Application Note

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Critical Quality Attribute Monitoring of mAbs at the Intact and Subunit Levels Using a Cost-Effective, Simple and Robust LC/MS Solution

Authors

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Abstract

Recombinant monoclonal antibodies (mAbs) represent a fast-growing biotherapeutics drug category. Due to the structural complexity of mAbs, a variety of tests are required to monitor Critical Quality Attributes (CQAs) throughout the development and manufacturing process, for example, monitoring molecular mass and major glycoforms of protein product. Single quadrupole liquid chromatography/mass spectrometry (LC/MS) has been used as an effective mass detection technique in the quality control (QC) environment for its robustness, simple operation, and cost-effective characteristics.

This Application Note describes use of the Agilent LC/MSD XT mass selective detector, a single quadrupole LC/MS system with a mass range of 10 to 3,000 *m/z*, for mass determination of mAbs at intact and subunit levels. Two mAbs, NISTmAb and an immunoglobulin 1 (IgG1) mAb (mAb1), were prepared using four different approaches:

- Intact protein dilution
- Deglycosylation
- IdeS digestion
- Reduction

Samples were generated with various molecular mass and glycosylation patterns and were analyzed using the Agilent InfinityLab Liquid Chromatography/Mass Selective Detector XT (LC/MSD XT), and deconvoluted using Agilent OpenLab ChemStation software to determine the molecular mass and monitor the major glycoforms.

Introduction

In the biotherapeutic industry, production of protein-based biotherapeutics is a complex process that often requires running a panel of tests to ensure product efficacy and safety. Protein molecular mass and glycosylation are important product quality attributes, which are closely monitored during the development and production process.[1] For this purpose, single quadrupole-based LC/MS has been adopted in the QC environment for monitoring complex biomolecules. Using single quadrupole-based LC/MS, analysis of an intact protein drug and its subunits in a QC environment has become feasible for identifying variations in protein mass and glycosylation pattern, providing a rapid and cost-effective approach to monitoring the product CQAs.

This study demonstrates that the InfinityLab LC/MSD XT, a single quadrupole-based LC/MS system with a mass range up to 3,000 *m/z*, is suitable for mass determination of mAbs at intact and subunit levels. Two mAbs, NISTmAb and mAb1, were selected as test cases. The samples were prepared using four approaches, intact protein dilution, deglycosylation, IdeS digestion, and reduction experiments to produce intact protein and various subunit fragments (Figure 1). These experiments produced protein compounds with molecular masses ranging from 23 to 148 kDa, and various glycoform patterns at three mass ranges including ~25, ~50, and ~148 kDa. All the samples were analyzed using the InfinityLab LC/MSD XT, coupled with an Agilent 1290 Infinity II LC and Agilent OpenLab ChemStation Software. The molecular mass of each compound peak was determined using the LC/MS deconvolution tool within OpenLab ChemStation software, and then compared to their theoretical average mass for mass accuracy evaluation.

