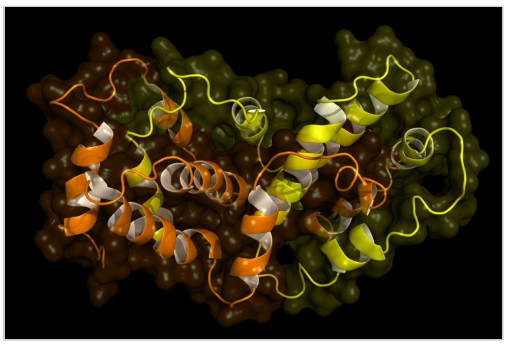
Waters™

Application Note

Increased Resolving Power for Acidic Glycans with an MS-Compatible Anion Exchange Reversed Phase Separation

Xiaoxiao Liu, Matthew A. Lauber

Waters Corporation



Abstract

Glycosylation 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 detected by fluorescence or mass spectrometry. Several challenges have, however, been encountered upon attempting to implement this approach, most particularly the incomplete recovery of acidic glycans and the lack of charge-based separations for MS-enhancing glycan labels. Herein, a novel mixed-mode, anion-exchange RPLC stationary phase has been used to afford a class separation of different charge states and additional resolution of acidic glycans. Combined with MaxPeak High Performance Surface (HPS) Technology, this new stationary phase and the ACQUITY PREMIER Glycan BEH C_{18} AX Column addresses these shortcomings and brings with improved capabilities for the analysis of acidic N-glycans.

Benefits

- Charge-based separation optimized for acidic N-glycans derivatized with both *Rapi*Fluor-MS and 2-AB labels
- Novel ACQUITY PREMIER Glycan BEH C_{18} AX Column with HPS Technology to provide improved recovery of analytes and optimal performance even with initial use
- Better resolution and MS compatibility than other commercially available AX RPLC columns
- QC-tested stationary phase batches to ensure reproducibility and acidic glycan resolution
- Integrated with MS-grade mobile phase concentrate and system performance check standard to provide a complete charged base separation solution
- Shared sample preparation consumables and procedures with HILIC mode separation

Introduction

Acidic N-linked glycans are often assigned as critical quality attributes in biotherapeutics because they can affect stability, efficacy, and immunogenicity. For instance, sialylated glycans alter anti-inflammatory effects,[1] and mannose-6-phosphate glycans facilitate lysosomal targeting.[2] In one widely adopted approach, N-glycans are enzymatically released and rapidly derivatized prior to being separated by hydrophilic interaction chromatography (HILIC) and detected by fluorescence or mass spectrometry.[3] At times, it is of interest to more directly ascertain charge specific information about a glycan profile. However, an LC separation with a charge-based separation mechanism optimized for recently introduced positive ion mode enhancing labels, like *Rapi*Fluor-MS, has not yet been developed. Even though a few anion exchange mixed mode columns are commercially available, none have been expressly optimized for MS enhancing, rapid labeling tags. Moreover, the fact that the commercially available options have been predominately constructed with metal hardware has meant that the incomplete recovery of acidic glycans has been commonplace.

To solve these problems, the novel ACQUITY PREMIER Glycan BEH C_{18} AX Column was developed and applied to enhance the analysis of acidic N-glycans. This new mixed-mode, anion-exchange RPLC stationary phase (95 Å, 1.7 µm), with its optimized ionizable modifier, has been used to afford an effective charge-based separation and additional resolution of acidic glycans derivatized with both traditional and MS enhancing labels. In addition, the column has been manufactured with High Performance Surface Technology, which has been demonstrated to minimize sample loss during the chromatographic analysis of acidic analytes.[4]

Experimental

Sample Description

- The *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard (p/n: 186008660) was prepared by reconstituting 1 vial of standard with 50 µL of water to give a final concentration of 8 pmol/µL
- The *Rapi*Fluor-MS Glycan Performance Test Standard (p/n: 186007983) was prepared by reconstituting 1 vial of standard sample with 50 µL of water to give a final concentration of 8 pmol/µL
- The AdvanceBio 2-AB Bovine Fetuin N-Glycan Library (Agilent, p/n: DP18I2601) was prepared by reconstituting 1 vial of standard sample with 20 µL of water to give a final concentration of 10 pmol/µL

