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Application Note

MaxPeak High Performance Surfaces Technology Improves HILIC Profiling of Released N-Glycans

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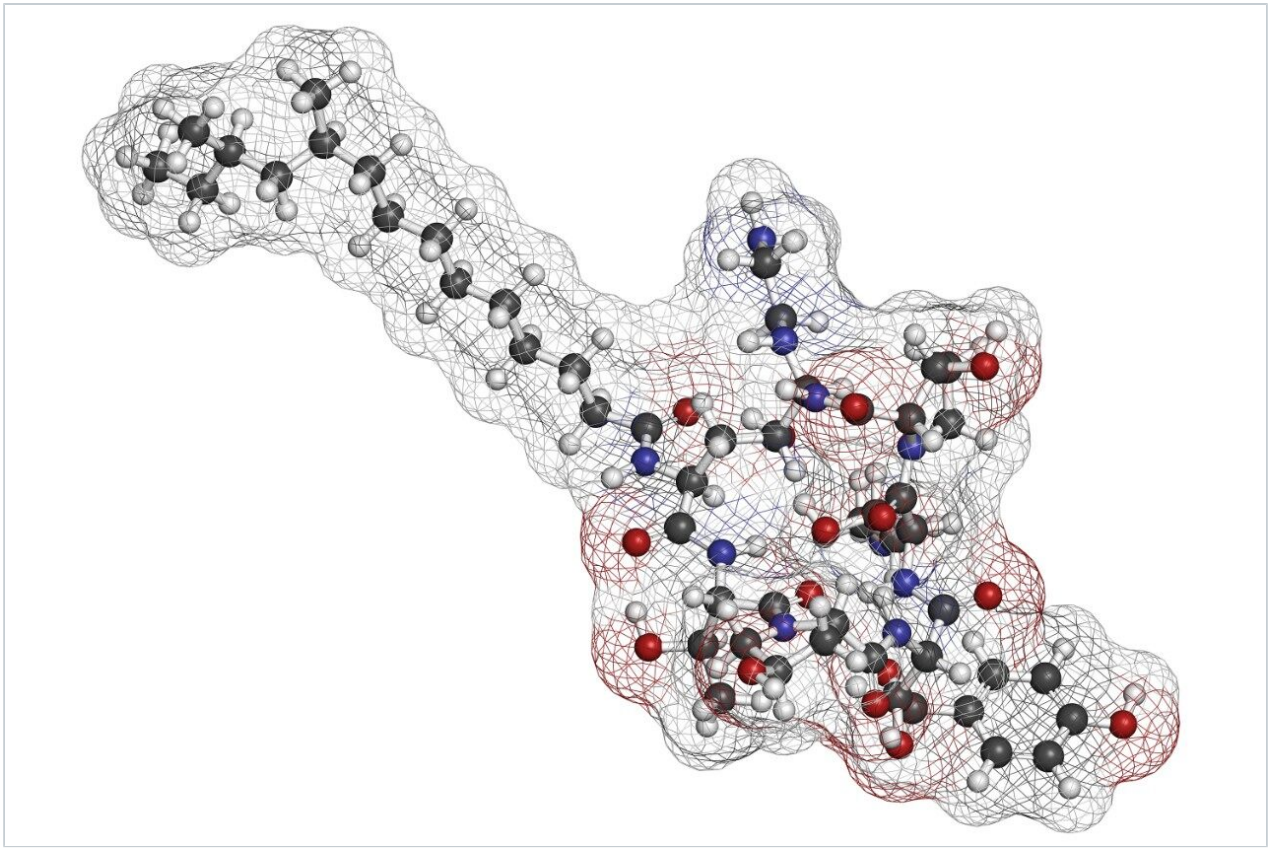

