SHIMADZU
Excellence in Science

Application News

No. AD-0212B

Biopharma / LCMS-9030 (Q-TOF)

Peptide Mapping of Monoclonal Antibody (mAb) Using LCMS™-9030 (Q-TOF) Mass Spectrometer with a Shim-pack™ GISS-HP Column

Yonghai Lu and Zhaoqi Zhan
Application Development & Support Centre, Shimadzu (Asia Pacific), Singapore

❑ Introduction

Monoclonal antibody (mAb)-based biotherapeutics are emerging as one of the fastest-growing categories of biologic drugs being developed today. Peptide mapping is a key analytical method for quality assurance of mAb products. It is employed for the elucidation of primary structure of mAb biosimilars. In the previous application news AD-0176 [1], we had developed a mAb peptide mapping workflow by using both Nexera™ Bio UHPLC and LCMS-9030 (Q-TOF) systems, yet the method is time consuming, taking 2 hours for each analysis. In the present report, we aim to optimize the workflow to reduce the running time and maintain the 100% peptide sequence coverage on Shimadzu LCMS-9030 (Q-TOF) mass spectrometer.

❑ Experimental

A 5 mg/mL of bevacizumab biosimilar sample solution was prepared in 50 mmol/L Tris-HCl (pH 8.0) buffer. A 20 µL aliquot of the sample was diluted with 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 60ºC for 60 minutes to denature and reduce disulfide bonds. The alkylation was done by adding 30 µL iodoacetamide (IAM, 0.2 M) followed by incubation at 37ºC for 60 minutes in the dark. The sample were diluted with 328 µL ABC solution (50 mM) before trypsin digestion. The sequencing grade modified trypsin was used for protein digestion at 37ºC for overnight. Stop the trypsin activity by adding 2 µL trifluoroacetic acid (TFA) to reduce the pH <4.0. The obtained sample was centrifuged and the supernatant was collected and injected to LCMS-9030 (Q-TOF) for peptide mapping. The analytical conditions are displayed in **Table 1**.

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

Column	: Shim-pack GISS-HP, 3 µm, 150 × 3.0 mm
Mobile phase	: (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

Table 2. Injection-to-injection repeatability of RT and TIC peak area of peptides from bevacizumab biosimilar (n=6)

Peak #	RT (min)	RSD (%)	Peak area	RSD (%)
Peak 1	3.76	0.08	1.30E+08	1.43
Peak 2	8.81	0.04	1.12E+07	2.89
Peak 3	11.24	0.03	2.86E+07	2.15
Peak 4	15.68	0.03	2.73E+07	2.12
Peak 5	20.93	0.04	3.22E+07	1.60
Peak 6	24.17	0.03	2.04E+07	2.69

❑ Results and Discussion

A. Optimization of analytical conditions

To reduce the running time of peptide mapping, Nexera X2 UHPLC combined with a Shim-pack GISS-HP C18 column with 3 µm particle size was used. The mobile phases and gradients were modified as well (**Table 1)**. With the optimization, the running time was decreased from 120 minutes to 45 minutes.

B. Repeatability of LCMS-9030 (Q-TOF) system

To provide reliable data for peptide mapping, errors from the analytical system must be minimized. Injection-to-injection repeatability of LCMS-9030 was evaluated. Overlay of the total ion chromatograms of six (TIC) replicates shows an excellent reproducibility (**Figure 1**). Six peaks (1-6) were selected to calculate variations of retention time (RT) and peak area. The results demonstrated a high repeatability of the system with %RSD <0.1 for RT and <3 for peak area (**Table 2**).