Method Conditions

LC Conditions

System:	ACQUITY UPLC H-Class Bio
Data acquisition:	MassLynx v4.1
Column:	ACQUITY PREMIER Glycan BEH C_{18} AX, 1.7 µm, 2.1 x 150 mm
Sample temp.:	8 °C
Sample injection volume:	1 µL
FLR wavelengths:	265 Ex/425 Em (RFMS-labeled glycans) 330 Ex/420 Em (2-AB-labeled glycans)
Column temp.:	60 °C
Seal wash:	30% ACN/70% 18.2 MΩ water v/v (seal wash interval set to 5 min)
Mobile phase A[1]:	18.2 MΩ water
Mobile phase B[2]:	10% IonHance Glycan C_{18} AX 1 M ammonium formate concentrate in 40%/60% water/acetonitrile (v/v)
Active preheater:	Enabled

Scan rate:	10 points/sec
Filter time constant:	Normal
Autozero on inject start:	Yes
Autozero on wavelength:	Maintain baseline

1. The water only mobile phase can be susceptible to bacterial growth. It is recommended to replace the mobile phase frequently (every 3 days) and periodically flush solvent line with 90/10 acetonitrile/water mixture.
2. Mobile Phase B is prepared by diluting 100 mL of IonHance Glycan C_{18} AX 1 M Ammonium Formate concentrate with 320 mL of MilliQ water and 580 mL of acetonitrile. It is recommended to replace this mobile phase B frequently (every 3 days) to avoid any potential performance change caused by acetonitrile evaporation.

Gradient - RapiFluor-MS Labeled Glycans (Figures 1, 2, 3)

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.4	100	0	Initial
36.0	0.4	78	22	6
36.3	0.4	0	100	6
37.3	0.4	0	100	6
38.0	0.4	100	0	6
45.0	0.4	100	0	6

Gradient – 2-AB Labeled Glycans (Figure 4)

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.4	100	0	Initial
36.0	0.4	70	30	6
36.3	0.4	0	100	6
37.3	0.4	0	100	6
38.0	0.4	100	0	6
45.0	0.4	100	0	6

MS Conditions

System:	Xevo G2-XS QTof
Ionization mode:	ESI, Positive
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 temp.:	120 °C
Desolvation temp.:	500 °C
Scan rate:	2 Hz

Data Management

Chromatography software:	MassLynx v4.1
MS software:	MassLynx v4.1
Informatics:	UNIFI v.1.8

Results and Discussion

The ACQUITY PREMIER Glycan BEH C_{18} AX Column is designed for a charge-based separation of RFMS-labeled glycans and to thereby provide an orthogonal selectivity compared to more traditional HILIC separations. Unlike other commercially available mixed mode stationary phases, ACQUITY PREMIER Glycan BEH C_{18} AX is based on a highly stable ionizable modifier and bonding procedures that make it ideally suited for separating glycans derivatized by MS-enhancing labels with amphiphilic, strongly basic moieties, such as *Rapi*Fluor-MS. Figure 1 highlights the specially designed attributes of this column technology as seen through a separation of RFMS-labeled N-glycans prepared from human IgG and bovine fetuin N-glycan, wherein panels A and B show chromatograms obtained with an ACQUITY PREMIER Glycan BEH C_{18} AX Column and a Glycan BEH Amide Column, respectively. Over 20 RFMS labeled glycans were resolved by both column chemistries. With the mixed mode separation, glycans were grouped by different charged states and retention was seen to increase according to analyte net charge. This mixed mode separation is achieved with a low ionic strength gradient from 0 to 22 mM ammonium formate that affords highly sensitive serial detection by fluorescence and mass spectrometric detection (Figure 1C). Indeed, this analysis strategy could be applied for the detection of glycans below 0.1% relative abundances with an absolute limit of detection within the femtomole level when paired with a Xevo G2-XS QToF.

