Analysis of N-glycan profile of purified monoclonal antibody IgG by nano LC/MS/MS

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Objective

To develop a comprehensive method for the characterization of the profile of N-glycans released from mAb IgG. The described integrated method covers sample preparation, mass spectrometry data acquisition and data analysis.

Methods: The N-glycans were released from mAb by PNGase-F and labeled using RapiFluor-MS N-Glycan Kit (Waters) and characterized by nano-LC/MS/MS using a New Objective HALO Glycan nano column on a Dionex Ultimate 3000 RSLCnano system coupled with a Thermo Q-Exactive mass spectrometer.

Results: The N-glycan profiles of Synagis, Biosimilar, and Waters intact mAb standard were analyzed using the proposed method. After N-glycan released by PNGase-F and labeling with RapiFluor-MS Kit, no further fractionation is required and the glycans released from the antibody can be analyzed directly by LC-MS. Glycans commonly present in mAbs are identified with high sensitivity and high selectivity with rapidity and their relative abundance is quantified by their peak intensities. Therefore the developed method provides a reliable approach for glycan analysis of antibodies.

Introduction

Glycoproteins are widely distributed in biological systems and play significant roles in many biological and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development (ref 1). Glycans can affect efficacy and safety of protein based drugs. For example, recombinant proteins and monoclonal antibodies (mAb) are often dependent on the structure and types of glycans attached to the proteins (ref 2). The structures of glycans are diverse, complex, and heterogeneous as a result of posttranslational modifications (PTMs) that can be affected by host cell type, stable clone diversity, and cell culture physiological conditions. Minor changes in glycan structure can result in striking differences in biological functions and clinical outcomes. The structural characterization of glycans is essential in bio-therapeutics and bio-pharmaceutical development projects (ref 3). Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans. The New Objective HALO Glycan is a high-performance nano liquid chromatography column based on a new

Fused-Core particle design. The Fused-Core particle provides a thin porous shell of high-purity silica surrounding a solid silica core. The HALO Glycan stationary phase is a highly polar ligand that possesses 5 hydroxyl groups tethered to the silica via novel proprietary linkage chemistry. This high performance material provides a column that can be used with the typical mobile phases for hydrophilic interactive liquid chromatography (HILIC) of oligosaccharides.

This application note presents a step-by-step method for the release, labeling, separation, and structural elucidation of N-glycans from glycoproteins by LC-MS/MS. The use of the Waters RapiFluor-MS N-Glycan Kit for the fast enzymatic release and rapid labeling of N-glycans make it much easier for profiling of glycans released from mAb.

This application note is validated by analyzing the glycans released from Waters intact mAb standard and commercial available monoclonal antibodies.

Materials and Methods

Samples and reagents

1. Waters Intact mAb Mass Check Standard
2. Synagis [palivizumab] (Syn, prepared by analyst A)
3. Synagis (J-Syn, prepared by analyst B)
4. Synagis biosimilar Clone 5 (J-C5, prepared by analyst B)
5. Synagis biosimilar Clone 35 (C35, prepared by analyst A)
6. Synagis biosimilar Clone 35 (J-C35, prepared by analyst B)

LC-MS/MS grade water, Methanol, and Acetonitrile were purchased from sigma.

GlycoWorks RapiFluor-MS N-Glycan Kit was purchased from Waters (Part No. 176003867).

Glycan release and labeling

Deglycosylation and labeling of N-glycans released from mAbs used the RapiFluor-MS labeling kit from Waters, and the labeled glycan samples were cleaned-up prior to LC analyses by using the solid-phase extraction (SPE) with the GlycoWorks HILIC µElution Plate.

Nanospray LC/MS/MS analysis and database search

The LC/MS/MS analysis was carried out using a Thermo Scientific Q-Exactive hybrid Quadrupole-Orbitrap Mass Spectrometer and a Thermo Dionex UltiMate 3000 RSLCnano System. Labeled free glycan mixtures released from mAb were loaded onto a HILIC trap cartridge at a flow rate of 5 µL/min. The trapped glycans were eluted onto a HALO Glycan 10 cm PicoFrit column (New Objective, Woburn, MA) using a linear gradient of acetonitrile (90-10%) in 0.1% formic acid. The elution duration was 60 min at a flow rate of 0.3 µL/min. Eluted

glycans from the PicoFrit column were ionized and sprayed into the mass spectrometer, using a Nanospray Flex Ion Source ES071 (Thermo) under the following settings: spray voltage, 1.6 kV, capillary temperature, 250°C.

