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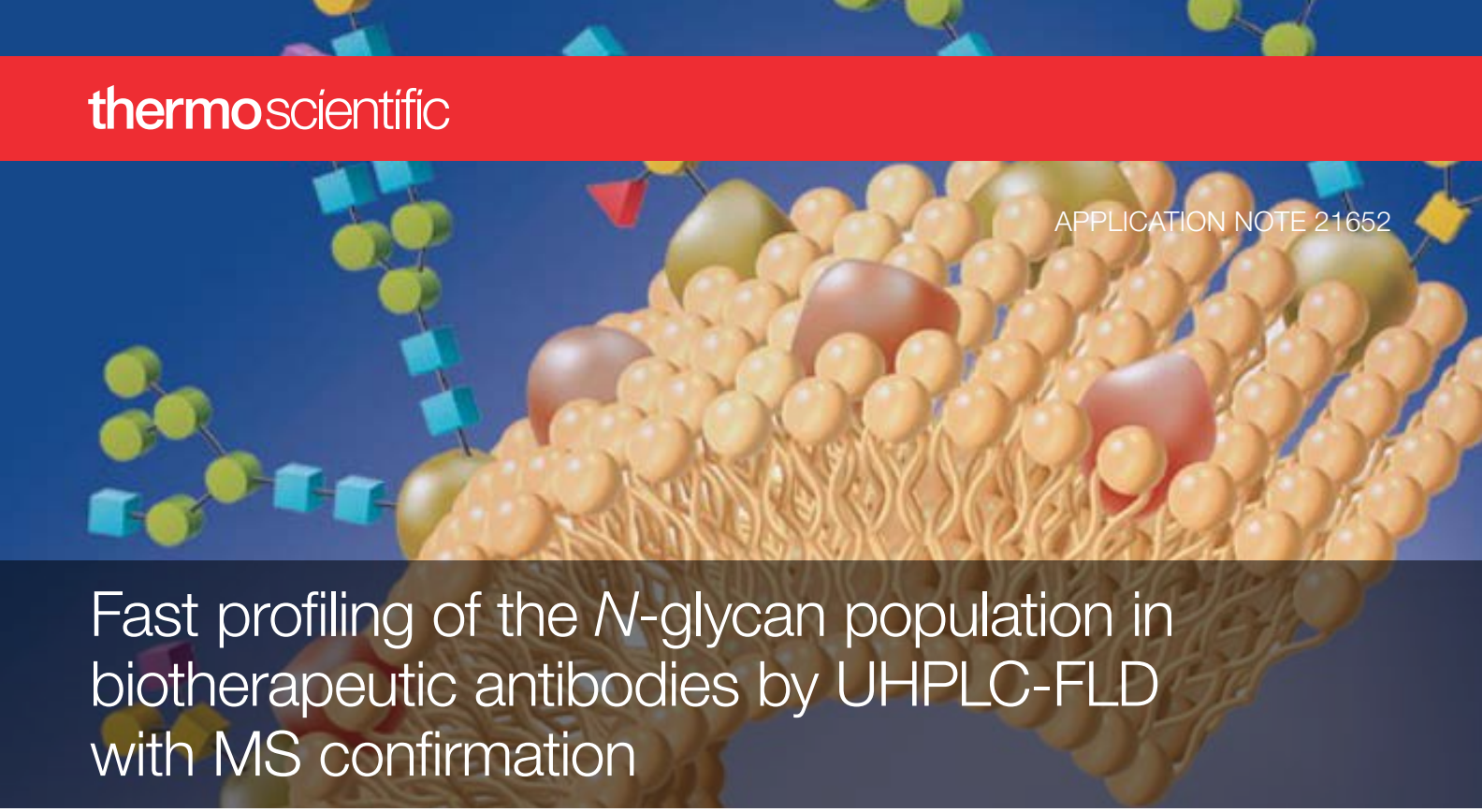
APPLICATION NOTE 21652



Fast profiling of the *N*-glycan population in biotherapeutic antibodies by UHPLC-FLD with MS confirmation

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Keywords

Glycosylation, Glycoprotein, Released *N*-glycan, Glycoform, Biosimilar, Innovator, Monoclonal Antibody, mAb, Biotherapeutics, IgG, Vanquish UHPLC, Accucore Amide HILIC, Solid Core, 2-AA, 2-AB, HRAMS MS, Q Exactive, Infliximab

