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Application News

No. AD-0190

Biopharma / LCMS-9030

Characterization of C-terminal and Disulfide Bond Peptides of Monoclonal Antibody (mAb) on Q-TOF Mass Spectrometer

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1. Introduction

In recent years, there is a significant surge in the market of biopharmaceutical products due to their popularity as alternative solution for many chronic diseases. Monoclonal antibody (mAb) is a highly complex biological macromolecule with specific therapeutic effects. It is produced from live cells in extremely complicated culture conditions. Quality control of biopharmaceuticals, especially biosimilars, is a critical step to elucidate any alteration in the primary structure as compared to the reference product (innovator). Peptide mapping and sequencing analysis of C-terminal and disulfide-bonds linked peptides are among the essential attributes for characterization of biosimilars. In this report, characterization of a bevacizumab biosimilar is described, with focusing on peptide mapping and MS/MS sequencing of C-terminal and cysteine-containing peptides of a bevacizumab biosimilar on LCMS-9030, a Q-TOF system.

2. Experimental

A bevacizumab biosimilar sample of 10 mg/mL was prepared in Tris buffer. Dithiothreitol (DTT) was added to the sample and the mixture was incubated for 30 minutes at 60°C for reducing the protein disulfide bonds. Alkylation of the sample was done by adding iodoacetamide (IAM) followed by incubation at 37°C for 30 minutes in the dark. Trypsin was added and the total volume of the sample was adjusted with ammonium bicarbonate (ABC) buffer to an optimized ratio for subsequent digestion at 37°C for 4 hours. Trifluoroacetic acid (TFA) solution was added and incubated for 5 minutes at room temperature. The obtained sample was centrifuged and the supernatant was collected and injected to LCMS-9030, for peptide mapping analysis and sequence confirmation in MS and MS/MS. The analytical conditions are compiled into **Table 1**.

Table 1. Analytical conditions of peptide mapping analysis on LCMS-9030 (Q-TOF)

Column	: Shim-pack GISS C18, 5 µm, 250 × 4.6 mm
Mobile phase	: (A) 0.1% formic acid in water (B) 0.1% formic in acetonitrile
Flow rate	: 0.3 mL/min
Gradient program	: B Conc. 0% (0 min) → 5% (0 min) → 50% (85 min) → 75% (95 – 100 min) → 0% (103 min) → 0% (115 min)
Column temp.	: 40°C
Injection volume	: 20 µL
Interface	: Heated ESI (positive mode)
MS Mode	: MS scan
TOF mass range	: 250 – 2500 (m/z)
Heat block temp.	: 400°C
DL temp.	: 250°C
Interface temp.	: 300°C
Nebulizing gas	: N2, 3 L/min
Drying gas	: N2, 10 L/min
Heating gas	: Zero air, 10L/min

3. Results and discussion

3.1 Disulfide bond linked and C-terminal peptides

The structure of IgG1 monoclonal antibody (mAb) is shown in **Figure 1**. A monoclonal antibody consists of 8 disulfide bond linked peptides: 2 in LC, 4 in HC, 1 inter LC-HC chain and 1 in hinge region. A pair of disulfide bonds in the hinge region is identical and thus considered as a single peptide sequence. By employing reducing agent (DTT), 15 cysteine-containing tryptic digest peptides could be produced from the structure. The C-terminal peptide from light chain, containing a cysteine (SFNRGEC), is also a disulfide bond linked peptide. The C-terminal peptide of HC (SLSLSPGK) is subjected to lysine truncation related to the production schemes [1]. Therefore, both sequences with and without

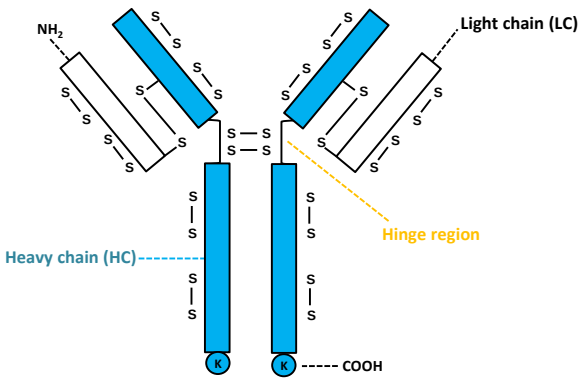


Figure 1. Schematic structure of IgG1 monoclonal antibody (mAb) and distribution of di-sulphide-bond linkages of inter- and intra-chain.

lysine are required to confirm with knowing their relative abundances in characterization of mAb biosimilar.

