Application Note

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Rituximab Biosimilar Analysis Using the Agilent InfinityLab Pro iQ Plus LC/MS

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Abstract

The biotherapeutics field is rapidly expanding, with recombinant monoclonal antibodies (mAbs) emerging as key products, particularly in the development of biosimilars. Analytical comparability is critical in biosimilar development to ensure structural consistency with the reference product. Intact and subunit liquid chromatography/mass spectrometry (LC/MS) workflows provide rapid assessment of molecular mass and glycosylation heterogeneity in monoclonal antibodies (mAbs). Single quadrupole LC/MS is frequently employed in quality control (QC) workflows for mass detection due to its reliability, straightforward operation, and favorable cost profile. This application note describes the application of the Agilent InfinityLab Pro iQ Plus mass selective detector, a single-quadrupole LC/MS system with a mass range of *m/z* 2 to 3,000 for molecular mass characterization of mAbs at both intact and subunit levels. Two biosimilars with their innovator compound were analyzed for intact and light and heavy chain subunits. Samples were generated with various molecular mass and glycosylation patterns. The results suggest that biosimilar 1 is more closely related to the innovator.

Introduction

Monoclonal antibody (mAb) therapeutics represent a fast-growing area in today's pharmaceutical market.[1] Innovator mAbs require substantial investments during discovery, development, and manufacturing. This has contributed to the high cost of treatment. As patents for many innovator molecules expire, demand for more affordable generic versions of innovators called biosimilars continues to increase.

To obtain regulatory approval, biosimilar manufacturers must establish that their products show no meaningful differences from the originator in terms of purity and potency.[2] A critical component of this process is a comprehensive analytical comparison to assess physicochemical similarities between the originator and biosimilars. Protein-based therapeutics production is inherently complex and ensuring product quality often requires various analyses. Among the most critical quality attributes are protein molecular weight and glycosylation profiles, which are closely monitored throughout the development and production process. Single quadrupole-based LC/MS platforms have been adopted in the quality control (QC) environment for monitoring complex biomolecules. These instruments enable rapid, cost-effective evaluation of intact proteins and their subunits to detect variations in mass and glycosylation forms

This application note demonstrates the suitability of the InfinityLab Pro iQ Plus single quadrupole LC/MS system, featuring a mass range up to *m/z* 3,000, for determining mAb masses at both intact and subunit levels. Two marketed rituximab biosimilars were evaluated against the innovator product as model systems.[3] The LC/MS analysis of protein species from 23 to 147 kDa molecular weight mAb species included glycoform patterns in both innovator and biosimilars. All samples were analyzed using the InfinityLab Pro iQ Plus coupled to Agilent 1290 Infinity II Bio LC and controlled via Agilent OpenLab software. Molecular masses for each chromatographic peak were obtained using the spectral deconvolution tool in OpenLab CDS software and compared against theoretical average masses to evaluate mass comparison between the innovator and the biosimilar samples.

Experimental

Materials and methods

Biosimilar and innovator samples of rituximab were purchased from a local distributor in Singapore. Dithiothreitol (DTT), guanidine HCl, formic acid, acetonitrile, and ammonium bicarbonate were purchased from Sigma (St. Louis, MO). Ultrapure water was collected from an in-house Millipore Sigma Milli-Q system (Billerica, MA). All mobile phase components were LC/MS grade.

Sample preparation

For intact mAb analysis, samples initially at 10 mg/mL were diluted to 500 ng/µL using the starting LC/MS buffer prior to injection. For reduced mAb heavy chain subunit analysis, samples were first diluted to 1 mg/mL in freshly prepared 50 mM ammonium bicarbonate (pH 8.0) containing 4 M guanidine HCl and 20 mM DTT. The mixtures were incubated at 60 °C for 60 minutes, then cooled to room temperature before processing. The final solutions used for LC/MS analysis were subsequently diluted to 250 ng/µL.

Instrumentation

For LC/MS analysis, the Agilent 1290 Infinity II Bio LC coupled to the Agilent InfinityLab Pro iQ Plus mass detector system was used, including:

- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent InfinityLab Pro iQ Plus mass detector (G6170A)

Software

Agilent OpenLab CDS software version 2.8 was used for data acquisition and data processing. The spectral deconvolution tool in OpenLab CDS was used to determine the molecular mass of the proteins.

