N-Glycan Analysis of mAbs and Other Glycoproteins with UHPLC and Fluorescence Detection

The Agilent 1290 Infinity Binary LC System with the Agilent AdvanceBio Glycan Mapping Column

Application Note

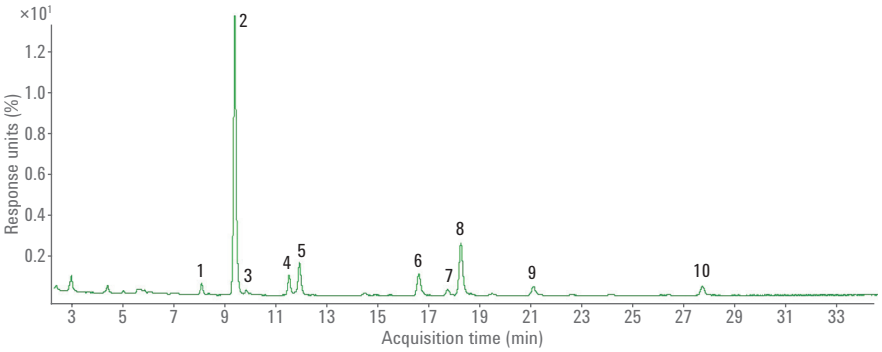
Biologics and Biosimilars

Authors

Sonja Schneider and Oscar Potter
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

This Application Note describes the analysis of N-linked glycans with hydrophilic interaction chromatography (HILIC) using the Agilent 1290 Infinity Binary LC together with the Agilent 1260 Infinity Fluorescence Detector and the Agilent 6530 Accurate-Mass Q-TOF LC/MS. Enzymatic glycan release with PNGase F followed by derivatization by 2-aminobenzamide (2-AB) was conducted on monoclonal antibodies (mAbs) and two other glycoproteins, fetuin and ovalbumin. The excellent resolution provided by the Agilent AdvanceBio Glycan Mapping column allowed detection and identification of all major N-glycans in the mAb sample. Furthermore, the highly complex N-glycans released from fetuin and ovalbumin were well resolved.



Agilent Technologies

Introduction

Glycosylation is one of the most frequently observed post-translational modifications. Mammalian glycoproteins contain three major types of glycans: N-linked, O-linked, and glycosylphosphatidylinositol (GPI) lipid anchors, which consist of one or more monosaccharide units. A single glycosylation site can generate considerable heterogeneity of the mass and charge of glycoproteins. These oligosaccharides are involved in many biological regulation and recognition processes, for example, protein sorting, immune and receptor recognition, inflammation, pathogenicity, metastasis, and other cellular processes[1,2]. In addition, properties such as safety, efficacy, and the serum half-life of therapeutic proteins can be affected by their glycosylation pattern.

Recombinant monoclonal antibody therapeutics (mAbs) represent the largest group of therapeutic proteins. The efficacy of these therapeutics is highly dependent on the correct glycosylation pattern of the mAbs and, so far, all licensed therapeutic mAbs are immunoglobulins G (IgGs)[3]. Human IgG has a single conserved N-linked glycosylation site located on the Fc region of each heavy chain at Asn297[4] (Figure 1), resulting in the presence of two N-glycans per IgG. This typically consists of a handful of major structures and numerous minor structures[5]. The combination of glycans at each of the two glycosylation sites on the Fc region leads to large numbers of different glycoforms in each batch of mAb production.

The glycan structure plays a critical role in complement activation and receptor affinity[6], which affect the efficacy of therapeutic mAbs. Moreover, non-human glycans are a safety issue due to induced immune responses. Therefore, analysis of the glycan pattern is an important part of the characterization of therapeutic glycoproteins, especially mAbs.

This Application Note uses symbolic glycan structures according to the Consortium for Functional Glycomics (CFG), as shown in Figure 2. Assigned glycans are also described by the Oxford glycan nomenclature and by another style of nomenclature, which is popular for mAb glycans, shown here in italics.

Figure 2A shows the general nomenclature used to describe sugar residues of different glycan structures on proteins. Figure 2B shows some predominant glycan structures present on the Asn-297 site in human IgG. In general, N-glycans have a core structure, containing two β-D-N-acetylglucosamine (GlcNac) and three mannose (Man) units. IgG Fc N-glycans are predominantly biantennary complex-type structures, partially core-fucosylated (for example, FA2 or G0F).

