SHIMADZU
Excellence in Science

Application News

No. AD-0191

Biopharma / Nexera Bio UHPLC / LCMS-9030

N-glycan Profiling of monoclonal Antibody (mAb) on Nexera Bio UHPLC Coupled with Fluorescence Detector and Q-TOF Mass Spectrometer

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

1. Introduction

N-linked glycosylation on Asn residue with consensus sequence Asn-X-Ser/Thr (where X is any amino acid except Pro) plays a critical role in stability, bioactivity, and immunogenicity of monoclonal antibodies (mAbs). The N-glycan moieties of therapeutic mAbs, especially biosimilar products, must be adequately and routinely characterized to ensure product quality. In this report, we established a robust, sensitive, and reproducible analytical platform that contains a Nexera Bio UHPLC system, a fluorescence detector (RF-20A), and a Q-TOF mass spectrometer (LCMS-9030) for N-glycan profiling of bevacizumab biosimilar. N-glycans were released from bevacizumab biosimilar with PNGase F, labeled with 2-aminobenzamide (2-AB), and subsequently detected via RF-20A and LCMS-9030. LCMS-9030 was applied for peak assignment using an accurate mass of corresponding N-glycans, while peak areas from RF-20A were used for N-glycan quantitation.

2. Experimental

Protein Solubilization: 1 mg/mL of bevacizumab biosimilar solution was prepared in Tris buffer. A 100 µL aliquot was loaded into a 10 kDa molecular weight cut-off (MWCO) to remove salts of the sample buffer. The recovered sample (~20 µL) was diluted with 25 mM ammonium bicarbonate solution to 100 µL.

Reduction and Alkylation: 2 µL dithiothreitol (DTT, 1M) solution was added to reduce disulfide bonds. The sample was incubated at room temperature for 60 min. 4 µL iodoacetamide (IAA, 1M) solution was added for alkylation, and incubated at room temperature in dark for 60 min.

Deglycosylation: 2 µL PNFase F (1000U) was added to release N-glycans from bevacizumab biosimilar, and incubated at 37 ºC for overnight.

Extraction of N-glycans: The N-glycans were extracted using LudgerClean™ EB10 cartridge by eluting with 4 × 200 µL of 50% acetonitrile with 0.1% trifluoroacetic acid. For details see the LudgerClean™ EB10 cleanup protocol [1]. The obtained sample was dried down by a centrifugal evaporator and reconstituted in 50 µL of acetonitrile.

2-AB Labeling : 10 µL 2-AB/acetic acid/ DMSO/sodium cyanoborohydrate mixture with defined composition was used for labeling [2].

Purification of 2-AB Labeled N-glycans: LudgerClean™ S cartridge was applied to remove the excess labeling reagent. For details see the LudgerClean™ S cleanup protocol [3]. The obtained sample was dried down by a freeze dryer and reconstituted in 50 µL of 50% acetonitrile for LC/Fluorescence/MS analysis (**Table 1**).

Table 1. LC/Fluorescence/MS conditions

LC conditions	
LC system:	Shimadzu Nexera Bio UHPLC
Column:	HALO®Glycan, 2.7 µm, 150 × 2.1 mm
Column temperature:	60 ºC
Flow rate:	0.4 mL/min
Mobile phase A:	50 mM ammonium formate
Mobile phase B:	Acetonitrile
Gradient program:	0 min, 78% B, 50min, 55% B, 51 min, 20% B, 56 min, 20% B, 57 min, 78% B.
Injection volume:	5 µL
Fluorescence conditions	
Fluorescence detector:	Shimadzu RF-20A
Excitation:	330 mm
Emission:	420 mm
Data rate:	1 pts/s
Gain:	1
MS conditions	
MS system:	Shimadzu LCMS-9030 (QTOF)
Interface:	Heated ESI (+)
Interface voltage:	4 kV
Interface temperature:	300 ºC
Nebulizing gas:	N2, 3 L/min
Heating gas flow:	Zero air, 10L/min
DL temperature:	250 ºC
Drying gas flow:	N2, 10 L/min
Heat block temperature:	400 ºC
MS mode:	MS scan
Mass range:	500 - 2500 m/z
MS mode:	MS/MS scan
Collision Energies:	50 ± 17 V
Mass range:	100 - 2500 m/z

3. Results and discussion

3.1 UHPLC/RF injection-to-injection reproducibility

The main purpose of UHPLC/RF analysis is to relatively quantify N-glycans. Injection-to-injection variability of UHPLC/RF system was evaluated as shown in **Figure 1**. Variations in peak area (**Table 2**) and retention time (**Table 3**) of three injections of the sample were less than 2% RSD for all peaks.

