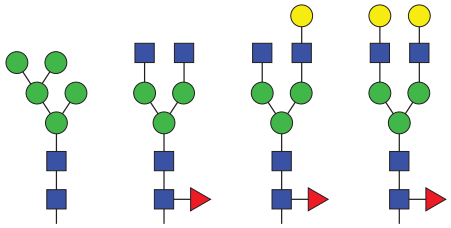
Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XT

Application Note

Biotherapeutics & Biologics



Authors

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Agilent LC/MSD XT

Introduction

N-glycosylation of therapeutic proteins is monitored during product development, process development, and QC. Many analysts perform enzymatic N-glycan release followed by labeling with a fluorescent tag to run HILIC-FLD analysis. The InstantPC tag from Prozyme Inc. allows manual sample prep within 1 hour, or alternatively, allows high-throughput parallel processing with the Agilent AssayMap Bravo liquid handling platform. High-resolution separation can be completed in short cycle times using the Agilent AdvanceBio Glycan Mapping column (see 5991-4886EN).

While fluorescence detection is a popular detection technique, analysts running HILIC-FLD workflows may face difficult challenges in the form of ambiguous peak assignment, particularly in the case of new sample types or unexpected peaks. In such cases, analysts might resort to sending the samples through complementary analyses such as LC-Q-TOF or MALDI-TOF, but this loses time, and incurs additional expenses.

We previously demonstrated an enhanced workflow where a HILIC-FLD system was hyphenated online to a high-resolution Q-TOF mass spectrometer (see 5991-6958EN). This approach couples the benefits of robust fluorescence based quantitation with the powerful structural elucidation capabilities of mass spectrometry. However, analysts who seek a routine cost-effective solution may prefer to work with a rugged, small-footprint mass selective detector (MSD) based on single quadrupole technology. Therefore, we present a method where a HILIC-FLD system is coupled to a rugged, sensitive, and stackable Agilent LC/MSD XT single quadrupole. The MSD data provide mass information to solve ambiguous peak assignments and detect coeluting structures. The method operates at less than 600 bar to maintain compatibility with a range of existing LC systems.

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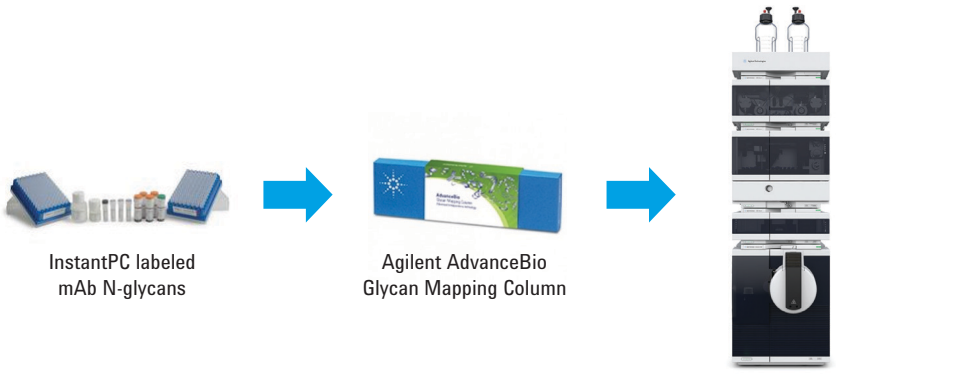

Figure 1. Flow chart of the sample preparation, LC column, and instruments used for identification and quantification of InstantPC labeled N-glycans.

Experimental

Sample preparation

Samples of monoclonal antibody (mAb) were expressed in our own lab (CHO mAb 1) or purchased from Sigma-Aldrich (SiLu Lite P/N MSQC4) and from NIST (NIST mAb, Reference Material 8671). All samples were adjusted to 1 µg/µL prior to processing by GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) from Prozyme Inc. (GPPNG-PC). The sample handling was automated using the AssayMap Bravo Liquid Handling Platform (G5542A). The final step of this protocol elutes the labeled glycans in 50 µL, so that each µL of this final sample solution represents N-glycans released from 1 µg of mAb. Conveniently, this workflow has the advantage of not requiring any centrifugation or dry down steps.

