Application Note

Biopharma/Pharma

Agilent

Trusted Answers

Analysis of Sialylated Glycan Modification in Protein Therapeutics

Using InstantPC labeling and anion exchange chromatography

Authors

YuFei Lu and JianQiu Mi
Agilent Technologies (China) Ltd., Beijing, China

Abstract

This application note introduces a new method for the analysis of sialylated glycan modification in protein therapeutics. The workflow uses Agilent AdvanceBio Gly-X N-glycan prep with InstantPC and anion exchange chromatography to provide a reproducible and high-throughput solution for the quantitation of sialylated N-glycans.

Introduction

Glycosylation is an important post-translational modification in protein. Over 60% of proteins are glycosylated. Some proteins, such as fusion protein and erythropoietin (EPO), are sialylated glycoproteins.[1] The modification of sialic acids in therapeutic glycoproteins significantly impacts the half-life, safety, and biological function of these drugs. Therefore, glycosylation is one of the critical quality attributes of biomolecules.

N-glycans are usually labeled by fluorescent dye and analyzed by hydrophilic interaction chromatography (HILIC). However, positional isomers exist for sialylated glycans and make the control of sialylated N-glycans in products exceptionally challenging. Presented here is a new method for sialylated N-glycans based on AdvanceBio Gly-X N-glycan prep with InstantPC and anion exchange chromatography. The workflow enables reliable and reproducible high-throughput sample preparation for the quantitation of sialylated N-glycans.

Experimental

Materials

- Agilent 1260 Infinity II LC system configured with an Agilent 1260 Infinity II quaternary pump and an Agilent 1260 Infinity II fluorescence detector (FLD)
- Agilent Buffer Advisor software
- Agilent Bio SAX column, NP5, 4.6 × 250 mm, 5 µm, PEEK (part number 5190-2467)
- Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96-ct (formerly ProZyme) (part number GX96-IPC)
- Agilent AdvanceBio InstantPC α(2,3) Sialylated tetraantennary N-glycan library (formerly ProZyme) (part number GKPC-234)
- Tris, Tris hydrochloride, sodium chloride – purchased from MilliporeSigma
- Acetonitrile, formic acid – purchased from Dikma Technologies
- Millipore Ultrapure Water System
- Fusion protein

Sample preparation

For detailed sample pretreatment protocol, please see the user manual for the Agilent AdvanceBio Gly-X N-Glycan Prep with InstantPC Kit, 96-ct (formerly ProZyme).[2] To summarize, protein solution was mixed with the denaturant solution in the deglycosylation plate and incubated uncapped at 90 °C for three minutes. N-Glycanase working solution was then added to each sample solution after cooling down to room temperature. Afterwards, the sample solution was incubated at 50 °C for 5 minutes. InstantPC labeling was conducted by adding 5 µL of InstantPC dye solution and incubated uncapped at 50 °C for 1 minute. Finally, the labeled glycans were purified using a cleanup plate. The schematic workflow is demonstrated in Figure 1.

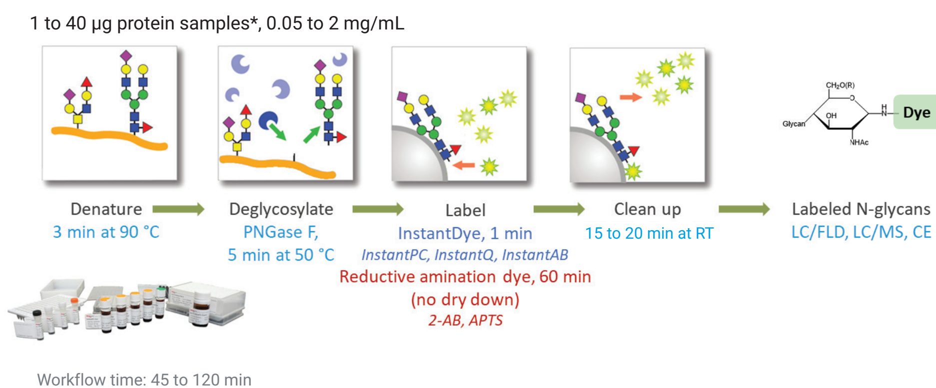

Figure 1. Workflow for InstantPC labeling of N-glycans.

