Sub-nanogram Level Intact Monoclonal Antibody Quantitation by LC/Q-TOF

Application Note

Authors

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Introduction

Quantifying biopharmaceuticals is often approached by targeted multiple reaction monitoring (MRM) techniques focusing on signature peptides from enzymatically digested samples. While this technique does offer the highest level of analytical sensitivity, it has two significant limitations: the inability to observe unexpected molecular species as well as possible artifacts introduced through sample handling. Quantifying at the intact protein level avoids these limitations, but faces separate challenges of analytical sensitivity and reproducibility. Using the latest developments in chromatography and the high performance Agilent 6545XT AdvanceBio LC/Q-TOF, this study establishes a highly sensitive and reproducible quantitative analytical method that spans an impressive linear dynamic range.

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

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Experimental

Reagents and chemicals

Formulated Trastuzumab monoclonal antibody (mAb) standard was sourced from Genentech (So. San Francisco, California, USA). Bovine serum albumin (BSA) and formic acid (FA) were sourced from Sigma-Aldrich.

Sample preparation

Stock 24 µg/µL concentration of glycosylated Trastuzumab was diluted in DI water containing 0.01 % BSA (w/v) and 0.1 % FA to cover a range from 0.01 ng/µL to 100 ng/µL. The dilution ratio was constant at 1:3.162, the square root of 10, resulting in a half order of dynamic range per dilution level.

Equipment and software

Separation was carried out using an Agilent 1290 Infinity II UHPLC System consisting of:

- Agilent 1290 Infinity II Binary Pump (G4220B)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Thermostatted Column Compartment (G7116B)

The UHPLC system was coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with a Dual Agilent Jet Stream electrospray ionization source. Agilent MassHunter Acquisition (B.08.01) workstation software with large molecule SWARM autotune feature was used to acquire data. Agilent MassHunter BioConfirm (B.08.00) and Agilent MassHunter Quantitative Analysis (B.08.00) were used for data analysis.

Methods

Table 1 summarizes the Agilent 1290 Infinity II UHPLC conditions. The gradient method used included a stop flow period of 0.9 minutes. Table 2 summarizes the mass spectrometer conditions. SWARM autotune was used to optimize large molecule transmission and detection.

Table 1. Agilent 1290 Infinity II UHPLC conditions.

LC Conditions		
Column	Agilent ZORBAX RRHD 300-Diphenyl 1.8 µm, 2.1 × 50 mm, 857750-944	
Column temperature	80 °C	
Injection volume	1 µL	
Multisampler temperature	4 °C	
Multiwash	30 seconds of 90/10 ACN/H_2O, then 10 seconds of starting condition	
Mobile phase	A) H_2O, 0.1 % formic acid B) 90/10 Acetonitrile/H_2O, 0.1 % formic acid	
Flow rate	0.500 mL/min	
Gradient	0.0 minutes 0.1–1.0 minutes 2.0 minutes 3 minutes	34.6 % B Stop flow for 0.9 minutes 34.6 % B 90 % B
Stop time	4 minutes	
Post time	1.5 minutes	
Turn-around time	~6.5 minutes (including injection)	

Table 2. Agilent 6545XT AdvanceBio LC/Q-TOF method parameters.

Parameter	Value
Ion mode	AJS, Positive
Gas temperature	290 °C
Drying gas flow	13 L/min
Nebulizer gas	45 psi
Sheath gas temperature	380 °C
Sheath gas flow	12 L/min
Capillary voltage	5,500V
Nozzle voltage	2,000 V
Fragmentor	380 V
Mass range	600–5,000
CE	0
Quad AMU	350
Acq rate	2 spec/s

Results and Discussion

Analytical Sensitivity

This method was able to detect injections down to a quantity of 0.0316 ng of intact mAb on-column. Extracted ion chromatograms (EICs) were generated that summed a total of the 12 most intense glycoform peaks over three charge states, with an extraction window of ±2 *m/z* for each peak. Figure 1 shows the overlaid extractions of six replicate injections for each dilution level, along with deconvoluted spectra at each level as a demonstration of consistent spectral results. The excellent intact protein sensitivity of the 6545XT AdvanceBio LC/Q-TOF can be attributed to a variety of design attributes in optics, vacuum, and heater systems that are fully realized when paired with the SWARM autotune feature to optimize for large molecules. While not shown here, this experiment was also performed on an Agilent 6550 iFunnel Q-TOF LC/MS system, which features a hexabore capillary design to maximize ion sampling. The 6545XT uses a single-bore capillary design, and is still able to achieve greater than 4x improved sensitivity compared to the 6550.

