

Comprehensive protein glycosylation comparison of an innovator monoclonal antibody to a candidate biosimilar by HILIC UHPLC analysis

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Keywords

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

Goal

- To apply a fast, hydrophilic interaction UHPLC approach to the comprehensive glycan profiling of 2-AA labelled candidate biosimilar and an innovator mAb
- To determine variations in glycan profile are observable between samples
- To confirm the glycan profiles by exoglycosidase enzyme digestion and high-resolution, accurate-mass (HRAM) mass spectrometry

Introduction

Biosimilars are biologic products that receive authorization based on an abbreviated regulatory application containing comparative quality, non-clinical and clinical data that demonstrate similarity to a licensed biological product (ICH Q5E/Q6B, USP 129). Regulatory authorities have generally reached the consensus that extrapolation of similarity from one indication to other approved indications of the reference product can be permitted if it is scientifically justified.¹ Monoclonal antibody (mAb) products are extraordinarily heterogeneous due to the presence of a variety of enzymatic and chemical modifications, such as deamidation, isomerization, oxidation, glycosylation, glycation, and terminal cyclization. The modifications in different domains of the antibody molecule can result in different biological consequences.

Therefore, characterization and routine monitoring of domain-specific modifications are essential to ensure the quality of the therapeutic antibody products.

Glycosylation is considered a critical quality attribute (CQA) and therefore, should be within an appropriate limit, range or distribution to ensure desired product quality, safety and efficacy.² Different glycosylation variants have been shown to influence the pharmacodynamics and pharmacokinetic behavior, while other glycan structures may be involved in adverse immune reactions.³⁻⁵ Comprehensive glycan profiling may be achieved using a variety of techniques, including use of oligosaccharide standards, enzymatic digests and lectin affinity in combination with liquid chromatography, or capillary electrophoresis coupled with fluorescence detection and mass spectrometry.⁶

Trastuzumab (Herceptin[®]) is a commercially available recombinant IgG1 kappa, humanized monoclonal antibody biotherapeutic produced in Chinese hamster ovary (CHO) cell culture. The early stage development biosimilar candidate has been produced using transient expression in a CHO cell line (Thermo Scientific[™] ExpiCHO[™] expression system). In this work, comprehensive glycan profiling of 2-AA labelled mAbs was achieved using HILIC chromatography coupled to a Thermo Scientific[™] Vanquish[™] UHPLC system with fluorescence (FLD) detection and subsequent structural confirmation by exoglycosidase digestions and high-resolution, accurate-mass mass spectrometry.

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 (purchased from a reputable supplier)
- 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)
- 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)

Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer

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

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

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 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 and 2-AA labeling of released *N*-glycans

1. 100 μg of protein was denatured using 8 M urea in 0.1 M tris buffer pH 8.0 and subsequently reduced and alkylated using 10 mM DTT and 50 mM IAA prepared in UA solution, respectively.
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 1000 units of PNGase F overnight at 37 °C.
3. Released glycans were collected from the 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.
5. Glycan samples were derivatized with 5 μL 2-aminobenzoic acid (2-AA) via reductive amination with sodium cyanoborohydride in 30% v/v acetic acid in DMSO at 60 °C for 5 hours.
6. Excess labelling reagent was removed by HILIC purification using an UltiMate 3000RS system. Samples were loaded in 80% acetonitrile, 20% 50 mM ammonium formate pH 4.4 (v/v) onto an Accucore 150-Amide-HILIC 2.1 × 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, $\lambda_{\text{ex/em}} = 350/425$ nm and evaporated to dryness.

Exoglycosidase digestions

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 labelled *N*-glycan pool was placed in a 0.2 mL PCR tubes (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) was 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) was 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 × 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 $\alpha(2-3)$, $\alpha(2-6)$ and $\alpha(2-8)$ lined non-reducing terminal sialic acids (NeuNAc and NeuNGc)	1 μL
BKF	Releases $\alpha(1-2)$ and $\alpha(1-6)$ linked non-reducing terminal fucose residues more efficiently than $\alpha(1-3)$ and $\alpha(1-4)$ linked fucose. Used for release of core $\alpha(1-6)$ fucose residues, can also remove $\alpha(1-3)$, but less efficiently.	1 μL
SPG	Hydrolyses non-reducing terminal $\beta(1-4)$ linked galactose residues	2 μL
GUH	Recombinantly expressed in <i>E. coli</i> . Releases β -linked GlcNAc but not bisecting GlcNAc $\beta(1-4)$ Man	2 μL

UHPLC-fluorescence *N*-glycan profiling

Labeled *N*-glycans were separated by hydrophilic interaction UHPLC-FLD on a Vanquish Horizon UHPLC. 2-AA labelled glycans were separated using a linear gradient. Glycans were injected in 20 μL 80% v/v acetonitrile and stored at 10 °C prior to injection.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 μm , 2.1 \times 150 mm (P/N 16726-152130)
Mobile phase A:	Ammonium formate 50 mM, pH 4.5
Mobile phase B:	Acetonitrile
Flow rate:	1.3 mL/min
Column temperature:	60 $^{\circ}\text{C}$
Sample volume:	20 μL
Backpressure:	Approximately 500 bar

Table 2. Mobile phase gradient for UHPLC-fluorescence N-glycan profiling.

