Analysis of Intact Monoclonal Antibodies Using an M3 MicroLC with the TripleTOF® 6600

Robust and Sensitive Workflow for Qualitative and Quantitative Analysis of Biotherapeutic IgGs

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Overview

Who Should Read This: Senior Scientists, Lab Directors

Focus: Analysis of intact monoclonal antibodies (mAbs) using microflow liquid chromatography-mass spectrometry (LC-MS) with on-column desalting.

Goal: Determine accuracy and sensitivity of the microflow LC-MS method for characterization and quantitation of intact monoclonal antibodies.

Problem: Traditional LC-MS methods for analyzing intact monoclonal antibodies and similar biotherapeutics require large amounts of sample and frequently deliver insufficient sensitivity. Microflow LC-MS, in spite of its inherent sensitivity advantage, has not been used extensively because the necessary off-line desalting and sample clean up result in possible sample loss and long sample preparation cycles.

Results: The SCIEX M3 MicroLC coupled with a SCIEX TripleTOF 6600 mass spectrometer provided accurate and highly sensitive characterization and quantitation of intact monoclonal antibodies. On-column desalting simplified and minimized sample preparation.

Key Challenges:

- Quantitation of intact mAbs over a wide subnanogram-to-nanogram dynamic range often requires greater sensitivity than can be achieve with LC-MS at standard LC flow rates.
- Microflow LC-MS can achieve the necessary sensitivity, but requires extensive desalting and sample cleanup.
- Traditional off-line (on-membrane) desalting methods are time consuming and result in sample loss.

Key Features:

- Single-run characterization and quantitation of intact mAbs and similar biologics
- Robust quantitation over a linear dynamic range of 3 orders of magnitude (0.1-100 ng on column)
- Quantitation at 5x lower concentration than can be achieved with standard-flow LC-MS
- High throughput intact mass analysis of mAbs (~140 samples/day)
- Fewer sample preparation steps, eliminating off-line desalting (~30 min process) and removal of carbohydrate (overnight digestion process)
- Reduced solvent, desalting, enzyme and operation costs

Fast-Growing Field: Protein biotherapeutics such as immunoglobulin G (IgG)-derived monoclonal antibodies occupy a rapidly increasing share of the pharmaceutical industry. Understanding the primary structure, heterogeneity, and post-translational modifications of these biologics are essential to understanding function, to developing novel therapeutics and to ensuring product safety and quality. Modifications (Figure 1) can directly affect protein activity, immunogenicity and stability.

LC-MS analysis has become an essential tool for the identification, characterization and quantification of intact mAbs) and similar high-molecular-weight proteins.[1, 2] In this note, we describe a robust and sensitive workflow using a M3 MicroLC coupled to a TripleTOF 6600 mass spectrometer for characterization and quantitation of intact mAbs. The method

takes advantage of on-column desalting to decrease sample preparation time and increase throughput.

Three different IgG1 molecules were used for method evaluation.

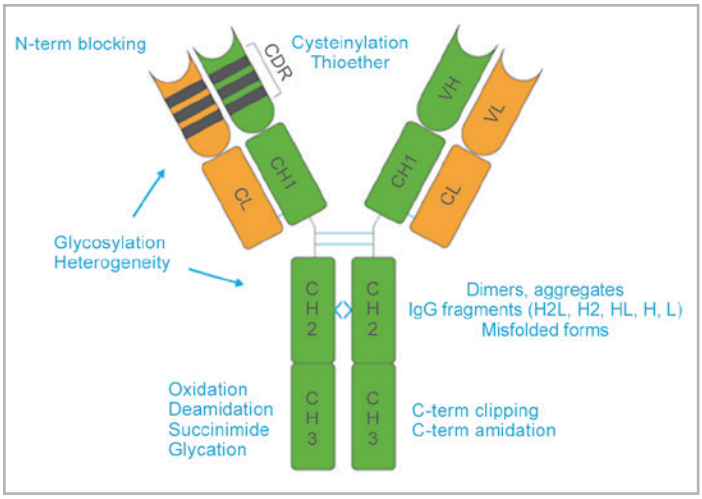


Figure 1. IgG1 Structure Heterogeneity. Therapeutic antibodies are generally complex, heterogeneous and subject to a variety of enzymatic or chemical modifications during expression, purification and long-term storage. These modifications are listed in blue.

