Comparative analysis of innovator and biosimilar monoclonal antibodies using a multi-attribute method

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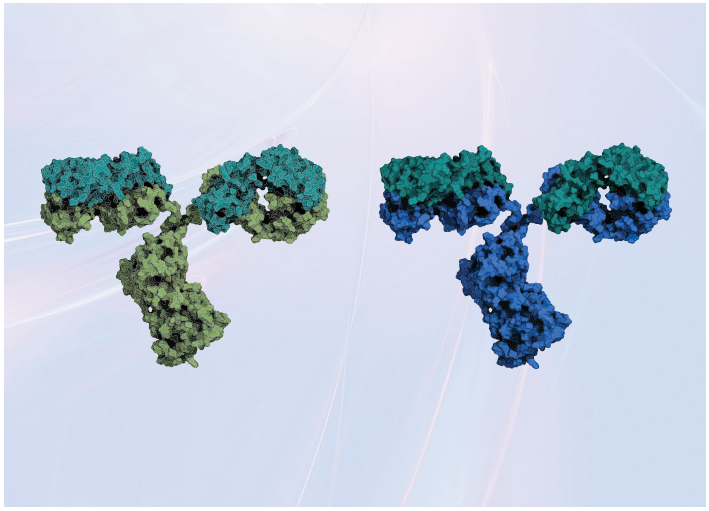

Application benefits

- High resolution Multi-Attribute Method (HR-MAM) provides a streamlined workflow for the identification, robust relative quantitation, and monitoring of product quality attributes (PQAs).
- Enables efficient and confident analytical comparability studies between innovator and biosimilar antibodies, and/or among different production batches.
- Ensures data integrity with an enterprise compliance-ready Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software.

Introduction

A biosimilar is a biological medicinal product that is highly similar to the reference molecule (the innovator)[1]. Regulatory authorities such as the US Food and Drug Administration (US FDA), European Medicines Agency (EMA), and National Medical Products Administration (NMPA) of China have set guidance on requirements needed to demonstrate the similarity between two biological products in terms of safety and efficacy[1]. Due to the complexities of the structure and manufacturing process of the biologics, biosimilar developers must conduct in-depth physicochemical characterization to verify that the quality attributes are comparable between

the biosimilar and the innovator products. To ensure the desired product quality and the molecular similarity with the innovator, identification and monitoring of PQAs are essential for biosimilar development, manufacturing, and quality control[2].

High-resolution accurate mass (HRAM) mass spectrometry (MS) has become an indispensable analytical tool in the characterization of innovator and biosimilar mAbs, such as confirmation of the amino acid sequence and identification of product variants such as chemical and post-translational modifications (PTMs)[3]. In addition, many conventional assays such as enzyme-linked immunosorbent assay (ELISA), hydrophilic-interaction liquid chromatography (HILIC), size-exclusion chromatography (SEC), cation-exchange chromatography (CEX), and capillary electrophoresis (CE) have been employed for both direct and indirect PQAs monitoring throughout biopharmaceutical development stages and manufacturing of innovator and biosimilar mAbs. In 2015, Rogers *et al.*[2] introduced a peptide mapping based Multi-Attribute Method (MAM) for concurrent monitoring and quantifying multiple PQAs as well as for new peak detection[2], providing extensive information about product quality and improving productivity. MAM has gained increasing attention from the biopharmaceutical industry and regulatory agencies for its potential as a replacement method in quality control (QC) labs[2-4]. Recently, Rogstad *et al.* from the US FDA suggested several conventional QC approaches, such as HILIC for glycan profiling, CEX for charge variant analysis, and reduced capillary electrophoresis-sodium dodecyl sulfate (rCE-SDS) for clipped variant analysis could be replaced when employing MAM as a QC method[4].

In this study, we assessed the comparability of biosimilar vs. innovator rituximab under untreated and forced degradation conditions by utilizing the Thermo Scientific™ HR-MAM workflow (Figure 1) to efficiently identify, relatively quantify, and monitor the selected PQAs to reduce the analytical testing and increase productivity.

Experimental

Instrumentation

- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (P/N 0726030)
- Thermo Scientific™ Vanquish™ Binary Flex UHPLC system consisting of:
  - Thermo Scientific™ Vanquish™ System Base (P/N VF-S01-A-02)
  - Thermo Scientific™ Vanquish™ Binary Pump F (P/N VF-P10-A-01)
  - Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
  - Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)

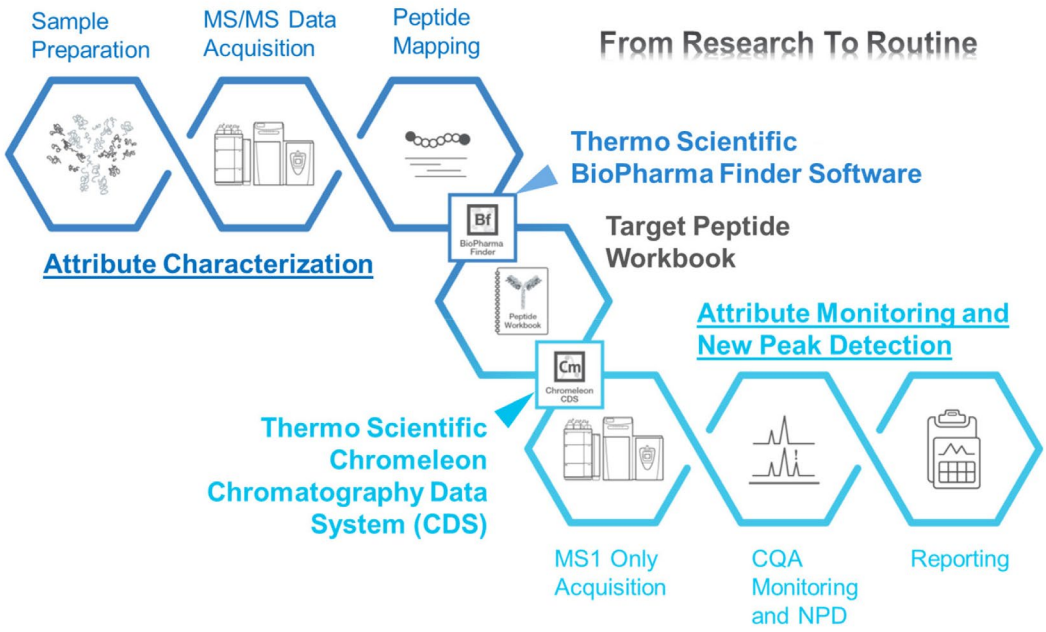

Figure 1. Schematic of Thermo Scientific HR Multi-Attribute Method Workflow

Software

- Thermo Scientific™ BioPharma Finder™ 4.0 QF1 Software (OPTON-30986)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) with the following components:
  - Chromeleon Enterprise Client (P/N 7200.0300)
  - Biopharma QC Package (P/N 7200.0044)
  - Thermo Scientific Instrument Control (P/N 7200.1000)
  - License Key New (P/N 7050.0104A)