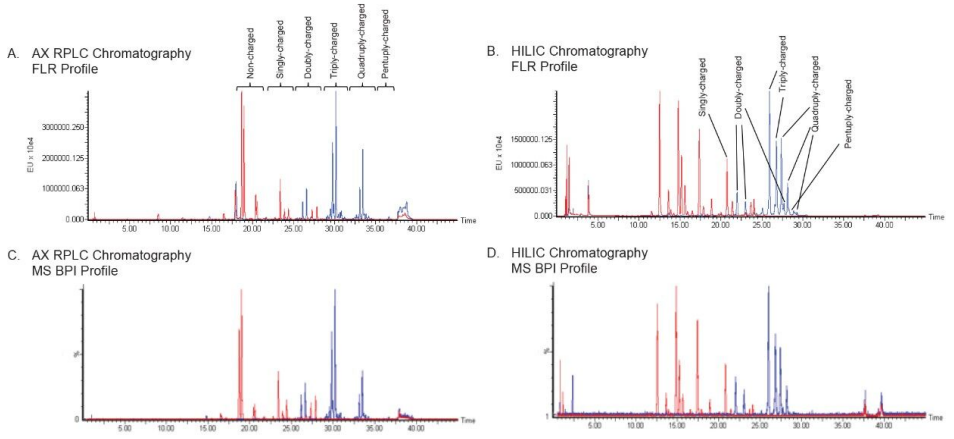


Figure 1. FLR Chromatograms of RFMS-labeled glycans from human IgG (red) and Fetuin (blue) using different LC chromatography. Stainless hardware with High Performance Surface were used to pack all columns. Figure 1 A, C: FLR profiling (A) and MS BPI profiling (C) using an anion exchange reversed phase liquid chromatography (AX RPLC) mixed mode separation with BEH C_{18} AX stationary phase. Figure 1 B, D: FLR profiling (B) and MS BPI profiling (D) using HILIC separation achieved with amide bonded BEH stationary phase.

Additional resolution of isomeric glycan species within the same charged group can be achieved due to the RP properties of the stationary phase. For example, N-acetylneuraminic acid (NeuAc) and N-Glycolylneuraminic acid (NeuGc) are the most prevalent sialic acids found in mammalian cells. Being a non-human form of sialic acid, NeuGc is often considered as a CQA for biotherapeutics since it can be immunogenic and cause the neutralization and rapid clearance of a biopharmaceutical upon dosing in a patient.[5] However, with only one extra oxygen atom in the N-glycolyl group of NeuGc than NeuAc, it is very difficult to separate NeuGc-glycans from NeuAc-glycans using HILIC chromatography. In contrast, the novel Glycan BEH C_{18} AX column successfully resolves NeuGc variants from predominant NeuAc glycan species. Figure 2 presents an analysis of RFMS-labeled N-glycans from bovine fetuin as separated on an ACQUITY PREMIER Glycan BEH C $_{18}$ AX Column using an optimized 45 min LC gradient (details in Experimental section). A 10x zoomed view of the obtained chromatogram (Figure 2B) shows two of the most abundant A2G2S2 species and that they eluted at 26.42 and 26.89 min, with an *m/z* value of 845.69 Da (Figure 2C). Meanwhile, two glycan peaks that eluted around 25.57 and 25.76 min showed *m/z* values of 851.03 Da (Figure 2D), which corresponds to an A2G2S2 species with one NeuGc and one NeuAc. Similar elution patterns were observed for both A3G3S3 and A3S1G3S3 which provide evidence of a NeuGc residue in place of NeuAc in the higher antennary structures too. For mixed mode chromatography, it can be confirmed that N-glycolyl species will elute ahead of their N-acetyl counterparts and that there is a compelling amount of resolution to parse apart individual glycan species within the different net charge states.

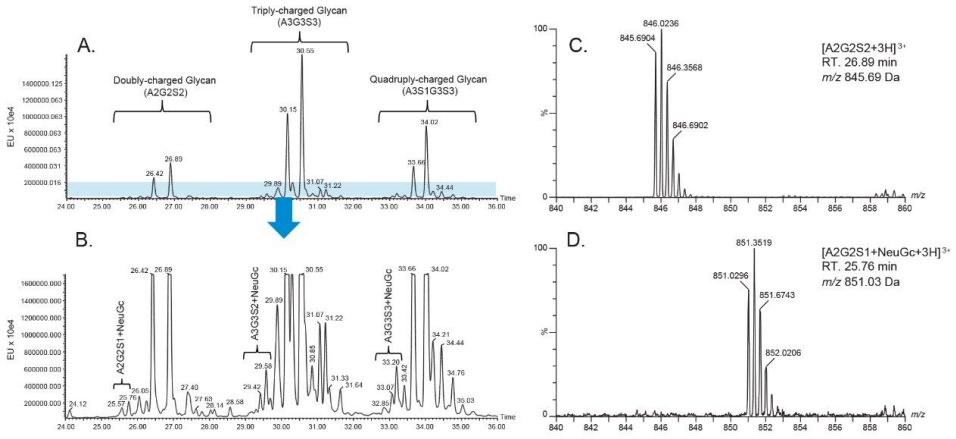


Figure 2. Improved resolution of acidic glycan structure variants with mixed mode separation. (A) FLR profile of RFMS-labeled Glycans from Fetuin. (B) Zoom-in view of (A), with additional separation of NeuGc versus NeuAc Glycan Species. Structural assignment of Glycans were based on MS spectra of each species. (C) MS spectrum of A2G2S2. (S indicates the NeuAc) (D) MS spectrum of A2G2S1+NeuGc.

