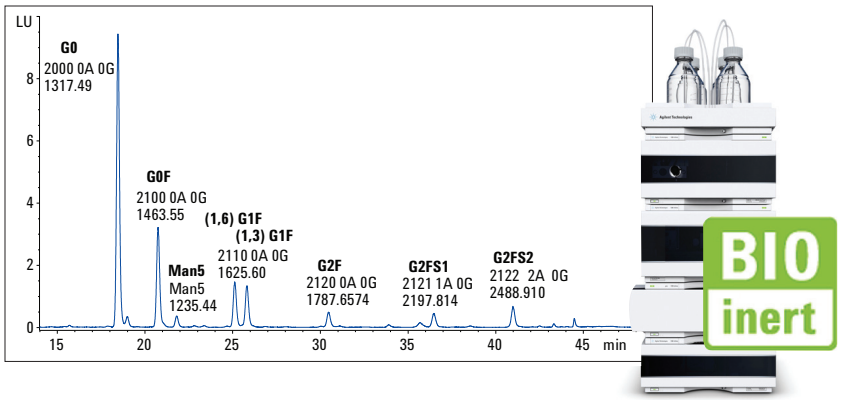
N-Glycan analysis of monoclonal antibodies and other glycoproteins using UHPLC with fluorescence detection

Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Infinity Fluorescence Detector

Application Note

Biopharmaceuticals



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Abstract

This Application Note shows the analysis of N-linked glycans with hydrophilic interaction chromatography (HILIC) separation using the Agilent 1260 Infinity Bio-inert Quaternary LC System together with the Agilent 1260 Infinity Fluorescence Detector. Enzymatic glycan release with PNGase F followed by derivatization by 2-aminobenzamide (2-AB) was conducted on monoclonal antibodies (mAbs) and two other glycoproteins from avian egg white: ovalbumin and conalbumin. The glycan pattern of the monoclonal antibody showed very good resolution and all major N-glycans occurring in mAbs could be detected with good signal-to-noise ratios. In addition, the complex glycan patterns of the two major types of avian egg glycoproteins were well resolved, resulting in over 30 to 35 detected peaks. The 1260 Infinity Bio-inert Quaternary LC System together with the 1260 Infinity Fluorescence Detector is an ideal solution for sensitive and high resolution analysis of 2-AB derivatized glycans released from mAbs and other glycoproteins.

Verified for Agilent
1260 Infinity II LC Bio-inert System

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Introduction

Protein glycosylation is one of the most frequently observed post translational modifications. Mammalian glycoproteins contain three major types of glycans: N-linked, O-linked, and glycosylphosphatidyinositol (GPI) lipid anchors, which consist of one or more monosaccharide units. A single glycosylation site can generate considerable heterogeneity regarding 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. Therefore, certain glycosylation patterns can be associated to the diseased or healthy state of a patient[1, 2]. In addition, properties like 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 as a major new class of drug. The efficacy of these therapeutics is highly dependent on the correct glycosylation patterns of the mAbs and so far, all licensed therapeutic mAbs are immunoglobulines G (IgGs)[3]. Human IgG has a single conserved N-linked glycosylation site located on the Fc region of each heavy chain at Asn-297[4] (Figure 1). This fact results in two sugar moieties per IgG, which are highly heterogeneous and contain up to 30 different glycan types[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.