Abstract

The chromatographic analysis of metal sensitive compounds can be negatively impacted by the stainless-steel column hardware. Because of adsorption and secondary interactions, such compounds can exhibit asymmetric peaks and poor recovery, especially under demanding, low ionic strength separations. To address this issue, MaxPeak High Performance Surfaces (HPS) were developed. This technology provides a highly effective surface barrier that prevents analytes from undergoing undesired interactions with the metal surfaces of a chromatographic column. In terms of released N-glycans, these sorts of problems can arise with acidic N-linked glycans, like those containing multiple sialic acids or phosphorylated mannose residues. Acidic N-linked glycans are often assigned as critical quality attributes in certain biotherapeutics because they can affect stability, efficacy, and immunogenicity. To carefully control and monitor these moieties, N-glycans are frequently released and analyzed by hydrophilic interaction chromatography (HILIC) and mass spectrometry. In some situations, with this technique, it has been a challenge to achieve good peak shape and recovery of acidic N-linked glycans while maintaining MS compatibility. With ACQUITY Premier Glycan BEH Amide Columns that are equipped with MaxPeak HPS Technology, it is now possible to recover highly-sialylated and phosphorylated glycans from ultrahigh pressure HILIC chromatography without any dependence or requirement for column conditioning.

Benefits

- Novel ACQUITY Premier Glycan BEH Amide Column with MaxPeak HPS Technology to provide desired performance and improved recovery of glycans starting with the first injection on a new column
- Accurate profiling of highly sialylated and phosphorylated glycans without time-consuming column condition and passivation
- Better reproducibility of retention times for sialylated glycans versus a conventional stainless-steel hardware column
- MS-grade ammonium formate concentrate and the *Rapi*Fluor-MS Glycan Performance Test Standards to help establish an easy-to-implement released N-glycan approach

Introduction

Because of the ever increasing pace of the biotherapeutics pipeline, there is always a need to hasten the accurate monitoring of critical quality attributes (CQAs) of the products. Glycosylation is one type of CQAs that can affect drug stability, efficacy, and immunogenicity. It has been preferred in glycan assay by combining a rapidly released glycan sample preparation with HILIC chromatographic separation.[1] It has often, however, been a challenge to analyze acidic glycans due to their propensity to be lost to the metallic surfaces of columns. The use of mobile phase with spiked-in low concentration of chelator with high ionic strength can help prevent this issue, but these separation conditions are not MS-friendly and they could cause problems for glycan characterization using ESI MS. Column passivation by loading large quantities of analytes is another way to help address this problem. This approach, however, is limited by the time consuming column conditioning effort and availability of excess sample. In many cases, column passivation is reversible and irreproducible thus not reliable for achieving the desired chromatographic performance. To address this issue directly, MaxPeak High Performance Surfaces (HPS) were developed and utilized to manufacture the ACQUITY Premier Columns.[2,3] These surfaces are based on hybrid inorganic-organic silica, serving as a highly effective surface barrier that prevents acidic glycans from undergoing undesired secondary interactions with the metal surfaces inside chromatographic columns. In this application, the column performance before and after passivation has been carefully investigated for both an ACQUITY Premier Glycan BEH Amide Column and a conventional stainless steel version of the column. The obtained data demonstrate advantages from using ACQUITY Premier Columns with MaxPeak HPS Technology to achieve improved recovery, obtain better reproducibility, and do away with column conditioning for released glycan separations.

Experimental

Sample Description

- The *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard (Waters, p/n 186008660 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186008660-rapifluor-ms-sialylated-glycan-performance-test-standard.html>) was prepared by reconstituting 1 vial of standard with 50 µL of water to give a final concentration of 8 pmol/µL. < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186008660-rapifluor-ms-sialylated-glycan-performance-test-standard.html>
- The *Rapi*Fluor-MS Glycan Performance Test Standard (Waters, p/n 186007983 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186007983-rapifluor-ms-glycan-

performance-test-standard.html>) was prepared by reconstituting 1 vial of standard with 50 µL of water to give a final concentration of 8 pmol/µL.

- Fetuin Protein Solution was prepared by dissolving 10 mg of Bovine Fetuin with 1 mL of water to give a final concentration of 10 mg/mL.
- N-glycans of 15 µg agalsidase was released, labeled, and purified using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit following the protocol described in Care and Use Manual (715004793EN < https://www.waters.com/webassets/cms/support/docs/715004793en.pdf>).[1] The final concentration of released glycan was about 0.5 pmol/µL.