Reagents and consumables

- Thermo Scientific™ Accucore™ Vanquish™ C18+ UHPLC column, 1.5 µm, 2.1 × 150 mm (P/N 27101-152130)
- Thermo Scientific™ Water, UHPLC-MS grade (P/N W8-1)
- Thermo Scientific™ Acetonitrile, UHPLC-MS grade (P/N A956-1)
- Thermo Scientific™ Pierce™ Trypsin Protease MS grade (P/N 90058)
- Thermo Scientific™ Pierce™ Formic acid, LC-MS grade (P/N 28905)
- Invitrogen™ UltraPure™ 1 M Tris-HCl buffer, pH 7.5 (P/N 15567027)
- Sigma-Aldrich, 8.0 M Guanidine Hydrochloride Solution (P/N G7294-100ML)
- Bio-Rad Bio-Spin™ P-6 Gel Columns, Tris Buffer (P/N 732-6227)
- Sigma-Aldrich, Sodium Iodoacetate (IAC) BioUltra >98% purity (P/N I-9148)
- Sigma-Aldrich, DL-Dithiothreitol (DTT) BioXtra ≥99% purity (P/N D-5545)

Sample preparation

Three samples were analyzed in this study:

- Innovator rituximab
- Two separate batches of biosimilar rituximab

All starting concentrations were 10 µg/µL and volumes were 10 µL. The stress conditions the drug samples were subjected to are described in Table 1.

Table 1. Thermal and oxidative stress conditions

Stress condition	Samples		
	Innovator	Biosimilar batch 1	Biosimilar batch 2
Thermally stressed	50 °C for 2 weeks		
Oxidatively stressed	0.015% H_2O_2 at room temperature for 24 hours in the dark		

The concentration of stressed samples was brought to 1 µg/µL by adding denaturing solution (7 M Guanidine HCl, 100 mM Tris, pH 8.3). Subsequently, all samples were digested following the protocol described in Application Note 72916[3].

Liquid chromatography

For each analysis, 8 µL of tryptic digest (5 µg) was loaded onto a 2.1 × 150 mm Accucore Vanquish C18+ UHPLC column with 1.5 µm particle size (P/N 27101-152130) and separated with a linear gradient using a Vanquish Flex Binary UHPLC system. The autosampler temperature was set to 5 °C while the column temperature was held at 50 °C (Still Air Thermostatting Mode).

The LC gradient used in this study is shown in Table 2.

- Mobile phase A: 0.1% formic acid in water
- Mobile phase B: 0.1% formic acid in acetonitrile
- Flow rate: 0.250 mL/min

Table 2. LC gradient for tryptic peptides separation

Time [min]	%B
0.0	1.0
5.0	1.0
6.0	10.0
70.0	35.0
72.0	90.0
77.0	90.0
79.0	1.0
81.0	1.0
83.5	10.0
91.5	45.0
93.0	90.0
99.0	90.0
101.0	1.0
115.0	1.0

Mass spectrometry

All experiments presented in this application note were performed on the Q Exactive Plus mass spectrometer fully controlled by Chromeleon CDS 7.2.10 with Tune 2.9 SP4. Ion source settings and MS method parameters are summarized in Table 3.

The data for peptide mapping were acquired with a data-dependent Top5 tandem mass spectrometry (ddMS^2) method and processed in BioPharma Finder software. For targeted PQA relative quantitation and monitoring, data was acquired using a Full Scan MS only method and processed in Chromeleon software.

Table 3. Mass spectrometry tune and method settings

MS source setting	Value
Sheath gas	35
Aux gas	10
Sweep gas	0
Spray voltage (kV)	3.5
S-lens RF level (%)	50
Aux gas temperature (°C)	250
Capillary temperature (°C)	250
Properties of Full MS	**Value**
General	
Runtime	0 to 72 min
Polarity	Positive
Full MS	
Resolution	140,000
AGC target value	3.00E+06
Maximum injection time (ms)	200
Scan range (*m/z*)	300–1800
Properties of Full MS/dd-MS^2 (Top5)	
General	
Runtime	0 to 72 min
Polarity	Positive
Default charge state	2
Full MS	
Resolution	140,000
AGC target value	3.00E+06
Maximum IT	100 ms
Scan range (*m/z*)	300–1800
dd-MS^2	
Resolution	17,500
AGC target value	1.00E+05
Maximum IT	250 ms
TopN	5
Isolation window	1.2 Th
NCE (%)	27
dd settings	
Minimum AGC target	2.00E+03
Intensity threshold	8.00E+03
Charge exclusion	Unassigned, 1, >8
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion (s)	8.0 s

Data processing

The ddMS^2 data was processed by BioPharma Finder software using the peptide mapping workflow, as described in an earlier technical note[5]. The processing parameters are listed in Table 4.

Table 4. BioPharma Finder software parameter settings for peptide mapping

Database parameters	
Protease	Trypsin (C-term KR)
Specificity	High
Fixed Modification	Carboxymethylation (C)
Variable Modifications	Deamidation (N)
	Deamidation (Q)
	NH3 loss (NQ)
	Oxidation (MW)
	Lys (C-term)
	Gln→Pyro-Glu (N-term)
	N, O Glycans (CHO)
Component detection parameters	
Task to Perform	"Find All Ions in the Run"
Absolute MS Signal Threshold (MS Noise Level *S/N Threshold)	"Automatic determination by software"
MS Noise Level	"Automatic determination by software"
S/N Threshold	"Automatic determination by software"
Typical Chromatographic Peak Width (min)	"Automatic determination by software"
Maximum Chromatographic Peak Width (min)	"Automatic determination by software"
Maximum RT Shift	"Automatic determination by software"
Identification parameters	
Search by Full MS Only	No
Use MS/MS	Use All MS/MS
Maximum Peptide Mass	7,000
Mass Accuracy (ppm)	8
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	2
Enable Mass Search for Unspecified Modifications	Unchecked
Glycosylation	CHO
Search for Amino Acid Substitutions	None
Perform Disulfide Bond Search	No
Enable HDX	Unchecked

A workbook was created containing all the detected charge states of the selected PQAs, which was then imported into the Chromeleon CDS software for MAM data processing. The MAM processing method was created using the default MS Quantitative template available in Chromeleon CDS software. The basic settings for the MAM processing method are the same as previously described in Application Note 72916[3].

Results and discussion

It is important to compare the biosimilarity of the biosimilar drug batches to the innovator product under stressed conditions, including thermal and oxidative stress, to investigate the effects on the PQAs of the biosimilar batches.

Characterization and selection of PQAs by peptide mapping analysis

Peptide mapping is a widely used analytical approach for the comprehensive characterization of biotherapeutics, providing insights into the primary structure information such as sequence confirmation, sequence variants, PTM identification, and localization[6]. In this study, we first utilized a peptide mapping approach to confirm the sequence, identify and select the PQAs. Figure 2A displays the mirrored base peak chromatograms (BPCs) of rituximab innovator and one batch of a biosimilar. High chromatographic similarity was observed for these two samples. The high sequence coverages (LC: >96% and HC: >93%) were also achieved for both innovator (Figure 2B) and biosimilar products (data not shown). The regions that were not identified contain very short tryptic peptides. According to our MS method setting, these peptides are usually excluded in ddMS^2.