The ACQUITY PREMIER Glycan BEH C_{18} AX Column has not only proven to be useful for RFMS derivatized glycans but also those labeled with 2-AB. Representative chromatograms from the analysis of both RFMS and 2-AB labeled fetuin N-glycans are provided in Figure 3. Retention and selectivity of the species is evident and tuned to be achieved with relatively low concentrations of ammonium formate. In addition, with the same LC gradient, glycan profiles were found to be quite comparable regardless of which label was used. The weaker basicity of the 2-AB label led to slightly stronger adsorption with the stationary phase and increased retention times, which is consistent with our predictions. Charge-based separation of glycans tagged with weak cationic labels, this novel mixed mode column provided an extra resolution of isomeric glycans conjugated with several different labels, including rapid, strong cationic labels with enhancing MS signal.

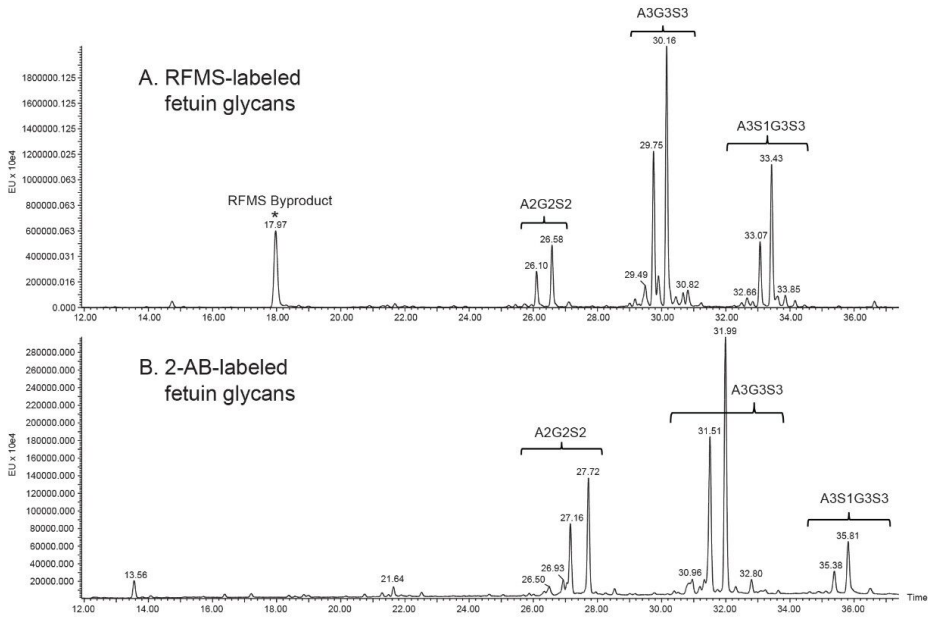


Figure 3. Suitable for both 2-AB and RFMS labeled glycans. (A) FLR profile of RFMS-labeled glycans from fetuin. (B) FLR profile of 2-AB-labeled glycans from fetuin. The asterisk (*) denotes a labeling byproduct peak.

The utility of the ACQUITY PREMIER Glycan BEH C_{18} AX Column technology is best highlighted through a comparison to other commercially available tools. To this point, we have compared its performance to that of a commercially available mixed mode column. Figure 4 presents a separation of 2AB labeled fetuin glycans as produced with an ACQUITY PREMIER Glycan BEH C_{18} AX Column (Figure 4A) versus a commercially available alternative (Figure 4B). Due to the strong retention of highly sialylated glycans with competitor's column, a higher ionic strength gradient from 0 to 30 mM ammonium formate was applied in this study. Even with the added ionic strength change, the ACQUITY PREMIER Glycan BEH C_{18} AX provided much better resolution, as evidenced by a Rs value for A3G3S3 of 3.1 versus 1.1. This testing also showed that an ionic strength greater than 30

mM ammonium formate was required to elute penta-sialylated glycans off the alternative mixed mode column, which is not as desirable for sensitive MS analysis.