3.2 Detection of C-terminal and disulfide bond peptides

The bevacizumab sample was prepared with addition of reducing agent (DTT) before trypsin digestion. **Figure 2(A)** shows the TIC of the sample acquired on LCMS-9030. Fifteen cysteine-containing peptides and three C-terminal peptides were detected. A quick detection method of these peptide was set up based on extract ion chromatograms (EICs) of accurate masses (<2 ppm). The results are shown in **Figure 2(B)** and **Table 2**.

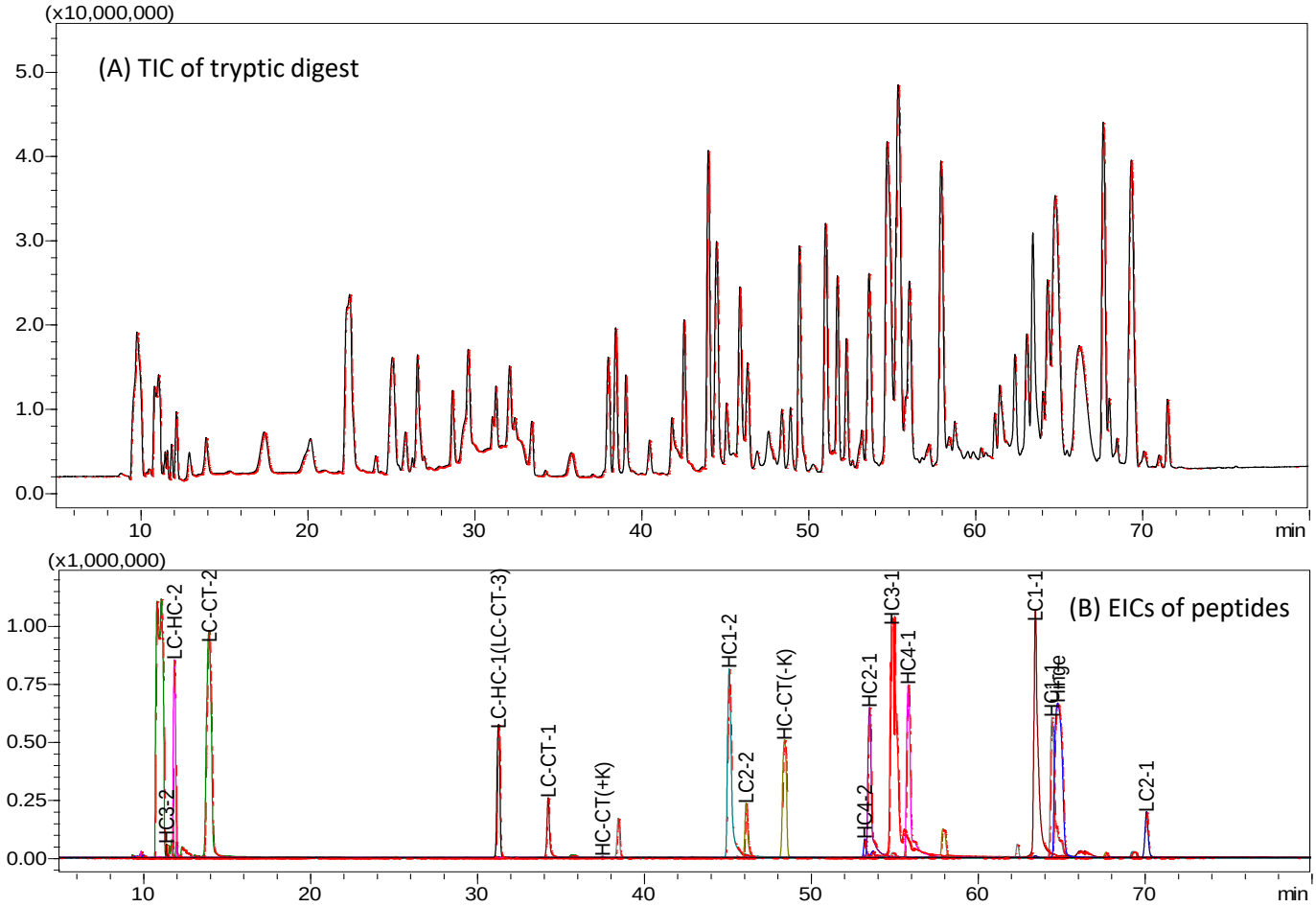


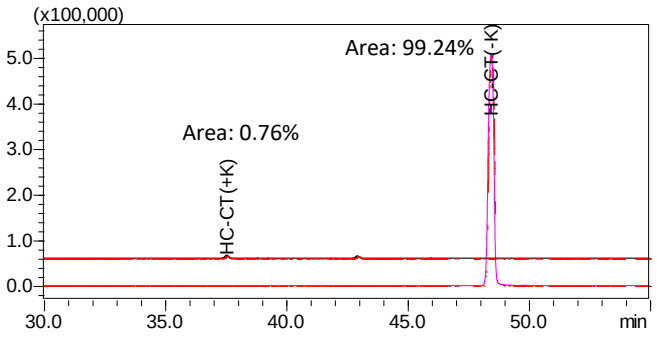
Figure 2. TIC of tryptic digest of bevacizumab biosimilar (A) and diagnosis ions (EICs) for C-terminal and cysteine-containing peptides (B)

Table 2: Detection and confirmation of C-terminal and disulfide-bond linked peptides of bevacizumab biosimilar

Type & Location		Sequence [1]	RT (min)	m/z, Theor	m/z, Mea.	Charge (+z)	Area %
Inter-chain di-S peptide	LC-HC-1 [2]	K.SFNRGEC.- [207, 213] (missed 1)	34.29	869.3570	869.3579	1	4.8
	LC-HC-2	K.SCDK.T [224, 227] (HC)	11.90	509.2024	509.2028	1	5.5
	Hinge	K.THTCPPCPAPELLGGPSVFLFPPKPK.D [228, 253]	64.83	948.8240	948.8255	3	14.0