3.2 Characterization of N-glycans using LCMS-9030

In total, we characterized nine 2-AB labeled N-glycans from bevacizumab biosimilar, including Man3, G0F-2GN, G0-GN, G0F-GN, G0, Man5, G0F, G1Fa, and G1Fb (**Figure 2**). Proposed structures for the 2-AB labeled N-glycans are shown in **Figure 3**. **Table 4** shows accurate mass data of LCMS-9030. MS/MS spectra of N-glycans are shown in **Figure 4**. Accurate mass combined with MS2 patterns provide high confidence in identification of N-glycans.

3.3 Relative quantitation of N-glycans

Figure 5 shows the relative abundance of N-glycans of bevacizumab biosimilar. As a result, G0F was found to be the most abundant N-glycan that make up 87.23% of the total N-glycans from bevacizumab biosimilar.

Table 2. Injection-to-injection repeatability of peak area (n = 3) of N-glycans from bevacizumab biosimilar

Peak #	Peak area	Std. Dev.	RSD (%)
Peak 1	18049	187	1.033
Peak 2	87783	993	1.131
Peak 3	101112	1082	1.070
Peak 4	505621	5588	1.105
Peak 5	308057	4351	1.412
Peak 6	559385	8982	1.606
Peak 7	23212699	260200	1.121
Peak 8	1117630	17295	1.547
Peak 9	701334	5500	0.784

Table 3. Injection-to-injection repeatability of retention time (n = 3) of N-glycans from bevacizumab biosimilar

Peak #	Average (min)	Std. Dev. (min)	RSD (%)
Peak 1	5.725	0.008	0.140
Peak 2	6.994	0.011	0.158
Peak 3	7.939	0.011	0.139
Peak 4	9.393	0.012	0.129
Peak 5	10.397	0.013	0.122
Peak 6	11.025	0.015	0.134
Peak 7	11.896	0.013	0.112
Peak 8	14.593	0.014	0.098
Peak 9	14.798	0.015	0.100

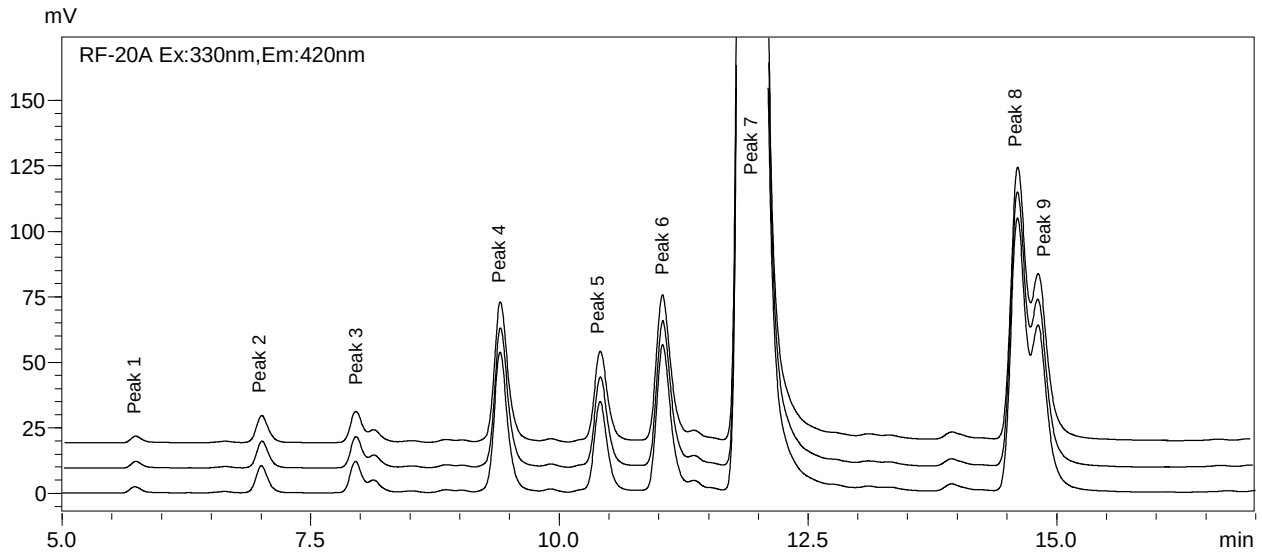

Figure 1. UHPLC-RF chromatograms of triplicate injections of 2-AB labelled N-glycans released from the same bevacizumab biosimilar product. It shows perfect alignment of chromatograms. The peak area and retention time variations were less than 2% RSD.