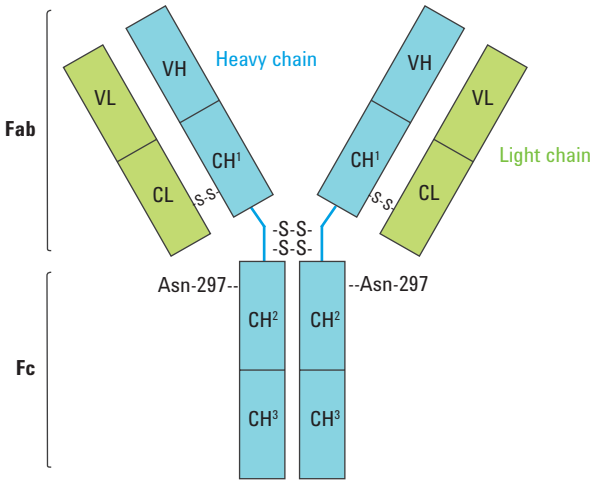


Figure 1. IgG antibody structure.

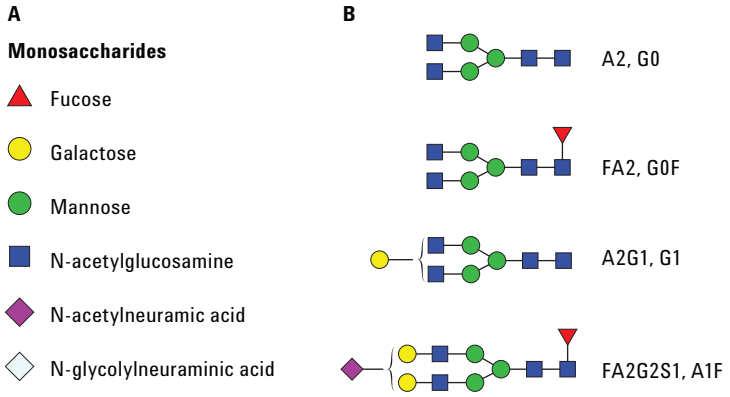


Figure 2. Glycan structure and isoforms. A) Monosaccharide description after the Consortium for Functional Glycomics, B) predominant glycan structures of human IgGs.

Different strategies for the analysis of N-glycans have been described. Many methods are based on enzymatic release of N-glycans from the protein by PNGase F. Due to the lack of intrinsic chromophores, it is also common to derivatize the glycans with a fluorescent label prior to analysis[7]. Each N-glycan contains a single reducing end site that can be reacted with an excess of fluorescent label, such that each N-glycan will be attached to one fluorophore. The processed sample is, therefore, appropriate for relative quantification by separation with fluorescence detection without the need for any quantitation standards or calibration. 2-AB is a stable, neutral label that is popular for N-glycan analysis[7,8,9]. Figure 3 illustrates 2-AB-labeling by reductive amination (Schiff's base intermediate not shown).

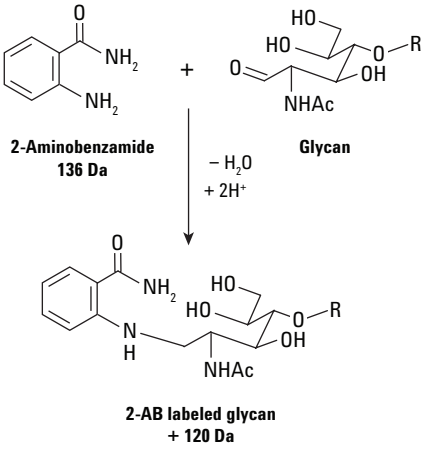


Figure 3. Labeling of a glycan with 2-aminobenzamide (2-AB).

Subsequent purification using hydrophilic interaction chromatography/solid phase extraction (HILIC/SPE) is performed to remove the large excess of 2-AB so that it does not interfere with the HILIC/FLD analysis.