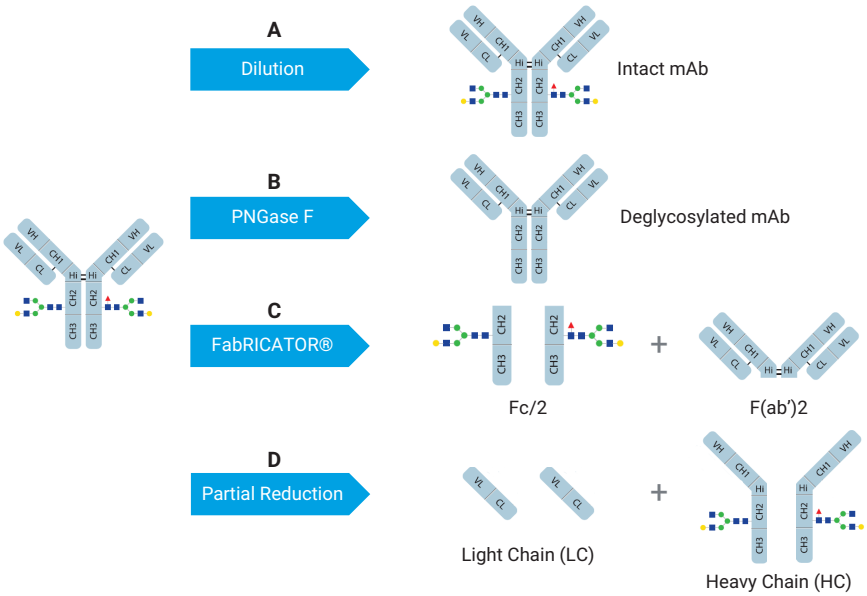

Figure 1. Scheme of sample preparation workflow. A: Dilution of Intact mAb. B: Deglycosylateion of mAb by PNGase F enzyme. C: Digestion of mAb by IdeS enzyme (FabRICATOR). D: Partial reduction of mAb. Image as kindly provided by Genovis.

Experimental

Materials

The NISTmAb reference material was purchased from the National Institute of Standards and Technology. The recombinant IgG1 mAb (mAb1) was obtained from a partner lab. PNGase F enzyme (P0705L) was purchased from New England BioLabs. IdeS enzyme (FabRICATOR) was purchased from Genovis.

Sample preparation

- **Intact mAbs:** Intact mAb stocks (10 µg/µL NISTmAb or 21 µg/µL mAb1) were diluted in H_2O to a final concentration of 0.5 µg/µL.
- **Deglycosylation of mAbs:** 20 µg mAb (NISTmAb or mAb1) was added into 2 µL of 10x G7 reaction buffer that was supplied with the PNGase enzyme by the vendor, diluted with H_2O to a total volume of 19 µL, then increased with 1 µL of PNGase F enzyme to give a final mAb concentration of 1 µg/µL. The resulting sample was incubated at 37 °C for one hour.
- **IdeS digestion:** 20 µg mAb (NISTmAb or mAb1) was incubated in 40 µL of reaction buffer containing 2 mM Na_2HPO_4, 6 mM NaCl, pH 6.6, and ~25 units of FabRICATOR at 37 °C for 30 minutes.
- **Partial reduction:** 40 µL of 0.5 µg/µL mAb (NISTmAb or mAb1) was incubated with 20 mM DTT at 60 °C for 30 minutes.

All the samples were injected immediately after sample preparation for LC/MS analysis.

LC/MS analysis

Liquid chromatography separation was carried out on a PLRP-S column (1,000 Å, 2.1 × 50 mm, 5 µm, p/n PL1912-1502) using an Agilent 1290 Infinity II LC, consisting of an Agilent 1290 Flexible Pump (G7104A), a Multisampler (G7167B) with sample cooler (Option 100), and a Thermostatted Column Compartment (G7116B). The MS system used was the Agilent LC/MSD XT (G6135BA) with the Agilent Jet Stream source (G1958-65138). Tables 1 and 2 list the LC/MS conditions.

Software

Agilent OpenLab ChemStation (version C 01.09) was used for data acquisition, data processing, and reporting.[2,3] The LC/MS deconvolution tool in ChemStation was used to determine the molecular mass of each protein compound. The theoretical average mass of each protein compound was calculated using NIST Mass and Fragment Calculator (v2.0) with NIST defined elemental average mass.[4]

Results and discussion

Agilent LC/MSD XT mass selective detector has an extended mass range up to 3,000 *m/z*, which covers the whole charge envelope of most mAb subunits and a portion of the charge envelope of intact mAb. To evaluate the reliability of mass determination of mAbs at both intact and subunit levels, NISTmAb and mAb1 were prepared by four difference methods as previously described, then injected onto the LC/MSD XT using the methods in Tables 1 and 2. To determine the mass of each protein compound peak, the deconvolution tool of the OpenLab ChemStation software was used to deconvolute the mass spectral data. Average spectra were selected at half peak height. Ion Peak Width at Half Height (PWHH) was set to 0.6 Da and Gaussian Curve fitting was used for molecular weight (MW) assignment.

