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Application News

No. LCMS-115

Liquid Chromatography Mass Spectrometry

Monoclonal Antibody Workflows on the Shimadzu Q-TOF LCMS-9030 Using the Protein Metrics Software Suite

■ Abstract

Accurate characterization of monoclonal antibodies is essential to development of biotherapeutics. Thorough understanding of biotherapeutic properties aids in the optimization of bioprocess production, product formulation, and product dosage. In this application note, we use the new Shimadzu Q-TOF LCMS-9030 to characterize the recombinant human IgG1κ NIST mAb reference standard as a model of biotherapeutic monoclonal antibodies.

High-resolution LC-MS instrumentation has become an important tool to resolve the complexity of mAb's primary-quaternary structure. The Shimadzu Q-TOF LCMS-9030 is an ideal instrument for qualitative analysis for biotherapeutic monoclonal antibodies. Protein Metrics software suite was used to deconvolute the intact protein mass and for comprehensive peptide identification of the digested protein.

■ Introduction

There are several strategies for complete mAb characterization due to the complexity of the intact protein. The typical construction of a mAb biopharmaceutical is shown in Figure 1a. This 4-chained protein contains the variable Fab sequence region which interacts with antigens, the Fc region which mediates cellular effector function, and the Complementarity Determining Region (CDR) which has direct contact to an antigen's surface[1]. Two identical heavy chains (HC) and two identical light chains (LC) are linked through both inter- and intra-chain disulfide bonds. Reduction of these disulfide bonds allows for the analysis of the HC and LC independently (Figure 1b). Enzyme digestion with trypsin, Glu-C or Lys-C will cleave the protein into peptide fragments (Figure 1d). The resulting masses can be used to confirm sequences compared to a theoretical sequence in silico using specialized software[2]. Oligosaccharides are covalently attached to the protein on the HC subunit during Post Translational Modification (PTM). The most common glycosylation in human mAbs occurs at an asparagine residue by an N-glyosidic bond. These N-glycans play an important role in regulation and recognition processes and can be cleaved by the addition of bacterial enzymes[3] (Figure 1c).

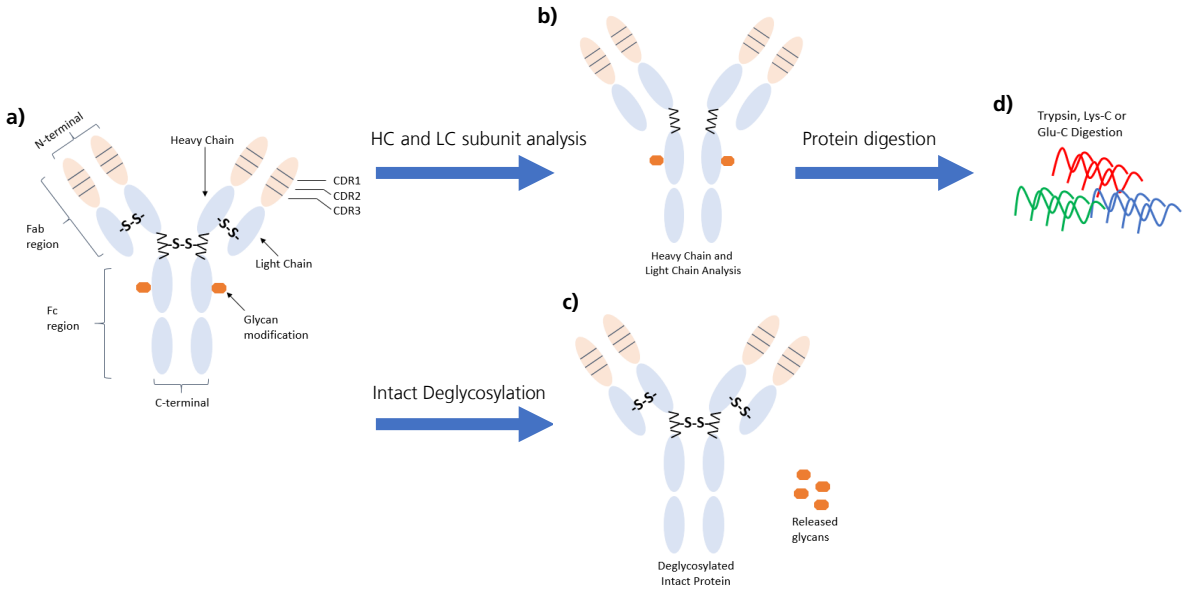

Figure 1: a) Typical mAb structure b) Reduction of mAb into HC and LC components c) deglycosylation of N-glycans d) protein digestion with various digestion enzymes

