Application Note

Biopharma/Pharma

Agilent

Trusted Answers

Monitoring Antibody Glycosylation at Intact and Subunit Levels Using a Single Quadrupole LC/MS

Author

Stephen Sciuto
Agilent Technologies, Inc.

Abstract

This application note describes the use of the Agilent InfinityLab Pro iQ Plus single quadrupole mass spectrometer (MS) to monitor antibody glycosylation relative abundances at the intact and subunit levels of trastuzumab. At the intact level, five glycosylated components were identified from the MS spectral deconvolution software with relative abundances that were consistent with that measured previously on Agilent high-resolution accurate mass systems. At the subunit level, the light chain of trastuzumab showed a single component, which was expected based on its amino acid sequence, while the trastuzumab heavy chain identified four peaks consistent with the G0, G0F, G1F, and G2F glycans. The combination of reliable relative abundances for glycosylation monitoring, as well as excellent MS spectral peak shapes highlights the performance of the Agilent Pro iQ Plus mass spectrometer.

Introduction

Glycosylation is an important parameter to monitor in the biopharmaceutical industry because glycans play a role in the structural stability, safety, and efficacy of a therapeutic drug.[1] A common quality metric that is monitored repeatedly is to ensure that the relative abundances of protein glycosylation fall within accepted tolerances. If relative abundances do not pass predefined standards established by the quality control (QC) team, additional investigation into the manufacturing process for the therapeutic drug is required. Since mass spectrometry provides sensitive and robust methods for the characterization of monoclonal antibodies (mAbs), it is ideal to use mass spectrometry for glycan characterization.

In this study, the Agilent InfinityLab Pro iQ Plus liquid chromatography/mass spectrometer (LC/MS) with improved mass range and superior mass spectral peak shape was used to detect glycosylation at the intact and subunit levels (HC, LC). The relative abundances of glycosylation after deconvolution illustrate that key information about protein glycosylation[2] can be measured on this instrument platform.

Experimental

Chemicals and preparation of solutions

The following solutions from Sigma-Aldrich (St. Louis, MO) were used for the reduction reaction:

A. 8 M guanidine hydrochloride, pH 8.5 (part number G7294, 100 mL)

B. 0.2 M DTT (DL-Dithiothreitol, part number D9779, 1 g)

C. 1 M Tris buffer, pH 8.0 (part number 648314, 100 mL)

Denaturing: 6.4 M guanidine-HCl, 200 mM tris-HCl buffer, pH 8.1

Add 80 mL of 8.0 M guanidine HCl solution to 20 mL of 1 M Tris-HCl buffer in a 100 mL volumetric flask. Mix thoroughly by inversion and measure the pH using a pipette and pH indicator strip and record. If the pH falls between 7.2 and 8.5, this will be sufficient to proceed with the reducing step.

Reduction: 200 mM DTT in 50 mM Tris-HCl solution

Measure 31 mg of DTT using an analytical balance and add 1 mL of 50 mM Tris-HCl solution to dissolve. Vortex solution prior to the reducing step.

Standards and sample preparation

The monoclonal antibody (mAb) trastuzumab at a concentration of 22 mg/mL was acquired from Genentech (South San Francisco, CA). This solution was diluted to a concentration of 1 µg/µL by adding 10 µL of the stock solution to 210 µL of 0.1% formic acid in water. The final solution used for LC/MS analysis was diluted to 250 ng/µL by adding 55 µL of the 1 µg/µL solution and adding it to 165 µL of 0.1% formic acid in water.

The sample preparation procedure used for reduction of trastuzumab was:

1. Take 2.3 µL of trastuzumab stock solution (22 mg/mL) and add to 27.4 µL of 6.4 M guanidine chloride, 200 mM Tris-HCl.
2. Add 6.3 µL of 200 mM DTT in 50 mM Tris-HCl to the trastuzumab solution. Mix gently and spin down, then incubate at 37 °C for 30 minutes (alternate: 60 °C for 30 minutes).
3. To quench the reduction reaction, the solution should be acidified to a final v/v% of 1% formic acid. Add 4 µL of 10% FA to the reduced solution from step 2. Vortex gently and spin down.
4. Add 160 µL of 0.1% formic acid to bring the final volume to 200 µL. The reduced trastuzumab solution has a concentration of 250 ng/µL and is ready for LC/MS analysis.

