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Improved Hydrophilic Interaction Liquid Chromatography for LC/FLD/MS Analysis of Released N-Glycans

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Abstract

Glycosylation directly impacts protein functionality and is frequently monitored as a critical quality attribute (CQA) or product quality attribute of biotherapeutics. Consistency in glycan profiles helps to ensure that the stability, safety, and serum half-life of biotherapeutic proteins are controlled. Therefore, it is essential that robust analytical tools and methods for profiling glycans are available to ensure the success of these therapeutics. Released N-glycans are frequently separated using hydrophilic interaction chromatography (HILIC). In this application note, the N-glycans of several monoclonal antibodies were profiled with the new Agilent AdvanceBio Amide HILIC column, which has been developed specifically for high-performance glycan separations. This column is designed to exhibit increased selectivity between glycans of differing charges, reducing the occurrence of glycan coelution in complex samples. This study shows that this novel column provides excellent peak shape and resolution for a wide diversity of glycan structures, including those that are common antibody-derived glycans. The column also enables diverse glycan mixtures containing sialylated species to be thoroughly profiled within one hour, providing performance that is highly competitive with other leading columns on the market while enhancing the ability to separate glycans of differing charge.

Introduction

Glycosylation is often a CQA for the development of biotherapeutic proteins, as the structure of N-linked glycans can strongly influence protein function.[1] Characterization of N-glycans is commonly performed using enzymatic release and labeling of N-glycans with a signal-enhancing tag, followed by LC/MS data collection and interpretation.[2] HILIC is the predominant chromatographic technique used for N-glycan analysis, normally with detection by fluorescence and/or mass spectrometry (MS).

Although HILIC separations are routinely applied to N-glycan analysis, technical challenges still arise when profiling proteins with complex N-glycan profiles, such as coelution of critical glycan pairs. Particularly when using a fluorescence detector (FLD), glycan coelution hampers an analyst's ability to conduct relative glycan quantitation, and may only be addressed by substantially lengthening the HILIC gradient time.

To address these and other common issues with HILIC separation of N-glycans, Agilent has developed the AdvanceBio Amide HILIC column—a novel HILIC stationary phase with enhanced charge-group selectivity. By allowing the user to independently modulate both HILIC retention and charge-group overlap, this column provides increased flexibility with method development, and enables analysts to reduce the occurrence of coeluting glycan peaks. The column has been released concurrently with the new Agilent AdvanceBio Ammonium Formate Mobile Phase concentrate, a HILIC mobile phase concentrate that can be easily diluted to produce ready-to-use HILIC mobile phase buffer with excellent batch-to-batch consistency.

Experimental

N-glycan sample preparation

Glycans were released from the glycoproteins cetuximab (Erbitux, lot number M160886), Agilent-NISTmAb reference standard, (part number 5191-5745, lot number 0006732507), and rituximab (Rituxan, lot number M120333), labeled with Agilent InstantPC dye, and cleaned up before analysis using the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit (part number GX96-IPC).

Instrumentation

Sample analysis was performed on an Agilent 1290 Infinity II LC system consisting of the following components:

- Agilent 1290 Infinity II Bio multisampler (G7137A)
- Agilent 1290 Infinity II Bio high-speed pump (G7132A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 fluorescence detector spectra (G7121B) with bio-inert FLD flow cell (part number G5615-60005)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549A)

The LC system was equipped with an Agilent bio ultralow dispersion kit (part number 5004-0007). LC separation was performed on the Agilent AdvanceBio Amide HILIC column (2.1 × 150 mm, 1.8 μm, part number 859750-913). The mobile phase buffer was prepared using the Agilent AdvanceBio Ammonium Formate HILIC Mobile Phase concentrate (part number G3912-00000) by adding a single bottle of concentrate (10 mL) to 1 L of MS-grade water. To reduce FLD cell backpressure, the LC flow was split just downstream of the cell (waste/MS) using a tee splitter. Other relevant method parameters are provided in Tables 1 and 2.

Software

The software used in this study was as follows:

- Agilent MassHunter Acquisition software, version 11.0
- Agilent MassHunter Qualitative Analysis software, version 10.0

Table 1. LC/FLD conditions for Agilent InstantPC-labeled released glycan analysis.