Intra-chain di-S peptide	LC1-1	R.VTITCSASQDISNYLNWYQQKPGK.A [18, 41]	63.49	915.4498	915.4495	3	12.7
	LC1-2	R.FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK.V [61, 102]	75.33	1151.5285	1151.5271	4	0.02
	LC2-1	K.SGTASVVCLLNNFYPR.E [126, 141]	70.14	870.9405	870.9405	2	1.8
	LC2-2	K.VYACEVTHQGLSSPVTK.S [190, 206]	46.15	606.9733	606.9732	3	1.9
	HC1-1	R.LSCAASGYTFTNYGMNWVR.Q [19, 37]	64.47	714.3242	714.3249	3	6.3
	HC1-2	R.AEDTAVYYCAK.Y [87, 97]	45.14	617.2764	617.2765	2	9.3
	HC2-1	K.STSGGTAALGCLVK.D [139, 152]	53.55	632.8319	632.8331	2	7.0
	HC2-2	K.DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTK.V [153, 215]	71.17	1343.4687	1343.4664	5	0.23
	HC3-1	R.TPEVTCVVVDVSHEDPEVK.F [261, 279]	54.89	694.6735	694.6739	3	5.8
	HC3-2	K.CK.V [326, 327]	11.43	250.1220	250.1221	1	0.15
	HC4-1	K.NQVSLTCLVK.G [366, 375]	55.87	552.8077	552.8079	2	9.1
	HC4-2	R.WQQGNVFSCSVMHEALHNHYTQK.S [422, 444]	53.26	915.4201	915.4194	3	0.60
C-terminal peptide	LC-CT2	K.SFNRGEC.- [207, 213] (missed 1)	31.32	406.6715	406.6714	2	1.9
	LC-CT4	R.GEC.- [211, 213]	13.98	365.1125	365.1128	1	13.4
	HC-CT+K	K.SLSLSPGK.- [445, 452]	37.55	394.7293	394.7292	2	0.04
	HC-CT-K	SLSLSPG	48.46	330.6818	330.6824	2	5.5

