Application Note

Pharma/Biopharma

Comparative Analysis of Agilent AdvanceBio Gly-X N-Glycan Prep

With InstantPC and Waters GlycoWorks *Rapi*Fluor-MS N-Glycan Kits using select biotherapeutics

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Abstract

Characterizing glycosylation is a necessary checkpoint during the development and manufacturing of biotherapeutics. Analysis of N-glycan content has become an essential part of the development process to ensure consistency in stability, safety, immunogenicity, and serum half-life of these products. Monoclonal antibodies (mAbs) are one of the main products in the pipeline of the biopharmaceutical industry. Released N-glycan analysis of a therapeutic antibody with characterization using hydrophilic interaction liquid chromatography (HILIC) and a labeling reagent that facilitates fluorescence (FLD) and mass spectrometry (MS) detection is commonly used as a checkpoint to ensure product quality and consistency from batch-to-batch preparations. There are N-glycan sample preparation kits that help simplify this analysis, such as the AdvanceBio Gly-X N-glycan prep with InstantPC kit from Agilent Technologies and the GlycoWorks *Rapi*Fluor-MS N-glycan kit from Waters Corporation. These kits offer end-to-end sample preparation for N-glycan analysis, consisting of three modules: release of N-glycans (denaturing/deglycosylation), labeling, and purification (cleanup). Although the deglycosylation and cleanup modules remain similar, the labeling modules differ in their fluorescent dye composition, affecting FLD and MS sensitivity of the released N-glycans. This application note presents a side-by-side comparison of the AdvanceBio and GlycoWorks kits for the preparation and analysis of released N-glycans from different biotherapeutic glycoproteins, such as MabThera, Enbrel, and Orencia, using HILIC mode separation and MS on Agilent LC/FLD/MS instrumentation, including the Agilent AdvanceBio Glycan Mapping column and detection by FLD and Q-TOF. This application note will help users assess the reproducibility and throughput of both workflows, particularly assessing fluorescence signal and MS ionization efficiency of released and labeled N-glycans from the same amount of glycoprotein, aiding in their biotherapeutics and drug development process.

Introduction

Glycosylation is a critical quality attribute (CQA) for the development of biotherapeutic proteins, as the structure of N-linked glycans can strongly influence protein function.[1] Characterization of N-glycans is commonly performed using enzymatic release and labeling of N-glycans with a signal-enhancing tag, and LC/MS data collection and interpretation.[2] This application note discusses the reproducibility and throughput of the AdvanceBio Gly-X N-glycan prep with InstantPC (IPC) kit and the GlycoWorks *Rapi*Fluor-MS (RFMS) N-glycan kit for assessing the N-glycan profile of different biotherapeutic glycoproteins, including monoclonal antibodies (mAbs) and Fc-fusion proteins. Both kits offer end-to-end workflows for N-glycan analysis, consisting of three modules—release of N-glycans (denaturing/deglycosylation), labeling, and purification (cleanup)—that allow 24 to 96 glycan samples to be prepared within 1 hour and characterized immediately after using HILIC with FLD and MS detection (Figure 1).

Although both kits have similar steps for enzymatic release and labeling of N-glycans, they differ in the composition of their fluorescent dyes, the amount of protein required for the workflow, and cleanup conditions, affecting FLD and MS sensitivity. In all experiments, N-glycans were released by PNGase F and fluorescently labeled with IPC or RFMS containing an N-hydroxysuccinimide carbamate functional group for rapid tagging of N-glycans (modifying the glycosylamine into a stable urea linkage), and a basic tertiary amine for high MS signal.[3] For the AdvanceBio kit, N-glycans were processed from 15 to 40 µg of protein and labeled with IPC, while for the GlycoWorks kit, N-glycans were processed from 15 µg of protein and labeled with RFMS (based on the user manual). The RFMS label had quinoline while the IPC label had procaine as fluorophores, resulting in a significantly higher (>3×) FLD signal for IPC-labeled N-glycans compared to RFMS-labeled N-glycans (depending on the model of FLD detector being used in the LC/MS setup).

