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Application News

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Biopharma / LCMS-9030 (Q-TOF)

Post-translational Modification Characterization of Monoclonal Antibody (mAb) by Q-TOF Mass Spectrometer

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❑ Introduction

The growing market for monoclonal antibodies (mAbs) brings new challenges to meet the increasing consumer demands for quality and safety of products. Much of this can be attributed to heterogeneity of mAbs caused by post-translational modifications (PTMs). PTMs such as glycosylation, deamidation, and oxidation are typical modifications for mAbs, but there are also many other PTMs that can occur during mAb production, storage, and post-administration. Identification and quantification of the PTMs are therefore critical to ensure the ultimate safety and potency of mAb products. In this study, we demonstrate a LC/MS-based peptide-mapping method on Shimadzu LCMS-9030 (Q-TOF) mass spectrometer for PTM characterization of mAbs.

❑ Experimental

A. Materials

The mAb sample was 5 mg/mL bevacizumab biosimilar in 50 mmol/L Tris-HCl (pH 8.0) buffer.

B. Sample preparation

A 20 µL aliquot of the mAb sample was diluted using 80 µL of ammonium bicarbonate (ABC) solution (50 mM), then mixed with 10 µL proteaseMAX™ (0.5%, w/w) and 10 µL Dithiothreitol (DTT, 0.2 M), incubated at 60°C for 1 h to denature and reduce disulfide bonds. Alkylation was done by adding 30 µL iodoacetamide (IAM, 0.2 M) followed by incubation at 37°C for 1 h in the dark. The sample were diluted using 328 µL of ABC solution (50 mM) before trypsin digestion. Sequencing grade trypsin was used for overnight digestion at 37°C. Finally, 2 µL trifluoroacetic acid was added to stop the reaction. The sample was centrifuged and supernatant was collected for LC/MS analysis.

C. LC/MS analysis

The digested sample was injected to a LCMS-9030 (Q-TOF) mass spectrometer for MS and MS/MS analysis. LC separation was achieved by a 45 min gradient on a Shim-pack GISS-HP C18 column (3 µm, 150 × 3.0 mm) (**Table 1**).

D. Data processing

Table 1. Analytical conditions on LCMS-9030 (Q-TOF)

LC conditions	
LC system:	Shimadzu Nexera X2 UHPLC
Column:	Shim-pack GISS-HP, 3 µm, 150 × 3.0 mm
Column temperature:	40 °C
Flow rate:	0.5 mL/min
Mobile phase A:	0.1% FA + 0.01% TFA in water
Mobile phase B:	0.1% FA + 0.01% TFA in acetonitrile
Gradient program:	B Conc. 0% (0-2 min) → 15% (10 min) → 35% (23 min) → 45% (30 min) → 75% (35-40 min) → 0% (40.1-45 min).
Injection volume:	20 µL
MS conditions	
MS system:	Shimadzu LCMS-9030 (QTOF)
Interface:	Heated ESI (+)
Interface voltage:	4.5 kV
Interface temperature:	300 °C
Nebulizing gas:	N2, 3 L/min
Heating gas flow:	Zero air, 10L/min
DL temperature:	250 °C
Drying gas flow:	N2, 10 L/min
Heat block temperature:	400 °C
MS mode:	MS
Mass range:	100 - 2000 m/z
MS mode:	MS/MS
Mass range:	100 - 2500 m/z
Collision Energies:	35 ± 17 V

Amino acid sequence of bevacizumab was submitted to the Skyline software for PTM identification. Parameters were set up as trypsin digest and maximum one missed cleavage. As we used IAM to alkylate cysteine residues in sample preparation, carbamidomethylation (+57.0215 Da) of cysteine was thus defined as a fixed modification. Glycosylation at asparagine 303 ($N_{303}ST$), deamidation, oxidation, N-terminal pyroglutamate, & C-terminal lysine cleavage were considered as variable modifications. Theoretical PTM-carrying peptide monoisotopic masses were calculated and used for searching PTMs in the MS data acquired on LCMS-9030. For matched MS peaks, MS/MS analysis was conducted for PTM confirmation.