Parameter	Value	
Column	Agilent AdvanceBio Amide HILIC, 2.1 × 150 mm, 1.8 μm (p/n 859750-913)	Waters Premier Glycan BEH Amide, 2.1 × 150 mm, 1.7 μm (p/n 186009524)
Column Temperature	60 °C	
Mobile Phases	A) 50 mM ammonium formate, pH 4.4, prepared from Agilent AdvanceBio Ammonium Formate HILIC Mobile Phase concentrate (p/n G3912-00000) B) Acetonitrile	
Flow Rate	0.6 mL/min	
Gradient Program	Time (min) %B 0 77 45 56 46 40 47 40 49 77 60 77	Time (min) %B 0 73 45 60 46 40 47 40 49 73 60 73
Injection Volume	1 μL	
Detection	λ_{Ex} 285 nm, λ_{Em} 345 nm	

Table 2. Agilent 6545XT AdvanceBio LC/Q-TOF parameters.

Parameter	Value
Source	Dual Agilent Jet Stream Electrospray ionization
Drying Gas Temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	10 L/min
Capillary Voltage	2,500 V
Nozzle Voltage	500 V
Data Acquisition Mode	2 GHz (extended dynamic range) Standard mass range (m/z 3,200)

Results and discussion

Profiling the less complex glycan mixtures derived from the NISTmAb (humanized IgG1κ monoclonal antibody) and rituximab (Figure 1) demonstrates that the new HILIC stationary phase is adept at resolving N-glycans that are most common to therapeutic antibodies, including G0F, Man5, G1[6]F, G1[3]F, and G2F. These neutral glycans were separated in approximately 15 minutes with excellent peak shape and resolution. The LC gradient was developed to suit a wide variety of sample types, including those with more complex glycan profiles, as described below. However, in instances where samples of low complexity are exclusively analyzed, a steeper gradient slope can be implemented to shorten analysis time while still resolving the major glycan species. The gradient shown in Table 1 also produces one coelution among minor NISTmAb glycan species (G2FGa2 and H5N3S1), which can be resolved with a steeper gradient.
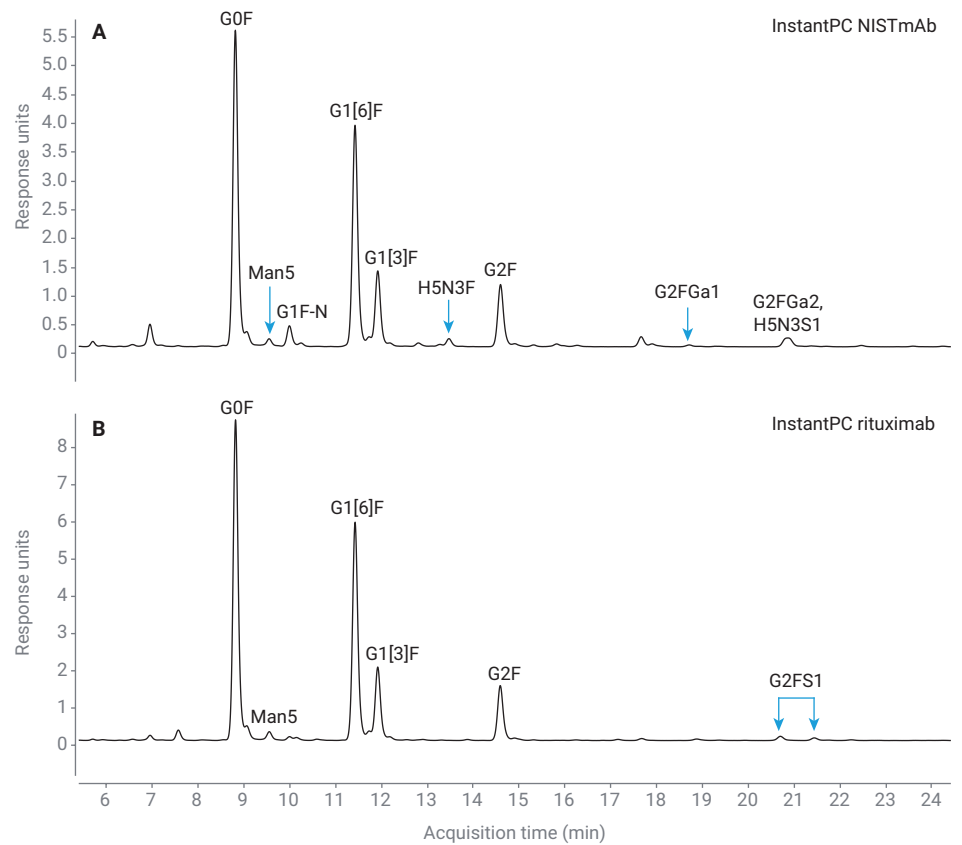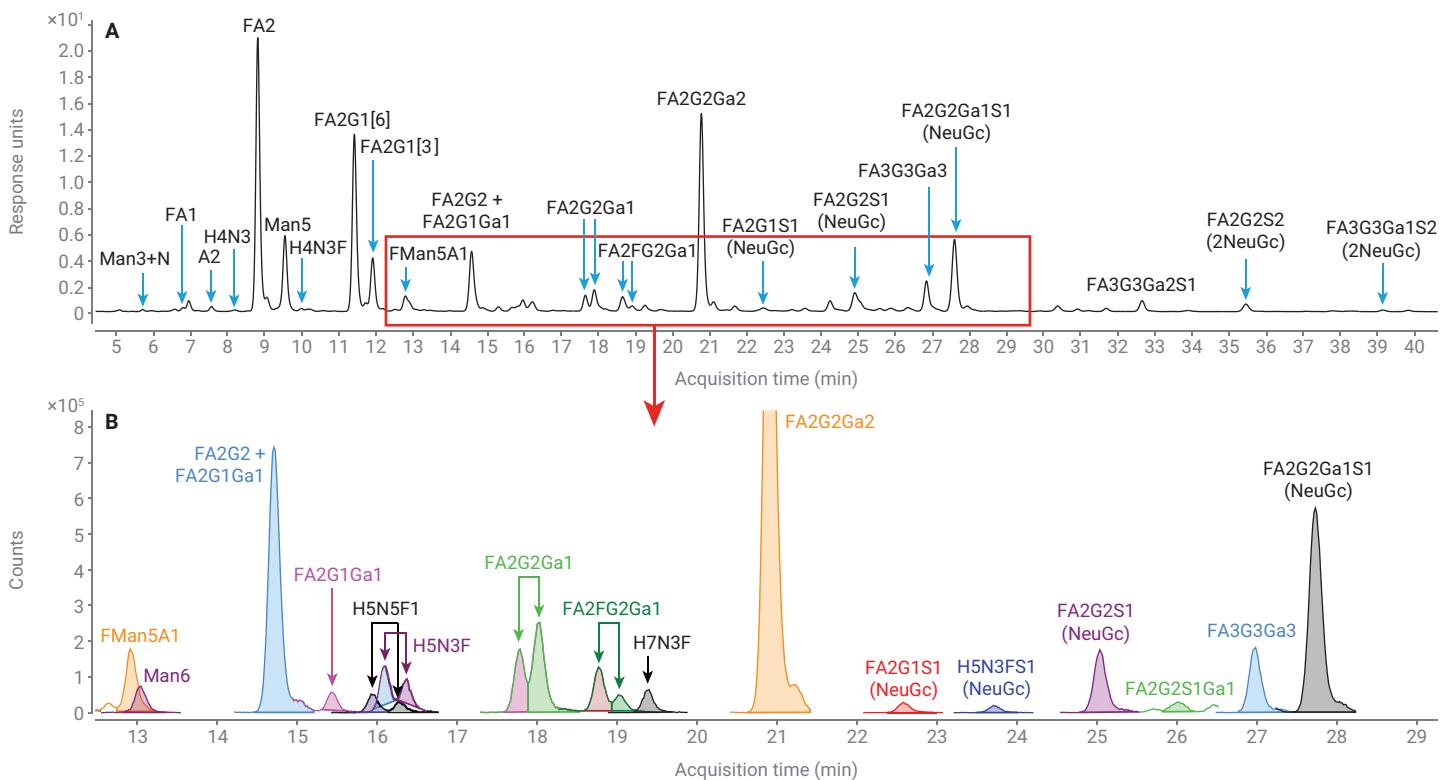

Figure 1. LC/FLD chromatograms of InstantPC labeled N-glycans from the NISTmAb and rituximab.

