Precise Characterization of Intact Monoclonal Antibodies by the Agilent 6545XT AdvanceBio LC/Q-TOF

Application Note

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Introduction

Monoclonal antibodies (mAbs) are a very important class of biopharmaceutical molecules. As a protein drug, thorough characterization of the mAb is required in each of the manufacturing steps. Intact mAb analysis offers rapid assessment on determining the accurate molecular weight of an mAb product and its degree of heterogeneity, such as post-translational modifications (PTMs), antibody-drug conjugate (ADC), mAb sequence variations, or degradation products. Quadrupole Time-of-flight (Q-TOF) LC/MS systems are often used to analyze intact proteins or antibodies due to excellent resolution at the high mass range[1-3]. The Agilent 6545XT AdvanceBio LC/Q-TOF system includes hardware and software features to significantly improve the measurement of biomolecules up to 30,000 *m/z*. This Application Note describes a seamless workflow using the Agilent 1290 Infinity II UHPLC system, 6545XT AdvanceBio LC/Q-TOF, and automatic data processing with Agilent MassHunter BioConfirm software to analyze a variety of mAb products.

Figure 1. Agilent 6545XT AdvanceBio LC/Q-TOF system.

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Experimental

Materials and methods

Monoclonal antibody standard RM 8671 was purchased from National Institute of Standards & Technology (NIST). The formulated Herceptin (Trastuzumab) and formulated ADC (T-DM1) were from Genentech (So. San Francisco, California, USA). All mAb samples were diluted with DI water to 1.0 µg/µL.

LC/MS analysis

LC/MS analyses were conducted on an Agilent 1290 Infinity II UHPLC system coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with a Dual Agilent JetStream source. LC separation was obtained with an Agilent PLRP-S 1000 Å column (2.1 × 50 mm, 5 µm). Table 1 and Table 2 list the LC/MS parameters used. Approximately 0.5 µg of mAb sample was injected for each analysis.

Data processing

All MS data of the mAbs were analyzed using the Protein Deconvolution feature of MassHunter BioConfirm B.08.00 software that uses the Maximum Entropy algorithm for accurate molecular mass calculation. Averaging spectra across the top 25 % of peak height over the mass range of 2,000 to 5,000 *m/z* was used for mass deconvolution. The deconvoluted mass range was set at 140,000 to 160,000 Daltons.

Results and Discussion

While multiple mAbs were analyzed, a common methodology led to excellent data quality for all samples examined. Figure 2 demonstrates the intact protein analysis on the NIST mAb standard. Approximately 0.5 µg of was injected (without sample desalting preparation) onto an Agilent PLRP-S column using a 4-minute gradient with a flow rate of 0.5 mL/min. High-quality MS spectra with multiply-charged ion envelopes of intact mAb were obtained over the mass range of 2,000 to 5,000 *m/z*. The zoom-in spectrum (Figure 2 inset) of each charge state clearly shows the six major glycoforms of the NIST mAb.

The BioConfirm B.08 Protein Deconvolution feature provides not only automatic mass range detection, but also accurate determination of zero-charge state spectra, resulting in excellent mass accuracy. Figure 3 illustrates the MS deconvolution result of the intact NIST mAb. Low ppm errors (average: <0.5 ppm) and superior MS resolution were achieved for all six major glycoforms of the NIST mAb. Beyond the major features of the mAb, other minor heterogeneities of glycosylation such as the loss of GlcNAc residues were easily identified. The raw data gathered by the LC/Q-TOF and the minimal processing of the Maximum Entropy deconvolution algorithm allow the user to detect and preserve fine details about the intact protein composition. This is important, especially when comparing more aggressive analysis techniques that employ high levels of data processing and manipulation that can obscure minor structures.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II UHPLC System	
Column	Agilent PLRP-S, 1000 Å, 2.1 × 50 mm, 5 µm (p/n PL1912-1502)
Thermostat	4 °C
Solvent A	0.1 % Formic acid in DI water
Solvent B	0.1 % Formic acid in 100 % acetonitrile
Gradient	0–1 minutes, 0–20 % B 1–3 minutes, 20–50 % B 3–4 minutes, 50–70 % B
Column temperature	60 °C
Flow rate	0.5 mL/min
Injection volume	0.5 µL