Table 1. LC conditions

LC parameters					
Analytical Column	PLRP-S 1000Å, 2.1 x 50 mm, 5 µm (p/n: PL1912-1502)				
Mobile Phase A	H_2O with 0.1% (v/v) formic acid				
Mobile Phase B	Acetonitrile with 0.1% (v/v) formic acid				
Column Temperature	80 °C				
Flow Rate	0.5 mL/min				
Gradient	Intact mAb				
	Time (minutes)	%A	%B	%C	%D
	0.0	90	10	0	0
	5.0	40	60	0	0
	6.0	90	10	0	0
	8.0	90	10	0	0
	mAb Subunits				
	Time (minutes)	%A	%B	%C	%D
	0	95	5	0	0
	1.0	80	20	0	0
	8.0	60	40	0	0
	8.1	50	50	0	0
	9.1	95	5	0	0
	11.0	95	5	0	0

Table 2. MS conditions.

MSD XT parameters	
Drying Gas Flow	12 L/min
Drying Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Sheath Gas Temperature	360 °C
Nebulizer Pressure	50 psi
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Peak Width	0.1 minutes
Scan	positive mode, step size 0.1, 1,000 to 3,000 *m/z* for intact mAb, 500 to 3,000 *m/z* for mAb subunits

Figure 2 shows intact NISTmAb and mAb1 analysis using the InfinityLab LC/MSD XT, including Total Ion Chromatogram (TIC), average mass spectra at half peak height, and deconvoluted mass. Both mAbs have a MW of ~148 kDa. Since the mass range of LC/MSD XT is up to 3,000 *m/z*, only a portion of the charge envelope of intact mAbs was detected, which falls within the range of 2,000 to 3,000 *m/z*. The spectral zoom-in views of one of the charge states (Figures 2C and 2D) show multiple peaks from NISTmAb and mAb1, which correspond to their major glycoforms observed on a high-resolution LC/Q-TOF.[5] Using this partial charge envelope, the deconvoluted mass yields five glycoforms of NISTmAb (Figure 2E) and three glycoforms of mAb1 (Figure 2F). Comparing the theoretical average molecular mass and experimental mass shows most intact protein glycoforms have a mass deviation of approximately 10 Da, ranging from −3.1 to −20.2 Da, that is, a mass accuracy of −21 to −136 ppm (Table 3).

To further test the application of the InfinityLab LC/MSD XT for large protein analysis, the PNGase F enzyme was used to remove N-linked oligosaccharides from mAbs. The deglycosylated mAbs have a MW of ~145 kDa. Figure 3 shows the analysis of deglycosylated NISTmAb and mAb1 using this system. It shows the multiple glycoforms were collapsed into one major peak (Figures 3C and 3D). The deconvoluted mass shows a mass error of −7.5 Da/−52 ppm for deglycosylated NISTmAb and 0.2 Da/1 ppm for deglycosylated mAb1 (Figures 3E and 3F; Table 3).