Results for all three mAbs showed improved sensitivity with a high level of accuracy and a good coefficient of variation (CV) for intact antibody quantitation across 3 orders of magnitude linear dynamic range (0.1-100 ng), and successful characterization of the glyco-isoforms at low nanogram levels down to 2.5 ng. The sensitivity gained with the M3 MicroLC, along with the high resolution and mass accuracy of the TripleTOF 6600, was essential for this comprehensive analysis.

Experimental Design

Sample Preparation: Intact mAb Mass Check Standard (Waters, P/N 186006552), trastuzumab and adalimumab (Myoderm, NDC #50242-0134-68, NDC# 0074-3799-02 ABB) were serially diluted with 1 pM/µl of BSA (bovine serum albumin) in 0.1% FA (formic acid, Thermo Scientific P/N 28905) from 200 µg/ml down to 0.02 µg/ml. All antibodies were desalted by our on-column desalting strategy for maximum recovery. The microflow LC utilizing on-column desalting method was compared to off-line on-membrane desalting of adalimumab on 10K cut off Amicon Ultra Centrifugal filters (Sigma-Aldrich, P/N Z677108-96EA). The adalimumab was desalted on membrane six times with 10% acetonitrile in 0.1% FA and then recovered with 0.1% FA. Serial dilutions of 0.02-10 µg/ml concentration were made for desalted adalimumab with 1 pM/µl of BSA in 0.1% FA.

Microflow Liquid Chromatography: The on-column desalting and separation of all three mAbs was performed on an M3 MicroLC (SCIEX) using Waters ACQUITY UPLC M-Class Protein BEH C4 column (300Å, 1.7 µm, 300 µm X 50 mm) at a 15 µl/min flowrate. All tests were performed in direct inject mode using the LC gradient in Table 1. The total LC run time was 10 minutes, of which the first 3 minutes was on-column desalting followed by 7 minutes of linear gradient separation from 20% B to 80% and back to 5% for maximum binding. The column temperature was maintained at 60° C. Five microliters of each serial dilution was loaded on column for each of the 3-4 replicate analyses. Mobile phase A was 100% water with 0.1% formic acid and 0.01% trifluoroacetic acid (TFA, Thermo Scientific P/N 28904). Mobile phase B was 100% acetonitrile with 0.1% formic acid and 0.01% TFA.

Time(min)	% Solvent B
0	20
3	20
5	80
6	80
6.5	20
7	20
7.5	5
10	5

Table 1. Microflow LC Gradient Used for Intact Antibody Analysis.

Mass Spectrometry: MS analyses were performed using a TripleTOF 6600 equipped with a DuoSpray™ Source and 25 µm I.D. electrode (SCIEX). The MS method was built in two periods: 3 min with ion spray voltage floating (ISVF) set to 0 during on-column desalting to avoid spraying salt into MS followed by 7 min with ISVF set to 5500 for sample analysis. The detailed MS and source parameters are shown in Table 2. A minimum of three replicate injections were performed for each serial dilution.

Data Processing: Analysis of intact mAbs, including spectral deconvolution, mass reconstruction and analysis of glycans and other post-translational modifications (PTMs), was performed using BioPharmaView™ (version 1.4.9170) software. mAbs quantitation analysis, including calibration curve, CV and accuracy, was performed using MultiQuant™ (version 3.0.2) software. The reproducibility of data was assessed visually with PeakView® (version 2.2.0.11391) software as well as quantitatively using MultiQuant software. The method was optimized by analyzing the data using a combination of BioPharmaView and PeakView.

MS Parameters	Values
Electrode ID	25 um
Curtain Gas	25
Collision Energy	25
IonSpray Voltage	5500
Temperature (°C)	300
Ion Source Gas 1	35
Ion Source Gas 2	35
Declustering Potential	240
Polarity	Positive
Mass Range	1000-5000
Accumulation Time (sec)	1.0
Time Bins to Sum	80
Scan Type	TOF MS
Intact Protein Mode	On

Table 2. MS and DuoSpray™ Source Parameters.

High Retention Time and Sensitivity Reproducibility

High reproducibility of retention time (RT) and sensitivity were observed. The M3 MicroLC-MS method provided highly reproducible retention time, spectra, peak height and peak area for all three antibodies.

