Application Note

Biopharma/Pharma

Sensitive and Repeatable Analysis of InstantPC-labeled N-Glycans from Human Immunoglobulin G

Enabling confident detection and quantification of low-abundance IgG glycans with UHPLC peak fidelity and excellent run-to-run precision

Author

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Abstract

Glycosylation is a post-translational modification of significant importance for biopharmaceuticals, especially antibodies, Fc fusion proteins, and other antibody-based proteins, and therefore represents one of the most important quality characteristics. Since it has a significant impact on stability, solubility, immunogenicity, pharmacokinetics, and effector functions of biopharmaceuticals, a suitable analysis device and method are essential. This application note demonstrates that the Agilent 1290 Infinity III Bio LC System with an Agilent 1290 Infinity III Fluorescence Detector (FLD) delivers reliable, high-confidence N-glycan profiles under UHPLC conditions—supporting detection and quantification of low-abundance glycans with excellent run-to-run reproducibility. The FLD provides high sensitivity and repeatability, while maintaining minimal peak dispersion volume for optimal peak shapes, which is critical for high-resolution glycan separations and robust characterization.

Introduction

Many biopharmaceuticals are glycosylated proteins such as monoclonal antibodies, Fc fusion proteins, and other antibody-based proteins These proteins are a powerful class of biopharmceuticals.[1,2]

A key characteristic of these proteins is their glycosylation, whereby a carbohydrate structure is bound to a specific asparagine residue within the amino acid sequence of the antibody. The resultant glycosylation is referred to as N-glycan. Glycosylation, a fundamental post-translational modification, exerts a substantial influence on stability, solubility, immunogenicity, pharmacokinetics, and effector functions of biopharmaceuticals, thereby rendering glycosylation a critical quality attribute. Therefore, it is essential to understand and control the glycosylation patterns to ensure product quality and consistency during manufacturing.[1,2,3]

To achieve these goals, a suitable analytical method for a protein glycosylation pattern analysis is required. However, this task is often quite challenging, because many glycan analytes occur only in low abundance in complex biological samples. To overcome this challenge, a sensitive and repeatable analytical method is needed.[4]

This application note demonstrates the high sensitivity for lowest limits of detection (LODs) and lowest quantification limits (LOQs), as well as repeatability, that can be achieved with an Agilent 1290 Infinity III Fluorescence Detector (FLD) within an Agilent 1290 Infinity III Bio LC System for N-glycan analysis—delivering UHPLC peak fidelity with minimal dispersion and high-confidence quantification of low-abundance glycoforms. This method requires derivatization of released N-glycans with a tag to allow detection by fluorescence. In this instance, N-glycans of human immunoglobulin G (HuIgG) derivatized with InstantPC (IPC) were used.

To assign glycans, glycan symbol structures were used according to the Consortium for Functional Glycomics (CFG) (see Figure 1). The Oxford glycan nomenclature and the biopharma mAb style were used for the assigned glycans.

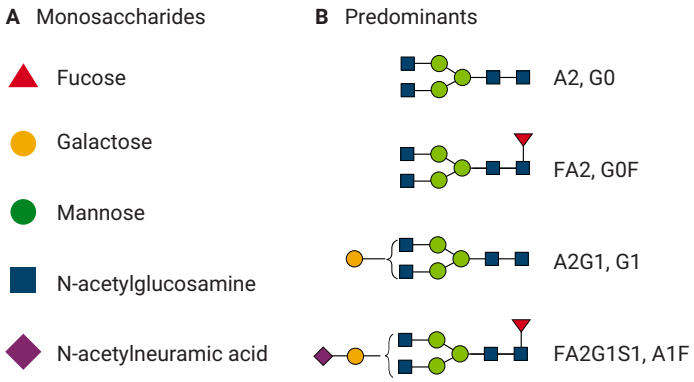


Figure 1. Glycan structures and isoforms. (A) Monosaccharide description after the Consortium for Functional Glycomics (CFG). (B) Predominant glycan structures of IgGs.

Experimental

Equipment

The Agilent 1290 Infinity III BioLC comprised the following modules:

- Agilent 1290 Infinity III Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity III Bio Multisampler (G7137A)
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) equipped with an Agilent Quick Connect Bio Heat Exchanger Standard Flow (G7116-60071)
- Agilent 1290 Infinity III Fluorescence Detector (FLD) (G7123B), equipped with a 2 µL FLD cell (G7123-60500)

Software

Agilent OpenLab CDS version 2.8 or later versions.

Columns

Agilent AdvanceBio Amide HILIC, Rapid Resolution HD, 2.1 × 150 mm, 1.8 Micron (part number 859750-913).