The theoretical average mass of the protein compounds was calculated using NIST Mass and Fragment Calculator (v2.0) with NIST defined elemental average mass.[4]

LC/MS analysis

Tables 1 and 2 list the acquisition parameters for LC and MS. Table 3 displays the deconvolution settings in OpenLab CDS data analysis.

Table 1. LC parameters.

Parameter	Agilent 1290 Infinity II Bio LC System
Column	Agilent PLRP-S, 2.1 × 50 mm, 5 µm (p/n PL1912-1502)
Thermostat	5 °C
Solvent A	Water with 0.1% formic acid
Solvent B	Acetonitrile with 0.1% formic acid
Flow Rate	0.4 mL/min
Gradient Program (Intact)	Time (min) %B 0 10 5 60 6 10 8 10
Gradient Program (Reduced/Subunit)	Time (min) %B 0 5 0.1 20 8 40 8.1 70 9.1 70 9.2 5 11 5
Post Time	4 min
Injection Volume	1 µL (intact) and 2 µL (reduced/subunit)
Column Temperature	60 °C

Table 2. MS data acquisition parameters.

Parameter	Agilent InfinityLab Pro iQ Plus LC/MS
Ion Source	Agilent Jet Stream ESI source
Polarity	Positive
Time Filter Window	0.1 min
Stop Time	As pump/No limit
MSI Scan Range	*m/z* 1500 to 3,000 (intact) *m/z* 600 to 2,500 (reduced)
Scan Time	2,000 ms
Detector Gain Factor	1
Fragmentor	275 V (intact) 175 V (reduced)
Fragmentor Ramp	Not checked
Data Storage	Profile
Gas Flow	12 L/min
Nebulizer	50 psi
Sheath Gas Flow	11 L/min
Capillary Voltage	4,500 v
Nozzle Voltage	2,000 v
Gas Temperature	350 °C
Sheath Gas Temperature	360 °C

Table 3. LC/MS deconvolution parameters.

Parameter	Value
Spectrum Extraction Mode	Average Peak
Background Mode	External background time range
Use *m/z* Range	Disabled
Low Molecular Weight	140,000 (intact), 10,000 (reduced)
High Molecular Weight	160,000 (intact), 60,000 (reduced)
Maximum Charge	60
Minimum Peaks in Set	15
MW Agreement (0.01%)	5 or 10
Absolute Noise Threshold	2,000
Relative Abundance Threshold	20%
MW Algorithm	Curve Fit
MW Algorithm Threshold	40
Envelope Threshold	50

Results and discussion

Single quadrupole mass spectrometry enables rapid identification of intact monoclonal antibodies (mAbs) and their major glycoforms with minimal sample preparation. Therapeutic mAbs typically contain one glycosylation site on each heavy chain, resulting in multiple glycoforms arising from different glycan pairings.

Figure 1 presents intact mass spectra of the innovator molecule and its biosimilars acquired using the InfinityLab Pro iQ Plus mass detector. The methods previously developed for mAbs were transferred to this analysis with ease.[5] Only minimal adjustments were needed in both acquisition and processing, demonstrating the robustness and user-friendly nature of the method and its strong transferability. The data include the total ion chromatogram (TIC), charge states from *m/z* 1,500 to 3,000, and a zoomed in view of selected charge states. Because the detector mass range extends to a maximum of *m/z* 3000, only a portion of the intact mAb charge envelope is captured, specifically the region between *m/z* 2,000 and 3,000. Closer examination of the zoomed-in charge states (Figures 1C) reveals multiple peaks per charge state for all mAbs analyzed. These peaks correspond to the main glycoforms previously characterized using high resolution quadrupole time-of-flight (LC/QTOF) analysis of the same molecules.[6] Despite using only the partial charge envelope, deconvolution provides clear results, identifying five glycoforms for the innovator mAb (Figure 2A) and four glycoforms for biosimilar 1 (Figure 2B) and many more glycoforms for biosimilar 2.

