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Application News

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Biopharma / LCMS-9030 (Q-TOF)

Disulfide Bond Characterization of Monoclonal Antibody (mAb) Using Q-TOF Mass Spectrometer

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❑ Introduction

Monoclonal antibody (mAb) is emerging as the fastest-growing category of biotherapeutics with a wide range of therapeutic and diagnostic applications. Higher-order structure of mAb plays a critical role in the efficacy and safety. For example, the number of disulfide bonds and their positions are critical quality attributes (CQAs) for mAb, because incorrect disulfide linkage formation can cause a loss of biological activity or even can elicit an immune response from the host. Herein, we report a LCMS-based method to precisely characterize disulfide bonds in mAb biosimilar by a comparative analysis of non-reduced and reduced conditions. The method uses ProteaseMAX™ surfactant to denature the protein, and trypsin to digest with/without reduction and alkylation. The peptides were gradient-eluted and analyzed using a Shimadzu LCMS™-9030 Q-TOF mass spectrometer for MS scan and MS/MS analysis.

❑ Experimental

A. Reduced condition

mAb solution: 5 mg/mL of bevacizumab biosimilar in 50 mmol/L Tris-HCl (pH 8.0) buffer.
Sample solution: the sample was prepared by taking 20 µL of mAb solution and diluted 5 times with 50 mM ammonium bicarbonate (ABC) solution.
Denaturation: 10 µL ProteaseMAX™(0.5%, w/w) was added and incubated at 60 ºC to denature the mAb.
Reduction and Alkylation: 10 µL Dithiothreitol (DTT, 0.2 M) was added to reduce disulfide bonds. Alkylation was done by adding 30 µL iodoacetamide (IAM, 0.2 M).
Dilution: dilute the sample with 50 mM ABC solution to the final volume of 478 µL.
Digestion: 20 µL sequencing grade trypsin was added for protein digestion at 37ºC for overnight.
Stop: 2 µL trifluoroacetic acid (TFA) was added to stop trypsin activity.
Analysis: the analytical conditions on LCMS-9030 (Q-TOF) are shown in **Table 1**.

B. Non-Reduced condition

The procedure of non-reduced condition is the same as it used in reduced condition except without the step of reduction and alkylation.

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

Column	: Shim-pack™ GISS-HP, 3 µm, 150 × 3.0
Mobile phase	mm : (A) 0.1% FA + 0.01% TFA in water
	(B) 0.1% FA + 0.01% TFA in acetonitrile
Flow rate	: 0.5 mL/min
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).
Column temp.	: 40ºC
Injection volume	: 20 µL
Interface	: Heated ESI (positive mode)
MS Mode	: MS scan
Interface voltage	: 4.5 kV
TOF mass range	: 100 – 2000 (m/z)
Heat block temp.	: 400ºC
DL temp.	: 250ºC
Interface temp.	: 300ºC
Nebulizing gas	: N2, 3 L/min
Drying gas	: N2, 10 L/min
Heating gas	: Zero air, 10L/min