N-glycosylation of biotherapeutics can influence efficacy and safety and therefore must be characterized and monitored throughout the development and manufacturing of biosimilar and innovator products. Figure 3 shows the MS^2 spectra of glycosylated and non-glycosylated peptides from the heavy chain. Figures 3A and 3B represent the most abundant glycopeptide (A2G0F) and the lowest abundant one (M8), respectively. Although the abundance of A2G0F is ~190-fold higher than that of M8, both spectra show great S/N and contain rich glycan fragment ions. Although the abundance of the non-glycosylated peptide is quite low (~0.48%), a confident identification could be achieved (Figures 3C and 3D).

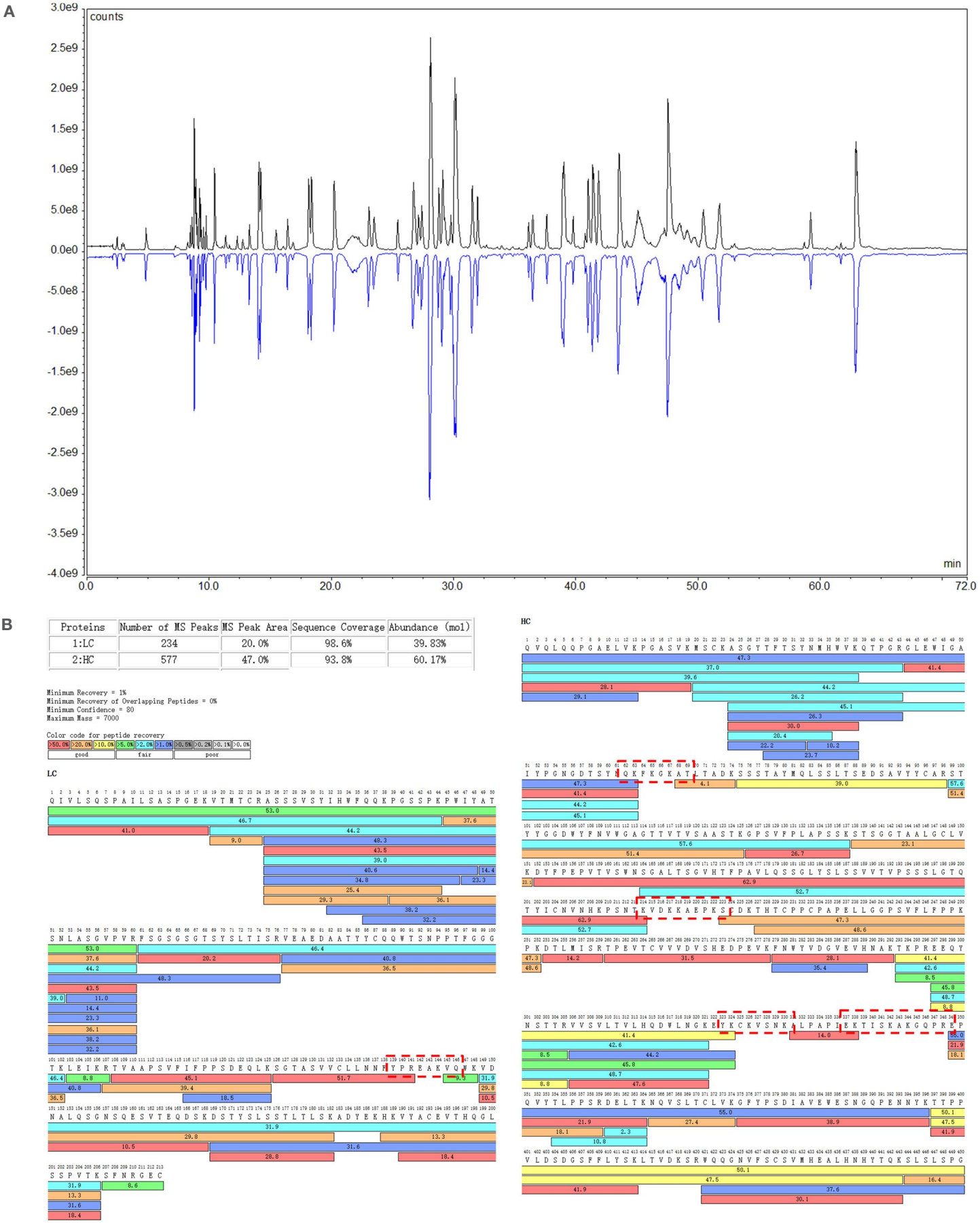

Figure 2. (A) Mirrored BPCs of rituximab innovator (black) and its biosimilar product (blue) and (B) sequence coverage map of the innovator. The regions that were not identified are marked with a red dotted line frame.