Here, we show enzymatic release of N-glycans using PNGase F with subsequent derivatization with 2-AB prior to separation by HILIC UHPLC, with fluorescence detection and identification by on-line quadrupole time-of-flight mass spectrometry (Q-TOF/MS).

Experimental

The Agilent 1290 Infinity Binary LC System consisted of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A) with 35-µL Jet Weaver
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B) with standard flow cell

MS system

Agilent 6530 Accurate-Mass Q-TOF LC/MS

Column

Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)

Software

Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems Rev. C.01.05 [38] and Agilent MassHunter Workstation Software, Version B.05.01, Build 4.0.479.0. Glycan structures were created with GlycoWorkbench, Version 2.1, stable (146).

Sample preparation

Deglycosylation procedure

N-glycans were released from a monoclonal antibody, fetuin, and ovalbumin using PNGase F. This enzyme cleaves asparagine-linked high mannose as well as hybrid and complex oligosaccharides from the glycoproteins and leaves the glycans intact. Fetuin has three N-glycosylation sites (Asn-81, Asn-138, and Asn-158) and four O-linked sites (Ser-253, Thr-262, Ser-264, and Ser-323)[11]. Ovalbumin has only one glycosylation site, whereas the mAb contains two glycosylation sites. The amount of PNGase F was adjusted to the amount of N-glycosylation sites. The proteins were deglycosylated according to instructions for 3 hours at 37 °C. The reaction was then stopped, and the sample was vacuum dried for further processing.

2-AB-labeling for fluorescence detection and sample cleanup

The dried glycan samples were labeled with 2-aminobenzamide according to the protocol for 3 hours at 65 °C. After the labeling procedure, the samples were purified using the HILIC cleanup cartridges according to the instruction manual. After the cleanup procedure, the samples were vacuum-dried and reconstituted in ultrapure water:acetonitrile 30:70 (v/v) for analysis.

Solvents and samples

Buffer A was 100 mM ammonium formate in water, pH 4.5 and buffer B was acetonitrile. All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). The monoclonal antibody was RAT Anti-DYKDDDDK Tag Antibody. Ammonium formate, fetuin and ovalbumin, PNGase F from *Elizabethkingia miricola*, GlycoProfil 2-AB Labeling Kit, and GlycoProfil Glycan Cleanup Cartridges were purchased from Sigma-Aldrich Corp., St. Louis, USA.

Instrumental conditions

	Antibody standard gradient	Fetuin gradient	Ovalbumin gradient
Starting flow rate	0.5 mL/min	0.5 mL/min	0.5 mL/min
Gradient	0 minutes 85 % B	0 minutes 75 % B	0 to 6 minutes 85 % B
	5 minutes 75 % B	45 minutes 50 % B	10 minutes 80 % B
	35 minutes 64 % B	47 minutes 40 % B, flow 0.5 mL/min	60 minutes 70 % B
	40 minutes 50 % B	47.01 minutes, flow 0.25 mL/min	65 minutes 50 % B, flow 0.5 mL/min
	42 minutes, flow 0.5 mL/min 42.01 minutes, flow 0.25 mL/min	49 minutes 0% B	65.01 minutes, flow 0.25 mL/min
	43 minutes 0 % B	51 minutes 0 % B	68 minutes 0 % B
	48 minutes 0 % B	51.01 minutes 75 % B, flow 0.25 mL/min	73 minutes 0 % B
	50 minutes 85 % B 50.01 minutes, flow 0.25 mL/min	52.00 minutes, flow 0.5 mL/min	74 minutes 85 % B, flow 0.25 mL/min
	51 minutes, flow 0.5 mL/min		75.00 minutes, flow 0.5 mL/min
Stop time	51 minutes	52 minutes	75 minutes
Post time	20 minutes	20 minutes	20 minutes
Injection volume	5 µL	1 µL	1 µL
Thermostat autosampler	5 °C		
Column temperature	60 °C		
FLD	Ex. 260 nm, em. 430 nm		
Peak width	> 0.013 minutes (0.25 seconds resp. time) (37.04 Hz)		

