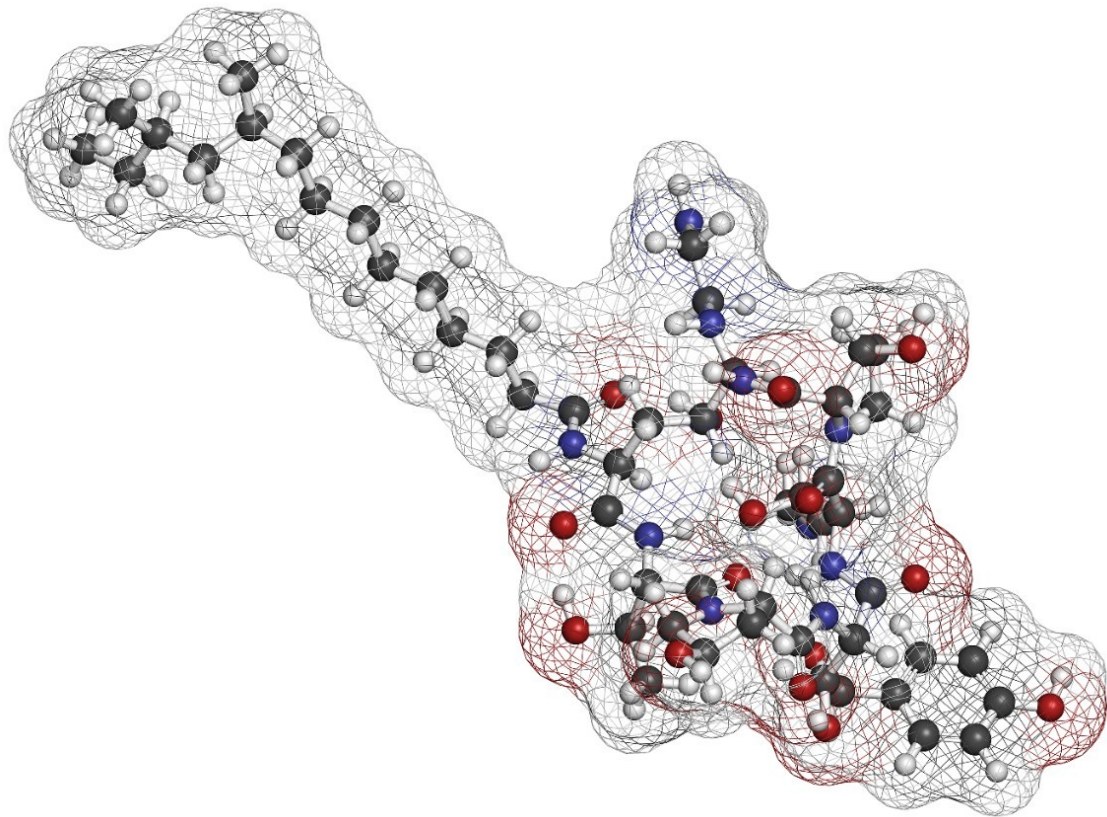


Improving Released N-glycan Analysis in Biotherapeutic Development Using the ACQUITY Premier Solution with MaxPeak High Performance Surfaces (HPS) Technology

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Abstract

Characterization and monitoring of glycosylation are necessary during the development and manufacturing of biotherapeutics to ensure that drug products are consistent, safe, and efficacious. As a critical quality attribute, glycosylation is commonly assessed via fluorescent labeling of released N-linked glycans, followed by hydrophilic interaction chromatography (HILIC), and fluorescence (FLR) detection. With the development of FLR and MS-friendly glycan labeling reagents, such as the *RapiFluor*-MS (RFMS), technology combined FLR/MS analysis has become increasingly common – even for routine analysis. Biopharmaceutical laboratories have developed robust standard analytical procedures for the analysis of glycosylation. However, acidic glycans, especially complex phosphorylated glycans, can demonstrate low recovery during HILIC-FLR/MS analysis due to ionic interactions with metal surfaces. To address this issue, we optimized the existing HILIC chromatographic separation method for RFMS labeled N-Glycans using the new BioAccord Premier System and ACQUITY Premier Glycan BEH Amide Column that can protect against these unwanted analyte-surface interactions.