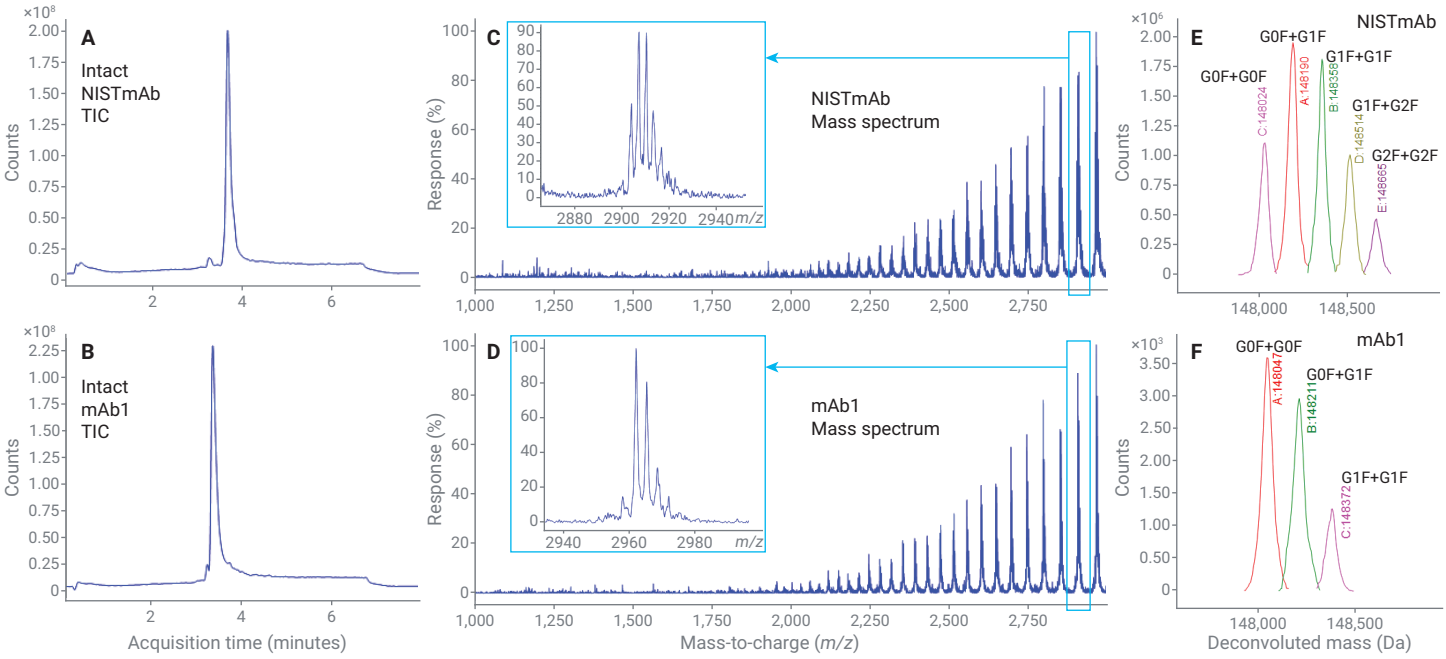

Figure 2. Intact NISTmAb and mAb1 analysis. Panels A,B: TIC. Panels C,D: Average Mass spectra at half peak height with the zoom-in view of one of the charge states in the insets. Panels E,F: Deconvoluted mass.

Table 3. Comparison summary of the theoretical average molecular mass and experimental mass as determined by deconvolution using Agilent ChemStation software.

Experiment	Subunits	Modification	Average Mass (Da)	Experimental Mass (Da)	Δ Mass (Da)	Mass Error (ppm)
Intact Protein Dilution	Intact NISTmAb	G0F + G0F	148,036.5	148,023.7	-12.8	-86
		G0F + G1F	148,198.6	148,189.5	-9.1	-62
		G1F + G1F	148,360.8	148,357.7	-3.1	-21
		G1F + G2F	148,522.9	148,514.2	-8.7	-59
		G2F+G2F	148,685.1	148,664.9	-20.2	-136
	Intact mAb1	G0F + G0F	148,055.9	148,047.3	-8.6	-58
		G0F + G1F	148,218.0	148,211	-7.0	-47
		G1F + G1F	148,380.2	148,372.2	-8.0	-54
PNGaseF Digest	Deglycosylated NISTmAb	None	145,145.8	145,138.3	-7.5	-52
	Deglycosylated mAb1	None	145,165.2	145,165.4	0.2	1
IdeS Digest	NISTmAb F(ab')2	None	97,608.6	97,606.3	-2.3	-24
	NISTmAb Fc/2	G0F	25,232.0	25,230.8	-1.2	-46
		G1F	25,394.1	25,393.1	-1.0	-39
		G2F	25,556.2	25,555.3	-0.9	-37
	mAb1 F(ab')2	None	97,628.0	97,622.1	-5.9	-61
	mAb1 Fc/2	G0F	25,232.0	25,230.8	-1.2	-46
		G1F	25,394.1	25,392.8	-1.3	-51
Partial Reduction	NISTmAb Light Chain	None	23,123.5	23,122.3	-1.2	-51
	NISTmAb Heavy Chain	G0F	50,898.8	50,900.7	1.9	38
		G1F	51,060.9	51,060.7	-0.2	-4
		G2F	51,223.1	51,222.3	-0.8	-15
	mAb1 Light Chain	None	23,438.8	23,437.8	-1.0	-42
	mAb1 Heavy Chain	G0F	50,593.2	50,593.2	0.0	0
		G1F	50,755.3	50,752.1	-3.2	-64