■ Experimental

Materials and Methods

NIST mAb Humanized IgG1κ monoclonal antibody reference material 8671 was purchased from the National Institute of Standards & Technology (NIST). For intact protein analysis, NIST mAb was diluted to 1mg/mL in 50mM ammonium bicarbonate. 100µg of the intact protein was reduced to its HC and LC subunits by adding 8M Urea, 50mM Tris-HCl, and 50mM DTT. Intact and subunit components were analyzed using a Restek Ultra C4 column with the conditions listed in Table 1.

HC and LC subunits were alkylated with 500mM IAA followed by digestion using trypsin, Lys-C, or Glu-C at a 1:25 enzyme:substrate ratio. The sample was desalted using Biotage Evolute® Express ABN SPE cartridges. The resulting peptides were analyzed using a Restek Raptor ARC-18 column and the Data Dependent Analysis (DDA) conditions in Table 2.

Intact protein was also treated PNGase F to remove glycosylation on the heavy chain region. The instrument conditions were the same as intact protein (Table 1).

Instrumentation

All data from this application note were obtained on a Shimadzu UHPLC in conjunction with a Q-TOF Mass Spectrometer, LCMS-9030. The specific configurations include LC-30AD x2 solvent delivery pumps, DGU-20A5R online degassing unit, SIL-30ACMP autosampler, CTO-20AC column oven, CBM-20A system controller, LCMS-9030 QTOF and LabSolutions Ver. 5.95 chromatography workstation.

Table 1: Intact and Subunit Instrument Parameters

Liquid Chromatography (LC) Conditions	
Column	Restek Ultra C4 5µm, 150mm x 2.1mm
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Flow Rate	0.4mL/min
Column Temp	50°C (Intact), 85°C (subunits)
Gradient (Intact)	B conc. 0.0-0.5min, 5%; 0.5-3.0min, 60%; 3.0-5.5min, 60%; 5.5-5.6min, 5%; end 10min
Gradient (Subunits)	B conc. 0.0-0.5min, 15%; 0.5-1.5min, 30%; 1.5-2.5min, 30%; 2.5-3.5min, 45%; 3.5-7.5min, 45%; 7.5-8.5min, 50%; 8.5-10.5min, 50%; 10.5-10.6min, 15%; end 15min
Q-ToF Conditions	
Mode	MS SCAN
TOF Start m/z	1000.0000 (intact); 800.0000 (subunits)
TOF End m/z	4000.0000
Event Time (s)	5.0 (intact); 2.0 (subunits)
Pulser Inj. Times	9993 (intact); 3993 (subunits)
Nebulizing Gas Flow	3.0L/min
Heating Gas Flow	10L/min
Interface Temp	300°C
Drying Gas Flow	10L/min

Table 2: Enzyme Digest Instrument Parameters

Liquid Chromatography (LC) Conditions	
Column	Restek Raptor C18 2.7µm, 150mm x 2.1mm
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Flow Rate	0.4mL/min
Column Temp	40°C
Gradient (Intact)	B conc. 0.0-0.5min, 5%; 0.5-10.5min, 60%; 10.5-12.5min, 60%; 12.5-12.6min, 5%; end 17min
Q-ToF Conditions	
Mode	DDA
TOF Start m/z	400.0000
TOF End m/z	2000.0000
DDA Start m/z	100.0000
DDA End m/z	2000.0000
CE	18
CE Spread	17
Event Time (s)	0.100
Pulser Inj. Time	194
Nebulizing Gas Flow	3.0L/min
Heating Gas Flow	10L/min
Interface Temp	300°C
Drying Gas Flow	10L/min

■ Results and Discussion

Intact NIST mAb

Figure 2 shows the intact TIC chromatogram, mass spectrum and deconvoluted spectrum of NIST mAb intact protein. The found masses were consistent to the theoretical NIST mAb masses, with less than 25ppm for all major glycoforms (Table 3). The intact mass was deconvoluted using Protein Metrics Intact Mass Workflow.

Light chain and heavy chain

Figure 3 shows the intact TIC chromatogram, mass spectrum, and deconvoluted spectrum of the LC and HC subunits for NIST mAb. The subunits' masses were deconvoluted using Protein Metrics Intact Mass Workflow.