❑ Results and Discussion

PTMs including N-glycosylation at $N_{303}ST$, deamidation (N), oxidation (M), N-terminal pyroglutamate (E), and C-terminal lysine (K) loss were observed (**Table 2**).

Table 2. Identified PTM-carrying peptides in bevacizumab biosimilar heavy chain.

PTM types	Peptide [AA numbers] with PTMs	PTM-carrying peptide				Wild type peptide			
		ID	RT (min)	*m/z*	ppm	ID	RT (min)	*m/z*	ppm
N-glycosylation	R.EEQYNSTYR.V [299, 307] (G0F)	PTM-1a	9.63	1317.5262++	-0.30	WT-1	10.20	595.2588++	-1.34
	R.EEQYNSTYR.V [299, 307] (G0F-GN)	PTM-1b	9.67	1215.9867++	-0.16				
	R.EEQYNSTYR.V [299, 307] (G1F)	PTM-1c	9.60	1398.5532++	0.14				
	R.EEQYNSTYR.V [299, 307] (Man5)	PTM-1d	9.67	1203.4715++	0.33				
Deamidation (N)	R.VVSVLTVLHQDWLNGK.E [308, 323] (isoAsp)	PTM-2a1	22.73	904.9990++	0.11	WT-2	22.90	904.5068++	-0.11
	R.VVSVLTVLHQDWLNGK.E [308, 323] (Asp)	PTM-2a2	23.13	904.9990++	0.11				
	R.VVSVLTVLHQDWLNGK.E [308, 323] (Succinimide)	PTM-2b	23.67	895.9936++	0.00				
Oxidation (M)	K.STAYLQMNSLR.A [77, 87] (Met sulfoxide)	PTM-3.1	13.33	650.3209++	-1.23	WT-3.1	16.00	642.3242++	-0.16
	K.DTLMISR.T [255, 261] (Met sulfoxide)	PTM-3.2	12.03	426.2185++	0.70	WT-3.2	13.27	418.2215++	1.91
N-terminal pyroglutamate (E)	-.EVQLVESGGGLVQPGGSLR.L [1, 19] (pyroGlu)	PTM-4	19.63	932.4994++	-0.43	WT-4	17.23	941.5052++	0.11
C-terminal lysine (K) loss	K.SLSLSPGK.- [446, 452] (K loss)	PTM-5	13.73	660.3566+	0.45	-			