LC method parameters

Parameter	Value
Instrument	Agilent 1260 Infinity II LC system
Column	Agilent Bio SAX column
Mobile Phase A	Tris HCl buffer 10 mM, pH 8.5
Mobile Phase B	Tris HCl buffer 10 mM with NaCl 500 mM, pH 8.5
Gradient	Time (min) %B 0 0 3 0 15 24 16 100 25 100
Stop Time	25 min
Post Time	15 min
Detection	Agilent 1260 Infinity II FLD; λ_{Ex} 285 nm, λ_{Em} 345 nm

Results and discussion

The separation of peak groups with different net charges

It has been reported that positional isomers exist for sialylated glycans released from proteins, resulting in unresolved multiple peaks with HILIC separation. The unresolved peaks make the control of sialylated N-glycans in products exceptionally challenging. The calculation of Z value from the results of anion exchange chromatography has been an alternate way to achieve this goal (Equation 1).[3]

Equation 1. Z value calculation.

$$Z = 1 \times SA1\% + 2 \times SA2\% + 3 \times SA3\% + 4 \times SA4\%$$

In a mobile phase with pH value greater than 7, the tertiary amine groups in the labeled sialylated N-glycans are partially positively charged, while the sialic acid parts are negatively charged; therefore, the labeled products have a net negative charge. Retention and separation are possible based on net charges with anion exchange chromatography. The separation could be optimized by fine-tuning the pH value of the mobile phase. As shown in Figure 2, for conbercept, a mobile phase with a pH value of 8.5 was chosen due to better resolution between the first two peaks (Figure 2).

InstantPC labeling

InstantPC labeling offers significantly better sensitivity for both fluorescence and mass spectrometry detections, compared with traditional 2-AB labeling.[4] The whole workflow of deglycosylation and InstantPC labeling could be finished within one hour, which greatly reduces the sample preparation time and effort.

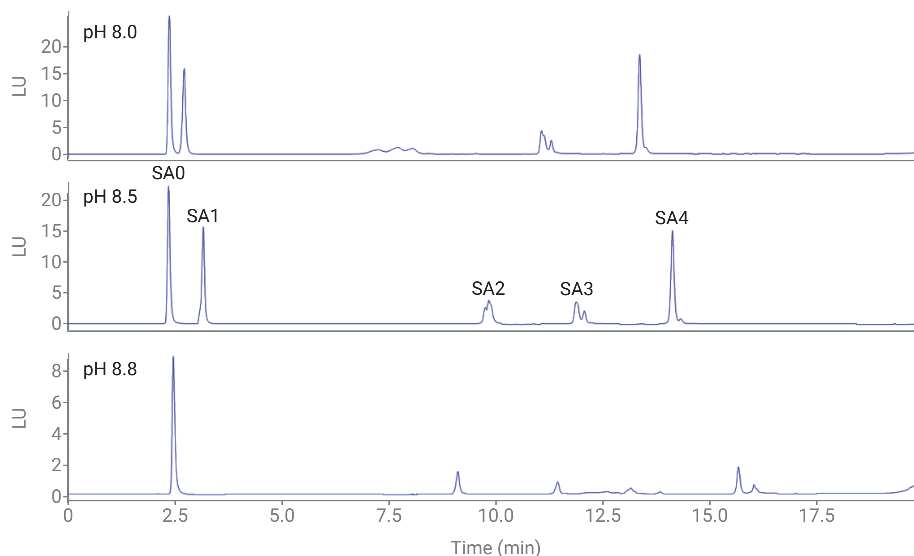

Figure 2. Optimization of mobile phase pH for the separation of InstantPC labeled sialylated N-glycans.