The Q Exactive instrument was operated in the data dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (m/z 300−2000) were acquired in the Orbitrap with 70,000 resolution (m/z 200) after accumulation of ions to a 1×10^6 target value based on predictive AGC from the previous full scan. Dynamic exclusion was set to 10 s. The 12 most intense multiply charged ions ($z \geq 2$) were sequentially isolated and fragmented in the octopole collision cell by higher-energy collisional dissociation (HCD) using normalized HCD collision energy 28% with an AGC target 1e5 and a maxima injection time of 100 ms at 17,500 resolution.

Raw data files were analyzed using the Xcalibur software (Thermo, San Jose, CA) and SimGlycan software (PREMIER Biosoft).

Calculation of relative abundance (%) of glycan forms

The MS1 peak intensity of each glycan molecule from samples were quantified by Xcalibur software. The relative abundance of each glycan form is calculated as percentage of its intensity to total intensity of all glycan forms.

Results

Profiling of N-glycans released from mAb by LC/MS/MS

The N-glycans released from Waters' Intact mAb, Synagis and Synagis biosimilar clone 5 and clone 35 were analyzed using the proposed protocol. The profile of N-glycans from each sample is summarized in the tables (Tables 1 through 6) below. The observed mass and retention time for each glycan form are similar among six samples analyzed.

Table 1: The N-Glycan profile of Waters Intact mAb mass check standard (anti-citrinin murine monoclonal IgG1) characterized by LC/MS/MS

Glycan Name	Mi (Da)	Obs m/z [Da]	Charge	Obsr MH+ [Da]	delta mass	Area	Relative (%)	RT [min]
M5	1545.608	773.81165	2	1546.61601	0.00801	4.413E6	0.14%	24.74
A2	1627.6611	814.83801	2	1628.66875	0.00765	1.375E7	0.43%	25.03
M6	1707.6609	854.83721	2	1708.67443	0.01353		0.00%	25.29
FA1G1	1732.6925	867.35388	2	1733.70049	0.00799	3.580E7	1.12%	25.92
FA2	1773.719	887.86761	2	1774.72795	0.00895	1.362E9	42.68%	25.46
A2G1	1789.714	895.86475	2	1790.72222	0.00822	2.437E7	0.76%	24.97
FA2G1	1935.7719	968.89484	2	1936.78240	0.01050	1.405E9	44.02%	26.01
A2G2	1951.7668	651.59601	3	1952.77347		9.556E5	0.03%	
FA2B	1976.7984	989.40686	2	1977.80644	0.00804	2.567E6	0.08%	25.15
FA2G2	2097.8247	1049.92090	2	2098.83452	0.00982	2.998E8	9.39%	26.19
FA2BG1	2138.8512	1070.43494	2	2139.86260	0.01140	6.055E6	0.19%	25.63
FA2G1S1	2226.8673						N/D	
FA2G2Ga1	2259.8775	1130.94836	2	2260.88945	0.01195	1.332E7	0.42%	26.34
FA2BG2	2300.9041	1151.45959	2	2301.91191	0.00781	3.047E6	0.10%	26.32
FA2G2S1	2388.9201						N/D	
FA2G2Sg1	2404.915	1203.46814	2	2405.92900	0.01400	1.090E7	0.34%	28.90
FA2G2Ga2	2421.9303	1211.97693	2	2422.94658	0.01628	1.028E7	0.32%	26.98
FA2BG2S1	2591.9995	1297.02539	2	2593.04350	0.04400		0.00%	22.31
FA2G2S2	2680.01500						N/D	
FA2BG2S2	2883.09490						N/D	

Table 2: The N-Glycan profile of Synagis (monoclonal IgG1) characterized by LC/MS/MS