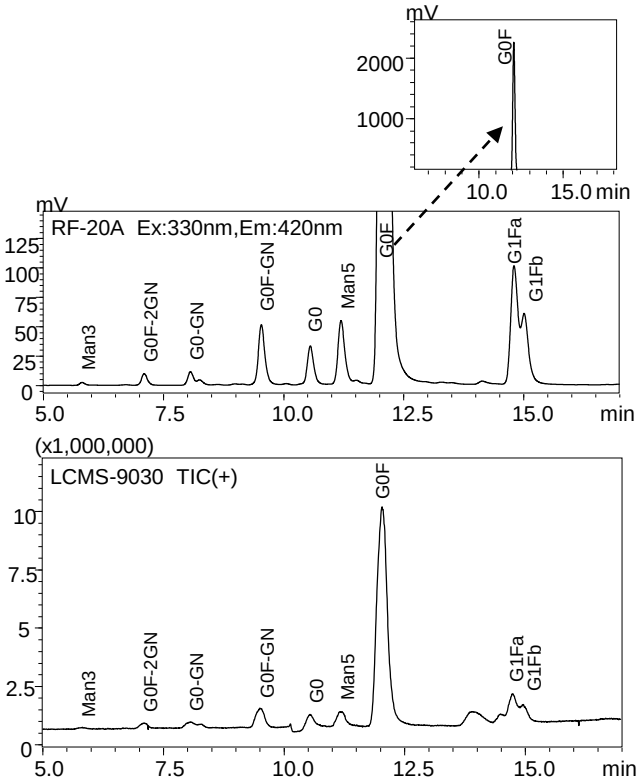

Figure 2. UHPLC/Fluorescence/MS analysis of 2-AB labeled N-glycans of bevacizumab biosimilar. Top chromatogram is fluorescence chromatogram; bottom is MS chromatogram.

4. Conclusions

✓ LCMS-9030 provides high sensitivity analysis of N-glycans with high accurate mass (< 2 ppm).

✓ The stability and repeatability of this analytical system is well (RSD < 2%).

In this report, we have demonstrated that Nexera Bio UHPLC coupled with RF-20A fluorescence detector and LCMS-9030 (Q-TOF) mass spectrometer comprise a robust and reliable system for N-glycan profiling and quantitation of bevacizumab biosimilar products. The results of injection-to-injection repeatability tests in peak area, retention time, and mass accuracy are satisfactory.

The demonstrated performance and features of both Nexera Bio UHPLC and LCMS-9030 (Q-TOF) signifies their high practicability for separation and assignment of N-glycans of antibody and biosimilar products and may become a tool of choice for biopharmaceutical mAb characterization.

Disclaimer: *The products and applications in this application news are intended for Research Use Only (RUO). Not for use in diagnostic procedures.*

Table 4. Mass accuracy of LCMS-9030 for N-glycan analysis

2-AB N-glycans	Accurate mass (m/z)	Exact mass (m/z)	Mass error (ppm)
Man3	1031.4033	1031.4038	-0.48
G0F-2GN	1177.4636	1177.4617	1.61
G0-GN	1234.4830	1234.4832	-0.16
G0F-GN	1380.5404	1380.5411	-0.51
G0	1437.5638	1437.5625	0.90
Man5	1355.5083	1355.5095	-0.89
G0F	1583.6195	1583.6205	-0.63
G1Fa	1745.6724	1745.6733	-0.52
G1Fb	1745.6724	1745.6733	-0.52