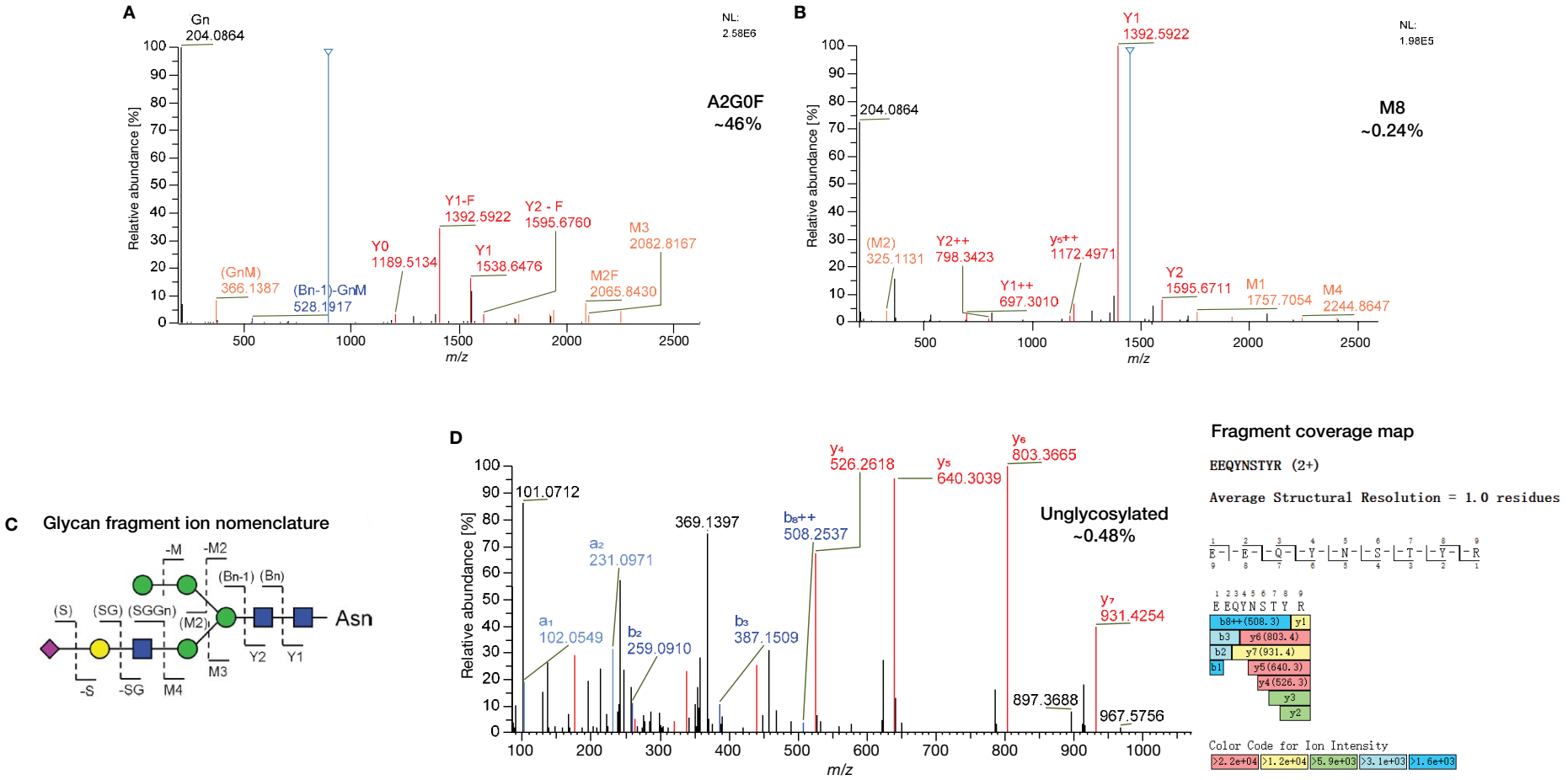

Figure 3. MS² spectra of EEQYNSTYR peptide of the heavy chain. (A) A2G0F, (B) M8, (C) schematic of glycan fragment ion nomenclature, and (D) non-glycosylated form and fragment coverage map of this peptide.

To identify and assess the level of PQAs involved in the degradation pathways, rituximab innovator samples were subjected to thermal and oxidative stress, respectively. Figure 4 shows MS^2 spectra for the peptide GLEWIGAIYPGN(55)GDTSYNQK from the heavy chain with and without deamidation at asparagine residue (N55) found in the innovator sample. The series of *b*- and *y*- fragment ions, with high S/N, in two MS^2 spectra, led to confident identification of endogenous as well as a deamidated peptide.

Using HR-MAM, the PQAs presented at low abundance ~0.1% could be identified and quantified with high reproducibility at the peptide level using Full MS. The following modifications, which may play important roles in product safety and efficacy, were chosen to demonstrate the capability of target quantitation in HR-MAM to assess the structural similarity between innovator vs biosimilar rituximab:

- HC N55 deamidation and succinimidation
- HC N388 and N393 deamidation
- HC N388 and N394 succinimidation
- HC M256 oxidation
- HC D284 isomerization
- N-glycosylation
- C-terminal lysine truncation and N-terminal pyroglutamate

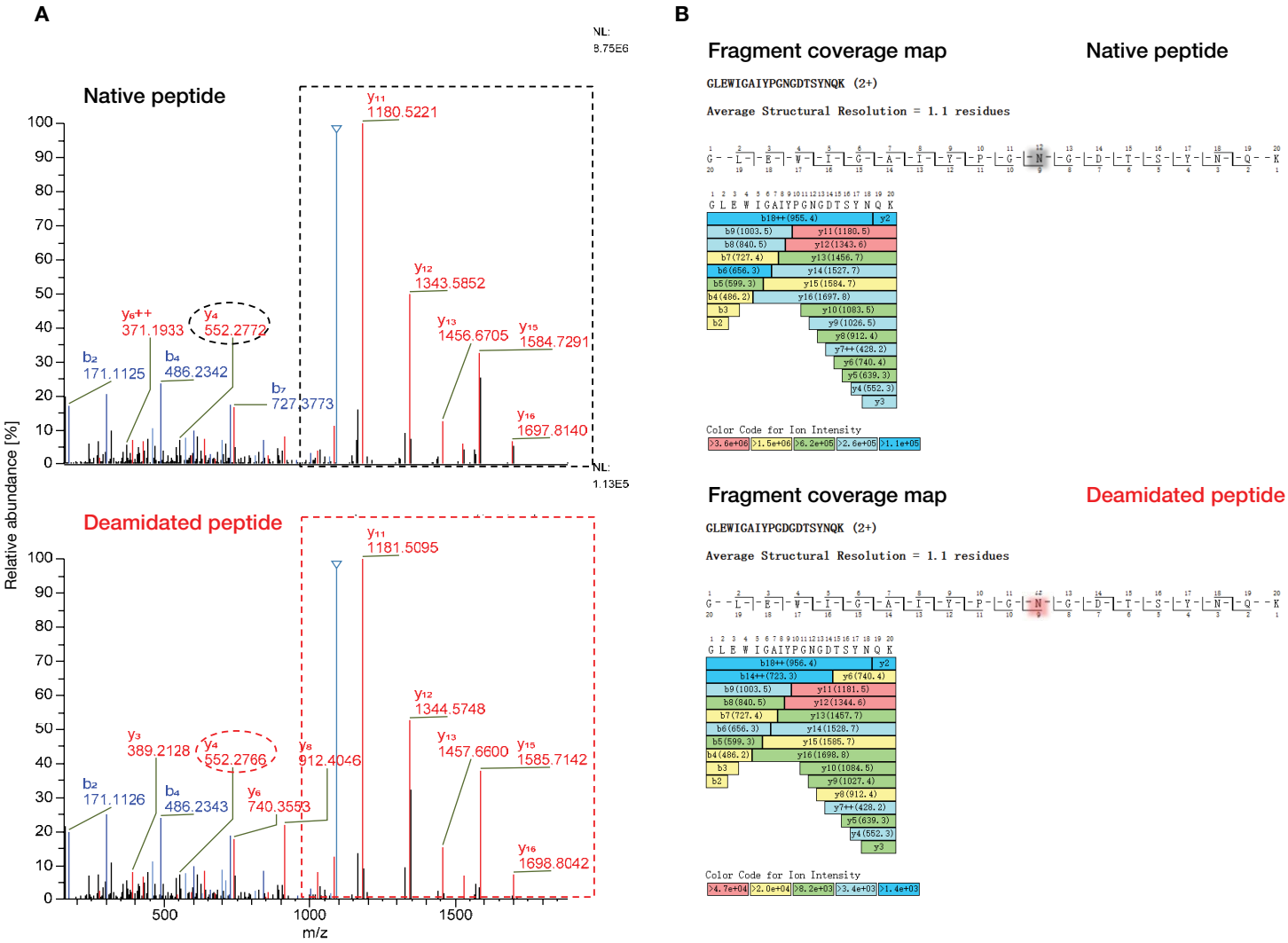

Figure 4. MS^2 spectra (A) and fragment coverage maps (B) of native and deamidated GLEWIGAIYPGN(55)GDTSYNQK detected in the innovator sample subjected to thermal stress. The series of y-ions confirming the deamidation of asparagine residue are highlighted in red.