LC/MS analysis

LC/MS analysis was performed on an Agilent 1290 Infinity II Bio LC system coupled to an Agilent InfinityLab Pro iQ Plus single quadrupole LC/MS system (Figure 1). An Agilent PLRP-S column (2.1 × 50 mm, 5 µm) was used for chromatographic separation. LC and MS parameters are listed in Tables 1 and 2.

Figure 1. Agilent 1290 Infinity II Bio LC system and Agilent Pro iQ Plus single quadrupole mass spectrometer.

Liquid chromatography

Table 1. Agilent 1290 Infinity II LC method.

Agilent 1290 Infinity II Bio LC System	
Column	Agilent PLRP-S, 2.1 × 50 mm, 5 µm (part number PL1912-1502)
Sampler Temperature	5 °C
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Flow Rate	0.5 mL/min
Injection Volume	2 µL
Column Temperature	80 °C
Gradient Program (Intact)	Time (min) %B 0 10 5 60 6 10 8 10
Gradient Program (Reduced)	0 5 0.1 20 8 40 8.1 70 9.1 70 9.2 5 11 5

Mass spectrometry

Table 2. MS parameters.

Agilent Pro iQ Plus Single Quadrupole Mass Spectrometer	
Ion Source	Agilent Jet Stream ESI source
Polarity	Positive
Time Filter Window	0.1 min
Stop Time	As pump/No limit
MS1 Scan Range	*m/z* 1,000 to 3,000 (intact) *m/z* 600 to 2,400 (reduced)
Scan Time	1,500 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
Divert Valve	Enabled; LC flow to waste from 0 to 1 min, LC flow to MS from 1 to 8 min (intact) or 1 to 11 min for reduced
Postrun Diverter Position	To waste

Data processing

The LC/MS data were processed using Agilent OpenLab CDS software, version 2.8. Intact deconvolution parameters are shown in Figure 2. For reduced mAb deconvolution, the external background time range was set from 3.0 to 4.3 minutes, automatic deconvolution RT window from 4.3 to 5.6 minutes, deconvoluted mass range from 10,000 to 60,000 Da, Absolute noise threshold to 2,000, relative abundance threshold to 25%, and MW algorithm to centroid.

Results and discussion

Total ion chromatogram for intact trastuzumab

The total ion chromatogram (TIC) for 500 ng of intact trastuzumab is illustrated in Figure 3A. As expected, the TIC shows a single peak that elutes at ~2.6 minutes. To prevent high aqueous content and salts from entering the mass spectrometer, the LC flow is diverted to waste during the first minute of the LC/MS method. From 1 to 8 minutes, the diverter valve was switched to direct flow to the Pro iQ Plus, where the mAb signal was measured.

Raw MS and deconvoluted spectrum for intact trastuzumab

The raw MS and deconvoluted spectra for intact trastuzumab are illustrated in Figures 3B and 3C, respectively. The Agilent Pro iQ Plus single quadrupole mass spectrometer has a scan range of *m/z* 3,000, which allowed for the detection of many charge states for intact trastuzumab and providing excellent deconvoluted spectra. In fact, the inset of Figure 3B shows the raw peak shapes for one of the charge states of trastuzumab. The relative abundances shown here match what was observed in the deconvoluted spectrum in Figure 3C, giving the user additional confidence in the deconvoluted spectrum and the five main glycosylated peaks identified.