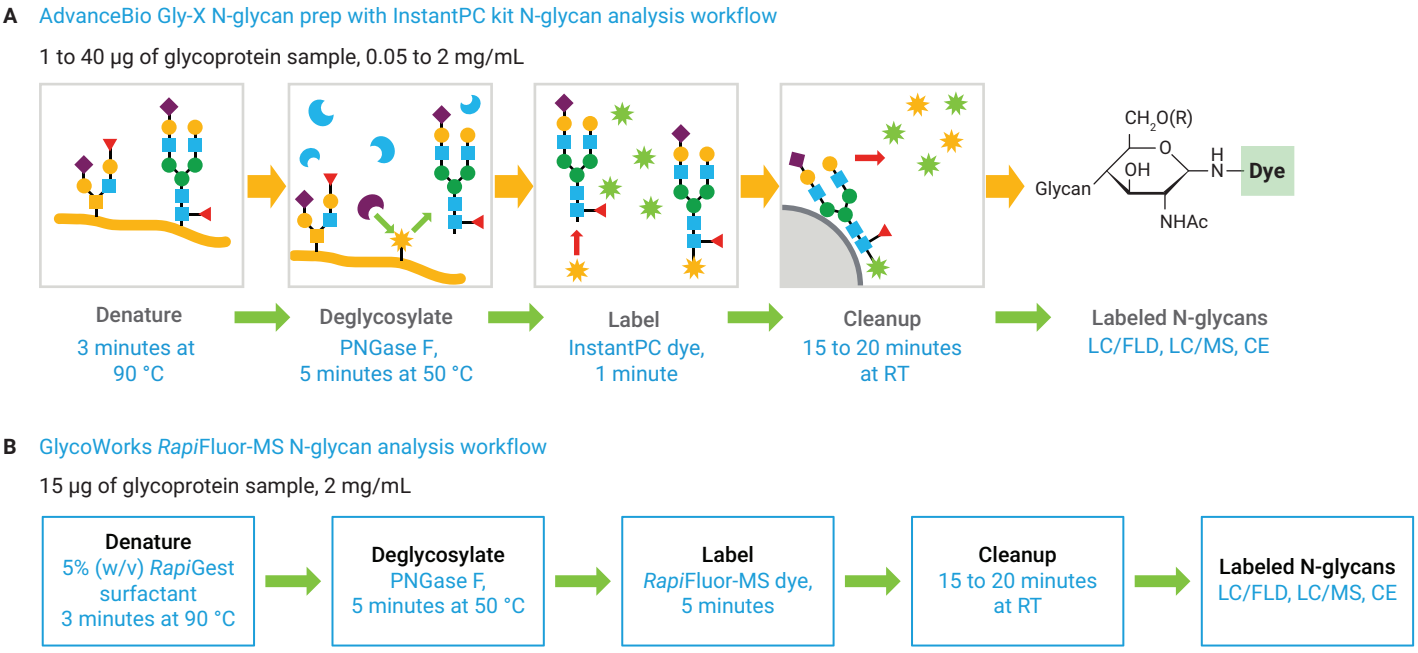

Figure 1. Released N-glycan analysis workflow for the (A) Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit and (B) Waters GlycoWorks *Rapi*Fluor-MS N-glycan kit.

Reagent storage guidelines provided in the manufacturer's user manual are essential for sample preparation flexibility and experiment planning (Table 1). In the AdvanceBio kit, the denaturant solution is stable for months at 4 °C, whereas, in the GlycoWorks kit, the *Rapi*Gest surfactant is stable for only one week at 2 to 8 °C, after reconstitution. Furthermore, the IPC dye solution is stable for three months at −20 °C and can withstand 10 freeze-thaw cycles compared to RFMS dye solution, which can only withstand one freeze-thaw cycle. Apart from storage guidelines, the AdvanceBio kit provides more flexibility in terms of the glycoprotein amount that can be used, and the minimum steps necessary for injection on LC/MS. The results in this application note will also summarize the advantage of higher fluorescence and MS signals that can be achieved using the AdvanceBio kit, and a simpler way of confirming the N-glycans using a range of IPC-labeled individual standards and libraries (Table 1).

Experimental

Materials

The Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit, 24 count (part number GX24-IPC) consists of three modules, including the Gly-X deglycosylation module (part number GX24-100), Gly-X InstantPC labeling module (part number GX24-101), and the Gly-X InstantPC cleanup module (part number GX96-102).

The Waters GlycoWorks *Rapi*Fluor-MS N-glycan kit, 24 count (part number 176003713) consists of three modules, including GlycoWorks deglycosylation module (part number 186008939), *Rapi*Fluor-MS labeling module (part number 186008091), and GlycoWorks RFMS cleanup module (part number 186008913).

Table 1. Features, advantages, and benefits of the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit compared to the Waters GlycoWorks *Rapi*Fluor-MS N-glycan kit.

Features of AdvanceBio Gly-X InstantPC	Benefits	Advantages of AdvanceBio Gly-X InstantPC Versus GlycoWorks *Rapi*Fluor-MS
1. AdvanceBio Gly-X denaturant is stable in solution (as shipped) for months at 4 °C	– Gly-X denaturant is shelf stable at 4 °C, no reconstitution needed – Flexibility to run 1 to 96 samples at a time; not limited by having to use an entire vial at one time – Enables lower throughput use	– GlycoWorks *Rapi*Gest can be stored for only one week, at 2 to 8 °C, after reconstitution – Four vials for a 96-count kit (4 × 24 samples), three vials for a 24-count kit (3 × 8 samples) – Limits flexibility for low-throughput use
2. Reconstituted InstantPC N-glycan dye can be stored for three months at −20 °C after reconstitution 3. Up to 10 freeze-thaw cycles	Flexibility with InstantPC dye use	– GlycoWorks RFMS allows up to one freeze-thaw cycle after reconstitution only. Suggested storage in 12 µL aliquots at −80 °C (no time frame for expiry). – Dye solvent must be used straight away after opening ampoule Dye reconstitution volume is incorrect for the protocol adapted for the use of 200 µL PCR tubes (66 µL) when, regardless of tube size, it should be 131 µL.
4. Cleanup plate storage at room temperature	Easy Gly-X cleanup plate storage	GlycoWorks cleanup plate storage is more complicated. After partial use, store open pouch, squeeze out any air, fold over the open end of the pouch, and seal with tape. Store in a desiccator.
5. Gly-X glycoprotein amount: Up to 40 µg standard protocol, can go up to 100 µg depending on the molecule	Flexibility to include more starting protein with Gly-X InstantPC	GlycoWorks kit can go up to 15 µg
6. Gly-X injection volume: Inject 1 µL eluent directly or dilute with DMF/ACN for larger injection volumes	Simpler route to injecting labeled glycan samples with Gly-X InstantPC	GlycoWorks: Dilute with DMF/ACN before injection, or dry down SPE eluent for 1 µL injections.
7. Fluorescence	Increased ability to examine less-abundant glycan species with Gly-X InstantPC using FLD	InstantPC has a brighter FLD signal than RFMS for some biotherapeutics (depending on the the model of FLD detector).
8. MS signal		Comparable to RFMS but Gly-X can take more protein into the preparation.
9. Glycan standards	Limited range or RFMS glycan standards (human IgG, fetuin, high-mannose libraries and ladder)	Extensive range of AdvanceBio InstantPC glycan standards and libraries.[4]

Controls tested

- Agilent AdvanceBio InstantPC maltodextrin ladder (part number GKPC-503)
- *Rapi*Fluor-MS dextran calibration ladder (part number 186007982)
- Agilent AdvanceBio InstantPC Human IgG N-glycan library (part number GKPC-005)
- *Rapi*Fluor-MS glycan performance test standard (human IgG) (part number 186007983)

Glycoproteins tested

- Agilent NISTmAb (part number 5191-5744)
- Agilent CHO mAb (part number GKP-020)
- Enbrel (etanercept; batch number X44248)
- Orencia (abatacept; batch number ABS2028)
- Intact mAb Mass Check Standard provided with the Waters kit

MabThera (rituximab; lot number M190170) HPLC-grade acetonitrile and water were purchased from Honeywell Research Chemicals.