Relative quantitation and monitoring of PQAs by HR-MAM

After selecting PQAs, the next step is to import these PQAs into Chromeleon software for relative quantitation and monitoring. The quantitation results of chosen PQAs in both innovator and biosimilars under different conditions were compared.

Terminal modifications, such as N-terminal pyroglutamate and C-terminal lysine truncation can affect charge heterogeneity of mAbs[6] and need to be monitored as part of structure comparability assessment. The levels of both LC and HC N-terminal pyroglutamate as well as C-terminal lysine variants are comparable between innovator and two batches of biosimilar rituximab products (Figure 5). The coefficients of variation (CVs) of three technical replicates were less than 2%, indicating great reproducibility.

N-glycosylation can affect the immunogenicity, potency, antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), serum clearance, and pharmacokinetics of the mAb therapeutics[6].

During biosimilar development and manufacturing, the N-glycosylation must be closely monitored and controlled to ensure product efficacy and safety. Consequently, the glycosylation heterogeneity profiles of the biosimilar and reference product must be comparable for biosimilar manufacturers to avoid extended clinical trials.

No significant differences were observed between innovator and biosimilar batches across all 15 N-glycoforms (Figure 6). Compared to the conventional released N-Glycan assay, the glycosylated level can also be monitored by HR-MAM. The data also showed great reproducibility for relative quantitation of all glycoforms monitored among technical replicates, including glycoforms with relative abundances lower than 0.5% (for example, CV<5.5% for A2S1G0F at ~0.3%,).

The stressed conditions employed in this work had a negligible effect on the C-terminal lysine truncation, N-terminal pyroglutamate, and N-glycoforms (data not shown), indicating that these modifications are neither temperature nor oxidant sensitive.

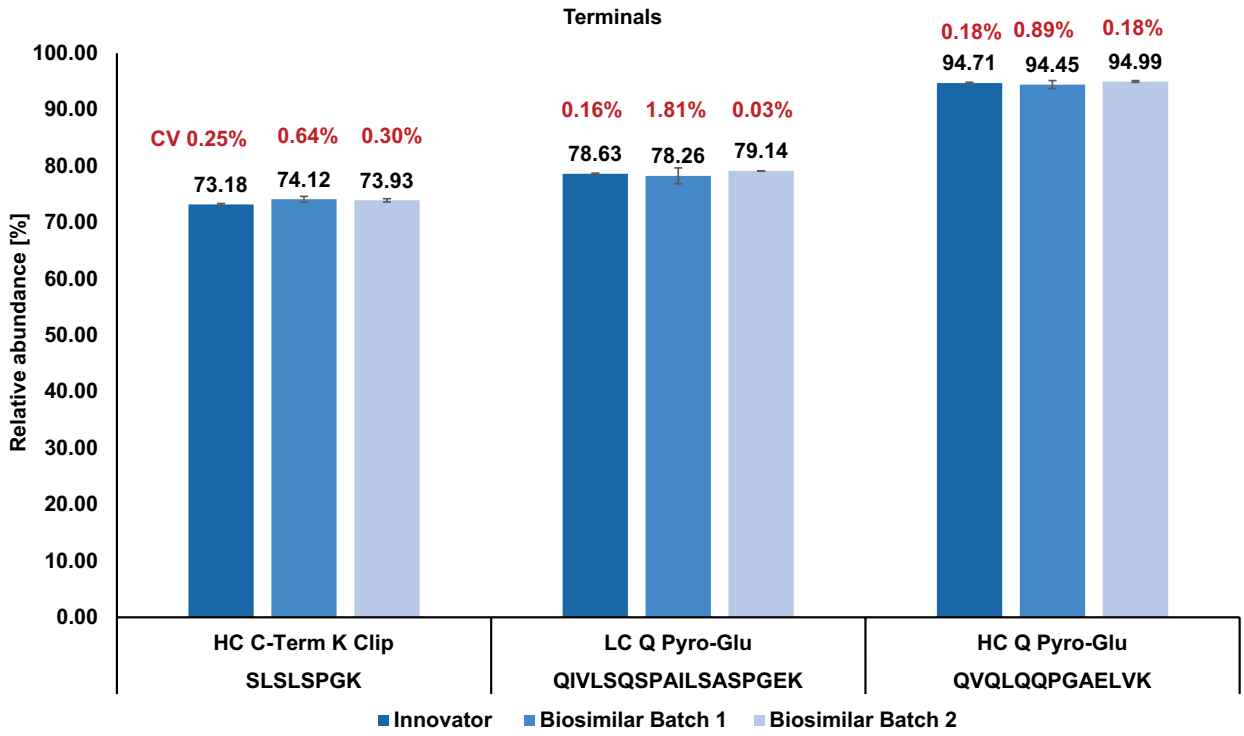

Figure 5. Relative quantitation of common terminal modifications in rituximab and biosimilars. N=3 technical replicates

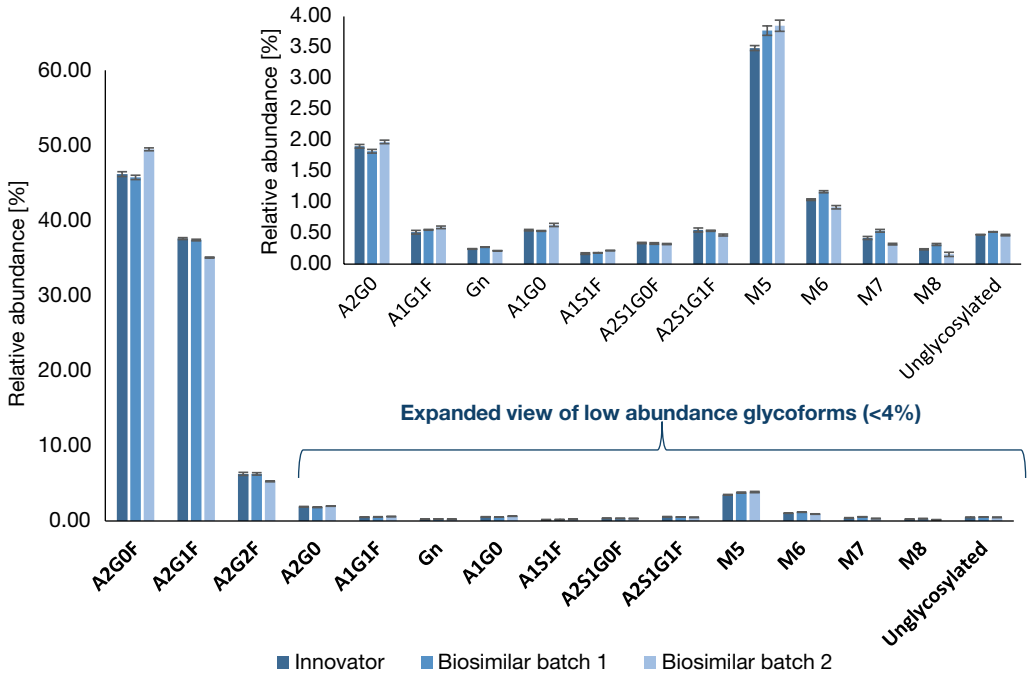

Figure 6. Relative quantitation of common 15 glycoforms of peptide EEQYNSTYR found in the heavy chain in rituximab and biosimilars. N=3 technical replicates.