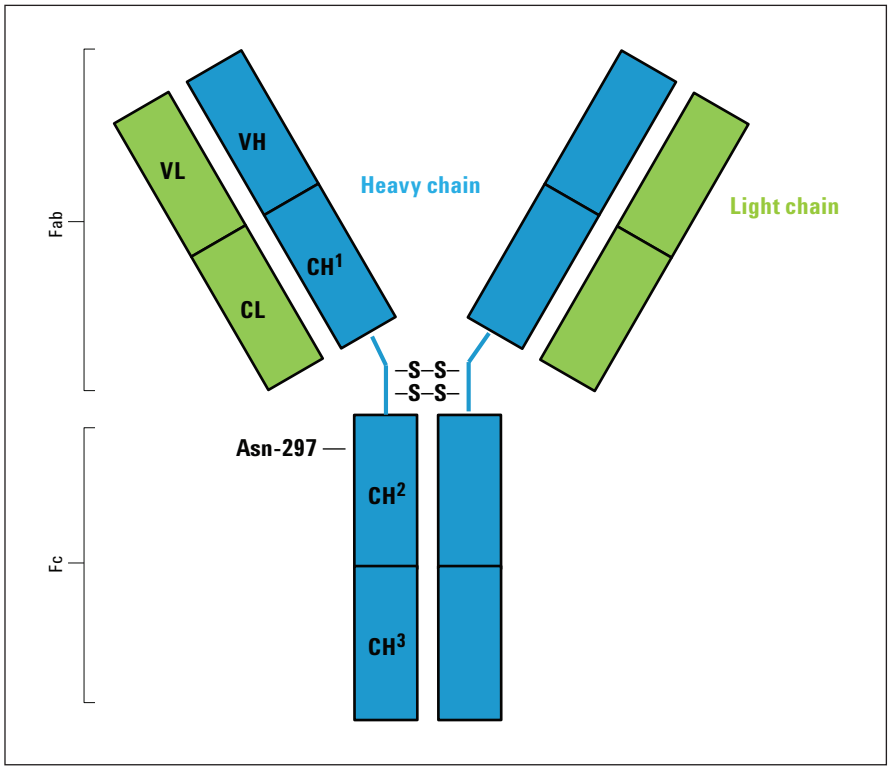

Figure 1
IgG antibody structure.

The glycan structure on this glycosylation site plays a critical role in complement activation and receptor affinity[6], which affects the efficacy of the therapeutic mAbs. Moreover, nonhuman glycans are a safety issue due to induced immune responses.

Therefore, analysis of the glycan pattern is an important part of characterization of therapeutic glycoproteins, especially mAbs. Figure 2a shows the general nomenclature used to describe sugar residues of different glycan structures on proteins. Figure 2b shows the predominant glycan structures present on the Asn-297 site in 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, G0F).

Different strategies for the analysis of glycans have been described. A large number of methods are based on protein-released and subsequently derivatized glycans[7] due to the lack of chromophores needed for optical detection methods, for example, UV detection. We have presented a combination of enzymatic release of N-glycans using PNGase F with subsequent derivatization with 2-aminobenzamide (2-AB) for fluorescence detection.

2-AB is a neutral and stable bonded label, numerously used in glycan analysis[7, 8, 10]. Figure 3 shows the 2-AB-labeling by reductive amination (Schiff's base intermediate not shown). The 2-AB labeling of the glycans results in the mass of the glycans +119 Da. Due to protonation, the resulting mass shift in the MS is 120 Da (Tables 1, 2, 3).

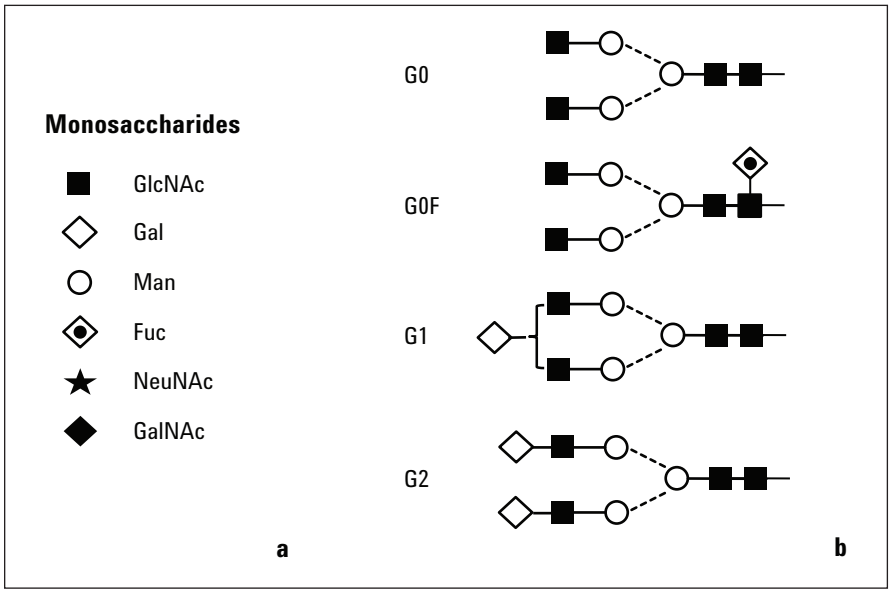

Figure 2
Glycan structure and isoforms. a) General nomenclature for glycans b) Predominant glycan structures of IgGs. G = Galactose units, F = Fucose units. Modified after Arnold *et al*[6].