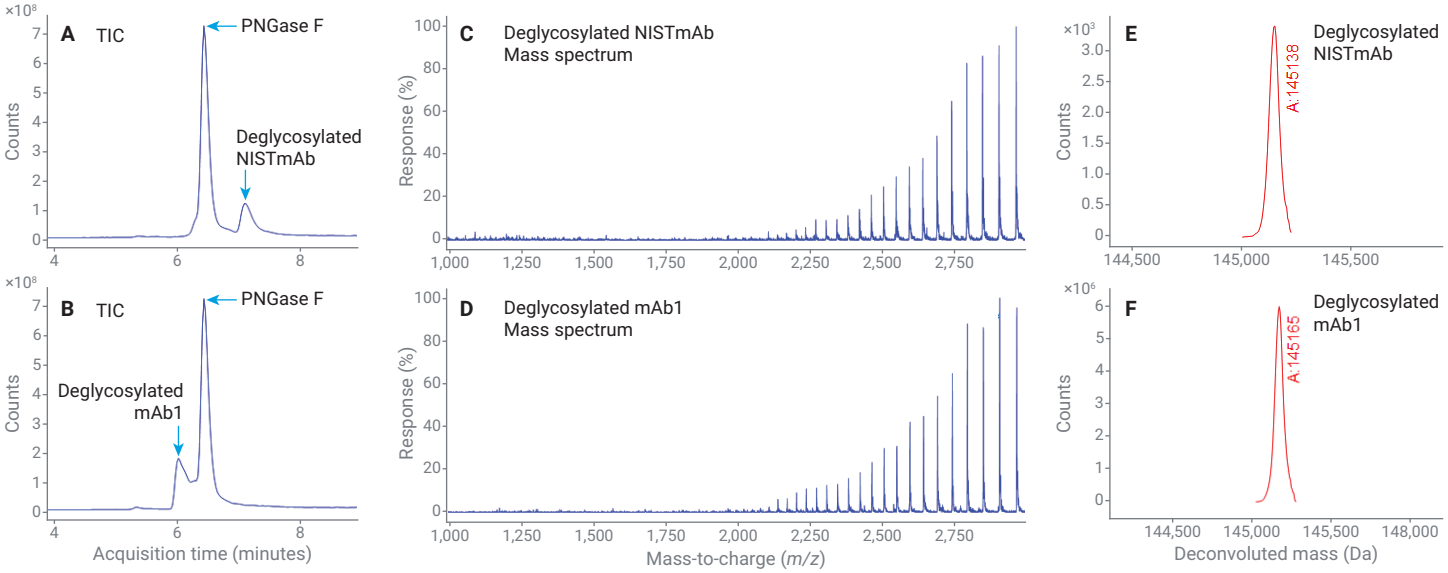

Figure 3. Deglycosylated NISTmAb and mAb1. Panels A,B: TIC. Panels C,D: Average mass spectra at half peak height. Panels E,F: Deconvoluted mass.

To assess the application of LC/MSD XT for subunit analysis, the commonly used IdeS enzyme was used to generate subunit fragments from NISTmAb and mAb1. By performing IdeS digestion of each mAb without reduction, two fragments–Fc/2 and F(ab')2–were produced, which are ~25 and ~98 kDa in size, respectively (Figures 4 and 5). Most of the charge envelope from the two fragments was observed by LC/MSD XT, even for the larger F(ab')2 fragment (Figures 4B, 4C, 5B, and 5C). After digestion, the N-glycosylation site was retained on the small fragment Fc/2. The Fc/2 fragment of NISTmAb shows three major peaks corresponding to G0F, G1F, and G2F glycoforms with the mass error ranging from −0.9 to −1.2 Da. The Fc/2 fragment of mAb1 shows two major peaks corresponding to G0F and G1F with the mass error of −1.2 and −1.3 Da, respectively. The F(ab')2 fragments from NISTmAb and mAb1 show a single major peak, and their deconvoluted mass have a mass error of −2.3 and −5.9 Da, that is, −24 and −61 ppm, respectively (Figures 4E and 5E; Table 3).