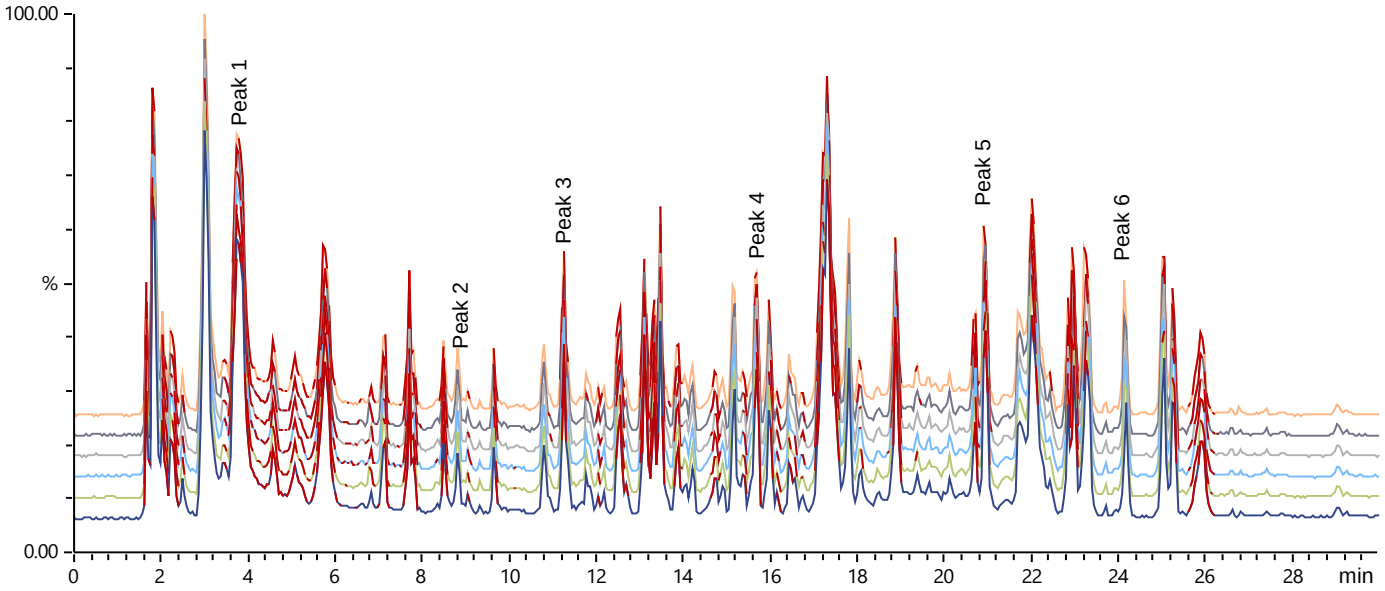

Figure 1. Overlay of total ion chromatograms (TIC, m/z100~2000) of six replicates of bevacizumab biosimilar tryptic digest on LCMS-9030