Example Sample List for Column Initial Performance Test:

Purpose	Sample name	Injection volume
Blank	Water blank	1 µL
Analysis – Glycan Inj. 1	*Rapi*Fluor-MS glycan performance test standard (8 pmol/µL)	1 µL
Analysis – Glycan Inj. 2		1 µL
Analysis – Glycan Inj. 3		1 µL
Analysis – Glycan Inj. 4		1 µL
Conditioning and Passivation	Fetuin Protein solution (10 mg/mL)	1 µL
Blank	Water blank	1 µL
Analysis (post passivation) – Glycan Inj. 5	*Rapi*Fluor-MS glycan performance test standard (8 pmol/µL)	1 µL

Calculation for Glycan Recovery (%) using Conventional Column:

Relative Recovery (%) of Glycan i = FLR Peak Area of Selected Glycan i obtained using Conventional Column/FLR Peak Area of Glycan i obtained using Passivated ACQUITY Premier Column X 100%

LC Conditions

LC system:	ACQUITY Premier System (QSM)
Data acquisition:	MassLynx v4.1
Column:	ACQUITY Premier Glycan BEH Amide Column (1.7 µm, 130 Å, 2.1 x 150 mm), p/n 186009524

	ACQUITY UPLC Glycan BEH Amide Column (1.7 µm, 130 Å, 2.1 x 150 mm), p/n 186004742
Sample temperature	8 °C
Sample injection volume:	1 µL
FLR wavelengths:	265 Ex./425 Em.
Column temperature:	60 °C
Seal wash:	30% ACN/70% 18.2 MΩ water v/v (seal wash interval set to 5 min)
Mobile phase A:	50 mM Ammonium formate, pH 4.4
Mobile phase B:	Acetonitrile (LC-MS Grade)
Active preheater:	Enabled
Scan rate:	10 points/sec
Filter time constant:	Normal
Autozero on inject start:	Yes
Autozero on wavelength:	Maintain baseline

Gradient

Time (min)	Flow (mL/min)	%A[1]	%B	Curve
0.0	0.4	25.0	75.0	Initial
11.7	0.4	46.0	54.0	6
13.2	0.2	100.0	0	6
14.2	0.2	100.0	0	6
16.4	0.2	25.0	75.0	6
23.0	0.4	25.0	75.0	6

1. 100% aqueous is applied in this study for fast column evaluation, while in practical, a mild wash condition of 80% A and 20% B is recommended to elongate column lifetime and performance.

MS Conditions

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Acquisition range:	700–3000 Da
Capillary voltage:	2.2 kV
Source offset:	50 V
Collision energy:	Off
Cone voltage:	75 V
Desolvation gas:	600 L/hr
Source temperature:	120 °C

Desolvation temperature:	500 °C
Scan rate:	2 Hz

Data Management

Chromatography software:	MassLynx v4.1
MS software:	MassLynx v4.1
Data processing:	UNIFI v.1.8

Results and Discussion

To investigate the initial performance of MaxPeak HPS-equipped ACQUITY Premier Column, an 8 pmol *Rapi* Fluor-MS Glycan Performance Test Standard was injected four times onto a brand new ACQUITY Premier Glycan BEH Amide Column. Subsequently, three high mass loading of 10 mg/mL fetuin, the highly sialylated glycoprotein, were injected at 1 µL, intended for column conditioning and passivation. After passivation, the *Rapi*Fluor-MS Glycan Performance Test Standard was injected again to check the column performance (refer to Experimental for an example sample list used in the study). For comparison, these tests were also performed on a brand new, conventional stainless steel column. Furthermore, the *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard was also adopted during the comparison test. In these experiments, we used an LC employed with MaxPeak Premier Technology to minimize LC hardware based impacts on the separations.