Benefits

The ACQUITY Premier Solution with MaxPeak High Performance Surfaces (HPS) technology offers:

- Enhanced recovery of acidic glycans that have stronger interactions with metal surfaces
- Improved recovery and peak shape of phosphorylated glycans that more affected by these interactions
- Automated glycan peak assignment capability assisted by calibrated glycan retention time using the existing UNIFI Glycan Scientific Library GU (Glucose Unit) library

Introduction

Glycosylation of biotherapeutics can impact drug safety, efficacy, and stability, therefore it is often designated as a critical quality attribute (CQA) in biopharmaceutical development.¹ Given its sensitivity to upstream processing conditions, glycosylation also serves as a key indicator of process stability and robustness.² In addition to the overall product glycoprofile, specific glycan species, such as high mannose and sialylated glycans, are particularly important as they can disproportionately influence the properties of the drug product. Glycosylation is commonly assessed by analyzing released and fluorescently labeled N-linked glycans via hydrophilic interaction chromatography (HILIC) coupled to fluorescence (FLR) and/or MS detection to provide comprehensive information (including the identity and relative abundance of glycan species).^{3,4} However, acidic glycans, especially

phosphorylated glycans, can suffer from low recovery during HILIC-FLR/MS analysis. Recent findings have attributed these losses, in part, to ionic interactions between the negatively charged functional group of glycans and metal surfaces of both the LC system and analytical column. This can be particularly challenging in the analysis of biotherapeutics with highly sialylated glycostructures such as fusion proteins, where the sialylated glycans can impact both the immunogenicity and clearance of the drug product. Phosphorylated glycans represent another challenge due to these unwanted interactions with metal surfaces, as in the case of mannose-6-phosphate glycans, which are known to affect cellular receptor uptake and lysosomal targeting in the treatment of lysosomal storage diseases.

To ensure product quality and consistency, accurate characterization and monitoring of glycosylation are required throughout the development and manufacturing of protein therapeutics.² To this end, it is highly desired to develop new methodologies to mitigate the metal mediated sample loss and improve the analysis of released glycans for process development and quality control of biotherapeutics.

The ACQUITY Premier Solution with MaxPeak High Performance Surface (HPS) technology mitigates non-specific adsorptive losses to improve recovery and analysis of metal-sensitive analytes.⁵⁻⁷ This is accomplished by adding a stable barrier layer on the surface of metal substrates to minimize analyte/surface interaction of metal sensitive species.⁵ Given this, and the adsorptive losses encountered with phosphorylated glycans, the ACQUITY Premier Solution should be especially well suited for complex glycan analysis.

This study reports an integrated solution to maximize the sample recovery and assay performance and robustness for released glycan analysis. The ACQUITY BEH Amide Column and the ACQUITY UPLC series have been used in various glycan assays to support the bioprocess development and release of biotherapeutics. To fully realize the potential for unbiased analysis of complex glycan samples, a BioAccord Premier System, comprised of an ACQUITY Premier BSM System and an ACQUITY RDa Time-of-flight Mass Detector, was used in conjunction with the ACQUITY Premier Glycan BEH Amide Column and QuanRecovery sample vials featuring MaxPeak HPS technology.⁸ In order to facilitate efficient and straightforward method development and data analysis, the integrated LC-FLR/MS platform was controlled by the compliant-ready waters_connect Informatics platform, enabling a system approach for N-glycans analysis that can be readily deployed across an organization for development, manufacturing, and release of therapeutic proteins.

The purpose of this application note is to:

- Compare the recovery of acidic glycans from the standard ACQUITY Glycan BEH Amide Column with ACQUITY UPLC I-Class based BioAccord System vs. ACQUITY Premier Glycan BEH Amide Column with BioAccord Premier System
- Demonstrate method transfer focusing on the continued utility of the UNIFI GU library across instrumental

Experimental

Sample Description

RapiFluor-MS Glycan Performance Test Standard (p/n 186007983 < <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186007983-rapifluor-ms-glycan-performance-test-standard.html>>). One vial of the standard sample was reconstituted in 20 μL water to give a final concentration of 20 pmol/ μL .