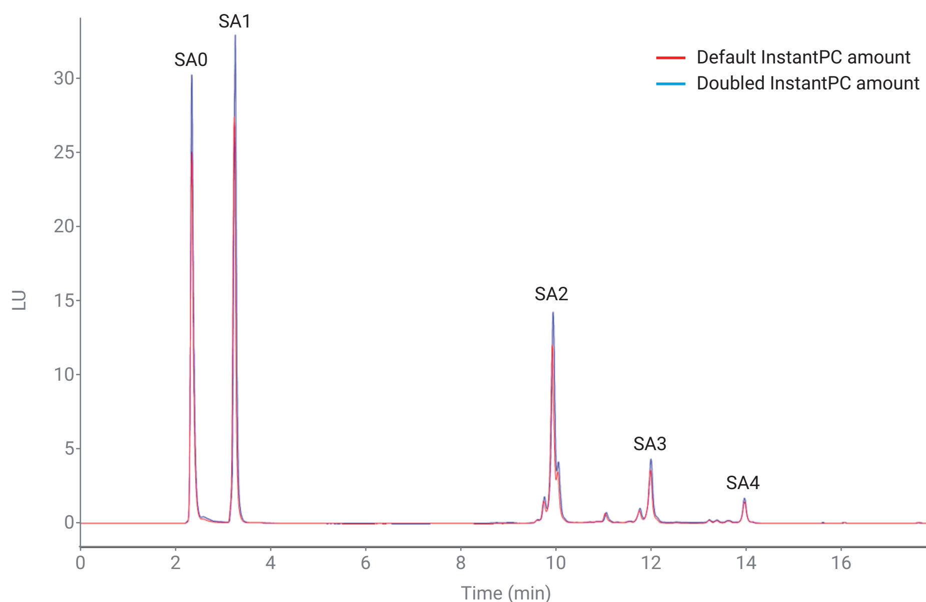

Figure 3. Comparison of different InstantPC amounts (red trace: default InstantPC amount; blue trace: doubled InstantPC amount).

The amount of InstantPC used in the labeling reaction was also studied. The comparison showed a slight increase of around 20% in peak area (Figure 3) with the InstantPC amount doubled.

Nevertheless, this peak area increase did not impact the calculated Z values, indicating that the labeling reaction did not discriminate different sialylated N-glycan groups (Table 1).

Recovery of labeled products

The final eluting step in the workflow protocol helped to remove the free dye in the system and purify the labeled products. Therefore, it was necessary to evaluate the eluting recovery of all the sialylated glycan forms to ensure the accuracy of the Z values. To verify the Z values, a second elution was conducted using the same eluent, right after the first one. The total peak areas of both collected solutions were analyzed. The results showed that the recovery of the sample preparation was more than 95%, and there is no bias in different N-glycan groups (Figure 4).

Reproducibility

The Buffer Advisor software provides excellent reproducibility for anion exchange separations. All mobile phases were prepared as stocks and mixed online by the quaternary pump. This gradient method could also be transferred onto an LC system with binary pump without much effort. Figure 5 demonstrates the highly reproducible overlaid results for six consecutive injections.

Table 1. Z value comparison of different InstantPC amounts.

	Peak Group	Area	Area Sum	Percentage	Z Value
Default InstantPC Amount	SA0	116.6	371.4	31.4%	1.21
	SA1	118.1		31.8%	
	SA2	89.8		24.2%	
	SA3	35.1		9.4%	
	SA4	11.8		3.2%	
Doubled InstantPC Amount	SA0	144.1	453.1	31.8%	1.21
	SA1	143.5		31.7%	
	SA2	108.3		23.9%	
	SA3	42.6		9.4%	
	SA4	14.6		3.2%	