Chromatography conditions

Pump

Agilent 1260 Infinity II Binary Pump G7112B

Mobile phase A

50 mM Formic acid adjusted to pH 4.5 with ammonium hydroxide

Mobile phase B

Acetonitrile

Sampler

Agilent 1260 Infinity II Multisampler (G7167A) with thermostat set at 11 °C

Columns

- AdvanceBio Glycan Mapping Column 1.8 µm, 2.1 × 100 mm used with method A
- AdvanceBio Glycan Mapping Column 2.7 µm, 2.1 × 150 mm used with method B
- AdvanceBio Glycan Mapping Column 1.8 µm, 2.1 × 150 mm used with method C

Column heater

Agilent 1260 Infinity II G7116 thermostatic column compartment with G7116-60015 solvent preheater set to 40 °C

Columns were plumbed using 100 µm id SSTL tubing to an Agilent 1260 Infinity Fluorescence Detector (G1321B) with a 8 µL flow cell (G1321-60005). The detector was set to λEx at 285 nm, λEm at 345 nm with PMT gain = 10.

LC/MSD XT Parameters

Parameter	Value
Ion source	
Source	Agilent Jet Stream in positive mode
Sheath gas	300 °C at 10.0 L/min
Dry gas temperature	150 °C at 9.0 L/min
Nebulizer pressure	35 psig
VCap	2,500 V Nozzle: 500 V
Acquisition settings	
Mass range	500–1,400 *m/z*
Fragmentor	100 V
Gain EMV	1.0
Step size	0.10
Peak width	0.2

Software

OpenLab CDS Chemstation Edition Rev C.01.07 SR3

Reagents

All reagents and solvents used were of the highest purity available.

Gradient tables

Method A (for 2.1 × 100 mm 1.8 µm column)

Timetable

Time (min)	%A	%B	Flow (mL/min)
0.00	25.0	75.0	0.70
0.50	27.0	73.0	0.70
4.00	28.0	72.0	0.70
9.00	33.0	67.0	0.70
9.20	50.0	50.0	0.70
9.70	50.0	50.0	0.70
10.00	25.0	75.0	0.70
11.50	25.0	75.0	0.70
11.80	25.0	75.0	0.80
14.50	25.0	75.0	0.90
15.30	25.0	75.0	0.70

Stop time = 18 minutes

Method B (for 2.1 × 150 mm 2.7 µm column)

Timetable

Time (min)	%A	%B	Flow (mL/min)
0.00	27.0	73.0	0.50
1.00	28.5	71.5	0.50
9.00	29.5	70.5	0.50
22.00	41.0	59.0	0.50
22.50	50.0	50.0	0.80
23.50	50.0	50.0	0.70
23.70	27.0	73.0	0.70
25.00	27.0	73.0	0.70
25.50	27.0	73.0	0.80
27.50	27.0	73.0	0.90
28.00	27.0	73.0	0.50

Stop time = 30 minutes

Method C (for 2.1 × 150 mm 1.8 µm column)

Time (min)	%A	%B	Flow (mL/min)
0.00	22.0	78.0	0.60
0.50	26.0	74.0	0.60
13.00	27.5	72.5	0.60
28.00	39.0	61.0	0.60
28.50	50.0	50.0	0.50
28.60	50.0	50.0	0.40
28.80	22.0	78.0	0.40
31.00	22.0	78.0	0.50
31.50	22.0	78.0	0.55
33.50	22.0	78.0	0.60

Stop time = 37 minutes

Results and Discussion

Separation

We optimized the separation of typical therapeutic mAb glycan profiles on three different columns, targeting various cycle times. The methods begin with a shallow gradient that maximizes resolution of the common neutral glycans that appear in most therapeutic mAb samples. The gradient slope was increased midway through the run to elute any larger, acidic glycan structures more efficiently. These methods were all designed to stay well under 600 bar to maximize robustness, as well as allowing for compatibility with a wide range of LC instruments. While all three separation methods are shown in Figure 2, we chose to use Method C for the remainder of the experiments because it was found to give the best overall performance for our three mAb N-glycan samples.