When profiling complex glycan mixtures, it is often challenging to consistently and completely resolve closely eluting glycan pairs, particularly in the absence of MS data. The AdvanceBio Amide HILIC column provides enhanced selectivity between glycans of differing charges, enabling users to resolve these critical glycan pairs more easily. Furthermore, peak shape and capacity have been dramatically improved over the legacy Agilent AdvanceBio Glycan Mapping column line (data not shown). Figure 2 shows the chromatographic profile of InstantPC-labeled cetuximab.

This antibody has a diverse glycan profile, owing to glycosylation being present on both Fc and Fab regions, including sialylated species with N-glycolylneuraminic acid (NeuGc or NGNA) as well as bi- and triantennary glycans with α1,3-linked galactose residues. Despite the sample's complexity, it was possible to collect a comprehensive glycan profile of cetuximab within a 40-minute retention time window using fluorescence detection alone (Figure 2A). The fluorescence trace shows numerous well-resolved peaks for glycans

containing the nonhuman Galα1-3Gal and NeuGc epitopes, which often require careful monitoring. If MS data are collected, peak areas for less abundant glycans can be calculated through extracted compound chromatograms (Figure 2B). Furthermore, tandem-MS data can provide more structural details that assist in characterizing uncommon or novel glycan peaks. For example, the y_4 and $y_4\alpha$ fragments of the glycan FA2G2Ga2, shown in Figure 3, provide convincing evidence of the Gal-Gal-GlcNAc sequence on the opposite/nonfragmented antenna.

Figure 2. LC/FLD chromatogram of InstantPC-labeled cetuximab N-glycans (A). Extracted compound chromatograms produced from MS data reveal additional glycan peaks not evident in the fluorescence trace (B).
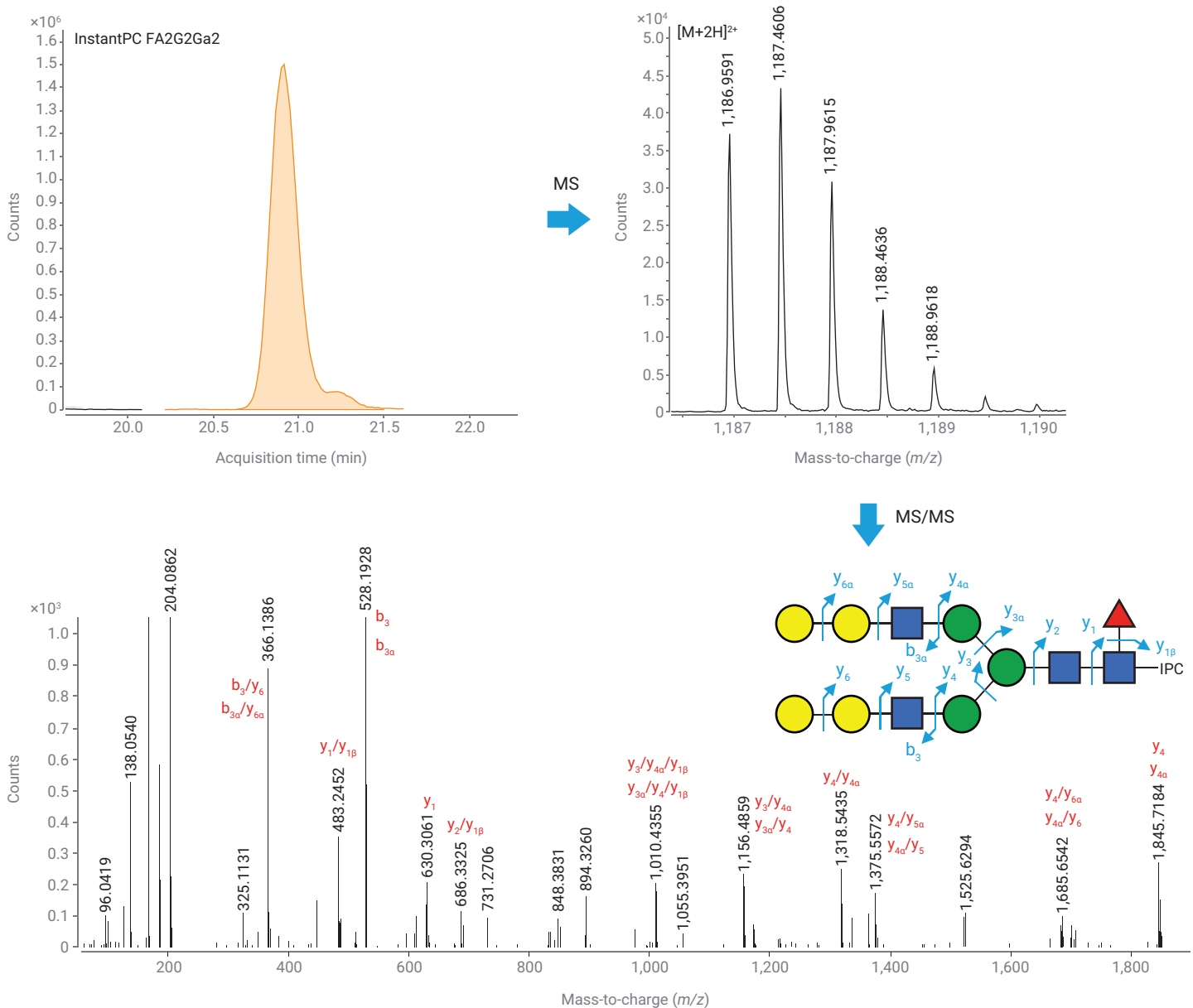
Figure 3. Extracted compound chromatogram, MS scan, and MS/MS scan of InstantPC FA2G2Ga2.

