Asparia Glycomics

Application note: Direct and robust identification of immunoglobulin N-glycosylation using fluorescent and MS-compatible N-glycan reference standards

Introduction

Glycosylation, a post-translational modification, involves the covalent bonding of complex polysaccharide molecules to proteins. Glycans manifest structural heterogeneity due to their monosaccharide composition and the different glycosidic bond types that link them. The same site of glycosylation may not necessarily have the same glycan structure, leading to different protein glycoforms. N-glycosylation is a critical quality attribute of therapeutic proteins, and it impacts the drug's safety, stability, and effectiveness. The European Medicines Agency (EMA) guideline (ICH Q6B) requires the characterization of monoclonal antibodies (mAbs) glycosylation profile during production and before the release of the drug. However, the task of characterizing glycan can be challenging due to the high number of isomers, which makes the detection of the right glycan sometimes ambiguous even with mass spectrometry (MS) analysis. To address this challenge, a new set of N-glycan standards has been developed at ***Asparia Glycomics***. These reference standards cover the typical bi-antennary N-glycans found in human antibodies. They have been purified and labeled with a fluorophore compatible with ***GlycoWorks™*** workflow. This labeling not only improves the mass spectrometry signal but also provides a strong fluorescence response. Our ***N-glycan standard compatible with GlycoWorks™ RapiFluor-MS™*** provides a simple and reliable way to measure the retention times of N-glycans, regardless of the chromatographic column chemistry, separation gradient, or chromatography system performance.

Material and methods

IgG samples including a human serum IgG and three monoclonal antibodies (mAbs) were denatured with SDS, and disulfide bonds were reduced with dithiothreitol at 95 °C for 5min. The denatured antibodies were washed with water through a 30kDa ultrafiltration filter (Amicon® Ultra Centrifugal Filters, Merck). An aliquot of 100U of PNGaseF (***Carboquant***) was added and incubated at 37 °C for 3 hours at pH 8 in sodium phosphate buffer. The released N-glycans were labeled with the ***GlycoWorks™ RapiFluor-MS™*** label reagent (Waters). N-glycans released from an equivalent of 200 ug of IgG were suspended in 100 µL of anhydrous DMF with 1 mg labeling ***GlycoWorks™ RapiFluor-MS™*** reagent for 4 hours at room temperature. All samples were reconstituted in acetonitrile/water (7:3) before the injection.

LC conditions for N-glycan profiling

Column: ACQUITY UPLC Glycan BEH Amide, 130 Å, 1.7 µm, 2.1 x 150 mm (Waters)

Temperature: 60 °C

Mobile phase A: 50 mM ammonium formate solution, pH 4.4

Mobile phase B: 100% acetonitrile

Flow rate: 0.4 mL/min

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

Injection vol: 10 µL

Fluorescence detection wavelengths: EX 265/EM 425 nm

MS detection: LCT Premier (Waters) in positive ionization mode, scan 100-4000 m/z

Results

1. A new set of bi-antennary N-glycan reference standards for the analysis of immunoglobulin glycosylation

Asparia Glycomics is pleased to announce the introduction of a new set of reference standard N-glycans that are compatible with the ***GlycoWorks™*** pipeline and have been specifically designed for immunoglobulin analysis. This set includes eight bi-antennary N-glycans: A0, G2, FG0, FG2, and the two sialic acid linkage isomers for G2S2 and FG2S2, namely α-2,3 and α-2,6, as shown in ***Figure 1***. The labeling of our reference N-glycans allows for a strong response factor with both mass spectrometry and fluorescence detectors.