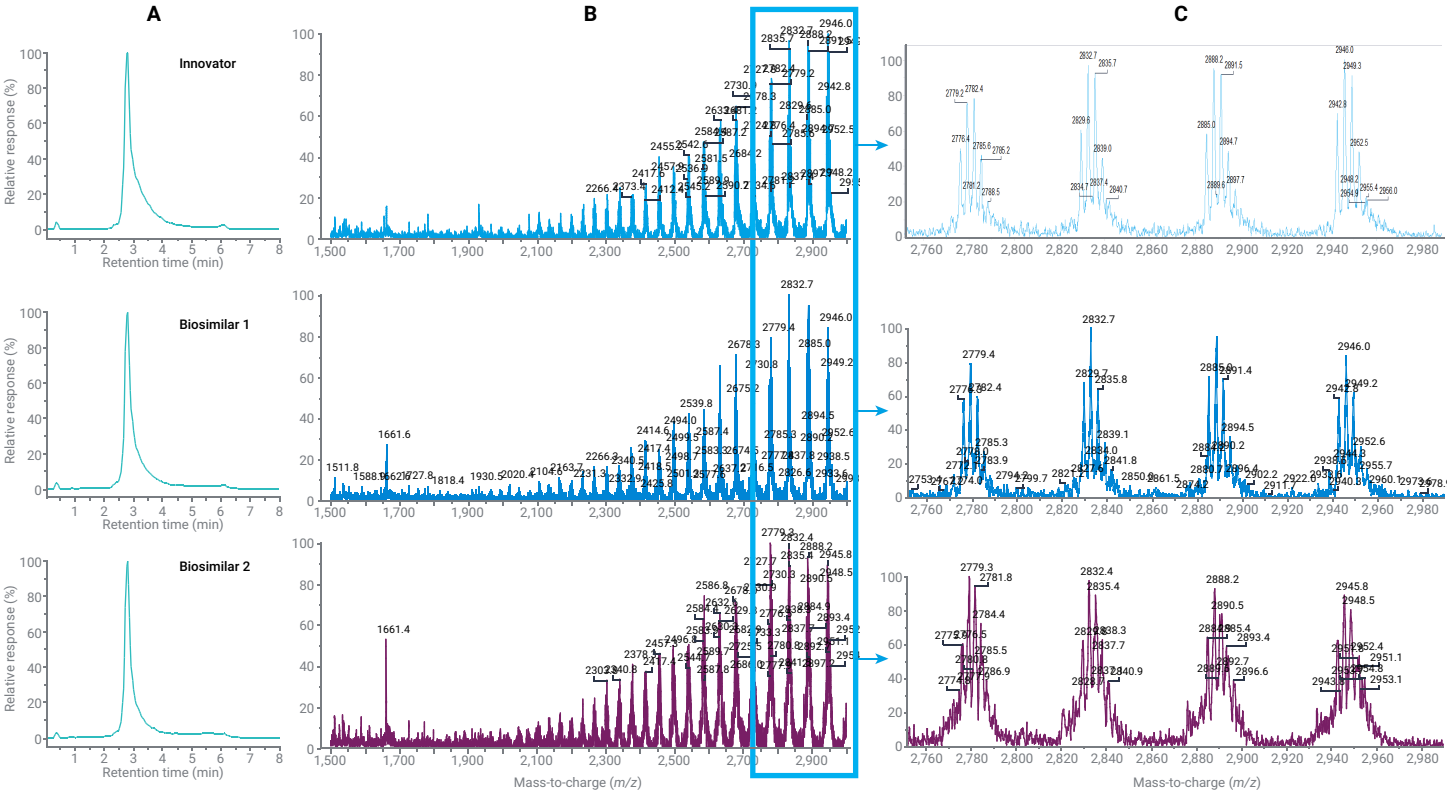

Figure 1. Total ion chromatogram (TIC) (A), charge states from *m/z* 1,500 to 3,000 (B), and zoomed in view of selected charge states (C) for innovator and biosimilars.

