Application Note

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Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS

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Abstract

This Application Note describes the preparation and analysis of released N-glycans from biotherapeutic glycoproteins using two labels, InstantPC and 2-aminobenzamide (2-AB). N-Glycan analysis is vital to the development and production of biotherapeutics, as glycosylation can influence the therapeutic function of the final drug product. The workflows described here use the Agilent AdvanceBio Gly-X with InstantPC and Gly-X 2-AB Express kits (formerly ProZyme) for the release of N-glycans using PNGase F followed by instant glycosylamine labeling with InstantPC or reductive amination labeling with 2-AB Express, respectively. Labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC), with detection using both fluorescence and mass spectrometry (MS). Gly-X sample preparation offers a high level of reproducibility and throughput, with a one hour preparation time for InstantPC and two hours for 2-AB Express. In addition, the InstantPC label offers improved fluorescence response and MS ionization efficiency.

Introduction

The characterization of N-glycans is an essential part of the biotherapeutic development process, as the structure of N-linked glycans can influence the function of glycosylated biotherapeutics, frequently making glycosylation a critical quality attribute (CQA).[1] N-Glycan analysis often involves the labeling of released glycans with a tag to allow for detection by fluorescence (FLD), and to enhance ionization for mass spectrometry (MS), followed by N-glycan separation, detection, and relative quantitation. Many of the frequently used fluorescent tags such as 2-AB[2] are limited concerning MS sensitivity compared with recently introduced dyes such as InstantPC, and pre-existing N-glycan sample preparation workflows can be time-consuming.[3] However, 2-AB has been used for over 20 years and so is well-established in the literature and in many laboratories.

This Application Note presents streamlined workflows for preparation of InstantPC and 2-AB labeled N-glycans coupled with analysis using Agilent LC/FLD/MS instrumentation. Gly-X N-glycan sample preparation kits for InstantPC or 2-AB Express labeling (formerly ProZyme) include all reagents for N-glycan sample preparation: denaturation, deglycosylation, labeling, and sample cleanup, as illustrated in Figure 1.

Experimental

N-Glycan sample preparation

Agilent AdvanceBio Gly-X N-glycan prep with InstantPC (p/n GX96-IPC) and Gly-X 2-AB Express (p/n GX96-2AB) Kits were used to prepare labeled N-glycans from monoclonal antibody rituximab (Rituxan, lot number M190170) and Fc fusion protein etanercept (Enbrel, lot number 1092537), 40 µg protein per preparation. Four replicates of each sample were analyzed with fluorescence/MS detection and relative percent glycan peak areas calculated.

InstantPC and 2-AB labeled samples were prepared by standard manual protocols. The Gly-X in-solution deglycosylation protocol uses a three-minute denaturation at 90 °C, opening up the glycoprotein target to enable a five-minute deglycosylation reaction at 50 °C with PNGase F. Following in-solution deglycosylation, InstantPC labeled samples are prepared by one-minute glycosylamine labeling of released N-glycans (Figure 2), followed by vacuum-driven cleanup of free dye using HILIC solid-phase extraction (SPE).

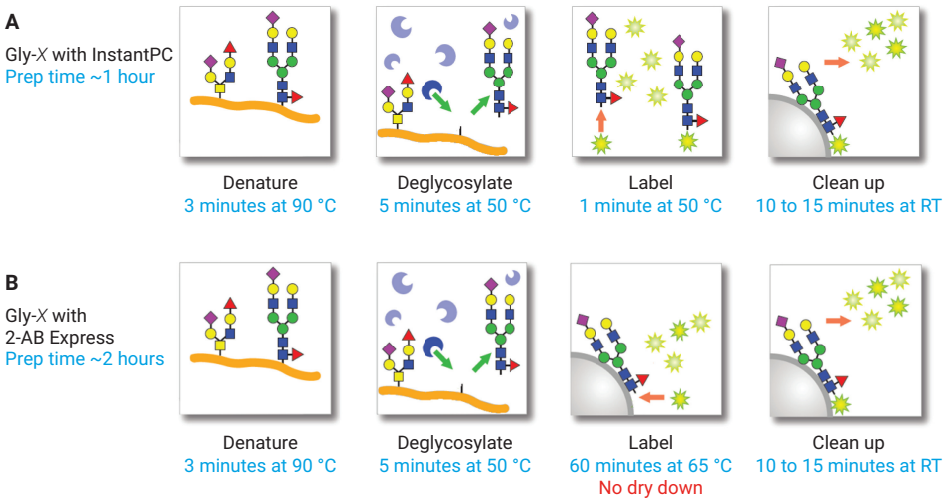

Figure 1. Gly-X N-glycan sample prep. A) InstantPC workflow with in-solution deglycosylation and labeling followed by on-matrix cleanup; B) 2-AB workflow with deglycosylation in-solution, followed by on-matrix labeling and cleanup.

