from Sigma-Aldrich. High-quality sequence grade trypsin was obtained from Agilent Technologies, Inc.

Sample preparation: trypsin digestion

Innovator and biosimilar mAbs were reduced/alkylated and trypsin-digested followed by desalting using the Agilent AssayMAP Bravo liquid-handling platform. Briefly, sample plates containing mAbs (10 µg/µL) were reconstituted in denaturation buffer (8 M guanidine-HCl with 5 mM TCEP and 150 mM Tris, pH 8) and incubated at 60 °C for 60 minutes. After denaturation and reduction of disulfide bonds, alkylation of free cysteines was carried out by adding 133 mM iodoacetamide (40 minutes at room temperature, protected from light). Guanidine-HCl concentration was reduced, and the pH of the solution was adjusted to pH 7 to 8 with 150 mM Tris-HCl before trypsin digestion. Trypsin digestion (20:1, protein to protease w/w) was performed overnight at 37 °C. The samples were later acidified with 0.1% formic acid to halt the action of trypsin using the AssayMAP reagent transfer utility. AssayMAP Bravo Peptide Cleanup protocol (desalting) was performed using C18 reversed-phase cartridges. The cartridges were primed with 100 µL of 60 % acetonitrile 0.1% trifluoroacetic acid equilibrated with 50 µL of 0.1% trifluoroacetic acid, loaded with 50 µL of mAb tryptic digested samples at a flow rate of 5 µL/min, washed with 50 µL of 0.1% trifluoroacetic acid, and eluted with 15 µL of 60 % acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 5 µL/min and mixed into 160 µL of 0.1% formic acid.

Instrumentation

LC system

Agilent 1290 Infinity II LC System including:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Multisampler (G7167B)

MS system

Agilent 6545XT AdvanceBio LC/Q-TOF

LC/MS analysis

LC/MS peptide map separation was performed on an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm) using a 30-minute gradient. The data were acquired in iterative MS/MS mode (Table 1).

Data analysis

The data were processed using the peptide mapping workflow in Agilent MassHunter BioConfirm 10. Briefly, molecular features were extracted and matched against the innovator mAb's sequence with fixed modifications (alkylation: C) and variable modifications (oxidation: M, deamidation: N, Q, lysine loss: K, and pyroglutamate: E). The mass tolerance was 10 ppm for MS, and 30 ppm for MS/MS. Sequence coverage was calculated based on peptide spectrum matches to MS/MS features and were filtered by a 1% false discovery rate (FDR).

Table 1. LC/MS conditions.

Parameter	Agilent 1290 Infinity II LC System
Column	Agilent AdvanceBio Peptide Mapping 2.1 × 150 mm, 2.7 µm, 120Å
Injection Volume	2 µL (2 µg/µL)
Sample Thermostat	5 °C
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Gradient	At 0 minutes → 3% B At 30 minutes → 40% B At 33 minutes → 90% B At 35 minutes → 90% B At 37 minutes → 3% B At 40 minutes → 3% B
Stop Time	40.1 minutes
Column Temperature	60 °C
Flow Rate	0.4 mL/min

Parameter	Agilent 6545XT AdvanceBio LC/Q-TOF
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Nebulizer	35 psi
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
Oct RF Vpp	750 V
Reference Mass	121.050873, 922.009798
Acquisition Mode	Data were acquired in Extended Dynamic Range (2 GHz)
Ms Mass Range	110 to 1,700 *m/z*
Acquisition Rate	8 spectra/sec
Auto Ms/Ms Range	50 to 1,700 *m/z*
Ms/Ms Acquisition Rate	3 spectra/sec
Isolation Width	Narrow (~1.3 *m/z*)
Precursors/Cycle	Top 10
Collision Energy	Charge state Slope Offset 2 3.1 1 3 and >3 3.6 −4.8
Threshold for MS/MS	1,000 counts and 0.001%
Dynamic Exclusion On	One repeat, then exclude for 0.1 or 0.2 minutes
Precursor Abundance Based Scan Speed	Yes
Target	25,000 counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Purity	100% stringency, 30% cutoff
Isotope Model	Peptides
Sort Precursors	By charge state then abundance; +2, +3, >+3

Results and discussion

MassHunter BioConfirm is a comprehensive and user-friendly data analysis tool for protein analysis. Figure 2 shows the BioConfirm 10.0 software peptide mapping workflow layout. For peptide mapping experiments, the software automatically extracts peptide characteristic features with a peptide find algorithm, and matches MS/MS spectra with possible PTMs to confirm and validate the peptide sequence.[2] In addition, the software performs relative quantification of PTMs automatically, and displays the information as histograms for quick data review, enabling faster decisions.