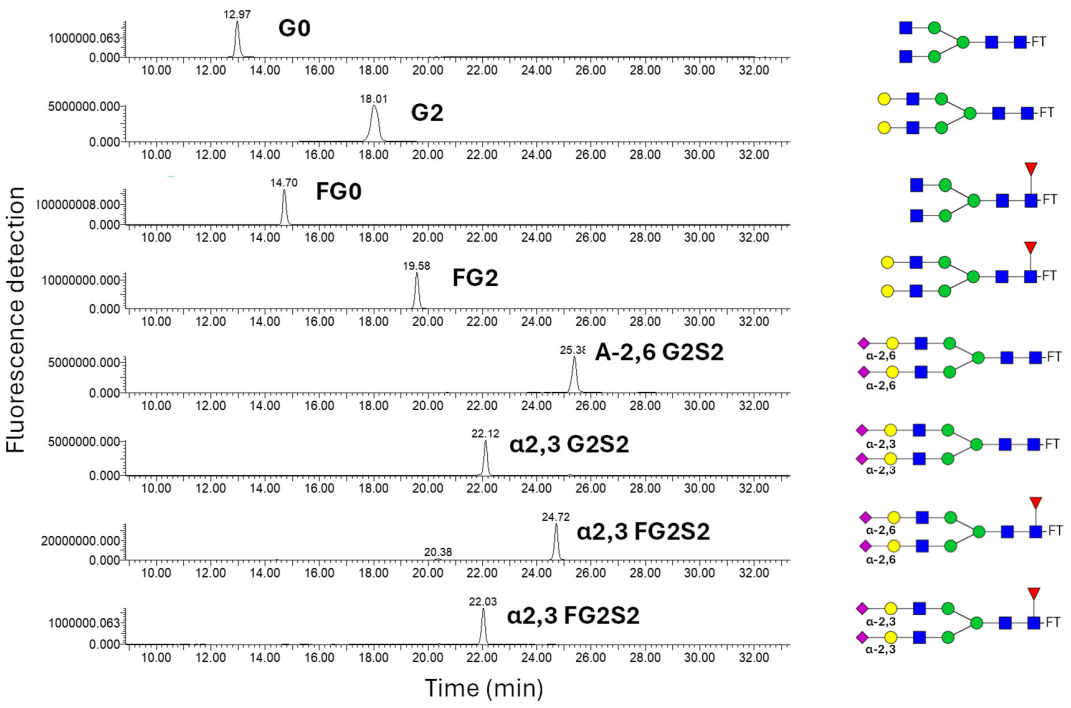


Figure 1: *Chromatograms of the eight* ***N-glycan standards compatible with GlycoWorks™ RapiFluor-MS™*** *obtained by UHPLC with an amide HILIC analytical column and fluorescence detection.*

N-glycans can exist in different forms of isomers, including positional, linkage, and constitutional isomers. These isomers can lead to isobaric or quasi-isobaric structures, as shown in ***Figure 2***. Identifying the correct structure of the N-glycans in chromatographic peaks usually requires extended analysis, such as MS/MS fragmentation, derivatization, or characterization by specific glycosidase digestion. Another approach involves using libraries of glycan relative retention times (GU units) calculated against a series of dextran of increasing masses. This approach potentially simplifies the identification of glycans as it is based only on the retention times. However, to be accurate, it requires a similar analytical setup as the one used to build the library. Changes in selectivity can occur with changes in the elution conditions, analytical column chemistry, or even with matrix effects. Using reference standards that are the exact isomers can help identify and align retention times more straightforwardly.

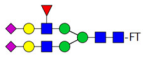
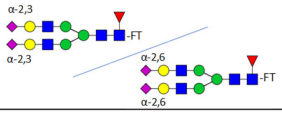
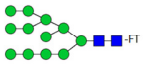
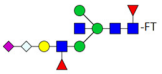
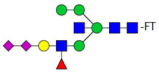
N-glycan structure	$[M+H]^+$	Mass error	Isomers
	2681.0228	0 ppm	Reference molecule
	2681.0228	0 ppm	Positional isomer
	2681.0228	0 ppm	Linkage isomer
	2680.9851	-14.0740 ppm	Isobaric structure
	2681.0228	-0.0036 ppm	Isobaric structure
	2681.0228	-0.0036 ppm	Isobaric structure
	2681.0228	-0.0036 ppm	Isobaric structure

Figure 2: *Illustration of the diversity of N-glycan structures with a mass equal to or close to a F2G2S2. Not all potential isomers are displayed.*

2. Application of N-glycan reference standards for the analysis of therapeutics mAbs N-glycosylation

We have demonstrated the potential of the N-glycan standards for analyzing the glycosylation of immunoglobulins. We used three therapeutic mAbs and one IgG extracted from human serum (see ***Figure 3***). We highlighted the detection and the correct peak assignment of the two linkage isomers of G2FS2, namely α-2,3 and α-2,6. Although the only difference between the structures lies in the sialic acid bonds with the terminal galactose, a significant difference in retention time can be observed. These N-glycans can be low in abundance and result in small peaks that can be difficult to assign, especially with the complex N-glycome of therapeutic mAbs. Nevertheless, we were able to observe both forms using both UV and MS detection.