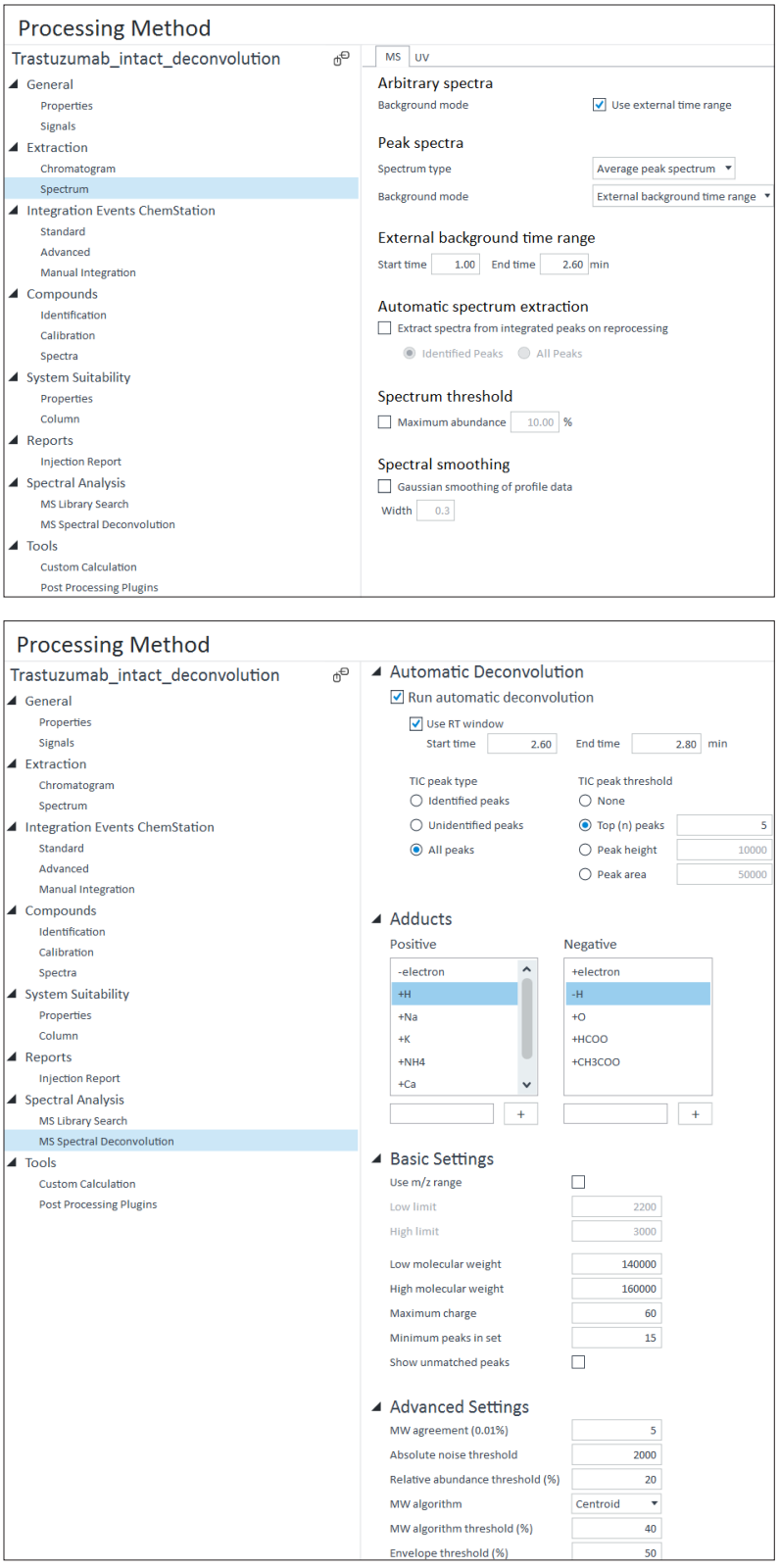


Figure 2. Agilent OpenLab CDS software, version 2.8 processing parameters that were used for intact mAb deconvolution.

A noteworthy processing parameter that reduced ghost peaks in the deconvoluted spectrum was the Minimum Peaks in Set parameter shown in the Basic Settings of the OpenLab CDS MS Spectral Deconvolution tab (Figure 2). For intact and reduced mAb deconvolution this setting was set to 15, which tells the software to deconvolute peaks that have at least 15 charge states. This is an important parameter because it improves the quality of peaks detected in the deconvoluted spectrum. This parameter can be adjusted based on the number of charge states observed in the raw mass spectrum for a molecule of interest.