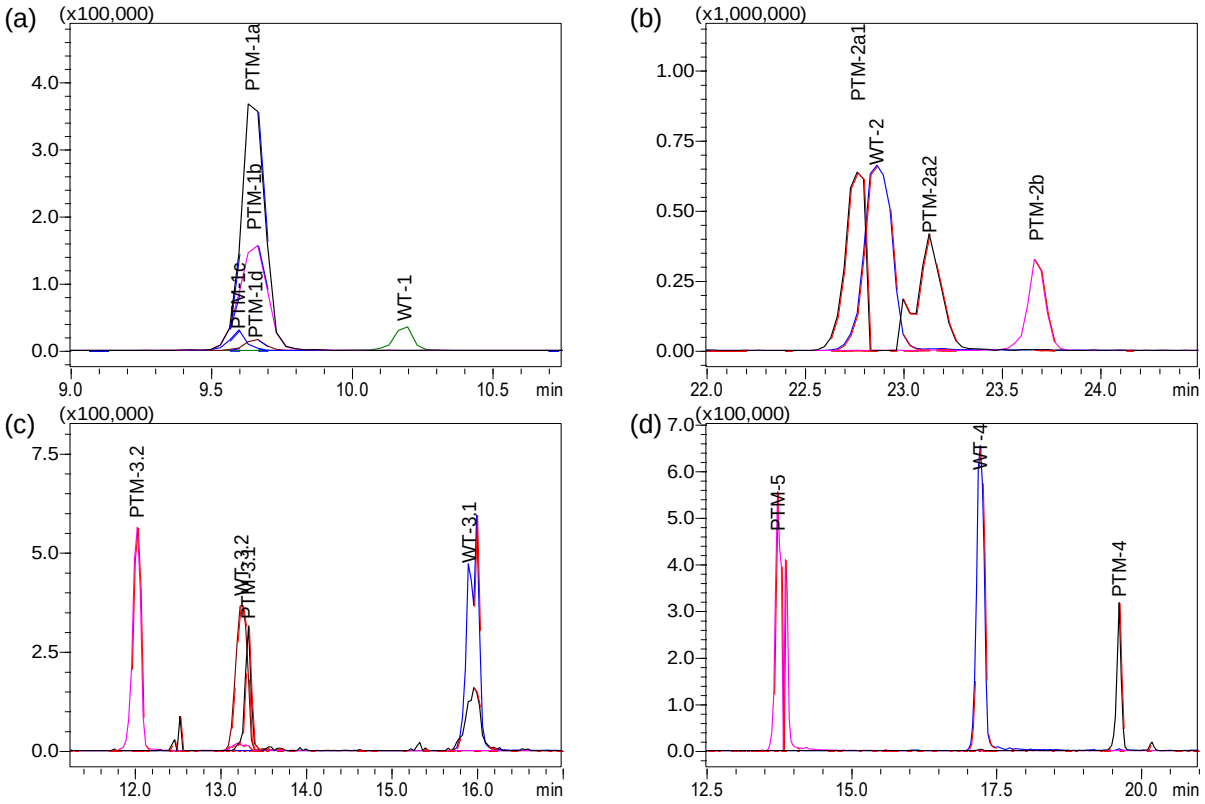


Figure 1. Extracted-ion chromatograms of identified PTMs and their wild type (WT) peptides. (a) N-glycosylation; (b) Deamidation (N) and Succinimide; (c) Oxidation (M); (d) N-terminal pyroglutamate and C-terminal lysine cleavage.

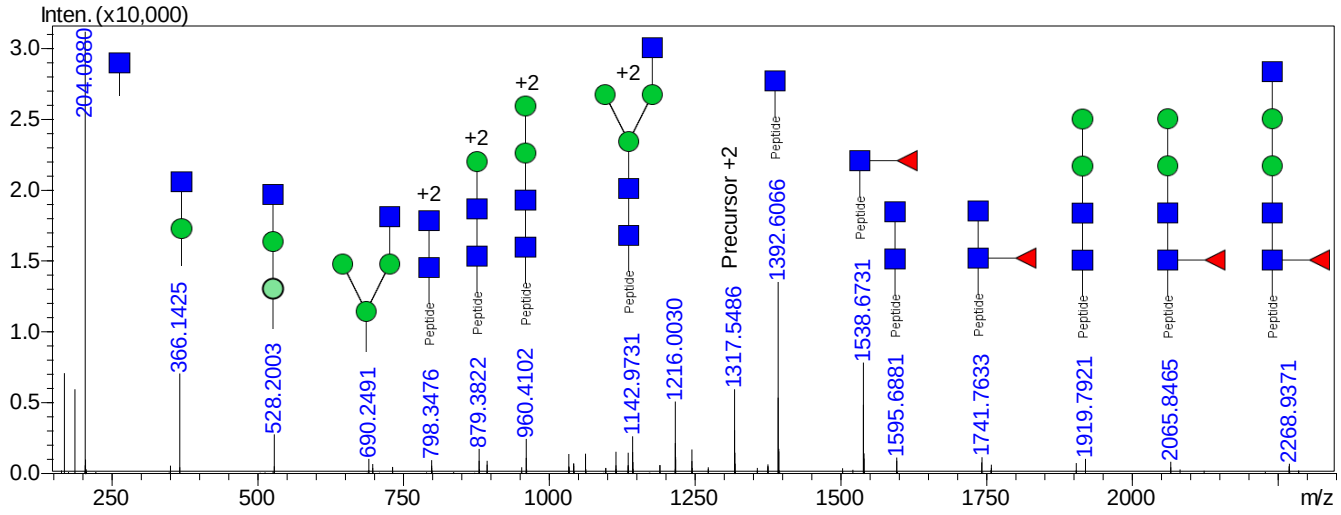


Figure 2. MS/MS spectrum of glycopeptide R.EEQYNSTYR.V [299, 307] with G0F.
N-acetylglucosamine; Manose; Fucose