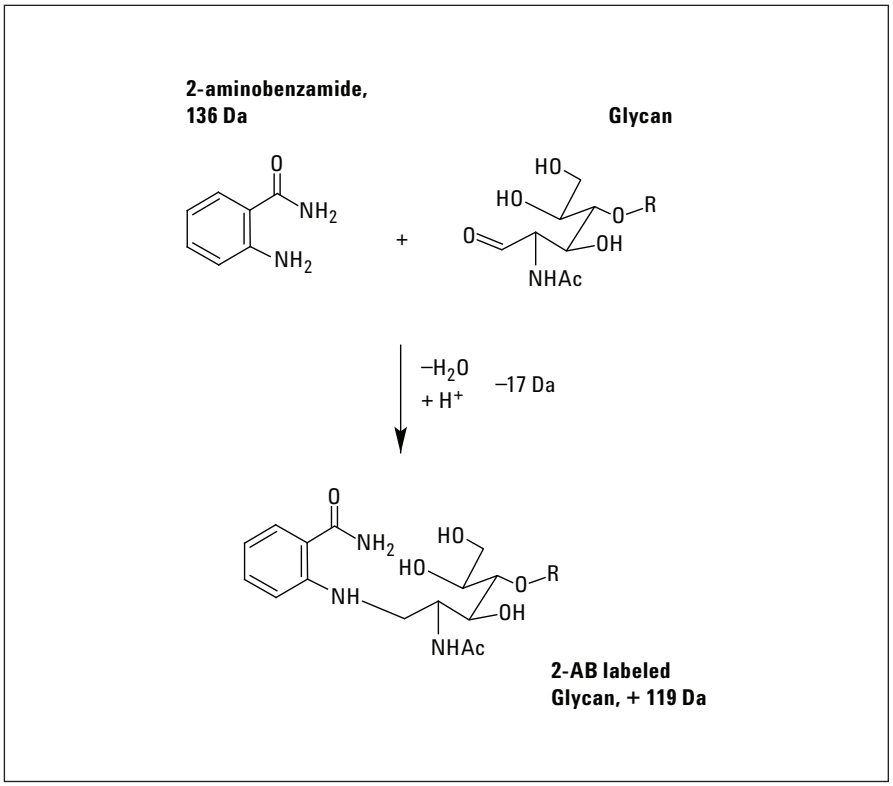

Figure 3
Labeling of a glycan with 2-aminobenzamide (2-AB).

Due to the hydrophilic properties of glycans, subsequent purification using Hydrophilic Interaction Chromatography – Solid Phase Extraction (HILIC-SPE) is added for removal of excess label[8].

Separation using HILIC with fluorescence detection is a robust method for glycan analysis[9]. Pauline Rudd and coworkers established a database (http://glycobase.nibrt.ie/) based on HIILIC retention properties, a frequently used technology for the analysis of protein glycosylation[8, 10]. To identify the monosaccharide composition of the glycans within a chromatographic peak, HILIC-LC can be coupled to ESI-QToF-MS for mass and structure information.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector VL (G1315D), with bio-inert standard flow cell, 10 mm
- Agilent 1200 Series Fluorescence Detector (G1321A), with standard flow cell

MS system
Agilent 6530 Accurate Mass QTOF LC/MS system

Column
HILIC Glycan Amide column, 2.1 × 150 mm, < 2 µm

Software
Agilent OpenLAB CDS, ChemStation Edition for LC & LC/MS Systems, Rev. C.01.02 [14]

Agilent MassHunter Workstation Software, Version B.04.00, Build 4.0.479.0

Sample preparation

Deglycosylation procedure
Deglycosylation of the monoclonal antibody and the glycoproteins was performed using PNGase F to release asparagine-linked oligosaccharides (N-glycans) from the glycoproteins. PNGase cleaves asparagine-linked high mannose as well as hybrid and complex oligosaccharides from the glycoproteins and leaves the glycans intact. The average glycosylation of proteins is 2–5 % with 1,000 Da as average molecular weight. Therefore, to release approximately 20 µg glycans 400 µg of glycoproteins were used. Ovalbumin and conalbumin have only one glycosylation site, whereas the mAb contains two glycosylation sites. The amount of PNGase F was adjusted to the amount of glycans. The proteins were deglycosylated according to instructions from Sigma-Aldrich for 3 hours at 37 °C. The reaction was then stopped and the sample was vacuum dried for further processing.