RapiFluor-MS Sialylated Glycan Performance Test Standard (p/n 186008660 < <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186008660-rapifluor-ms-sialylated-glycan-performance-test-standard.html>>). One vial of the sialylated standard sample was reconstituted in 20 μL water to give a final concentration of 20 pmol/ μL .

A phosphorylated glycoprotein, Cathepsin D, was purchased from Thermo Fisher. N-glycans were released from 15 μg of Cathepsin D and labeled using the GlycoWorks *RapiFluor-MS N-Glycan Kit* (p/n 176004082 < <https://www.waters.com/nextgen/us/en/shop/application-kits/176004082-glycoworks-rfms-n-glycan-kit---8-x-12.html>>) following the GlycoWorks Quick Start protocol for disulfide bond rich proteins (720006992EN < <https://www.waters.com/waters/library.htm?cid=511436&lid=135073129&lcid=135073128>>). Dithiothreitol (DTT) was purchased from Thermo Scientific (Waltham, MA) and used during denaturation to reduce the disulfide bonds.

Method Conditions

Analyses were performed using a BioAccord LC-MS System (with ACQUITY UPLC I-Class PLUS) or a BioAccord Premier LC-MS System (with ACQUITY Premier BSM System).

LC Conditions

System configurations	Standard configuration	ACQUITY Premier Solution
LC system:	ACQUITY UPLC I-Class PLUS	ACQUITY Premier BSM UPLC
Detection:	ACQUITY FLR Detector ($\lambda_{\text{excitation}}=265$ nm, $\lambda_{\text{emission}}=425$ nm, 2Hz)	ACQUITY Premier FLR Detector ($\lambda_{\text{excitation}}=265$ nm, $\lambda_{\text{emission}}=425$ nm, 2Hz)
Column(s):	ACQUITY Glycan BEH Amide Column, 1.7 μ m, 130 Å, 2.1 \times 150 mm (P/N 186004742)	ACQUITY Premier Glycan BEH Amide Column, 1.7 μ m, 130 Å, 2.1 \times 150 mm (P/N 186009524)
Vials:	QuanRecovery with MaxPeak HPS 300 μ L Vials (p/n 186009186)	
Column temp.:	60 °C	
Sample temp.:	6 °C	
Injection amount:	1 μ L	
Seal wash:	20% acetonitrile in water	
Mobile phase A:	H ₂ O with 50 mM NH ₄ HCO ₂	
Mobile phase B:	Acetonitrile	

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.4	25	75	6
35.00	0.4	46	54	6
36.50	0.2	80	20	6
39.50	0.2	80	20	6
43.10	0.2	25	75	6
47.60	0.4	25	75	6
55.00	0.4	25	75	6

MS Conditions

MS system:	ACQUITY RDa Mass Detector
Ionization mode:	ESI Positive
Acquisition range:	50–2,000 <i>m/z</i>
Capillary voltage:	1.5 kV

Cone voltage (CV): 45 V

Fragmentation CV: 70–90 V

Data Management

Informatics: waters_connect with UNIFI 1.9.4

Workflow: “Glycan FLR with MS confirmation”

Results and Discussion

Analysis of Common Acidic Glycans

The levels of acidic glycans (including NeuAC, NeuGC, phosphorylated, and sulfated glycans) can affect drug quality and efficacy and need to be well characterized and monitored during development and manufacturing of therapeutic proteins. In general, current HILIC-based methods using the ACQUITY BEH Amide Column work well for RFMS labeled mAb glycans, which are mostly neutral or mono-sialylated structures. However, new modalities, such as fusion proteins, typically have more complex glycoprofiles including highly sialylated, modified, and more extensively branched glycans. These glycans can suffer from low recovery during LC-FLR/MS analysis, biasing results within the product profile.