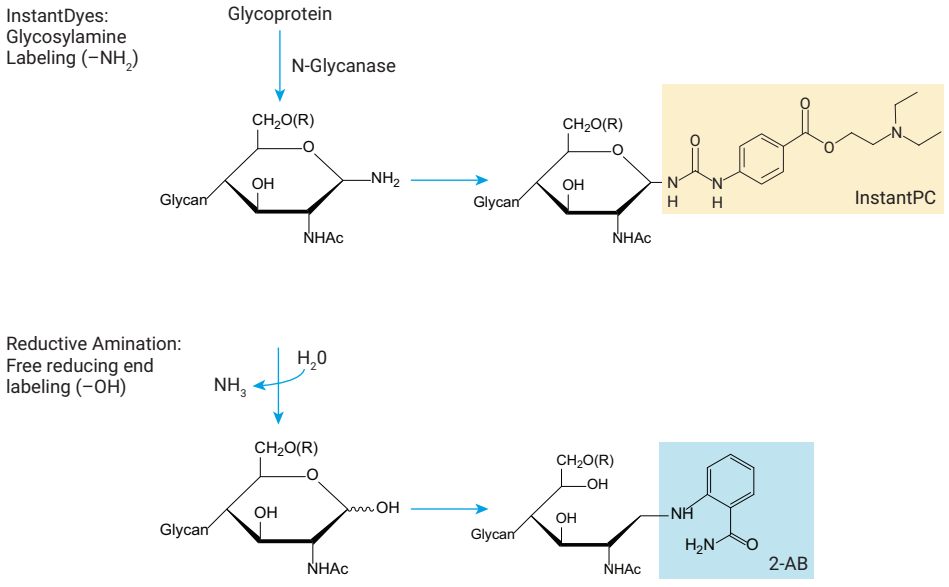

Figure 2. Comparison of InstantPC glycosylamine labeling and traditional reductive amination with 2-AB.

N-Glycan samples labeled with 2-AB were prepared using the standard Gly-X 2-AB Express protocol with reductive amination chemistry. Following the Gly-X five-minute deglycosylation with PNGase F, released N-glycans are converted from the glycosylamine form ($-NH_2$) to free reducing end form (–OH) to allow for 2-AB labeling with reductive amination. N-Glycans are then desolvated by vacuum filtration onto a solid-state matrix followed by an on-matrix 2-AB labeling step. This process eliminates the need for glycan drying prior to the 2-AB labeling step, thereby reducing total sample preparation time.

N-Glycan analysis

InstantPC and 2-AB labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC) using an Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913) with an Agilent 1290 Infinity II LC system with in-line fluorescence detection (Table 1) coupled to an Agilent AdvanceBio 6545XT LC/Q-TOF using the parameters shown in Table 2 (equivalent MS instruments may also be used in positive mode).

All HILIC separations were conducted under the conditions described in Table 1. A fixed flow splitter (IDEX Health & Science p/n UH-427) was used post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. Agilent MassHunter BioConfirm software was used for data processing, with a personal compound database (PCD).

Materials

LC/MS grade acetonitrile and water were purchased from Honeywell Research Chemicals.

Table 1. Agilent 1290 Infinity II UHPLC HILIC/FLD conditions.

Parameter	Value		
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)		
Column Temp	40 °C		
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) Acetonitrile		
Gradient Program	InstantPC and 2-AB labeled glycans		
	Time (minutes)	%B	Flow rate (mL/min)
	0	80	0.5
	2	75	0.5
	48	62	0.5
	49	40	0.5
	51.5	80	0.5
	52	80	0.5
	60	80	0.5
Injection Volume	1 µL (equivalent to glycans from 0.4 µg protein)		
Detection	Agilent 1260 Infinity II FLD InstantPC: λ_{Ex} 285 nm, λ_{Em} 345 nm 2-AB: λ_{Ex} 260 nm, λ_{Em} 430 nm		

Table 2. Agilent 6545XT Q-TOF parameters.