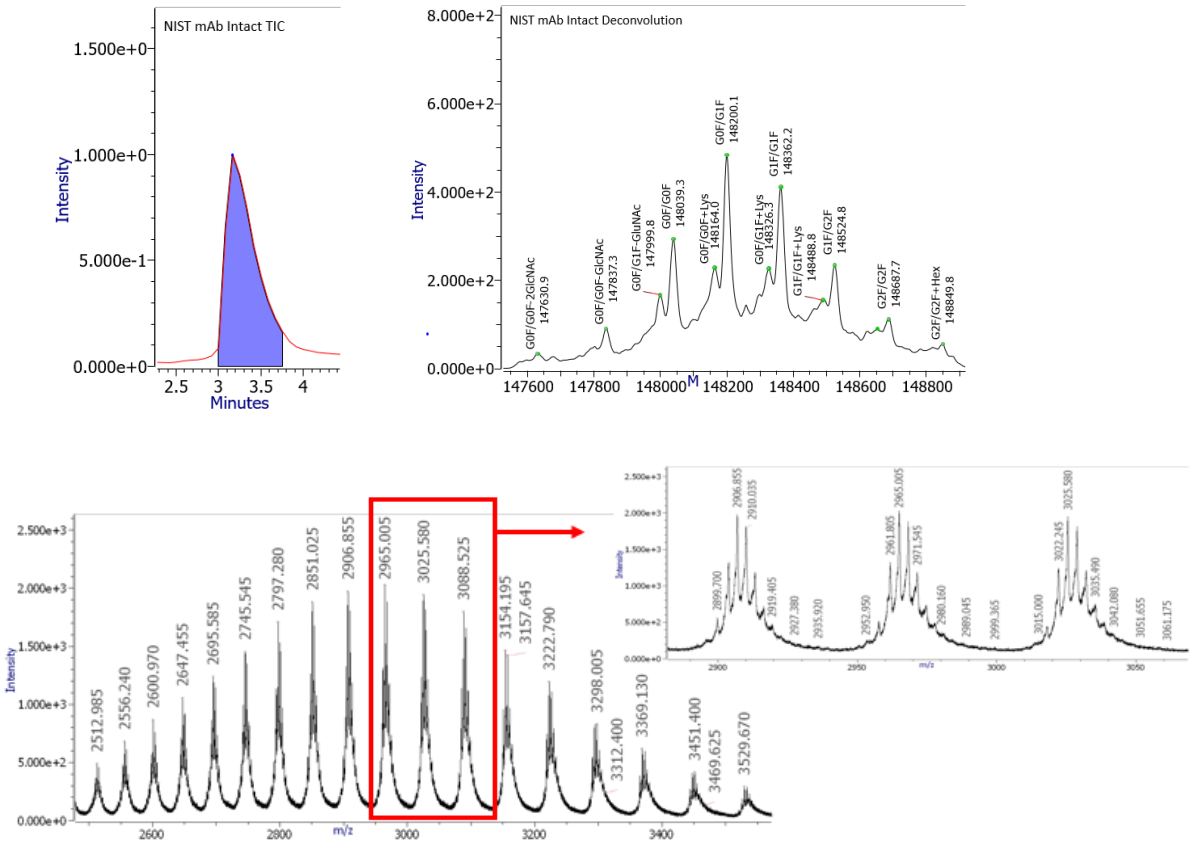

Figure 2: NIST mAb Intact TIC chromatogram (top, left), deconvoluted spectrum (top right), and mass spectrum (bottom)

Table 3: Theoretical vs Observed Mass for Intact NIST mAb on the Shimadzu LCMS-9030

			Sample name ←	NIST mAb
			Peak # ←	1
Name ↓	Expected mass ↑	Mass ↑		
G2F/G2F+Hex	148848	148850		1.8
G2F/G2F	148686	148688		1.9
G1F/G2F+Lys	148652	148653		1.6
G1F/G2F	148524	148525		1.0
G1F/G1F+Lys	148490	148489		-0.9
G1F/G1F	148362	148362		0.7
G0F/G1F-GluNAc	147996	148000		3.5
G0F/G1F+Lys	148328	148326		-1.4
G0F/G1F	148199	148200		0.7
G0F/G0F-GlcNAc	147834	147837		3.2
G0F/G0F-2GlcNAc	147631	147631		0.0
G0F/G0F+Lys	148165	148164		-1.5
G0F/G0F	148037	148039		2.0