AB-labeling for fluorescence detection and sample cleanup
The dried glycan samples were labeled with the fluorophore 2-AB (2-aminobenzamide) using the GlycoProfil 2-AB Labeling Kit according to the preparing protocol from Sigma-Aldrich for 3 hours at 65 °C. After the labeling procedure, the samples were purified using the GlycoProfil Glycan Cleanup Cartridges from Sigma-Aldrich according to the instructions manual. After the HILIC cleanup procedure, the samples were vacuum dried and reconstituted in 15 µL ultrapure water for LC analysis.

Chromatographic conditions

	Gradient antibody standard	Gradient glycoproteins
Starting flow rate:	0.5 mL/min	0.5 mL/min
Gradient:	0 minutes – 85% B	0–6 minutes – 85% B
	5 minutes – 75% B	10 minutes – 80%B
	35 minutes – 64%	60 minutes – 64% B
	40 minutes – 50%	65 minutes – 50% B
	42 minutes - Flow 0.25 mL/min	67 – Flow 0.25 mL/min
	43 minutes – 0% B	68 minutes – 0% B
	48 minutes – 0% B	73 – 0% B
	50 minutes – 85% B	75 minutes – 85% B
	55 minutes – Flow 0.5 mL/min	80 minutes – Flow 0.5 mL/min
Stop time:	55.01 minutes	80.01 minutes
Post time:	20 minutes	20 minutes
Injection volume:	10 µL	1 µL
Column temperature:	60 °C	
FLD:	Ex. 260 nm Em. 430 nm	
Peak width:	> 0.025 minutes (18.52 Hz)	

Solvents and samples

Buffer A: 100 mM ammonium formate, pH 4.5

Buffer B: acetonitrile

All solvents used 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 standard, that was used, is part of the mAb-Glyco Chip Kit (p/n G4240-64026). Ammonium formate, ovalbumin and conalbumin, PNGase F from Elizabethkingia miricola, GlycoProfil 2-AB Labeling Kit and GlycoProfil Glycan Cleanup Cartridges were purchased from Sigma-Aldrich, St.Louis, USA.

Results and discussion

Analysis of N-glycans from monoclonal antibodies

The glycans from the monoclonal antibody standard (included in Agilent mAb-Glyco Chip Kit) were AB-labeled and analyzed using HILIC-UHPLC. Figure 4 shows the separation of the mAb glycans. The resulting HILIC glycan pattern was compared to a mAb glycan pattern generated by Melmer *et al*,[10] and the single peaks were assigned to the corresponding glycan structures. For peak labeling, both the nomenclature used in various publications[4, 5, 10] and the nomenclature of the Agilent mAb-Glyco Chip manual was used. The mAb glycan pattern was optimally resolved, allowing separation of all major N-glycans occurring in mAbs: G0, G0F, Man5, (1,6)G1F and (1,3)G1F. In addition, two more peaks could be detected, representing two sialylated glycans (G2FS1, G2FS2) containing N-acetylneuramic acid (NANA).

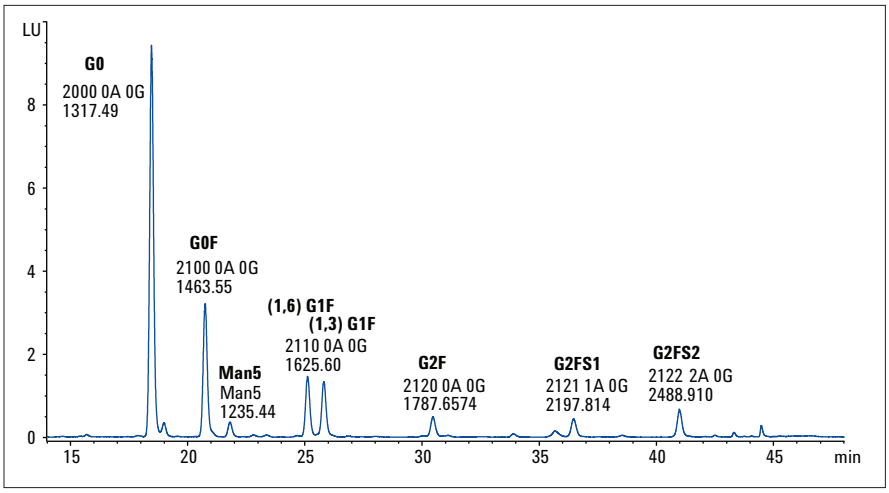

Figure 4
Separation of mAB N-glycans.