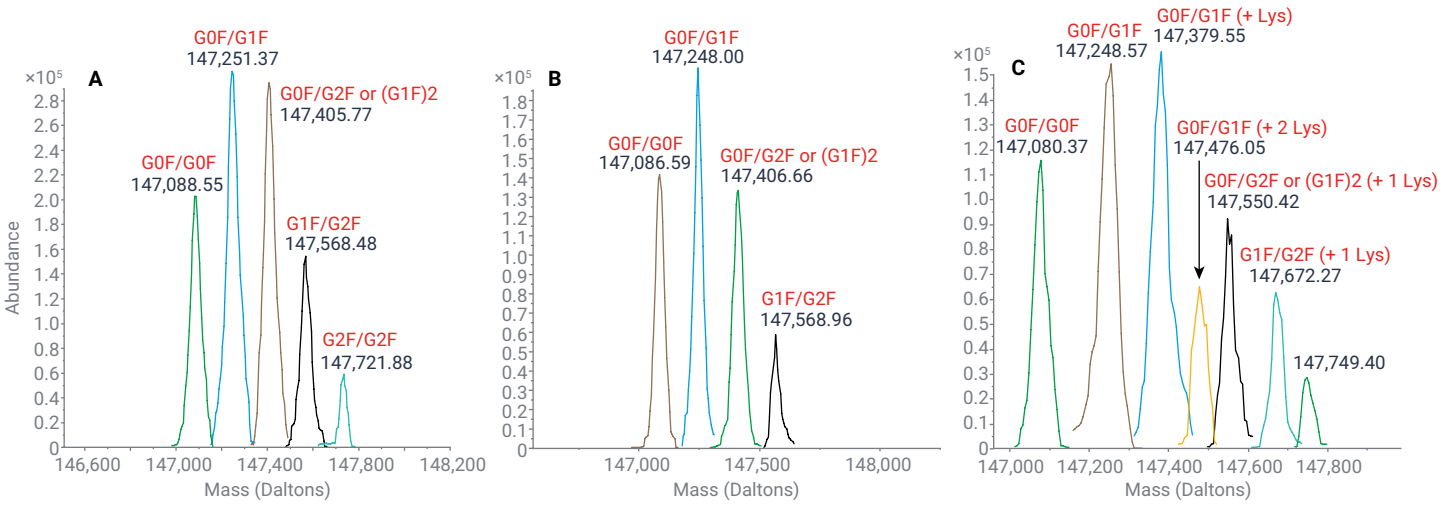

Figure 2. Deconvoluted intact mAb spectra showing major glycoforms (A) innovator, (B) biosimilar 1, and (C) biosimilar 2.

All mAbs analyzed have an approximate molecular weight of 147 kDa. Comparison of the theoretical average masses across innovator and biosimilars with experimentally determined values shows that most intact protein glycoforms exhibit mass deviations between −17.5 and 22.9 Da, corresponding to a mass accuracy of −119 to +155 ppm (Table 4). These measurements allow confident comparisons of the innovator molecule from its biosimilars based on their intact masses.

Inspection of the charge-state distributions and deconvoluted spectra (Figures 1 and 2) indicate that biosimilar 1 closely matches the innovator mAb across all major glycoforms. In contrast, biosimilar 2 displays a more complex spectrum, primarily due to heterogeneous C-terminal lysine truncation as noted in earlier studies.[6] Each lysine loss introduces an average mass shift of −128.2 Da, causing individual glycoforms to appear as multiple peaks rather than single well-defined species, complicating the data interpretation for unit mass resolution instruments. C-terminal lysine on the heavy chains are excised during cell culture due to endogenous carboxypeptidase activity.[7]

Table 4. Comparison of theoretical average intact molecular mass and experimentally observed molecular masses after deconvolution in Agilent OpenLab CDS software.