MS parameters	
Gas temperature	250 °C
Sheath gas temperature	250 °C
Gas flow	8 L/min
Sheath gas flow	8 L/min
Nebulizer	25 psi
Vcap	3,500 V
Nozzle	1,000 V
Fragmentor	200 V
Skimmer	45 V
Oct 1 RF Vpp	550
Collision energies	15 and 30 V
Mode	MS and targeted MS/MS

Results and Discussion

Analysis of N-glycans from monoclonal antibodies

Figure 4 shows the separation of the mAb N-glycans. The mAb glycan pattern was optimally resolved, allowing separation and integration of all major N-glycans. Relative quantification was made based on the calculation of the peak area percentage. High intensity of the labeled glycans was achieved by setting the optimal wavelengths for glycan detection on the Agilent 1260 Infinity Fluorescence Detector, using 260 nm as excitation wavelength and 430 nm as emission wavelength[10].

The resulting HILIC glycan profile was assigned to the corresponding glycan structures based on the parent ion mass observed and the related MS/MS spectra. The parent masses were entered into the GlycoMod tool from Expasy to find related glycan structures.

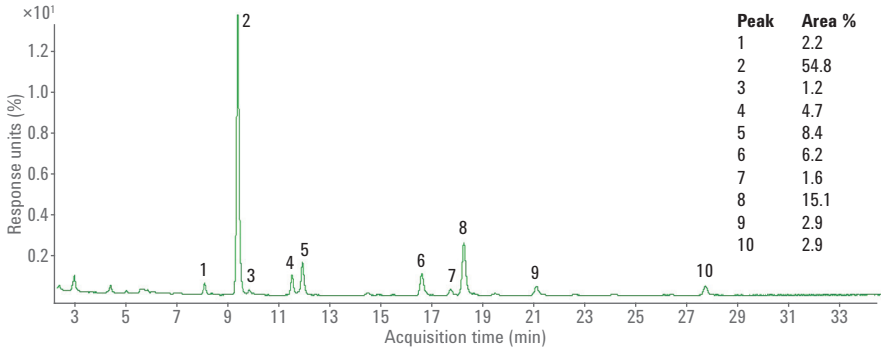


Figure 4. Separation of mAb N-glycans with fluorescence detection with 260 nm as the excitation wavelength.

GlycoMod predicts possible glycan structures (labelled or unlabeled) from the experimentally determined masses (http://web.expasy.org/glycomod/). Another helpful tool for glycan assignment and glycan structure design is GlycoWorkbench[15], which was used in this work to prepare glycan structure cartoons.

As an example of the workflow, the N-glycan FA2G1Sg1 with a parent mass of 1026.88 [z = 2] (peak 7 and 8) was chosen. The glycan databases revealed the two most likely glycan structures for this mass (Figure 5).

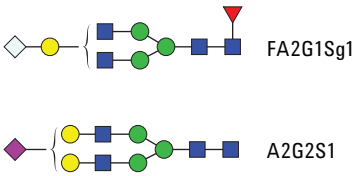


Figure 5. Two most likely glycan structures for the parent mass of 1,026.88 [z = 2], FA2G1Sg1 and A2G2S1.

MS/MS data was then used to distinguish between these two potential structures. Figure 6 shows the collision-induced dissociation (CID) MS/MS spectrum of the N-glycan FA2G1Sg1. The MS/MS data confirm the presence of a type of sialic acid, N-glycolylneuraminic acid (NeuGc), which results in strong signals for fragment ions at *m/z* 308 (NeuGc) and *m/z* 673 (NeuGc attached to galactose and N-acetylglucosamine). Meanwhile, there are no signals at *m/z* 292 or *m/z* 657, which would have indicated the presence of N-acetylneuraminic acid (NeuAc). Therefore, these results provided strong evidence that the structure was FA2G1Sg1 (containing NeuGc) rather than A2G2S1 (which contains NeuAc). The decision to assign a structure with a core fucose was also supported by the MS/MS data, based on the lack of strong fragment ion signals at *m/z* 512 or *m/z* 350, which would have been present if the fucose had instead been attached in the outer arm region.