Glycan Name	Mi (Da)	Obs m/z [Da]	Charge	Obsr MH+ [Da]	delta mass	Area	Relative (%)	RT [min]
M5	1545.608	773.81140	2	1546.61553	0.00753	1.214E8	2.27%	24.90
A2	1627.6611	814.83765	2	1628.66802	0.00692	9.755E6	0.18%	25.06
M6	1707.6609	854.83813	2	1708.66899	0.00809	1.816E6	0.03%	25.32
FA1G1	1732.6925	867.35358	2	1733.69988	0.00738	9.079E7	1.69%	25.50
FA2	1773.719	887.86688	2	1774.72649	0.00749	1.988E9	37.10%	25.40
A2G1	1789.714	895.86292	2	1790.71855	0.00455	1.026E7	0.19%	25.40
FA2G1	1935.7719	968.89386	2	1936.78044	0.00854	2.277E9	42.50%	25.94
A2G2	1951.7668		2				N/D	
FA2B	1976.7984	989.40833	2	1977.80937	0.01097	1.231E7	0.23%	25.12
FA2G2	2097.8247	1049.92078	2	2098.83428	0.00958	7.267E8	13.56%	25.82
FA2BG1	2138.8512	1070.43420	2	2139.86113	0.00993	1.904E7	0.36%	25.75
FA2G1S1	2226.8673						0.00%	
FA2G2Ga1	2259.8775	1130.94788	2	2260.88848	0.01098	5.771E7	1.08%	26.31
FA2BG2	2300.9041	1151.46130	2	2301.91533	0.01123	9.311E6	0.17%	26.27
FA2G2S1	2388.9201						0.00%	
FA2G2Sg1	2404.915	1203.46667	2	2405.92607	0.01107	8.874E6	0.17%	28.75
FA2G2Ga2	2421.9303	1211.97424	2	2422.94121	0.01091	2.518E7	0.47%	26.75
FA2BG2S1	2591.99950						N/D	
FA2G2S2	2680.01500						N/D	
FA2BG2S2	2883.09490						N/D	

Note: Preparation of RapiFluor-MS labeled N-Glycan by **analyst A**.

Table 3: The N-Glycan profile of Synagis (monoclonal IgG1) characterized by LC/MS/MS

Glycan Name	Mi (Da)	Obs m/z [Da]	Charge	Obsr MH+ [Da]	delta mass	Area	Relative (%)	RT [min]
M5	1545.608	773.81140	2	1546.61553	0.00753	1.214E8	2.27%	24.90
A2	1627.6611	814.83765	2	1628.66802	0.00692	9.755E6	0.18%	25.06
M6	1707.6609	854.83813	2	1708.66899	0.00809	1.816E6	0.03%	25.32
FA1G1	1732.6925	867.35358	2	1733.69988	0.00738	9.079E7	1.69%	25.50
FA2	1773.719	887.86688	2	1774.72649	0.00749	1.988E9	37.10%	25.40
A2G1	1789.714	895.86292	2	1790.71855	0.00455	1.026E7	0.19%	25.40
FA2G1	1935.7719	968.89386	2	1936.78044	0.00854	2.277E9	42.50%	25.94
A2G2	1951.7668	651.59833	3	1952.78043	0.01363	3.139E6	0.00%	25.90
FA2B	1976.7984	989.40833	2	1977.80937	0.01097	1.231E7	0.23%	25.12
FA2G2	2097.8247	1049.92078	2	2098.83428	0.00958	7.267E8	13.56%	25.82
FA2BG1	2138.8512	1070.43420	2	2139.86113	0.00993	1.904E7	0.36%	25.75
FA2G1S1	2226.8673						N/D	
FA2G2Ga1	2259.8775	1130.94788	2	2260.88848	0.01098	5.771E7	1.08%	26.31
FA2BG2	2300.9041	1151.46130	2	2301.91533	0.01123	9.311E6	0.17%	26.27
FA2G2S1	2388.9201						0.00%	
FA2G2Sg1	2404.915	1203.46667	2	2405.92607	0.01107	8.874E6	0.17%	28.75
FA2G2Ga2	2421.9303	1211.97424	2	2422.94121	0.01091	2.518E7	0.47%	26.75
FA2BG2S1	2591.9995						N/D	
FA2G2S2	2680.01500						N/D	
FA2BG2S2	2883.09490	1442.09302	2	2883.17876			0.00%	22.28

Note: Preparation of RapiFluor-MS labeled N-Glycan by **analyst B**.