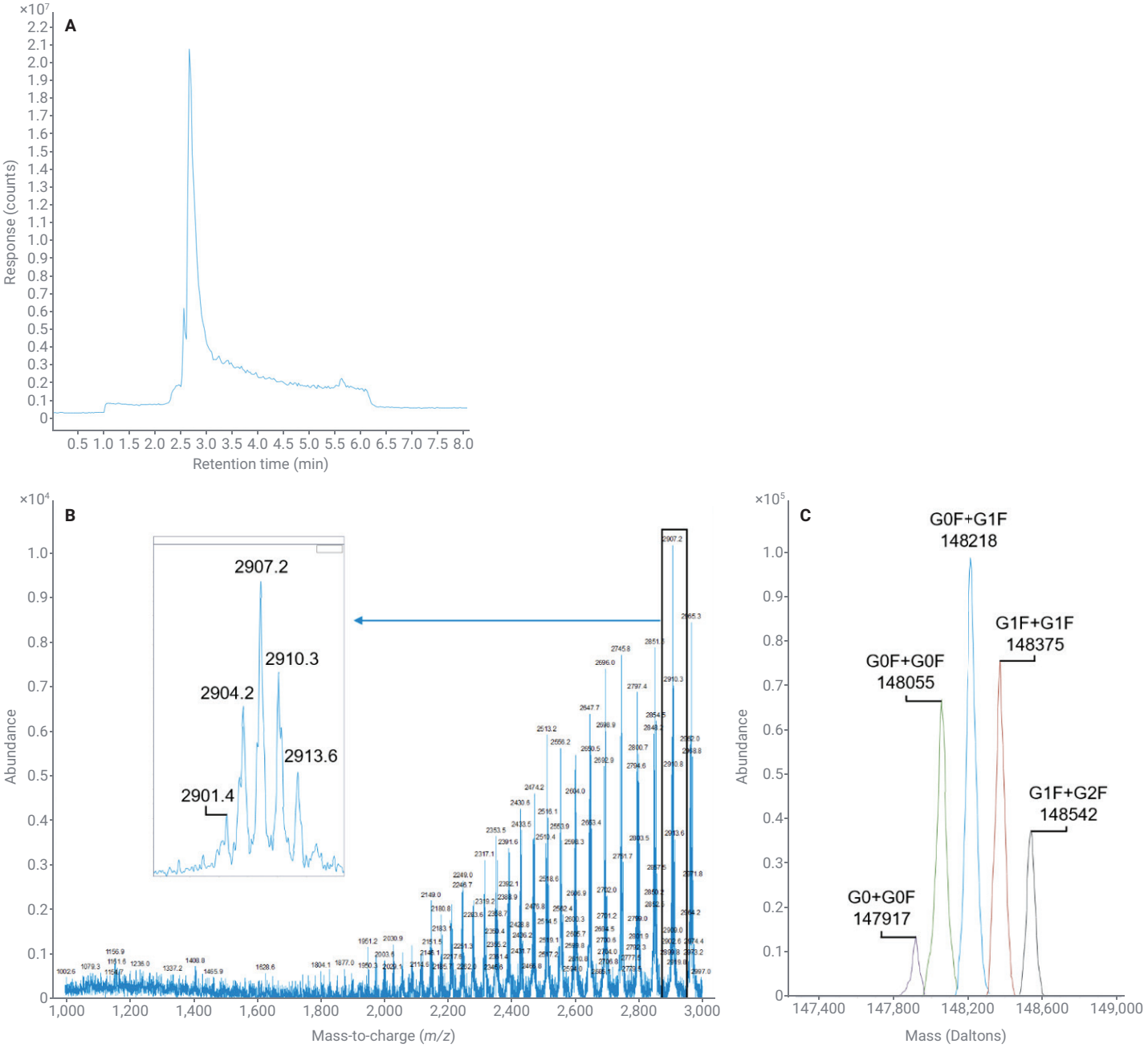


Figure 3. (A) TIC for intact trastuzumab measured on the Agilent Pro iQ Plus single quadrupole mass spectrometer. (B) Raw MS for trastuzumab. The inset shows the zoomed-in region for one of the charge states. (C) Deconvoluted mass spectrum for trastuzumab with measured masses and proposed glycoforms. LC/MS settings are listed in Tables 1 and 2, processing parameters are shown in Figure 2, and calculated mass errors in Table 3.