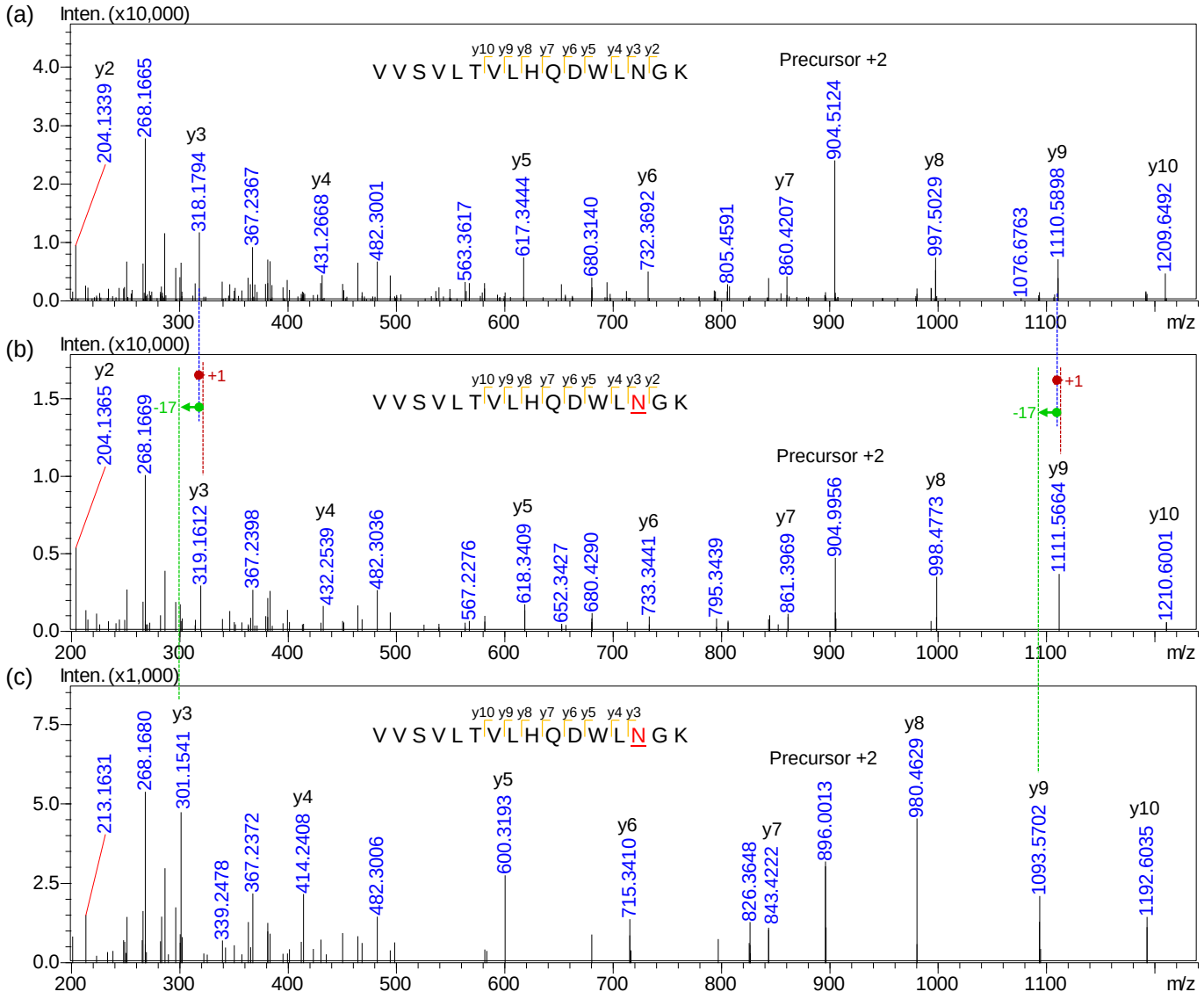


Figure 3. MS/MS spectra of peptide R.VVSVLTVLHQDWLNGK.E [308, 323].
(a) Wild type; (b) Deamidation; (c) Succinimide intermediate