Instrumentation

Labeled N-glycan samples were separated using an Agilent AdvanceBio Glycan Mapping column (Table 2 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 II fluorescence detector (G1321B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (parameters in Table 3)

Methods

N-glycan sample preparation

The AdvanceBio Gly-X N-glycan prep with InstantPC kit and the GlycoWorks *Rapi*Fluor-MS N-glycan kit were used to prepare labeled N-glycans from mAbs, CHO, Enbrel, NIST, Orencia, Intact mAb Mass Check Standard, and Ritux; 15 and 40 µg protein per preparation for the AdvanceBio Gly-X N-glycan prep with InstantPC kit and 15 µg for the GlycoWorks *Rapi*Fluor-MS N-glycan kit.

Deglycosylation module

The in-solution enzymatic deglycosylation of protein samples was carried out according to the instructions of the AdvanceBio Gly-X N-glycan prep with IPC kit or the GlycoWorks RFMS N-glycan kit.

1. Dilute protein samples (15 µg, 7.5 µL of 2 mg/mL for both kits and 40 µg, 20 µL of 2 mg/mL for the AdvanceBio kit) with water to make a final volume of 20 µL.
2. For the AdvanceBio kit, add 2 µL of Gly-X denaturant. For the GlycoWorks kit, add 3 µL of buffered 5% (w/v) *Rapi*Gest surfactant solution to the plate. Mix thoroughly.
3. Incubate samples at 90 °C for 3 minutes.
4. For the AdvanceBio kit, add 2 µL of N-glycanase to the 20 µL of protein sample and mix thoroughly. For the GlycoWorks kit, add 1.2 µL of GlycoWorks Rapid PNGase F and mix thoroughly.
5. Incubate at 50 °C for 5 minutes.

Rapid labeling with InstantPC/*Rapi*Fluor-MS

6. For the AdvanceBio kit, add 5 µL of the IPC dye solution to the prepared samples and mix thoroughly. For the GlycoWorks kit, add 6 µL of the RFMS reagent solution to the prepared samples and mix thoroughly.
7. For the AdvanceBio kit, incubate samples at 50 °C for 1 minute. For the GlycoWorks kit, incubate samples at room temperature for 5 minutes.

InstantPC-labeled glycan purification

8. Add 150 µL of the Load/Wash solution (2.5% formic acid/97.5% acetonitrile) to each sample, and mix thoroughly.
9. Transfer the entire sample (~172 µL) to each well of the Gly-X cleanup plate containing 400 µL of Load/Wash solution.
10. Wash samples three times with 600 µL of Load/Wash solution after passing the solution through the cleanup plate by applying a vacuum (based on the user manual).
11. Elute the IPC-labeled N-glycans with 100 µL of Gly-X InstantPC eluent (160 mM ammonium formate/10% acetonitrile (v/v), pH 4.4).
12. Analyze the collected N-glycan solutions (~100 µL) immediately without further treatment.

*Rapi*Fluor-MS-labeled glycan purification

13. Dilute samples with 179 µL of acetonitrile.
14. Equilibrate wells to be used on the GlycoWorks µElution plate with 200 µL of water, followed by 200 µL of 15:85 water:acetonitrile.
15. Transfer the entire sample (~200 µL) to each well of the GlycoWorks µElution plate.
16. Wash samples two times with 600 µL of wash solution (1:9:90 formic acid:water:acetonitrile) after passing the solution through the cleanup plate by applying a vacuum (based on the user manual).
17. Elute the RFMS-labeled N-glycans with three 30 µL volumes of GlycoWorks *Rapi*Fluor eluent (200 mM ammonium acetate/ 5% acetonitrile (v/v)).

18. Analyze the collected N-glycan solutions (90 µL).

HILIC/FLD analysis of InstantPC- and *Rapi*Fluor-MS-labeled N-glycans

The profiles of IPC- and RFMS-labeled N-glycans from the protein samples were determined by HILIC/FLD using an AdvanceBio Glycan Mapping column (2.1 × 150 mm, 1.8 µm, part number 859700-913), equipped with a 1290 Infinity II LC system with in-line fluorescence detection (Table 2), and coupled to an AdvanceBio 6545XT LC/Q-TOF (Table 3). The IPC- and RFMS-labeled glycan samples, without any further treatment, were injected at a volume of 1 µL. The N-glycans were separated with 50 mM ammonium formate (pH 4.4) as solvent A, and acetonitrile as solvent B. The HPLC system was equilibrated with 50 mM ammonium formate (pH 4.4) and acetonitrile, at a flow rate of 0.5 mL/min. After, the separation was carried out by a linear gradient of 80 to 40% acetonitrile (v/v) in a 50-minute analytical run, at a flow rate of 0.5 mL/min. Samples were maintained at 4 °C before injection, and the column temperature was set to 40 °C. All HILIC separations were conducted under the conditions described in Table 2. A fixed-flow splitter (IDEX Health & Science, part number UH-427) was used after 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.

Table 2. Agilent 1290 Infinity II LC HILIC/FLD conditions.

Parameter	Value
Column	Agilent AdvanceBio Glycan Mapping column, 300 Å, 2.1 mm × 150 mm, 1.8 µm (p/n 859700-913)
Column Temperature	40 °C
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) Acetonitrile
Gradient Program	InstantPC- and *Rapi*Fluor-MS-labeled glycans Time (min) %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 0.15 or 0.4 µg protein)
Detection	Agilent 1260 Infinity II fluorescence detector InstantPC: λ_{Ex} 285 and λ_{Em} 345 nm *Rapi*Fluor-MS: λ_{Ex} 265 and λ_{Em} 425 nm

Table 3. Agilent 6545XT AdvanceBio LC/Q-TOF parameters.

Agilent 6545XT AdvanceBio LC/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/s
Acquisition Mode	High resolution (4 GHz)

Results and discussion

HILIC Separation of InstantPC- and *Rapi*Fluor-MS-labeled N-Glycans

HILIC separation of N-glycans from six biotherapeutics (CHO mAb, NIST mAb, Rituxan, mouse IgG1 or GlycoWorks intact mAb mass check standard, Enbrel, and Orencia) labeled with IPC or RFMS resulted in well-resolved peaks for major glycan species with the 60-minute method from Table 2 (Figures 2 and 3). CHO mAb, NISTmAb, Rituxan, and IgG1 have N-glycan profiles similar to monoclonal antibodies with one N-glycosylation site in the Fc region. The profiles predominantly consist of neutral, complex biantennary N-glycans with core fucose, followed by a low abundance of Man5, and almost negligible presence of sialylated glycans (Figures 2 and 3A to 3D). The N-glycan profile of Enbrel and Orencia—Fc fusion proteins—contain a higher level of G2FS1 and G2FS2 sialylated glycans. This is due to two more N-glycosylation sites in their fusion partner—TNF-α receptor (TNFR) or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) respectively—of the extracellular domain, which is linked to the Fc portion consisting of a single N-glycan site (Figures 2 and 3E to 3F).[5] Orencia also had the highest amount of G2F glycan present in the glycosylation profile compared to the other five biotherapeutics (Figures 2F and 3F).