Agilent 6545XT Q-TOF	
Source	Dual AJS ESI
Gas Temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	10 L/min
Vcap	3,000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range	*m/z* 600 to 3,000
Scan Rate	1 spectra/sec
Acquisition Mode	High resolution (4 GHz)

Instrumentation

Labeled N-glycan samples were separated using an Agilent AdvanceBio Glycan Mapping column (Table 1 shows the method details) on an Agilent LC/MS setup composed of:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (parameters in Table 2, equivalent instruments may also be used)

Software

- Agilent MassHunter Acquisition
- Agilent MassHunter Qualitative Analysis software

Results and discussion

HILIC Separation of InstantPC and 2-AB N-Glycans

HILIC separation of labeled N-glycans from Rituxan and Enbrel labeled with InstantPC or 2-AB results in well resolved peaks for major glycan species with the 60-minute method used (Figures 3 and 4). Rituxan (Figure 3A, InstantPC; Figure 4A, 2-AB), an IgG, has an N-glycan profile typical of monoclonal antibodies with one N-glycosylation site in the Fc region produced in Chinese hamster ovary (CHO) cells: predominantly neutral biantennary complex N-glycans with core fucose, some Man5, and a relatively low proportion of sialylated glycans. The N-glycan profile of Enbrel (Figure 3B, InstantPC; Figure 4B, 2-AB), an Fc fusion protein, contains a higher level of sialylated glycans owing to two additional N-glycosylation sites in the fusion partner, TNF-α receptor (TNFR) extracellular domain, in addition to the single N-glycan site in the Fc portion.[5]

The HILIC retention time of 2-AB N-glycans is shorter than for InstantPC N-glycans, although the elution order of N-glycan species is comparable. Critical pairs such as G0F/Man5 and Man5/G1, which are often monitored during the development process of biotherapeutics, are well separated with both InstantPC and 2-AB labels, leading to confident determination of relative percentage composition. G1F isomers G1F[6] and G1F[3] are also separated. Relative percent areas, standard deviation, and relative standard deviation are reported in Tables 3 through 6, and show a low degree of variability between the four sample preparation replicates. This variability rises for lower abundance glycans.

An added benefit of InstantPC is the separation of isoforms G2S1[6]/[3] and G2FS1[6] from Enbrel (Figure 3B) compared to 2-AB (Figure 4B) using the previously described chromatography conditions. Analysis with fluorescence detection of InstantPC and 2-AB labeled N-glycans from biotherapeutics Rituxan and Enbrel results in comparable relative percent areas for major glycoforms G0F, G1F[6]/[3], G2F, G2S2, and G2FS2.