According to a previously published work, 19 glycans can be resolved from the Glycan Performance Test Standard using HILIC chromatography.[4] As shown in Figure 1A, only 13 of the 19 glycan species were identified with the conventional steel column from injection 1; 5 of the 7 sialylated glycans could not be detected from this initial injection. Furthermore, the relative recovery of glycans resolved from conventional column was compared with that obtained from the ACQUITY Premier Column and calculated based on related FLR peak areas using equations mentioned in Experimental. By comparing with the ACQUITY Premier Column, the recovery of the most abundant, singly-sialylated glycan FA2G2S1 was less than 40%, and none of

those doubly-sialylated glycans could be detected without conditioning based passivation (Figure 1B). It was noticed that a few neutral glycans also exhibited recovery issues at the first injection on the conventional stainless steel column. The recovery of FA2, FA2G1, and FA2G2 obtained from stainless steel column appeared to be 95%, 80%, and 60%, respectively (Figure 1B). The loss of glycans, especially acidic glycans, during these separations with the conventional steel column was believed to be a result of non-specific binding of analytes to metal surfaces. It has been shown that, despite best efforts, these types of undesired secondary interactions can sometimes be difficult to eliminate by column conditioning. On those column, 3 more injections of glycan standard were performed but still 6 glycans remained unresolved (Figure 1A), and their peak areas remained undesirably low (Figure 1B). Problematic glycan species could only be recovered after high mass load injections of fetuin protein to achieve conditioning based passivation. After passivation, all 19 glycans, including doubly-sialylated species, could be successfully detected. The effective peak capacity of the conventional steel column was improved from 50 to 66 upon conditioning based passivation (Figure 1C).

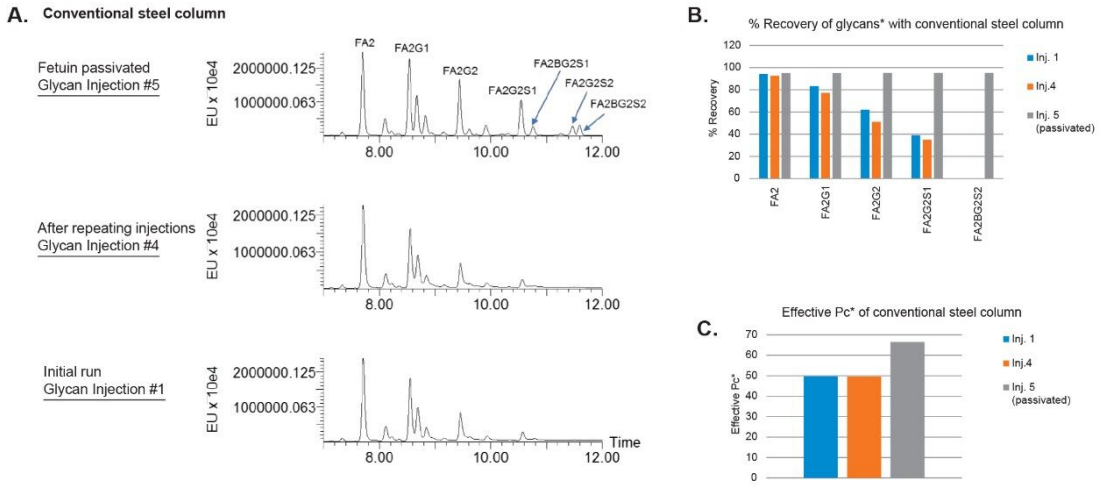

Figure 1. Impact of column conditioning on HILIC of N-Glycans when using conventional steel columns.

A) FLR profiling of the RapiFluor-MS Glycan Performance Test Standard using a 2.1 x 150 mm conventional steel 1.7 µm BEH amide column at injection 1 (initial run), Inj. 4 (after several repeat injections of the glycan standard) and Inj. 5 (after fetuin-based conditioning and passivation); B) % Recovery of selected glycans at different injections; C) Effective peak capacity (Pc) of conventional columns at different injections.*