Reduced mAb chromatogram, raw MS, and deconvoluted spectra

To add an additional dimension to glycosylation profiling, an intact mAb can be reduced into subunits to reduce complexity. The TIC chromatogram for reduced trastuzumab is shown in Figure 4A, where two peaks for the light chain and heavy chain are observed. The measured MS spectral intensity for the light chain was 4-fold higher than the heavy chain. However, considerable glycosylation information for the HC and excellent MS spectral quality was obtained on the Agilent Pro iQ Plus. The insets of Figures 4B and 4D show a zoomed in region for the trastuzumab LC and HC, respectively. Since the site of glycosylation follows a consensus sequence for trastuzumab (i.e., glycosylation occurs at asparagine residues that follow an NXS/T motif), it is expected that the LC would show a single peak since its amino acid

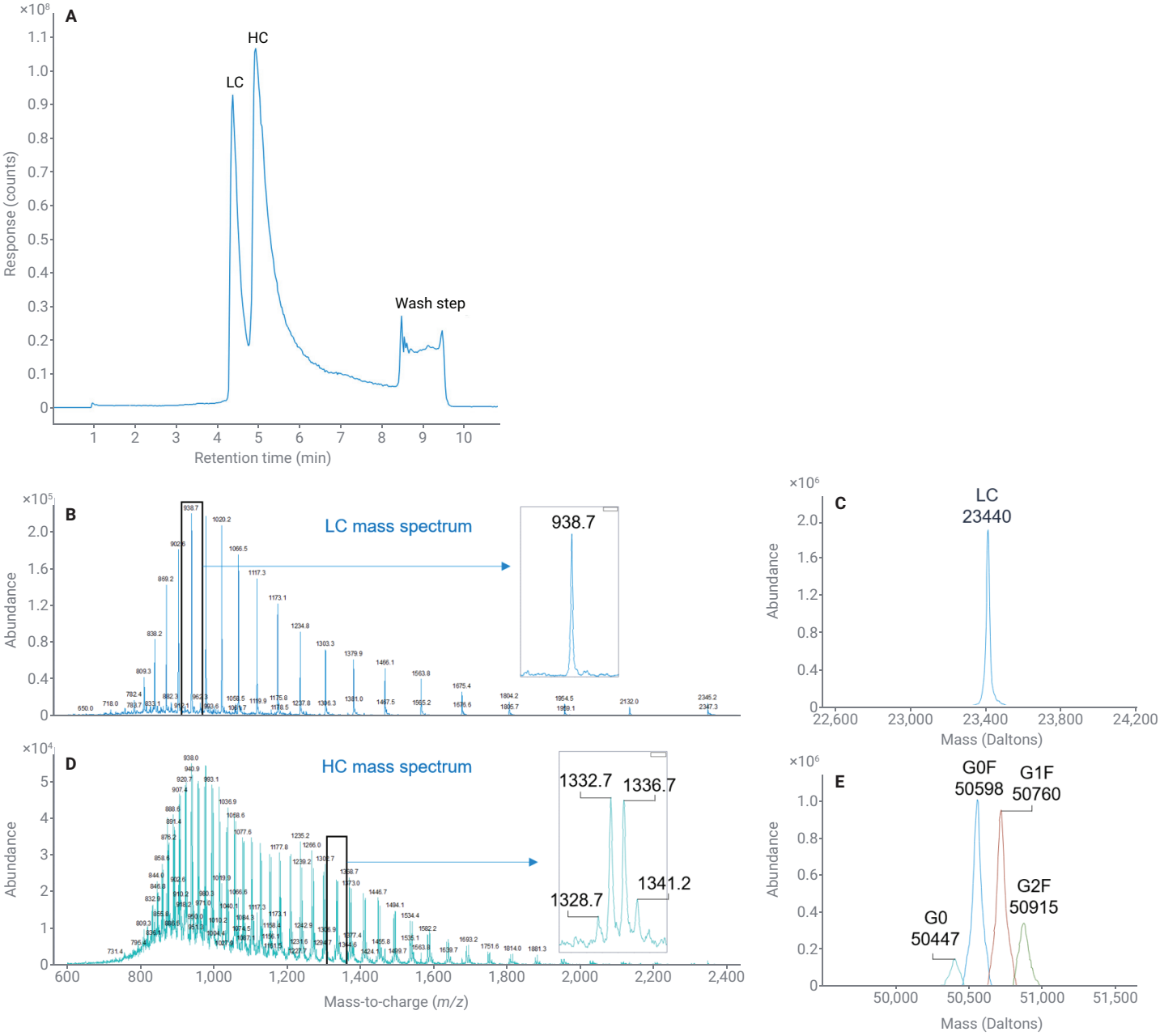


Figure 4. (A) TIC for reduced trastuzumab measured on the Agilent Pro iQ Plus single quadrupole mass spectrometer. Raw MS (B+D) for the trastuzumab light chain and heavy chain, respectively. The insets show the raw spectral quality on the instrument. Deconvoluted mass spectrum (C+E) for trastuzumab light chain and heavy chain, respectively. Calculated mass errors are displayed in Table 3.

sequence should not allow glycan modification. On the other hand, the trastuzumab HC has an NXS/T motif, which would permit glycosylation at the asparagine residue in the Fc region. The inset of Figure 4D shows four peaks in the raw MS spectrum that are consistent with the G0, G0F, G1F, and G2F glycans. The deconvoluted spectra for the LC and HC are shown in Figures 4C and 4E, respectively. The processing parameters used for reduced trastuzumab were similar to that shown in Figure 2 for the intact mAb. The only difference was that the deconvoluted mass range was changed to 10,000 to 60,000 Da and the relative abundance threshold was set to 25%. Noteworthy in the deconvoluted spectrum for the trastuzumab HC is that