Goal

- To develop a widely applicable UHPLC approach to fast, comprehensive profiling of 2-AA and 2-AB labeled glycans in IgG antibodies
- To validate the approach with a human serum IgG and a commercial chimeric IgG1 mAb (infliximab)
- To confirm the glycan profiles by exoglycosidase enzyme digestion and high-resolution, accurate-mass mass spectrometry (HRAMS MS)
- To increase throughput with assay optimization

Introduction

Glycans play an essential role in many biological processes, including cell development and differentiation, cell-cell or cell-matrix communication, and pathogen-host recognition. The glycan components of biotherapeutics can be important determinants of biological activity and therapeutic efficacy[1-3] and hence characterization of the glycan profile of a biomolecule is required under regulatory guidelines (ICH Q5E/Q6B and USP 129).[4-5] Therapeutic antibodies must be demonstrated to meet applicable quality requirements to ensure continued safety, purity, and potency of a drug product. An intensive biochemical characterization of the antibody itself is required, which includes a thorough examination of glycan distribution and potential

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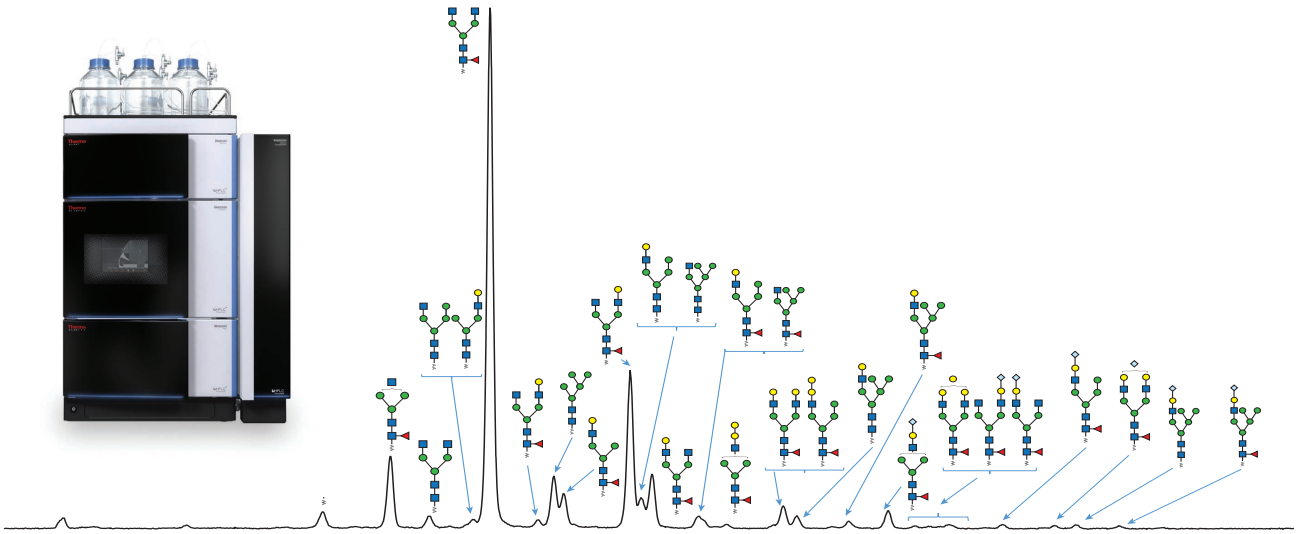
impacts of glycoforms on mAb function.[6] On the other hand, differences in glycan profiles between healthy and diseased states[7] are utilized for clinical diagnosis[8], providing targets for many novel classes of therapeutics including cancer chemotherapy, diabetes treatment, and antibiotic and anti-viral medicine.

The 'gold standard' for studying IgG glycosylation relies on enzymatic *N*-glycan release, subsequent fluorescent labeling by reductive amination, and analysis of the labeled glycans by high-performance liquid chromatography with fluorescence detection (HPLC-FLD), using hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection. In addition to characterization of the sugar sequence, the analysis must elucidate linkages and separate all isomeric, charge, and branching variations of glycans.

HILIC columns commonly used for glycan analysis are based on amide, amine, or zwitterionic packing materials. These columns separate glycans mainly by hydrogen bonding, resulting in separations based on size and composition. The Thermo Scientific™ Accucore™ 150-Amide-HILIC HPLC column is a solid particle core phase designed for the separation of hydrophilic biomolecules in HILIC mode, which offers an excellent choice for glycan separation.