In contrast, with the deployment of MaxPeak HPS Technology, the ACQUITY Premier Glycan BEH Amide Column provided excellent chromatographic recovery and peak resolution for all types of glycan species the

first injection and without any dependence on column conditioning. As shown in Figure 2A, all 19 glycans species were successfully resolved upon the first injection and run. Impressively, all 7 sialylated glycans in the sample were detected, even though some are present with relative abundance as low as 0.5%. Glycan profiles acquired using the ACQUITY Premier Column are comparable both before and after conditioning based passivation (injection 1, injection 4, and injection 5; Figure 2A). The recovery of individual glycan and the effective peak capacity of the separation remained consistent through injection 1 to injection 5 (Figure 2B and 2C). It is also worth mentioning that the glycan profile achieved using the ACQUITY Premier Column was comparable to that of generated with the fully passivated conventional steel column (Figure 3). The %RSD for the relative abundance of all the resolved glycans was less than 8% with three averaged data points from (1) the passivated conventional steel column (Inj. 5), (2) passivated ACQUITY Premier Column (Inj. 5), and (3) the very first run on the ACQUITY Premier Column (Inj. 1) (Table 1). This result demonstrates that MaxPeak HPS Technology attributes accurate glycan profiles and there is little effect of hybrid surfaces on the resolution or selectivity. With ACQUITY Premier Glycan BEH Amide Columns, glycan recoveries have been markedly improved and requirements for column conditioning upon the first time usage have been eliminated.

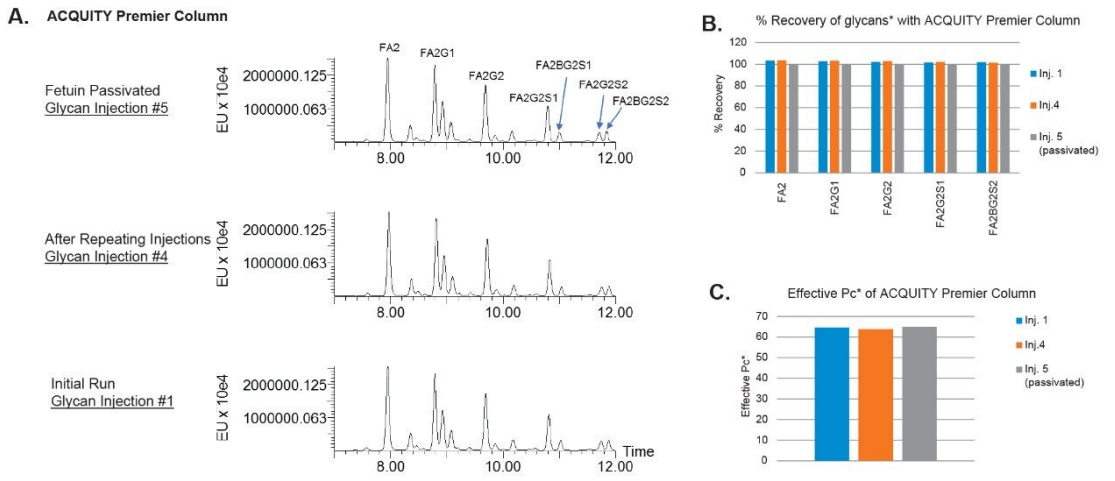

Figure 2. Impact of column conditioning on HILIC of N-Glycans when using ACQUITY Premier Columns.

A) FLR profiling of the RapiFluor-MS Glycan Performance Test Standard using a 2.1 x 150 mm ACQUITY Premier 1.7 µm BEH Amide Column at injection 1 (initial run), Inj. 4 (after several repeat injections of the glycan standard) and Inj. 5 (after fetuin-based conditioning and passivation); B) % Recovery of selected glycans at different injections; C) Effective peak capacity (Pc) of ACQUITY Premier Columns at different injections.*