Chemicals

Agilent InfinityLab Acetonitrile HPLC Gradient Grade (part number 5191-5100*) was used for all experiments. Fresh ultrapure water was obtained from a Milli-Q integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Agilent AdvanceBio Ammonium Formate (part number G3912-00000) was used to prepare mobile phase A (50 mM ammonium formate, pH 4.4).

*Only available in select countries

Standards were obtained from Agilent:

- AdvanceBio InstantPC HuIgG N-glycan library (part number GKPC-005)
- AdvanceBio InstantPC Man5 / M5 N-glycan standard (part number GKPC-103)
- AdvanceBio InstantPC G0 / A2 N-glycan standard (part number GKPC-301)
- AdvanceBio InstantPC G2F/ FA2G2 N-glycan standard (part number GKPC-305)
- AdvanceBio InstantPC G2FS2a(2,3) / FA2G2S2 N-glycan standard (part number GKPC-323)
- AdvanceBio InstantPC G0F / FA2 N-glycan standard (part number GKPC-302)

N-glycan sample preparation

IPC HuIgG N-glycan library was dissolved in 60 µL mobile phase A.

For the determination of linearity, LOQ, and LOD 200 pmol of each IPC-labeled N-glycan standard (M5, A2, FA2, FA2G, and FA2G2) were combined and dissolved in 20 µL in mobile phase A. The corresponding N-glycan standard mixture was diluted in the range from 10 pmol/µL to 0.16 fmol/µL.

Methods

Table 1. Chromatographic conditions for the analysis of InstantPC labeled N-glycans.

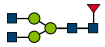
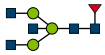
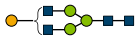
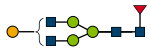
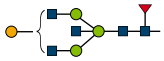
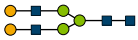
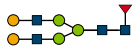
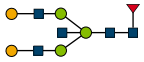
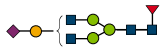
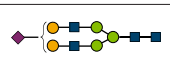
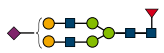
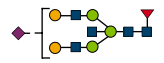
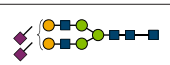
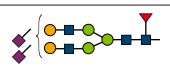
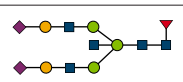
Parameter	Value
Column	Agilent AdvanceBio Amide HILIC, Rapid Resolution HD, 2.1 × 150 mm, 1.8 µm
Solvent	A) 50 mM ammonium formate, pH 4.4, prepared from p/n G3912-00000 B) Acetonitrile
Gradient	Time (min) % A % B 0 25 75 20 37 63 27 60 40 28 60 40 29 30 70 30 30 70 Stop time: 30 min Post time: 10 min
Flow Rate	0.700 mL/min
Temperature	60 °C
Detection	FLD: Excitation: 285 nm; emission: 345 nm PMT gain: Standard Peak width: > 0.1 min (5 Hz)
Injection	Injection volume: 1 µL Sample temperature: 10 °C

Results and discussion

Analysis of InstantPC-labeled N-glycans from human IgG

Figure 2 shows a chromatogram from the analysis of an IPC-labeled HuIgG N-glycan library. The chromatogram illustrates the effective separation of all predominant glycan structures within 30 minutes. Peaks 2, 5, and 9 are the most prominent glycan structures, which are fucosylated structures carrying none, one, or two galactose residues. The non-sialylated neutral glycans elute first, followed by the sialylated structures. The nomenclature used in various publications[5,6,7] was employed with respect to the assignments. These assignments are listed in Table 2.

Table 2. Structures of the assigned N-glycans in the human IgG glycan library and corresponding naming according to Oxford nomenclature and biopharma mAb acronyms.

Peak	Oxford	Biopharma mAb Style	Structure
1	A2	G0	
2	FA2	G0F	
3	FA2B	G0FB	
4	A2G1	G1	
5,6	FA2G1	G1F	
7	FA2BG1	G1FB	
8	A2G2	G2	
9	FA2G2	G2F	
10	FA2BG2	G2FB	
11	FA2G1S	G1FS1	
12	A2G2S1	A1	
13	FA2G2S1	A1F	
14	FA2BG2S1	G2FBS2	
15	A2G2S2	G2S2	
16	FA2G2S2	G2FS2	
17	FA2BG2S2	G2FBS2	

