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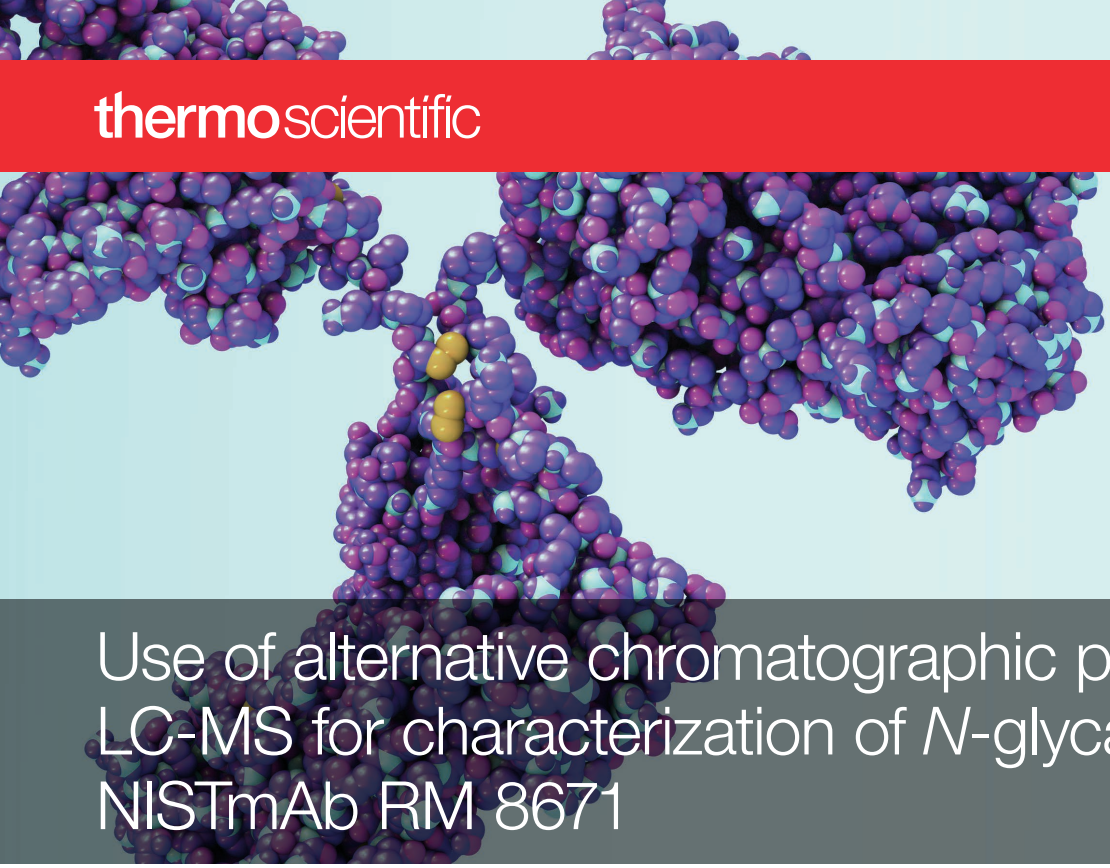

Use of alternative chromatographic phases and LC-MS for characterization of *N*-glycans from NISTmAb RM 8671

Authors

Amy Farrell, Silvia Millan Martin, Csaba Váradi, and Jonathan Bones, National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland

Keywords

NIBRT, Biopharmaceutical, Bio-production, QA/QC, Biotherapeutic, IgG, Monoclonal antibody (mAb), Glycans, Glycosylation, NISTmAb RM 8671, Vanquish Flex UHPLC, Accucore 150 Amide HILIC, GlycanPac AXH-1, Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer with Biopharma Option

Application benefits

- Demonstrates the use of LC-MS for identification of *N*-glycans released from monoclonal antibodies
- Demonstrates alternative chromatographic stationary phases for *N*-glycan analysis of therapeutic monoclonal antibodies

Goal

To evaluate chromatographic stationary phases for separation of oligosaccharides released from NIST monoclonal antibody reference material. To demonstrate the high mass accuracy achievable with Orbitrap-based mass spectrometry instrumentation for annotation of glycan moieties.