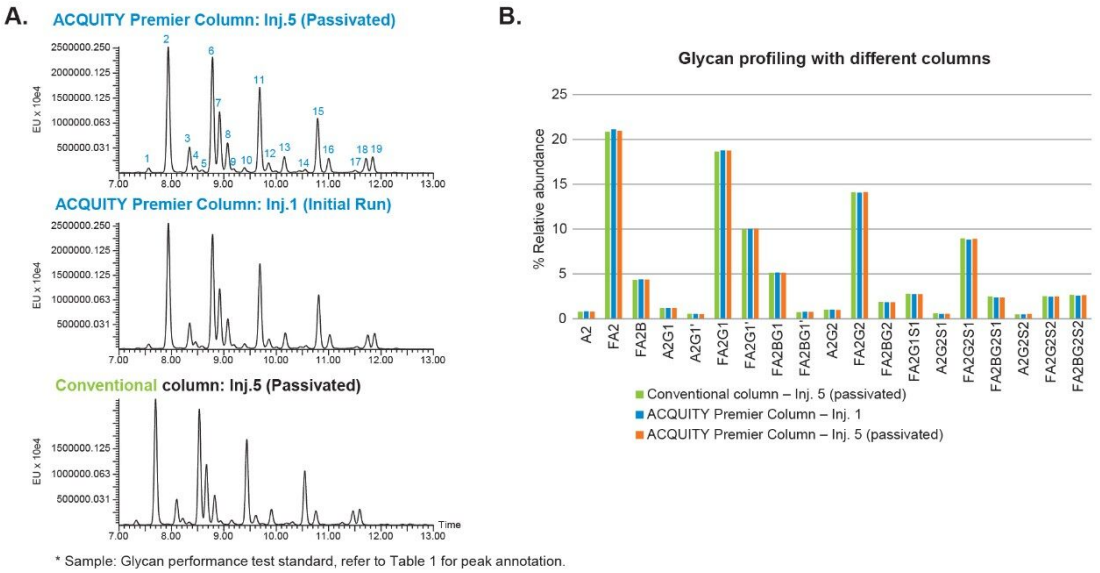

Figure 3. Comparable glycan profiles obtained using passivated conventional steel versus ACQUITY Premier HILIC Column.

A) FLR profiling of RapiFluor-MS Glycan Performance Test Standard using a passivated conventional steel column at injection 5 (bottom) and ACQUITY Premier Column at inj. 1 (middle) and Inj. 5 (top); B) Relative abundance of resolved glycans using different columns

Glycans performance test standard			%Relative abundance			% RSD of glycan profile between conventional vs. ACQUITY Premier Column		
			ACQUITY Premier Column		Conventional column			
Peak No.	Glycan	RFMS labeled glycan composition	1st injection	5th injection (passivated)	5th injection (passivated)	Avg. rel. abundance	Std. dev.	%RSD
1	A2	C67H105O37N9	0.86	0.82	0.80	0.83	0.03	3.83
2	FA2	C73H115O41N9	21.13	20.98	20.84	20.98	0.15	0.69
3	FA2B	C81H128O46N10	4.40	4.38	4.35	4.38	0.03	0.60
4	A2G1	C73H15O42N9	1.22	1.20	1.21	1.21	0.01	0.64
5	A2G1'	C73H15O42N9	0.54	0.53	0.57	0.55	0.02	4.42
6	FA2G1	C79H125O46N9	18.78	18.77	18.65	18.73	0.07	0.39
7	FA2G1'	C79H125O46N9	10.03	10.05	10.01	10.03	0.02	0.21
8	FA2BG1	C87H138O51N10	5.17	5.15	5.14	5.16	0.02	0.30
9	FA2BG1'	C87H138O51N10	0.80	0.78	0.75	0.78	0.02	2.75
10	A2G2	C79H125O47N9	1.03	1.01	1.04	1.03	0.02	1.74
11	FA2G2	C85H135O51N9	14.07	14.15	14.12	14.11	0.04	0.30
12	FA2BG2	C93H148O56N10	1.87	1.86	1.90	1.88	0.02	1.15
13	FA2G1S1	C90H142O54N10	2.75	2.76	2.80	2.77	0.03	0.92
14	A2G2S1	C90H142O55N10	0.55	0.57	0.64	0.59	0.05	7.95
15	FA2G2S1	C96H152O59N10	8.83	8.92	8.97	8.91	0.07	0.82
16	FA2BG2S1	C104H165O64N11	2.40	2.41	2.49	2.43	0.05	2.07
17	A2G2S2	C101H159O63N11	0.51	0.55	0.51	0.52	0.02	3.70
18	FA2G2S2	C107H169O67N11	2.46	2.49	2.53	2.49	0.03	1.38
19	FA2BG2S2	C115H182O72N12	2.60	2.64	2.67	2.64	0.04	1.35

Table 1. Glycan profiles with conventional steel versus ACQUITY Premier Columns – before (Inj. 1) and after Passivation (Inj. 5).

