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Nota de aplicación

Released Glycan Analysis of Erythropoietin Using the ACQUITY Premier Glycan BEH C $_{18}$ AX Column and BioAccord System with ACQUITY Premier

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Abstract

The abundance and structure of highly sialylated glycans can factor into the clearance rate and *in-vivo* activity of many glycosylated biotherapeutics. Given this, accurate analytical characterization and monitoring methods are required to ensure drug product quality. For released glycan characterization, Hydrophilic Interaction Chromatography (HILIC) with fluorescence and mass spectrometry detection is widely used. However, the abundance, heterogeneity, and various isomeric forms of highly sialylated glycans limit the chromatographic resolving power when using traditional HILIC-based separations. In this work, we demonstrate how analyte charge can be leveraged to improve the chromatographic selectivity and detection of fluorescently tagged highly sialylated glycans by using mixed mode chromatography within the integrated LC-FLR-MS BioAccord System with ACQUITY Premier.

Benefits

The BioAccord System with ACQUITY Premier and the mixed-mode ACQUITY Premier Glycan BEH C_{18} AX Column offers:

- Improved chromatographic resolution for highly sialylated, highly complex glycans

- Charge-based glycan profile for highly sialylated glycans improved compared to traditional HILIC methods
- Improved sensitivity for highly sialylated glycans using FLR and MS detection
- Fragmentation capability of the BioAccord System provides additional glycan structural information for assignments

Introduction

Protein glycosylation plays an important role in the safety and efficacy of biotherapeutics. Glycans attached to the protein can directly impact the clearance rate and immune response of the drug product.[1] Given the role glycosylation plays, and that more than 50% of commercialized protein-based biotherapeutics are glycosylated, considerable effort has been given in the development of methods for the analysis and monitoring of glycan modified proteins. Of these methods, those that rely on release and labeling approaches continue to dominate due to the resolving power and sensitivity afforded by these methods. However, glycoproteins such as EPO (Erythropoietin) which contain highly branched and sialylated structures continue to be challenging for established methods due to the structural complexity and heterogeneity in glycans present on these types of samples.

The traditional labeled released glycan HILIC method that is widely used for mAb glycan analysis does not have enough selectivity and effective chromatographic resolving power to provide sufficient separation for the most complicated EPO N-glycans. Current practices use a weak anion exchange technique first to fractionate fluorescently labeled glycans, followed by a HILIC separation of each isolated fraction.[2] The ion exchange fractionation is primarily charge based since sialic acid contains a negative charge under physiological pH, similar to a phosphate moiety. The two-stage separation method not only takes more time to develop, but also costs more per sample to analyze. Mixed mode chromatography (IEX and RP) has been used to improve the peak resolution for highly sialylated glycans for 2AB labeled glycans, however, such methods are mainly coupled with FLR detection due to low MS response.[3]

Recently, Waters introduced the Premier brand mixed mode column, the ACQUITY Premier Glycan BEH C_{18} AX Column, featuring MaxPeak High Performance Surfaces (HPS) Technology, which has been proven to reduce analyte/surface interaction of metal sensitive analytes via a stable barrier layer.[4] This column, in conjunction with Waters established *Rapi*Fluor-MS (RFMS) glycan labeling technology for improved FLR-MS sensitivity, offers the potential to address the challenges associated with the analysis of highly branched and sialylated glycoproteins.

In this study, we evaluate a method developed using the ACQUITY Premier C_{18} AX Column and its ability to analyze RFMS-labeled sialylated structures. In addition to RFMS labeling technology and the ACQUITY Premier C

$_{18}$ AX Column, mass confirmation of the labeled glycans and their fragments will be performed using a BioAccord System with the ACQUITY System (Figure 1). This configuration is comprised of an ACQUITY Premier LC System, for optimal chromatographic performance of metal sensitive analytes such as acidic glycans, in line with mass detection performed using the ACQUITY RDa Mass Detector. When operated in Fragmentation mode, the system can generate alternating spectra containing both precursor and fragment ion data, a form of data independent acquisition (DIA) that provides mass compositional data for the full length glycan and related fragments, to further assist with glycan structural assignments.