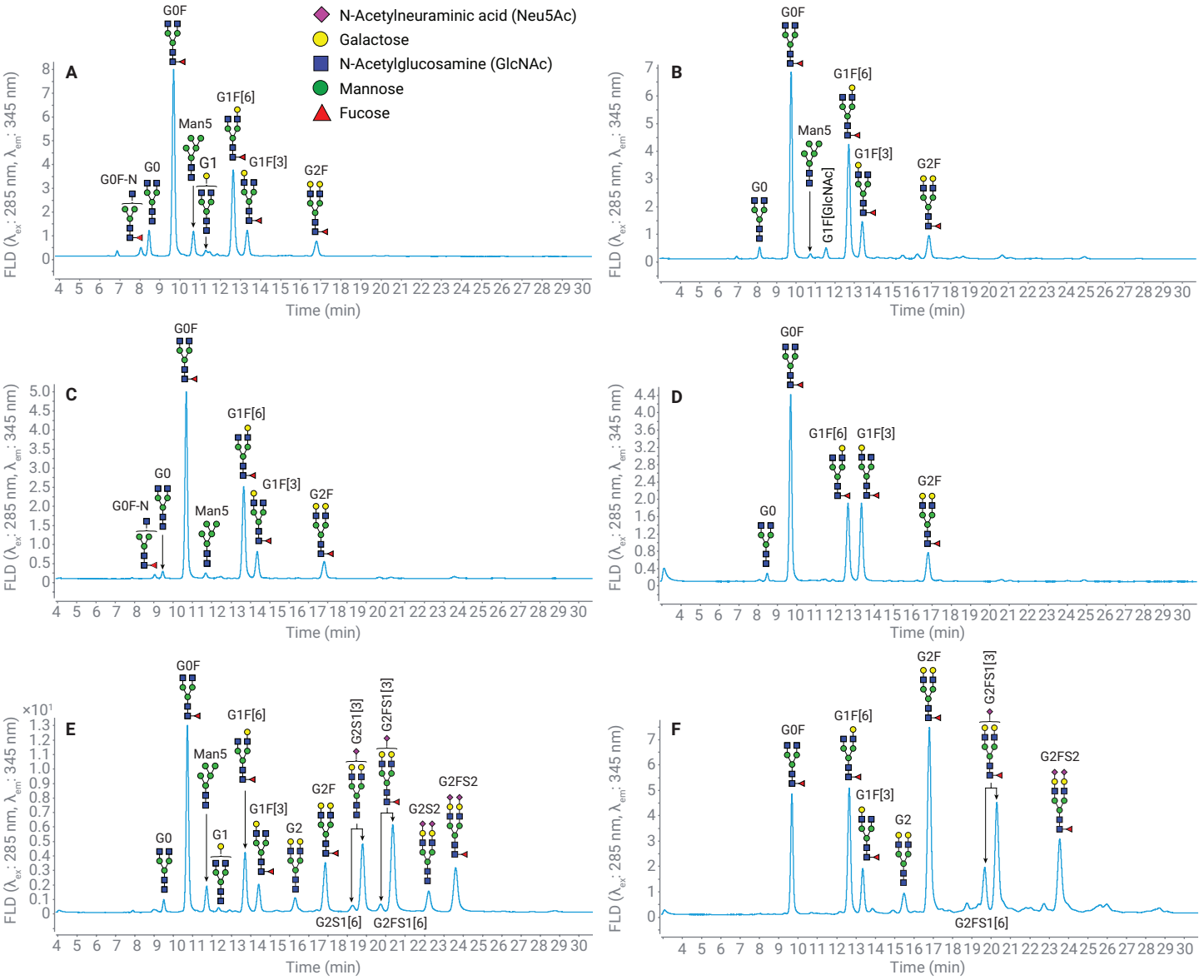

Figure 2. HILIC-UHPLC fluorescence profile of (A) CHO mAb, (B) NIST mAb, (C) Ritux, (D) IgG1, (E) Enbrel, and (F) Orencia labeled with InstantPC (15 µg). N-glycan relative areas (%) are shown in Table 4, n = 3.