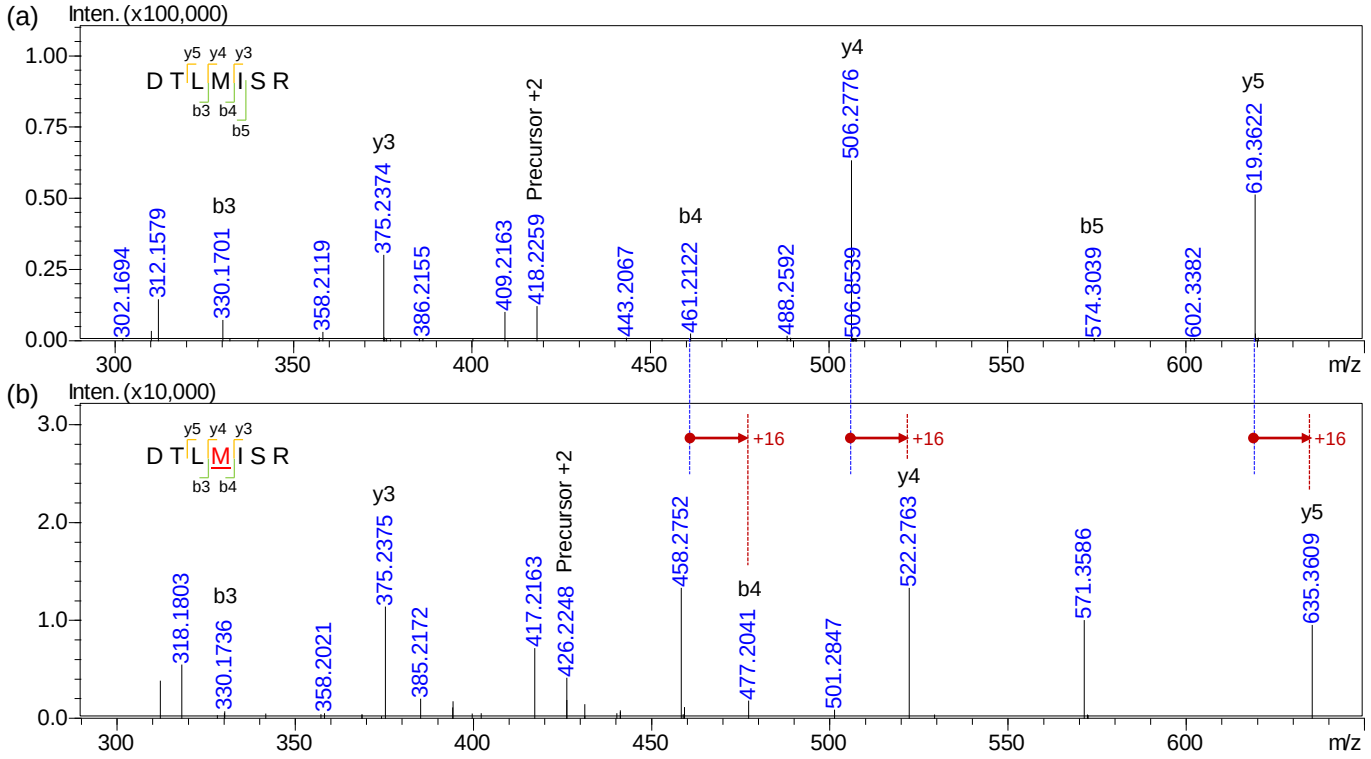
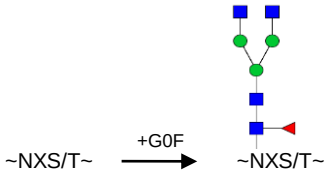


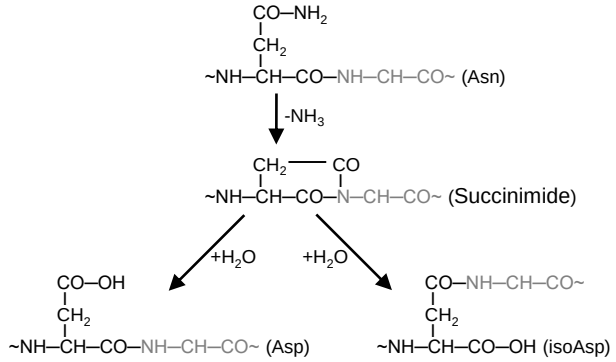
Figure 4. MS/MS spectra of peptide K.DTLMISR.T [255, 261]. (a) Wild type; (b) Oxidation.