the relative intensities of the glycosylated peaks are similar to that observed in the raw MS spectrum inset. While each charge state for the HC will show different relative intensities for the glycosylated peaks, being able to correlate the relative abundances from the input MS spectrum to the deconvoluted spectrum provides confidence in the deconvoluted spectra. The ability to report on the relative abundances of glycosylation at the reduced and intact levels is an important measurement because deviation of these abundances from established tolerances will prompt the QA/QC team in a biopharmaceutical lab to perform additional investigation.

Relative abundances at intact/subunit levels and MS Spectral Deconvolution report

Table 4 shows the measured relative abundances for trastuzumab at the intact and subunit levels, respectively. The relative abundance (%) is calculated by normalizing abundances relative to the most intense component, whereas the relative quantitation (%) will measure the absolute abundance of a component relative to the total abundance of all components. Both the relative abundance (%) and relative quantitation (%) can be used to monitor product reproducibility and quality.

Table 3. Comparison of theoretical average molecular mass and experimentally observed molecular masses after deconvolution in Agilent OpenLab CDS software.

Measured Experimental Errors on the Agilent Pro iQ Plus Mass Spectrometer					
Molecule	Modification	Theoretical Mass (Da)	Experimental Mass (Da)	Δ Mass (Da)	Mass Error (ppm)
Intact Trastuzumab	G0+G0F	147,912.7	147,916.7	4.0	27
	G0F+G0F	148,058.8	148,055.1	–3.7	–25
	G0F+G1F	148,221.0	148,217.7	–3.3	–22
	G1F+G1F	148,383.1	148,374.7	–8.4	–57
	G1F+G2F	148,545.3	148,542.0	–3.3	–22
Trastuzumab HC	G0	50,456.1	50,447.3	–8.8	–174
	G0F	50,602.2	50,597.9	–4.3	–85
	G1F	50,764.4	50,759.9	–4.5	–89
	G2F	50,926.5	50,915.4	–11.1	–218
Trastuzumab LC	None	23,443.3	23,440.5	–2.8	–119

Table 4. Relative abundances after deconvolution at the (A) intact and (B) reduced levels.