Multiple peptide mapping files can be processed and compared across different protein digest samples. With these powerful and user-friendly features, MassHunter BioConfirm software is well suited for comparative assessment of innovator and biosimilar peptide mapping experiments.

Figure 2. Screenshot of Agilent MassHunter BioConfirm 10.0 software for peptide mapping analysis.

Peptide mapping is routinely used to identify proteins by confirming the amino acid sequence. It is also the preferred approach for the characterization and monitoring of PTMs. This study chose an innovator and two biosimilar mAbs for comparability assessment. Figure 3 shows the LC/MS of tryptic peptide map of innovator and biosimilar mAbs. The 30-minute gradient on the AdvanceBio Peptide Mapping column produced a high-resolution peptide separation. Using the automatic peptide mapping workflow in MassHunter BioConfirm software, peptide masses from the LC/MS/MS run were matched with the theoretical digest with preferred modifications included in the mAb sequence. The BioConfirm software automatically identified and assigned the peptides for the MS/MS data. The result of iterative MS/MS gives >97% sequence coverages for heavy chain and light chain sequences (Table 2).

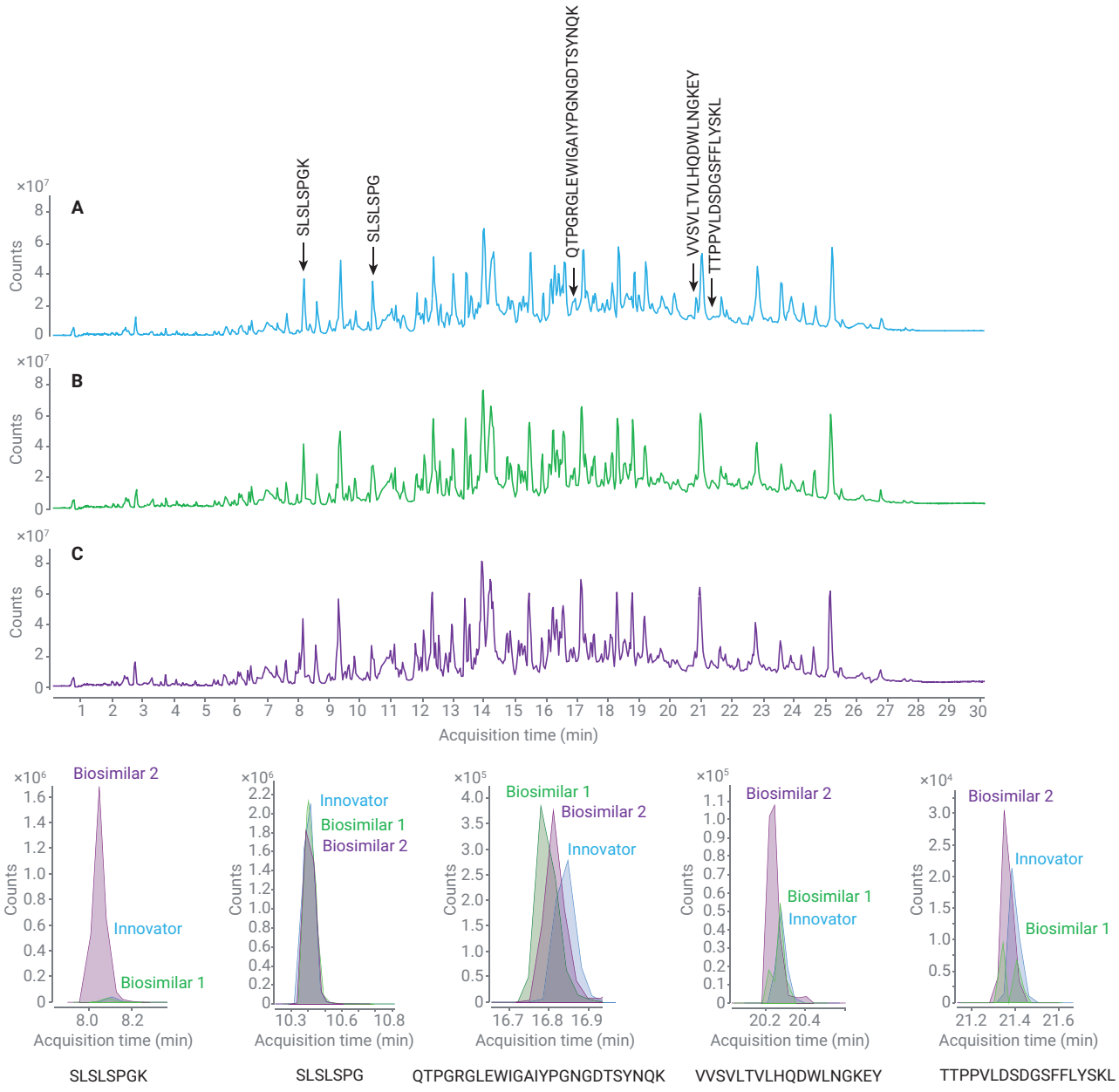

Figure 3. Comparison of tryptic digested mAbs. TIC of (A) Innovator, (B) Biosimilar 1, and (C) Biosimilar 2. The arrows highlight the difference in TIC profiles, and the below ECCs represent the corresponding peptides.

Table 2. Average sequence coverage.

mAb Samples	Sequence Coverage	
	Heavy Chain	Light Chain
Innovator	98.23	100
Biosimilar 1	98.10	99.06
Biosimilar 2	97.59	100

