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

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Introduction

Glycosylation is one of the most common post-translational modifications found on therapeutic proteins, and the presence and composition of these glycans can profoundly affect protein structure, functionality, and efficacy.[1] As such, the glycan profiles of therapeutics are frequently denoted as critical quality attributes,[2] and robust analytical tools are essential to characterize and monitor glycan profiles.

Hydrophilic interaction liquid chromatography (HILIC) is the predominant LC technique for asparagine-linked (N-linked) glycan analysis, typically connected to to a fluorescence detector or a mass spectrometer. Following enzymatic release from the protein backbone, glycans are commonly derivatized to impart fluorescence or enhanced MS sensitivity prior to chromatographic separation.

Technical issues often encountered in glycan analysis include co-elution of critical glycan pairs, loss of peak shape due to undesirable metal-glycan interactions in the LC flow path, and the need for a volatile mobile phase buffer which is compatible with mass spectrometry yet still facilitates high chromatographic resolution and selectivity. To address these issues, we have developed a novel HILIC stationary phase with enhanced charge group selectivity, and we have shown that this selectivity can be modulated by adjusting mobile phase buffer concentration within an MS-compatible window. Further, we have tested the column with an MS-compatible deactivator additive, which passivates metal sites within the LC flow path, thus preventing peak shape deterioration for acidic glycans without impacting MS data quality.

Experimental

Glycan profiling of therapeutic antibodies.

InstantPC-labeled samples and glycan standards were analyzed with the Agilent 6545XT AdvanceBio LC/Q-TOF, equipped with a 1290 Infinity II Bio LC stack, as detailed below.

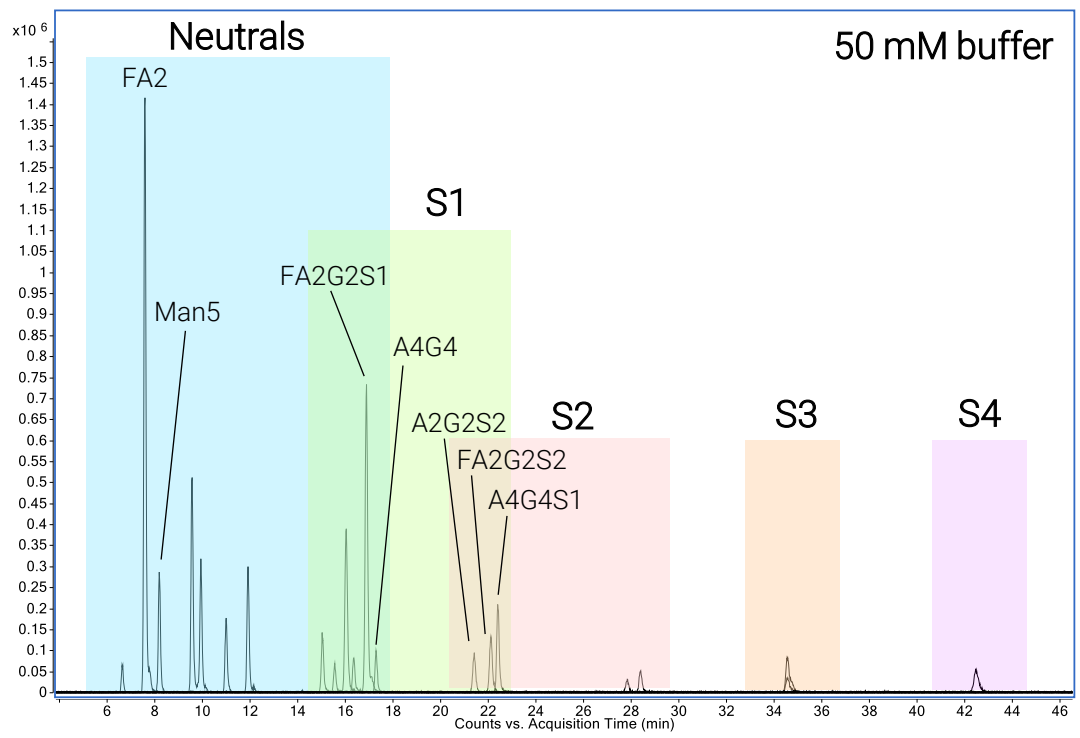
6545XT AdvanceBio LC-MS Conditions	
Column	Prototype Amide HILIC, 2.1 x 150 mm, 1.8 µm
Column Temperature	60 °C
Mobile phase	A = 50 mM ammonium formate, pH 4.4 B = Acetonitrile
Flow rate	0.6 mL/min
Gradient program	Time (min) %B 0 77 45 48 46 40 47 40 49 77 60 77
Drying gas temp.	150 °C
Drying gas flow	9 L/min
Nebulizer	35 PSI
Sheath gas temp.	300 °C
Sheath gas flow	10 L/min
Vcap	2500 V
Nozzle voltage	500 V
Data acquisition mode	2 GHz (Extended dynamic range) Standard mass range (m/z 3200)