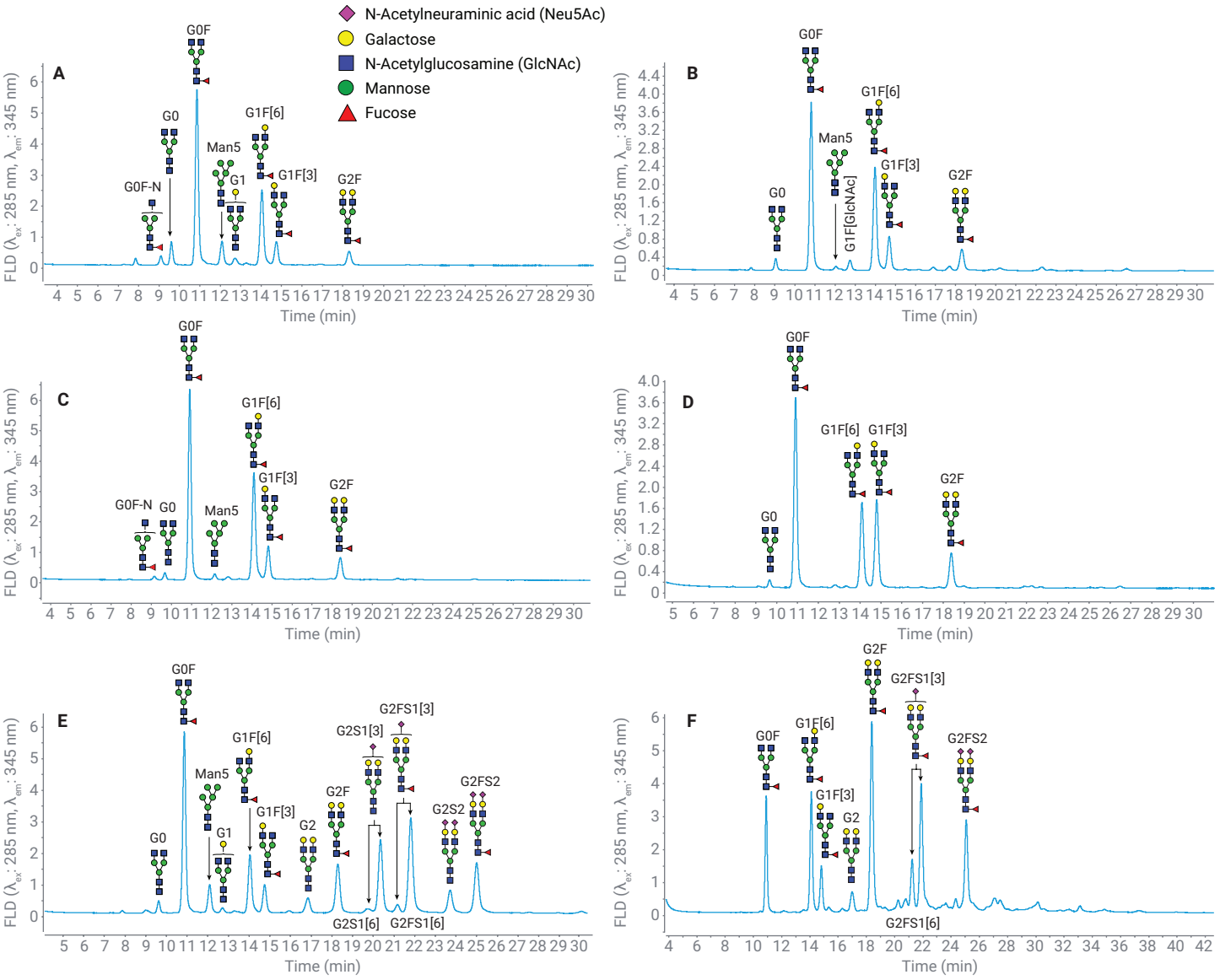

Figure 3. HILIC-UHPLC fluorescence profile of (A) CHO mAb, (B) NIST mAb, (C) Ritux, (D) IgG1, (E) Enbrel, and (F) Orencia labeled with *Rapi*Fluor-MS (15 µg). N-glycan relative areas (%) are shown in Table 4, n = 3.

The HILIC retention time of IPC N-glycans is shorter than for RFMS N-glycans, with the former eluting approximately one minute earlier than the latter. The elution order was comparable for both IPC- and RFMS-labeled N-glycan species. For both IPC and RFMS labels, CHO mAb, NIST mAb, and Rituxan showed well-separated glycan critical pairs such as G0F/Man5 and Man5/G1, which are often monitored for biotherapeutic development process. Furthermore, IgG1 mAb showed G1F isomers, G1F[6] and G1F[3], present in equal abundance, and were well separated using both labels. The Fc fusion proteins, Enbrel and Orencia, helped us to analyze the HILIC separation of IPC- and RFMS-labeled sialylated N-glycans. Both showed separation of G2S1 and G2FS1 isomers, G2S1[6] and G2S1[3]/G2FS1[6] and G2FS1[3] respectively, as well as separated G2S2 and G2FS2 glycans, leading to confident determination of relative percent composition. Relative percent areas, standard deviation, and relative standard deviation (%CV) are reported in Tables 4 and 5, for three sample preparation replicates.

Table 4. Figure 2 relative area (%), SD, and %CV values for A) CHO mAb, B) NIST mAb, C) Ritux, D) IgG1, E) Enbrel, and F) Orencia, labeled with InstantPC and *Rapi*Fluor-MS (15 µg), n = 3.

A

	IPC			RFMS		
CHO mAb	Average Relative Area (%)	SD	%CV	Average Relative Area (%)	SD	%CV
G0F-N	1.91	0.10	5.20	2.40	0.15	6.36
G0	5.71	0.19	3.29	5.37	0.08	1.56
G0F	46.66	1.15	2.46	47.95	0.80	1.68
Man5	6.48	0.39	6.04	6.84	0.19	2.77
G1	2.27	0.04	1.81	1.89	0.19	9.86
G1F[6]	25.14	0.76	3.03	23.91	0.29	1.21
G1F[3]	6.93	0.70	10.05	6.75	0.07	1.07
G2F	4.90	0.39	8.01	4.89	0.10	1.96

B

	IPC			RFMS		
NIST mAb	Average Relative Area (%)	SD	%CV	Average Relative Area (%)	SD	%CV
G0	2.58	0.52	19.99	2.62	0.15	5.77
G0F	46.24	1.87	4.03	45.62	0.87	1.91
Man5	0.93	0.09	9.89	0.73	0.16	22.12
G1F-GlcNAc	2.68	0.13	4.75	2.39	0.58	24.43
G1F[6]	31.21	1.34	4.28	31.63	0.72	2.27
G1F[3]	9.35	0.64	6.83	9.75	0.25	2.61
G2F	7.00	0.66	9.41	7.28	0.39	5.40

C

	IPC			RFMS		
Ritux	Average Relative Area (%)	SD	%CV	Average Relative Area (%)	SD	%CV
G0F-N	0.90	0.09	10.10	1.47	0.12	8.23
G0	1.64	0.02	1.51	48.54	0.43	0.88
G0F	52.14	0.29	0.56	1.28	0.06	4.87
Man5	1.43	0.12	8.61	32.23	0.39	1.21
G1F[6]	30.15	0.19	0.63	9.26	0.09	0.92
G1F[3]	8.37	0.24	2.84	7.23	0.25	3.40
G2F	6.29	0.13	2.12	1.47	0.12	8.23

D

	IPC			RFMS		
IgG1	Average Relative Area (%)	SD	%CV	Average Relative Area (%)	SD	%CV
G0	1.59	0.06	3.65	1.71	0.23	13.29
G0F	45.33	0.40	0.89	43.62	1.06	2.44
G1F[6]	21.91	0.49	2.25	21.94	0.30	1.36
G1F[3]	21.93	0.63	2.89	22.82	0.37	1.64
G2F	9.25	0.09	0.92	9.91	0.21	2.07