Figure 1. BioAccord System with ACQUITY Premier, and the ACQUITY Premier Glycan BEH C_{18} AX Column, standards and mobile phase concentrates. The system is controlled, and data processed and managed by the compliance ready waters_connect informatics platform.

To document the performance of the system described above, this application note compares the data from mixed-mode C_{18} AX LC-FLR-MS to the HILIC-FLR-MS for EPO released N-glycan analysis. Both column chemistries utilized MaxPeak HPS Technology to minimize interaction of labeled glycans with metal surfaces. Different selectivity and peak resolution of these chemistries are illustrated and discussed, for direct application to human EPO sample characterization.

Experimental

Sample Description

- *Rapi*Fluor-MS (RFMS) 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.
- Erythropoietin (EPO) was purchased from European Pharmacopeia (reference material E1515000) and buffer exchanged to Milli-Q water prior to glycan release. Additional EPO samples were purchased from StemCell Inc. and reconstituted to a concentration at 1.5 mg/mL (p/n: 78007). Ammonium acetate was purchased from Sigma-Aldrich. N-glycans were released from 15 µg of EPO and labeled using the GlycoWorks RFMS 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 RFMS Quick Start protocol (720005470EN < https://www.waters.com/webassets/cms/library/docs/720005470en.pdf>). Please note that a quenching step is added to the Quick Start protocol: *after 5 minutes labeling, quench the excessed RFMS reagent by adding 5 µL of 1 M ammonium acetate solution to the sample mixture. Allow the quench reaction to proceed at room temperature for 5 minutes.*

LC Conditions (ACQUITY Premier)

System:	BioAccord System with ACQUITY Premier
LC system:	ACQUITY Premier BSM UPLC
Detection:	ACQUITY Premier FLR Detector ($\lambda_{excitation}$=265 nm, $\lambda_{emission}$=425 nm, 2 Hz)
Column(s):	1. Mixed mode: ACQUITY Premier Glycan BEH C $_{18}$ AX Column, 1.7 µm, 95 Å, 2.1 × 150 mm (p/n: 186009760) 2. HILIC mode: ACQUITY Premier Glycan Amide Column, 1.7 µm, 130 Å, 2.1 × 150 mm (p/n: 186009524)

Vials:	QuanRecovery with MaxPeak HPS 300 µL vials (p/n: 186009186)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection amount:	1 µL
Mobile phase A:	Mixed mode: Milli-Q H_2O HILIC mode: Milli-Q H_2O with 50 mM NH_4HCO_2*
Mobile phase B:	Mixed mode: 100 mM ammonium formate, 100 mM formic acid in 40/60 (v/v) water/acetonitrile** HILIC mode: LC-MS grade acetonitrile

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**: mobile phase A in HILIC mode was prepared by diluting 10 mL ammonium formate concentrate (pH=4.4, p/n: 186007081 <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186007081-waters-ammonium-formate-solution--glycan-analysis.html>) to 50 mM concentration in 1 L water.*

***: mobile phase B in mixed mode was prepared by diluting 100 mL IonHance Glycan C_{18} AX ammonium formate concentrate (p/n: 186009762 <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009762-ionhance-glycan-c18-ax-ammonium-formate-concentrate.html>) to 100 mM concentration in 320 mL Milli-Q water and 580 mL LC-MS grade acetonitrile.*

Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.4	95	5	6
40.00	0.4	77	23	6
40.30	0.4	0	100	6
41.30	0.4	0	100	6
42.00	0.4	95	5	6
49.00	0.4	95	5	6

Mixed mode

Gradient

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

HILIC mode

MS Conditions (RDa Detector)

MS system:	ACQUITY RDa Mass Detector (Figure 1)
Ionization mode:	ESI+
Acquisition range:	50–2,000 *m/z*
Capillary voltage:	1.5 kV
Cone voltage (CV):	45 V
Fragmentation CV:	70–90 V