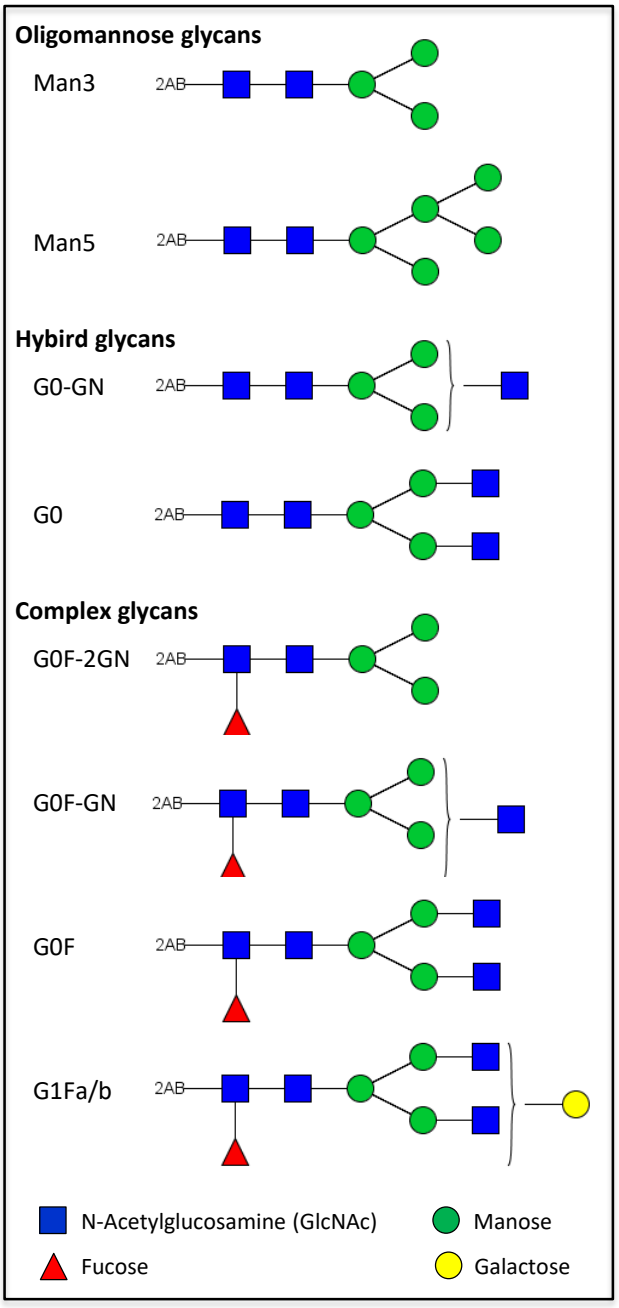

Figure 3. Proposed structures for 2-AB labeled N-glycans from bevacizumab biosimilar. GN = GlcNAc