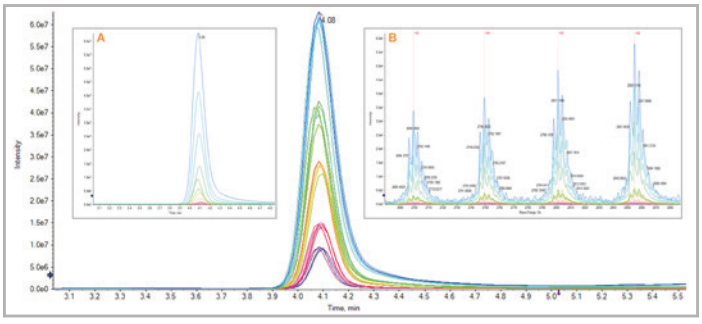


Figure 2. TIC Chromatogram for Waters Intact mAb Standard. Overlays of microflow LC replicates for mAb at 50-1000 ng load on column shows high reproducibility of RT and intensity (STDEV.P of 0.01). Peak height corresponding to 0.1-1000 ng mAb load on column is shown in Figure 2a. The spectra for +51 charge species of Waters mAb in 0.1-1000 ng range are shown in Figure 2b.

Advantages of On-Column Desalting

Commercial mAbs require buffering salt for stabilization in solution. However, to gain sensitivity in LCMS analysis of intact mAbs, it is critically important to remove the salt before LC-MS analysis. This is typically done either by off-line desalting or a trap-elute workflow. Instead, we used on-column desalting to minimize sample preparation time and avoid sample loss. Ion spray voltage floating (ISVF) was set to 0 during the on-column desalting period to avoid spraying salt into the MS. To confirm the viability of on-column desalting, we compared this approach against on-membrane desalted adalimumab (50 ng load on column). We observed less sample loss and greater sensitivity using the on-column desalting method (Figure 3).

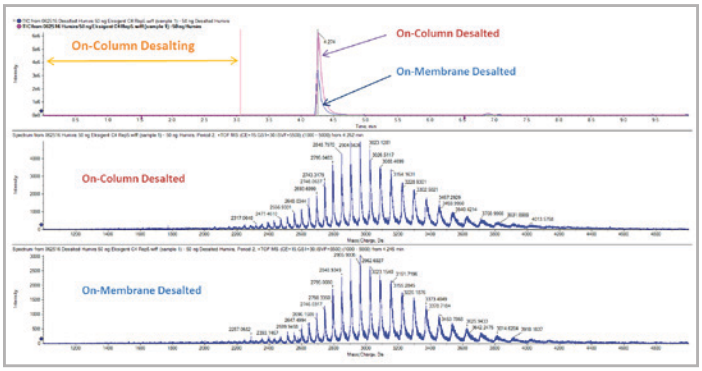


Figure 3. Advantage of On-Column Desalting Method. TIC chromatogram and TOF MS spectra for intact adalimumab (50 ng load on column) clearly shows the gain in sensitivity and efficient desalting using an on-column approach (pink spectra) vs on-membrane desalting (blue spectra).

Quantitation of mAbs with Increased Dynamic Range

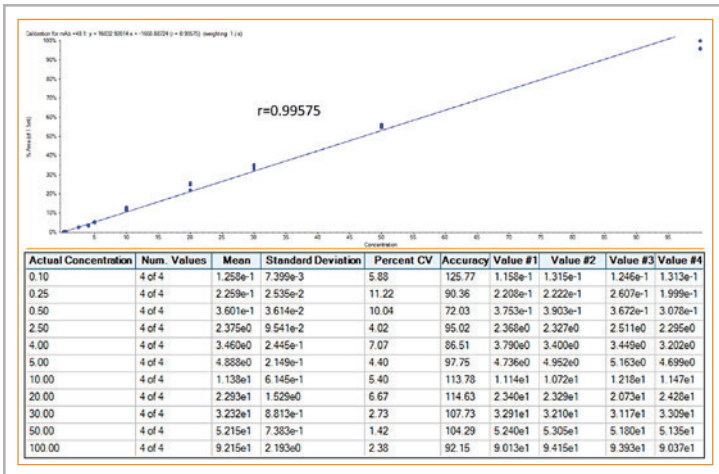
Quantitation of intact proteins was evaluated based on linear dynamic range of the method and the lower limit of detection (LLOD) and quantitation reproducibility and accuracy. Three different intact antibodies: Waters Intact mAb Standard, adalimumab (Humira) and trastuzumab (Herceptin), were used to evaluate the analytical performance of our integrated M3 MicroLC coupled to a high-resolution TripleTOF 6600. The MS spectra were analyzed with MultiQuant using protein charge states peak area with linear regression and 1/x weighting (Figure 4).