To evaluate the potential benefits of ACQUITY Premier Instrument and Column technology for the analysis of acidic glycans, both neutral and sialylated RFMS labeled glycan performance test standards were analyzed using the standard ACQUITY Column with the BioAccord System and the ACQUITY Premier Column with the BioAccord Premier System. The side-by-side comparison of the analysis results is shown in Figure 1A. While the chromatographic profiles were highly comparable, the recovery of sialylated glycans was elevated when using the ACQUITY Premier Column with BioAccord Premier System. As shown in Figure 1B, based on the integrated peak area in three consecutive injections, the average relative abundance of sialylated glycans was 2.22% in ACQUITY Premier Solution as compared to 2.11% in standard configuration, whereas the average relative abundances of neutral glycans were consistent (20.5% vs. 20.5% for FA2). To confirm the observed difference in glycan abundances, a more systematic comparison was conducted using the four representative peaks from the sialylated glycan standard sample, including A2G2S2, A3G3S3, A2S1G3S3, and A2S2G3S3. As shown in Figure 1C, higher

FLR signal responses were observed for all four glycans with the ACQUITY Premier Solution, confirming the increased recovery of sialylated glycans. These results demonstrated the consistent improvement of using ACQUITY Premier Solutions with MaxPeak HPS technology to maximize glycan recovery, which can be particularly beneficial to the therapeutics with highly sialylated glycans such as fusion proteins.

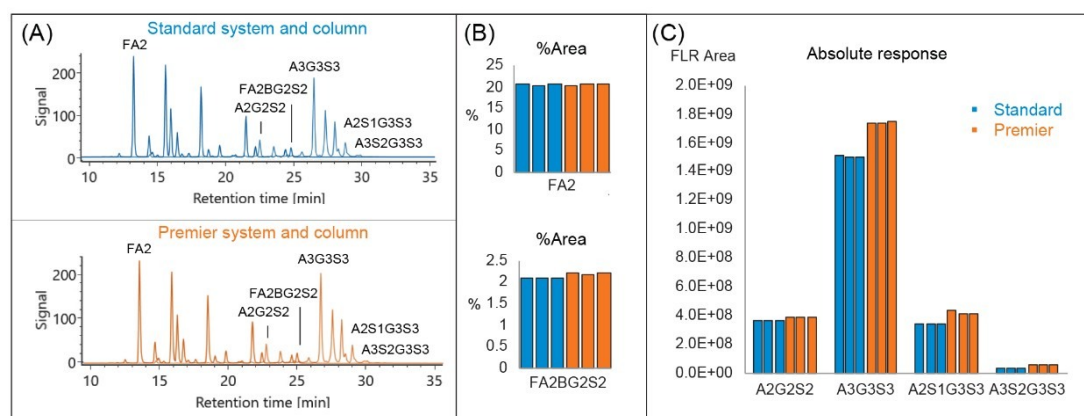


Figure 1. The recovery of sialylated glycans are slightly better using the ACQUITY Premier BioAccord System with ACQUITY Premier Column. (A) overlaid chromatograms of the separation of neutral and sialylated RFMS labeled glycan performance test standards (GPTS) on Standard vs. ACQUITY Premier System and Column. (B) Comparison of relative abundances of a neutral glycan, FA2, vs. a sialylated glycan, FA2BG2S2 in the neutral GPTS. (C) Comparison of the absolute response of the representative glycans in the sialylated GPTS.

Analysis of Phosphorylated Glycans In many lysosomal enzyme replacement therapies, the abundance of phosphorylated glycans in the drugs is a critical quality attribute requiring monitoring during development and manufacturing because it determines the efficiency of lysosomal enzyme delivery.⁹ However, phosphorylated glycans also face the challenge of low recovery in LC-MS analysis due to the affinity of phosphate groups towards metal surfaces. To demonstrate that ACQUITY Premier Solution with HPS technology also mitigates these effects, a lysosomal enzyme, Cathepsin D, was used as a model protein to investigate the recovery of phosphorylated glycans for HILIC-FLR/MS analysis.