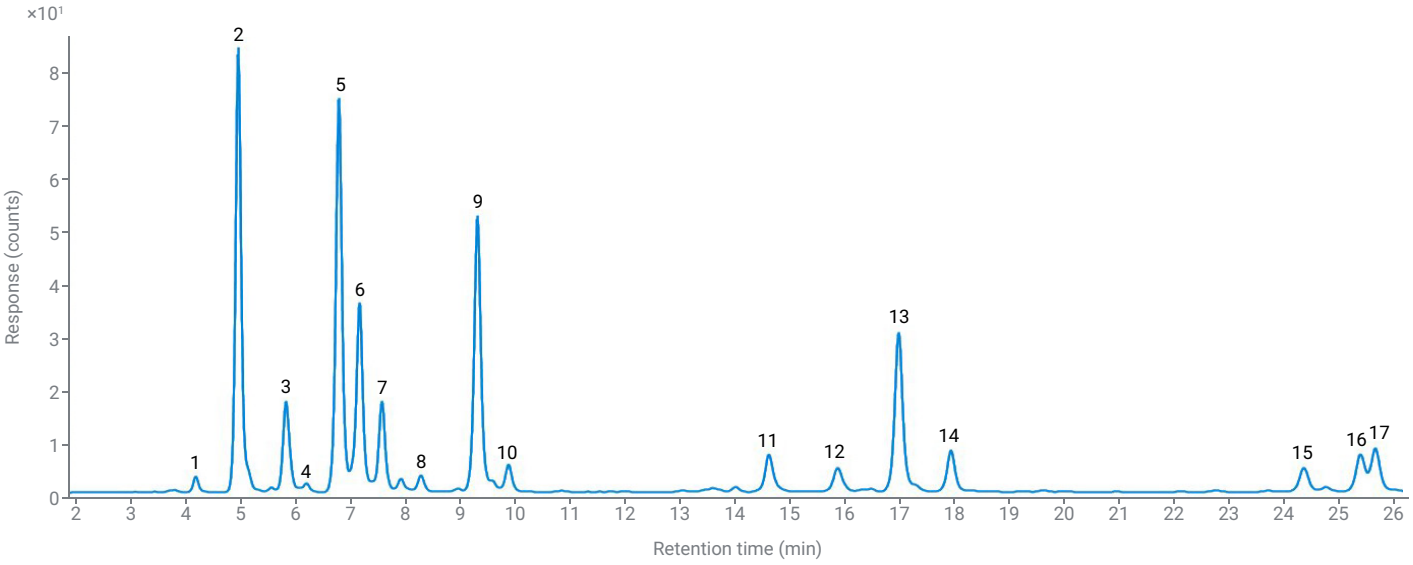


Figure 2. Chromatogram from analysis of an IPC-labeled HuIgG N-glycan library. Peak identification is given in Table 3.

The retention time (RT) and peak area precision were determined by 10 consecutive injections (n = 10) of the IPC-labeled HuIgG N-glycan library. For all glycans excellent RT and peak area RSD were obtained, showing values 0.16% or less for RT RSD and 0.57% or less for peak area RSD (see Table 3).

Table 3. Precision of N-glycan analysis of an IPC-labeled human IgG glycan library in terms of retention time and peak area for ten consecutive injections.

Peak	Oxford	Biopharma mAb Style	RT RSD (%)	Area RSD (%)
1	A2	G0	0.16	0.53
2	FA2	G0F	0.15	0.43
3	FA2B	G0FB	0.14	0.42
4	A2G(4)1	G1	0.16	0.57
5	FA2[6]G1	G1F	0.14	0.44
6	FA2[3]G1	G1F	0.14	0.43
7	FA2BG1	G1FB	0.13	0.40
8	A2G2	G2	0.12	0.43
9	FA2G2	G2F	0.11	0.39
10	FA2BG2	G2FB	0.10	0.35
11	FA2[3]G1S1	G1FS1	0.05	0.38
12	A2G2S1	A1	0.05	0.44
13	FA2G2S1	A1F	0.05	0.36
14	FA2BG2S1	G2FBS2	0.05	0.25
15	A2G2S2	G2S2	0.07	0.29
16	FA2G2S2	G2FS2	0.07	0.27
17	FA2BG2S2	G2FBS2	0.06	0.26

Determination of linearity, LOD, and LOQ

To determine linearity, LOD, and LOQ, the glycan standard mixtures were analyzed at concentrations ranging from 0.16 fmol/µL to 10 pmol/µL, and a calibration curve was generated. Figure 3 shows a chromatogram of the glycan standard mixture, and the respective structures and nomenclature are illustrated in Table 4. The calibration curves exhibited remarkable linearity, with correlation coefficients exceeding 0.999. Results suggest that linearity is maintained for all five glycans over a considerable range, at least from 0.8 fmol/µL to 10 pmol/µL. Corresponding calibration results and subsequent linearity are shown in Table 5. Furthermore, exceptional sensitivity was achieved for all N-glycans, with LODs of 0.3 fmol/µL or less and LOQs of predominantly 1 fmol/µL or less. The LOD was determined by employing a threshold of three times the S/N value. The LOQ was determined by employing a threshold of ten times the S/N value.