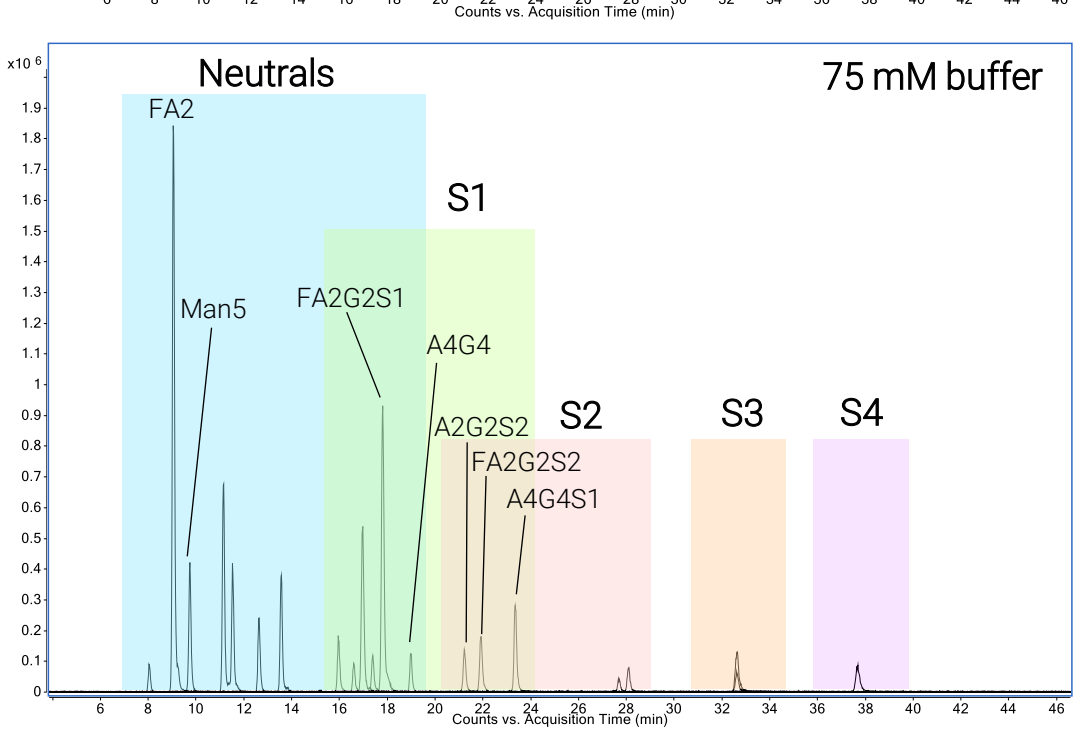
Mitigating metal-glycan interactions with the Agilent InfinityLab Deactivator Additive.

InstantPC-labeled glycans were analyzed with the Agilent 6230 LC/TOF, equipped with a 1290 Infinity II LC stack.

6230 LC-MS Conditions	
Column	Prototype Amide HILIC, 2.1 x 150 mm, 1.8 µm
Column Temperature	60 °C
Mobile phase	A = 50 mM ammonium formate, pH 4.4 + 5 µM InfinityLab Deactivator Additive B = Acetonitrile
Flow rate	0.5 mL/min
Gradient program	Time (min) %B 0 77 36 42.5 37 40 37.8 40 38 77 52 77
MS conditions	Same as above

Charge group selectivity modulation.