	mAb Type	Glycoform	Calculated Average Mass (Da)	Experimental Mass (Da)	Δ Mass (Da)	Mass Error (ppm)
Intact mAb	Innovator	G0F/G0F	147,075.0	147,088.6	13.5	92
		G0F/G1F	147,237.1	147,251.4	14.3	97
		G0F/G2F or (G1F)2	147,399.3	147,405.8	6.5	44
		G1F/G2F	147,561.4	147,568.5	7.1	48
		G2F/G2F	147,723.6	147,721.9	−1.7	11
	Biosimilar 1	G0F/G0F	147,075.0	147,086.6	11.6	79
		G0F/G1F	147,237.1	147,248.0	10.9	74
		G0F/G2F or (G1F)2	147,399.3	147,406.7	7.4	50
		G1F/G2F	147,561.4	147,569.0	7.5	51
	Biosimilar 2	G0F/G0F	147,075.0	147,080.4	5.4	37
		G0F/G1F	147,237.1	147,248.6	11.4	78
		G0F/G1F (+ 1 Lys)	147,365.3	147,379.6	14.3	97
		G0F/G1F (+ 2 Lys)	147,493.5	147,476.0	−17.5	−119
		G0F/G2F or (G1F)2 (+ 1 Lys)	147,527.5	147,550.4	22.9	155
		G1F/G2F (+ 1 Lys)	147,689.6	147,672.3	−17.3	−117

Subunit analysis can simplify data interpretation by reducing the size and complexity of intact mAb samples. This approach is achieved by cleaving disulfide bonds using reducing agents such as DTT. Figure 3 shows the TIC, mass spectrum of light chain (LC) and heavy chain (HC). Figure 4 presents the deconvoluted mass spectra of light (~ 23 kDa) and heavy chains (~50 kDa) generated through complete disulfide bond reduction with DTT. As expected, deconvolution of the light chain produces a single, well-defined peak, whereas deconvolution of the heavy chain reveals three major glycoforms derived from the mAbs. The mass accuracy obtained for the light and heavy chains ranges from –62.0 to +28.0 ppm (Table 5). The heterogeneity of the different lysine species observed in intact biosimilar 2 is also reflected in the heavy chains (HC-G1F + Lys and HC-G2F + Lys).

Overall, these findings demonstrate that the InfinityLab Pro iQ Plus mass detector consistently provides mass errors below ±100 ppm for the majority of tested mAbs and their subunits. Only three glycoforms from the intact mAb analysis showed deviations exceeding this threshold. This deviation is likely due to the additional lysine residues and compounded by glycan heterogeneity and prevents clear separation of the 0-, 1-, and 2-Lys forms present in biosimilar 2, which impacts mass accuracy for unit mass resolution instruments.