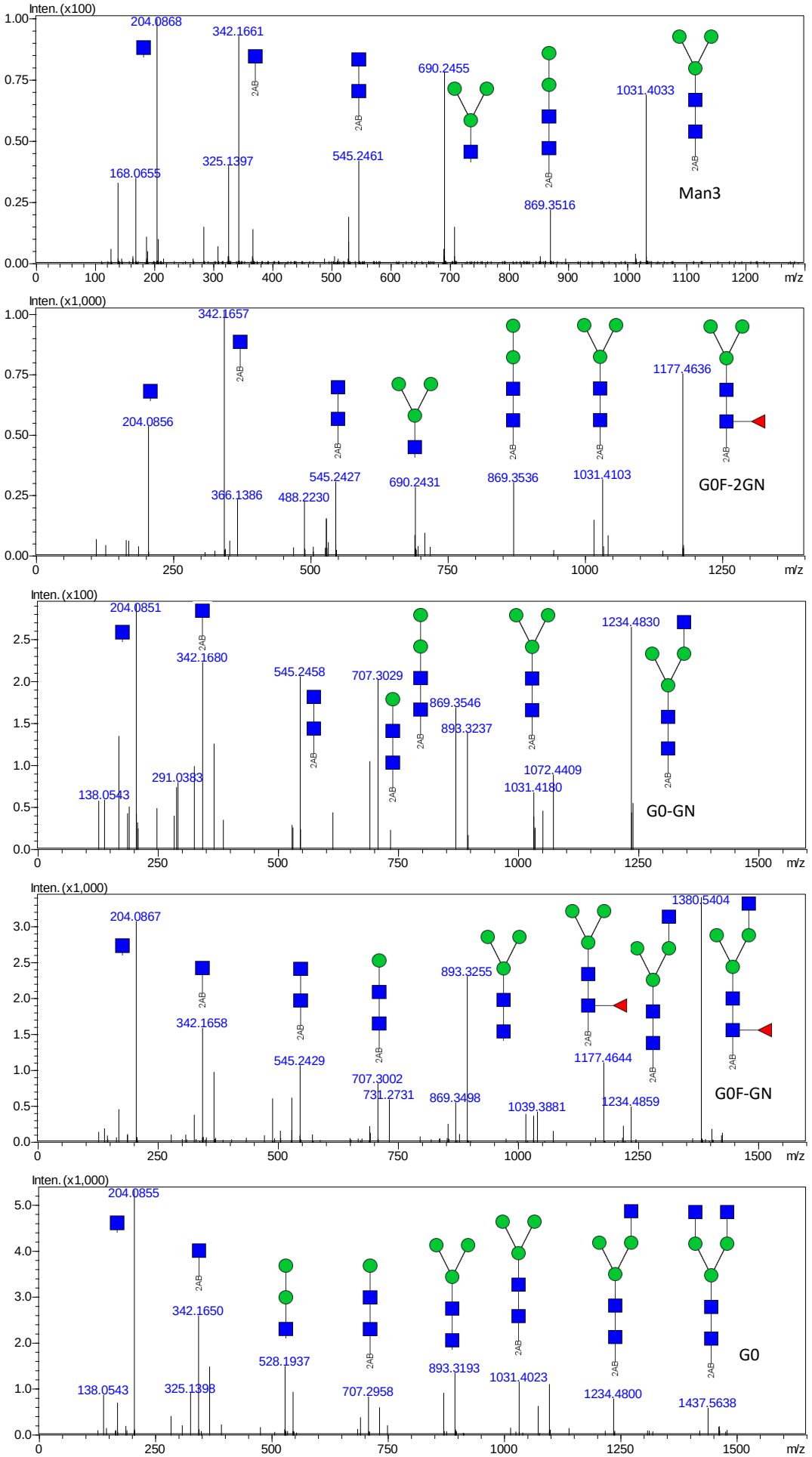

Figure 4. MS/MS spectra of 2-AB labeled N-glycans obtained by LCMS-9030 in positive ion mode.