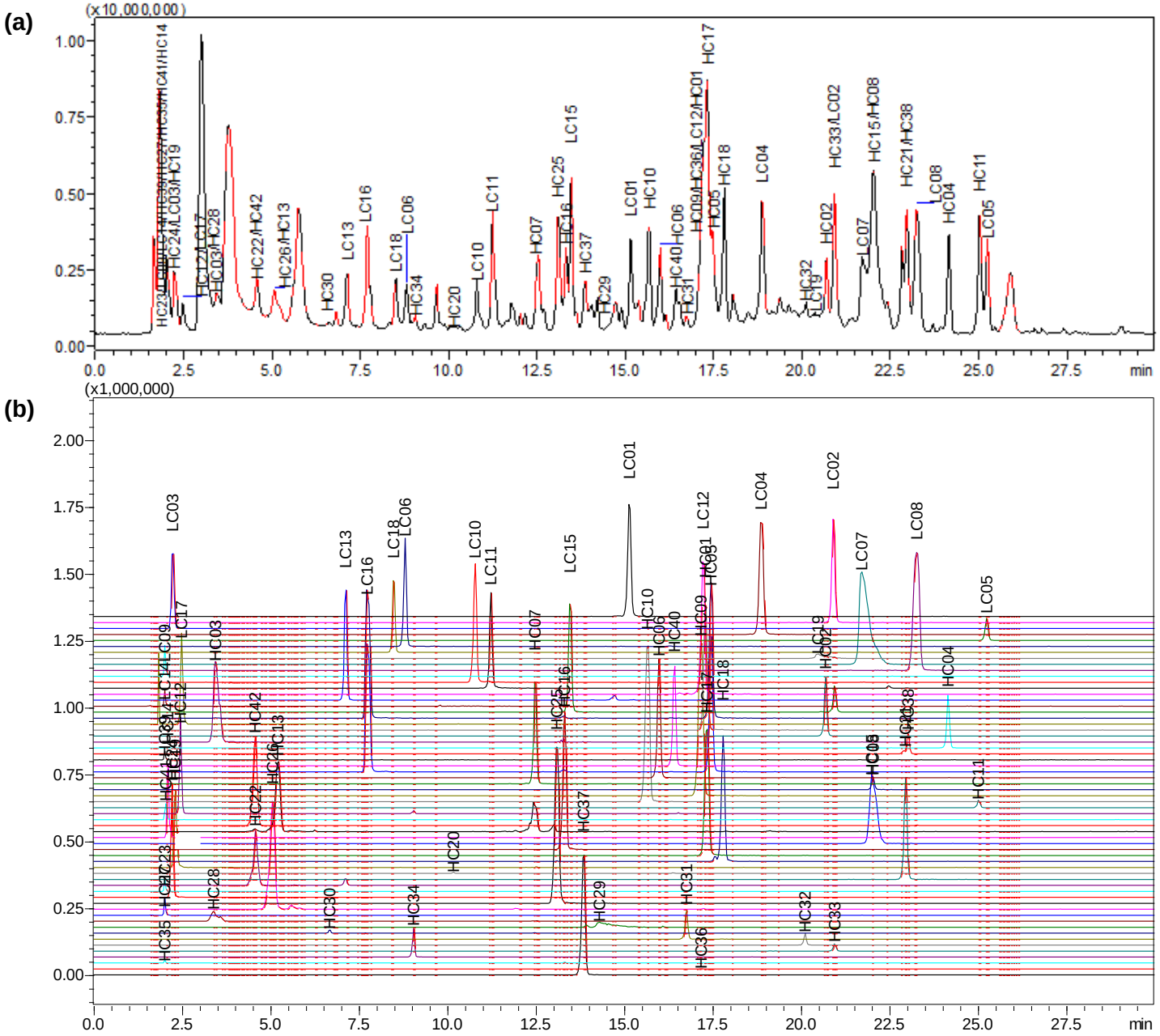

Figure 2. Tryptic peptide profiles of bevacizumab biosimilar on LCMS-9030 (Q-TOF). (a) Total ion chromatogram (m/z100~2000). (b) Extracted ion chromatograms of peptides. The peak # refers to Tables 3 & 4

Table 3. Full sequence confirmation of bevacizumab biosimilar light chain by accurate mass matching of tryptic peptides on LCMS-9030 (Cys→camCys)

Peak No.	RT (min)	Peptide [AA numbers]	Peptide m/z	Adduct Ion
LC01	15.15	-.DIQMTQSPSSLSASVGDR.V [1, 18]	939.9461	[M+2H]2+
LC02	20.92	R.VTIT**C**SASQDISNYLNWYQQKPGK.A [19, 42]	934.4559	[M+3H]3+
LC03	2.24	K.APK.V [43, 45]	315.2025	[M+H]+
LC04	18.88	K.VLIYFTSSLHSGVPSR.F [46, 61]	881.9774	[M+2H]2+
LC05	25.25	R.FSGSGSGTDFTLTISSLQPEDFATYY**C**QQYSTVPWTFGQGTK.V [62, 103]	1554.0406	[M+3H]3+