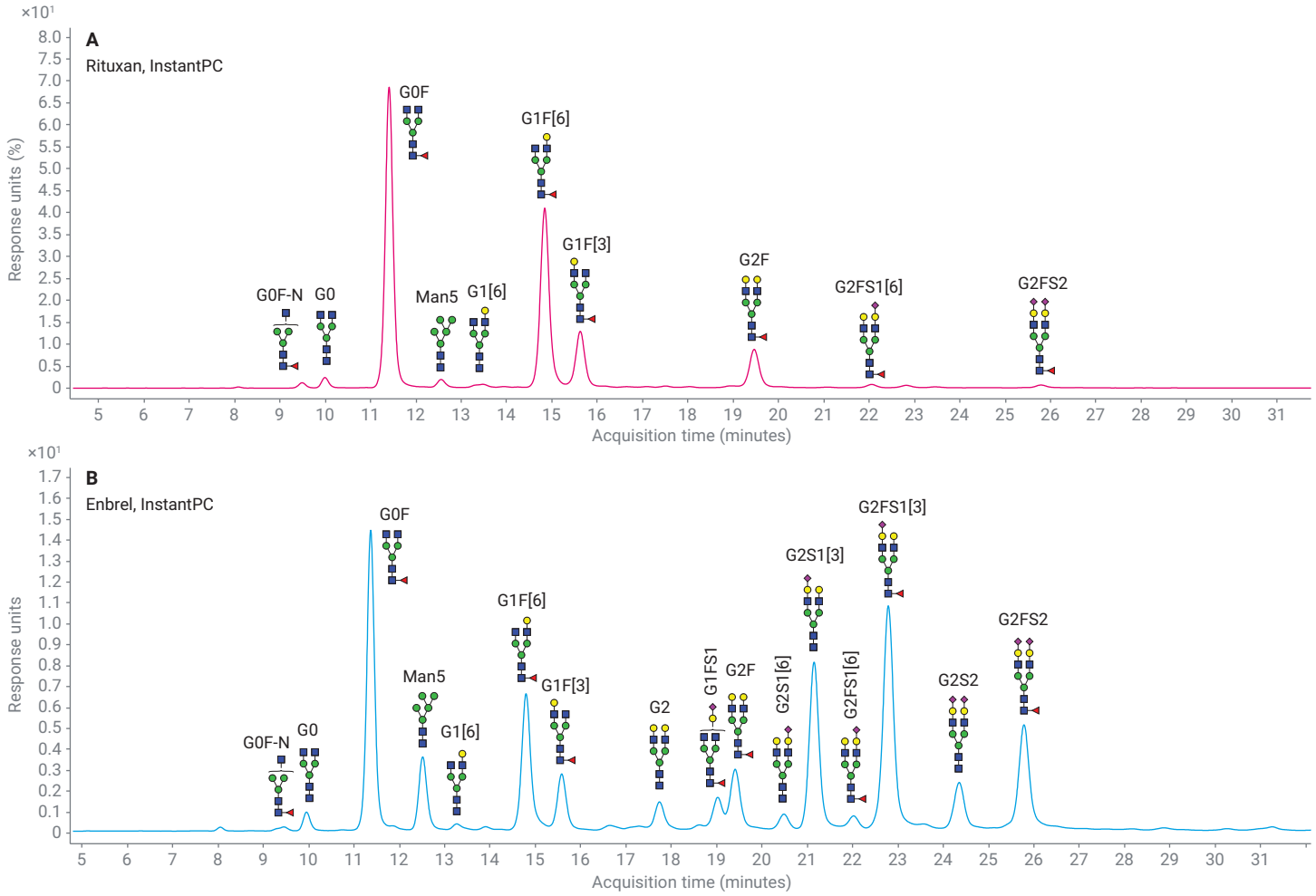

Figure 3. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with InstantPC. N-Glycan relative percent areas are shown in Table 3 and Table 4, n = 4.

Table 3. Figure 3A relative % area, SD, and %CV values for Rituxan N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

Table 4. Figure 3B relative % area, SD, and %CV values for Enbrel N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