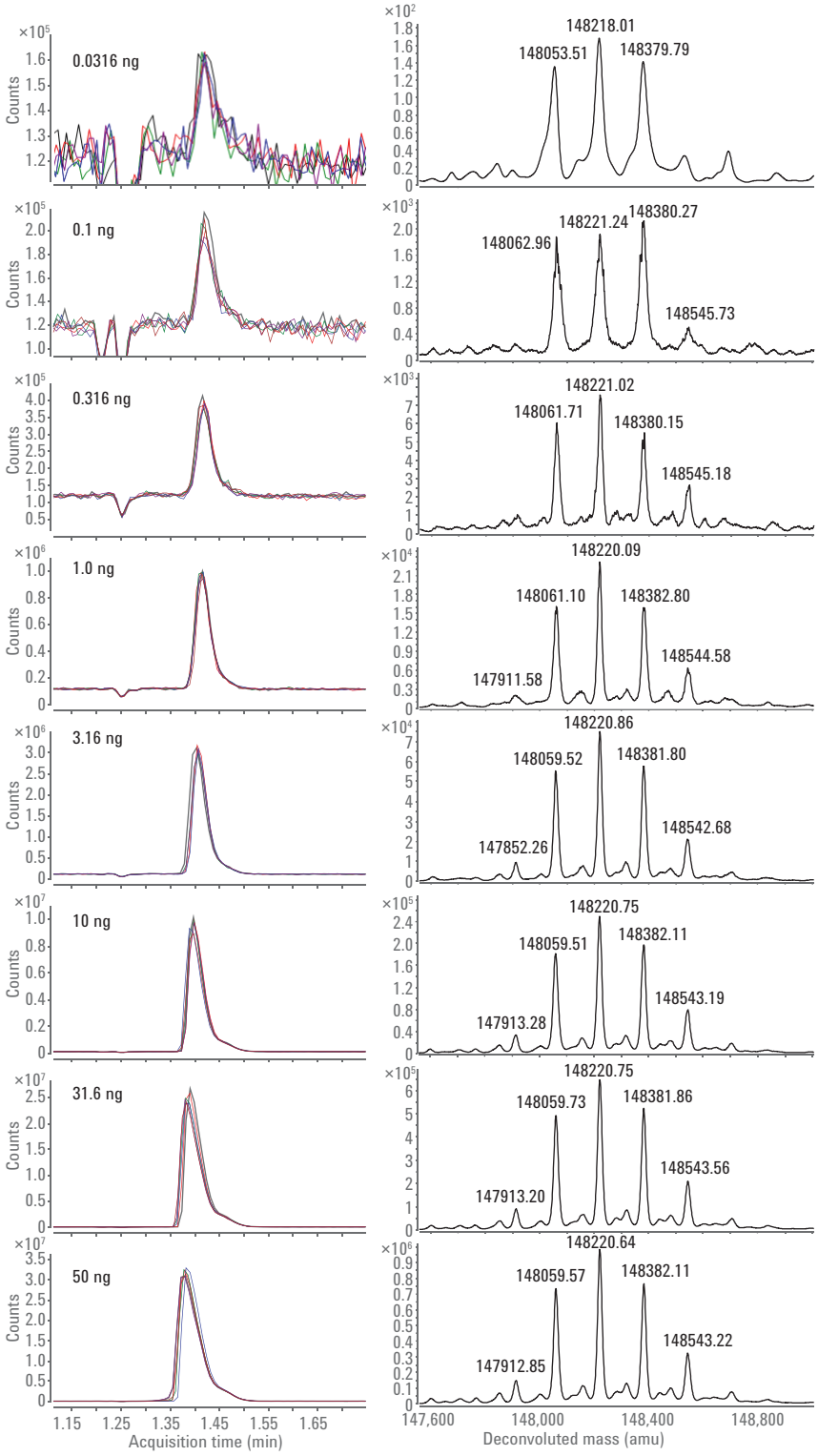

Figure 1. Reproducibility of chromatographic response for all dilution levels.

Reproducibility

Reproducibility of peak response was also evaluated over 100 injections of 10 ng on-column. Figure 2 plots the EIC peak areas obtained over the course of the experiment. The recorded areas showed a precision level of 1.5 % RSD, even for this low quantity on-column. This experiment was carried out without any internal standard for response correction, and reflects the stability of both the 1290 Infinity II UHPLC, as well as the detection by the LC/Q-TOF.

Mass accuracy

For confirmation of identification, spectral deconvolution was performed using the Maximum Entropy algorithm in BioConfirm. A series of 100 replicate 10 ng injections was performed to understand the reproducibility of mass accuracy for the neutral charge mass assignment. Figure 3 shows the mass errors recorded over the course of the 100 injections for the two most intense glycoforms. Every data point was within 10 ppm mass error, with an average error for the two most intense glycoforms of -1.78 ppm and -5.68 ppm, respectively. Equally impressive was the standard deviation of these errors, below 2.0 ppm. This low level of variability establishes a basis for larger scale experiments.