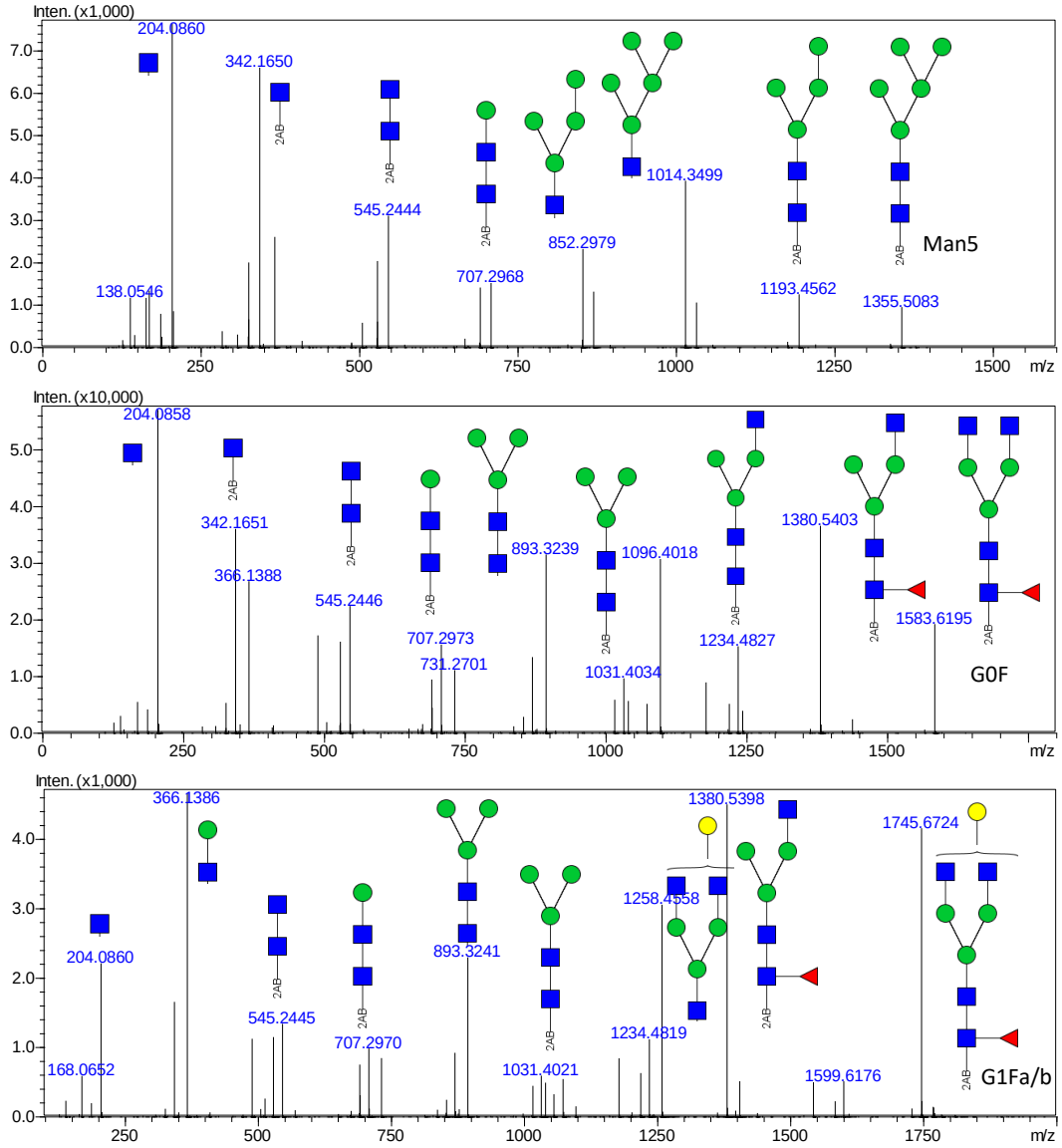

Figure 4. MS/MS spectra of 2-AB labeled N-glycans obtained by LCMS-9030 in positive ion mode (continued).

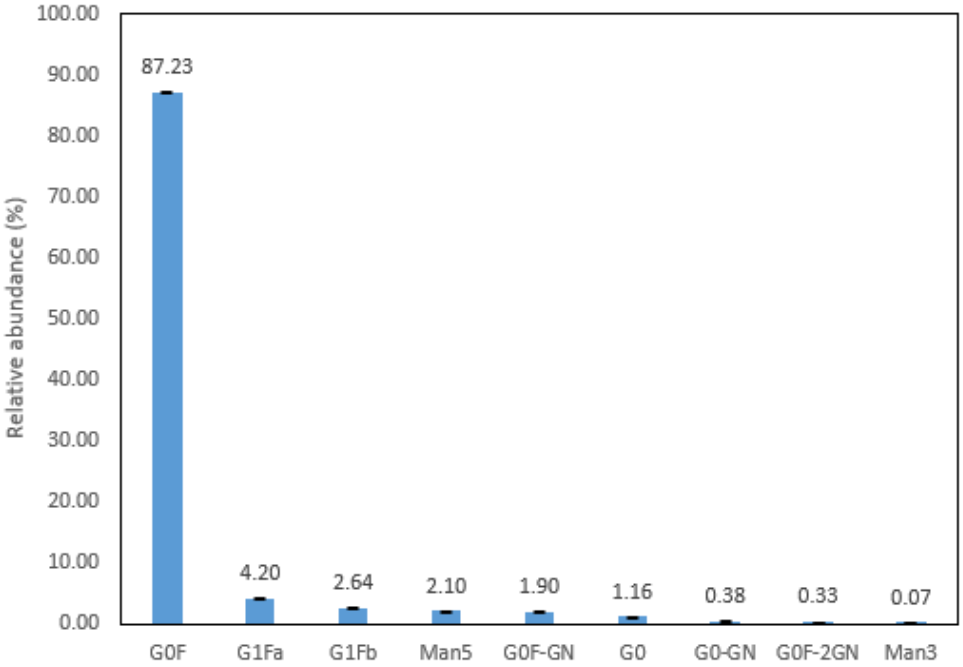

Figure 5. Relative abundance of 2-AB labeled N-glycans from bevacizumab biosimilar. Each relative abundance value has error bars based on triplicate analyses.

References

[1] https://www.ludger.com/docs/products/lc/eb/ludger-lc-eb10-ax-guide.pdf

[2] Keser T, Pavić T, Lauc G, Gornik O. Comparison of 2-Aminobenzamide, Procainamide and RapiFluor-MS as Derivatizing Agents for High-Throughput HILIC-UPLC-FLR-MS N-glycan Analysis. *Front Chem* 2018 6:324. .

[3] https://www.ludger.com/docs/products/lc/s/ludger-lc-s-ax-guide.pdf

SHIMADZU
Excellence in Science

***Disclaimer**: The products and applications in this application news are intended for Research Use Only (RUO). Not for use in diagnostic procedures.*

Application Development & Support Centre, 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

Copyright © 2019 SHIMADZU (Asia Pacific) Pte. Ltd.
All rights reserved. No part of this document may be reproduced in any form or by any means without permission in writing from SHIMADZU (Asia Pacific) Pte. Ltd.