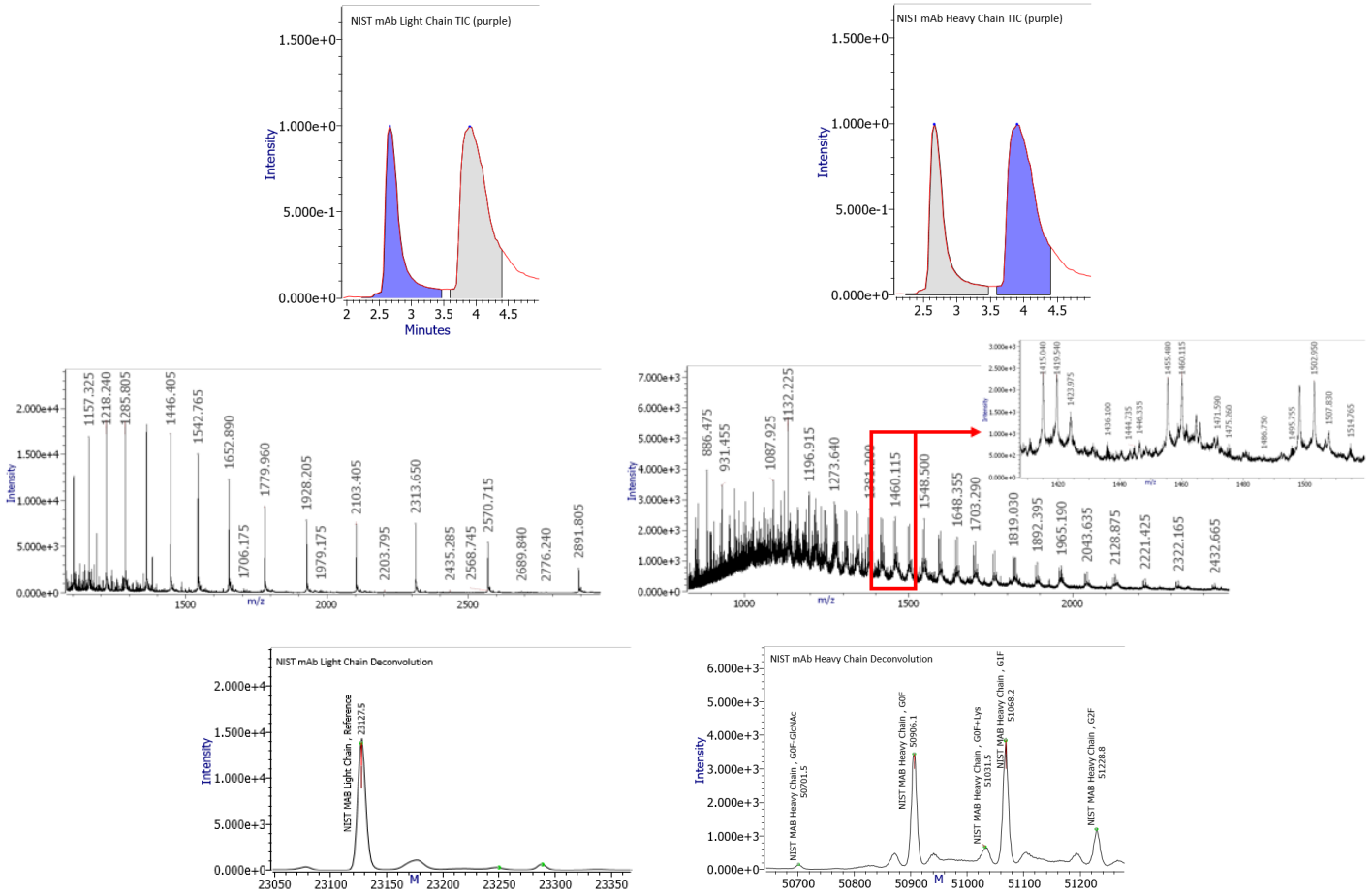

Figure 3: NIST mAb LC and HC separation (top), LC and HC mass spectra (middle), LC and HC deconvolution spectra (bottom)

Peptide Mapping Digestion Results

HC and LC subunits were digested with either trypsin, Glu-C or Lys-C proteases. Trypsin specifically cleaves the carboxyl side of amino acids lysine or arginine, except when either is followed by proline. Glu-C preferentially cleaves peptide bonds C-terminus to glutamic acid residues, and Lys-C cleaves on the C-terminal side of lysine residues. Using different enzymes can selectively cleave different amino acid residues of the protein giving more confidence in the protein sequence.