Introduction

N-linked glycosylation is an important post-translational modification (PTM) that imparts structural heterogeneity to recombinant monoclonal antibodies (mAbs). Oligosaccharides (glycans) attached to the C_H2 domain of an IgG1 can impact the pharmacodynamic and pharmacokinetic behavior of therapeutic proteins and are often considered to be critical quality attributes (CQAs). Detailed characterization of the glycosylation profile of mAbs is a regulatory requirement to ensure the safety, quality, and efficacy profile of therapeutic proteins. Characterization of the glycan profile of

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biosimilar products is also required to demonstrate the comparability of an innovator therapeutic protein and related biosimilar candidate. Analysis of glycans attached to mAbs is challenging as *N*-glycosylation is heterogeneous as a result of the activity of the individual enzymes that construct the oligosaccharides at each glycosylation site. Consequently, extensive sample handling and full detailed analysis, often including orthogonal technologies, are necessary. These requirements have resulted in an increased demand for reliable and robust analytical technologies for the comprehensive characterization of protein glycosylation.

A common strategy for *N*-glycan analysis usually involves removal of *N*-glycan species from therapeutic proteins using peptide *N*-glycosidase F (PNGase F), followed by modification of the free reducing terminus of released glycans with an appropriate fluorophore to improve detectability, chromatographic retention, and/or ionization efficiency. Hydrophilic interaction (HILIC) liquid chromatography is the most frequently applied separation strategy for glycan analysis during which glycan retention increases with increasing glycan size and associated reduction in hydrophobicity. Introduction of an additional and complementary separation dimension has been employed to increase resolution of oligosaccharides. Several hybrid chromatographic phases are available, such as the Thermo Scientific™ GlycanPac™ AXH-1 column, which has both weak anion exchange and HILIC properties, enabling separation of molecules based on charge, polarity, and size. After liquid chromatography (LC) or capillary electrophoresis separation, labeled glycan species are detected using fluorescence (FLD) or mass spectrometry (MS) detection to enable identification and relative quantitation of oligosaccharides.

Glyco-analytical standards are of critical importance in evaluating the suitability of analytical methods for glycosylation analysis of therapeutic mAbs throughout a therapeutic protein product life-cycle. The NISTmAb reference material 8671 (NISTmAb) is a recombinant humanized $IgG1_K$ that may be used as a reference standard for system suitability or analytical method evaluation for mAb characterization. NISTmAb is a ~150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process-related impurities.

During production, NISTmAb undergoes various PTMs including glycosylation, and thus it is a suitable analytical standard for glycan analysis methodologies.

In this application, the glycosylation profile of NISTmAb was analyzed using liquid chromatography-mass spectrometry (LC-MS). Released and labeled glycans were analyzed using an LC-MS platform composed of a Thermo Scientific™ Vanquish™ Flex UHPLC system with FLD, coupled to a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer with the BioPharma option. Separation of oligosaccharides was achieved using a Thermo Scientific™ Accucore™ Amide-150 HILIC, 2.1 × 150 mm (HILIC) column or a Thermo Scientific GlycanPac AXH-1 (AXH), 1.9 µm, 2.1 × 150 mm column. Excellent reproducibility of the sample preparation and resulting chromatographic separation of NISTmAb glycans was observed on both columns. Subsequent identification of glycan moieties was performed based on the mass of the species detected upon LC-MS analysis. Use of a high-resolution, accurate-mass MS enabled high-confidence identification of oligosaccharides, with < 3 ppm error in mass accuracy for all *N*-glycan species determined.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Acetonitrile, Optima™ LC-MS grade (Fisher Chemical) (P/N 10001334)
- Water, Optima™ LC-MS grade (Fisher Chemical) (P/N 10505904)
- Formic acid, Optima™ LC-MS grade (Fisher Chemical) (P/N 10596814)
- Ammonium hydroxide (Honeywell Fluka) (P/N 44273)
- PNGase F (New England BioLab) (P/N P0705L)
- Amicon® Ultra 0.5 mL centrifugal filters MWCO 10 kDa (Fisher Scientific) (P/N 10088753)
- Sodium cyanoborohydride (Acros Organics) (P/N 168550100)
- Glacial acetic acid (Fisher Chemical) (P/N 10394970)
- Dimethylsulfoxide (DMSO) (Thermo Scientific, Pierce) (P/N 13494279)