The same MS data were used for characterization of glycans and their abundances using BioPharmaView software. The M3 MicroLC provided increased sensitivity for analysis of intact proteins as compared to standard-flow LC due to improved

electrospray ionization and sampling efficiency of protein charged species and decreased matrix suppression effect.

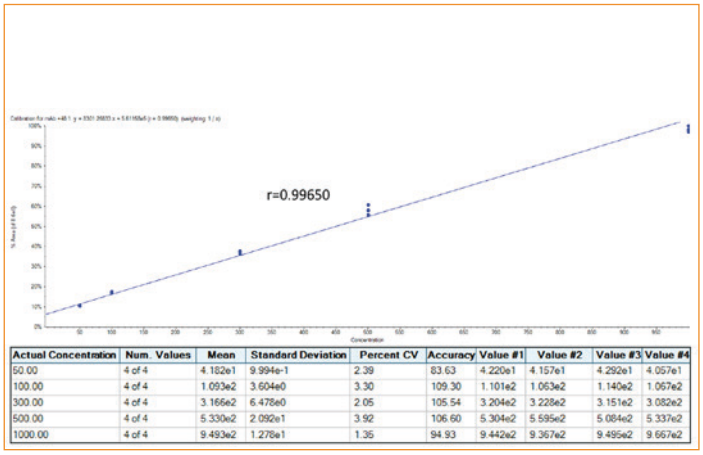
Quantitation over 3 orders of magnitude dynamic range (0.1-100 ng/column) was achieved for the Waters Intact mAb Standard (Figure 4a), with extended linear response for 50-1000 ng (Figure 4b). Quantitation over 3 orders of magnitude linear dynamic range (0.1-100 ng/column) was achieved for intact adalimumab (Figure 4c) and 2.5 orders of magnitude linear dynamic range (0.5-100 ng/column) was achieved for trastuzumab, (Figure 4d).

Results showed improved sensitivity with high accuracy and CV for intact antibody quantitation across 3 orders of magnitude linear dynamic range (0.1-100 ng). The sensitivity gained using the M3 MicroLC coupled with the high resolution and mass accuracy of TripleTOF 6600 was essential for this comprehensive analysis. In addition, we compared the linear regression with a Wagner logarithmic regression[3] which worked better for the nature of intact proteins at higher concentration and allowed quantification over 4 orders of magnitude dynamic range.



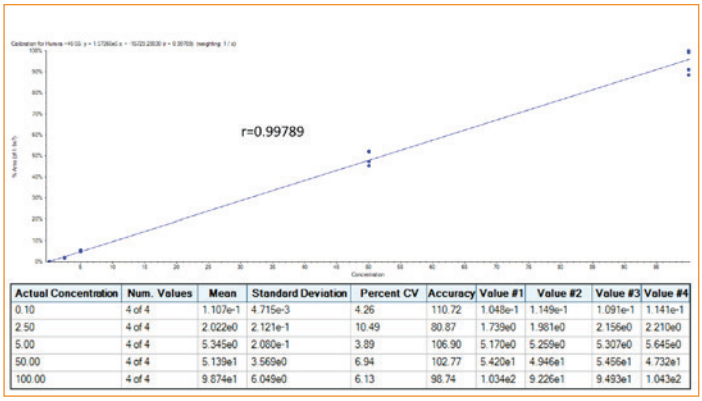
Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3	Value #4
0.10	4 of 4	1.258e-1	7.399e-3	5.88	125.77	1.158e-1	1.315e-1	1.246e-1	1.313e-1
0.25	4 of 4	2.259e-1	2.535e-2	11.22	90.36	2.208e-1	2.222e-1	2.607e-1	1.999e-1
0.50	4 of 4	3.601e-1	3.614e-2	10.04	72.03	3.753e-1	3.903e-1	3.672e-1	3.078e-1
2.50	4 of 4	2.375e0	9.541e-2	4.02	95.02	2.368e0	2.327e0	2.511e0	2.295e0
4.00	4 of 4	3.460e0	2.445e-1	7.07	86.51	3.790e0	3.400e0	3.449e0	3.202e0
5.00	4 of 4	4.888e0	2.149e-1	4.40	97.75	4.736e0	4.952e0	5.163e0	4.699e0
10.00	4 of 4	1.138e1	6.145e-1	5.40	113.78	1.114e1	1.072e1	1.218e1	1.147e1
20.00	4 of 4	2.293e1	1.529e0	6.67	114.63	2.340e1	2.329e1	2.073e1	2.428e1
30.00	4 of 4	3.232e1	8.813e-1	2.73	107.73	3.291e1	3.210e1	3.117e1	3.309e1
50.00	4 of 4	5.215e1	7.383e-1	1.42	104.29	5.240e1	5.305e1	5.180e1	5.135e1
100.00	4 of 4	9.215e1	2.193e0	2.38	92.15	9.013e1	9.415e1	9.393e1	9.037e1