Name	Calculated mass	Mass + AB	Monosaccharide composition	Structure
G0	1317.5	1437.6	$GlcNAc_2Man_3GlcNAc_2$	
G0F	1463.6	1583.6	$GlcNAc_2Man_3GlcNAc_2Fuc$	
Man5	1235.4	1355.5	$Man_5GlcNAc_2$	
(1,6)G1F	1625.6	1745.7	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
(1,3)G1F	1625.6	1745.7	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
G2F	1787.6	1907.7	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
G2FS1	2077.8	2197.8	$NeuNAcGal_2GlcNAc_2Man_3GlcNAc_2Fuc$	
G2FS2	2368.9	2488.9	$NeuNAc_2Gal_2GlcNAc_2Man_3GlcNAc_2Fuc$	

Table 1
This table gives an overview of the detected glycan structures of the monoclonal antibody.

The glycan pattern of the mAb standard (Figure 3) showed a high amount of glycan G0, which is added as internal standard for system checkout. Table 1 shows an overview of the detected glycan structures.

High intensity of the detected labeled glycans was achieved by setting the optimal wavelengths for glycan detection on the 1260 Infinity Fluorescence Detector, using 260 nm as excitation wavelength and 430 nm as emission wavelength. Usually, an excitation wavelength of 330 nm is preferred for the analysis of 2-AB labeled glycans[11]. We used the lower 260 nm, due to higher intensity, resulting in better signal-to-noise ratios, as described by Melmer *et al*[10].

Two additional glycoproteins (ovalbumin and conalbumin) were deglycosylated, the glycans derivatized and analyzed using HILIC-UHPLC. Figure 5 shows the separation of ovalbumin glycans. Ovalbumin is N-glycosylated only at one site (Asn-292), but a complex glycosylation pattern can be associated to this site[11]. Due to the complexity of the glycan pattern, the gradient had to be adjusted to achieve higher resolution. Six glycans were identified with ESI-QTOF detection and mass correlation to the glycan library published by Harvey *et al* 2000[12], see Table 2 for detailed glycan information. Over 35 peaks could be resolved in the glycan sample, released from ovalbumin.

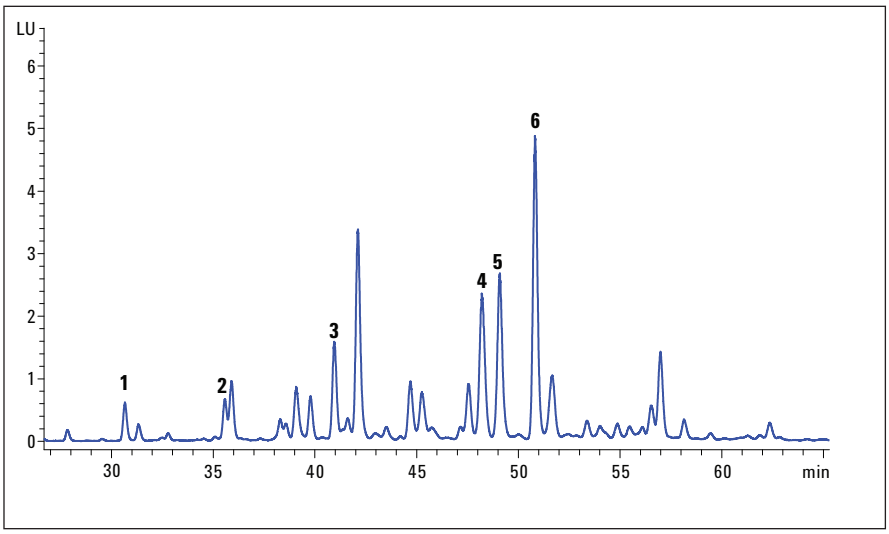

Figure 5
Separation of N-glycans, released from ovalbumin.

Peak	Retention time	Calculated mass	Mass + AB	Name	Structure
1	30.66	1114.5	1234.5	$GlcNAcMan_3GlcNAc_2$	
2	35.59	1317.6	1437.6	$GlcNAc_2Man_3GlcNAc_2$	
3	40.95	1235.5	1355.5	$Man_5GlcNAc_2$	
4	48.21	1397.6	1517.6	$Man_6GlcNAc_2$	
5	49.09	1438.6	1558.6	$GlcNAcMan_5GlcNAc_2$	
6	50.81	1927.8	2047.8	$GlcNAc_5Man_3GlcNAc_2$	

Table 2
Detailed information identified N-glycans ovalbumin. For more and detailed information, see 5990-9774EN.

