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Application News

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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

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❑ 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.

❑ 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 from the sample buffer. The recovered sample (~20 µL) was diluted to 100 µL with 25 mM ammonium bicarbonate solution.

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. Then, 4 µL iodoacetamide (IAA, 1M) solution was added for alkylation, and incubated in the dark for 60 min at room temperature.

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

Extraction of *N*-glycans: *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

Results and Discussion

A. UHPLC/RF injection-to-injection reproducibility

The 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.

B. 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.

C. 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 makes 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

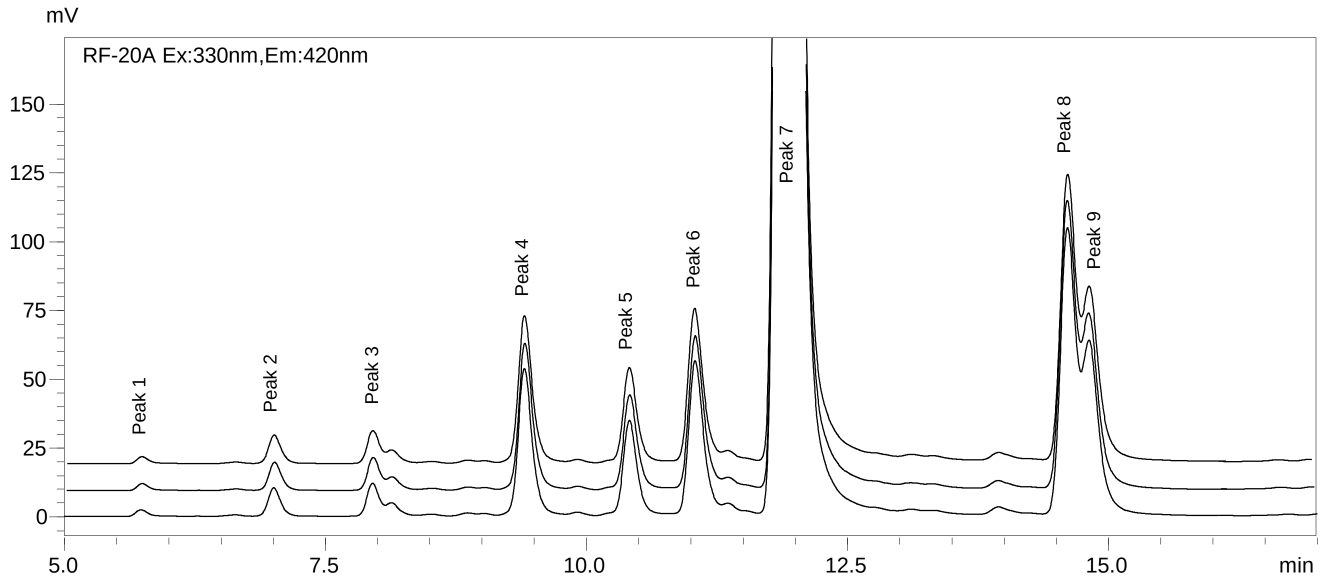

Figure 1. UHPLC-RF chromatograms of triplicate injections of 2-AB labelled *N*-glycans released from the same bevacizumab biosimilar product. It shows perfect alignment of chromatograms. The peak area and retention time variations were less than 2% RSD.