Table 4: The N-Glycan profile of Synagis biosimilar clone 5 (mouse IgG1) characterized by LC/MS/MS

Glycan Name	Mi (Da)	Obs m/z [Da]	Charge	Obs MH+ [Da]	delta mass	Area	Relative (%)	RT [min]
M5	1545.608	773.81238	2	1546.61748	0.00948	1.403E7	1.61%	24.89
A2	1627.6611	814.83600	2	1628.66472	0.00362	1.596E6	0.18%	24.53
M6	1707.6609						N/D	
FA1G1	1732.6925	867.35437	2	1733.70146	0.00896	2.452E7	2.82%	25.14
FA2	1773.719	887.86646	2	1774.72563	0.00663	2.953E8	33.97%	24.79
A2G1	1789.714	895.86530	2	1790.72331	0.00931	1.664E6	0.19%	25.05
FA2G1	1935.7719	968.89423	2	1936.78118	0.00928	3.132E8	36.03%	25.61
A2G2	1951.7668						N/D	
FA2B	1976.7984	989.40594	2	1977.80461	0.00621		0.00%	25.10
FA2G2	2097.8247	1049.92053	2	2098.83379	0.00909	1.062E8	12.22%	25.84
FA2BG1	2138.8512	1070.42981	2	2139.85234	0.00114	4.352E6	0.50%	25.58
FA2G1S1	2226.8673						N/D	
FA2G2Ga1	2259.8775	1130.94641	2	2260.88555	0.00805	1.535E7	1.77%	26.28
FA2BG2	2300.9041	1151.45874	2	2301.91020	0.00610	2.495E6	0.29%	26.07
FA2G2S1	2388.9201						N/D	
FA2G2Sg1	2404.915	1203.46692	2	2405.92656	0.01156	8.315E7	9.56%	28.90
FA2G2Ga2	2421.9303	1211.96973	2	2422.93218	0.00188	7.395E6	0.85%	26.73
FA2BG2S1	2591.99950						N/D	
FA2G2S2	2680.01500						N/D	
FA2BG2S2	2883.09490	1442.60352	2	2884.19975	0.10485		0.00%	22.17

Note: Preparation of RapiFluor-MS labeled N-Glycan by **analyst B**.

Table 5: The N-Glycan profile of Synagis biosimilar clone 35 (mouse IgG1) characterized by LC/MS/MS

Glycan Name	Mi (Da)	Obs m/z [Da]	Charge	Obs MH+ [Da]	delta mass	Area	Relative (%)	RT [min]
M5	1545.608	773.81160	2	1546.62320	0.01520	4.800E5	0.13%	24.82
A2	1627.6611						N/D	
M6	1707.6609						N/D	
FA1G1	1732.6925	867.35480	2	1733.70232	0.00982	2.432E6	0.68%	25.44
FA2	1773.719	887.86664	2	1774.72600	0.00700	1.451E8	40.46%	25.22
A2G1	1789.714						N/D	
FA2G1	1935.7719	968.89502	2	1936.78276	0.01086	1.370E8	38.20%	25.54
A2G2	1951.7668						N/D	
FA2B	1976.7984						N/D	
FA2G2	2097.8247	1049.92078	2	2098.83428	0.00958	3.815E7	10.64%	26.06
FA2BG1	2138.8512	1070.43140	2	2139.85552	0.00432	7.998E5	0.22%	25.70
FA2G1S1	2226.8673						N/D	
FA2G2Ga1	2259.8775	1130.95032	2	2260.89336	0.01586	5.328E6	1.49%	26.39
FA2BG2	2300.9041	1151.44507	2	2301.88286	-0.02124		0.00%	27.89
FA2G2S1	2388.9201	1195.55115	2	2390.09502	0.17492	1.308E7	3.65%	20.42
FA2G2Sg1	2404.915	1203.46509	2	2405.92290	0.00790	1.449E7	4.04%	28.74
FA2G2Ga2	2421.9303	1211.97839	2	2422.94951	0.01921	1.836E6	0.51%	26.83
FA2BG2S1	2591.9995						N/D	
FA2G2S2	2680.01500						N/D	
FA2BG2S2	2883.09490	1442.59985	2.00000	2884.19243	0.09753		0.00%	23.05930

Note: Preparation of RapiFluor-MS labeled N-Glycan by **analyst B**.