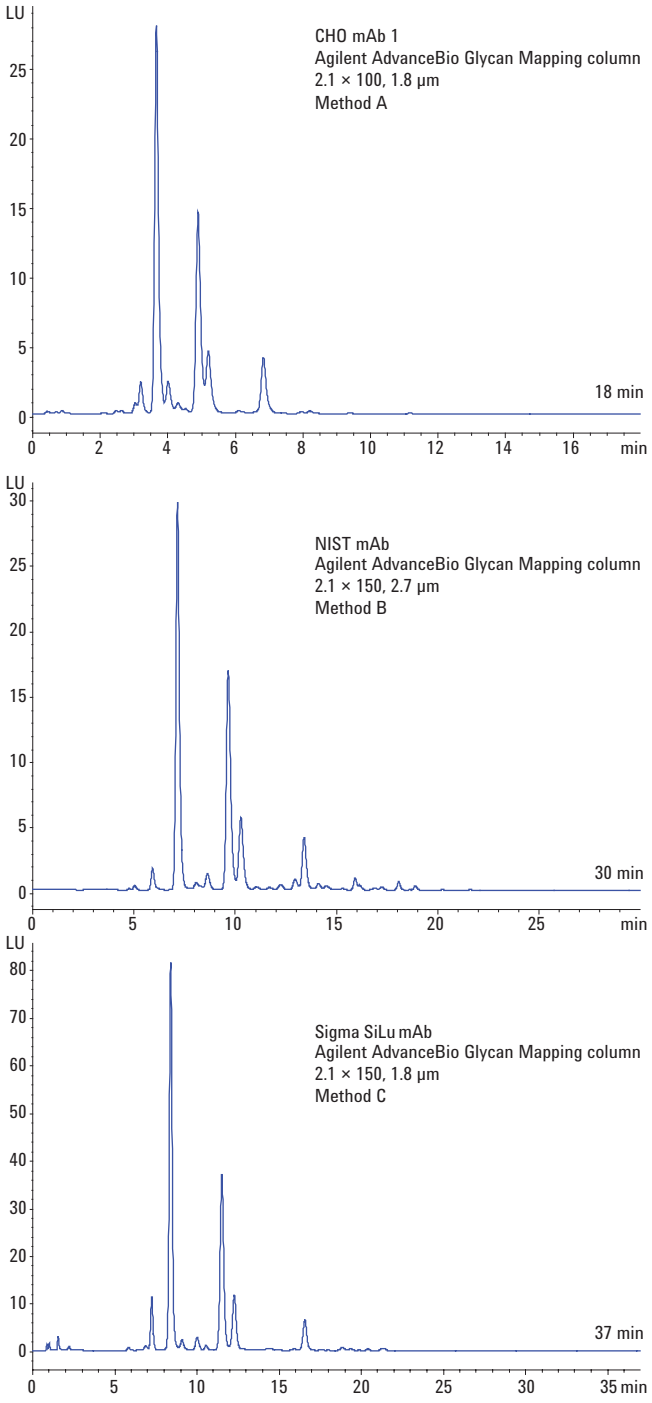

Figure 2. Three separation methods using different column formats with three mAb samples.

LC/MSD XT Sensitivity

Traditionally, analysts have avoided using single quadrupole mass spectrometers for LC/MS analysis of N-glycans due to concerns about sensitivity. However, two technical innovations have led to dramatic improvements in the limit of detection for N-glycans. The first is the availability of a highly sensitive ion source, Agilent Jet Stream, which uses a super-heated sheath gas flow around the electrospray plume to dramatically improve ionization. The second innovation is the availability of a InstantPC tag from Prozyme Inc. that radically increases ionization efficiency versus traditional fluorescent labels.

We optimized the MSD parameters to maximize sensitivity. InstantPC-labeled N-glycans were observed almost exclusively as doubly charged ions of the forms $[M+2H]^{2+}$, with some $[M+NH_4+H]^{2+}$, and $[M+H+Na]^{2+}$. Increasing the dry gas temperature or fragmentor voltage can cause collision-induced dissociation of the N-glycans, offering powerful structural elucidation strategies similar to those achievable with a Q-TOF (see 5991-5253EN).

Diagnostic signals were obtained for glycans making up as little as 0.1 % of the profile. When comparing this sensitivity with alternative workflows, remember that these results were achieved without adding a preconcentrating step to the end of the sample preparation workflow, therefore saving considerable time. Sensitivity could be boosted even further by drying the samples and reconstituting them in a lower volume of sample matrix.