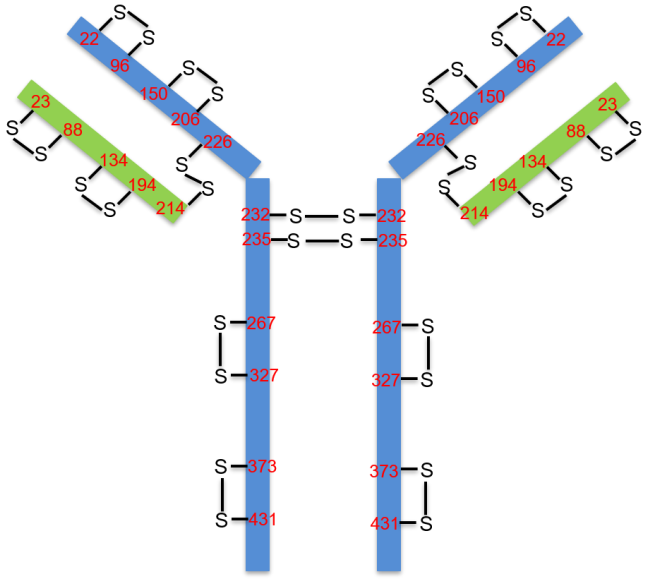

Figure 1. Disulfide bond linkage structure of bevacizumab

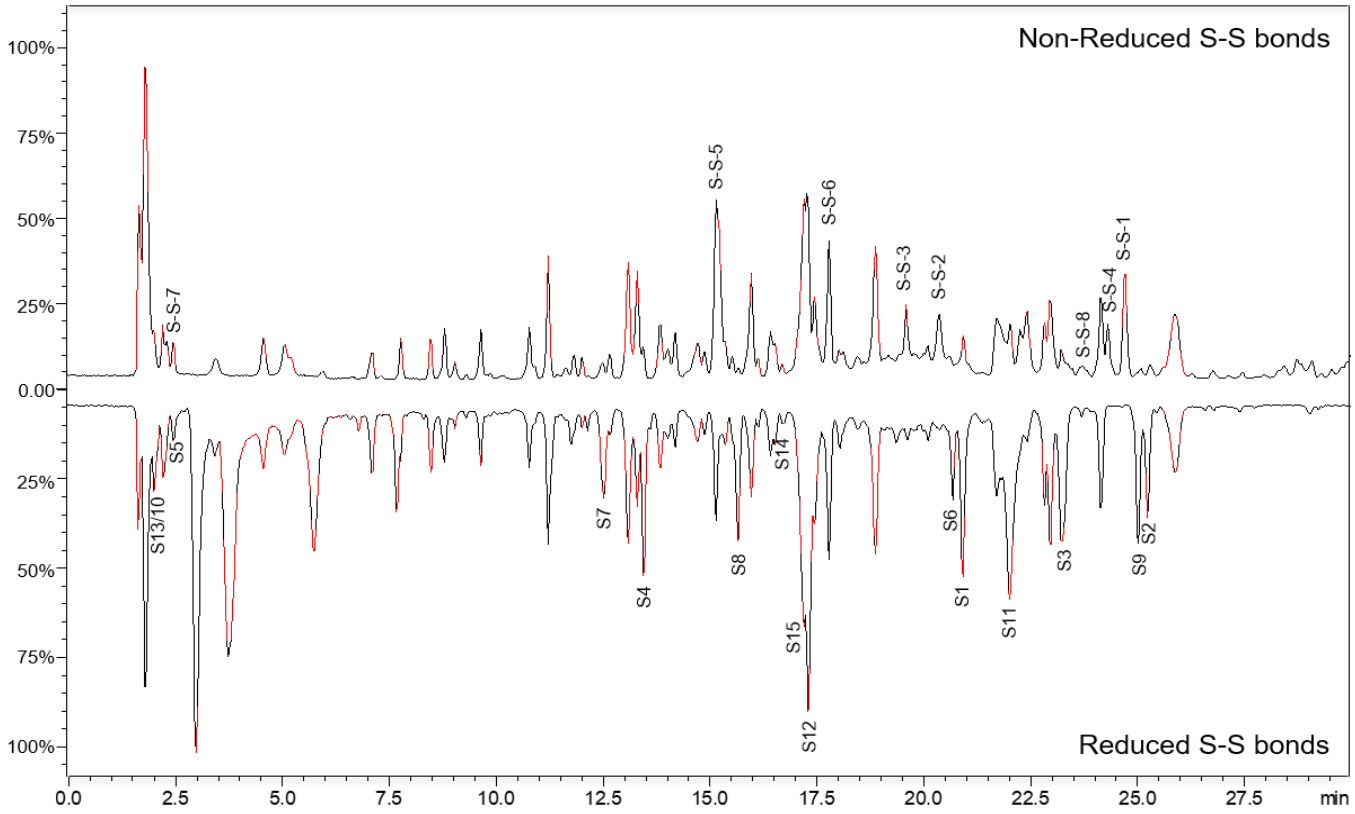

Figure 2. Comparative total ion chromatograms (TIC) of disulfide bond peptides of bevacizumab biosimilar under non-reduced and reduced conditions. The peak # refers to tables 3 and 4.