- 2-Aminobenzoic acid (2-AA) (Honeywell Fluka) (P/N 10678)
- Urea (Fisher Chemical) (P/N 10687422)
- Tris-HCl (Fisher Chemical) (P/N 10060390)
- Dithiothreitol (DTT) (Fisher Bioreagents) (P/N 10386833)
- Iodoacetamide (IAA) (Acros Organics) (P/N 10408660)
- Ammonium bicarbonate (Acros Organics) (P/N 10207183)
- Sodium hydroxide (NaOH) (Fisher Chemical) (P/N 10488790)
- Thermo Scientific GlycanPac AXH-1, 1.9 µm column, 2.1 × 150 mm (P/N 082472)
- Thermo Scientific Accucore 150 Amide HILIC column, 2.1 × 150 mm (P/N 16726-152130)
- Thermo Scientific Accucore 150 Amide HILIC column, 2.1 × 50 mm (P/N 16726-052130)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ vial identification system (P/N 60180-VT100)

Instrumentation

- Vanquish Flex UHPLC system, including:
  - Quaternary Pump F (P/N VF-P20-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A) with 25 µL (V = 50 µL) sample loop
  - Fluorescence Detector F (P/N VF-D50-A)
  - System Base Vanquish Flex (P/N VF-S01-A)
- Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with the BioPharma option
- Thermo Scientific™ SpeedVac™ Concentrator (P/N SPD121p)

Sample preparation

Buffer preparation

- Ammonium formate (50 mM, pH 4.4): 1.8 g of formic acid was dissolved in 1 L of DI water. The pH was adjusted to 4.4 with ammonium hydroxide solution.
- 8 M urea in 0.1 M Tris-HCl, pH 8.5 (UA buffer): 1.57 g of Tris-HCl was dissolved in 100 mL DI water and the pH was adjusted to 8.5 with 1 M NaOH. 24 g of urea was dissolved in 50 mL 0.1 M Tris-HCl, pH 8.5.
- Ammonium bicarbonate (50 mM, pH 7.8): 0.39 g of ammonium bicarbonate was dissolved in 100 mL of DI water.

Release of N-glycans from proteins

500 µg of protein was denatured using 8 M urea in 0.1 M Tris buffer pH 8.0 (UA solution) and subsequently reduced and alkylated using 10 mM DTT and 50 mM IAA prepared in UA solution. Following buffer exchange into 50 mM ammonium bicarbonate using 10 kDa MWCO filters, N-glycan release was performed by incubation of the reduced and alkylated sample with 2500 units of PNGase F overnight at 37 °C. The released glycans were collected from the deglycosylated proteins by centrifugation through 10 kDa MWCO filters and subsequently reduced to dryness via vacuum centrifugation. The dried glycans were reconstituted in 50 µL of 1% (v/v) aqueous formic acid to ensure complete conversion to the reducing sugar form prior to derivatization and subsequently reduced to dryness.

2-AA labeling of released N-glycans

1. 2-AA labeling reagent (100 µL) was prepared by dissolving 2-aminobenzoic acid (5 mg) and sodium cyanoborohydride (6 mg) in 70/30 DMSO/glacial acetic acid.
2. 10 µL of 2-AA labeling reagent solution was added to the dried *N*-glycans.
3. The solution was incubated at 65 ºC for 5 hours.

Cleanup of fluorescently labeled N-glycans

Excess labeling dye removal was carried out by HILIC purification. Samples were loaded in 80% acetonitrile, 20% 50 mM ammonium formate pH 4.4 (v/v) onto an Accucore 150-Amide-HILIC 2.1 x 50 mm column at 0.5 mL/min for 2.5 minutes. Labeled glycans were eluted in 20% aqueous acetonitrile for 2.5 minutes, monitored by fluorescence detection, $\lambda_{ex/em}$ = 350/425 nm, and evaporated to dryness.

Sample preparation for analysis using UHPLC-FLD and UHPLC-FLD-MS

1. 25 µL of purified labeled *N*-glycans resuspended in DI water (at 2.5 µg/µL) was diluted with 75 µL of acetonitrile.
2. The total solution was transferred to the autosampler vial for analysis.