a) Waters Intact mAb Standard 0.1-100 ng on column



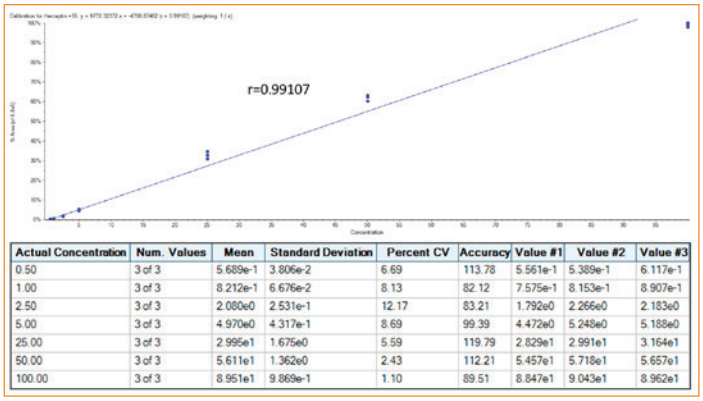
Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3	Value #4
50.00	4 of 4	4.182e1	9.994e-1	2.39	83.63	4.220e1	4.157e1	4.292e1	4.057e1
100.00	4 of 4	1.093e2	3.604e0	3.30	109.30	1.101e2	1.063e2	1.140e2	1.067e2
300.00	4 of 4	3.166e2	6.478e0	2.05	105.54	3.204e2	3.228e2	3.151e2	3.082e2
500.00	4 of 4	5.330e2	2.092e1	3.92	106.60	5.304e2	5.595e2	5.084e2	5.337e2
1000.00	4 of 4	9.493e2	1.278e1	1.35	94.93	9.442e2	9.367e2	9.495e2	9.667e2

b) Waters Intact mAb Standard 50-1000 ng on column



Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3	Value #4
0.10	4 of 4	1.107e-1	4.715e-3	4.26	110.72	1.048e-1	1.149e-1	1.091e-1	1.141e-1
2.50	4 of 4	2.022e0	2.121e-1	10.49	80.87	1.739e0	1.981e0	2.156e0	2.210e0
5.00	4 of 4	5.345e0	2.080e-1	3.89	106.90	5.170e0	5.259e0	5.307e0	5.645e0
50.00	4 of 4	5.139e1	3.569e0	6.94	102.77	5.420e1	4.946e1	5.456e1	4.732e1
100.00	4 of 4	9.874e1	6.049e0	6.13	98.74	1.034e2	9.226e1	9.493e1	1.043e2

c) Adalimumab 0.1-100 ng on column



Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
0.50	3 of 3	5.689e-1	3.806e-2	6.69	113.78	5.561e-1	5.389e-1	6.117e-1
1.00	3 of 3	8.212e-1	6.676e-2	8.13	82.12	7.575e-1	8.153e-1	8.907e-1
2.50	3 of 3	2.080e0	2.531e-1	12.17	83.21	1.792e0	2.266e0	2.183e0
5.00	3 of 3	4.970e0	4.317e-1	8.69	99.39	4.472e0	5.248e0	5.188e0
25.00	3 of 3	2.995e1	1.675e0	5.59	119.79	2.829e1	2.991e1	3.164e1
50.00	3 of 3	5.611e1	1.362e0	2.43	112.21	5.457e1	5.718e1	5.657e1
100.00	3 of 3	8.951e1	9.869e-1	1.10	89.51	8.847e1	9.043e1	8.962e1

d) Trastuzumab 0.5-100 ng on column

Figure 4. Linear Quantitation Curve for mAbs. Calibration curves for 3-4 replicate analysis of different amount of Waters Intact mAb Standard (Figure 4a and 4b) with r =0.99 (charge state +48). Similar data and r values were obtained for quantitation of adalimumab (sum of charge states +46 to +55) (Figure 4c) and trastuzumab (charge state +55) (Figure 4d).