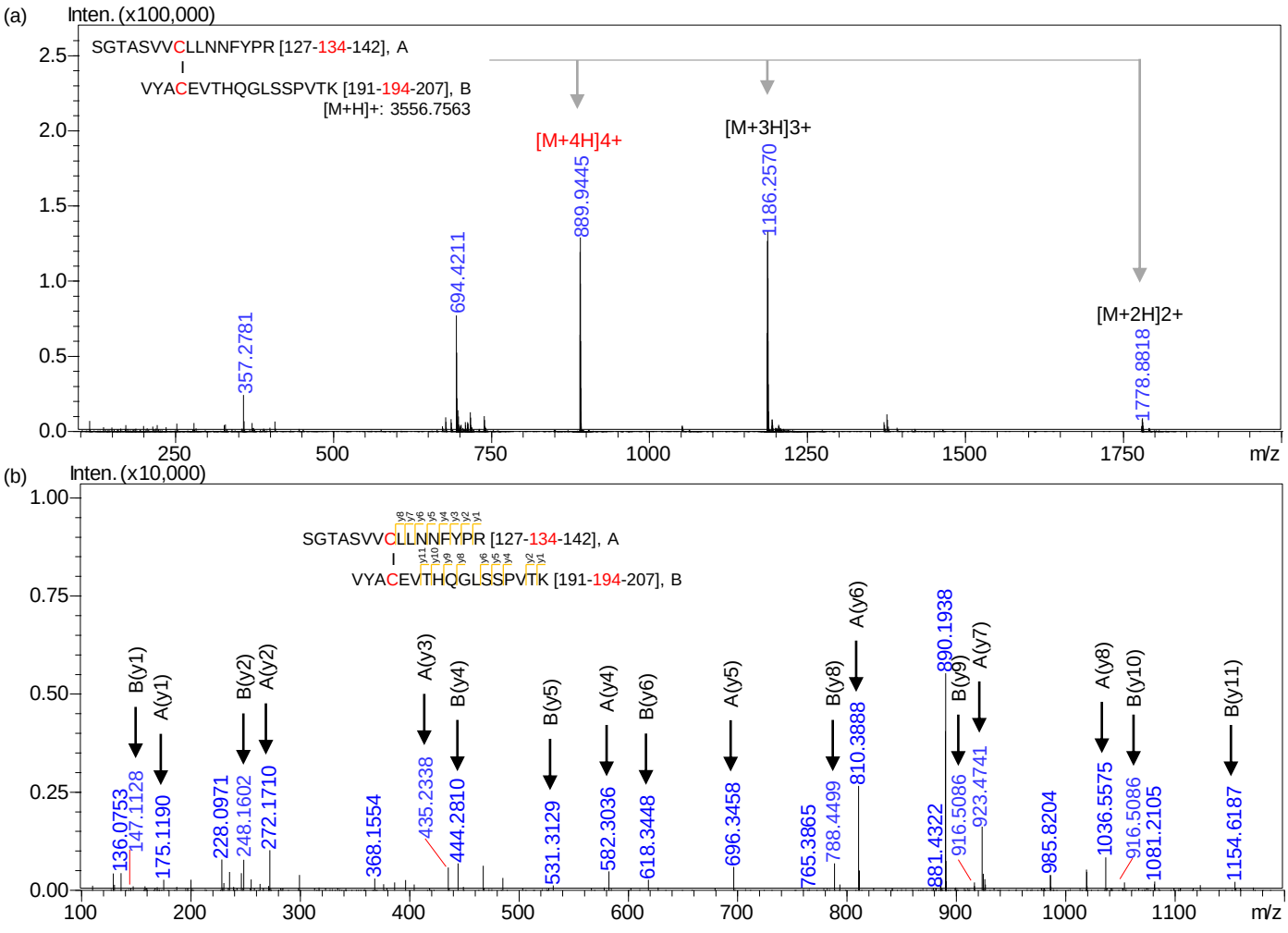

Figure 3. MS (a) and MS/MS (b) spectra of the non-reduced disulfide bond peptide (S-S-2) of bevacizumab biosimilar.