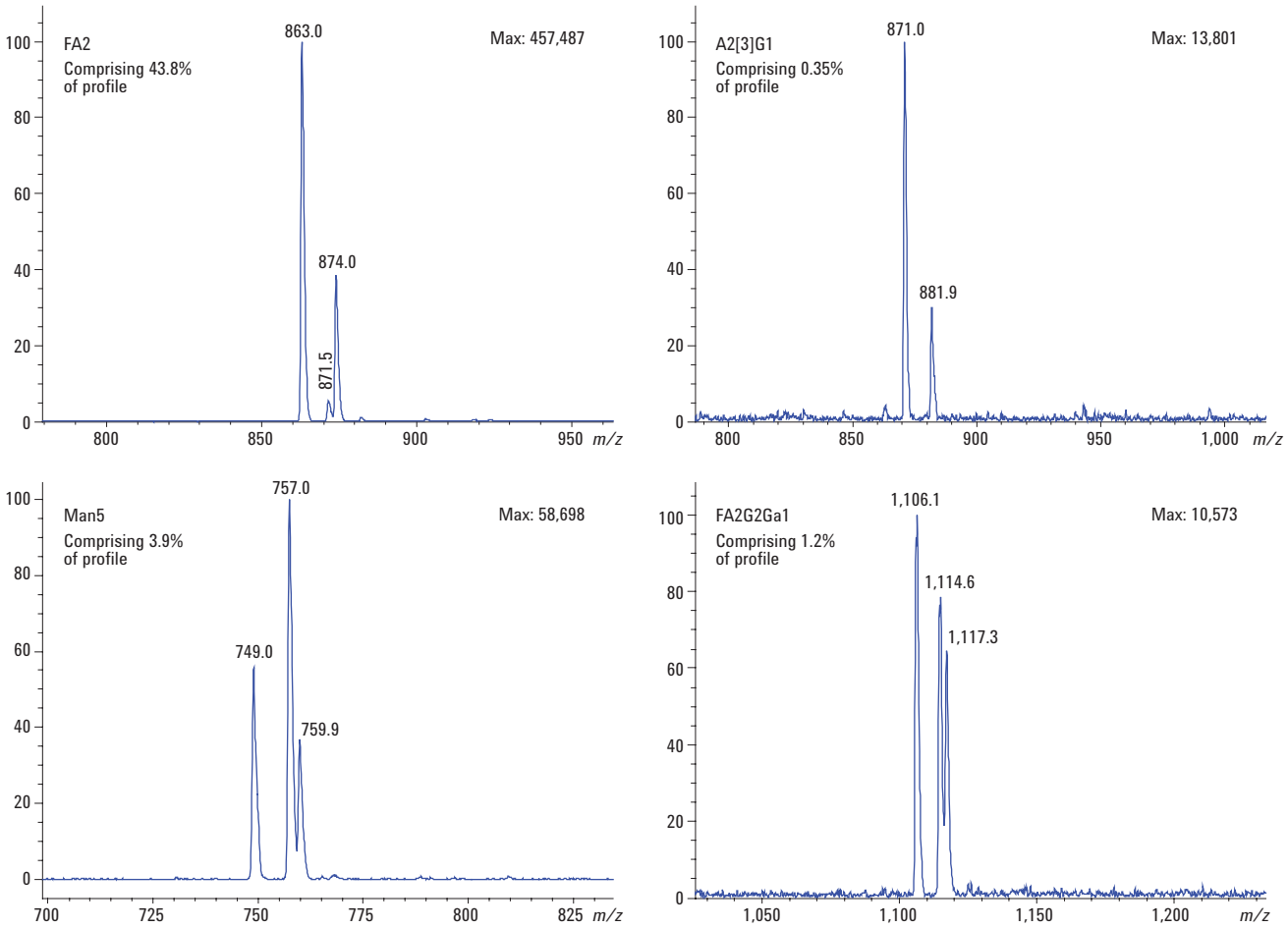

Figure 3. Mass spectra of four representative glycans from 3 µg of mAb at various levels of relative abundance. The signal for A2[3]G1, which makes up just 0.35 % of the glycan profile, is clearly observable with a high signal-to-noise ratio.

Peak assignment

The data from all three samples (using method C) were integrated, and the peaks were assigned to glycan compositions using an approach similar to that shown in application note 5991-5253EN.