Analytical conditions for UHPLC analysis

Analytical conditions for UHPLC analysis are listed in Tables 1-3.

Table 1. UHPLC parameters used for all analysis.

Parameter	Condition
Mobile phase	A: Ammonium formate 80 mM, pH 4.4 B: Acetonitrile
Flow rate	0.4 mL/min
Column temperature	30 ºC
Autosampler temperature	10 ºC
Sample volume	25 µL
FLD excitation/emission wavelength	342 / 423 nm
Mobile phase gradient	Refer to Tables 2 and 3

Table 2. Mobile phase gradient for analysis performed using an Accucore Amide-150 HILIC column.

Time (min)	% A	% B	Curve
0	25	75	5
30	45	55	5
30.5	60	40	5
32	60	40	5
32.5	25	75	5
40	25	75	5

Table 3. Mobile phase gradient for analysis performed using a GlycanPac AXH-1 column.

Time (min)	% A	% B	Curve
0	22	78	5
30	30	70	5
35	40	60	5
40	50	50	5
40.5	22	78	5
50	22	78	5

Analytical conditions for MS analysis

Glycan samples were analyzed using a Q Exactive Plus hybrid quadrupole-Orbitrap MS with BioPharma option equipped with a HESI ion source (Table 4) using a Vanquish Flex UHPLC system to enable identification of glycan species.

Table 4. MS parameters utilized for all LC-MS analysis.

Parameter	Setting
Method duration	40 min (HILIC); 50 min (AXH)
Polarity	Negative
In-source CID	20 eV
Default charge state	2
Full MS	
Number of microscans	1
Resolution	70,000 Da
AGC target	3.00E+06
Maximum IT	50 ms
Scan range	380–2000 *m/z*
dd-MS2 / dd-SIM	
Number of microscans	3
Resolution	17,500
AGC target	1.00E+05
Maximum IT	120 ms
TopN	3
Isolation window	2.0 m/z
Scan range	200–2000 *m/z*
(N)CE/stepped (N)CE	NCE: 30, 42, 72
dd settings	
Minimum AGC target	1.00E+03
Intensity threshold	8.30E+03
Charge exclusion	Unassigned, 1, 5-8, >8
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	10.0 s
Tune file settings	
Spray voltage	3.5 kV
Capillary temperature	320 °C
Sheath gas pressure	40
Aux gas pressure	10
Probe heater temp.	400 °C
S-lens RF value	50

Data processing and software

Thermo Scientific™ Xcalibur™ software version 2.2 SP1.48 was used for UHPLC-FLD-MS data acquisition and analysis.

Results and discussion

Regulatory agencies require that state-of-the-art, robust, reproducible analytical methods for glycan analysis be included in regulatory filings for glycoprotein-based biotherapeutics to ensure accuracy and consistency of reported results.[1,2] The demand placed on these methods has steadily increased as better technologies have been developed, including high-resolution, accurate-mass MS instrumentation. There is also an appetite for simplified and standardized methodologies that may provide additional assurance that glycoanalytical methods used are transferrable between testing sites, ensuring quality and efficiency in manufacturing. In order to evaluate the reproducibility of a sample preparation method for glycosylation of mAbs, three separate preparations of glycan samples were prepared from NISTmAb. *N*-glycans released from NISTmAb using PNGase F were derivatized with 2-aminobenzoic acid (2-AA) and cleaned using an Accucore Amide-150 HILIC column before LC-MS analysis. Excellent reproducibility of the labeling, cleanup, and LC-MS method were observed following analysis of 2-AA labeled NISTmAb *N*-glycans, despite sample preparation by different analysts. Figure 1 shows fluorescence traces of the NISTmAb glycan profile following separation on an Accucore Amide-150 HILIC column, while Figure 2 shows corresponding analysis performed using a GlycanPac AXH-1 column.