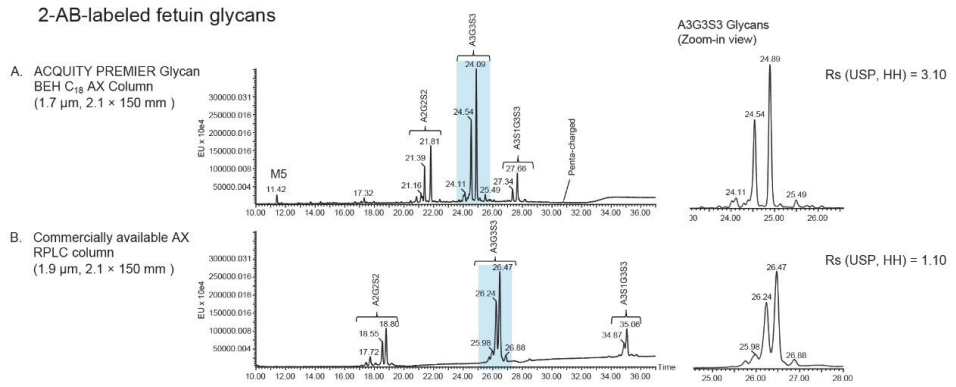


Figure 4. A stationary phase tuned for labeled glycan separations. FLR profiling of 2-AB-labeled fetuin glycans with (A) an ACQUITY PREMIER Glycan BEH C_{18} AX Column, and (B) a commercially available AX RPLC column.

Finally, there is a key aspect to manufacturing of the ACQUITY PREMIER Glycan BEH C_{18} AX Column that warrants attention. It has often been a challenge to analyze acidic due to their propensity to be lost to the metallic surfaces of columns. Where it has become a critical issue, some investigators have resorted to the use of mobile phase conditions that are not MS-friendly, like those containing chelator additives. To more directly address this issue, the ACQUITY PREMIER Glycan BEH C_{18} AX Column hardware has been outfitted with specialized inert surfaces, referred to as MaxPeak High Performance Surfaces. This treatment introduces a barrier layer to prevent acidic glycans from undergoing undesired secondary interactions inside column. In turn, glycan recoveries have been markedly improved and requirements for sample conditioning upon first time use have been eliminated. It is worth mentioning that this HPS technology has also provided breakthrough performance for phosphorylated glycans and that additional work is underway to further explain its significance to the analysis of enzyme replacement therapies for lysosomal storage disorders.

Conclusion

In addition to providing an orthogonal technology to HILIC, the novel ACQUITY PREMIER Glycan BEH C_{18} AX Column provides a charge-based separation for acidic glycans that is uniquely optimized for species derivatized with MS-enhancing labels, like *Rapi*Fluor-MS. The carefully designed anion exchange RP stationary phase contained within this technology is suitable for use with different glycan labels and offers additional resolution for isomeric glycans within the same charge group. With the benefit of High Performance Surface Technology, this column provides improved recoveries and improved first-time use performance compared with other commercially available products, making it more feasible than ever to analyze complicated, yet important, glycan profiles.

References

1. Varki, A. Sialic Acids in Human Health and Disease. *Trends in Mol. Med*. 2008, 14(8):351-360.

2. Bones, J.; Mittermayr, S.; McLoughlin, N.; Hilliard, M.; Wynne, K.; Johnson, G. R.; Grubb, J. H.; Sly W. S.; Rudd, P. M. Identification of N-glycans Displaying Mannose-6-phosphate and Their Site of Attachment on Therapeutic Enzymes for Lysosomal Storage Disorder Treatment. *Anal Chem.* 2011, 83(13), 5344-52.

3. Lauber, M. A.; Yu, Y. Q.; Brousmiche, D. W.; Hua, Z.; Koza, S. M.; Magnelli, P.; Guthrie, E.; Taron, C.H.; Fountain, K. J. Rapid Preparation of Released N-glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection. *Anal Chem*. 2015, 87(10), 5401-9.

4. Lauber, M. A.; Walter, T. H.; DeLano, M.; Jung, M.; Boissel, C.; Gilar, M.; Smith, K.; Birdsall, R.; McDonald, T.; MacLean, J.; Nguyen, J.; Donegan, M.; Lame, M.; Rainville, P.; Belanger, J.; Wyndham, K. Waters Application Note, 720006930EN, 2020.

5. Nguyen, D. H.; Tangvoranuntakul, P.; Varki, A. Effects of Natural Human Antibodies Against a Nonhuman Sialic Acid that Metabolically Incorporates into Activated and Malignant Immune Cells. *J. Immunol.* 2005, 175(1), 228-36.

Featured Products

ACQUITY UPLC H-Class PLUS Bio System

Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry

UNIFI Scientific Information System

MassLynx MS Software

720007038, October 2020