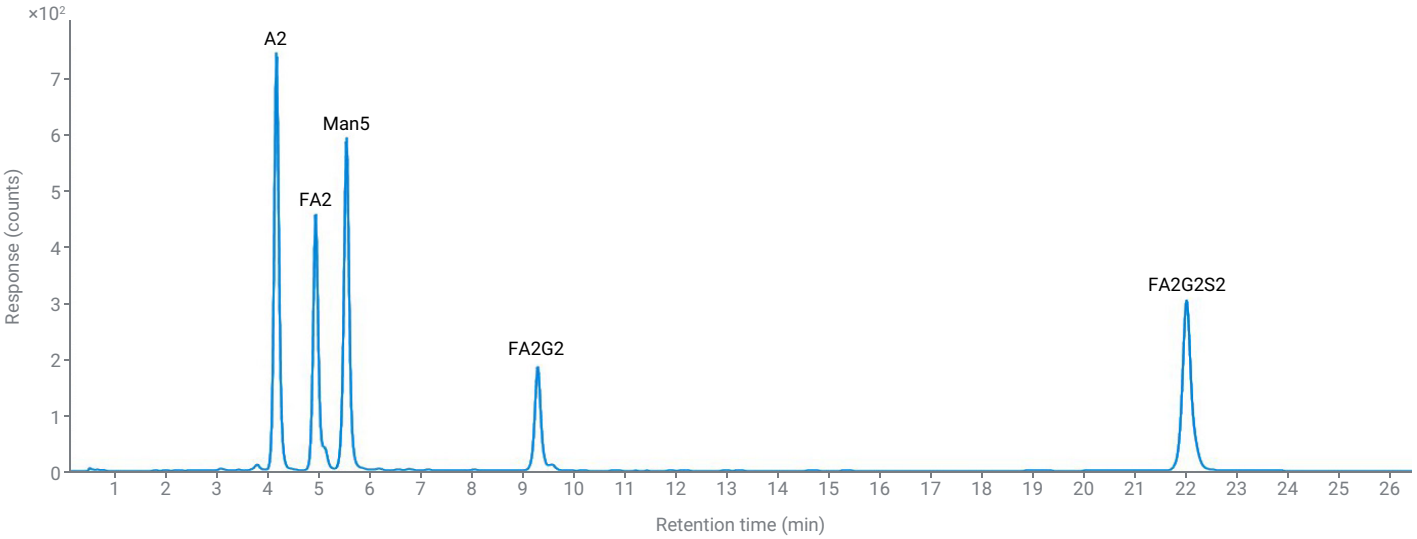


Figure 3. Chromatogram of IPC-labeled glycan standard mixture.

Table 4. Structures of the assigned glycans in the standard mixture and corresponding naming according to Oxford nomenclature and biopharma mAb acronyms.

Oxford	Biopharma mAb Style	Structure
M5	Man5	
A2	G0	
FA2	G0F	
FA2G2	G2F	
FA2G2S2	G2FS2a(2,3)	

Table 5. Linearity, LOD, and LOQ of N-glycan analysis. LOD was determined using a threshold of three times the S/N value. The LOQ was determined using a threshold of ten times the S/N value.

Oxford	Biopharma mAb Style	LOD (fmol/µL)	LOQ (fmol/µL)	Calibration Range (per µL)	Correlation Coefficient (R^2)
A2	G0	0.14	0.47	0.16 fmol to 10 pmol	0.9991
FA2	G0F	0.07	0.23	0.16 fmol to 10 pmol	0.9995
M5	Man5	0.08	0.27	0.16 fmol to 10 pmol	0.9999
FA2G2	G2F	0.32	1.05	0.8 fmol to 10 pmol	0.9994
FA2G2S2	G2FS2	0.30	1.01	0.8 fmol to 10 pmol	0.9994

Conclusion

The Agilent 1290 Infinity III Bio LC System, equipped with an Agilent 1290 Infinity III FLD, has been demonstrated to exhibit excellent overall performance in terms of repeatability, sensitivity, and low peak dispersion for the analysis of IPC-labeled N-glycans. These results are substantiated by the low retention time and peak area relative standard deviations, as well as the low LOD and LOQ values. The 1290 Infinity III FLD is distinguished by its high linear dynamic range, enabling accurate quantification across a wide concentration range and supporting both abundant and low-level glycoforms in a single method. By combining UHPLC peak fidelity with high sensitivity and repeatability, the system reduces reruns and troubleshooting, lowers the cost per compliant result, and recovers analytical capacity. Importantly, the achieved LOD/LOQ and precision provide the confidence needed for defensible decisions at the limit—positioning the 1290 Infinity III FLD as a fluorescence detector of choice for UHPLC N-glycan characterization workflows.

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