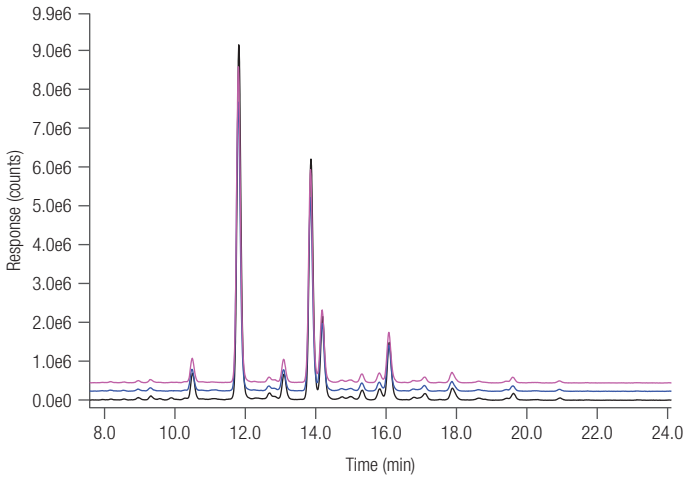

Figure 1. Overlay of replicate analysis of N-glycans released from NISTmAb following separation on an Accucore Amide-150 HILIC column and a Vanquish Flex UHPLC.

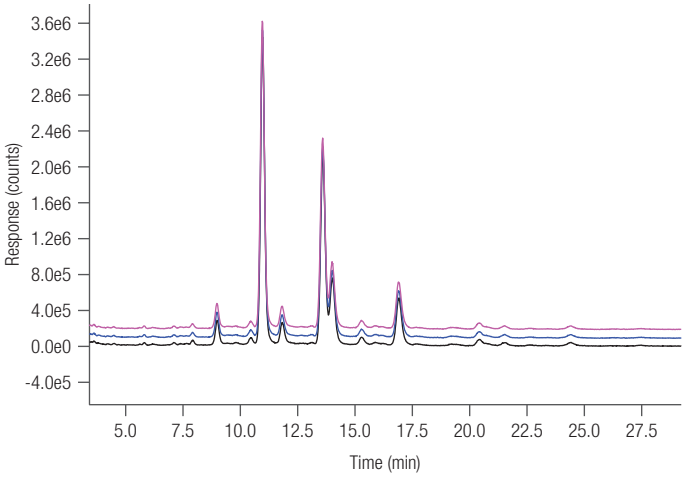

Figure 2. Separation of N-glycans released from NISTmAb following separation on a GlycanPac AXH-1 column and a Vanquish Flex UHPLC.

Use of high-resolution, accurate-mass LC-MS analysis enables detailed characterization of *N*-glycans attached to mAbs. High-resolution MS instruments, such as the Q Exactive Plus hybrid quadrupole-Orbitrap MS with the BioPharma option, allows for highly accurate mass measurements, thereby ensuring confidence in assignment of proposed glycan structures based on detected mass. Figures 3 and 4 show the *N*-linked glycan structures that were identified after analysis of NISTmAb glycans. Associated chromatographic and MS data are outlined in Table 5. Similar glycan structures were observed in a published book series outlining the characterization of NISTmAb;[3] here only those species identified with low mass errors are reported (<3 ppm). The LC-MS platform incorporating an Accucore Amide-150 HILIC column enabled the determination of 32 proposed glycan structures for NISTmAb. One additional glycan structure was identified following analysis with a GlycanPac AXH-1 column, namely A2G1 (circled, Figure 4).