In addition to the preceding application,

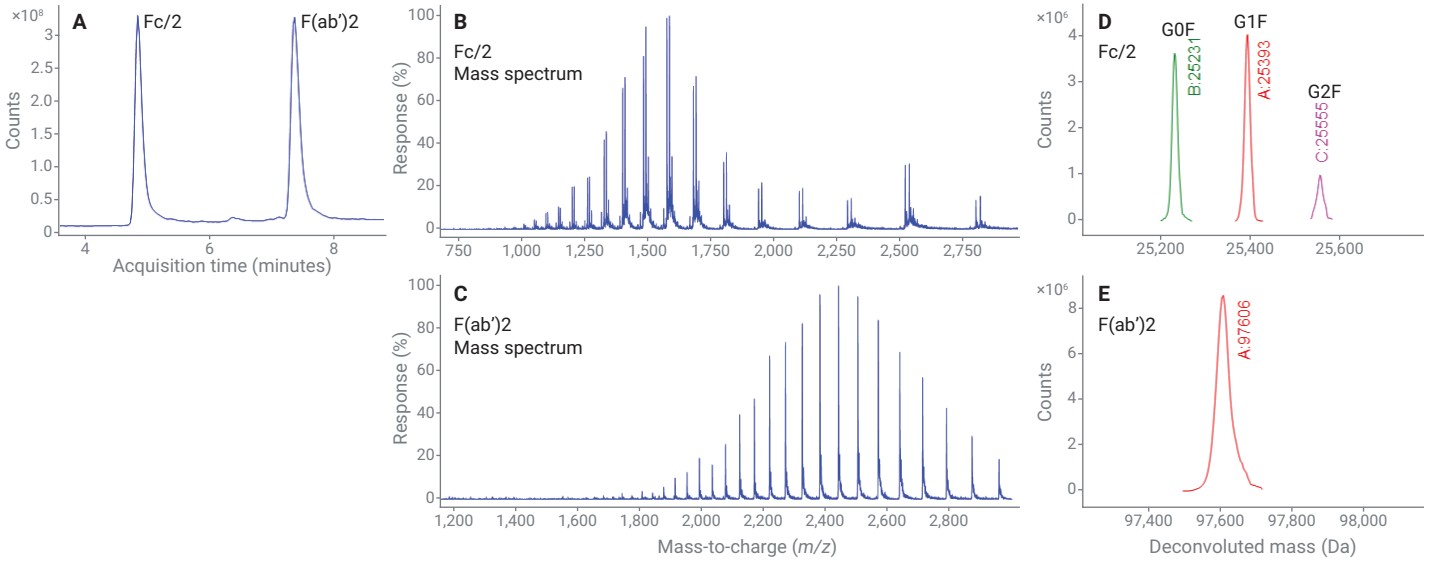

Figure 4. NISTmAb fragments after IdeS digestion. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