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

N-glycan analysis by LC-MS

Glycan samples were injected on a Q Exactive Plus hybrid quadrupole-Orbitrap MS equipped with H-ESI ion source. Samples were diluted in 75% acetonitrile prior to analysis.

Separation conditions

Column:	Accucore 150-Amide-HILIC 2.6 μm , 2.1 \times 150 mm
Mobile phase A:	Ammonium formate 50 mM, pH 4.5
Mobile phase B:	Acetonitrile
Flow rate:	0.4 mL/min
Column temperature:	40 $^{\circ}\text{C}$
Sample volume:	11 μL

Table 3. 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 $^{\circ}\text{C}$
Sheath gas flow:	20 Arb
Auxiliary gas flow:	10 Arb
Spray voltage:	3.8 kV
Capillary temperature:	320 $^{\circ}\text{C}$

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

A candidate biosimilar mAb was compared in detail to a commercially available IgG1 innovator product. Separation of *N*-glycan structures for innovator (black trace, a) and biosimilar candidate produced in house (blue trace, b) are displayed as a mirror chromatogram in Figure 1. Thirteen *N*-glycan features were assigned for commercial IgG1 mAb (trastuzumab, Herceptin) and 16 *N*-glycan features for the biosimilar candidate.

Annotation of the *N*-glycans structures and glycosidic linkages present in each chromatographic peak were deduced using exoglycosidase arrays as detailed in Figures 2 and 3. Use of exoglycosidase arrays involves the application of a panel of enzymes with high specificity for different monosaccharides to samples of released glycans. Here, a panel of four exoglycosidases, namely sialidase (ABS), α (1-2,3,4,6) fucosidase (BKF), beta(1-4)-galactosidase (SPG), and β -*N*-acetylhexosaminidase (GUH), were applied to facilitate removal of sialic acid, fucose, galactose, and hexose glycan constituents, respectively. After removal of specific monosaccharides, differences in retention time and peak area were observed upon LC analysis, as is shown in Figures 2 and 3, enabling the structural annotation of glycan species in the mAb samples under analysis. The oligosaccharide composition, outlined in Table 4, was also confirmed by accurate-mass MS data.