Data Management

Informatics:	waters_connect with UNIFI v1.9.4

Results and Discussion

HILIC vs Mixed-mode Chromatography

A HILIC FLR or FLR-MS method is generically used for labeled mAb released N-glycan separations, as these N-glycans are generally comprised of biantennary neutral species with less than 5% of associated glycans being mono or di-sialylated. When comparing the HILIC and the new C_{18} AX Column performance, a clear selectivity difference was observed for N-glycans released from a humanized IgG mAb (Figure 2). The HILIC column was observed to separate glycans primarily by hydrophilicity, while the elution profile from the C_{18} AX Column showed a distinct charge-based primary separation of neutral, singly, and doubly sialylated glycans. Within each charge group, the more hydrophilic glycans eluted slightly earlier than glycans with similar branch structure (hydrophobicity driven), for example, FA2G1 elutes earlier than FA2. It was also observed that the Man5 glycan is more hydrophilic compared to a complex type glycan with similar molecular weight. This was noted in Figure 2 where the Man5 (M5) peak elutes earlier than A2 glycan in the C_{18} AX chromatogram, while the opposite elution order was observed using the HILIC Column (Man5 elutes later than A2). In addition to observed differences in selectivity, the peaks from the

C_{18} AX separation are narrower than those from the HILIC separation (~20% on average), suggesting higher peak capacity can be reached with the mixed-mode column.