Intact mAb Heterogeneity Characterization and PTM Analysis

The sensitivity gained using the M3 MicroLC coupled with the high resolution and mass accuracy of TripleTOF 6600 enabled comprehensive analysis of intact mAbs at low ng level. We demonstrated successful characterization of Waters Intact mAb Standard in low nanogram amounts (2.5 ng) and characterized different PTMs, including N-terminal glutamate to pyroglutamate conversion and different glycosylations (M*+G0F+G0F, M*+G0F+G1F, M*+G1F+G1F, M*+G1F+G2F, M*+G2F+G2F). Characterization of mAbs glycoforms and other PTMs using BioPharmaView provided routine molecular weight determination and characterization of mAbs (Figure 5).

Results show the potential application of this workflow, for both quantitation and characterization in low nanogram amounts, to other mAbs and IgG classes. Additional modifications such as oxidation, deamidation, pyroglutamic acid formation at the N-terminus and N-terminal lysine loss were identified on adalimumab (Figure 6) and trastuzumab (Figure 7). Our data identified potential major glycoforms. Deeper characterization may be further evaluated with glycopeptides analysis at MS/MS and MS/MRM and level.[4]

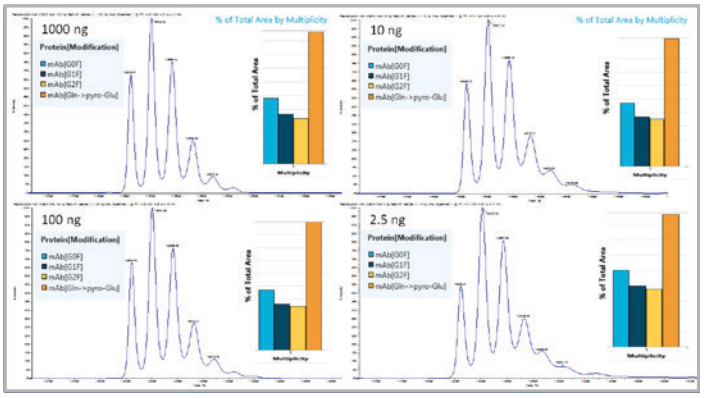
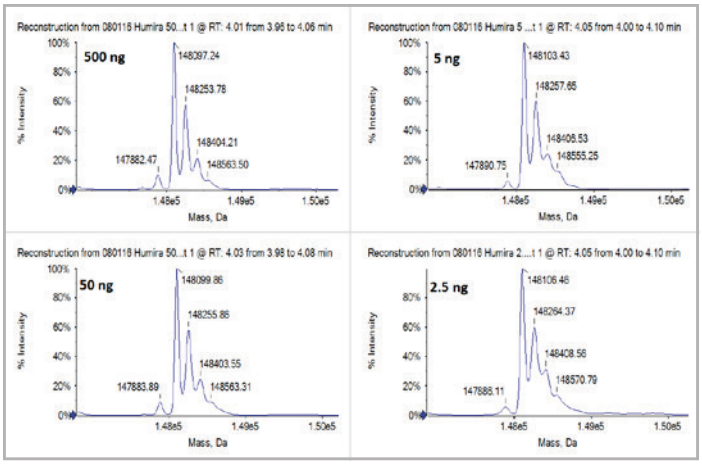


Figure 5. Quantitative Multiplicity Characterization of mAb Glycoforms. Intact mAbs mass analysis, including spectral deconvolution, mass reconstruction and analysis of glycans and other PTMs was performed. We demonstrated successful characterization using only 2.5 ng of Waters Intact mAb Standard.