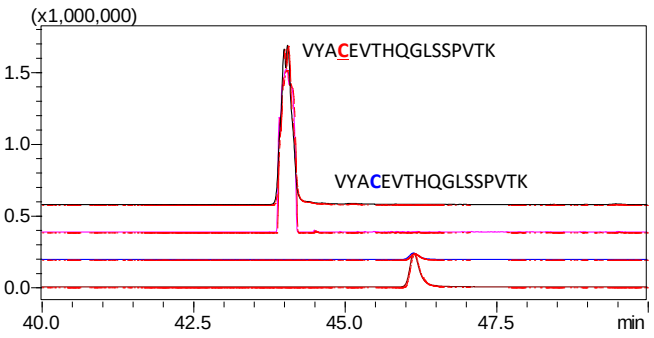
[1] C represents cysteine with carbamidomethyl modification; [2] Also C-terminal peptide (LC-CT1)

C-terminal lysine variants of the HC were observed as shown in **Figure 3,** with both peptides being confirmed by the accurate masses and charge status. The peak areas of EICs indicate the dominance of the clipped lysine (-K) sequence in the sample [1]. **Figure 4** shows a common phenomenon of peptides – carbamidomethyl modification. Both sequences without and with the modification were found and confirmed firmly by accurate masses and charge numbers of the peptides. Moreover, LC1-2 and HC2-2 are the largest cysteine-containing peptides. As shown in **Figure 5**, detection and conformation of their presences are highly reliable due to the accurate masses and charge statues measured on LCMS-9030 Q-TOF. The EICs based method is an easy and reliable approach in detection and confirmation of interested peptides in peptide mapping of mAb.



Location	Sequence	RT (min)	m/z (+z), mea.		Area %
HC-CT(+K)	SLSLSPG**K**	37.55	788.4512+	394.7293++	0.76
HC-CT(-K)	SLSLSPG	48.46	660.3563+	330.6818++	99.24

Figure 3. Heavy chain C-terminal peptide with and without lysine and their relative abundances based on peak areas



Location	Sequence	RT (min)	m/z (+z), meas.	
LC2-2	VYA**C**EVTHQGLSSPVTK	44.06	938.4646(+2)	625.9842(+3)
	VYACEVTHQGLSSPVTK	46.17	909.9558(+2)	606.9732(+3)

Figure 4. Quick Confirmation of di-sulfide bond linked peptide of LC2-2 with and without carbamidomethyl modification

3.3 MS/MS Sequencing of C-terminal and cysteine-containing peptides

Figure 6 shows an example of de novo sequencing of the main C-terminal peptide (SLSLSPG) with clipped lysine through MS/MS fragmentation and ion annotation. There are various de novo sequencing software available for quick and easy sequencing analysis [2,3].