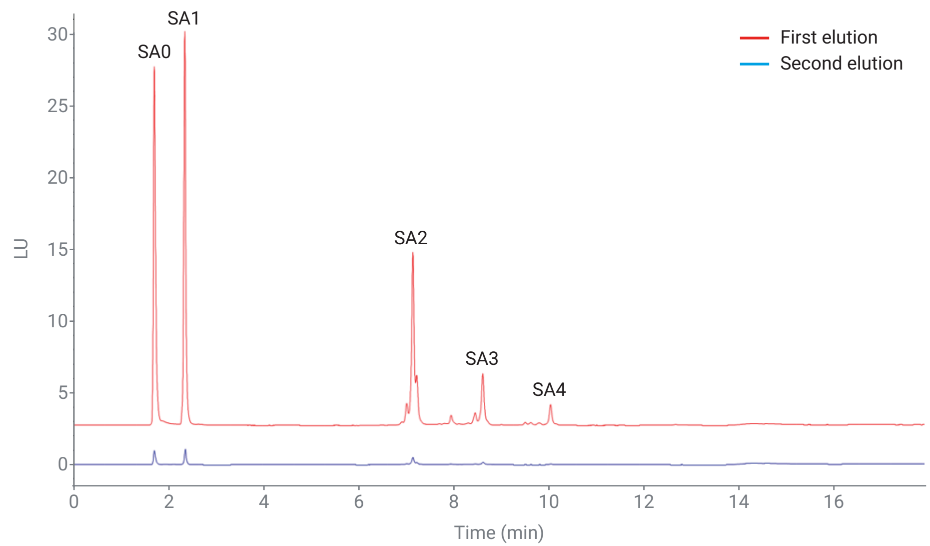

Figure 4. Investigation on recovery of sample preparation.

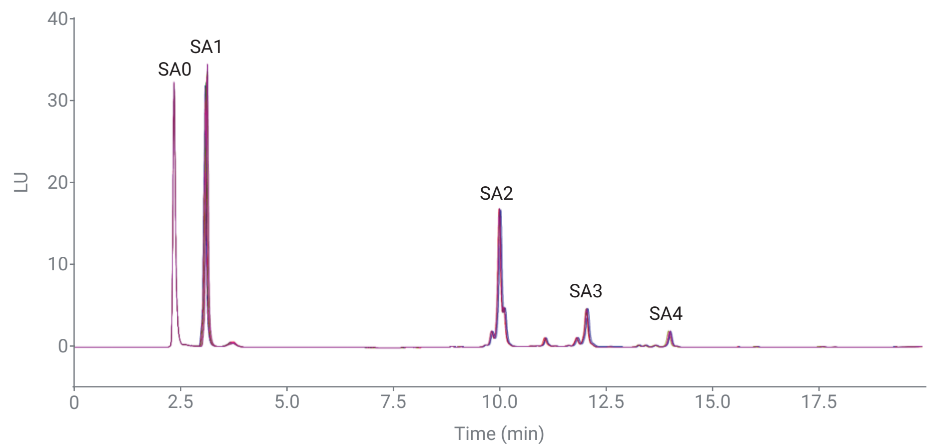

Figure 5. Chromatogram overlay of six consecutive injections with the conbercept sample.

Conclusion

This application note introduces a new method for the analysis of sialylated glycan modification in protein therapeutics. Sample preparation and labeling by Agilent AdvanceBio Gly-X N-glycan prep with InstantPC offers a high level of reproducibility and throughput, with a one hour preparation time for InstantPC. Sufficient fluorescence response can be obtained using InstantPC as the fluorescent dye. Based on net charges, the sialylated glycan separated into groups using anion exchange chromatography. This workflow makes the control of sialylated N-glycans in products easy. This method has good reproducibility and is suitable for routine QC tests.

References

1. Yuen, C. T. *et al.* Glycan Analysis of Glycoprotein Pharmaceuticals: Evaluation of Analytical Approaches to Z Number Determination in Pharmaceutical Erythropoietin Products. *Biologicals* **2011**, *39(6)*, 396–403.
2. Agilent AdvanceBio Gly-X N-Glycan Prep with InstantPC Kit, 96-ct (formerly ProZyme). *Agilent Technologies user manual,* publication number 5994-1231EN, **2019**.
3. Pharmacopoeia of the People's Republic of China: Volume III. **2020**.
4. Yan, J.; Jones, A. Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS. *Agilent Technologies application note*, publication number 5994-1348EN, **2021**.

www.agilent.com

DE39314734

This information is subject to change without notice.

© Agilent Technologies, Inc. 2022
Printed in the USA, May 24, 2022
5994-4934EN

Agilent
Trusted Answers