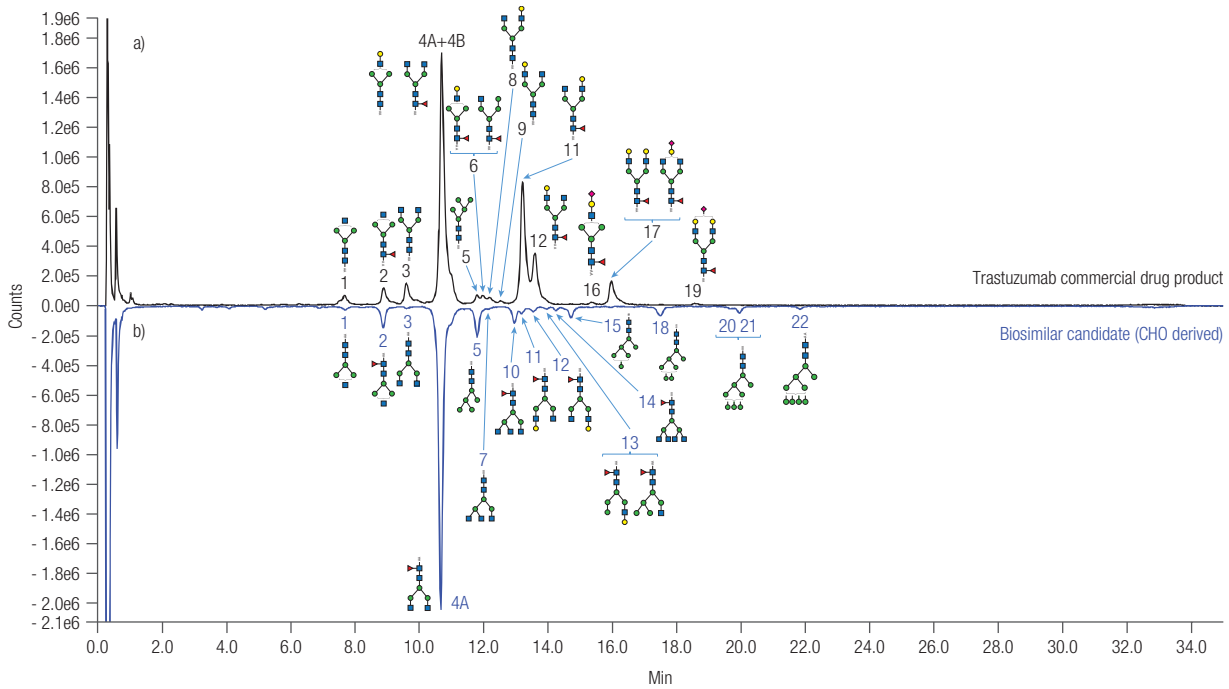


Figure 1. Mirror plot for the chromatographic separation on an Accucore 150-Amide-HILIC column (2.1 × 150 mm, 2.6 μm) of *N*-glycans from commercial IgG1 mAb (a) and biosimilar candidate (b) produced in CHO cell lines.

Relative areas for the identified glycan peaks showed few important differences in terms of glycosylation as defined in Table 4. Innovator drug product showed 92.5% of core fucosylated *N*-glycan mainly bi-antennary structures and 5.6% of sialylated structures. The biosimilar candidate

produced ‘in house’ showed 80.9% of core fucosylation and a high content of high mannose *N*-glycan structures (14.5%). Non-sialylated structures were detected for the biosimilar candidate.

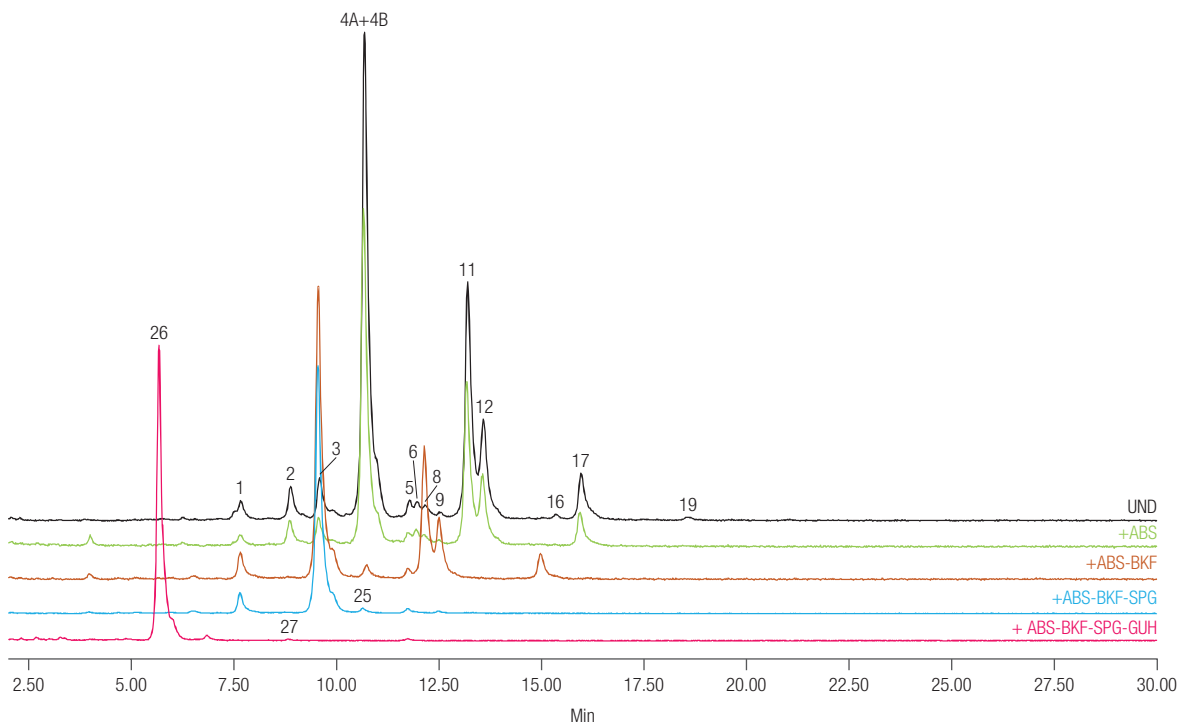


Figure 2. HILIC chromatograms of trastuzumab commercial drug product 2-AA labelled *N*-glycan pool (undigested, UND) and after digestion with a range of exoglycosidase enzymes on an Accucore 150-Amide-HILIC 2.1 × 150 mm column. ABS removes all sialic acids (α 2-3, -6 and -8), BKF removes α (1-6) linked 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.