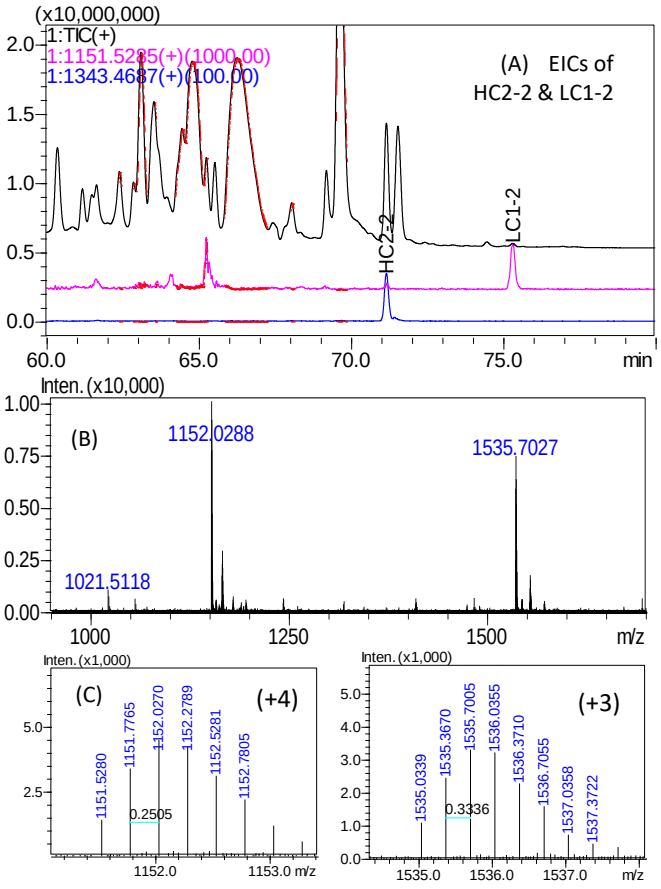


Figure 5. Confirmation of peptide LC-1-2 by EIC (A), accuracy (< 2ppm) and charge status of two ions (B, C).

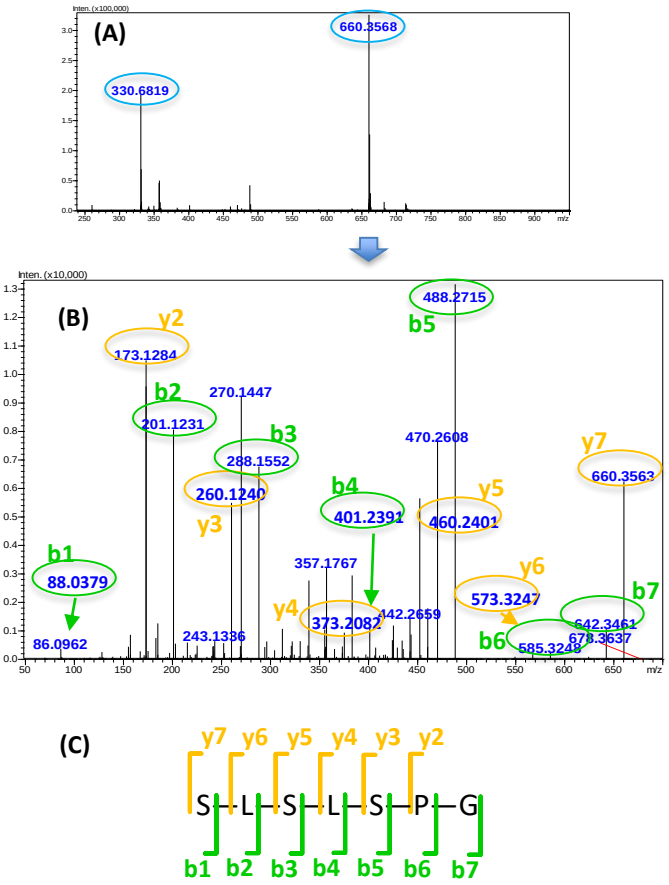


Figure 6. MS detection, MS/MS fragmentation of 660.3568+ ion and de novo sequencing of C-terminal peptide (SLSLSPG) of HC.