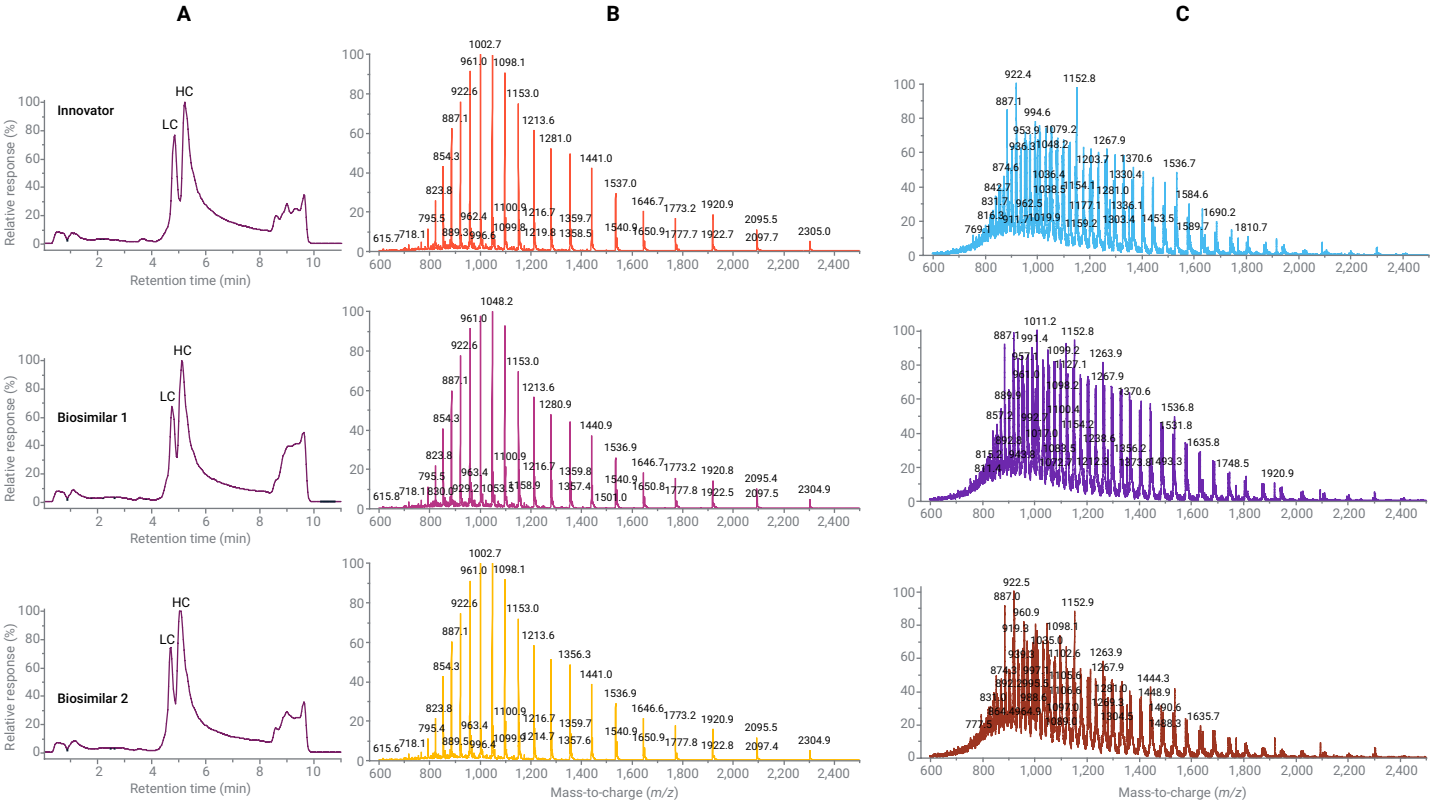

Figure 3. (A) TIC chromatogram showing separation of light and heavy chains in innovator and biosimilars. (B) Charge envelope of innovator and biosimilars light chain peak. (C) Charge envelope of innovator and biosimilars heavy chain peak.