The benefit of the enhanced ability of the AdvanceBio Amide HILIC column to separate glycans of differing charges is well illustrated in a head-to-head comparison of cetuximab chromatographic profiles. In Figure 4, a gradient was developed on the Waters Premier Glycan BEH Amide column (2.1 × 150 mm, 1.7 µm, part number 186009524) that closely matches the retention time window used on the AdvanceBio Amide HILIC column. While both columns give competitive separations, the competitor column shows two instances of overlap between neutral and singly sialylated species. Even when using MS-derived chromatograms, the FA2G2S1(NeuGc)/ FA2G2Ga2 pair can be difficult to distinguish because the $[M+H+NH_4]^{2+}$ ion of FA2G2S1 differs from the doubly protonated adduct of FA2G2Ga2 by only 5 ppm. While ammonium adducts are generally not a major species, this similarity could confound peak area calculations when glycans are identified through automated search algorithms that consider multiple charge adducts.
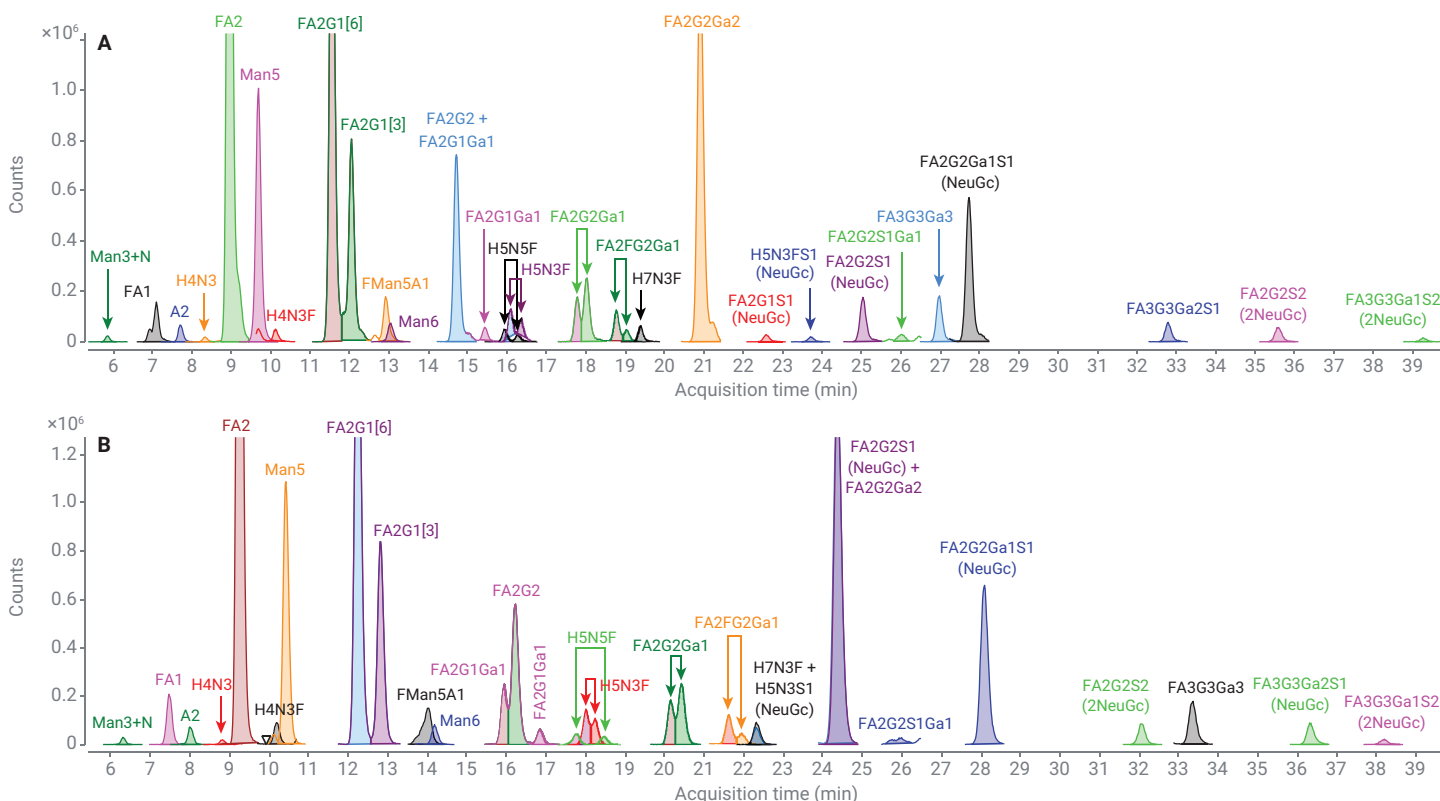
Figure 4. InstantPC cetuximab separations on an Agilent AdvanceBio Amide HILIC (A) and a Waters Premier BEH Amide (B).