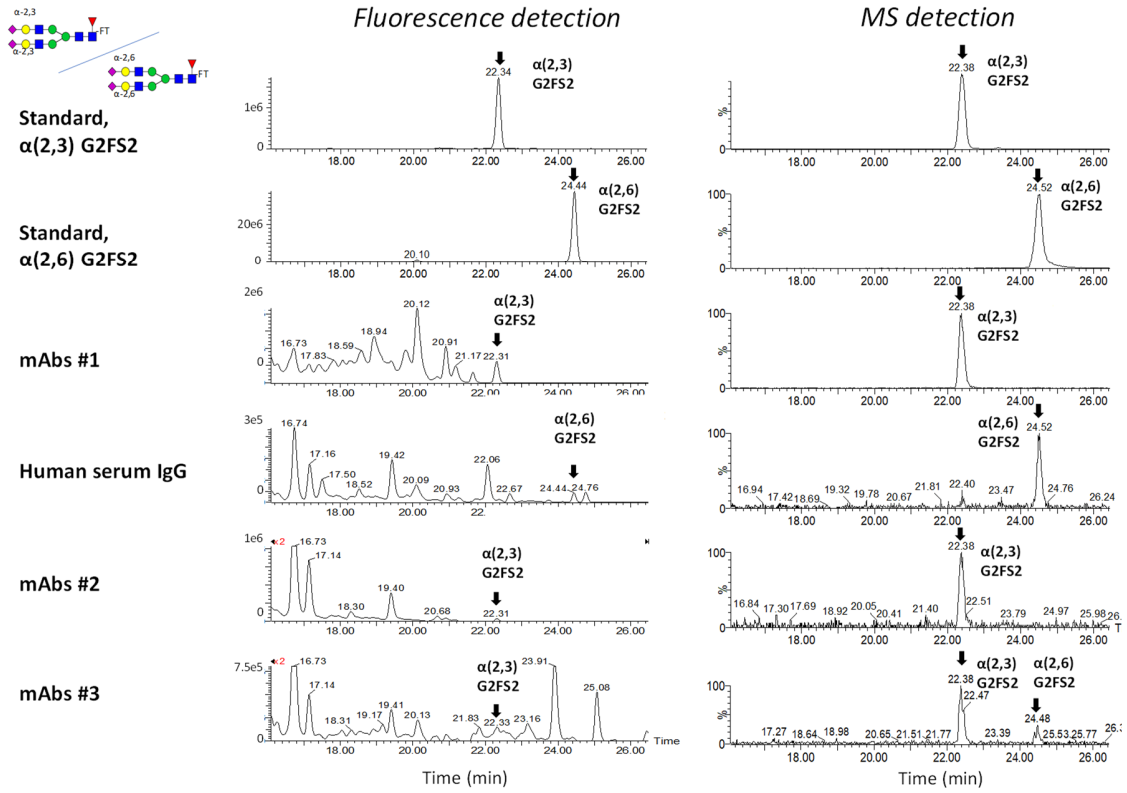


Figure 3: *Fluorescence chromatograms and extracted ion chromatograms of the α-2,3 and α-2,6 G2FS2 N-glycan in 3 therapeutics mAbs and a human serum extracted IgG.*

Conclusions

Asparia Glycomics has launched a new series of labeled purified N-glycans that are compatible with the ***GlycoWorks™*** analytical pipeline. In this application note, we have demonstrated that these new standards enable the rapid and unambiguous identification of N-glycans released from immunoglobulins. By utilizing the exact retention time of the exact isomers, the peak assignment becomes easy and limits the need for MS analysis. This methodology is independent of HILIC column chemistry or dimension and can be used with most HPLC and UHPLC stationary phases. We plan to expand the coverage of these standards with new standards in the future. Additionally, you can contact us to get a custom preparation of your glycan standards.