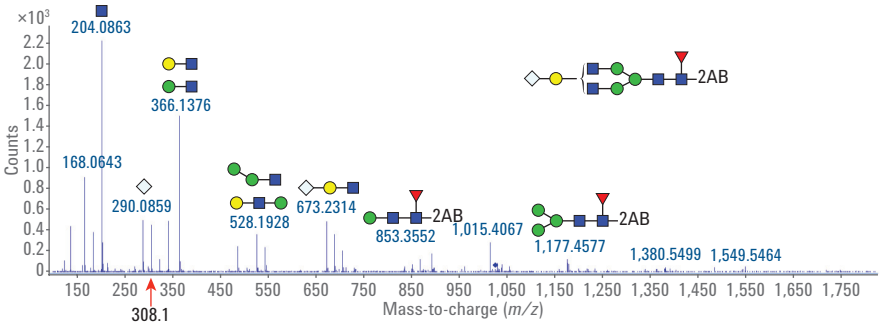


Figure 6. MS/MS spectra of FA2G1Sg1 - 1026.88 [z = 2] - 1931.6876 Da.

All other peaks were similarly assigned using their MS and MS/MS spectra. The assigned structures are given in Table 1. The results show that the mAb mainly contains core fucosylated complex glycans, including several structures with NeuGc. These findings are typical for an IgG antibody produced by rat cells. NeuGc does not normally occur in human glycoproteins and is undesirable in therapeutic proteins[12]. The sialic acids occurring in human glycoproteins are typically N-acetylneuramic acids.

Table 1. Overview of masses and assigned 2AB- glycan structures of rat monoclonal antibody.

Peak	Oxford	Biopharma mAb style	Mass detected (Da)	Structure
1	FA1	G0-GlcNAc	1,380.537 [z = 1]	
2	FA2	G0F	792.3130 [z = 2]	
3	M5	Man5	1,355.5 [z = 1]	
4,5	FA2G1	G1F	873.34 [z = 2	
6	FA1G1Sg1	G1FSg1-GlcNAc	925.34 [z = 2]	
7,8	FA2G1Sg1	G1FSg1	1,026.88 [z = 2]	
9	FA2G2Sg1	Ag1F	1,107.9135 [z = 2]	
10	FA2G2Sg2	Ag2F	1,261.446 [z = 2]	

Analysis of antibodies from fetuin and ovalbumin

N-glycans from two more proteins, fetuin and ovalbumin, were released by PNGase F, derivatized with 2-AB and analyzed using HILIC/UHPLC with online MS. Figure 7 shows the separation of bovine fetuin N-glycans. This glycosylation profile was dominated by complex non-fucosylated biantennary and triantennary glycans containing NeuAc. Nine major peaks could be assigned using Q-TOF/MS detection. Table 2 shows the assigned glycan structures.

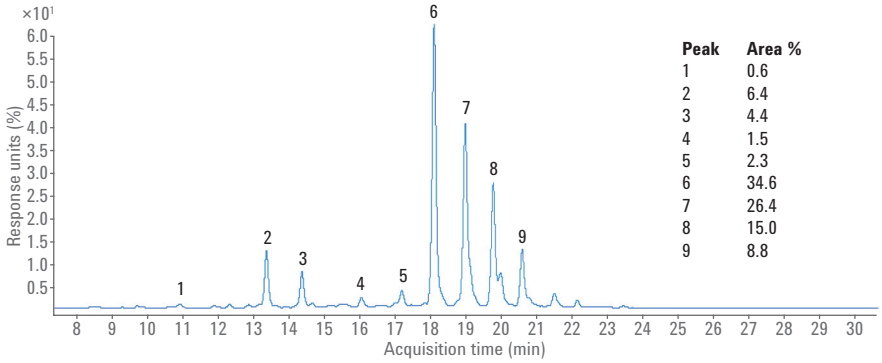


Figure 7. Separation of 2-AB-labeled fetuin.

Table 2. Detailed information of N-glycan ovalbumins.