The total ion chromatogram (TIC) of tryptic digest obtained from auto MS/MS was compared to find the similarity/differences between the innovator and biosimilar mAbs. Figure 3 shows that inspection of the ion chromatograms reveal differences in the expression levels of peptides. The C-terminal sequence (SLSLSPGK) is enriched in biosimilar 2 (~eight minutes) compared to innovator and biosimilar 1. Conversely, the lysine-truncated peptide form (SLSLSPG) was enriched in the innovator mAb (~10.4 minutes). The three other peptides, QTPGRGLEWIGAIYPGNGDTSYNQK, VVSVLTVLHQDWLNGKEY, and TTPPVLDSDGSFFLYSKL show different intensity levels between innovator and biosimilar mAbs.

PTMs are CQAs that may be linked to drug efficacy and safety. Therefore, it is important to identify and compare the levels of PTMs between the biosimilar and innovator mAb pairs. Data on two commonly found modifications, oxidation and deamidation, between innovator and biosimilar mAbs were presented.

Oxidation

Oxidation is a common PTM of mAbs, and can occur at various stages of bioprocessing development such as purification, formulation, and storage. To compare the oxidation levels of innovator and biosimilar mAbs, a representative heavy chain DLTMISR peptide was selected. Figure 4 compares MS/MS spectra, separation of oxidized and unmodified DLTMISR peptides, and the relative quantification of M256 oxidation. Inspection of y_4 and y_5 ion masses shows an increase of 16 Da in oxidized MS/MS spectra, suggesting oxidation of the Met residue. Figure 4C shows the triplicate measurements of relative oxidation levels of M256 as a histogram, a useful feature of MassHunter BioConfirm software, for all the mAbs. The examination of histograms for M256 oxidation levels for innovator and biosimilar 1 mAbs were comparable (1.48% and 1.27% respectively), whereas for biosimilar 2, a slightly higher level of oxidation (2.5%) was observed.