Using the Waters GlycoWorks RapiFluor-MS N-Glycan Kit, N-linked glycans were released from Cathepsin D, labeled with RFMS, and then analyzed via HILIC-FLR/MS using the ACQUITY Glycan BEH Amide Column and BioAccord System (Standard System). As shown in Figure 2A, the released N-glycans of Cathepsin D were separated using a 35 min gradient with the major peaks being identified as high mannose structures (3–7) based on their accurate masses. The m/z corresponding to mannose-6-phosphate (M6P) was observed at approximately 20.7

min with a good signal-to-noise ratio (S/N) in the ESI spectra (Figure 2A inset), resulting in the mass error of only 1.4 ppm for the measured monoisotopic mass of 1788.6461 Da. As shown in the extracted ion chromatogram (XIC) of M6P (Figure 2B), two peaks were observed with the same m/z , which were identified as a pair of structural isomers and annotated as M6P-1 and M6P-2, respectively. The peak for M6P-2 showed significant tailing, suggesting the presence of secondary interactions, possibly caused by metal chelation. As a comparison, the released N-glycans of Cathepsin D were analyzed in the same fashion using the ACQUITY Premier Column with BioAccord Premier System (ACQUITY Premier Solution). As shown in Figure 2C, highly similar chromatographic profiles were obtained, suggesting the overall comparability of these two instrument configurations on released glycan analysis. However, while the m/z of M6P was observed at a similar retention time at 25.8 min (Figure 2C inset), the MS response of M6P-2 was enhanced approximately 3-fold in the XIC of M6P (Figure 2D) with significantly improved peak shape. This demonstrates the ability of ACQUITY Premier Solution with MaxPeak HPS technology to reduce adsorptive losses of phosphorylated glycans to metal surfaces. The %RSD of the MS response of M6P over 15 injections was at 3.96%, demonstrating the high reproducibility of the analysis. Hence, quantification of phosphorylated glycans can be more accurate in released glycan analysis using the ACQUITY Premier technology.

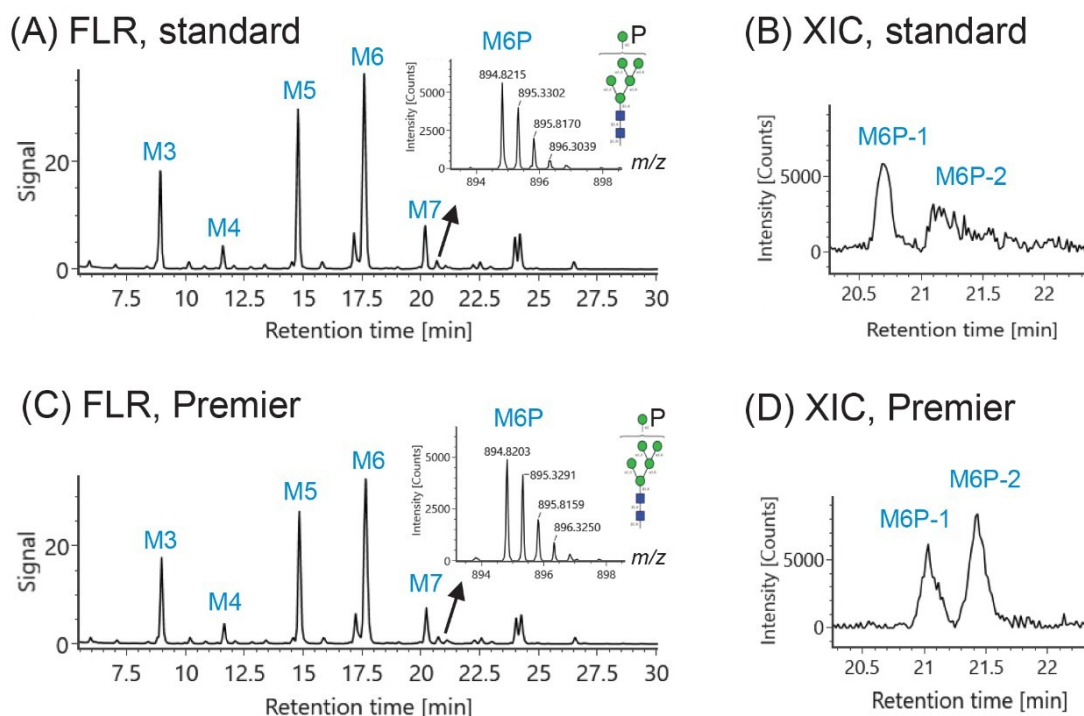


Figure 2. Comparison of signal response of a phosphorylated glycan, Mannose-6-Phosphate (M6P), analyzed on different analytical platforms. Released N-glycan profile of Cathepsin D on (A) a standard BioAccord System with an ACQUITY BEH Amide Column; and (C) a Premier BioAccord System with an ACQUITY Premier BEH Amide Column. Figure inset shows the MS spectra of M6P. (B) and (D) are the extracted ion chromatograms of M6P on the two system configurations. The same mass were observed for M6P-1 and M6P-2.