E

	IPC			RFMS		
Enbrel	Average Relative Area (%)	SD	%CV	Average Relative Area (%)	SD	%CV
G0	1.33	0.03	2.46	1.43	0.08	5.67
G0F	23.83	0.29	1.22	25.08	0.94	3.74
Man5	3.58	0.15	4.15	3.92	0.15	3.85
G1	0.63	0.00	0.66	0.61	0.11	18.02
G1F[6]	9.14	0.06	0.60	9.27	0.30	3.27
G1F[3]	4.10	0.01	0.26	4.14	0.09	2.19
G2	2.62	0.04	1.54	2.66	0.06	2.13
G2F	8.51	0.09	1.02	8.57	0.50	5.83
G2S1[6]	1.18	0.07	5.72	0.92	0.55	60.07
G2S1[3]	12.33	0.24	1.94	12.03	0.66	5.52
G2FS1[6]	1.20	0.02	1.46	1.00	0.10	9.78
G2FS1[3]	17.46	0.10	0.56	16.70	0.98	5.87
G2S2	4.11	0.03	0.62	3.61	0.10	2.81
G2FS2	9.98	0.16	1.59	9.99	0.32	3.24

F

	IPC			RFMS		
Orencia	Average Relative Area (%)	SD	%CV	Average Relative Area (%)	SD	%CV
G0F	11.95	0.19	1.56	12.04	0.27	2.25
G1F[6]	15.00	0.12	0.77	13.90	0.27	1.92
G1F[3]	5.16	0.16	3.01	5.32	0.05	0.98
G2	2.89	0.17	6.04	3.02	0.02	0.63
G2F	27.16	0.70	2.58	26.30	0.61	2.32
G2FS1[6]	5.58	0.27	4.83	6.04	0.20	3.37
G2FS1[3]	17.24	0.25	1.45	17.54	1.05	5.99
G2FS2	13.96	0.12	0.84	14.76	0.10	0.69

FLD detection of InstantPC- and *Rapi*Fluor-MS-labeled N-glycans

Generally, IPC and RFMS display similar fluorescence signal (Figure 4), when using the same amount of glycoprotein starting material (15 µg) and injecting a similar relative volume for HILIC separations (1 µL of 100 µL AdvanceBio kit eluent or 1 µL of 90 µL GlycoWorks kit eluent) on the Agilent system. However, slightly higher fluorescence signal for CHO mAb, NIST mAb, Enbrel, and Orencia labeled with IPC was observed compared to their RFMS counterparts (15 µg). By increasing the glycoprotein amount to 40 µg, as directed in the AdvanceBio kit, the biotherapeutics displayed a significantly higher fluorescence signal, with Enbrel showing the highest response (Figures 4 and 5).

In comparison to the Agilent system, using a Waters system for 15 µg of glycans from Rituxan or Enbrel shows a ~3× increase in fluorescence signal for IPC glycans compared to RFMS (Figure 6). However, for both, higher fluorescence signal was achieved when the biotherapeutics were labeled with IPC from 40 µg of glycoprotein.

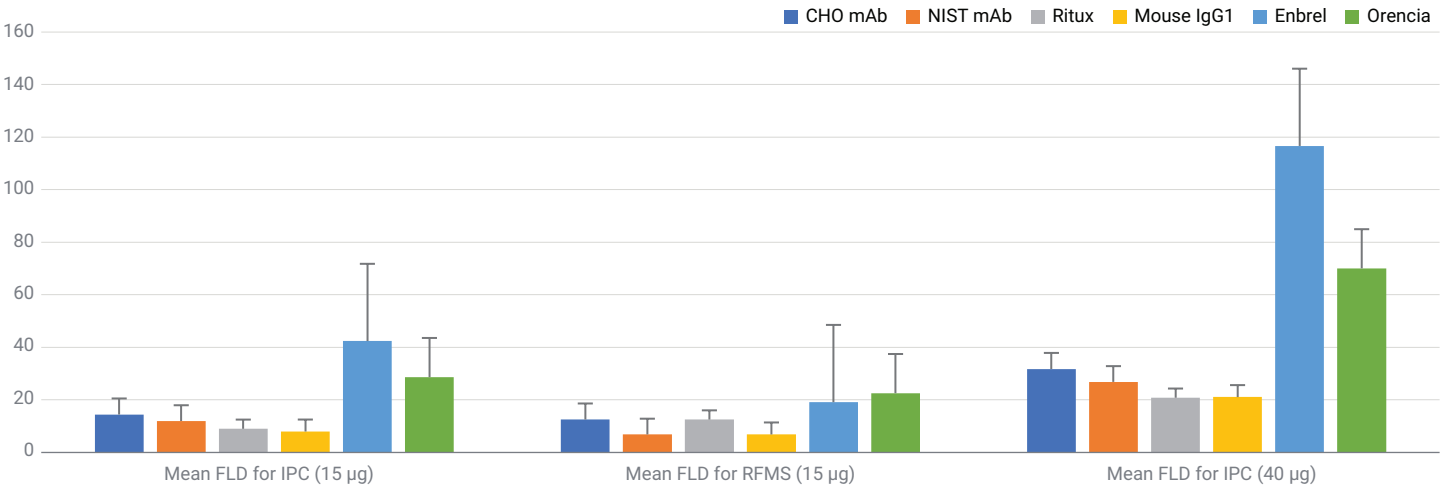

Figure 4. Total fluorescence signal for all biotherapeutics labeled with InstantPC (15 and 40 µg) and *Rapi*Fluor-MS (15 µg) from Figures 2 to 3, and 7, respectively, using the Agilent AdvanceBio Glycan Mapping column and 1290 Infinity II LC system with in-line fluorescence detection.