This application note presents a step-by-step method for release, labeling, separation, and exoglycosidase-based structural elucidation of *N*-glycans from human serum IgG and commercial chimeric IgG1 mAb (infliximab) using Thermo Scientific™ Vanquish™ Horizon UHPLC-FLD and confirmation of the structures by a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer.



Experimental

Chemicals and reagents

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Acetonitrile, HPLC grade (P/N 10407440)
- Fisher Scientific™ Optima™ Formic acid, LC-MS (P/N 10596814)
- Fisher Scientific Ammonium hydroxide (P/N 10508610)
- Fisher Scientific Ammonium bicarbonate (P/N 10207183)
- Thermo Scientific™ Virtuoso™ Vial kit (P/N 60180-VT402)
- PNGase F (New England Biolabs®, Ipswich, MA, USA)
- Amicon® Ultra 0.5 mL centrifugal filters MWCO 10 kDa (purchased from a reputable supplier)
- Fisher Scientific Sodium cyanoborohydride (P/N 10082110)
- Fisher Scientific Glacial acetic acid (P/N A/0360/PB17)
- Fisher Scientific Dimethylsulfoxide (DMSO) (P/N 10213810)
- Anthranilamide (2-AB) (purchased from a reputable supplier)
- Anthranilic acid (2-AA) (purchased from a reputable supplier)
- Fisher Scientific Tris(hydroxymethyl)methylamine hydrochloride, (P/N 10060390)

- Fisher Scientific Urea (P/N 10132740)
- Fisher Scientific Ethanol (P/N 10644795)
- ABS Sialidase/NANase III (purchased from a reputable supplier)
- BKF α(1-2,3,4,6) Fucosidase (bovine kidney) (purchased from a reputable supplier)
- SPG beta(1-4)-Galactosidase (*Streptococcus pneumoniae*) (purchased from a reputable supplier)
- GUH β-*N*-Acetylhexosaminidase / Hexase I (purchased from a reputable supplier)

Equipment

Thermo Scientific™ UltiMate™ 3000 RS system, including:

- LPG-3400RS Rapid Separation Quaternary Pump (P/N 5040.0036)
- WPS-3000TRS Rapid Separation Thermostatted Well Plate Autosampler (P/N 5840.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- FLD-3400RS Fluorescence Detector with Dual-PMT (P/N 5078.0025)
- SR-3000 Solvent Rack (P/N 5035.9200)
- 2 µL Micro Flow Cell (P/N 6078.4330)

Thermo Scientific™ Vanquish™ Horizon UHPLC system, including:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler HT (P/N VH-A10-A) with 25 µL (V=50 µL) sample loop
- Fluorescence Detector F (P/N VF-D50-A)

Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer

Thermo Scientific™ SpeedVac™ Concentrator (P/N SPD121p)

Accucore 150-Amide-HILIC, 2.6 µm, 2.1 × 50 mm (P/N 16726-052130)

Accucore 150-Amide-HILIC, 2.6 µm, 2.1 × 150 mm (P/N 16726-152130)

Accucore 150-Amide-HILIC, 2.6 µm, 2.1 × 250 mm (P/N 16726-252130)

Preparation of buffers

- Ammonium formate (50 mM, pH 4.4): 1.8 g of formic acid was dissolved in 1 L of DI water. pH was adjusted pH 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 pH 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

1. 100 µg of protein were 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.
2. 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 500 units of PNGase F overnight at 37 °C.
3. Released glycans were collected from deglycosylated proteins by centrifugation through 10 kDa molecular weight cut-off (MWCO) filters and subsequently reduced to dryness via vacuum centrifugation.
4. 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 reaction 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. 5 µL of 2-AA labeling reagent solution were added to the mixture (dried *N*-glycans released from 100 µg glycoprotein).
3. The solution was incubated at 60 °C for 5 hours.

2-AB labeling reaction of released *N*-glycans

1. 2-AB labeling reagent (100 µL) was prepared by dissolving 2-aminobenzamide (5 mg) and sodium cyanoborohydride (6 mg) in 70/30 DMSO/glacial acetic acid.

2. 10 µL of 2-AB labeling reagent solution were added to the mixture (dried *N*-glycans released from 100 µg glycoprotein).
3. The solution was incubated at 65 °C for 2 hours.