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Source	Dual Agilent Jet Stream
Gas temperature	350 °C
Gas Flow	12 L/min
Nebulizer	60 psig
Sheath gas temperature	400 °C
Sheath gas flow	11 L/min
Capillary voltage	5,500 V
Nozzle voltage	2,000 V
Fragmentor	380 V
Skimmer	140 V
Quad AMU	500 *m/z*
Mass range	100–10,000 *m/z*
Acquisition rate	1.0 spectra/s
Reference mass	922.0098
Acquisition mode	Positive, Extended (10,000 *m/z*) Mass Range

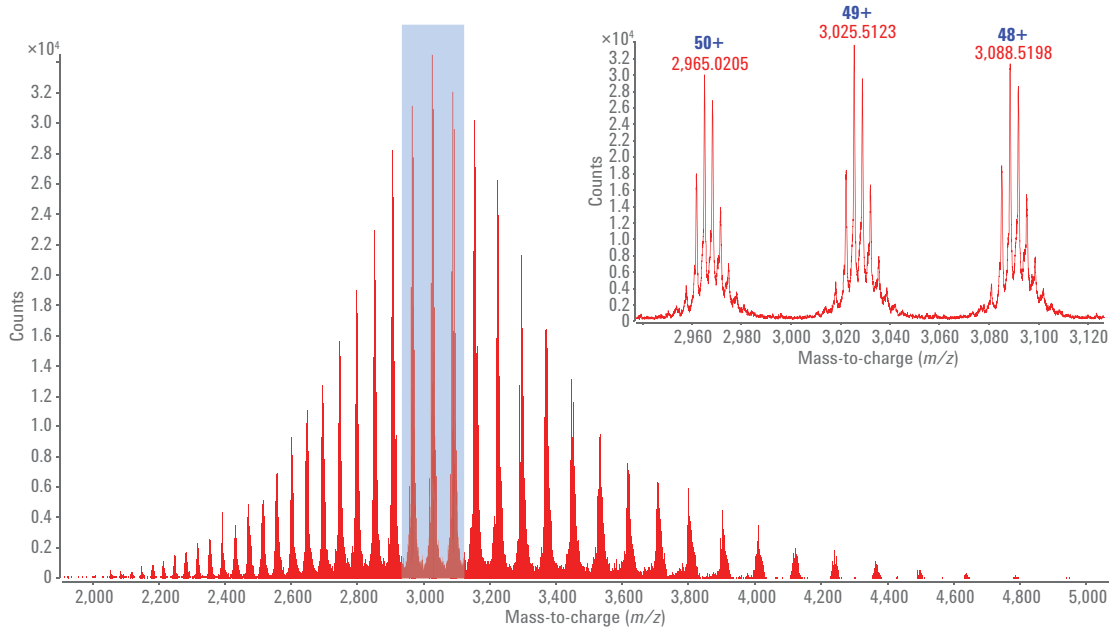

Figure 2. Intact NIST mAb analysis (0.5 µg injection).