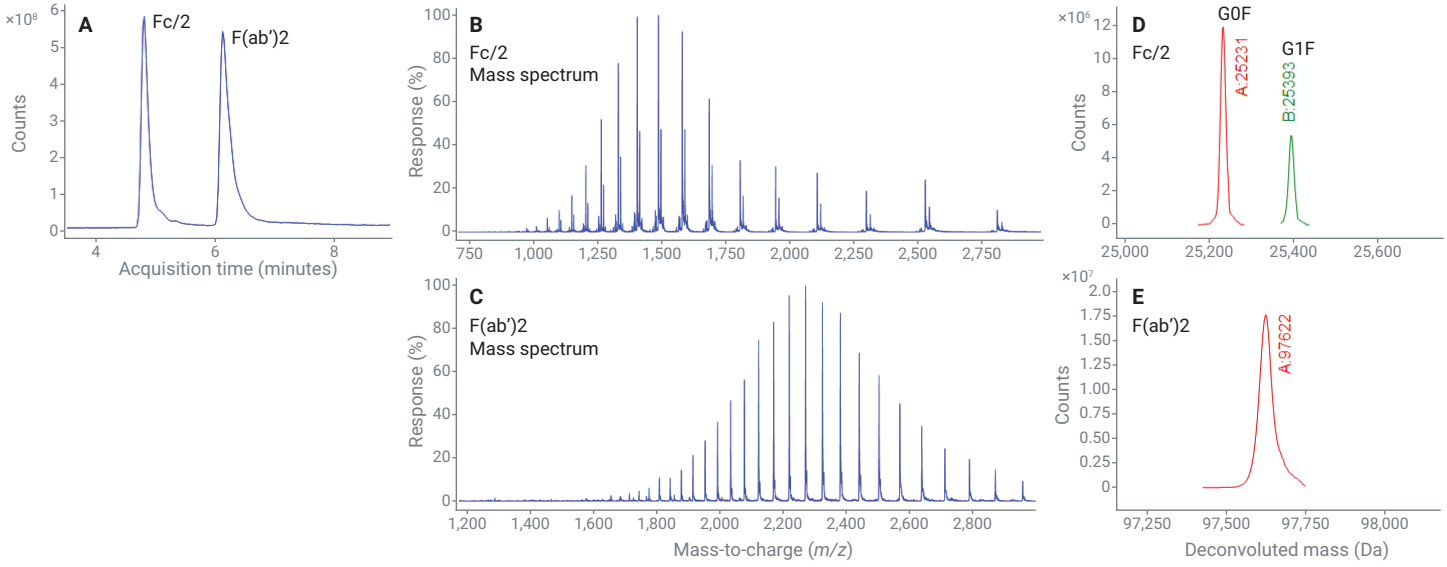

Figure 5. mAb1 fragments after IdeS digestion. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

the mAbs were also partially reduced as described in the experimental method to obtain data for the light and heavy chains. As expected, the deconvolution of the light chain collapses the charge envelope to a single peak while the deconvolution of the heavy chain identifies three major glycoforms from NISTmAb and two major glycoforms from mAb1 (Figures 6 and 7). The mass accuracy of the light and heavy chains from these two mAbs ranges from −64 to 38 ppm (Table 3).

In summary, these results show that the InfinityLab LC/MSD XT yields a mass error less than ±70 ppm for most protein and subunits tested. Only two glycoforms from intact mAbs showed a mass error greater than ±70 ppm.