In addition, the ACQUITY Premier Glycan BEH Amide Column has also provided breakthrough performance for the analysis of phosphorylated glycans. Phosphorylated glycans with mannose-6-phosphate residue on their terminal ends serve as a signaling tag for lysosomal enzymes, which is critical to the uptake of enzyme replacement therapies.[5] Due to the presence of phosphate residue, however, this group of analytes is notoriously difficult to recover through LC analysis since they tend to adsorb onto metal surfaces. To evaluate whether the MaxPeak HPS column hardware is beneficial for phosphorylated glycan analysis, N-glycans released from agalsidase were analyzed via the same testing protocol described in the experimental section. The same batch of the stationary phase was packed in both an ACQUITY Premier Column and a conventional stainless steel column. The separation of a singly phosphorylated glycan, Man7-1P, with different columns is summarized in Figure 4. Like other acidic glycan species, the Man7-1P cannot be identified with a conventional steel column until conditioning based passivation is applied. Even after fetuin passivation, the peak response of Man7-1P from the conventional steel column is around 70% of that obtained with the ACQUITY Premier Column.

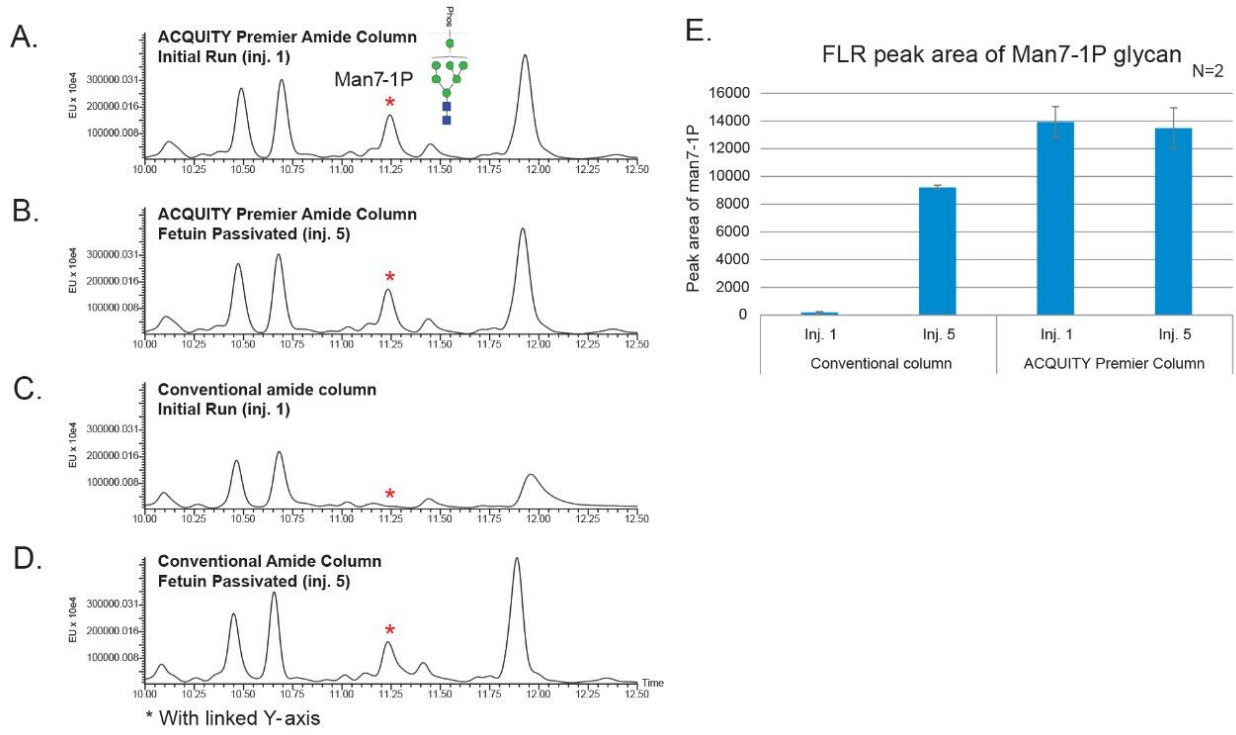

*Figure 4. Improved recovery of phosphorylated glycans using ACQUITY Premier Glycan Amide Column. A, B) FLR detection of a singly phosphorylated glycan (Man7-1P, labeled with *) using an ACQUITY Premier Glycan BEH Amide Column before and after passivation (A versus B); C, D) FLR detection of singly phosphorylated glycan (Man7-1P, labeled with *) using a conventional steel column before and after passivation (C versus D); E) Recovery of Man7-1P glycan based on FLR peak area.*