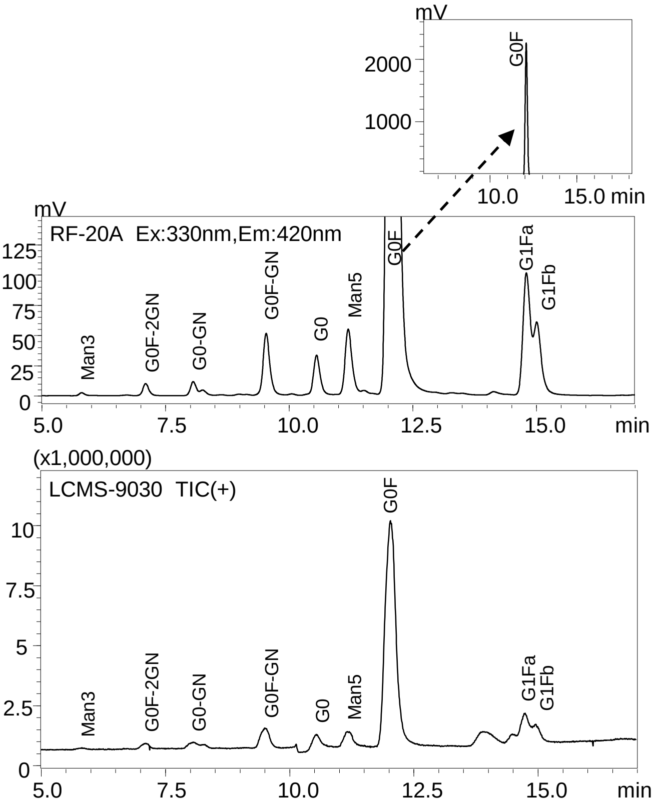

Figure 2. UHPLC/Fluorescence/MS analysis of 2-AB labeled *N*-glycans of bevacizumab biosimilar. Top chromatogram is fluorescence chromatogram; bottom is MS chromatogram.

❑ 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 satisfactory (RSD < 2%).

In this report, we have demonstrated that the system comprising of Nexera Bio UHPLC coupled with RF-20A fluorescence detector and LCMS-9030 (Q-TOF) mass spectrometer is robust and reliable for N-glycan profiling and quantitation of bevacizumab biosimilar products. The 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.

❑ Reference

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

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

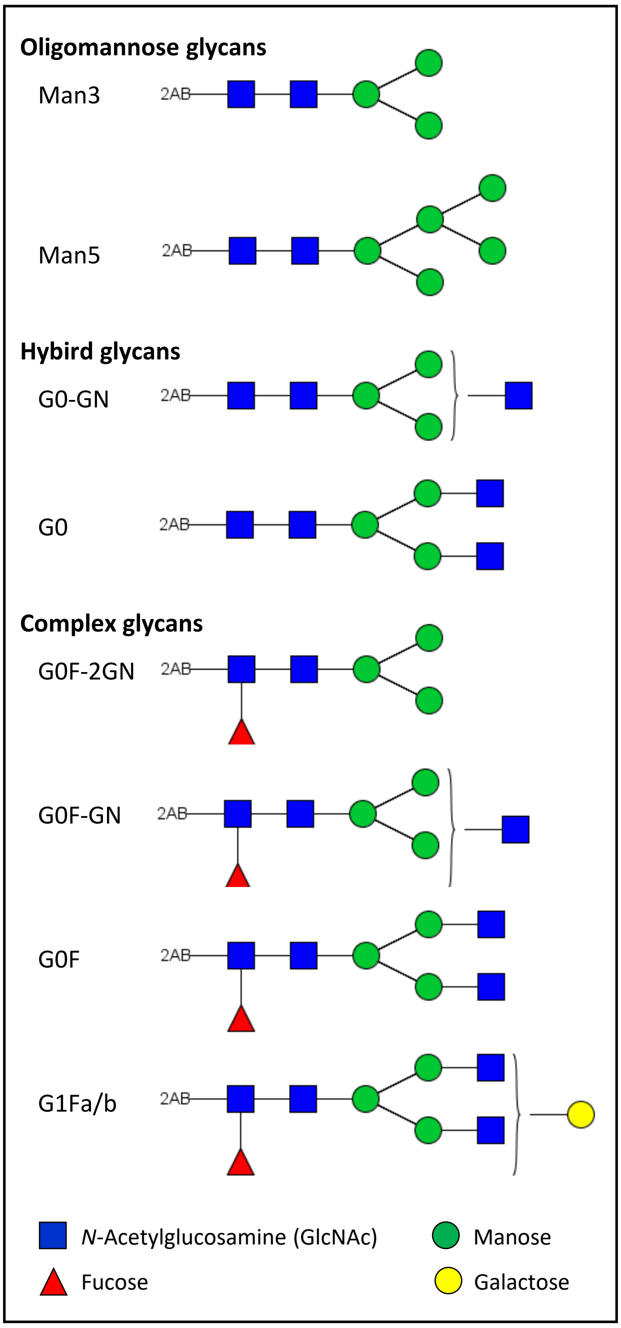

Figure 3. Proposed structures for 2-AB labeled *N*-glycans from bevacizumab biosimilar. GN = GlcNAc