Deamidation is a common degradation of proteins and it can significantly impact protein structure and function. At neutral and basic pH, deamidation proceeds via the formation of a five-membered ring intermediate succinimide[6,7]. Figure 7A shows the relative abundance of deamidated forms of GLEWIGAIYPGN(55)DTSYNQK on the heavy chain. The level of deamidation at N55 increased in the thermally stressed samples compared to the untreated samples, whereas no significant differences were observed between innovator and biosimilars. Succinimidation of N55 follows the same trend, albeit to a lesser degree (Figure 7B). Within Chromeleon CDS, the ability to obtain consistent quantitation of a variety of PQAs without any need to modify the processing method has already been demonstrated[8]. Figure 7C shows an example of the N55 succinimidation peak extracted ion chromatogram (XIC) using Chromeleon CDS at the peptide level.

Next, deamidation and succinimidation of GFYPSDIAVEWESN(388)GQPEN(393)N(394)YK were investigated. Target quantitation of the “PENNYK” peptide could be a challenge because of multiple potential modification sites on this peptide. Deamidation and succinimidation are thermally driven mechanisms. No deamidated form of N388 was detected under untreated conditions. In contrast, % deamidation of both sites (N388 and N393) increased after two weeks of thermal stress (Figure 8A). Succinimidation of N388 and N394 also slightly increased under thermal stress (Figure 8B).

Besides deamidation and succinimidation, the most significant PQA variations for rituximab and biosimilars are isomerization under thermal stress and methionine oxidation with oxidative stress.

Although the isomerization of the aspartic acid does not affect the net charge of the antibody, the addition of one carbon to the peptide backbone changes the length and folding of the side chain, thereby changing the charge distribution on the surface of the antibody. Here we chose to monitor D284 of the heavy chain. The exposure to thermal stress (50 °C, two weeks) had a significant effect on the isomerization of aspartic acid. The ratio of isoAsp on FNWYVD(284)GVEVHNAK increased by >30-fold (from 0.08–0.11% to 3.2–3.7%, Figure 9).

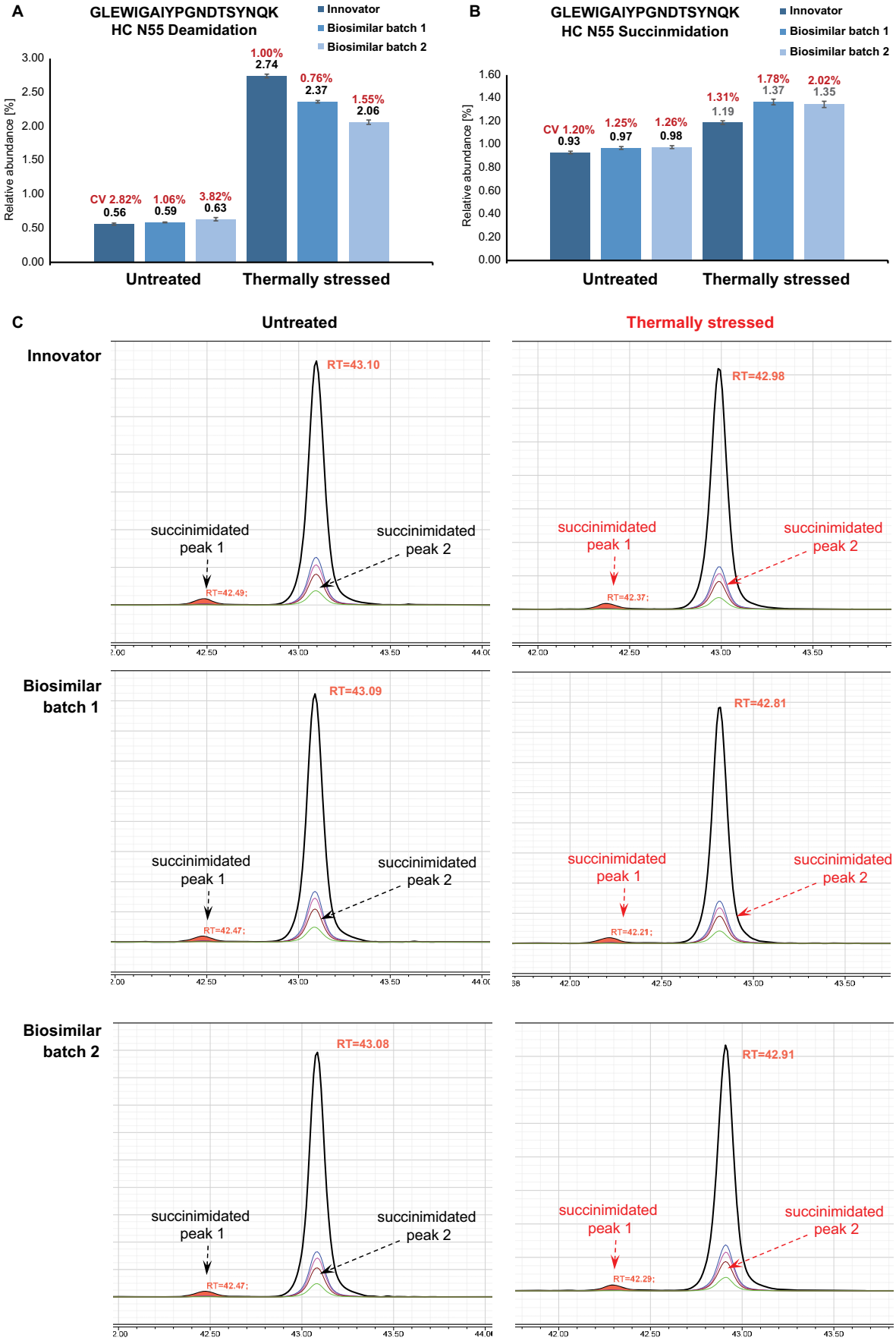

Figure 7. Relative quantitation of deamidation and succinimidation of peptide GLEWIGAIYPGN(55)DTSYNQK on the heavy chain in rituximab and biosimilars with and without subjection to thermal stress. N=3 technical replicates. (A) % deamidation. (B) % succinimidation. (C) XIC of succinimidated peptides in Chromeleon software. Plots normalized to 100% signal level.