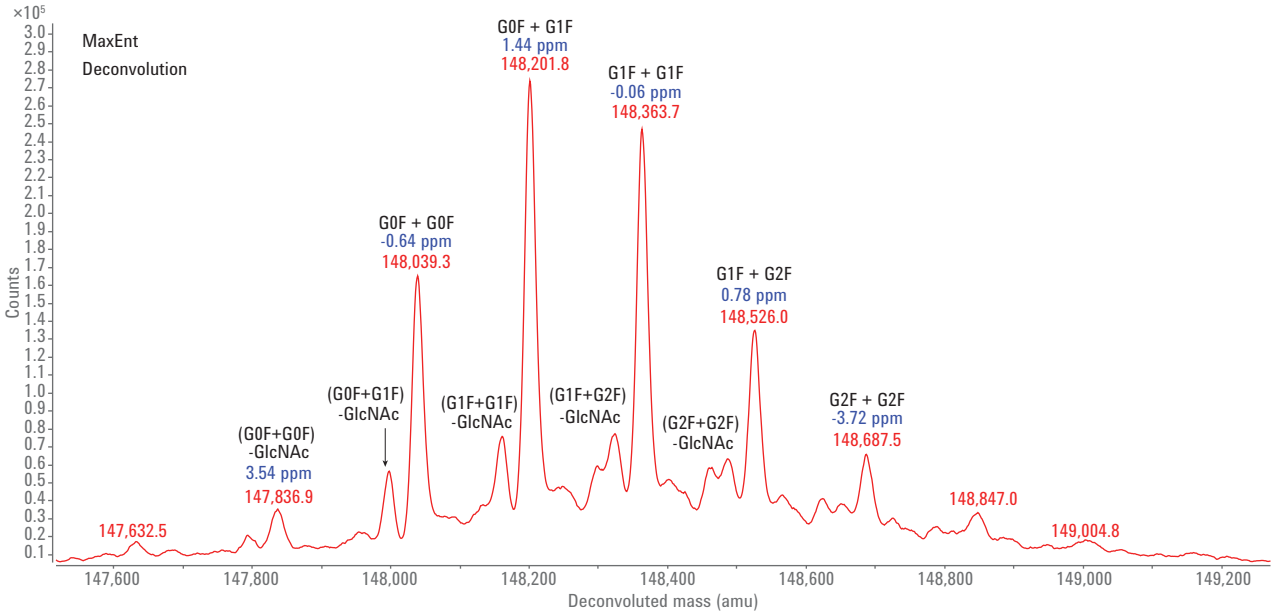

Figure 3. MS Deconvolution of intact NIST mAb (0.5 µg injection).

To confirm the reproducibility of mass accuracy of the 6545XT system on mAb analysis, several mAbs were also analyzed with the same amount of sample injection and HPLC conditions. Table 3 shows the deconvoluted mass results of the top six glycoforms of NIST mAb and Herceptin. Impressive low-ppm mass accuracy and clear representation of all glycoforms was consistently shown.

ADCs represent a new generation of effective biotherapeutics that are target-specific. It is crucial to obtain the accurate drug-to-antibody ratio (DAR) to optimize the efficacy, and minimize the toxicity of the ADC. They present an added challenge due to the increased complexity presented by variable levels of drug conjugation. Figure 4 shows the deconvoluted spectra of an intact glycosylated ADC. Nine mass clusters were observed with masses matching D0–D8 of ADC. The three major peaks in each cluster group correspond with the G0F/G0F, G0F/G1F, and G1F/G1F glycoforms. Most importantly, the average DAR value calculated using the BioConfirm DAR calculator was 3.5, which is consistent with the DAR values of the intact ADC reported previously using data from other analytical methods.

Table 3. Summary of intact mAbs analysis.

	NIST mAb		Herceptin	
mAb	Cal. MW (Da)	Mass error (ppm)	Cal. MW (Da)	Mass error (ppm)
G0 + G0F			147,912.6887	0.76
(G0F + G0F) - GlcNAc	147,836.3503	3.54		
G0F + G0F	148,039.4297	-0.64	148,058.8326	4.76
G0F + G1F	148,201.5729	1.44	148,220.9758	0.16
G1F + G1F	148,363.7162	-0.06	148,383.1191	-5.07
G1F + G2F	148,525.8595	0.78	148,545.2623	-6.74
G2F + G2F	148,688.0027	-3.72	148,707.4056	-13.23

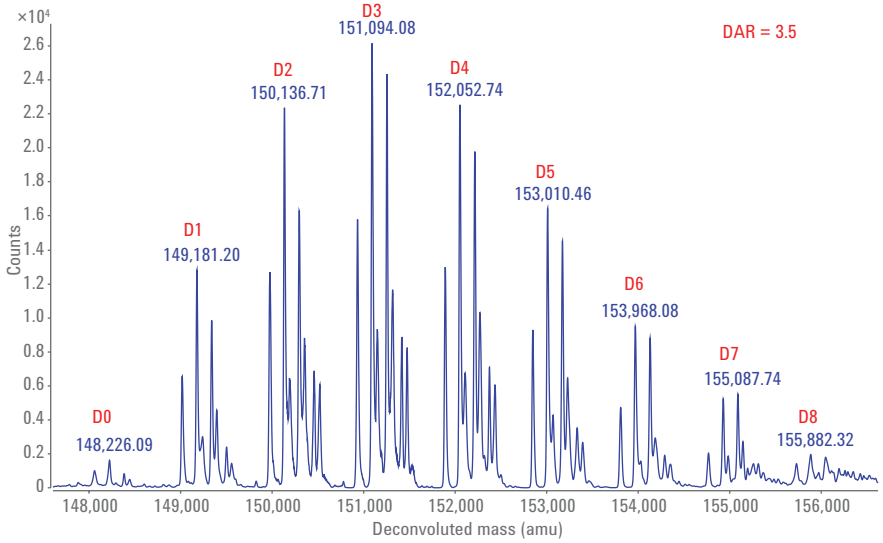

Figure 4. Intact T-DM1 analysis (0.5 µg injection).