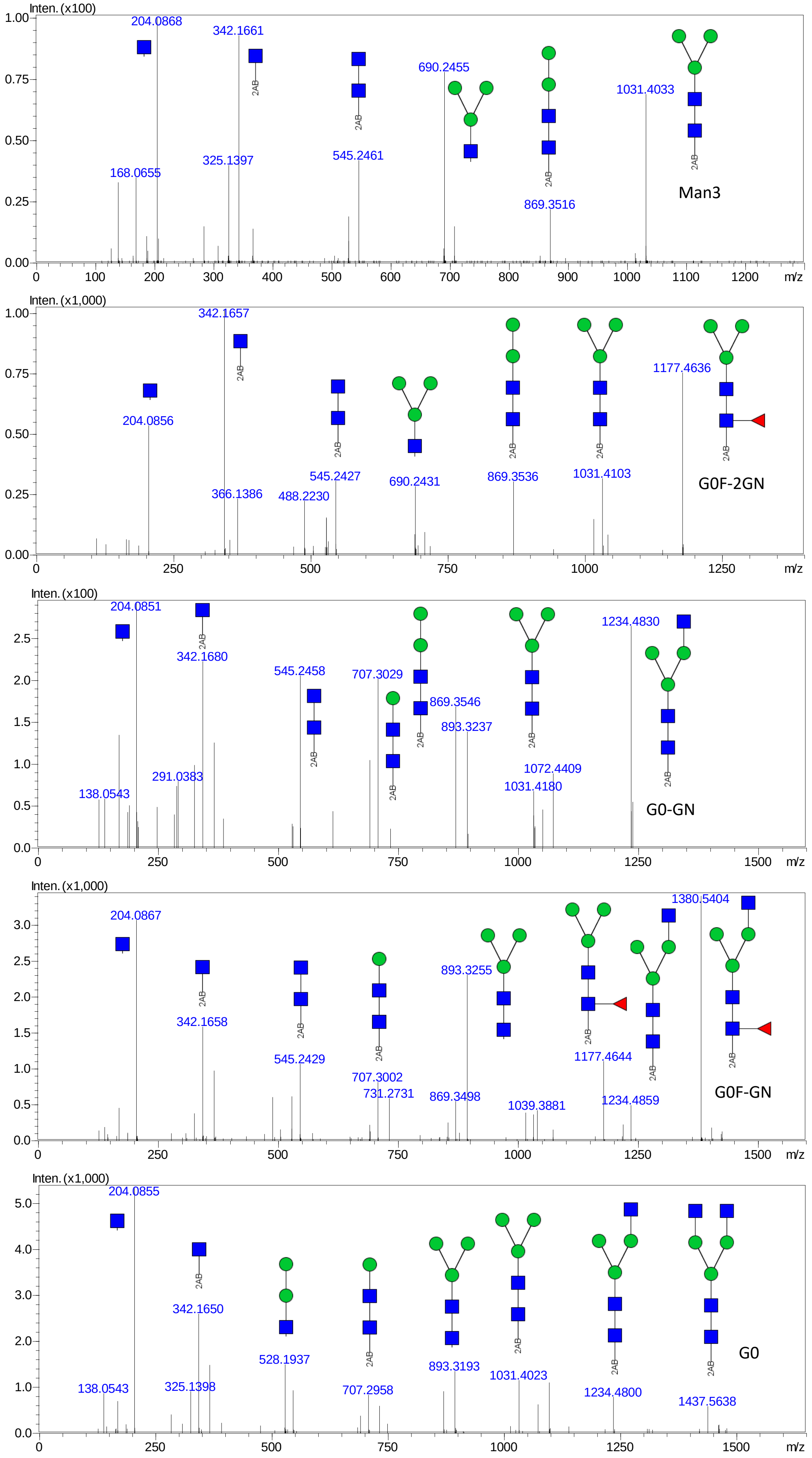

Figure 4. MS/MS spectra of 2-AB labeled *N*-glycans obtained by LCMS-9030 in positive ion mode.