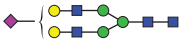
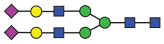
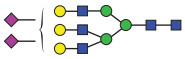
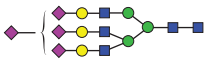
Peak	Oxford	Structure
1	A2G2S1	
2,3	A2G2S2	
4	A3G3S2	
5	A3G3S3, A3G3S2 (trace)	
6	A3G3S3, A3G3S2 (trace)	
7	A3G3S3, A3G3S4 (trace)	
8	A3G3S4, A3G3S3	
9	A3G3S4	

Figure 8 shows the separation of ovalbumin glycans. Ovalbumin is N-glycosylated only at one site (Asn292), but a complex glycosylation pattern can be associated to this site[13]. Due to the complexity of the glycan profile, the gradient had to be adjusted to a longer separation time to achieve higher resolution. The high performance of the AdvanceBio Glycan Mapping column allowed resolution of over 50 peaks with a good signal-to-noise (S/N) ratio. Twenty major peaks were assigned based on the parent ion-mass data (Table 3). Detailed structural conclusions were not achievable due to the high chance of isobaric structures occurring, several of which cannot necessarily be distinguished from the MS/MS data. Instead, the N-glycans are described in terms of their monosaccharide composition.

Compared to the relatively simple glycan pattern of the mAb, the two other glycoproteins had a greater variety of glycan structures. No fucosylated glycans were detected in ovalbumin in contrast to the mAb glycans, which matches previously reported findings that avian egg glycoproteins are non-fucosylated[14].

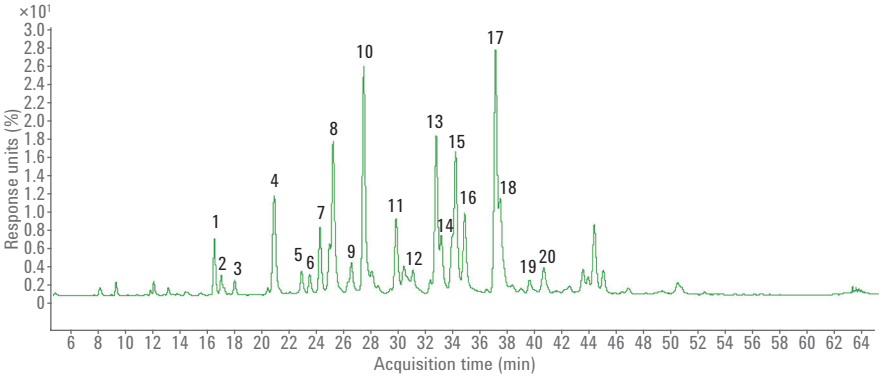


Figure 8. Separation of N-glycans released from ovalbumin.

Table 3. Assigned masses and monosaccharide composition of ovalbumin N-glycans; H = hexoses, i.e. galactose or mannose; N = N-acetylglucosamine.

Peak	Mass + 2AB (Da)	Calculated mass (Da)	Composition (short form)	Composition (long form)
1,2	1,234.48 [M]	1,114.48	H3N3	$(HexNAc)_1+(Man)_3(GlcNAc)_2$
3	1,193.45 [M]	1,073.45	H4N2	$(Hex)_4(HexNAc)_2$
4	1,437.56 [M]	1,317.56	H3N4	$(HexNAc)_2+(Man)_3(GlcNAc)_2$
5	1,396.53 [M]	1,276.53	H4N3	$(Hex)_1(HexNAc)_1+(Man)_3(GlcNAc)_2$
6,7	1,640.64 [M]	1,520.64	H3N5	$(HexNAc)_3+(Man)_3(GlcNAc)_2$
8	1,355.506 [M]	1,235.51	H2N5	$(Hex)_2+(Man)_3(GlcNAc)_2$
9	1,599.61 [M]	1,479.61	H4N4	$(Hex)_1(HexNAc)_2+(Man)_3(GlcNAc)_2$
10	1,843.73 [M]	1,723.73	H3N6	$(HexNAc)_4+(Man)_3(GlcNAc)_2$
11	1,802.74 [M]	1,682.74	H4N5	$(Hex)_1(HexNAc)_3+(Man)_3(GlcNAc)_2$
12,14	2,046.884 [M]	1,926.88	H3N7	$(HexNAc)_5+(Man)_3(GlcNAc)_2$
13	1,517.56 [M]	1,397.56	H6N2	$(Hex)_3+(Man)_3(GlcNAc)_2$
15	1,761.6574 [M]	1,641.66	H5N4	$(Hex)_2(HexNAc)_2+(Man)_3(GlcNAc)_2$
16	2,005.8098 [M]	1,885.81	H4N6	$(Hex)_1(HexNAc)_4+(Man)_3(GlcNAc)_2$
17	2,249.9728 [M]	2,129.97	H3N8	$(HexNAc)_6+(Man)_3(GlcNAc)_2$
18	1,964.82 [M]	1,844.82	H5N5	$(Hex)_2(HexNAc)_3+(Man)_3(GlcNAc)_2$
19,20	2,208.87 [M]	2,088.87	H4N7	$(Hex)_1(HexNAc)_5+(Man)_3(GlcNAc)_2$