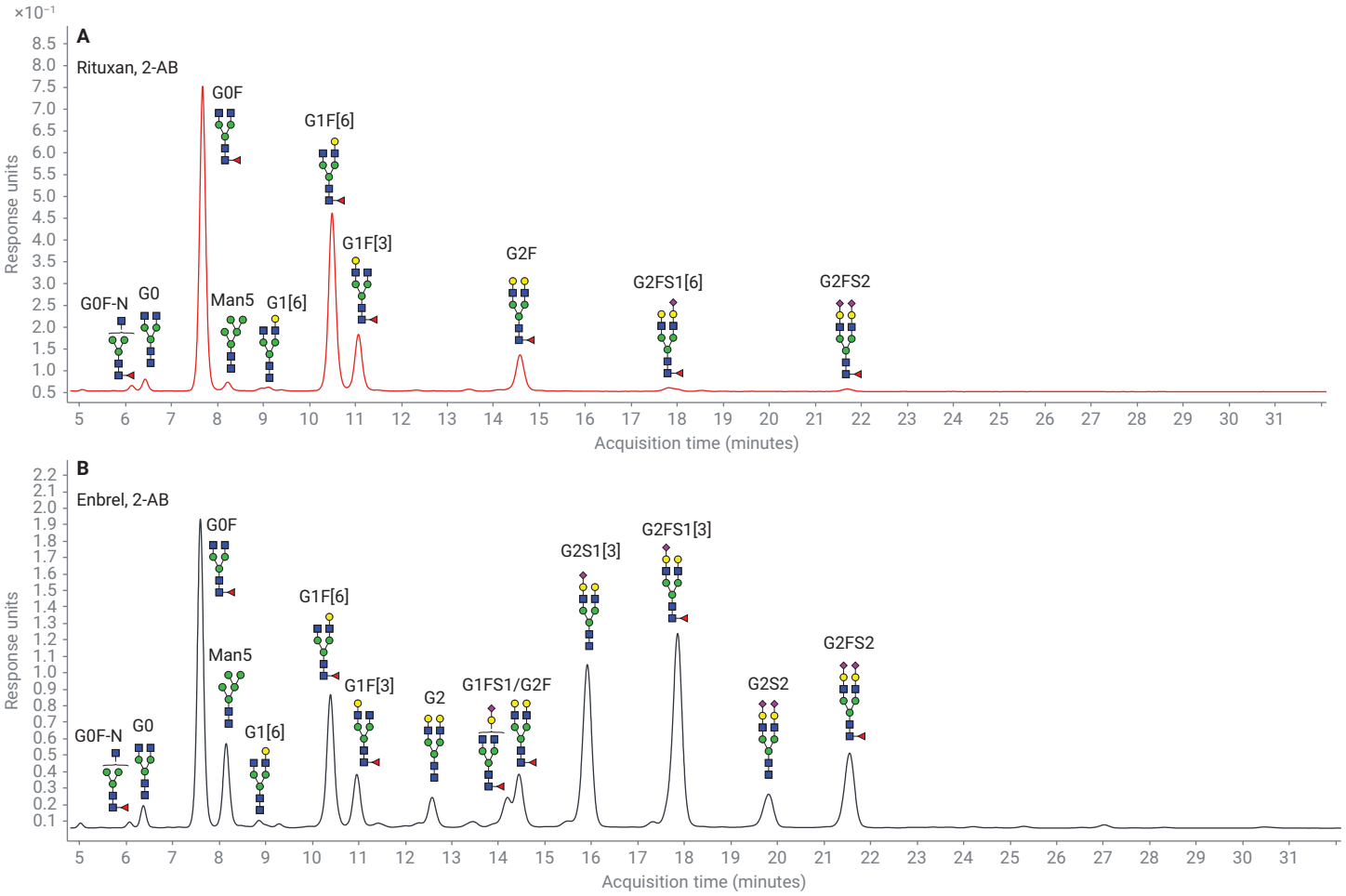

Figure 4. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with 2-AB. N-Glycan relative percent areas are shown in Table 5 and Table 6, n = 4.

Table 5. Figure 4A relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.78	0.09	11.94
G0	1.64	0.05	3.12
G0F	44.89	0.39	0.87
Man5	1.54	0.14	8.83
G1F[6]	31.39	0.09	0.27
G1F[3]	10.40	0.14	1.34
G2F	7.52	0.16	2.10
G2FS1	1.17	0.03	2.13
G2FS2	0.67	0.02	3.58

Table 6. Figure 4B relative % area, SD, and %CV values for Enbrel N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.32	0.02	7.44
G0	1.27	0.07	5.34
G0F	20.18	0.45	2.22
Man5	5.50	0.34	6.17
G1[6]	0.45	0.02	3.89
G1F[6]	10.35	0.33	3.18
G1F[3]	3.92	0.17	4.39
G2	2.21	0.15	6.78
G2F/G1FS1	7.00	0.25	3.63
G2S1	15.19	0.17	1.09
G2FS1	20.10	0.32	1.59
G2S2	4.19	0.25	5.95
G2SF2	9.35	0.74	7.93

FLD and MS detection of InstantPC and 2-AB N-Glycans

InstantPC displays higher fluorescence and MS signal compared to 2-AB (Figure 5), when using the same amount of glycoprotein starting material (40 µg), and injecting the same relative volume for HILIC separations (1 µL of 100 µL kit eluent). Individual spectra for InstantPC and 2-AB labeled Man5 illustrates the higher MS signal of InstantPC (Figure 6).