LC06	8.81	K.VEIK.R [104, 107]	488.3074	[M+H]+
LC18	8.49	K.VEIKR.T [104, 108] (missed 1)	644.4083	[M+H]+
LC19	20.49	K.RTVAAPSVFIFPPSDEQLK.S [108, 126] (missed 1)	1051.5659	[M+2H]2+
LC07	21.72	R.TVAAPSVFIFPPSDEQLK.S [109, 126]	973.5162	[M+2H]2+
LC08	23.28	K.SGTASVV**C**LLNNFYPR.E [127, 142]	899.4511	[M+2H]2+
LC09	2.02	R.EAK.V [143, 145]	347.1917	[M+H]+
LC10	10.79	K.VQWK.V [146, 149]	560.3188	[M+H]+
LC11	11.24	K.VDNALQSGNSQESVTEQDSK.D [150, 169]	1068.4873	[M+2H]2+
LC12	17.24	K.DSTYSLSSTLTLSK.A [170, 183]	751.8828	[M+2H]2+
LC13	7.13	K.ADYEK.H [184, 188]	625.2823	[M+H]+
LC14	2.02	K.HK.V [189, 190]	284.1709	[M+H]+
LC15	13.47	K.VYA**C**EVTHQGLSSPVTK.S [191, 207]	938.4662	[M+2H]2+
LC16	7.73	K.SFNR.G [208, 211]	523.2622	[M+H]+
LC17	2.50	R.GE**C**.- [212, 214]	365.1117	[M+H]+