Cleanup of 2-AB/2-AA labeled *N*-glycans

1. Excess labeling dye removal was carried out by HILIC purification using an UltiMate 3000RS system.
2. Samples were loaded in 85% acetonitrile, 15% 50 mM ammonium formate pH 4.4 (v/v) (for 2-AB labeled *N*-glycans) or in 80% acetonitrile, 20% 50 mM ammonium formate pH 4.4 (v/v) (for 2-AA labeled *N*-glycans) onto an Accucore 150-Amide-HILIC 2.1 × 50 mm column at 0.5 mL/min for 2.5 minutes.
3. Labeled glycans were eluted in 20% aqueous acetonitrile for 2.5 minutes, monitored by fluorescence detection, $\lambda_{ex/em}$ = 330/420 nm (for 2-AB labeled *N*-glycans) or $\lambda_{ex/em}$ = 350/425 nm (for 2-AB labeled *N*-glycans), and evaporated to dryness.

Sample preparation for analysis using UHPLC-FLD

1. 5 µL of purified labeled *N*-glycans re-suspended in DI water (at 2.5 µg/µL) were mixed with 20 µL of acetonitrile.
2. The total solution was transferred to the auto-sampler vial for analysis. Note: Store the standard at -20 °C.

Exoglycosidase digests

All exoglycosidase digestions were performed in 50 mM ammonium acetate buffer, pH 5.5 in a final volume of 10 µL at 37 °C overnight. Amounts of enzyme are indicated in Table 1.

1. For each enzyme digestion, 5 µL of labeled *N*-glycan pool were placed in a 0.2 mL PCR tube (for GUH digest, sample was dried in vacuum centrifuge and re-suspended in 3 µL water).
2. Required volume of buffer (1 µL), water, and enzyme(s) (Table 1) were added to each tube mixing by pipette after each addition (10 µL final volume).
3. Samples were incubated at 37 °C overnight (16 hours).
4. Cleanup was performed by ethanol precipitation: 90 µL ethanol (-30 °C) were added to the sample tubes and mixed thoroughly. Samples were kept at -30 °C for 30 min and spun for 10 min at 16.1 x 1000 rcf, 10 °C. Supernatant was placed into a new tube and sample was dried completely in a vacuum centrifuge.
5. Digested samples were re-suspended in 5 µL DI water and 20 µL acetonitrile for UHPLC-FLD analysis.

Table 1. Enzyme specificity and required volume per exoglycosidase digestion.

Enzyme	Specificity	Volume per Digest
ABS	Releases α(2-3), α(2-6) and α(2-8) lined non-reducing terminal sialic acids (NeuNAc and NeuNGc)	1 µL
BKF	Releases α(1-2) and α(1-6) linked non-reducing terminal fucose residues more efficiently than α(1-3) and α(1-4) linked fucose. Used for release of core α(1-6) fucose residues, can also remove α(1-3), but less efficiently.	1 µL
SPG	Hydrolyses non-reducing terminal β(1-4) linked galactose residues	2 µL
GUH	Recombinantly expressed in E. coli. Releases β-linked GlcNAc but not bisecting GlcNAc β(1-4) Man	2 µL

Separation conditions

Columns:	Accucore 150-Amide-HILIC #1: 16726-252130 (2.1 × 250 mm) #2: 16726-152130 (2.1 × 150 mm) #3: 16726-052130 (2.1 × 50 mm)
Mobile phase A:	Ammonium formate 50 mM, p 4.4
Mobile phase B:	Acetonitrile
Flow rate (mL/min):	Refer to Tables 2–4
Column temperature:	60 °C
Sample volume:	33 µL (2.1 × 250 mm) 20 µL (2.1 × 150 mm) 11.5 µL (2.1 × 50 mm)
Mobile phase gradient:	Refer to Tables 3–5

Table 2. Mobile phase gradient for column #1 (P/N 16726-252130).

Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1.4	5
48.10	40	60	1.4	5
49.88	50	50	1.4	5
51.19	50	50	1.4	5
51.31	20	80	1.4	5
55.00	20	80	1.4	5

Table 3. Mobile phase gradient for column #2 (P/N 16726-152130).

Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1.3	5
31.05	40	60	1.3	5
32.23	50	50	1.3	5
33.08	50	50	1.3	5
33.15	20	80	1.3	5
35.00	20	80	1.3	5

Table 4. Mobile phase gradient for column #3 (P/N 16726-052130).

Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1	5
13.45	40	60	1	5
13.97	50	50	1	5
14.33	50	50	1	5
14.37	20	80	1	5
15.00	20	80	1	5

N-glycan analysis by LC-MS

Glycan samples were injected on a Q Exactive Plus Hybrid Quadrupole-Orbitrap MS equipped with a HESI source. Samples were diluted in 75% acetonitrile prior to analysis.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 150 mm
Mobile phase A:	Ammonium formate 50 mM, pH 4.4
Mobile phase B:	Acetonitrile
Flow rate:	0.4 mL/min
Column temperature:	50 °C
Sample volume:	11 µL

Table 5. Mobile phase gradient for *N*-glycan analysis by LC-MS.

Time (min)	%A	%B	Flow (mL/min)	Curve
0	25	75	0.4	5
30.0	50	50	0.4	5
30.5	55	45	0.4	5
32.0	55	45	0.4	5
32.5	25	75	0.4	5
40.0	25	75	0.4	5

MS conditions

Ionization:	HESI Negative Ion
Scan range:	500 to 2000 *m/z*
Source temperature:	300 °C
Sheath gas flow:	20 Arb
Auxiliary gas flow:	10 Arb
Spray voltage:	3.8 kV
Capillary temperature:	320 °C

Data processing and software

Chromatographic software:	Thermo Scientific™ Chromeleon™ CDS 7.2 SR4
MS data acquisition:	Thermo Scientific™ Xcalibur™ software 2.2 SP1.48

Results and discussion

Separation of 2-AB-labeled glycan from human IgG

A sample of human serum IgG glycans was analyzed on Accucore 150-Amide-HILIC HPLC columns of different lengths. The glycan separation and elution is based on size and polarity by HILIC interaction. Figure 1 shows the separation of neutral and acidic 2-AB labeled *N*-glycans from human IgG on different column lengths. FLD chromatograms reveal 18 well-resolved *N*-glycan peaks for 2.1 × 250 and 2.1 × 150 mm columns. Despite some loss in peak resolution when column length is reduced to 50 mm, it may be sufficient for fast analysis of less-complex glycan profiles, enabling high-throughput analysis within 15 minutes. The structural assignment for each identified peak was confirmed by accurate MS data and listed in Table 6. The oligosaccharides present in the polyclonal IgG, attached at Asn297, are of the complex bi-antennary type and are comprised of a chitobiose core with variable addition of fucose, galactose, bisecting *N*-acetylglucosamine, and sialic acid. Sialylation is modest, with < 15% of structures being monosialylated or disialylated.

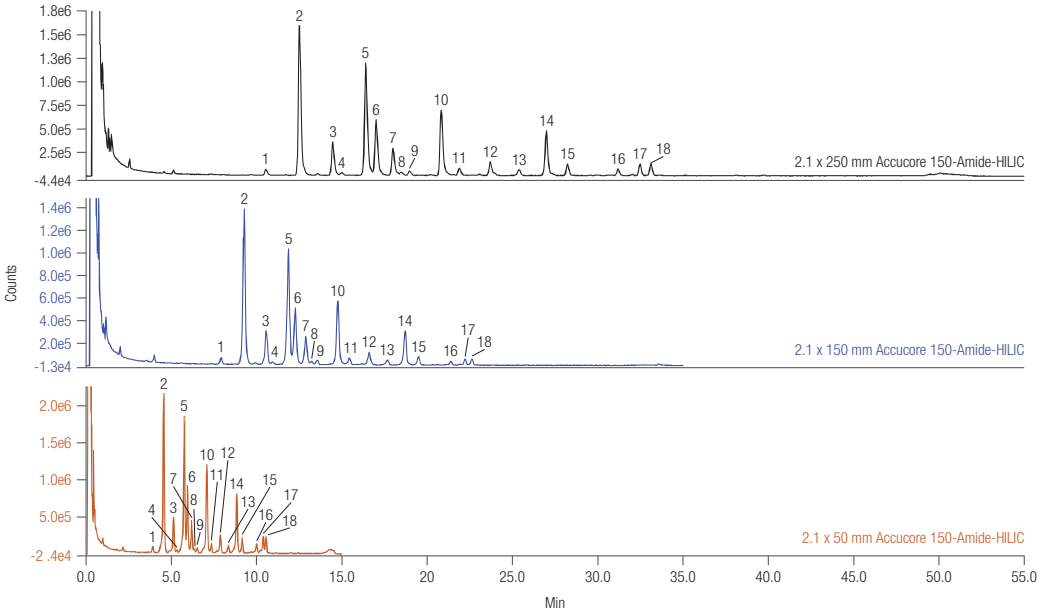


Figure 1. Chromatographic separation of human serum IgG 2-AB-labeled *N*-glycans on Accucore 150-Amide-HILIC columns of varying lengths.