A

Trastuzumab Intact Relative Abundances				
Component	Measured Mass	Proposed Identity	Relative Abundance (%)	Relative Quantitation (%)
A	148,218 Da	G0F+G1F	100	33.77
B	148,375 Da	G1F+G1F	76.63	25.88
C	148,055 Da	G0F+G0F	67.72	22.87
D	148,542 Da	G1F+G2F	38.00	12.83
E	147,917 Da	G0+G0F	13.79	4.66

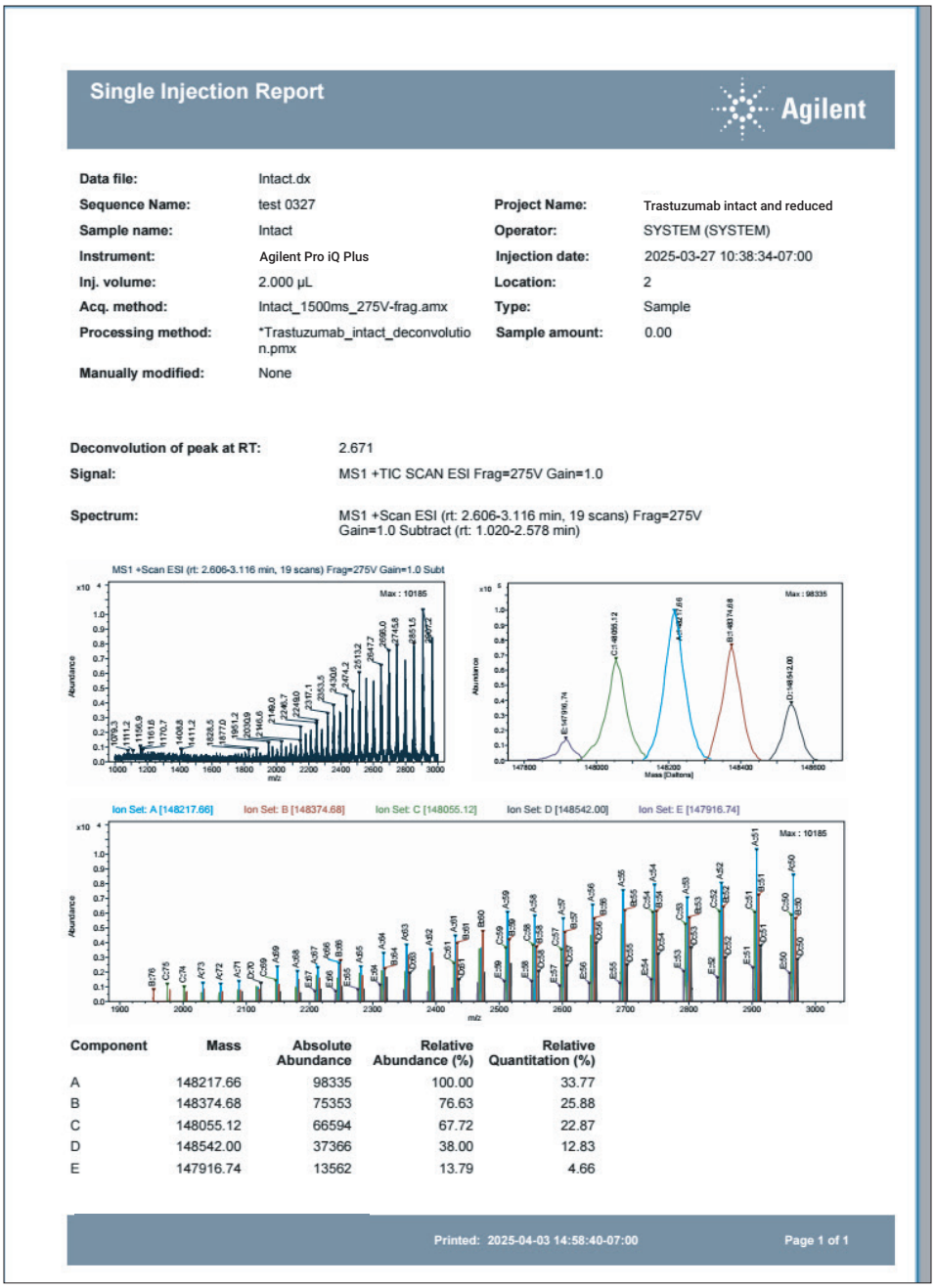
B

Trastuzumab Heavy Chain Relative Abundances				
Component	Measured Mass	Proposed Identity	Relative Abundance (%)	Relative Quantitation (%)
A	50,598 Da	G0F	100	41.22
B	50,760 Da	G1F	94.13	38.80
C	50,915 Da	G2F	33.71	13.90
D	50,447 Da	G0	14.73	6.07

Figure 5 shows an example MS spectral deconvolution software report for intact trastuzumab. The report is easily customizable in the report editor tab of OpenLab CDS software. Here, the report displays details about the acquisition and processing methods, injection volume, vial position, and injection date for traceability. In addition, raw MS and deconvoluted spectra with detected components are shown. The report will also list relative abundance (%) and relative quantitation (%) of the components in the deconvoluted spectrum.