Note: InstantPC labeling causes a mass increment of 261.1477 *m/z* versus the free reducing-end form of the glycans.

Table 1 was constructed showing the composition of the glycans that were detected across the three samples. Glycan compositions are shown in the form: HxNxFxSgx + Core (where H = Galactose or Mannose, N = N-acetylglucosamine, F = Fucose, Sg = N-glycolylneuraminic acid, and Core = trimannosyl, a core common to all N-glycans). In many cases, these compositions allowed us to propose glycan structures by supplementing basic knowledge of therapeutic mAb glycosylation patterns and HILIC retention order. Since most therapeutic mAbs contain a similar, limited set of common N-glycan structures, Table 1 can serve as a useful reference for assigning N-glycans in this workflow.

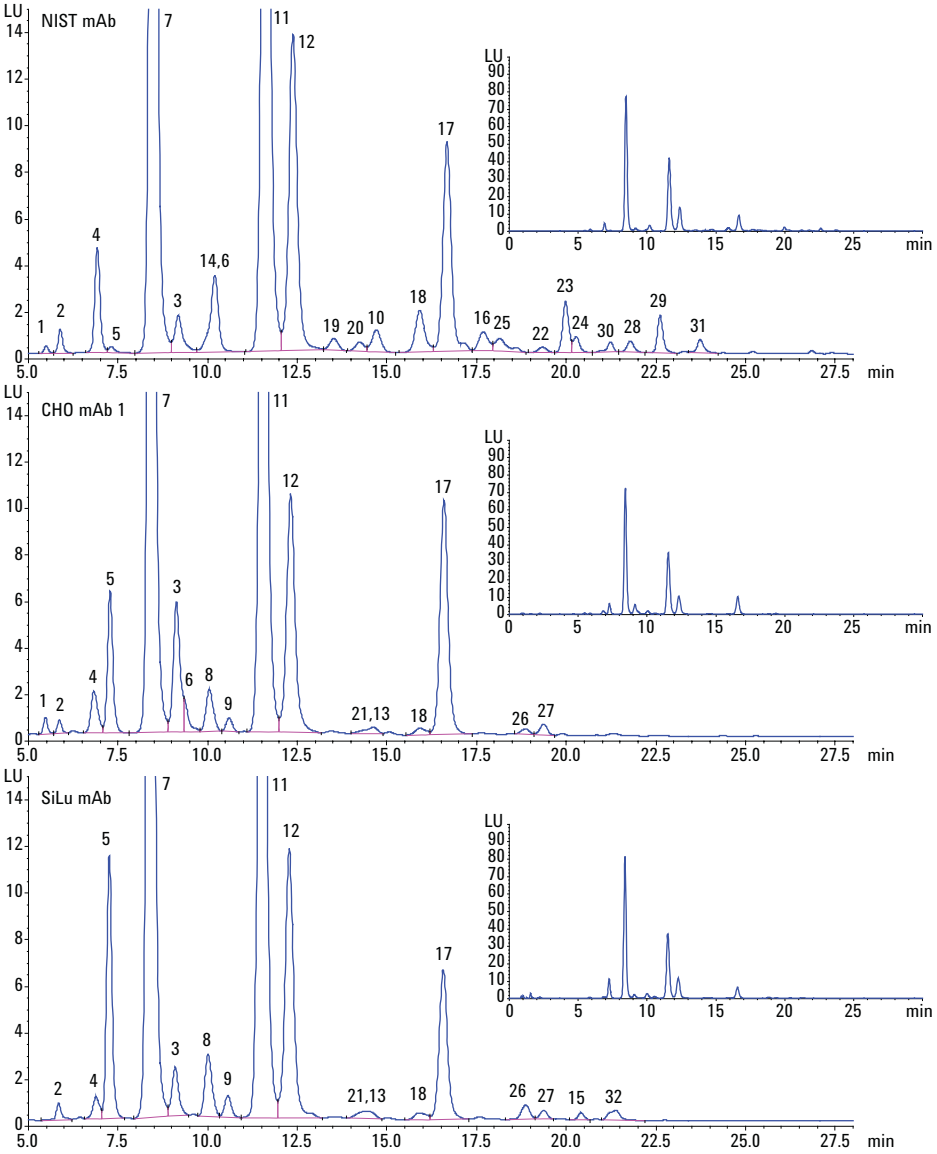

Figure 4. Zoomed FLD chromatograms of the three mAb N-glycan samples showing detection of major and minor glycans for method C. Insets show the zoomed-out data. Annotations refer to Table 1.