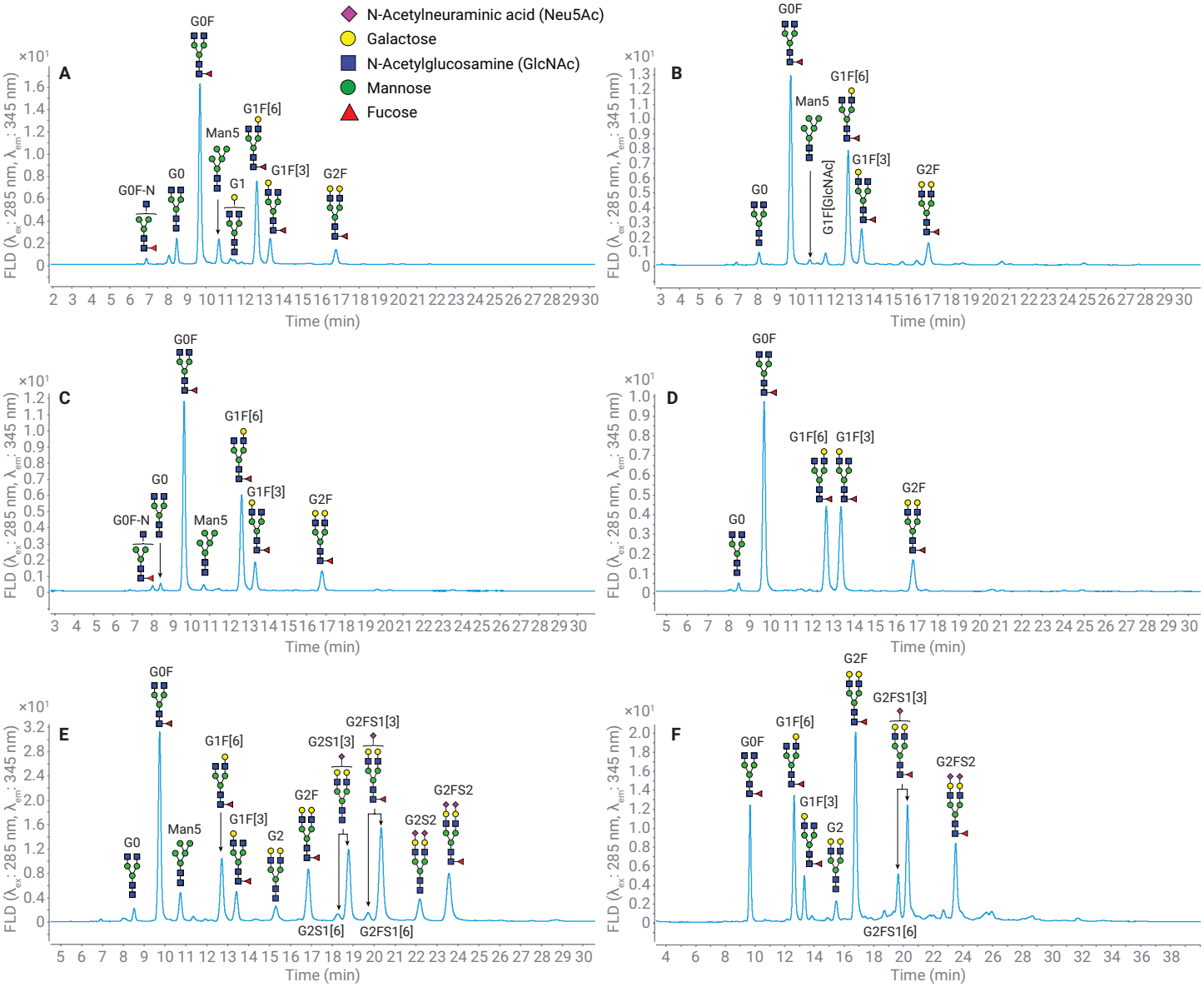

Figure 5. HILIC-UHPLC fluorescence profile of A) CHO mAb, B) NIST mAb, C) Ritux, D) IgG1, E) Enbrel, and F) Orencia labeled with InstantPC (40 µg). N-glycan relative areas (%) are shown in Table 5, n = 3.

Table 5. Figure 7 relative area (%), SD, and %CV values for (A) CHO mAb, (B) NIST mAb, (C) Ritux, (D) IgG1, (D) Enbrel, and (E) Orencia labeled with InstantPC (40 µg), n = 3.

A

CHO mAb	Average Relative Area (%)	SD	%CV
G0F-N	2.23	0.20	8.85
G0	5.68	0.29	5.10
G0F	45.81	1.18	2.57
Man5	6.74	0.14	2.03
G1	2.33	0.06	2.76
G1F[6]	25.04	0.86	3.43
G1F[3]	7.07	0.25	3.57
G2F	5.09	0.48	9.40

B

NIST mAb	Average Relative Area (%)	SD	%CV
G0	2.36	0.32	13.69
G0F	44.03	1.07	2.44
Man5	0.98	0.05	4.88
G1F-GlcNAc	2.71	0.14	5.03
G1F[6]	32.50	0.70	2.15
G1F[3]	9.91	0.38	3.80
G2F	7.52	0.53	7.08

C

Ritux	Average Relative Area (%)	SD	%CV
G0F-N	1.00	0.07	6.52
G0	1.58	0.01	0.48
G0F	49.86	0.48	0.97
Man5	1.43	0.17	11.97
G1F[6]	31.09	0.54	1.74
G1F[3]	8.94	0.23	2.59
G2F	7.09	0.08	1.11

D

IgG1	Average Relative Area (%)	SD	%CV
G0	1.49	0.03	1.77
G0F	43.25	0.13	0.31
G1F[6]	22.66	0.21	0.93
G1F[3]	22.96	0.15	0.66
G2F	9.63	0.07	0.74

E

Enbrel	Average Relative Area (%)	SD	%CV
G0	1.31	0.04	3.21
G0F	22.77	0.18	0.81
Man5	3.63	0.06	1.72
G1[6]	0.60	0.01	2.02
G1F[6]	8.99	0.09	1.05
G1F[3]	4.12	0.03	0.64
G2	2.67	0.01	0.47
G2F	8.67	0.12	1.41
G2S1[6]	1.36	0.04	3.02
G2S1[3]	12.51	0.03	0.25
G2FS1[6]	1.24	0.01	1.12
G2FS1[3]	17.65	0.13	0.73
G2S2	4.24	0.10	2.39
G2FS2	10.25	0.12	1.21