Conclusion

Intact mass analysis by LC/MS is a technique that is commonly employed in the biopharmaceutical lab to monitor the relative abundances of glycosylation. These analyses are important for product efficacy and safety. The Agilent 1290 Infinity II Bio LC system coupled to an Agilent InfinityLab Pro iQ Plus provides a small and cost-effective LC/MS instrument for labs that require robust systems for routine analysis. In this study, the Pro iQ Plus LC/MS system was used to report on the relative abundances of glycosylated peaks at the intact and reduced levels in a therapeutic drug. Five glycosylated peaks were identified at the intact level with relative abundances that were consistent with results measured on Agilent high-resolution accurate mass spectrometry systems. Relative abundances were also monitored for the trastuzumab heavy chain, where four peaks were identified that are consistent with the G0, G0F, G1F, and G2F glycans. The combination of reliable relative abundances for monitoring glycosylation as well as excellent MS spectral peak shapes highlights the performance of the Agilent Pro iQ Plus single quadrupole mass spectrometer.



Single Injection Report

Agilent

Data file:	Intact.dx		
Sequence Name:	test 0327	**Project Name:**	Trastuzumab intact and reduced
Sample name:	Intact	**Operator:**	SYSTEM (SYSTEM)
Instrument:	Agilent Pro iQ Plus	**Injection date:**	2025-03-27 10:38:34-07:00
Inj. volume:	2.000 µL	**Location:**	2
Acq. method:	Intact_1500ms_275V-frag.amx	**Type:**	Sample
Processing method:	*Trastuzumab_intact_deconvolution.pmx	**Sample amount:**	0.00
Manually modified:	None		

Deconvolution of peak at RT: 2.671

Signal: MS1 +TIC SCAN ESI Frag=275V Gain=1.0

Spectrum: MS1 +Scan ESI (rt: 2.606-3.116 min, 19 scans) Frag=275V Gain=1.0 Subtract (rt: 1.020-2.578 min)

MS1 +Scan ESI (rt: 2.606-3.116 min, 19 scans) Frag=275V Gain=1.0 Subt

Ion Set: A [148217.66] Ion Set: B [148374.68] Ion Set: C [148055.12] Ion Set: D [148542.00] Ion Set: E [147916.74]

Component	Mass	Absolute Abundance	Relative Abundance (%)	Relative Quantitation (%)
A	148217.66	98335	100.00	33.77
B	148374.68	75353	76.63	25.88
C	148055.12	66594	67.72	22.87
D	148542.00	37366	38.00	12.83
E	147916.74	13562	13.79	4.66

Printed: 2025-04-03 14:58:40-07:00 Page 1 of 1

Figure 5. MS spectral deconvolution report for intact trastuzumab.

References

1. Higel, F.; Seidl, A.; Sörgel, F.; Friess, W. N-glycosylation Heterogeneity and the Influence on Structure, Function and Pharmacokinetics of Monoclonal Antibodies And Fc Fusion Proteins. *Eur. J. Pharm. Biopharm.* **2016**, *100*, 94–100. https://doi.org/10.1016/j.ejpb.2016.01.005
2. Wang, D.; Baudys, J.; Bundy, J.; Solano, M.; Keppel, T.; Barr, J. Comprehensive Analysis of the Glycan Component of SARS-CoV-2 Spike Proteins Using Signature Ions-Triggered Electron-Transfer/Higher-Energy Collisional Dissociation (EThcD) Mass spectrometry. *Anal. Chem.* **2020**, *92*, *21*, 14730–14739. https://doi.org/10.1021/acs.analchem.0c03301

www.agilent.com

DE-006222

Printed in the USA, May 13, 2025
5994-8345EN

Agilent
Trusted Answers