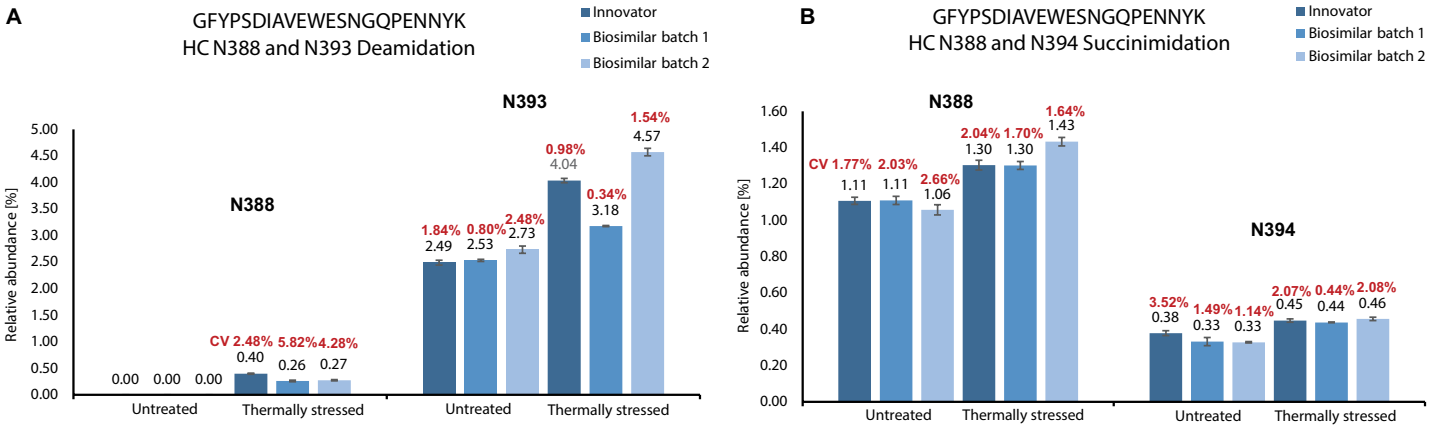

Figure 8. Relative quantitation of deamidation and succinimidation of GFYPSDIAVEWESN(388)GQPEN(393)N(394)YK on the heavy chain in rituximab and biosimilars under thermal stress. N=3 technical replicates. (A) %deamidation of N388 and N393, (B) %succinimidation of N388 and N394.

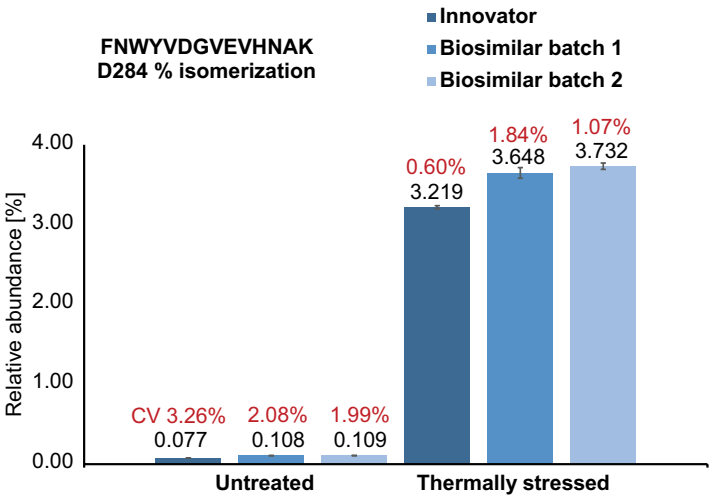

Figure 9. Relative quantitation of isomerization of peptide FNWYVD(284)GVEVHNAK on the heavy chain in thermally stressed and untreated rituximab and two biosimilars samples. N=3 technical replicates.

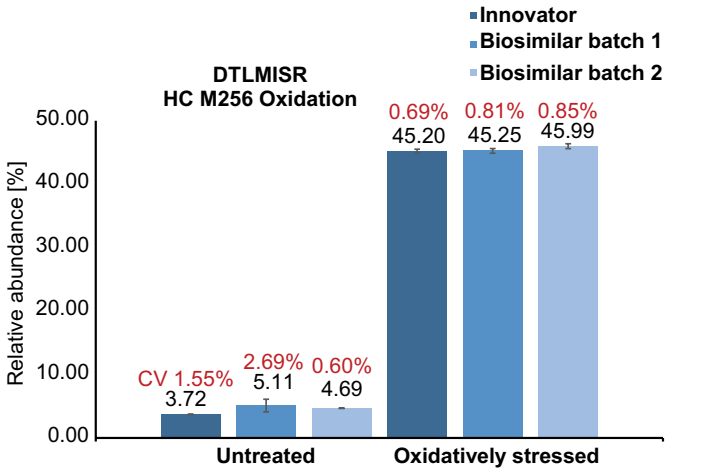

Figure 10. Relative quantitation of oxidation of DTLM(256)ISR on the heavy chain in rituximab and biosimilars under untreated condition or oxidative stress. N=3 technical replicates.

Oxidation is a common protein modification, mainly occurring on methionine, cysteine, histidine, and tryptophan residues. The two methionine oxidation sites found in the human IgG1 monoclonal antibody are M256 on the CH2 domain and M432 on the CH3 domain of the Fc region. Oxidation of both sites could be observed under the treatment of t-butyl hydroperoxide (tBHP), hydrogen peroxide, ultraviolet light, and high-temperature conditions[6]. After the oxidation of these sites, the hydrophobicity of the product decreased. Here we chose M256 oxidation under H_2O_2 stress as an example to observe the effect of oxidative stress. Thermal stress resulted in a moderate increase in the level of oxidation in DTLMISR peptide (from 3–5% to 8–9%, data not shown), while the percent abundance of M256 oxidation increased significantly (from 3–5% to ~45%) under oxidative stress (Figure 10).

PQA profiling report in Chromeleon software

Chromeleon CDS provides spreadsheet-like reporting with extensive options for customization, offering flexibility to users for constructing the report templates to meet their needs.

The example report displayed in Figure 11 shows the selected information of M256 oxidation of innovator and biosimilar rituximab under oxidative stress conditions. The report contains information on data acquisition sequence, peak integration area, % modification, XICs, and MS1 spectra of native and M256 oxidized peptides.