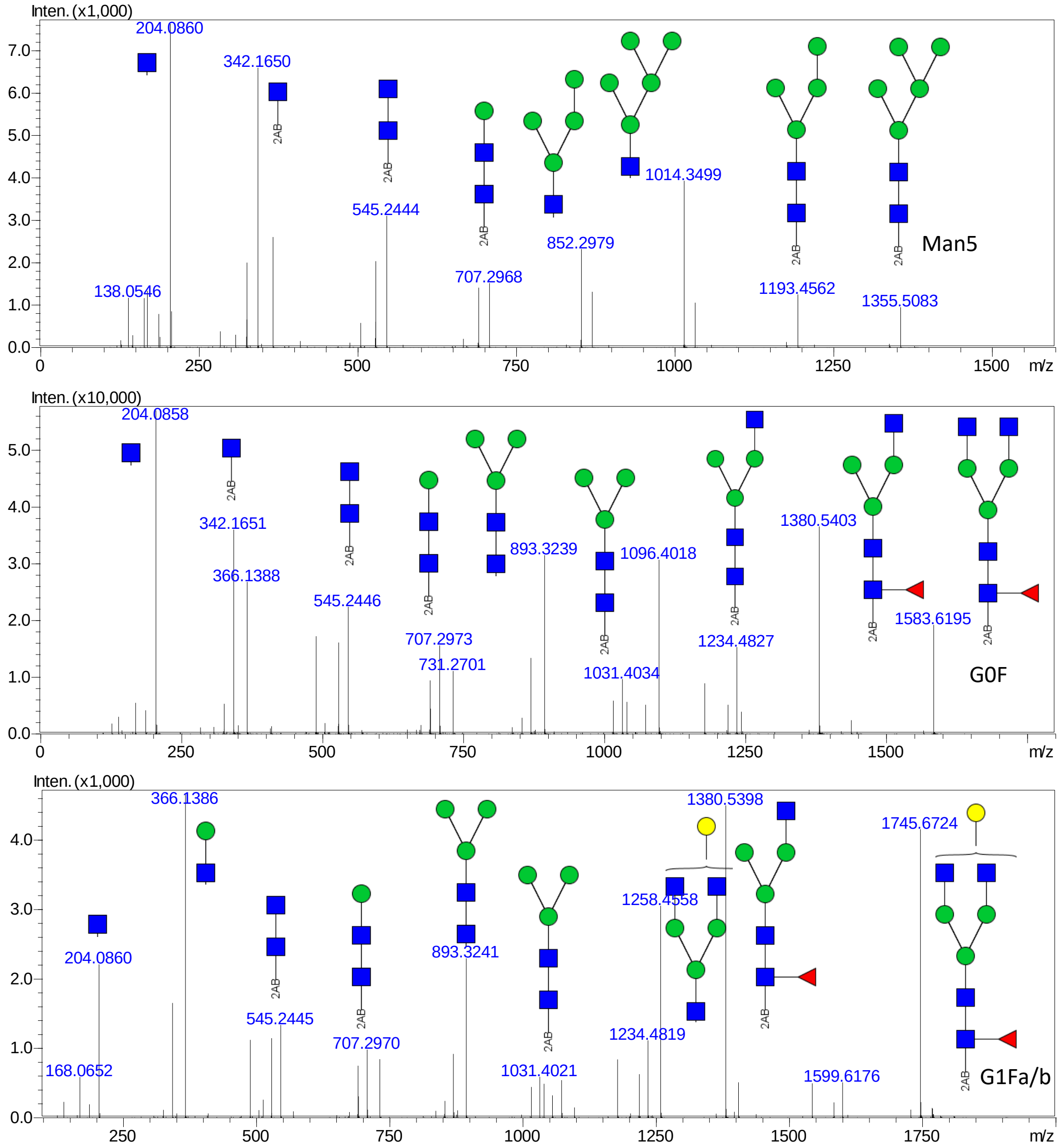

Figure 4. MS/MS spectra of 2-AB labeled *N*-glycans obtained by LCMS-9030 in positive ion mode (continued).

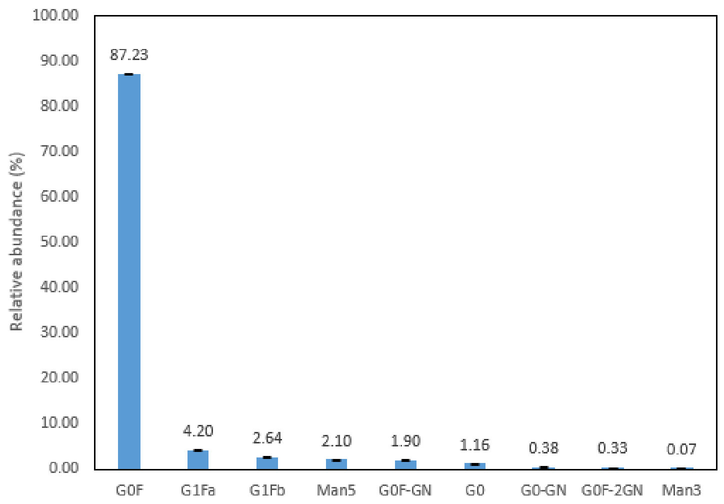

Figure 5. Relative abundance of 2-AB labeled *N*-glycans from bevacizumab biosimilar. Each relative abundance value has error bars based on triplicate analyses.

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