Linear dynamic range

The last aspect of this experiment was to establish the linear dynamic range of which this method was capable, a linear response over 3.2 orders of magnitude. It has been reported that linear response of intact protein analysis by LC/MS has more limitations than that of traditional small molecule analysis.[1] With this method, linear response was maintained from 0.0316 ng on-column up to 50 ng on-column. This represents a linear dynamic range of 3.2 orders, which is greater than anything currently published. Figure 4 shows the calibration curve generated in MassHunter Quantitative Analysis, along with statistics for the replicates acquired at each level.

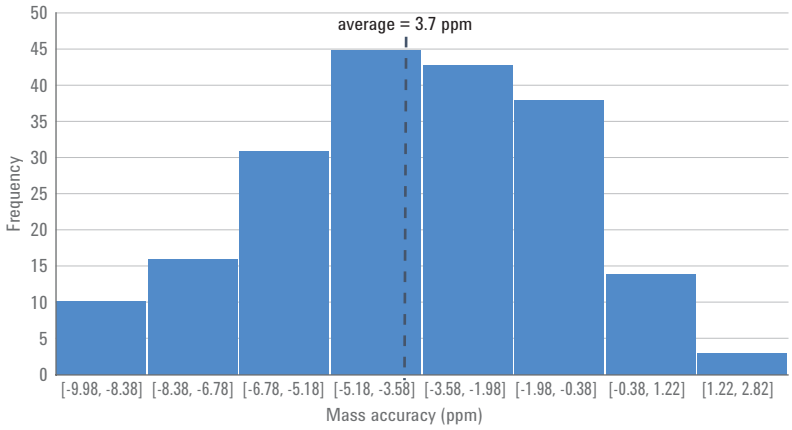

Figure 2. Histogram of mass accuracy measurements for the top two glycoforms in 100 injections.

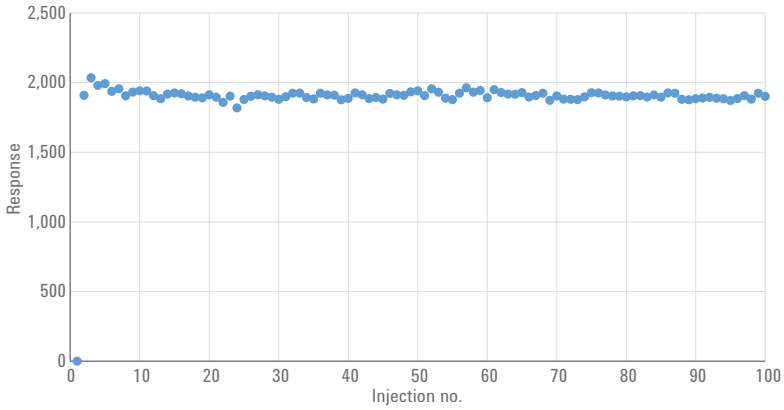

Figure 3. Intensity reproducibility over 100 injections of 10-ng on-column.

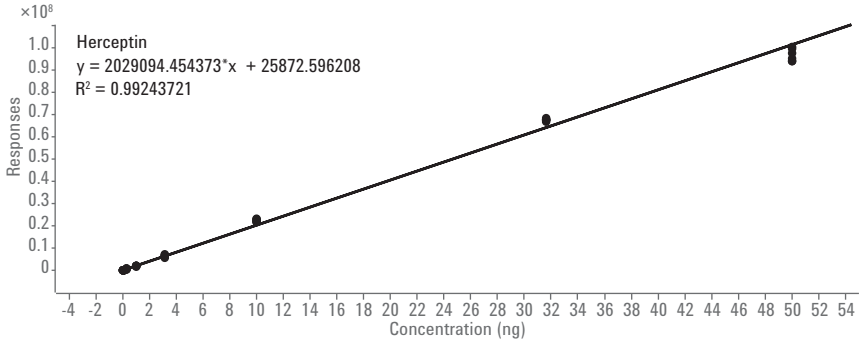

Figure 4. Calibration curve showing 3.2 orders of linear response for the intact glycosylated mAb analysis.

Conclusion

This method shows the combination of excellent analytical sensitivity, reproducibility, and accuracy necessary for confident quantitation of intact glycosylated monoclonal antibodies. The automatic optimization of the Agilent 6545XT AdvanceBio LC/Q-TOF for large molecule analysis by SWARM autotune showed remarkable improvements in detection limits as well as dynamic range compared to the Agilent 6550 iFunnel Q-TOF LC/MS system. The reproducibility can also be attributed to the level of performance obtained from the Agilent 1290 Infinity II UHPLC system. The hundreds of samples injected and precise results lay a foundation for larger scale quantitative analysis of intact mAbs and other large biomolecules.

Reference

1. van den Broek, I.; van Dongen, W. D. LC-MS-based quantification of intact proteins: perspective for clinical and bioanalytical applications. *Bioanalysis* **2015**, *7.15*, 1943-1958.

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