A

Oxidation						
No.	Injection Name	Summed Quantitation counts*min (+1) DTLMISR	Summed Quantitation counts*min (+2) DTLMISR	Summed Quantitation counts*min (+1) DTLM[Oxidation]ISR	Summed Quantitation counts*min (+2) DTLM[Oxidation]ISR	% Oxidation M256
1	20200528_S1_Oxi_MSOnly01	7.702e+06	4.874e+07	4.379e+06	4.183e+07	45.02
2	20200528_S1_Oxi_MSOnly02	6.578e+06	4.725e+07	3.968e+06	4.011e+07	45.02
3	20200528_S1_Oxi_MSOnly03	6.350e+06	4.564e+07	3.905e+06	3.960e+07	45.56
4	20200528_S2_Oxi_MSOnly01	8.577e+06	5.403e+07	4.834e+06	4.771e+07	45.63
5	20200528_S2_Oxi_MSOnly02	8.712e+06	5.282e+07	5.139e+06	4.500e+07	44.90
6	20200528_S2_Oxi_MSOnly03	8.093e+06	5.338e+07	5.254e+06	4.545e+07	45.20
7	20200528_S3_Oxi_MSOnly01	7.753e+06	5.579e+07	4.828e+06	4.972e+07	46.19
8	20200528_S3_Oxi_MSOnly02	8.015e+06	5.199e+07	4.825e+06	4.685e+07	46.27
9	20200528_S3_Oxi_MSOnly03	7.461e+06	5.341e+07	4.605e+06	4.622e+07	45.50
10	20200528_S1_MSOnly01	1.177e+07	7.753e+07	1.843e+05	3.207e+06	3.66
11	20200528_S1_MSOnly02	1.188e+07	7.482e+07	1.884e+05	3.178e+06	3.74
12	20200528_S1_MSOnly03	1.169e+07	7.539e+07	1.924e+05	3.214e+06	3.76
13	20200528_S2_MSOnly01	1.488e+07	9.614e+07	3.288e+05	5.704e+06	5.15
14	20200528_S2_MSOnly02	1.593e+07	9.097e+07	3.619e+05	5.220e+06	4.96
15	20200528_S2_MSOnly03	1.569e+07	9.244e+07	3.521e+05	5.601e+06	5.22
16	20200528_S3_MSOnly01	1.631e+07	1.018e+08	3.446e+05	5.494e+06	4.71
17	20200528_S3_MSOnly02	1.603e+07	9.978e+07	3.383e+05	5.358e+06	4.69
18	20200528_S3_MSOnly03	1.599e+07	9.891e+07	3.530e+05	5.263e+06	4.66

B

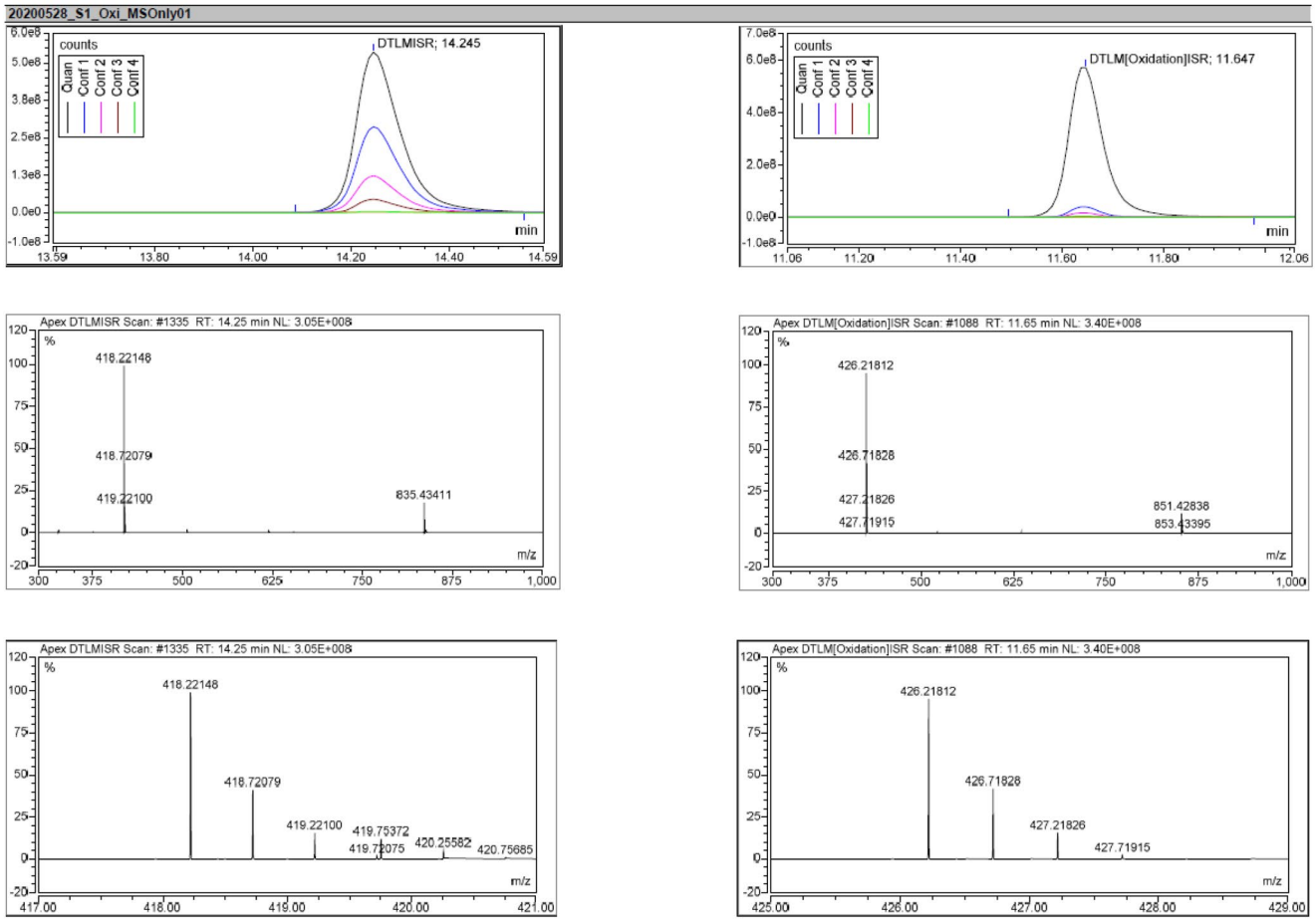

Figure 11. M256 oxidation of heavy chain profiling report generated in Chromeleon CDS. (A) Expanded view of the % modification table. (B) Expanded view of the XICs and MS1 spectra of native and oxidized peptides of the innovator rituximab under oxidative stress condition.

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Conclusion

In this study, we demonstrated how the Thermo Scientific HR-MAM workflow can be used for relative quantitation and monitoring of multiple PQAs simultaneously to improve productivity when assessing the comparability between rituximab innovator vs. biosimilar products and across different batches.

- No significant differences were observed among all PQAs monitored and their response to stress conditions between innovator and biosimilar rituximab, suggesting their high structural similarity.
- The high sensitivity and selectivity of the Q Exactive Plus mass spectrometer, combined with the reproducible separation offered by the Vanquish Flex UHPLC system and Accucore Vanquish C18+ column, enables robust and reproducible quantitation of all PQAs monitored.
- Chromeleon CDS, a full enterprise compliance-ready solution, offers a streamlined workflow for automated data acquisition, processing, and reporting.
- Overall, the HR-MAM workflow was found to be an excellent analytical solution for confident and productive comparability assessment that can be deployed during biosimilar mAb development, manufacturing, and quality control.

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