Table 6. Structural identification of 2-AB labeled *N*-glycans from human IgG using Accucore 150-Amide-HILIC columns and a Q Exactive Plus mass spectrometer.

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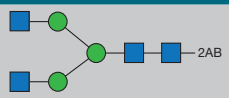
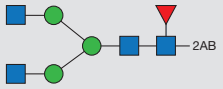
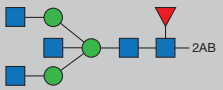
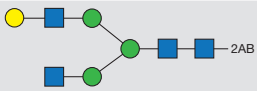
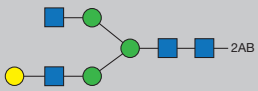
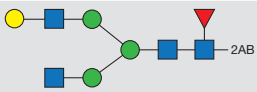
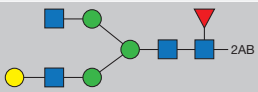
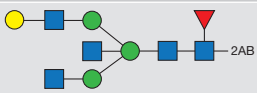
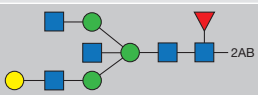
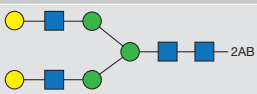
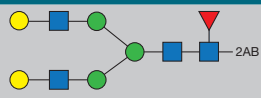
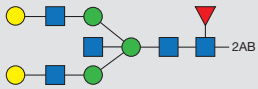
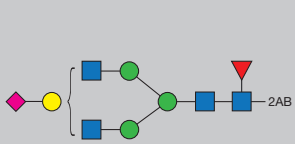
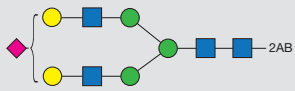
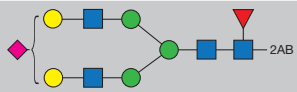
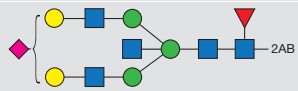
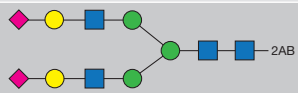
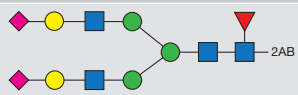
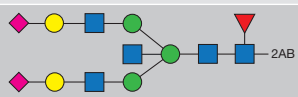
1	
2	
3A	
3B	
4	
5	
6	
7	
8	
9	

PEAK number (Figure 1)	Glycan structure
10	
11	
12	
13	
14	
15	
16	
17	
18	

▼ Fucose ■ *N*-Acetylglucosamine ● Mannose ○ Galactose ◆ *N*-Acetyl Neuraminic acid

Separation of 2-AA-labeled glycan from commercial chimeric IgG1

A commercial chimeric IgG1 was also analyzed on Accucore 150-Amide-HILIC columns with the aim to demonstrate appropriate separation for a more complex glycan sample. Figure 2 shows the separation of neutral and acidic 2-AA labeled *N*-glycans from chimeric IgG1 on different column lengths. FLD chromatograms reveal 24 *N*-glycan peaks for 2.1 × 250 and 2.1 × 150 mm columns. Annotation of the *N*-glycans structures and glycosidic linkages present in each chromatographic peak were deduced using exoglycosidase arrays as detailed in Figure 3 and Table 7. The oligosaccharide composition was also confirmed by high-resolution, accurate-mass (HRAM) MS data and listed in Table 7. Thirty-eight *N*-glycan structures were annotated, including high mannose, hybrid and complex bi-antennary glycans with variable degrees of core fucosylation, galactosylation, sialylation, and galactose α1-3 linked galactose epitopes.