A total of 5 dependent events were monitored with 1-3 charge states. The DDA chromatograms are shown in Figure 4a. The results were analyzed using Protein Metrics PTM Workflow. Complete coverage for HC and LC was observed by using a combination of digestion enzymes (Figure 4b).

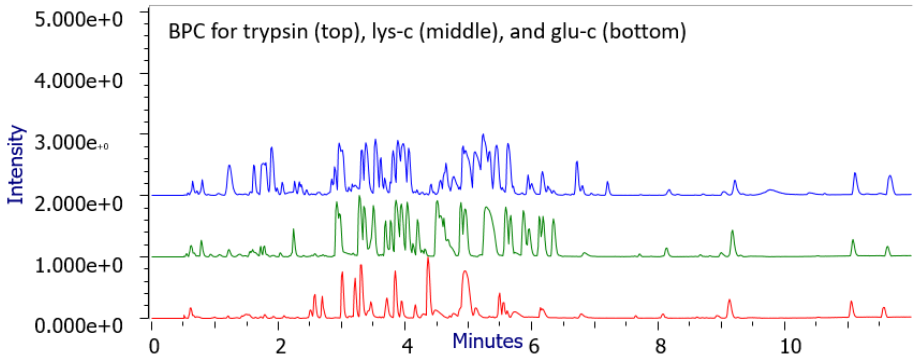

Figure 4a: Base Peak Chromatogram (BPC) for trypsin (top, blue), lyc-c (middle, green) and glu-c (bottom, red)

Figure 4b: NIST mAb peptide coverage using Lys-C (red), Glu-C (black) and Trypsin (blue)

Deglyosylation

N-linked glycolylation is a common post-translational modification (PTM) that imparts structural heterogeneity to mAb therapeutics. PNGase F was used to release the N-glycans from the HC component of intact NIST mAb (Figure 5a) and from the reduced NIST mAb subunit (Figure 5b).

The deglycosylated intact protein and subunit were analyzed using Protein Metrics Intact and Reduced Mass Workflow.

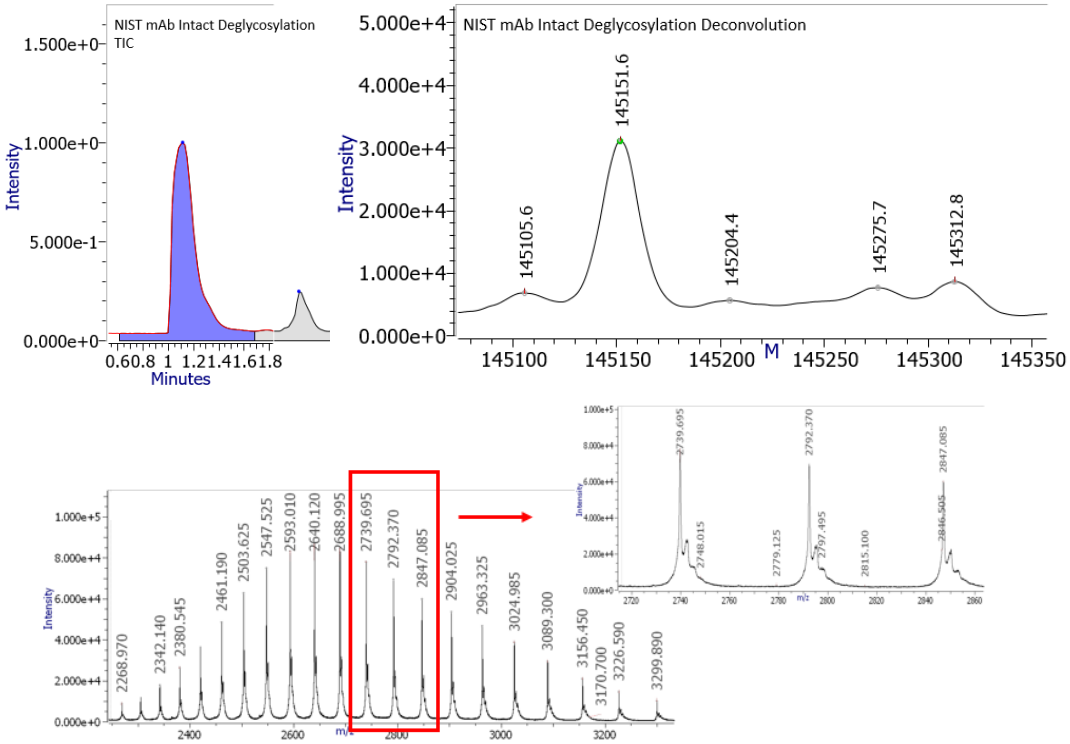

Figure 5a: NIST mAb intact deglycosylation TIC chromatogram (top left), deconvolution (top right) and mass spectrum (bottom)