Conclusion

The Agilent AdvanceBio Amide HILIC column presented in this application note is a powerful tool for generating high-quality glycan data for both simple and complex N-glycan profiles. The increased charge-group selectivity provides avenues to eliminate the most troublesome coelutions that are commonly seen with HILIC separations of labeled N-glycans.

Excellent temperature stability, longevity, peak shape, and reproducibility mean that this column is an effective option for users looking to separate N-glycans from a wide variety of biotherapeutic glycoprotein samples. Use of the Agilent AdvanceBio Ammonium Formate Mobile Phase concentrate further reduces variability and reduces preparation time for LC separations.

References

1. Delobel, A. Glycosylation of Therapeutic Proteins: A Critical Quality Attribute BT - Mass Spectrometry of Glycoproteins: Methods and Protocols; Delobel, A., Ed.; Springer US: New York, NY, **2021**; pp 1–21. https://doi.org/10.1007/978-1-0716-1241-5_1.

2. Zhang, X.; Vimalraj, V.; Patel, M. Routine Analysis of N-Glycans Using Liquid Chromatography Coupled to Routine Mass Detection BT - Mass Spectrometry of Glycoproteins: Methods and Protocols; Delobel, A., Ed.; Springer US: New York, NY, **2021**; pp 205–219. https://doi.org/10.1007/978-1-0716-1241-5_15.

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