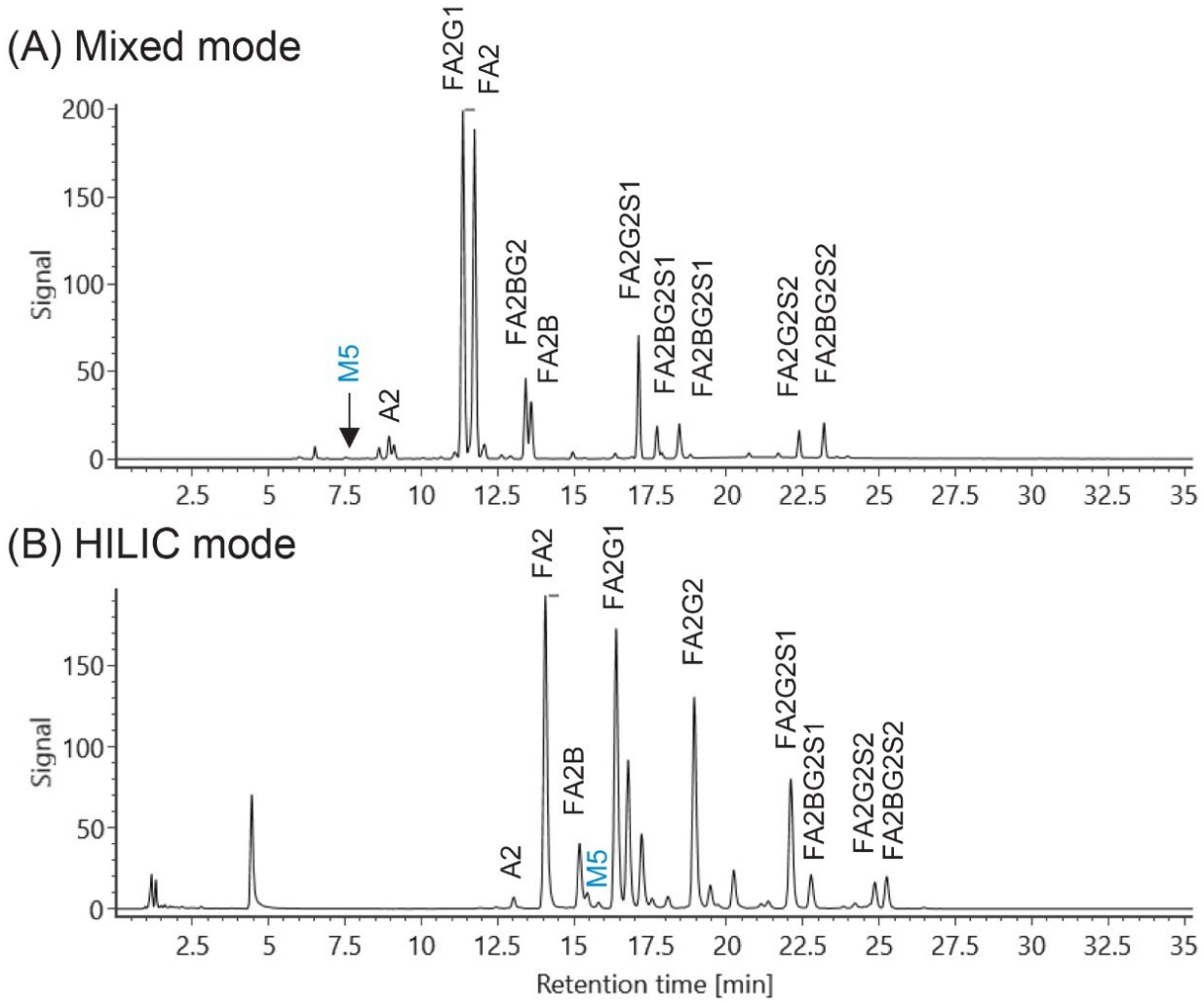

Figure 2. Change in selectivity when comparing (A) mixed mode (C_{18}/AX) separation and (B) HILIC mode separation. Sample: Waters glycan performance test standard with the RapiFluor-MS label. Both the mixed mode and the HILIC separations show that Man 5 (M5) is more hydrophilic than the A2 (or G0) glycan.

Broader applicability of the C_{18} AX Column was evaluated using human EPO as an exemplar molecule with a complex glycosylation profile, one containing many highly sialylated species. In this instance, HILIC offered limited resolving power with “crowded” separation of glycans exhibiting highly branched structures with various level of sialic acids. In contrast, the C_{18} AX Column was able to increase chromatographic separation of the same structures, given its ability to resolve glycan species with incremental sialic acids (Figure 3). Again, it was observed that the peak width from the C_{18} AX chromatographic separation is narrower compared to the peaks from

the HILIC separation, indicating the mixed-mode column has the potential to increase chromatographic performance in terms of higher peak capacity and column efficiency for highly sialylated samples. Using an optimized method, a total of 82 fluorescently labeled N-glycans with unique mass from EPO were observed using the C_{18} AX Column. The glycans loaded on column was released from 1 µg of EPO protein by estimation.

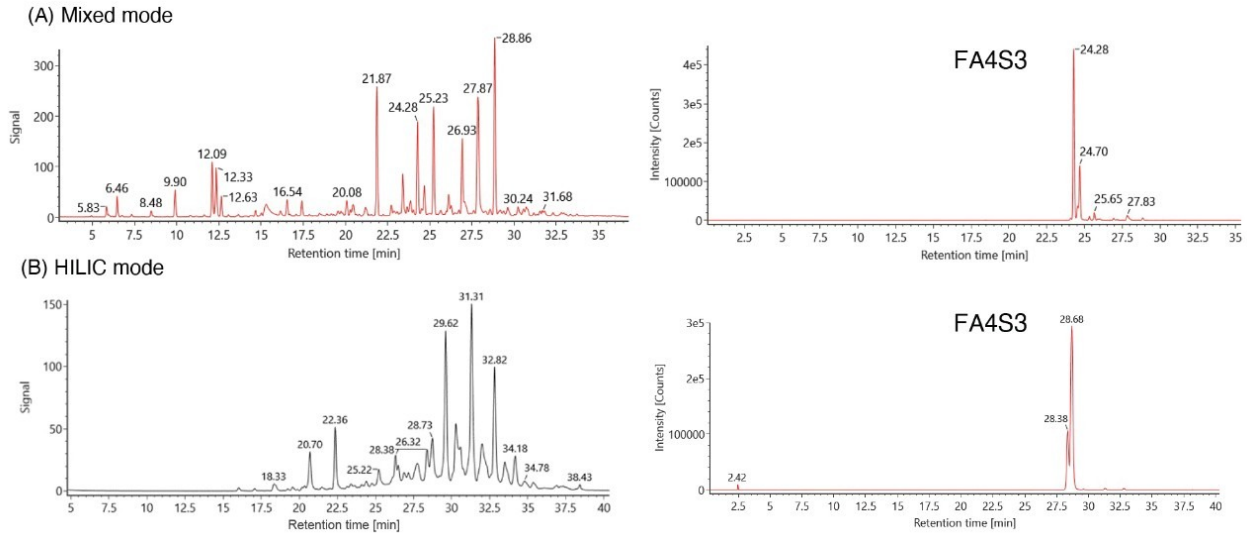

Figure 3. Comparison of EPO released glycan analyses using (A) HILIC mode and (B) mixed mode separation. The charge based mixed mode separation provides higher resolution for isomeric sialylated glycans. One example is the separation of fucosylated tetra-antennary glycans bearing 3 sialic acids, FA4S3.