A. N-glycosylation at $N_{303}ST$



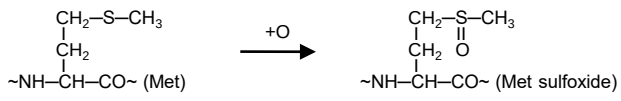
N-glycosylation at the consensus sequence ~NXS/T~ is a natural modification in mAbs, and N-glycan profile has been recognized as a critical quality attribute (CQA) for mAb drug development. Bevacizumab has one known N-glycosylation site at ~N_{303}ST~. In combination of MS (**Fig. 1**) and MS/MS (**Fig. 2**) results, four glycopeptides (R.EEQYN_{303}STYR.V [299, 307]) with different glycans (G0F, G0F-GN, G1F, and Man5) were identified (**Table 2**). As shown in the **Figure 1**, the glycopeptide with G0F was found to be the most abundant peak. The result was highly similar to our previous finding obtained by 2-AB labeling/HILIC UHPLC method [1]. Moreover, we noted that glycosidic bond was much easier to be broken than peptide bond in the collision-induced dissociation (CID) MS/MS. Thus, the method described here can be used for N-glycan profiling of mAbs as well.

B. Deamidation (N)



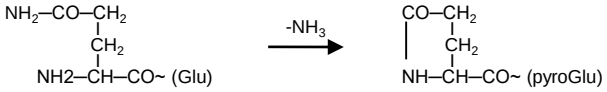
Deamidation of asparagine (Asn, N) is one of the most frequently observed modifications in mAbs. It is a major source of instability in the formulation and storage. Asn is deamidated through a cyclic succinimide intermediate to form aspartate (Asp) or iso-aspartate (isoAsp). In this study, a deamidation (N) at position 321 was identified (**Table 2**). Meanwhile, the succinimide intermediate at the same position was measured as well. As shown in **Figure 1**, although there is only a 0.9840 Da shift for deamidation, our method can achieve a high separation accuracy for Asn and Asp isomers. The MS/MS spectra were shown in **Figure 3**.

C. Oxidation (M)



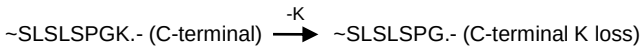
Methionine (Met, M) oxidation is a common degradation pathway of mAbs, in which the side-chain sulfur of Met is oxidized to Met sulfoxide. As Met oxidation affects the drug's efficacy and safety, it should be strictly controlled during storage period. In this study, the oxidation (M) at positions 83 and 258 was identified (**Table 2**). Here we used peptide K.DTLMISR.T [255, 261] to demonstrate the identification progress. Its MS and MS/MS data are shown in **Figures 1** and **4**, respectively.

D. N-terminal pyroglutamate (E)



Pyroglutamate (pyroGlu) is a cyclic amino acid found at N-terminal of mAbs, via the rearrangement of glutamate (Glu, E) or glutamine (Gln, Q) residues. In this study, cyclization of N-terminal Glu to pyroGlu is observed in the heavy chain (**Table 2**). **Figure 1** shows the MS data (MS/MS data not shown).

E. C-terminal lysine (K) loss



C-terminal lysine (Lys, K) loss is commonly observed in mAbs. This modification can be important as it is found to be sensitive to the production process. In particular, C-terminal K loss is one of the most commonly detected sources of product charge-heterogeneity. In this study, peptide SLSLSPG was detected as C-terminal peptide of heavy chain after lysine loss (**Table 2, Figure 1**).

❑ Conclusions

An integrated MS and MS/MS approach on LCMS-9030 (Q-TOF) mass spectrometer has been demonstrated for PTM characterization of mAbs. This method can be used to quickly and accurately identify the PTMs at any stage during the development and manufacture of mAb biotherapeutics, and it indicates a potential to determine the relative expression of PTMs.

❑ Reference

1. Shimadzu (Asia Pacific), "N-glycan Profiling of monoclonal Antibody (mAb) on Nexera Bio UHPLC Coupled with Fluorescence Detector and Q-TOF Mass Spectrometer", Application News, No. AD-0191, 2018

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