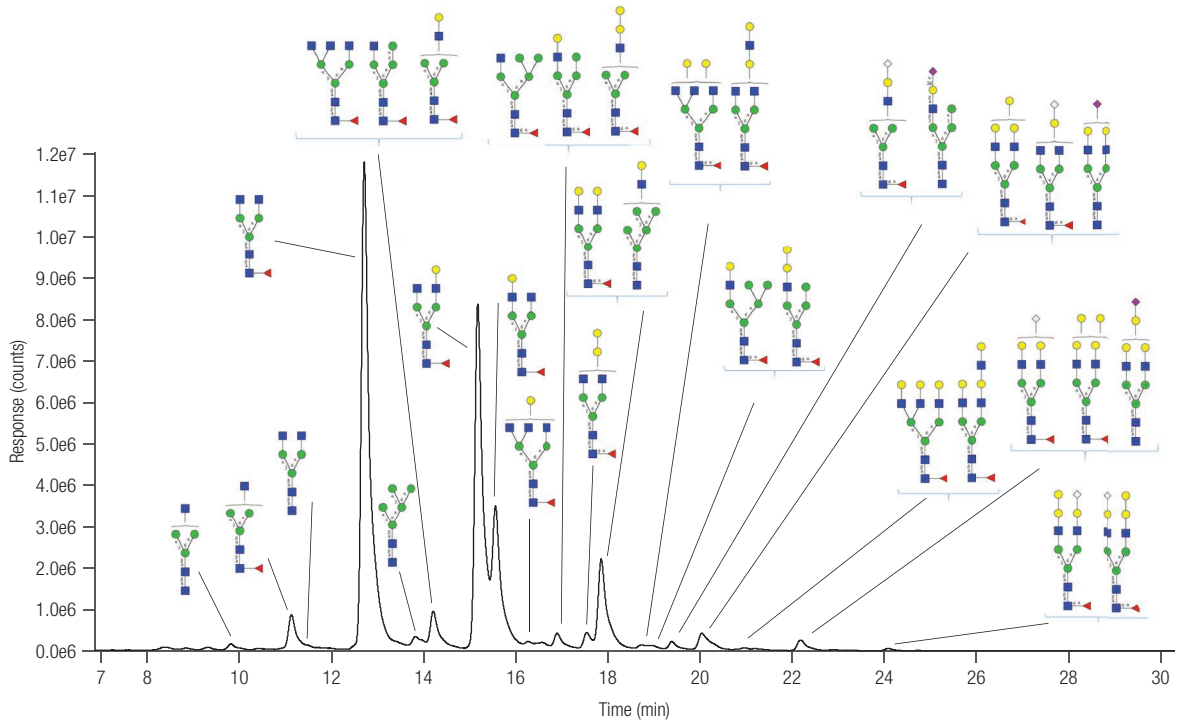

Figure 3. Identification of N-glycans released from NISTmAb following separation on an Accucore Amide-150 HILIC column and a Vanquish Flex UHPLC coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with the Biopharma option. Glycan structures are annotated according to the Consortium for Functional Glycomics (CFG) system.[4]