Table 4. Full sequence confirmation of bevacizumab biosimilar heavy chain by accurate mass matching of tryptic peptides on LCMS-9030 (Cys→camCys)

Peak No.	RT (min)	Peptide [AA numbers]	Peptide m/z	Adduct Ion
HC01	17.30	-.EVQLVESGGGLVQPGGSLR.L [1, 19]	941.5049	[M+2H]2+
HC02	20.70	R.LS**C**AASGYTFTNYGMNWVR.Q [20, 38]	1099.4920	[M+2H]2+
HC03	3.45	R.QAPGK.G [39, 43]	500.2819	[M+H]+
HC04	24.16	K.GLEWVGWINTYTGEPTYAADFK.R [44, 65]	840.0673	[M+3H]3+
HC38	23.03	K.GLEWVGWINTYTGEPTYAADFKR.R [44, 66] (missed 1)	892.1007	[M+3H]3+
HC39	2.02	K.RR.F [66, 67] (missed 1)	331.2192	[M+H]+
HC40	16.42	R.RFTFSLDTSK.S [67, 76] (missed 1)	601.3134	[M+2H]2+
HC05	17.46	R.FTFSLDTSK.S [68, 76]	523.2636	[M+2H]2+
HC06	15.99	K.STAYLQMNSLR.A [77, 87]	642.3237	[M+2H]2+
HC07	12.49	R.AEDTAVYY**C**AK.Y [88, 98]	645.7864	[M+2H]2+
HC08	22.04	K.YPHYYGSSHWYFDVWGQGTLVTVSSASTK.G [99, 127]	1108.5177	[M+3H]3+
HC09	17.18	K.GPSVFPLAPSSK.S [128, 139]	593.8275	[M+2H]2+
HC10	15.67	K.STSGGTAALG**C**LVK.D [140, 153]	661.3435	[M+2H]2+
HC11	25.02	K.DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI**C**NVNHKPSNTK.V [154, 216]	1679.0808	[M+4H]4+
HC12	2.46	K.VDK.K [217, 219]	361.2075	[M+H]+
HC41	2.05	K.VDKK.V [217, 220] (missed 1)	489.3020	[M+H]+
HC42	4.57	K.KVEPK.S [220, 224] (missed 1)	600.3708	[M+H]+
HC13	5.22	K.VEPK.S [221, 224]	472.2758	[M+H]+
HC14	2.11	K.S**C**DK.T [225, 228]	509.2017	[M+H]+
HC15	22.01	K.THT**C**PPCPAPELLGGPSVFLFPPKPK.D [229, 254]	948.8226	[M+3H]3+
HC16	13.32	K.DTLMISR.T [255, 261]	418.2207	[M+2H]2+
HC17	17.34	R.TPEVT**C**VVVDVSHEDPEVK.F [262, 280]	713.6805	[M+3H]3+
HC18	17.80	K.FNWYVDGVEVHNAK.T [281, 294]	839.4042	[M+2H]2+
HC19	2.31	K.TKPR.E [295, 298]	501.3136	[M+H]+
HC20	10.18	R.EEQYNSTYR.V [299, 307]	595.2581	[M+2H]2+
HC21	22.96	R.VVSVLTVLHQDWLNGK.E [308, 323]	904.5058	[M+2H]2+
HC22	4.58	K.EYK.C [324, 326]	439.2177	[M+H]+
HC23	2.02	K.**C**K.V [327, 328]	307.1427	[M+H]+
HC24	2.22	K.VSNK.A [329, 332]	447.2557	[M+H]+
HC25	13.10	K.ALPAPIEK.T [333, 340]	419.7559	[M+2H]2+
HC26	5.06	K.TISK.A [341, 344]	448.2763	[M+H]+
HC27	2.02	K.AK.G [345, 346]	218.1493	[M+H]+
HC28	3.40	K.GQPR.E [347, 350]	457.2508	[M+2H]2+
HC29	14.31	R.EPQVYTLPPSR.E [351, 361]	643.8391	[M+2H]2+
HC30	6.68	R.EEMTK.N [362, 366]	637.2850	[M+H]+
HC31	16.77	K.NQVSLT**C**LVK.G [367, 376]	581.3173	[M+2H]2+
HC32	20.12	K.GFYPSDIAVEWESNGQPENNYK.T [377, 398]	848.7138	[M+3H]3+
HC33	20.95	K.TTPPVLDSDGSFFLYSK.L [399, 415]	625.3106	[M+3H]3+
HC34	9.05	K.LTVDK.S [416, 420]	575.3390	[M+H]+
HC35	2.02	K.SR.W [421, 422]	262.1503	[M+H]+
HC36	17.18	R.WQQGNVFS**C**SVMHEALHNHYTQK.S [423, 445]	934.4272	[M+3H]3+
HC37	13.86	K.SLSLSPG.- [446, 452]	660.3559	[M+H]+