Moreover, the ACQUITY Premier Glycan BEH Amide Column provided more reproducible retention times for sialylated glycans. Reproducibility was assessed through four continuous injections of Sialylated Glycan Performance Test Standard on fetuin-passivated conventional steel and ACQUITY Premier Columns. Three passivated columns from each column type, with the same batch of stationary phase, were used for this study. Retention times and the corresponding %RSD values were recorded and calculated for the six most abundant sialylated glycan species in the test standard (Figure 5). As demonstrated, the %RSD of glycan retention times across three ACQUITY Premier Columns were within the range of 0.12–0.22% while they increased to 1.15–1.96% for the same sialylated glycans when analyzed with conventional steel columns.

Average retention time (min), n=12

	Conventional steel columns		ACQUITY Premier Glycan BEH Amide Columns	
	Avg. RT ± Std. Dev. (min)	%RSD	Avg. RT ± Std. Dev. (min)	%RSD
Peak 1	10.89± 0.13	1.17	10.87± 0.01	0.12
Peak 2	11.26± 0.13	1.15	11.23± 0.01	0.12
Peak 3	12.15± 0.15	1.24	12.13± 0.02	0.15
Peak 4	12.55± 0.18	1.43	12.52± 0.02	0.16
Peak 5	12.87± 0.21	1.64	12.84± 0.02	0.19
Peak 6	13.42± 0.26	1.96	13.39± 0.03	0.22

* Peak assignment: A2G2S2 (Peak 1 and 2); A3G3S3 (Peak 3 and 4); A3S1G3S3 (Peak 5 and 6)

Example chromatogram of the *Rapi*Fluor-MS sialylated glycan performance test standard

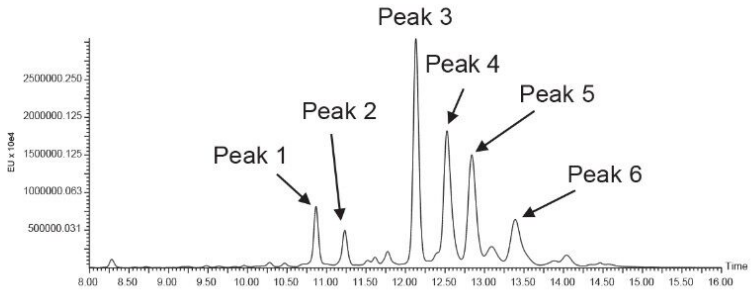

Figure 5. Column-to-Column retention times and RSD values for 6 major sialylated glycan species resolved using either conventional steel or ACQUITY Premier Glycan BEH Amide Columns after passivation. Three conventional and three ACQUITY Premier Glycan BEH Amide Columns were fetuin passivated and then used to separate 4 repeat injections of RapiFluor-MS Sialylated Glycan Performance Test Standard, respectively. An example chromatogram of the sialylated glycan profile is shown below.

Conclusion

The innovative MaxPeak High Performance Surfaces Technology is used for various column chemistry to improve chromatographic performance, including the HILIC mode separation of glycans with the ACQUITY Premier Glycan BEH Amide Column. Improved glycan chromatographic recoveries and a new level of out-of-box performance, which is most pronounced for acidic glycans, were illustrated in this application note. More accurate profiling of released glycans can be easily achieved and tedious column conditioning and passivation procedures can be circumvented. Additionally, these columns can facilitate the recovery of

challenging phosphorylated glycans and provide improved reproducibility for the retention times of sialylated glycans. As a result, released glycan profiling LC-MS assay is more robust, which is becoming more critical as protein therapeutics with increasingly complex glycan profiles emerge from the development pipeline.

References

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