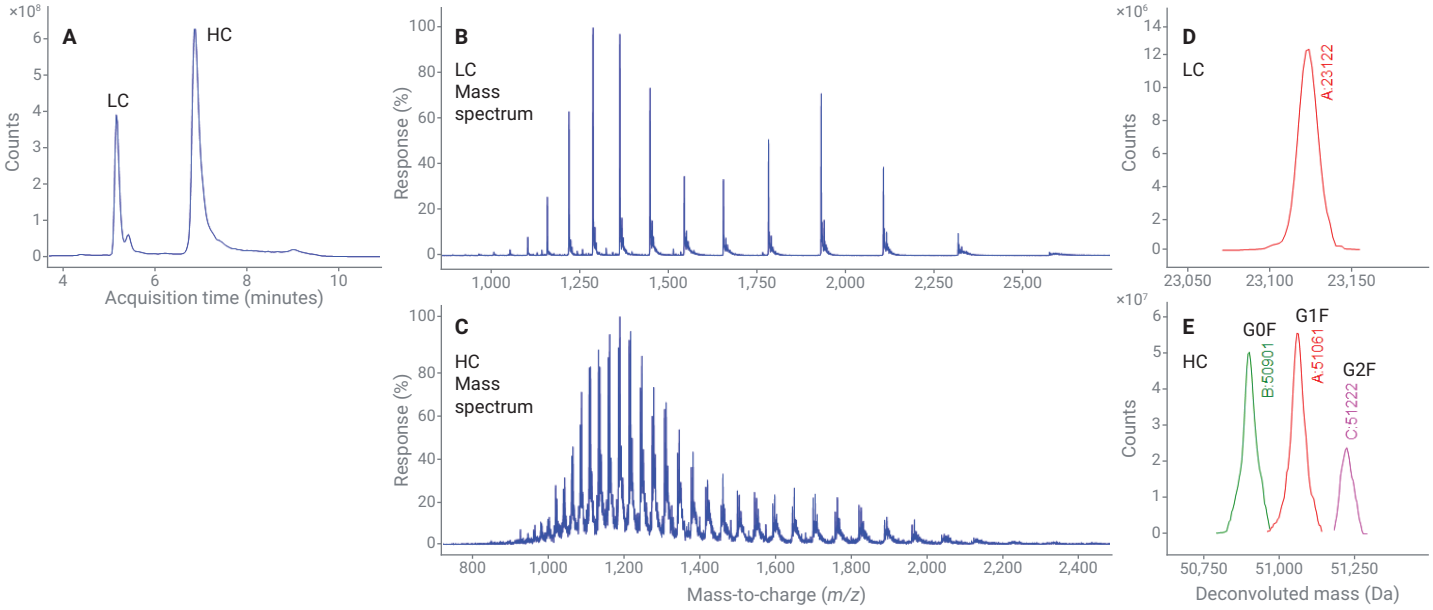

Figure 6. NISTmAb Light Chain (LC) and Heavy Chain (HC) subunits after partial reduction. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

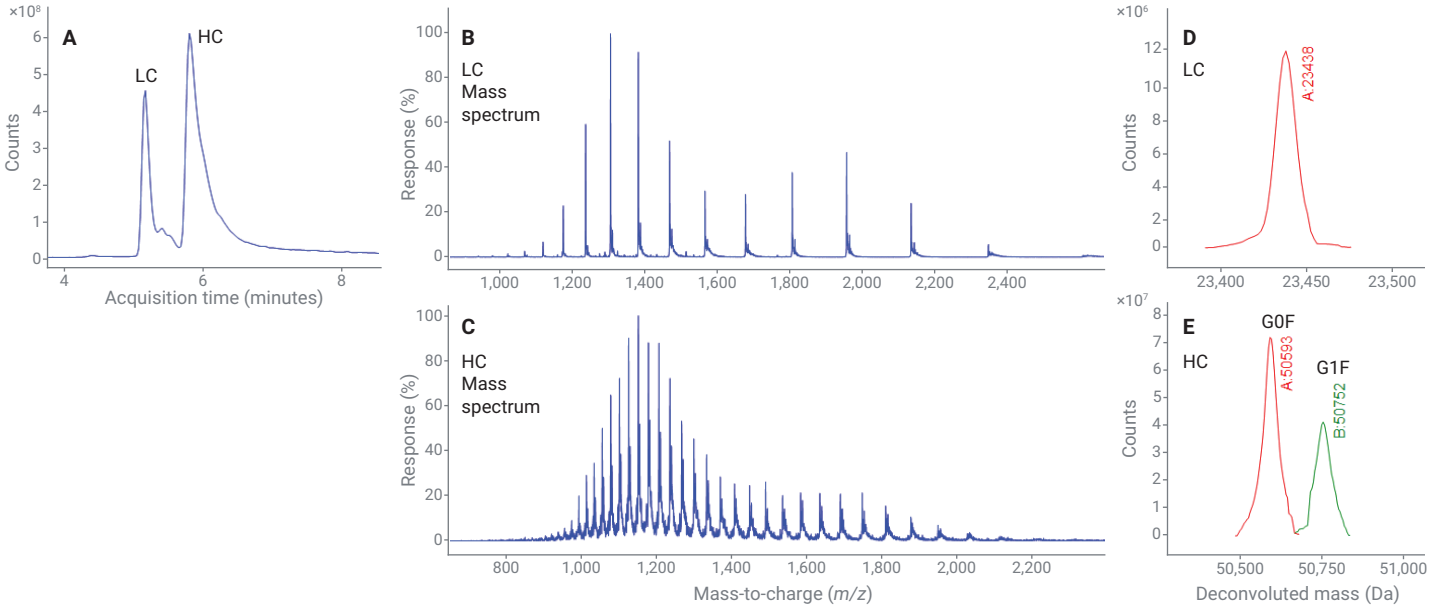

Figure 7. mAb1 subunits after partial reduction. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

Conclusion

This Application Note demonstrates that the Agilent InfinityLab LC/MSD XT can be used for quick and reliable mass determination of mAbs at both intact and subunit levels using four different sample preparations. These four procedures produced various mAb samples, with the molecular weight ranging from ~23 to ~148 kDa. This mass range covers most protein samples at intact and subunit levels. This study shows that the InfinityLab LC/MSD XT can provide a simple, rapid, and cost-effective way to monitor variations in the molecular weight and in the glycoform pattern in an analytical development and quality control environment.

References

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