An added benefit of the RFMS labeling technology used in this method was the observed increase in MS response. In this instance, the mass spectrometry signals enabled mass confirmation of glycans as low as 0.1% (relative concentration), using only 15 micrograms of EPO as starting material for the complete sample preparation process (denaturing, deglycosylation and labeling with RFMS tag). The extracted ion chromatograms (XICs) of glycan FA4S3 (contains one fucose, four antennary branches and three sialic acids) was used to further investigate column selectivity and resolution differences between HILIC and C_{18} AX. As shown in Figure 3, (right-side panel) both XICs showed two well separated peaks, presumably structural isomers, however, only the C_{18} AX Column exhibited near baseline separation of the two isomers.

The charged-resolved profile of the RFMS labeled EPO N-glycans separated using the mixed mode column is shown in Figure 4A where groups of glycans were separated based on their number of sialic acids. Interestingly, high mannose phosphate glycans eluted before the complex glycans that were similar in size and number of charges. This was presumably driven by their increased hydrophilicities.

The increased chromatographic resolution afforded by the enhanced selectivity was also observed to directly simplify

the interpretation of data independent MS fragmentation spectra obtained using the BioAccord System in Fragmentation Mode. An example (Figure 4B) where fragment ion data exhibited a series of consecutive neutral monosaccharide losses that supported the proposed glycan composition based assignment as an M7P2 glycan (a high mannose type with 2 phosphates).