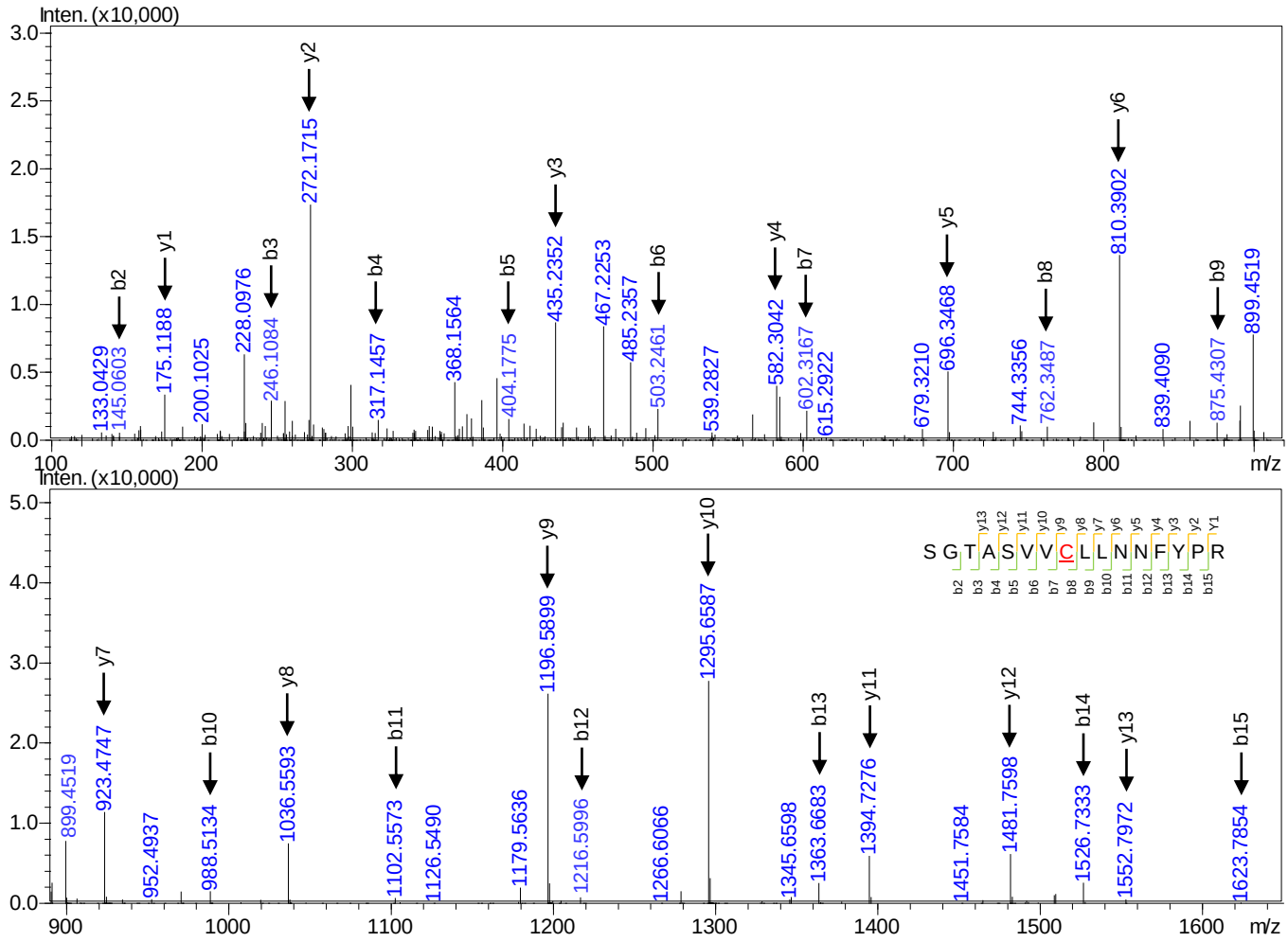

Figure 4. MS/MS spectra of the reduced disulphide bond (i.e., Cys-containing) peptide (S3) of bevacizumab biosimilar.

Table 3. Detection of non-reduced disulphide bond peptides of bevacizumab biosimilar

Peak No.	RT (min)	Peptide [AA numbers]	Peptide m/z	Adduct Ion
S-S-1	24.7	Light chain, [19-23-42] VTITCSASQDISNYLNWYQQKPGK I FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK [62-88-103], Light chain	1469.6837	[M+5H]5+
S-S-2	20.4	SGTASVVCLLNNFYPR [127-134-142], Light chain I VYACEVTHQGLSSPVTK [191-194-207], Light chain	889.9428	[M+4H]4+
S-S-3	19.6	LSCAASGYTFTNYGMNWVR [20-22-38], Heavy chain I AEDTAVYYCAK [88-96-98], Heavy chain	1124.4967	[M+3H]3+
S-S-4	24.3	Heavy chain, [140-150-153] STSGGTAALGCLVK I DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTK [154-206-216], Heavy chain	1584.3884	[M+5H]5+
S-S-5	15.2	TPEVTCVVVDVSHEDPEVK [262-267-280], Heavy chain I CK [327-328], Heavy chain	777.0393	[M+3H]3+
S-S-6	17.8	NQVSLTCLVK [367-373-376], Heavy chain I WQQGNVFSCSVMHEALHNHYTQK [423-431-445], Heavy chain	962.2111	[M+4H]4+
S-S-7	2.5	GEC [212-214], Light chain I SCDK [225-226-228], Heavy chain	379.1272	[M+2H]2+
S-S-8	23.7	THTCPPCPAPELLGGPSVFLFPPKPK [229-232-235-254], Heavy chain I I THTCPPCPAPELLGGPSVFLFPPKPK [229-232-235-254], Heavy chain	1364.7007	[M+4H]4+