Table 1.

ID	Observed $[M+2H]^{2+}$	Proposed composition	Theoretical $[M+2H]^{2+}$	Proposed structure	
				Oxford	Alternative
1	659.9	F1+Core	659.774	F1M3	G0F-2GlcNAc
2	688.4	N1+Core	688.284	A1	G1-GlcNAc
3	748.9	H2+Core	748.798	M5	Man5
4	761.5	N1F1+Core	761.313	FA1	G0F-GlcNAc
5	790.0	N2+Core	789.824	A2	G0
6	842.4	H1N1F1+Core	842.340	FA1G1	G1F-GlcNAc
7	863.0	N2F1+Core	862.853	FA2	G0F
8	871.0	H1N2+Core	870.851	A2[6]G1	G1
9	871.0	H1N2+Core	870.851	A2[3]G1	G1'
10	923.6	H2N1F1+Core	923.366	-	-
11	944.0	H1N2F1+Core	943.879	FA2[6]G1	G1F
12	944.0	H1N2F1+Core	943.879	FA2[3]G1	G1F'
13	952.0	H2N2+Core	951.877	A2G2	G2
14	964.7	N3F1+Core	964.393	FA2B	G0FB
15	991.9	H5+Core	991.877	M8	Man8
16	996.1	H1N1Sg1F1+Core	995.885	FA1G1Sg1	G1Sg1F-GlcNAc
17	1025.1	H2N2F1+Core	1024.906	FA2G2	G2F
18	1025.1	H2N2F1+Core	1024.906	FA2G1Ga1	G1F+αGal
19	1045.6	H1N3F1+Core	1045.419	FA2[6]B1G1	G1FB
20	1045.7	H1N3F1+Core	1045.419	FA2[3]B1G1	G1FB'
21	1089.5	H1N1F1S1+Core	1089.427	FA1G1S1	G1S1F-GlcNAc
22	1097.7	H1N2F1Sg1+Core	1097.425	FA2G1Sg1	G1Sg1F
23	1106.1	H3N2F1+Core	1105.932	FA2G2Ga1	G2F+αGal
24	1106.1	H3N2F1+Core	1105.932	FA2G2Ga1 iso	G2F+αGal'
25	1126.5	H2N3F1+Core	1126.446	FA2BG2	G2FB
26	1170.4	H2N2F1S1+Core	1170.454	FA2G2S1	A1F
27	1170.4	H2N2F1S1+Core	1170.454	FA2G2S1 iso	A1F iso
28	1178.7	H2N2F1Sg1 + Core	1178.451	FA2G2Sg1	Ag1F
29	1187.1	H4N2F1+Core	1186.959	FA2G2Ga2	G2F+(αGal)2
30	1207.7	H3N3F1+Core	1207.472	FA2BG2Ga1	G2FB+αGal
31	1259.4	H3N2F1Sg1+Core	1259.477	FA2G2Sg1Gal1	Ag1F+αGal
32	1316.2	H2N2F1S2+Core	1316.001	FA2G2S2	A2F

Result tables

Peaks in the FLD chromatograms were reported as relative sum % of the total glycan profiles. Figure 5 shows the results.

Some of the profiles contained peaks representing coeluting glycan structures. These coelutions involved minor glycans, and quantitation based on FLD alone would report the peak area as the combined contribution of two structures.