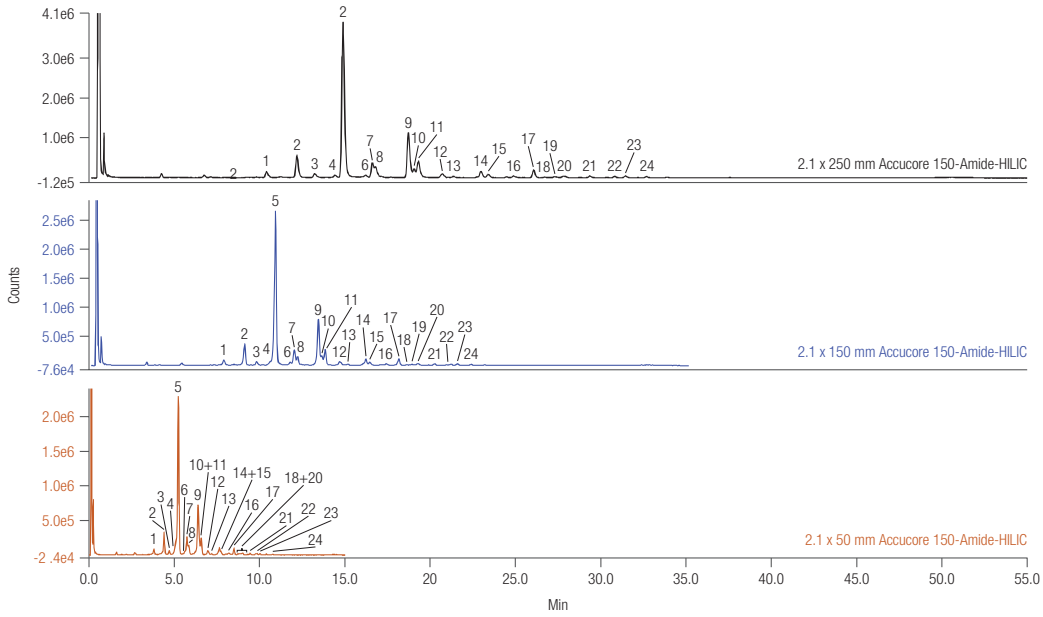
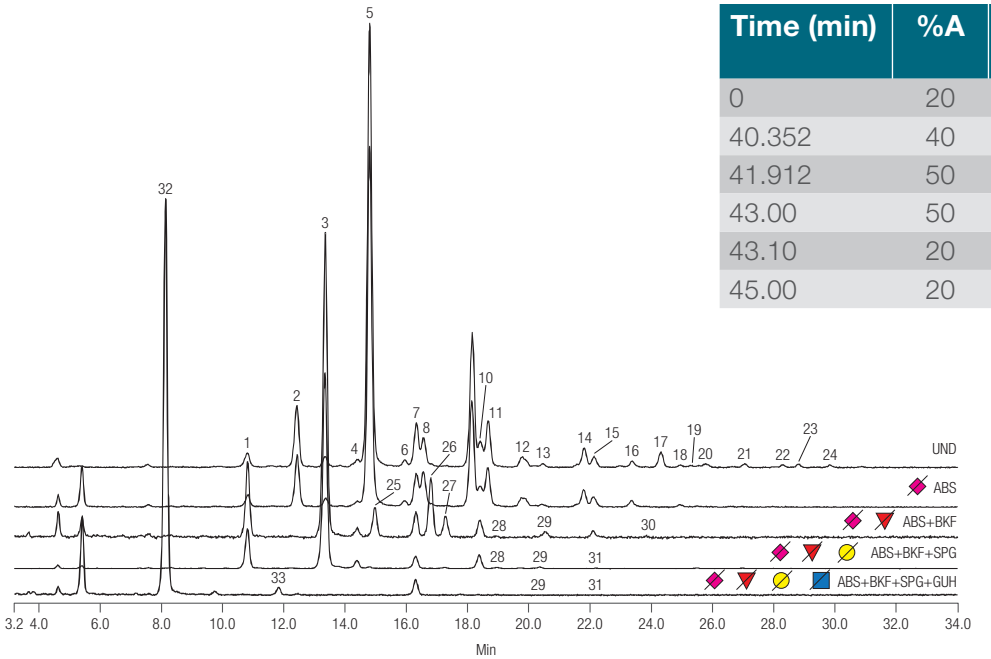


Figure 2. Chromatographic separation of commercial chimeric IgG1 mAb (infliximab) 2-AA-labeled *N*-glycans on Accucore 150-Amide-HILIC columns of varying lengths.