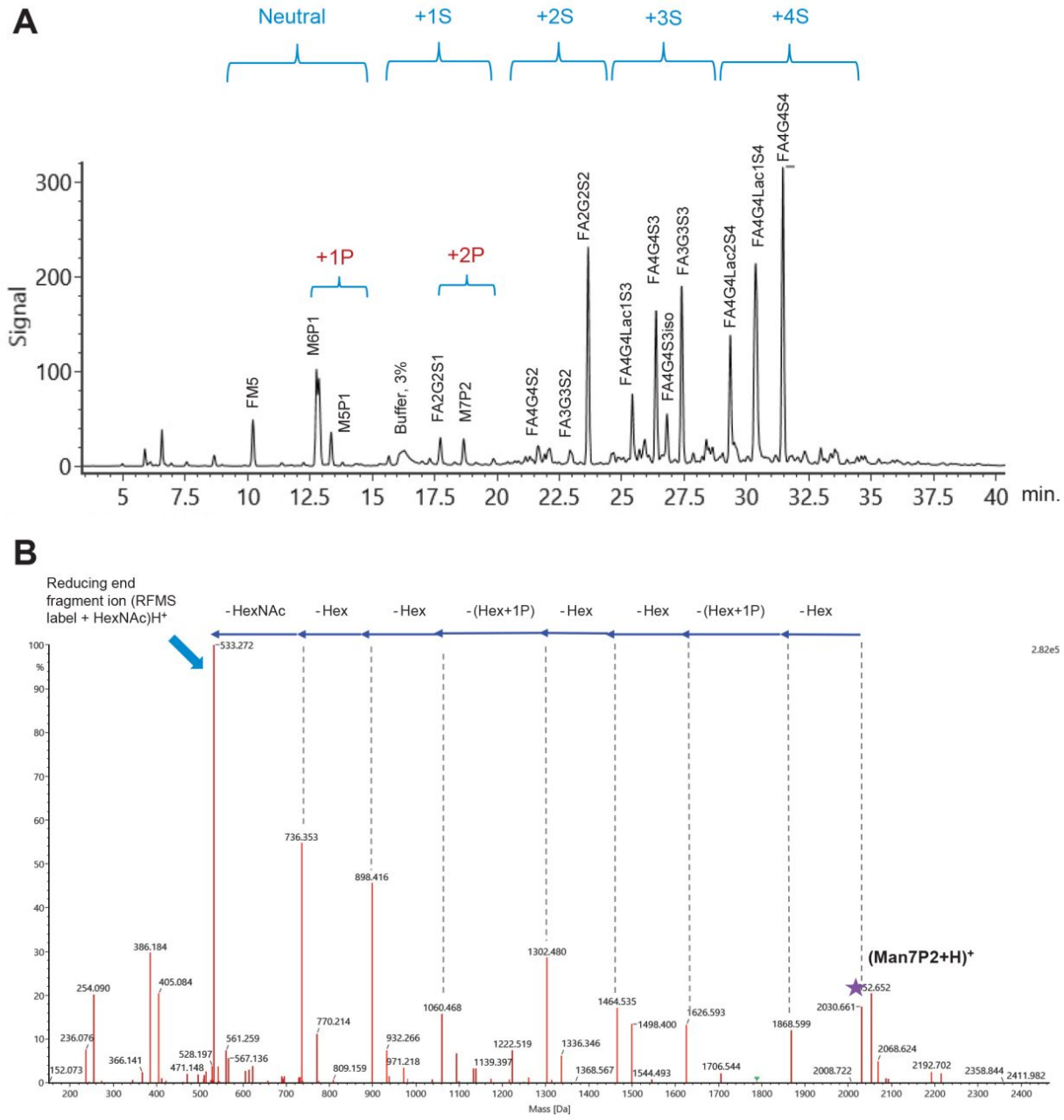

Figure 4A. C_{18}/AX separation of EPO N-glycans. Distinct charge-based separation is observed for charges = 0-4. Within each charge grouping, glycan structures are further separated based on their hydrophobicity. Note that high mannose phosphate glycans have earlier elution time compared to complex type glycans of equivalent charge.
B. The BioAccord System can alternately generate spectra of low energy (precursor) ions and higher energy (fragment) ions. Data is shown for fragmentation of the high- mannose doubly phosphorylated M7P2 glycan. Consecutive neutral losses from monosaccharides are annotated to confirm the glycan composition. The star labels the $(M+H)^+$ precursor ion in the MaxEnt 3 charge deconvolved spectrum.

The optimized C_{18} AX LC Method was applied to perform comparability analysis using glycans derived from multiple sources of human EPO (Figure 5). The charge profile remained consistent for the two N-glycan samples, suggesting this developed method can be generally used for comparative charge-based profiling of complex glycoprotein samples.