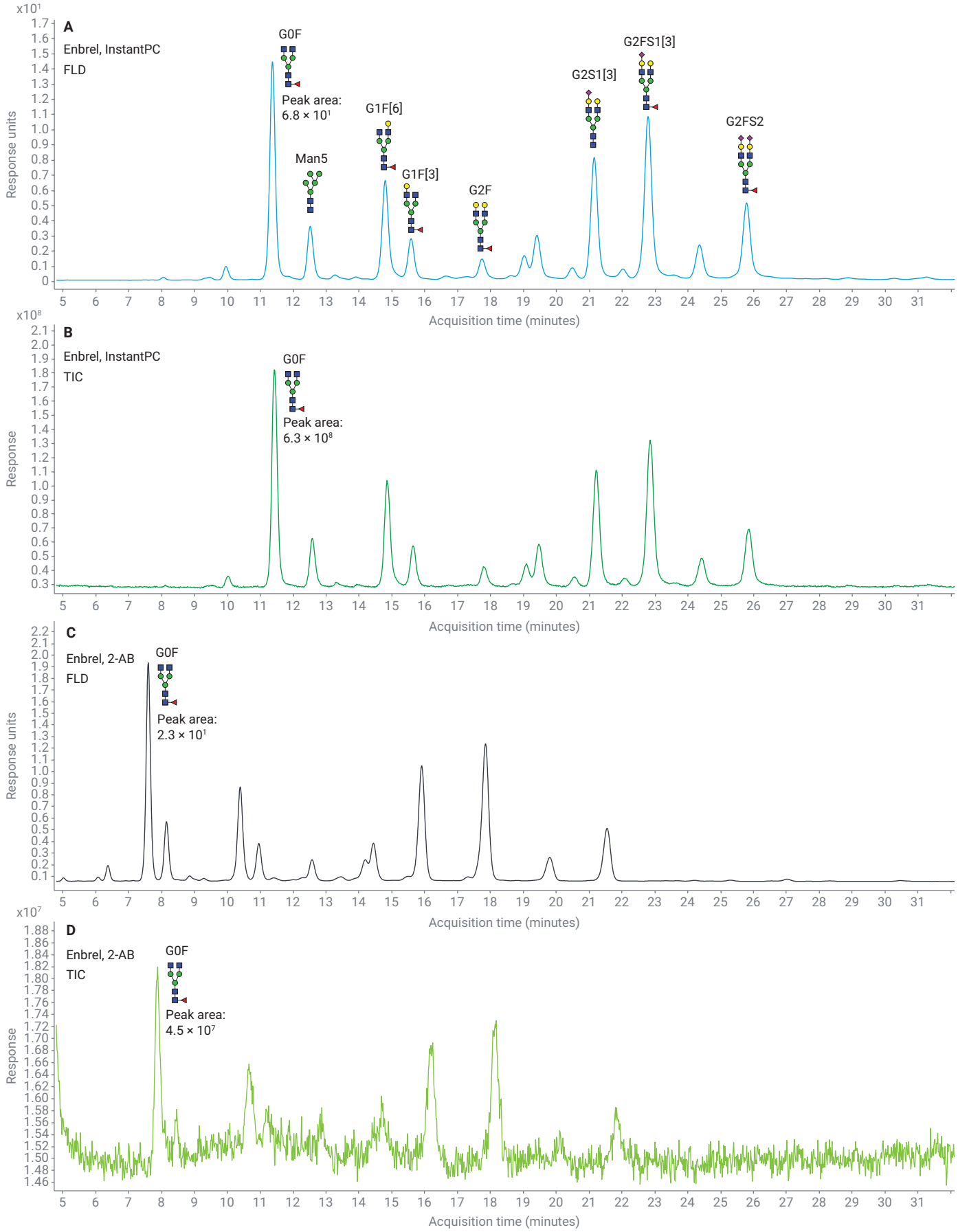

Figure 5. FLD and MS of InstantPC and 2-AB labeled N-glycans from Enbrel. A) InstantPC FLD; B) InstantPC TIC (total ion chromatogram); C) 2-AB FLD; D) 2-AB TIC.