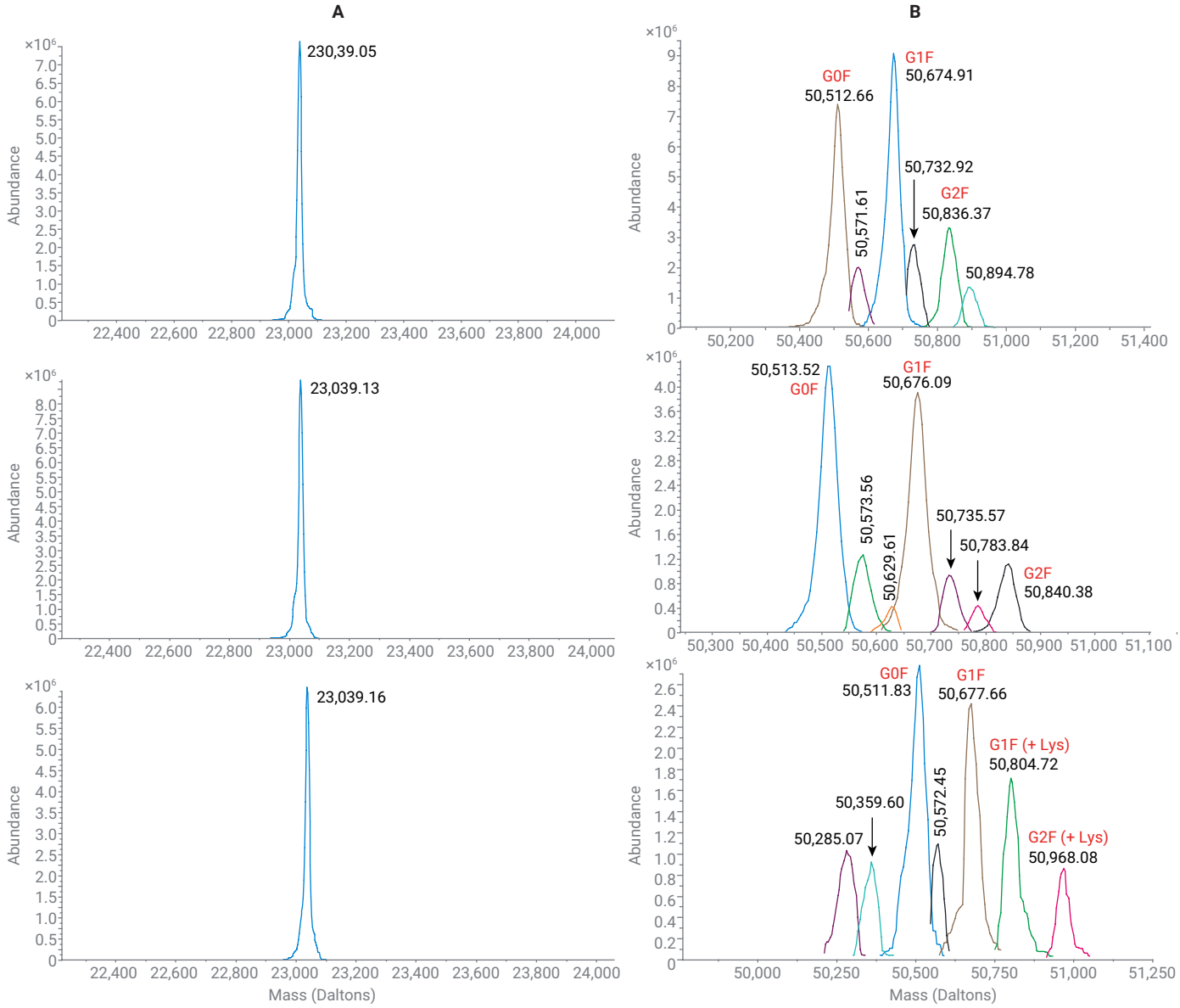

Figure 4. Deconvoluted mass spectra of reduced mAb for innovator and biosimilars. (A) Deconvoluted mass spectra of reduced mAb light chain. (B) Deconvoluted mass spectra of reduced mAb heavy chain.

Table 5. Comparison of theoretical average molecular mass and experimentally observed molecular masses after deconvolution of reduced monoclonal antibody in Agilent OpenLab CDS software.

	mAb Type	Glycoform	Calculated Average Mass (Da)	Experimental Mass (Da)	Δ Mass (Da)	Mass Error (ppm)
Reduced Subunits	Innovator	LC	23,039.40	23,039.05	−0.4	−15
		HC-G0F	50,514.20	50,512.66	−1.5	−30
		HC-G1F	50,676.30	50,674.91	−1.4	−27
		HC-G2F	50,839.50	50,836.37	−3.1	−62
	Biosimilar 1	LC	23,039.40	23,039.13	−0.3	−12
		HC-G0F	50,514.20	50,513.52	−0.7	−13
		HC-G1F	50,676.30	50,676.09	−0.2	−4
		HC-G2F	50,839.50	50,840.38	0.9	17
	Biosimilar 2	LC	23,039.40	23,039.16	−0.2	−10
		HC-G0F	50,514.20	50,511.83	−2.4	−47
		HC-G1F	50,676.30	50,677.66	1.4	27
		HC-G1F +Lys	50,804.50	50,804.72	0.3	5
		HC-G2F +Lys	50,966.65	50,968.08	1.4	28

Conclusion

The result of this study demonstrated minimal sample preparation, deconvolution for molecular mass, and major glycoform profiling using InfinityLab Pro iQ Plus mass detector. Both intact and reduced subunit deconvoluted spectra showed highly comparable mass profiles between the innovator and the biosimilars. The profiles of the innovator and biosimilar 1 were closely aligned, whereas biosimilar 2 exhibited greater variance. Reduced subunit analysis enabled clear assignment of LC and HC glycoforms. Observed masses matched theoretical values with good agreement across intact, LC, and HC measurements. Consistent detection of G0F, G1F, and G2F species demonstrated workflow robustness and reproducibility. The InfinityLab Pro iQ Plus LC/MS platform enabled rapid, reliable intact and subunit analysis for biosimilar comparability studies. The InfinityLab Pro iQ Plus mass detector demonstrated the capability to resolve more complex IgG variants as seen with biosimilar 2. The compact, cost-effective design make the platform well suited for routine applications such as lot release testing, early clone screening, and biosimilar development.

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