Figure 6 shows the glycosylation pattern of conalbumin (synonym: ovotransferrin). Like ovalbumin, conalbumin has only one N-linked glycosylation site per mol protein[13]. Despite the complexity of the glycan pattern, high resolution resulting in successful glycan separation with good peak shape was achieved. Three glycans were identified with ESI-QTOF detection, see Table 3 for detailed glycan information. Overall, more than 30 peaks could be detected with good signal-to-noise ratio in the glycan sample from conalbumin.

Compared to the relative simple glycan pattern of the mAb, the two egg white glycoproteins have a higher variety of glycan structures, although they are only assigned to a single glycosylation site on both proteins. No fucosylated glycans were detected in ovalbumin and conalbumin in contrast to the mAb glycans, due to the fact that all avian egg glycoproteins are not fucosylated[14].

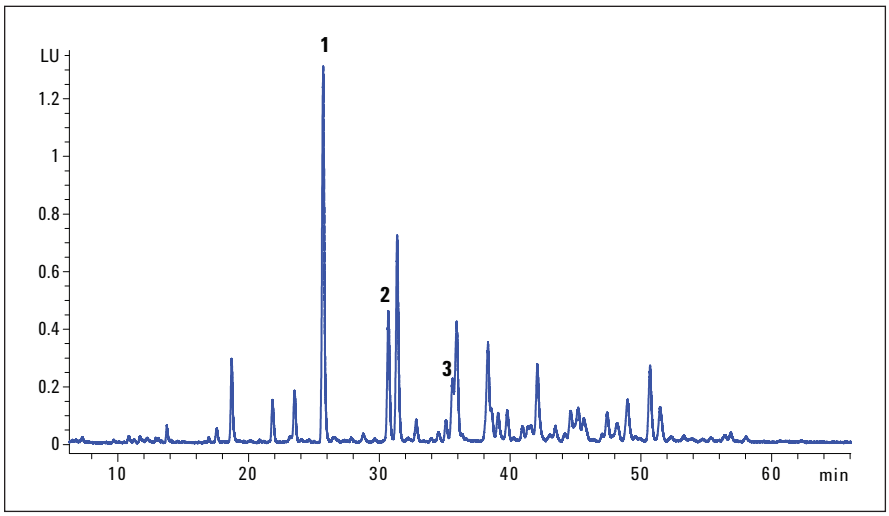

Figure 6
Separation of N-glycans released from conalbumin.

Peak	Retention time	Calculated Mass	Mass + AB	Name	Structure
1	25.6	911.4	1031.4	$Man_3GlcNAc_2$	
2	30.66	1114.5	1234.5	$GlcNAcMan_3GlcNAc_2$	
3	35.59	1317.6	1437.6	$GlcNAc_2Man_3GlcNAc_2$	

Table 3
Retention time, masses, monosaccharide composition and structure of the conalbumin glycans identified.

Conclusion

This Application Note demonstrates, that the Agilent 1260 Infinity Bio-inert Quaternary LC System together with the Agilent 1260 Infinity Fluorescence Detector is an ideal solution for the analysis of protein-released glycans, derivatized with 2-aminobenzamide (2-AB). Sample preparation using PNGase F for the release of N-linked glycans following 2-AB derivatization with subsequent HILIC sample cleanup was shown for the monoclonal antibody standard and two glycoproteins from avian egg white.

The glycan pattern of a monoclonal antibody standard was optimally resolved, allowing separation of all major N-glycans occurring in mAbs: G0, G0F, Man5, (1,6)G1F, (1,3)G1F, G2F, even G2FS1 and G2FS2 could be detected with good signal-to-noise ratios.

With fluorescence wavelengths optimized for our system, higher intensities and, therefore, better signal-to-noise ratios were achieved using the 1260 Infinity Fluorescence Detector. Instead of an excitation wavelength of 330 nm (as recommended by Anumula 2005[11]) the lower wavelength 260 nm was used.

In addition, the complex glycan patterns of two avian egg glycoproteins, ovalbumin and conalbumin, were well resolved, resulting in over 30 to 35 detected peaks with good peak shape and signal-to-noise ratio. Subsequent ESI-QTOF analysis enabled the identification of different glycan masses and related monosaccharide composition.

The 1260 Infinity Bio-inert Quaternary LC System together with fluorescence detection provides an optimal system for sensitive and high resolving analysis of 2-AB derivatized glycans released from mAbs and other glycoproteins.

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