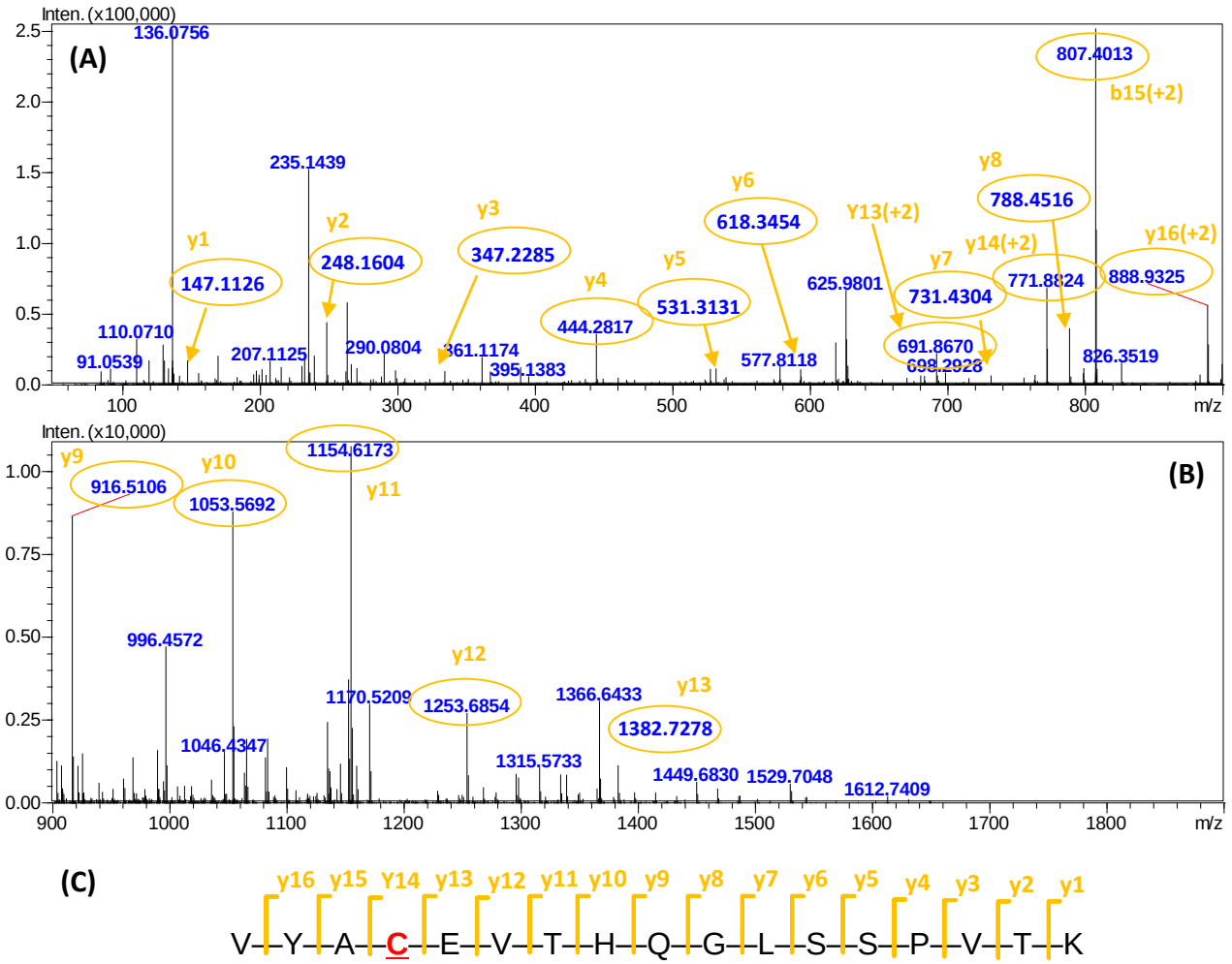


Figure 7. MS/MS spectra of peptide LC2-2 (VYACEVTHQGLSSPVTK, m/z 938.4677++) for m/z range: 50-900 (A) and 901-1900 (B), and annotation of all y ions (y1-y16).

Another representative example of de novo peptide sequencing is displayed in **Figure 7**. The results highlight MS/MS fragmentation of a cysteine-containing peptide LC2-2 in the light chain (VYACEVTHQGLSSPVTK). As illustrated in **Figure 4**, both modified (carbamidomethyl) and unmodified 'VYACEVTHQGLSSPVTK' ions were found in the sample at different retentions. The former ion had higher signal and was selected for MS/MS analysis. The MS/MS spectra of 938.4677(+2) ion are displayed**,** and annotation of the fragment ions covering b and y-ions from amino acid residues of 1 to 16 (b-ions are not shown) are carried out successfully.

4. Conclusions

Mapping and sequencing of C-terminal and disulfide bond linked peptides are the essential analyses, which are among the critical quality attributes (CQA) to be characterized in biosimilar production. In this study, all the three C-terminal peptides and fifteen cysteine-containing peptides released from disulfide bond linked sequences of bevacizumab biosimilar were successfully detected, identified and sequenced on LCMS-9030 Q-TOF. An easy and reliable approach based on the EICs of accurate mass (<2ppm) and charge status was set up and used in quick peptide identification and mapping. This report serves as continuation of application news series [4] and confirms LCMS-9030 applicability for biosimilar characterization.

References

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