Table 6: The N-Glycan profile of Synagis biosimilar clone 35 (mouse IgG1) characterized by LC/MS/MS

Glycan Name	Mi (Da)	Obs m/z [Da]	Charge	Obs MH+ [Da]	delta mass	Area	Relative (%)	RT [min]
M5	1545.608	773.81220	2	1546.62440	0.01640	4.200E5	0.09%	24.82
A2	1627.6611						N/D	
M6	1707.6609						N/D	
FA1G1	1732.6925	867.35486	2	1733.70244	0.00994	9.483E6	2.11%	25.02
FA2	1773.719	887.86603	2	1774.72478	0.00578	2.095E8	46.54%	24.85
A2G1	1789.714						N/D	
FA2G1	1935.7719	968.89288	2	1936.77849	0.00659	1.785E8	39.65%	25.35
A2G2	1951.7668						N/D	
FA2B	1976.7984	989.40887		1977.81047	0.01207	5.318E5	0.12%	25.22
FA2G2	2097.8247	1049.92175	2	2098.83623	0.01153	3.868E7	8.59%	25.89
FA2BG1	2138.8512		2				0.00%	
FA2G1S1	2226.8673						N/D	
FA2G2Ga1	2259.8775	1130.94873	2	2260.89018	0.01268	2.876E6	0.64%	26.40
FA2BG2	2300.9041	1151.93250	2	2302.85771	0.95361		0.00%	27.92
FA2G2S1	2388.9201						0.00%	
FA2G2Sg1	2404.915	1203.46692	2	2405.92656	0.01156	9.498E6	2.11%	28.83
FA2G2Ga2	2421.9303	1211.97778	2	2422.94829	0.01799	6.745E5	0.15%	26.86
FA2BG2S1	2591.9995						N/D	
FA2G2S2	2680.01500						N/D	
FA2BG2S2	2883.09490						N/D	

Note: Preparation of RapiFluor-MS labeled N-Glycan by **analyst A**.

Evaluation of the similarity of N-glycan profile between the reference and biosimilar

The N-glycan profiles from Synagis and from Biosimilar were analyzed and compared. The composition of dominant glycans from two group mAb samples is shown in Figure 1. The difference of their relative abundance among these mAbs is similar and shown in Figure 1 and Figure 2.

Figure 1: Comparison of glycan compositions between the reference and biosimilar.

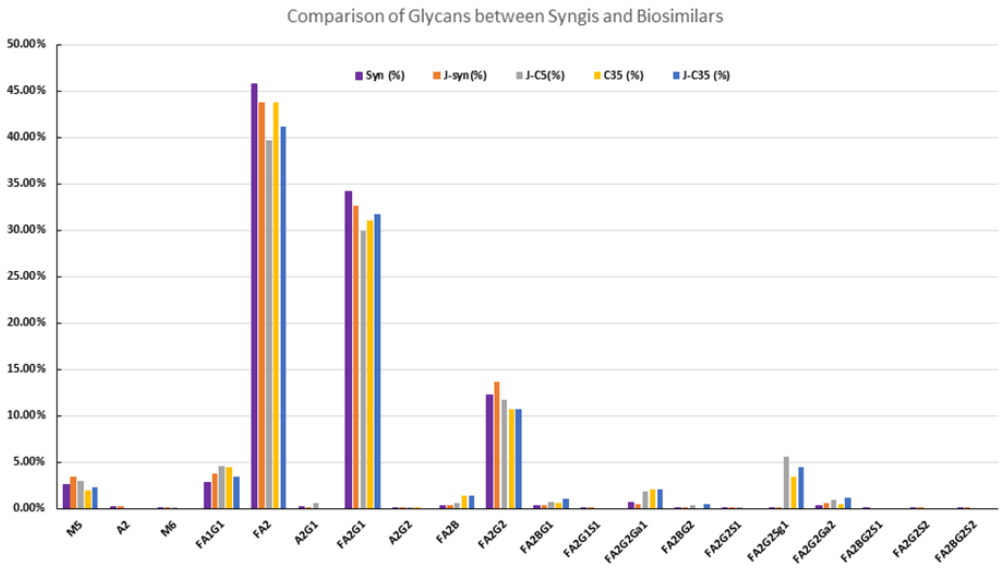


Figure 2: Comparison of glycans spectra between reference Synagis (upper panel) and biosimilar (lower panel).