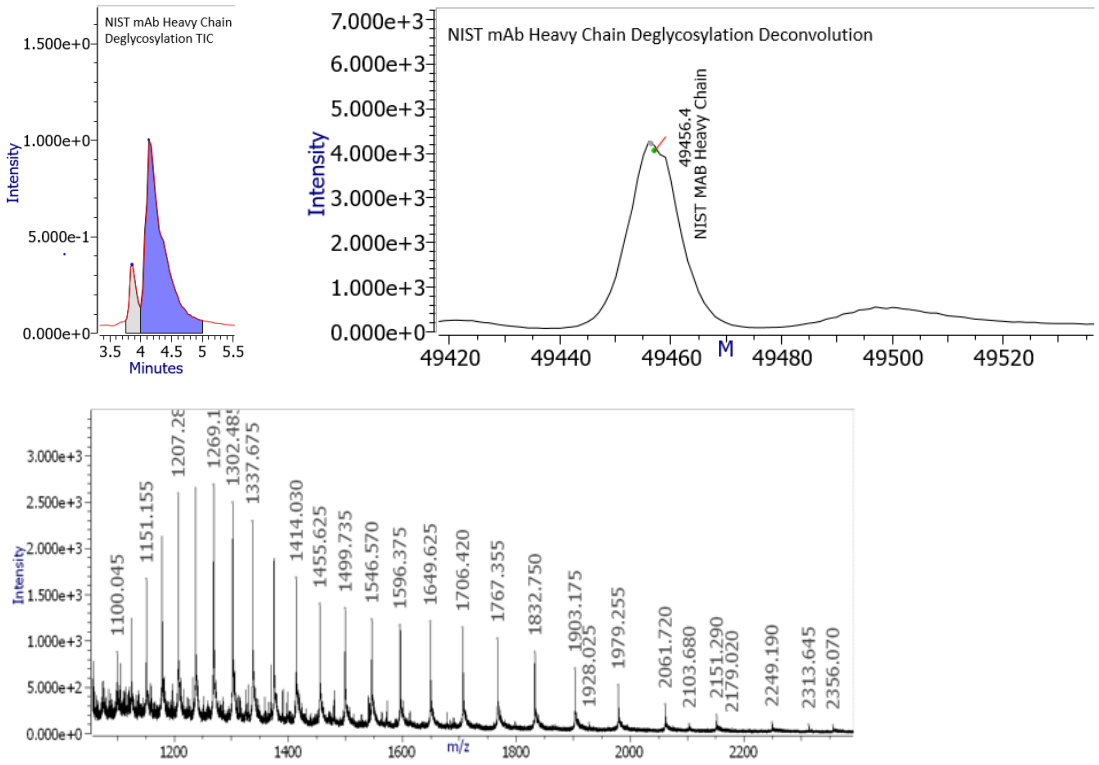

Figure 5b: NIST mAb reduced deglycosylation TIC chromatogram (top left), deconvolution (top right) and mass spectrum (bottom)

■ Conclusion

The Shimadzu Q-TOF LCMS-9030 plus Protein Metrics software offers a complete workflow solution for monoclonal antibody characterization. Customizable workflows can be created for any type of analysis including intact data, subunit, peptide fragments and deglycosylation analysis.

■ References

1. Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm*. 1999; **185**(2): 129- 188. https://doi.org/10.1016/S0378-5173(99)00152-0.
2. Chen, G. (2013) Characterization of Protein Therapeutics using Mass Spectrometry. New York, NY: Springer
3. Varki, A., Cummings, RD., Esko, JD., Freeze, HH., Stanly, p., Bertozzi, CR., Hart, GW., Etzler, ME. (2009) Essentials of Glycobiology, Second Edition. Cold Spring Harbor, NW: Cold Spring Harbor Laboratory Press

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Q-TOF LCMS-9030

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