Table 4. Detection of reduced disulphide bond (i.e., Cys-containing) peptides of bevacizumab biosimilar

Peak No.	RT (min)	Peptide [AA numbers]	Peptide m/z	Adduct Ion
S1	20.9	R.VTITCSASQDISNYLNWYQQKPGK.A [19, 42] Light chain	934.4560	[M+3H]3+
S2	25.3	R.FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK.V [62, 103] Light chain	1554.0400	[M+3H]3+
S3	23.3	K.SGTASVVCLLNNFYPR.E [127, 142] Light chain	899.4510	[M+2H]2+
S4	13.6	K.VYACEVTHQGLSSPVTK.S [191, 207] Light chain	938.4664	[M+2H]2+
S5	2.5	R.GEC.- [212, 214] Light chain	365.1119	[M+H]+
S6	20.7	R.LSCAASGYTFTNYGMNWVR.Q [20, 38] Heavy chain	1099.4914	[M+2H]2+
S7	12.5	R.AEDTAVYYCAK.Y [88, 98] Heavy chain	645.7863	[M+2H]2+
S8	15.7	K.STSGGTAALGCLVK.D [140, 153] Heavy chain	661.3433	[M+2H]2+
S9	25.0	K.DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTK.V [154, 216] Heavy chain	1679.0810	[M+4H]4+
S10	2.1	K.SCDK.T [225, 228] Heavy chain	509.2016	[M+H]+
S11	22.0	K.THTCPPCPAPELLGGPSVFLFPPKPK.D [229, 254] Heavy chain	948.8226	[M+3H3]+
S12	17.3	R.TPEVTCVVVDVSHEDPEVK.F [262, 280] Heavy chain	713.6804	[M+3H]3+
S13	2.0	K.CK.V [327, 328] Heavy chain	307.1429	[M+H]+
S14	16.8	K.NQVSLTCLVK.G [367, 376] Heavy chain	581.3172	[M+2H]2+
S15	17.2	R.WQQGNVFSCSVMHEALHNHYTQK.S [423, 445] Heavy chain	934.4253	[M+3H]3+