Generating Glycan Structure Assignments using UNIFI GU Library

In the released glycan analytical workflow, the UNIFI GU Library was designed to automatically identify glycan peaks based on Glucose Unit (GU) and accurate mass, thus improving the productivity and confidence of released glycan analysis. The GU values were generated based on the calibrated retention times of glycans at a specified method condition which relies on column chemistry and chromatographic method conditions. Therefore, it is necessary to confirm that the comparability of GU values collected from two LC instrument configurations (Standard vs. ACQUITY Premier Columns).

Using the released glycan workflow in the waters_connect platform, the data previously collected from the glycan performance standard were processed to compare GU values. As shown in Table 1, the GU values of the 19 glycans in the standard sample are consistent with the expected GU values and are comparable across different instrument configurations (BioAccord vs. BioAccord Premier System). Therefore, the released glycan workflow and

established GU libraries can be seamlessly transferred to the ACQUITY Premier System configuration. The standard deviation was at 0.001 GU based on three consecutive injections, demonstrating the high reproducibility of the analyses. To this end, the ACQUITY Premier Solution with MaxPeak HPS technology can be used in combination with the well-established released glycan workflow in UNIFI for streamlined N-glycan analysis with high productivity and sample recovery.

Peak	Name	Expected GU	Standard GU	Premier GU
1	A2	5.46	5.53±0.001	5.50±0.001
2	FA2	5.79	5.84±0.001	5.81±0.001
3	FA2B	6.12	6.19±0.001	6.16±0.001
4	A2G1a	6.25	6.27±0.001	6.25±0.001
5	A2G1b	6.29	6.39±0.001	6.37±0.001
6	FA2G1a	6.53	6.58±0.001	6.55±0.001
7	FA2G1b	6.66	6.71±0.001	6.68±0.001
8	FA2BG1a	6.80	6.86±0.001	6.83±0.001
9	FA2BG1b	6.91	6.98±0.001	6.94±0.001
10	A2G2	7.10	7.15±0.001	7.13±0.001
11	FA2G2	7.43	7.46±0.001	7.43±0.001
12	FA2BG2	7.61	7.65±0.001	7.61±0.001
13	FA2G1S1	7.84	7.94±0.001	7.88±0.001
14	A2G1S1	8.40	8.37±0.001	8.32±0.001
15	FA2G2S1	8.55	8.66±0.001	8.60±0.001
16	FA2BG2S1	8.82	8.94±0.001	8.87±0.001
17	A2G2S2	9.42	9.56±0.001	9.47±0.001
18	FA2G2S2	9.69	9.85±0.002	9.75±0.001
19	FA2BG2S2	9.86	10.03±0.002	9.92±0.001

Table 1. GU values of the separated peaks in Glycan Performance test standard obtained using the ACQUITY Premier System and standard version of BioAccord System and Column. The range in ACQUITY Premier Standard and Standard GU values represented the standard deviation, which was calculated based on 3 consecutive injections.

Conclusion

This study demonstrates the new ACQUITY Premier System and Column technology improves the LC-FLR-MS data quality for released N-glycan analysis. The recovery and peak shape of phosphorylated glycans were significantly improved, and the recovery of sialylated glycans was enhanced. With this released glycan workflow under the waters_connect informatics platform, the data analysis was streamlined via automated peak assignments against the existing UNIFI GU library. The GU values obtained from the BioAccord Premier System are consistent with values obtained from the standard system, enabling the transfer of previously developed ACQUITY UPLC methods to the new ACQUITY Premier System. Collectively, this application note demonstrated the enhanced recovery of released glycans by the ACQUITY Premier Solution with MaxPeak HPS technology, which helps develop robust and unbiased glycan assays for biotherapeutic development, manufacture, and release.

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