The high level of detail provided by the 6545XT AdvanceBio LC/Q-TOF has a direct benefit when developing and analyzing biosimilar therapeutics. BioConfirm permits a direct mirror-plot comparison of deconvoluted spectra (Figure 5) to allow quick visualization of differences between protein samples. In this example, the two samples appear to be very similar in nature. Differences that could alter the quality of the drug product, such as protein sequence (mutation), glycosylation, or protein truncation could quickly be observed.

Conclusion

Monoclonal antibodies have a high level of complexity associated with them, requiring high resolution, precision, and dynamic range to fully characterize them with confidence. This Application Note demonstrates a high-throughput intact mAb analysis workflow solution integrating high-performance chromatography technologies, the Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software for automatic data processing.

- The workflow permits excellent mass accuracy down to the single ppm level for glycoforms measured during the intact mAb mass analysis.
- Highly detailed information was obtained about the heterogeneous composition of mAb proteins. In addition to major glycoforms, minor components of low intensity and similar molecular weight were clearly resolved. Variations such as all major glycoforms with loss of a GlcNAc sugar moiety and the full length protein sequences with a C-terminus lysine of the heavy chain were easily distinguished.

The total analysis time needed with this method is very short, allowing it to be used for large sample sets. With just a 4-minute LC gradient, and the Agilent BioConfirm automatic data processing, it is possible to run an entire 96-well plate in 8 hours.

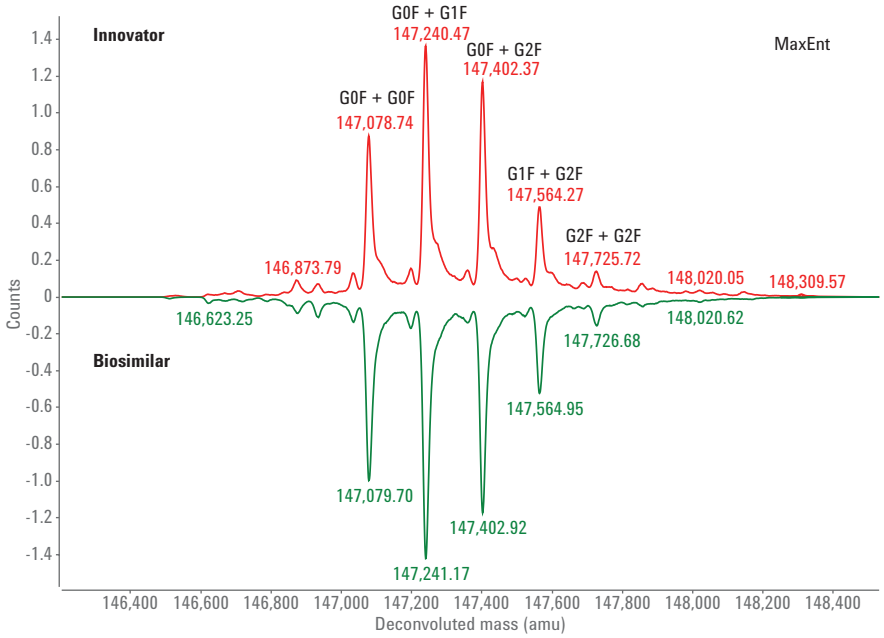

Figure 5. Intact Rituximab analysis (Innovator versus Biosimilar) (0.5 µg).

References

1. Intact Protein Analysis Using an Agilent 6550 Q-TOF Mass Spectrometer, *Agilent Technologies*, publication number 5991-2116EN.

2. Analysis of Monoclonal Antibody (mAb) Using Agilent 1290 Infinity LC System Coupled to Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF), *Agilent Technologies*, publication number 5991-4266EN.

3. An Integrated Workflow for Automated Calculation of Antibody-Drug Conjugate (ADC) Drug-to-Antibody Ratio (DAR), *Agilent Technologies*, publication number 5991-7366EN.

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