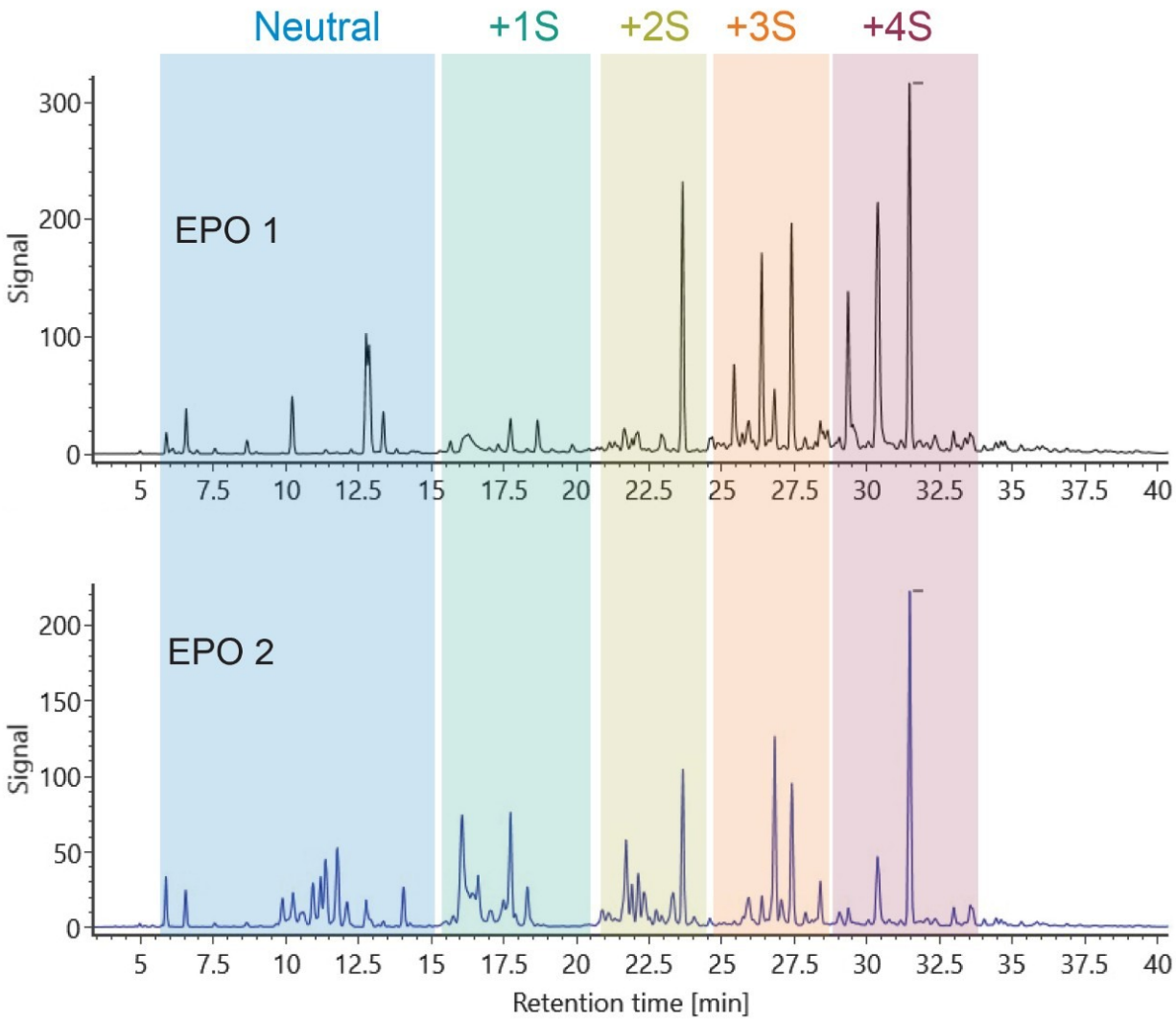

Figure 5. Optimized C_{18}/AX LC method for the analysis of EPO from two difference sources.

Conclusion

An LC-FLR-MS method was optimized on the BioAccord System with ACQUITY Premier LC fitted with an

ACQUITY Premier Glycan C_{18} AX Column. Compared to a traditional HILIC-based method, the new mixed-mode C_{18} AX Column offered more resolving power for complex N-glycans with higher levels of sialic acid. The optimized LC-FLR-MS method enabled distinct charge-based separation and compositional assignments based on the number of sialic acids and mass data from the BioAccord System. More glycan structures were further resolved based on their hydrophilicity within the retention time window for the same charge (based on number of sialic acid). In summary, the combination of MaxPeak HPS Technology and the SmartMS functionality of the BioAccord LC-MS System enables the generation of high-quality data with respect to peak shape, resolution, and MS sensitivity in the analysis of highly sialylated complex N-glycans.

References

1. Zhang P. *et al.* Challenges of Glycosylation Analysis and Control: An Integrated Approach to Producing Optimal and Consistent Therapeutic Drugs. *Drug Discovery Today*, Volume 21, 2016; 5, 740–765.
2. Bones J. *et al*. 2D-LC Analysis of BRP 3 Erythropoietin N-glycosylation Using Anion Exchange Fractionation and Hydrophilic Interaction UPLC Reveals Long poly-N-acetyl Lactosamine Extensions. *Anal. Chem*. 2011 June; 83(11):4154–4162.
3. Udayanath A. *et al*. Separation of 2AB labeled N-glycans from Bovine Fetuin on a Novel Mixed-Mode Stationary Phase. Thermo Fisher Scientific Application Note 2075A.
4. Liu X. *et al.* Increased Resolving Power for Acidic Glycans with an MS-Compatible Anion Exchange Reversed-Phase Separation. Waters Application Note, 720007038, 2020.

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