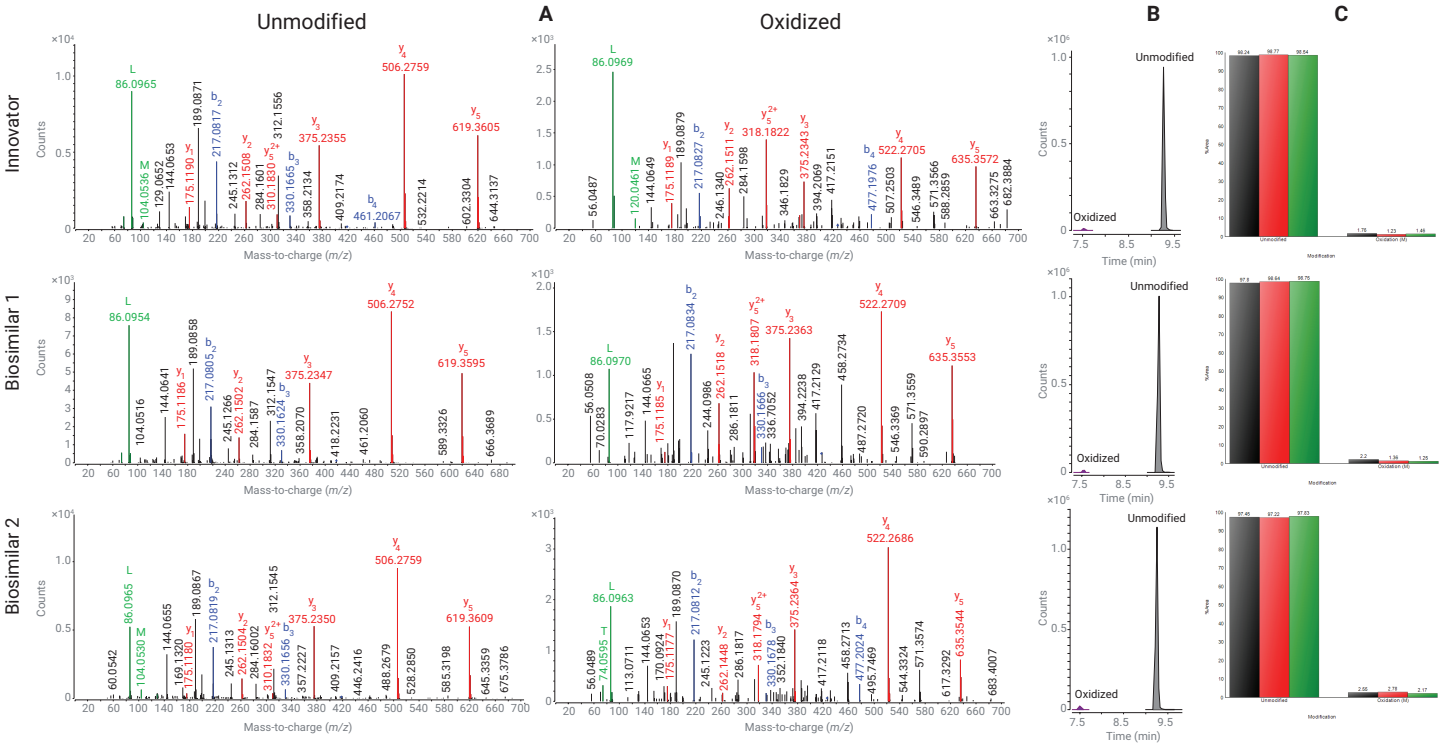

Figure 4. Oxidation analysis of DTLMISR peptide. (A) MS/MS spectrum of unmodified and oxidized peptide, (B) ECC overlay, and (C) relative quantification of M256 (three replicate measurements).

Deamidation

Deamidation is another commonly found modification that occurs primarily at asparagine (N) and glutamine (Q) residues. Deamidation on N residues occurs more rapidly than on Q residues. To compare the deamidation levels of innovator and biosimilar mAbs, the heavy chain GFYPSDIAVEWESNGQPENNYK peptide sequence (375–396) was used. Figure 5 shows the GFYPSDIAVEWESNGQPENNYK analysis results. Analysis of MS/MS spectra showed a +1 Da mass shift of y_9 product ions onwards, while y ions from y_8 downwards are the same, indicating Asn388 deamidation. The analysis of the results of relative quantification showed higher Asn388 deamidation levels in biosimilars 1 and 2 (19.99% and 19.83% respectively) compared to the innovator mAb (10.92%).