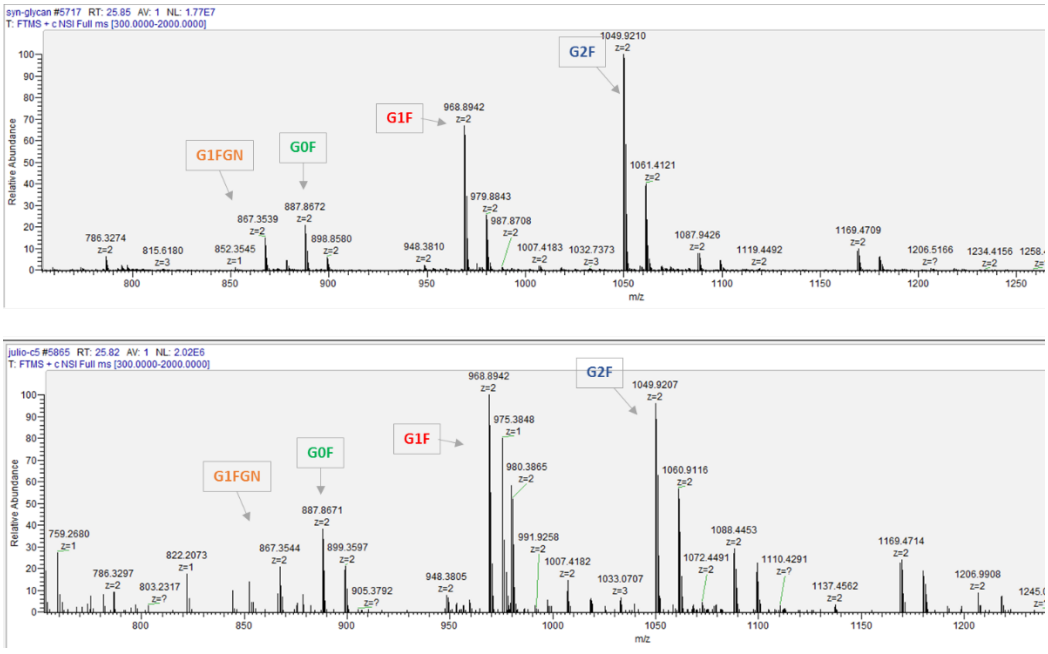
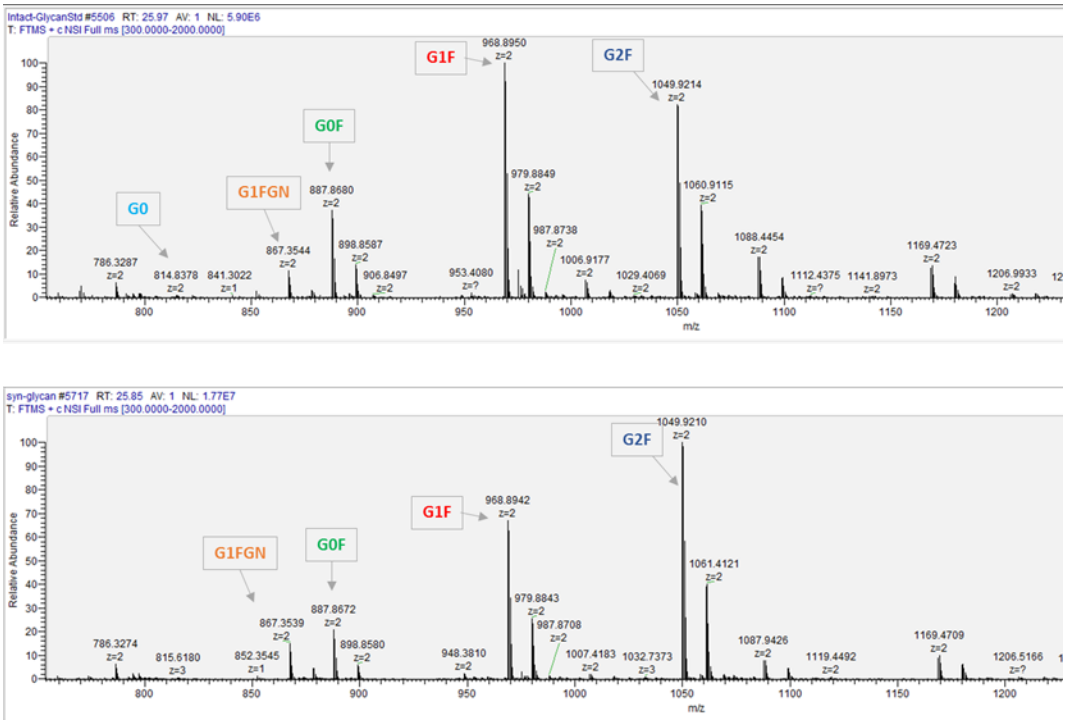


Figure 3: Comparison of glycans spectra between Waters Standard (upper panel) and Synagis (lower panel).



The Synagis and Waters standard mAb were both originated from murine sources. Their N-glycan profiles (dominant glycans) are similar as demonstrated in figure 3.

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