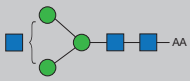
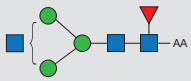
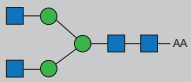
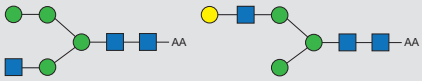
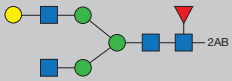
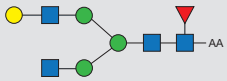
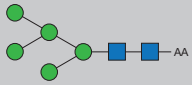
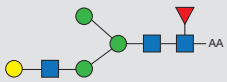
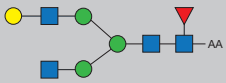


Time (min)	%A	%B	Flow (mL/min)	Curve
0	20	80	1.0	5
40.352	40	60	1.0	5
41.912	50	50	1.0	5
43.00	50	50	1.0	5
43.10	20	80	1.0	5
45.00	20	80	1.0	5

Figure 3. HILIC chromatograms of the infliximab 2-AA labeled *N*-glycan pool (undigested, UND) and after digestion with a range of exoglycosidase enzymes. Separations were performed on an Accucore 150-Amide-HILIC 2.1 × 150 mm column using the gradient conditions outlined in the figure legend table.

ABS removes all sialic acids (α2-3, -6 and -8), BKF removes α(1-6) linked core fucose and outer arm α(1-2 and 1-6) linked fucose, SPG removes β(1-4) linked galactose, and GUH removes β linked GlcNAc.

Table 7. Structural identification of 2-AA labeled *N*-glycans from commercial chimeric IgG1 mAb (infliximab) using Accucore 150-Amide-HILIC columns, exoglycosidase digests, and an offline Q Exactive Plus mass spectrometer.

PEAK Number (Figures 2 & 3)	Glycan structure
1	
2	
3	
4	
5	
6	
7	
8	
9	
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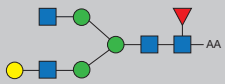
11	
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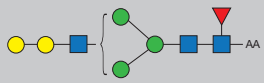
13	
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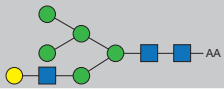
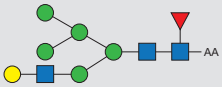
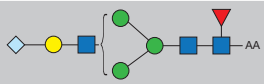
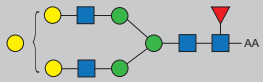
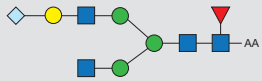
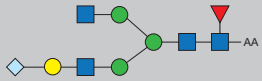
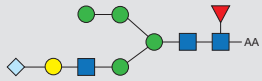
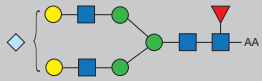
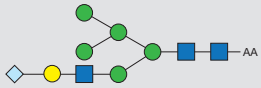
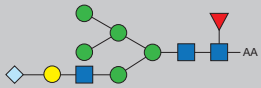
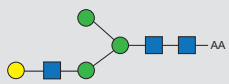
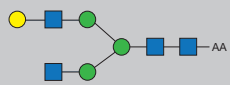
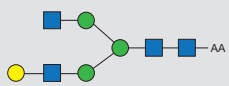
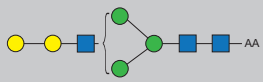
15	
16	
17	

PEAK Number (Figures 2 & 3)	Glycan structure
18	
19	
20	
21	
22	
23	
24	
25	
26	
27	
28	
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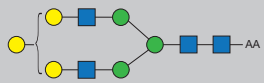
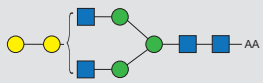
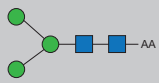
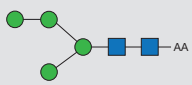
30	
31	
32	
33	

Fucose *N*-Acetylglucosamine Mannose Galactose *N*-Acetyl Neuraminic acid

Conclusions

- A fully integrated workflow for glycan profiling and structural characterization of fluorescently labeled *N*-glycans released was demonstrated successfully.
- The combination of the Vanquish Horizon UHPLC system equipped with fluorescence detection and the Accucore 150-Amide-HILIC column provided the opportunity for assay speed up and associated increase in throughput without compromising the quality of the analytical data.
- The chromatographic resolution, reproducibility, and sensitivity enabled analysis of minor glycoforms, which are otherwise challenging to assign.
- Structural confirmation of glycans with exoglycosidase enzyme digestion followed by determination using the Q Exactive Plus Hybrid Quadrupole-Orbitrap MS allowed rapid and unambiguous profiling.

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