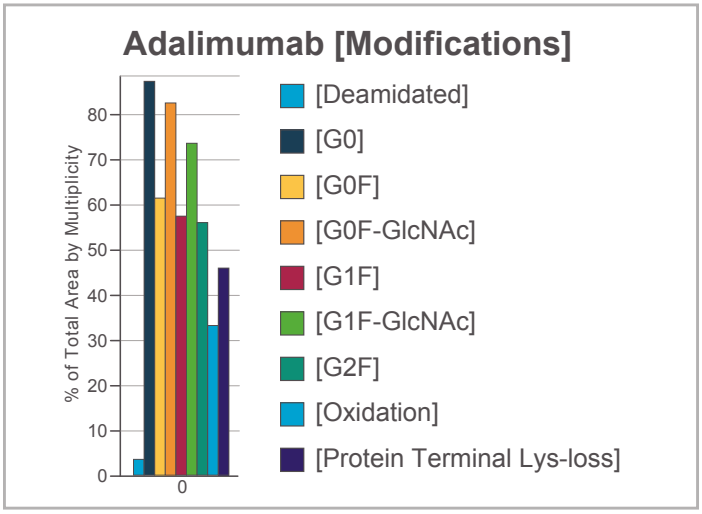
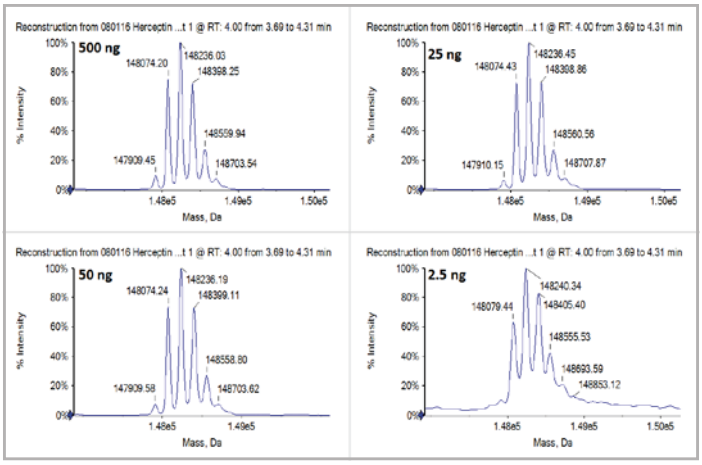


Figure 6. Characterization of Adalimumab by BioPharmaView. Intact adalimumab mass analysis including spectral deconvolution, mass reconstruction and analysis of glycans and other PTMs was performed using BioPharmaView.

This workflow was developed to confirm the intact mass of the target biotherapeutic antibodies and to profile product-related variants such as N-glycosylation of major glycan isoforms and antibody drug conjugates (ADCs) or other PTMs. The SCIEX M3 MicroLC coupled with a SCIEX TripleTOF 6600 mass spectrometer and aided by the advanced, easy-to-use MultiQuant and BioPharmaView software provided accurate quantitation of intact monoclonal antibodies at subnanogram-to-nanogram levels. The method and instrumentation also demonstrated successful characterization of multiple PTMs and glycosylation states on intact antibodies at very-low-nanogram levels. On-column desalting simplified and minimized sample preparation, enabling the use of the more sensitive microLC-MS technique.



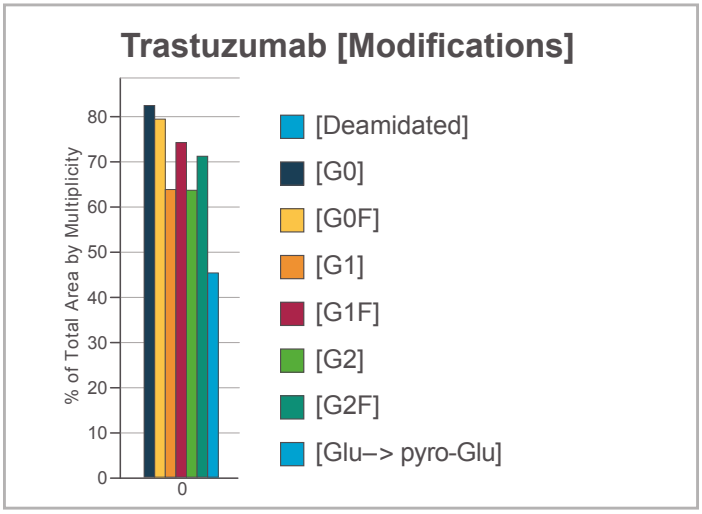


Figure 7. Characterization of Trastuzumab by BioPharmaView Intact adalimumab mass analysis including spectral deconvolution, mass reconstruction and analysis of glycans and other PTMs was performed using BioPharmaView.

References

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4. Jenny Albanese, Christie L. Hunter and Ningombam Sanjib Meitei, Profiling the Distribution of N-Glycosylation in Therapeutic Antibodies using the QTRAP® 6500 System. SCIEX Technical Note, Document Number RUO-MKT-02-2590-A

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