F

Orencia	Average Relative Area (%)	SD	%CV
G0F	12.00	0.18	1.49
G1F[6]	15.21	0.35	2.29
G1F[3]	5.28	0.03	0.62
G1FS1[3]	2.97	0.01	0.37
G2F	26.66	0.09	0.33
G2FS1[6]	5.78	0.33	5.64
G2FS1[3]	17.32	0.22	1.29
G2FS1	1.06	0.02	1.48
G2FS2	13.72	0.20	1.46

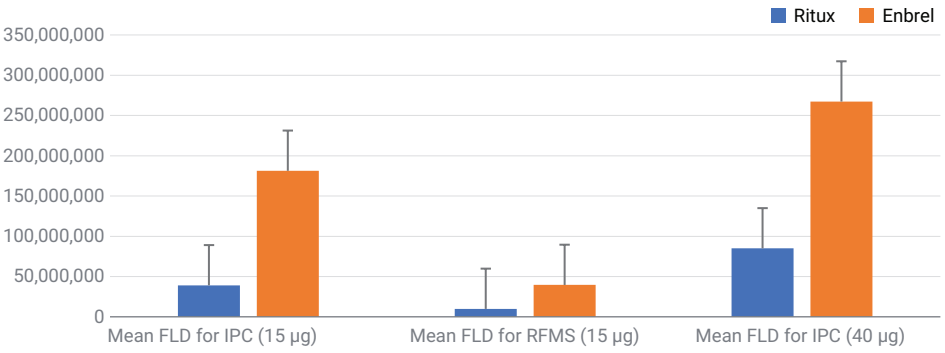

Figure 6. Total fluorescence signal for Ritux and Enbrel labeled with InstantPC (15 and 40 µg) and *Rapi*Fluor-MS (15 µg), using the Waters ACQUITY UPLC H-Class system with ACQUITY UPLC Glycan BEH Amide column (130 Å, 2.1 × 150 mm, 1.7 µm, part number 186004742, Waters).

Glycoprotein amount flexibility with Gly-X InstantPC sample preparation

The ability of the AdvanceBio kit to take more protein into preparation (40 to 100 µg; Figure 7) and yield higher FLD response was beneficial for detecting less-abundant structural isomers of N-linked glycans.

The recommended starting glycoprotein amount for the AdvanceBio kit is 15 to 40 µg, whereas the recommendation for the GlycoWorks kit it is 15 µg. Figure 7 shows that the AdvanceBio kit can be used for glycoprotein amount starting from as little as 2 µg up to 100 µg for Enbrel, with detection of all the major N-glycans. Higher glycoprotein amount (>10 µg) was useful to detect less abundant glycans such as G2S2, whereas lower glycoprotein amount (<10 µg) was sufficient to detect major glycans such as G0F and G1F isomers.

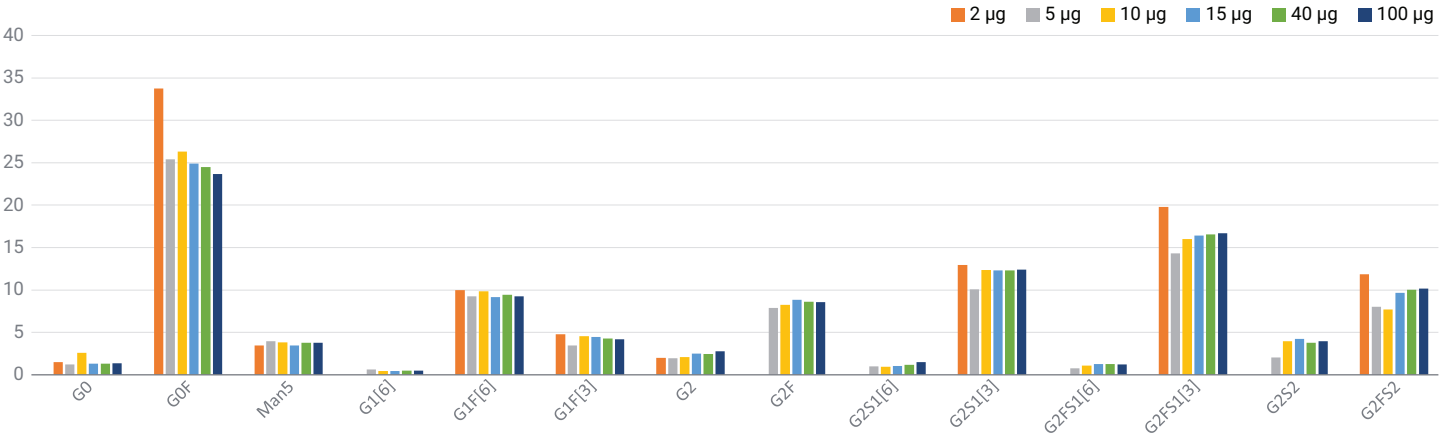

Figure 7. Relative area (%) for Enbrel labeled with InstantPC (1 to 100 µg).

Conclusion

Glycosylation is an important feature of biotherapeutics that is often monitored as a CQA for their development and production process. Both the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit and the Waters GlycoWorks *Rapi*Fluor-MS N-glycan kit demonstrated workflows that allowed rapid release of N-glycans, labeling of glycosylamine with InstantPC and *Rapi*Fluor-MS respectively, and analysis of glycan species by relative fluorescence peak area (%) and peak assignments confirmed by high-resolution mass spectrometry. Both IPC- and RFMS-labeled glycans displayed similar FLD signal when using 15 µg of glycoprotein for sample preparation; however, IPC allowed for higher FLD signal to be achieved when 40 µg of glycoprotein was used with greater MS ionization efficiency in positive mode, and confident detection of less-abundant glycan species. Although the performance of IPC and RFMS labels for various biotherapeutics are clear, the AdvanceBio kit offers flexibility for its reagents such as the Gly-X denaturant, which is stable for months, and the reconstituted IPC solution, which can go through 10 freeze-thaw cycles compared to *Rapi*Gest surfactant (one week) and RFMS label (one freeze-thaw cycle), respectively. Moreover, the AdvanceBio kit can go up to 100 µg for some biotherapeutics, whereas the GlycoWorks kit is limited to 15 µg. Lastly, the extensive range of IPC individual glycan standards and libraries increases throughput and makes glycan analysis simpler for the user.

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