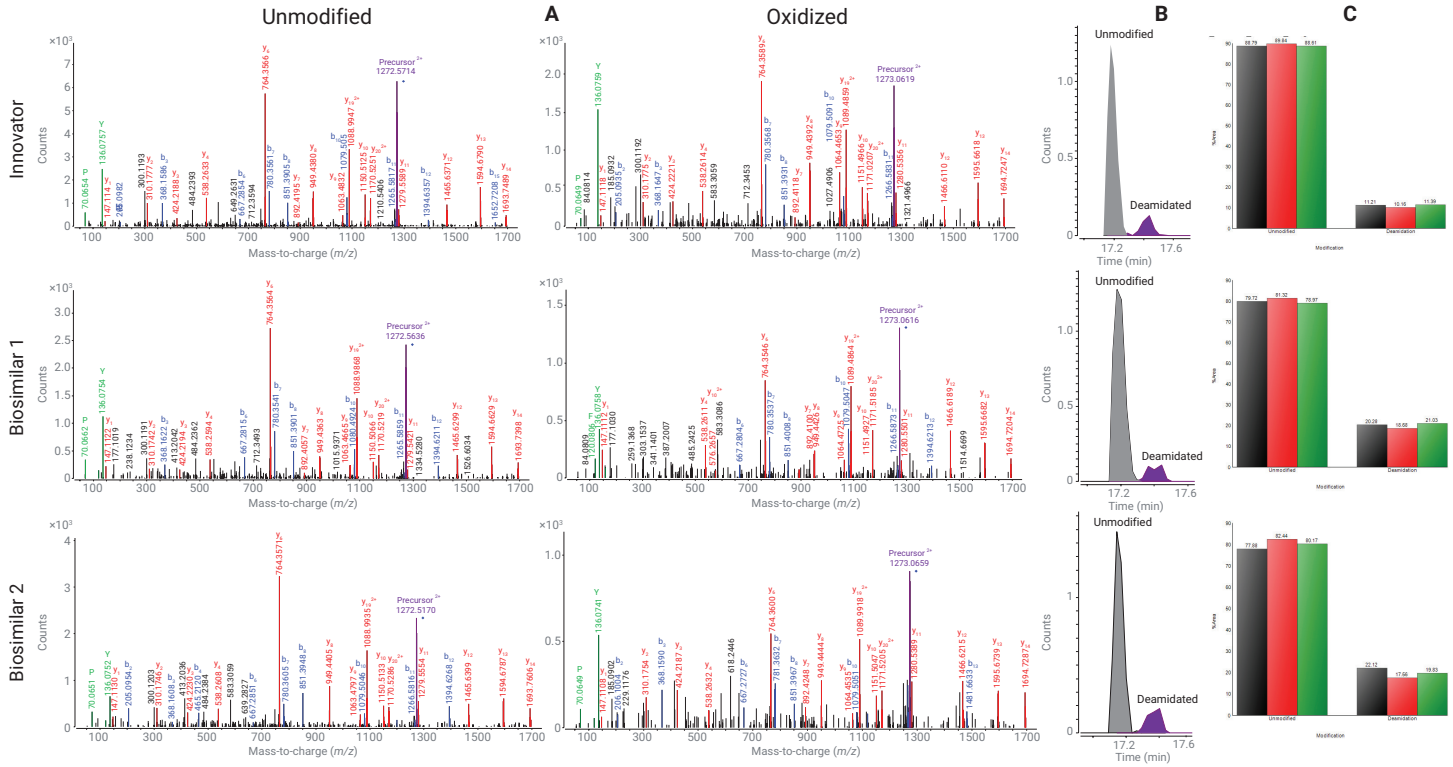

Figure 5. Deamidation analysis of GFYPSDIAVEWESNGQPENNYK peptide sequence (375–396). (A) MS/MS spectrum of unmodified and deamidated peptide, (B) ECC overlay, and (C) relative quantification of N388 (three replicate measurements).

PTMs on other peptides were also analyzed using the histogram feature of BioConfirm. Figure 6 shows the representative relative quantification for other modified residues of innovator and biosimilar mAbs. The histogram comparison display provides an easy and quick way of monitoring the levels of quality attributes across a series of samples.