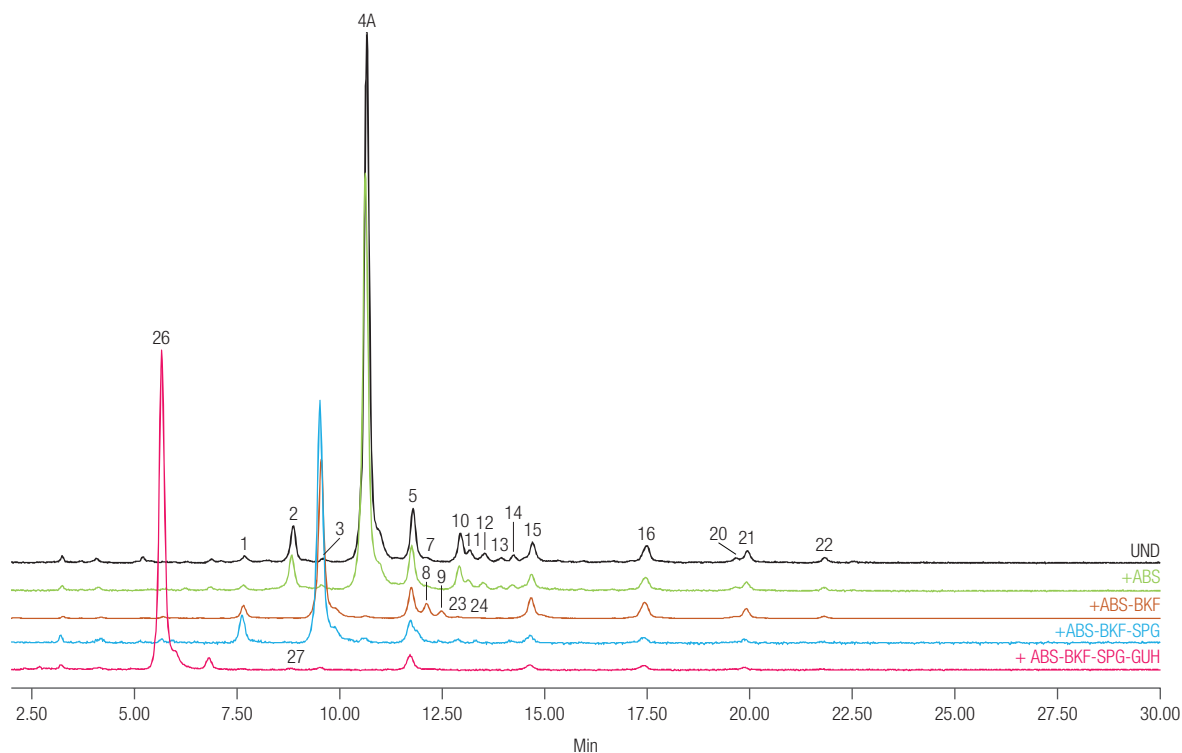


Figure 3. HILIC chromatograms of trastuzumab biosimilar candidate 2-AA labelled *N*-glycan pool (undigested, UND) and after digestion with a range of exoglycosidase enzymes on Accucore 150-Amide-HILIC 2.1 × 150 mm column. ABS removes all sialic acids (α 2-3, -6 and -8), BKF removes α (1-6) linked core 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 4. Structural identification of 2-AA labeled *N*-glycans from trastuzumab commercial drug product and related biosimilar candidate using an Accucore 150-Amide-HILIC column, exoglycosidase digestions, and offline Q Exactive Plus MS. Green shading signifies the presence of that glycan moiety in either mAb, whereas red shading indicates the absence of a glycan species in either mAb sample.

Glycan peak number	Glycan structure	Present in commercial mAb	Present in candidate biosimilar
1			
2			
3			
4A		A + B	A
4B		A + B	B
5			
6A		A + B	A + B
6B			
7			
8			
9			

Glycan peak number	Glycan structure	Present in commercial mAb	Present in candidate biosimilar
10		Red	Green
11		Green	Green
12		Green	Green
13 A		Red (A + B)	Green (A + B)
13 B			
14		Red	Green
15		Red	Green
16		Green	Red
17 A		Green (A + B)	Red (A + B)
17 B			
18		Red	Green
19		Green	Red
20		Red	Green
21		Red	Green
22		Red	Green
23 A		Red (A + B)	Green (A + B)
23 B			
24		Red	Green
25		Green	Red
26		Green	Green
27		Green	Green

Fucose
 N-Acetylglucosamine
 Mannose
 Galactose
 N-Acetyl Neuraminic acid

Conclusions

- A fully integrated workflow for glycan profiling for an innovator and candidate biosimilar was successfully demonstrated and differences between biomolecules were identified.
- The combination of the Vanquish Horizon UHPLC system equipped with fluorescence detection and the Accucore 150-Amide-HILIC column provided fast resolution of *N*-glycoforms from biotherapeutics for comprehensive glycan profiling.
- 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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