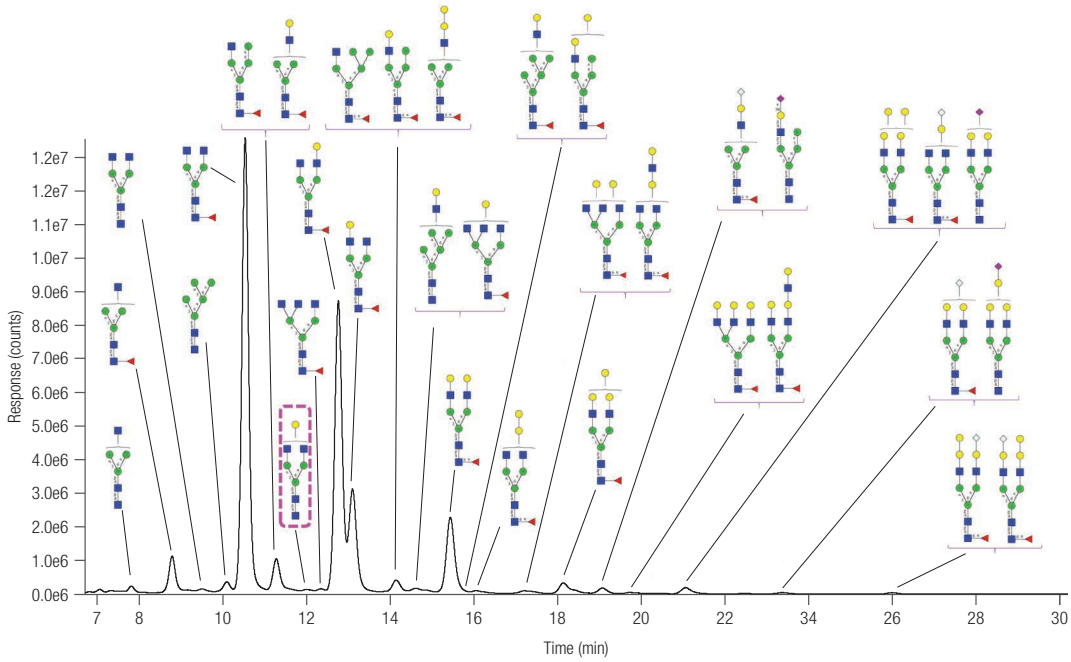

Figure 4. Identification of N-glycans released from NISTmAb following separation on a GlycanPac AXH-1 column and a Vanquish Flex UHPLC coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with the Biopharma option. Glycan structures are annotated according to the Consortium for Functional Glycomics (CFG) system.[4] The glycan structure A2G1 is highlighted in pink.

Table 5: Proposed N-linked glycan structures and associated LC-MS data used for annotation of glycan structures shown in Figure 4.

Retention time (min)	Experimental mass (*m/z*)	Theoretical mass (*m/z*)	Mass accuracy (ppm)	Ion	Proposed Structure
7.91	616.2230	616.2227	0.49	[M-2H] $^{2-}$	A1
8.99	689.2522	689.2516	0.87	[M-2H] $^{2-}$	FA1
9.79	717.7631	717.7624	0.98	[M-2H] $^{2-}$	A2
10.45	676.7362	676.7358	0.59	[M-2H] $^{2-}$	M5
10.96	790.7920	790.7913	0.89	[M-2H] $^{2-}$	FA2
11.82	770.2786	770.2780	0.78	[M-2H] $^{2-}$	FA1G1/FM4A1
12.69	798.7897	798.7888	1.13	[M-2H] $^{2-}$	A2G1
13.07	892.3316	892.3310	0.67	[M-2H] $^{2-}$	FA3/FA2B
13.59	871.8183	871.8177	0.69	[M-2H] $^{2-}$	FA2G1
14.00	871.8183	871.8177	0.69	[M-2H] $^{2-}$	FA2G1
15.27	851.3052	851.3045	0.82	[M-2H] $^{2-}$	FM5A1/FM4A1G1/FA1G1Ga1
15.89	973.3583	973.3574	0.92	[M-2H] $^{2-}$	FA3G1/FA2BG1
15.89	859.3032	859.3019	1.51	[M-2H] $^{2-}$	M5A1G1
16.89	952.8445	952.8441	0.42	[M-2H] $^{2-}$	FA2G2
17.31	932.3323	932.3309	1.50	[M-2H] $^{2-}$	FM5A1G1/FM4A1G1Ga1
17.68	952.8449	952.8441	0.84	[M-2H] $^{2-}$	FA2G1Ga1
19.28	1054.385	1054.3840	0.95	[M-2H] $^{2-}$	FA3G2/FA2BG2
20.39	1033.871	1033.8710	0.10	[M-2H] $^{2-}$	FA2G2Ga1
21.52	923.8241	923.8232	0.97	[M-2H] $^{2-}$	FA1G1Sg1/M4A1G1S1
22.62	1135.4110	1135.4100	0.79	[M-2H] $^{2-}$	FA3G3/FA2G2Lac1
24.38	1025.3640	1025.3630	0.59	[M-2H] $^{2-}$	FA2G1Sg1/A2G2S1
24.38	1114.8970	1114.8970	0.09	[M-2H] $^{2-}$	FA2G2Ga2
27.48	1106.3900	1106.3890	0.54	[M-2H] $^{2-}$	FA2G2Sg1/A2G2Ga1S1
31.11	1187.4160	1187.4160	0.25	[M-2H] $^{2-}$	FA2G2Ga1Sg1

Conclusions

- An LC-MS method for detailed characterization of *N*-glycans released from mAbs was described. Use of a Vanquish Flex UHPLC system coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with the BioPharma option enabled identification of *N*-glycans with < 2 ppm mass error.

- Two chromatographic stationary phases were demonstrated for analysis of NISTmAb *N*-glycosylation. In addition to the commonly used HILIC stationary phase, the GlycanPac AXH-1 column, incorporating weak anion exchange and HILIC properties, was evaluated.

- The GlycanPac AXH-1 column may be a powerful alternative to Amide HILIC chromatographic phases for glycosylation analysis of therapeutic mAbs.

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