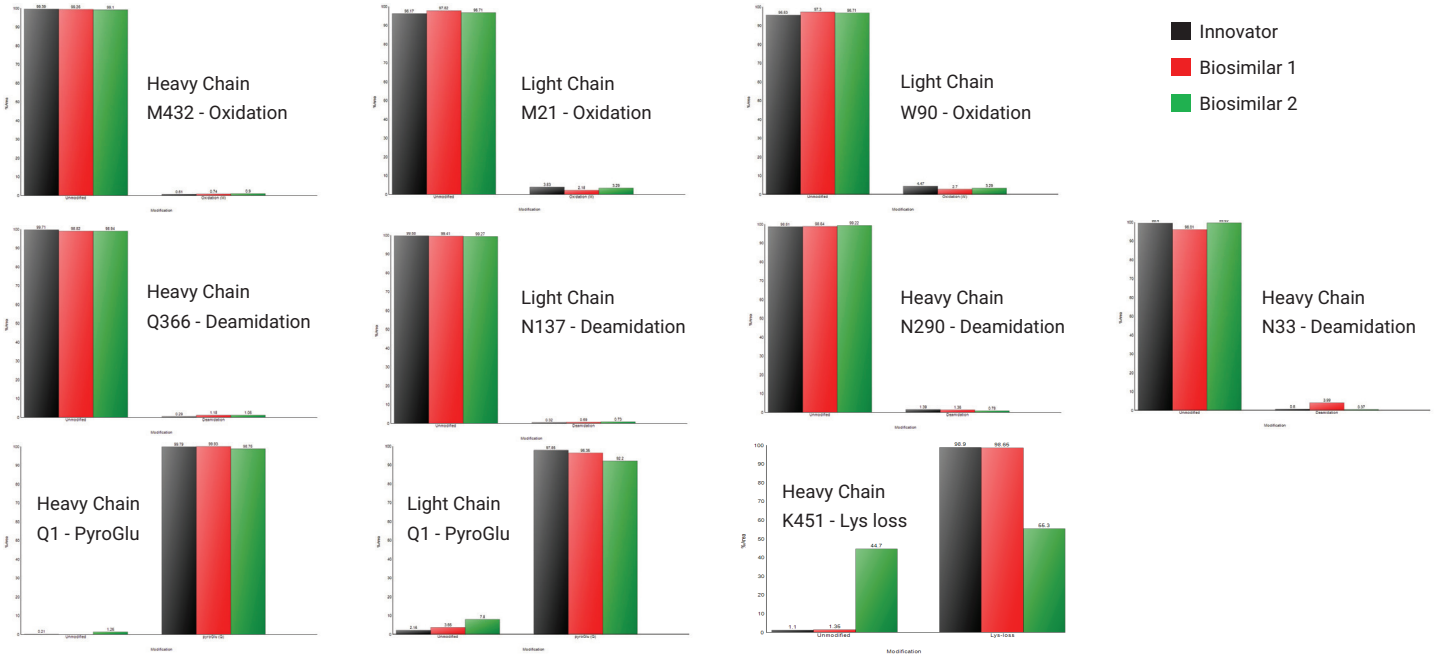

Figure 6. Representative histograms showing relative quantitation of PTMs (oxidation, deamidation, Lys truncation, and N-terminal cyclizations of Gln to pyroGlu).

Conclusion

This Application Note demonstrates an integrated peptide mapping workflow to study innovator and biosimilar mAbs using an Agilent AssayMAP Bravo platform, Agilent Accurate Mass 6545XT AdvanceBio LC/Q-TOF, and easy-to-use Agilent MassHunter BioConfirm software for data analysis. The results obtained on peptide mapping provides excellent sequence coverage for innovator and biosimilar mAbs. Precise characterization of PTMs was achieved due to high-efficiency chromatographic separation and high-quality MS/MS spectra. This integrated approach also allowed relative quantification of various PTMs between innovator and biosimilar mAbs.

References

1. Ecker DM1, Jones SD, Levine HL. The Therapeutic Monoclonal Antibody Market. MAbs. 7(1):9-14 **2015**
2. *Agilent Technologies*, publication number 5991-8552EN.

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