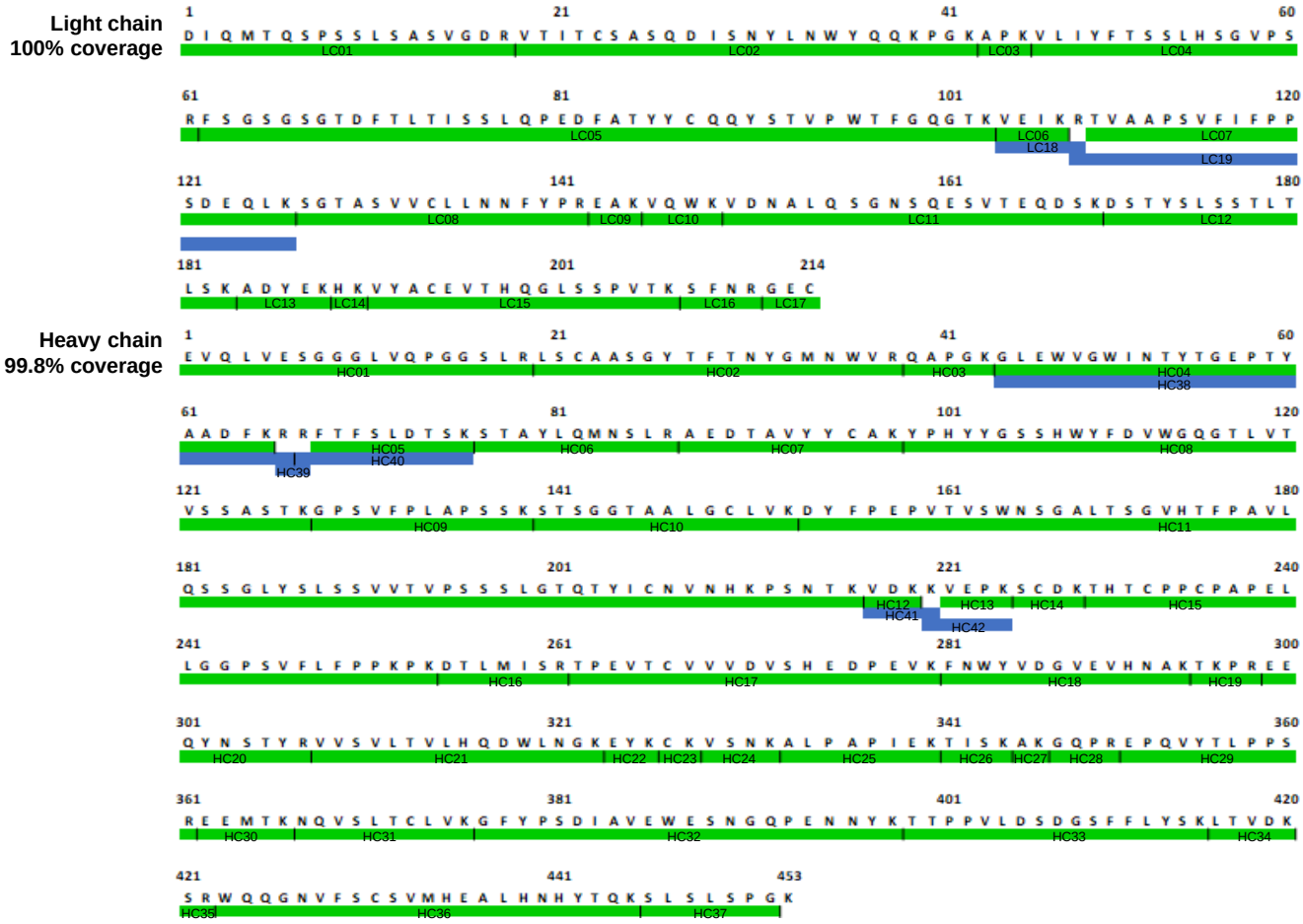

Figure 3. Sequence coverage view: green color represents 0 missing cleavage peptides; blue color represents 1 missing cleavage peptides. The peptide # refers to the peak # in Tables 3 & 4

C. Characterization of peptides using LCMS-9030

In total, we characterized 19 tryptic peptides from light chain (LC01-19) of bevacizumab biosimilar and 42 peptides from heavy chain (HC01-42). As shown in **Figure 2**, all of the 61 peptides were eluted out in 30 minutes. **Tables 3** and **4** show the accurate mass data of measured peptides. In comparison with theoretical masses of bevacizumab tryptic peptides using Skyline s/w [2], all the peptides of bevacizumab biosimilar were measured with <3 ppm mass error. A peptide map with 100% sequence coverage is shown in the **Figure 3**. Notably, peptides with two consecutive enzyme target sites (including KR, RR, and KK) were measured with 1 missing cleavage type, such as LC18, LC19, HC38, HC39, HC40, HC41, and HC42. The C-terminal peptide (SLSLSPGK) of heavy chain was detected as SLSLSPG (HC37) due to the occurrence of lysine truncation, resulting in 99.8% true coverage of heavy chain.

❑ Conclusions

Nexera UHPLC combined with Shim-pack GISS-HP C18 column of 3 µm particle size is proven to be robust and reliable for peptide mapping of mAb. Moverover, peptide analysis on LCMS-9030 (Q-TOF) mass spectrometer provides an in-depth understanding of primary structure of mAb products with 100% peptide sequence coverage. In summary, the demonstrated performance of LCMS-9030 system signifies the advantages of accurate mass in peptide sequence characterization of mAb.

❑ Reference

1. Shimadzu (Asia Pacific), “Peptide Mapping of Monoclonal Antibody (mAb) Using Nexera Bio with Q-TOF Mass Spectrometer for Full Sequence Confirmation”, Application News, No. AD-0176, 2018
2. https://skyline.ms/wiki/home/software/Skyline/page.view?name=default

❑ Acknowledgement

The authors sincerely thank Dr Atsuhiko Toyama from Marketing Innovation Centre, Shimadzu and Tadayuki Yamaguchi from global marketing department at Shimadzu Corporation for their valuable suggestions and discussion during this work.

SHIMADZU (Asia Pacific) Pte. Ltd
79 Science Park Drive, #02-01/08 Cintech IV, Singapore 118264,
www.shimadzu.com.sg; Tel: +65-6778 6280 Fax: +65-6778 2050