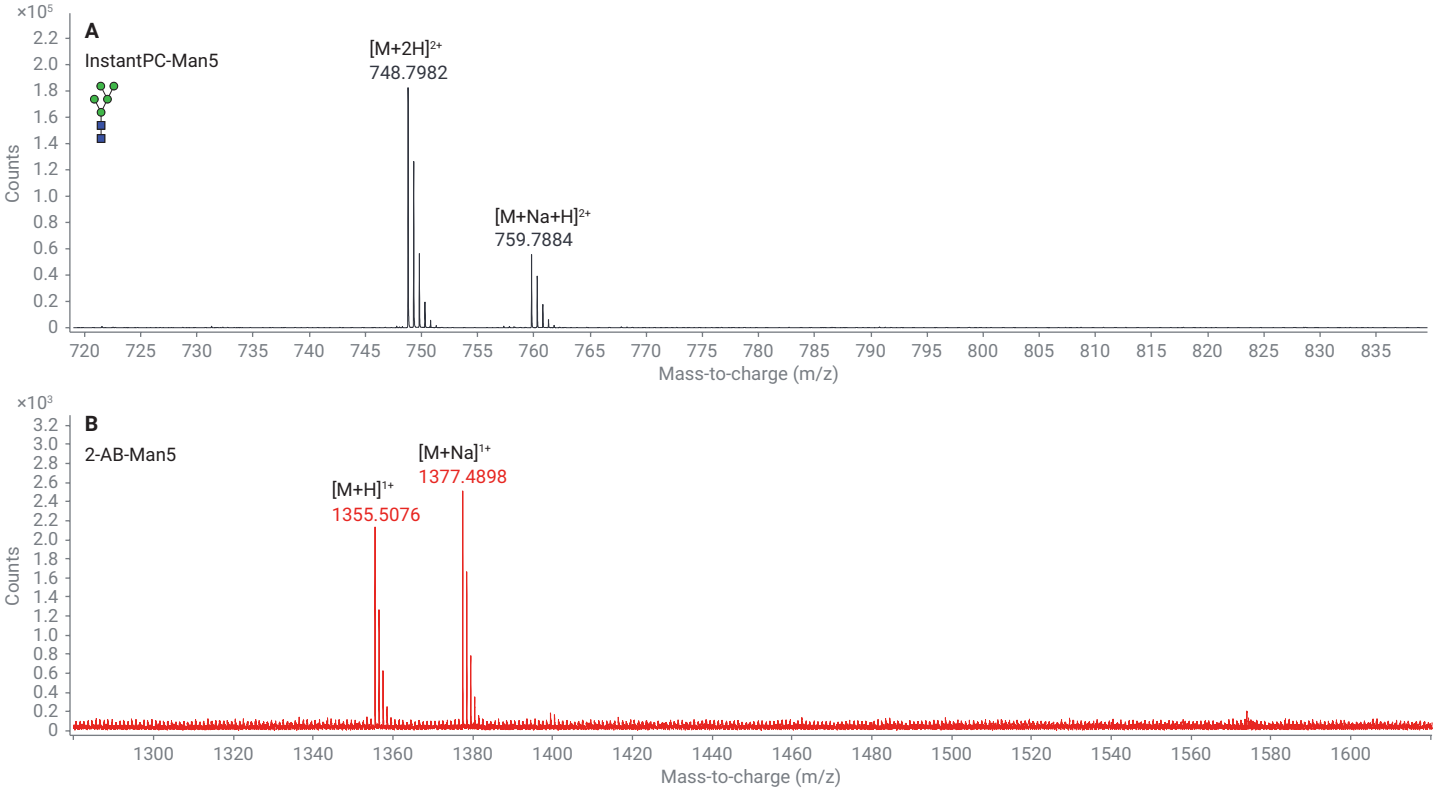

Figure 6. Mass spectrum comparison of Man5 from Enbrel, labeled with A) InstantPC and B) 2-AB.

Conclusion

Glycosylation is a feature of many biotherapeutic proteins and is often a CQA that must be monitored. N-Glycan analysis is important in the development and production of therapeutic proteins. Gly-X N-glycan sample preparation workflows enable a five minute release of N-linked glycans suitable for labeling both by glycosylamine labeling with InstantPC and reductive amination chemistry with 2-AB. These workflows allow for instant glycosylamine labeling with InstantPC or no dry down on-matrix reductive amination labeling with 2-AB. Glycan species were profiled by relative fluorescence peak area % and peak assignments confirmed by high resolution mass spectrometry. Compared to 2-AB, InstantPC labeled glycans display higher FLD signal and greater MS ionization efficiency in positive mode, allowing for confident detection of low abundance glycan species. Although the performance benefits of InstantPC are clear, 2-AB is an N-glycan label that has been used for many years. Therefore, a rapid 2-AB workflow enables continuity with historical 2-AB N-glycan data sets.

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