Conclusions

The Agilent 1290 Infinity Binary LC System, together with the Agilent 1260 Infinity Fluorescence Detector and Agilent 6530 Accurate-Mass Q-TOF LC/MS was an ideal combination for the analysis of released N-glycans that were derivatized with 2-aminobenzamide. Sample preparation using PNGase F for the release of N-linked glycans followed by 2-AB derivatization with subsequent HILIC sample cleanup was demonstrated for one monoclonal antibody and two other glycoproteins.

The Agilent AdvanceBio Glycan Mapping column demonstrated excellent resolving power, allowing separation and identification of all major N-glycans in a rat mAb sample. Complex biantennary and triantennary N-glycans from fetuin and ovalbumin were also analyzed with very high resolution. Optimized fluorescence excitation and emission wavelengths of 260 and 430 nm provided better S/N ratios. Electrospray ionization Q-TOF MS analysis allowed assignment of different glycan structures or monosaccharide compositions, depending on the complexity of the sample.

References

1. Rademacher, T. W; Williams, P; DwekMark, R. A. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *P. Natl. Acad. Sci*. **1994**, *91*, pp 6123-6127.
2. Peracaula. R; *et al.* Glycosylation of human pancreatic ribonuclease: differences between normal and tumor states. *Glycobiology* **2003**, *13*, pp 227-244.
3. Jefferis, R. Glycosylation of recombinant antibody therapeutics. *Biotechnol. Progr.* **2005**, *21*, pp 11-16.
4. Arnold, J. N; *et al.* Human immunoglobulin glycosylation and the lectin pathway of complement activation. *Adv. Exp. Med. Biol.* **2005**, *564*, pp 27-43.
5. Fernandes, D. Demonstrating comparability of antibody glycosylation during biomanufacturing. *Euro. Biopharm. Re*. **2005**, Summer, pp 106-110.
6. Abès, R; Teillaud, J. L. Impact of Glycosylation on Effector Functions of Therapeutic IgG. *Pharmaceuticals* **2010**, *3*, pp 146-157.
7. Ruhaak, L. R; *et al.* Glycan labeling strategies and their use in identification and quantification. *Anal. Bioanal. Chem*. **2009**, *397*, pp 3457-3481.
8. Royle, L; *et al.* HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal. Biochem*. **2008**, *376*, pp 1-12.
9. Huhn, C; *et al.* IgG glycosylation analysis. *Proteomics* **2009**, *9*, pp 882-913.
10. Melmer, M; *et al.* HILIC analysis of fluorescence-labeled N-glycans from recombinant biopharmaceuticals. *Anal. Bioanal. Chem*. **2010**, *398*, pp 905-914.
11. Ding, W; *et al.* Identification and Quantification of Glycoproteins Using Ion-Pairing Normal-phase Liquid Chromatography and Mass Spectrometry. *Mol. Cell. Proteom.* **2009**, *8*, pp 2170-2185.
12. Leibiger, H; *et al.* Variable domain-linked oligosaccharides of a human monoclonal IgG: structure and influence on antigen binding. *Biochem. J*. **1999**, *338*, pp 529-538.
13. Anumula, K. R. Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. *Anal. Biochem.* **2006**, *350*, pp 1-23.
14. Montreuil, J; Vliegenthart, J. F. G; Schachter, H., Eds.; Glycoproteins II. Elsevier B. V., Amsterdam, **1997**.
15. Ceroni, A; *et al.* GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. *J. Proteome Res*. **2008**, *7*, pp 1650-1659.

www.agilent.com/chem

Published in the USA, October 1, 2014
5991-5253EN

Agilent Technologies