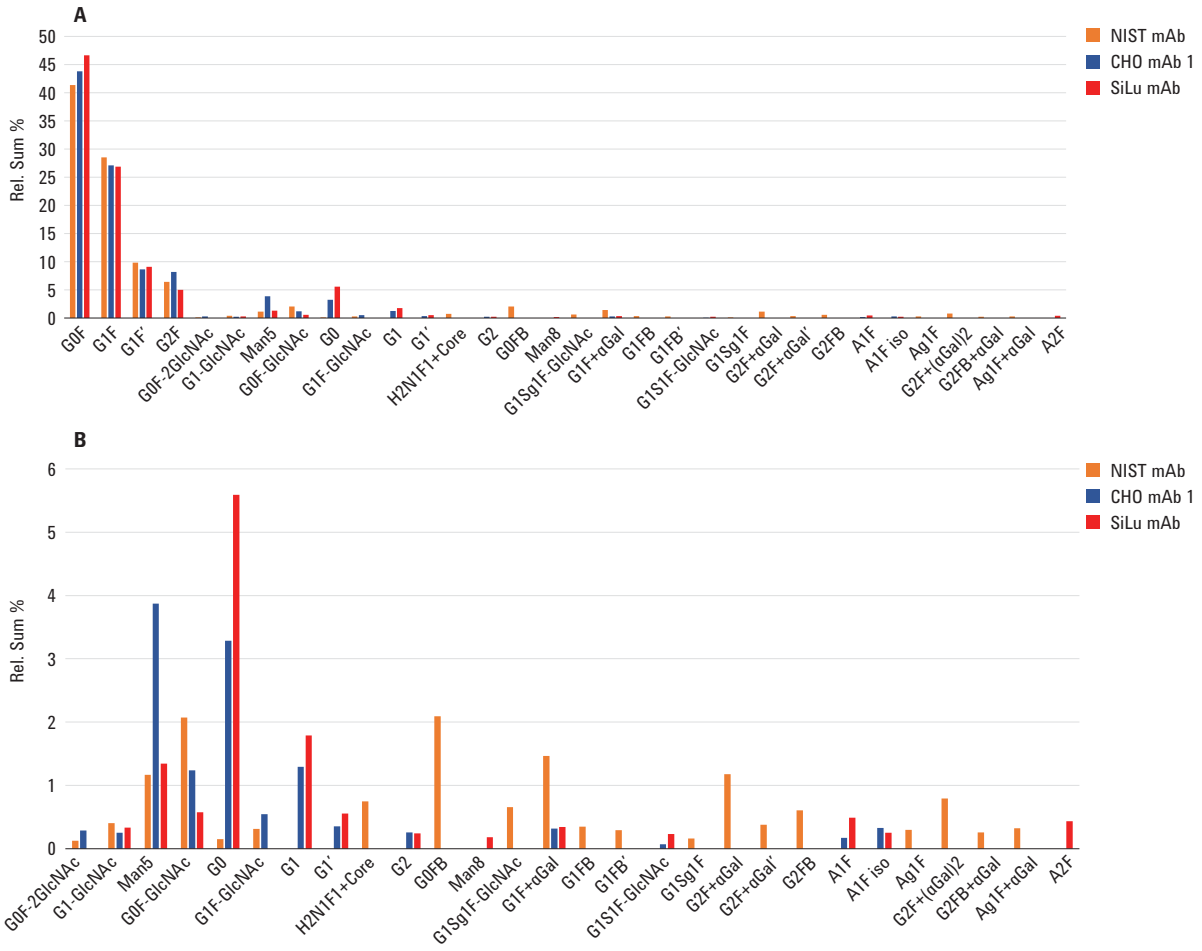

Figure 5. A) Relative abundance of N-glycans in the three mAb samples. B) Zoomed-in chart showing only the minor components. Minor glycans were detected easily at less than 1 %.

For analysts who need accurate quantitation of these low abundance coeluting structures, we propose using the LC/MSD XT data to supplement the FLD. The mass spectrum can be integrated across the time period corresponding to the coeluting FLD peaks. The FLD area can then be apportioned to the different structures based on the combined relative intensity of the doubly charged N-glycan ions in this spectrum. We have previously shown that the MS signals for InstantPC labels closely correspond to their true abundance as defined by fluorescence intensity (see 5991-6958EN).

Conclusion

The Agilent LC/MSD XT based on single quad technology can be hyphenated online to a typical UHPLC HILIC-FLD system to provide the option of mass spec-based identification for every peak in every sample. Using an Agilent Jet Stream ion source in combination with the InstantPC glycan tag provides ample sensitivity to detect MS signals for both major and minor peaks in typical antibody samples without needing to concentrate the sample. For typical monoclonal antibody samples, this allows confident assignment of glycan structures. While this application note used the traditional approach of basing relative quantitation on robust fluorescence detection, the MS data can be used to assist quantitation in the case of coeluting peaks.

For More Information

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