❑ Results and Discussion

A. Disulfide bond linkages in bevacizumab

Typical structure of bevacizumab is shown in **Figure 1**. It contains a total of 16 disulfide bonds, 12 of which are intra-chain linkages (four in the light chain and eight in the heavy chain), whereas the other 4 disulfide bonds are inter-chain linkages (two links light chain and heavy chain, and two links the two heavy chain in the hinge region). By employing reduction reagent DTT, 15 Cys-containing tryptic peptides could be produced from the mAb (reduced disulfide bonds). Without reduction by DTT, 8 intact disulfide bond linked peptides could be produced (non-reduced disulfide bonds).

B. Detection of disulfide bond peptides

The S-S bond of bevacizumab biosimilar were cleaved in reduced sample, while the intact S-S linked peptides remained in the non-reduced sample. To identify these disulfide bond peptides, both reduced and non-reduced samples were prepared and analyzed by LCMS-9030 (Q-TOF) under the same analytical conditions.

Figure 2 shows the total ion chromatograms (TIC) of both non-reduced and reduced samples. The mirror plot clearly indicates the major different peaks between the two samples. Compared to the reduced sample, there were several large peptide molecules with longer elution times measured in the non-reduced sample. By comparing with theoretical masses of tryptic disulfide bond peptides using the Skyline s/w, all the 8 intact S-S linked peptides (S-S-1/8) and the 15 Cys-containing peptides (S1/15) from bevacizumab biosimilar were correctly measured by LCMS-9030 (Q-TOF) with <3 ppm mass error. The peptide sequences and their accurate masses are shown in **Tables 3** and **4**.

C. MS/MS sequencing of disulfide bond peptides

De novo sequencing analysis of all the disulfide bond peptides listed in Tables 3 and 4 were performed. Here we show the results of non-reduced S-S-2 and reduced S-3 as representative examples only. **Figure 3** shows *de novo* sequencing of non-reduced disulfide bond peptide (S-S-2) from light chain of bevacizumab biosimilar through a MS/MS spectrum with the fragment ion annotation. The peptide sequence and accurate *m/z* values of multiple-charged ions (2+, 3+, 4+) are displayed in the top panel. MS/MS spectrum of the 4+ ion (*m/z* 889.9445) is shown in the bottom panel, with the explanations (y ion) of matching fragments based on the peptide sequence. In addition to the non-reduced disulfide bond peptides, we also conduct *de novo* sequencing for reduced disulfide bond peptides. **Figure 4** shows the MS/MS spectra of the reduced peptide (S3) form the S-S-2 in the reduced sample.

❑ Conclusions

A straightforward LCMS-based method for accurate disulfide bond peptide characterization of mAb biosimilar was established on LCMS-9030 (Q-TOF). The MS/MS spectra with fragmentation data provide high confidence results on sequencing analysis. The demonstrated performance for bevacizumab biosimilar in detection and *de novo* sequencing of non-reduced and reduced disulfide bonds signifies its practicability for the structural characterization of mAb biosimilars.

❑ Acknowledgement

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