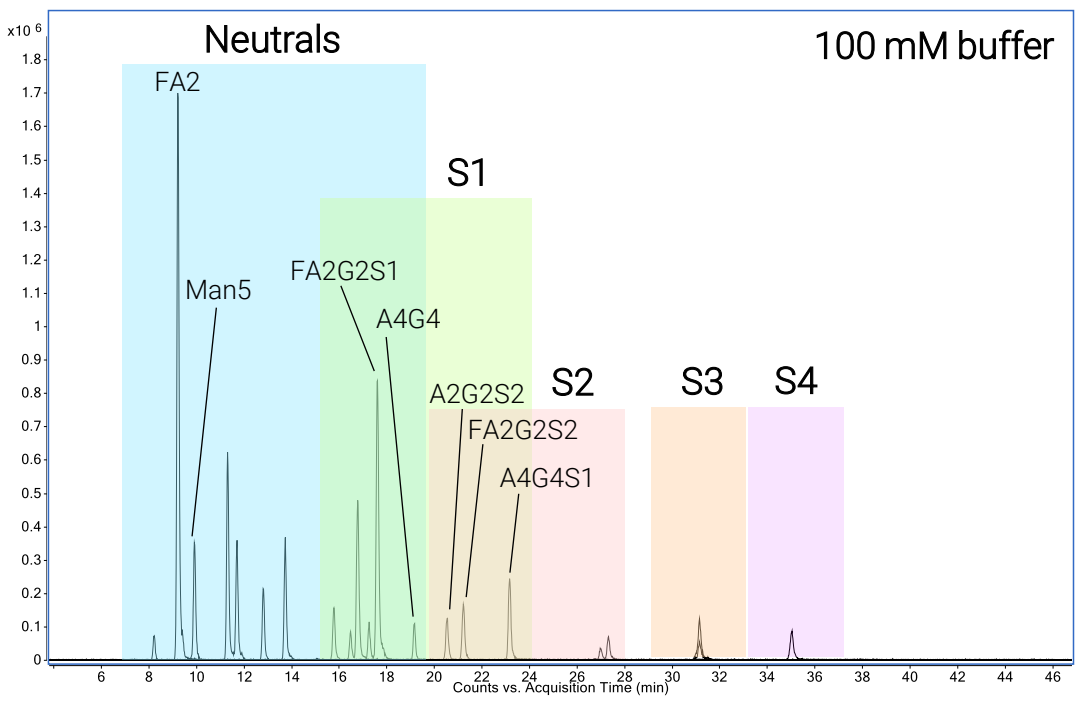


Figure 1. Chromatographic separation of InstantPC glycan standards performed with 50 mM (top), 75 mM (middle) and 100 mM (bottom) buffer in mobile phase bottle A. Buffer concentration can be varied as needed to optimize charge group spacing and critical pair selectivity.

Achieving chromatographic separation of critical glycan pairs via adjustment of the HILIC gradient slope alone is often challenging or impractical. To mitigate this roadblock, we have developed a HILIC stationary phase which allows charge group selectivity to be modulated through adjustment of the mobile phase buffer concentration (Figure 1). By varying the buffer concentration within an MS-compatible window, it is possible to resolve critical glycan pairs of differing charge. The capability to adjust charge group selectivity also enables total gradient times to be reduced by eluting highly-charged glycans earlier without sacrificing resolution of the earliest-eluting glycans in mixtures with a diversity of neutral and singly-sialylated structures.

N-glycan separations of therapeutic proteins.

When using 50 mM ammonium formate as the aqueous mobile phase component, our novel stationary phase demonstrates relatively little charge group overlap, particularly between neutral and S1 (mono-sialylated) glycans. When analyzing samples with a high diversity of neutral structures, this increased charge group separation reduces the occurrence of peak overlap without the need for a lengthy chromatographic gradient. Samples with high-complexity glycan profiles, such as abatacept or cetuximab (Figure 2) can be profiled within a 30-minute window with high peak capacity. Neutral glycans elute in the order typically seen with amide columns (FA2 < Man5 < FA2[6]G1 < FA2[3]G1) and isomer separation of larger neutral structures, such as FA2FG2Ga1, is feasible.

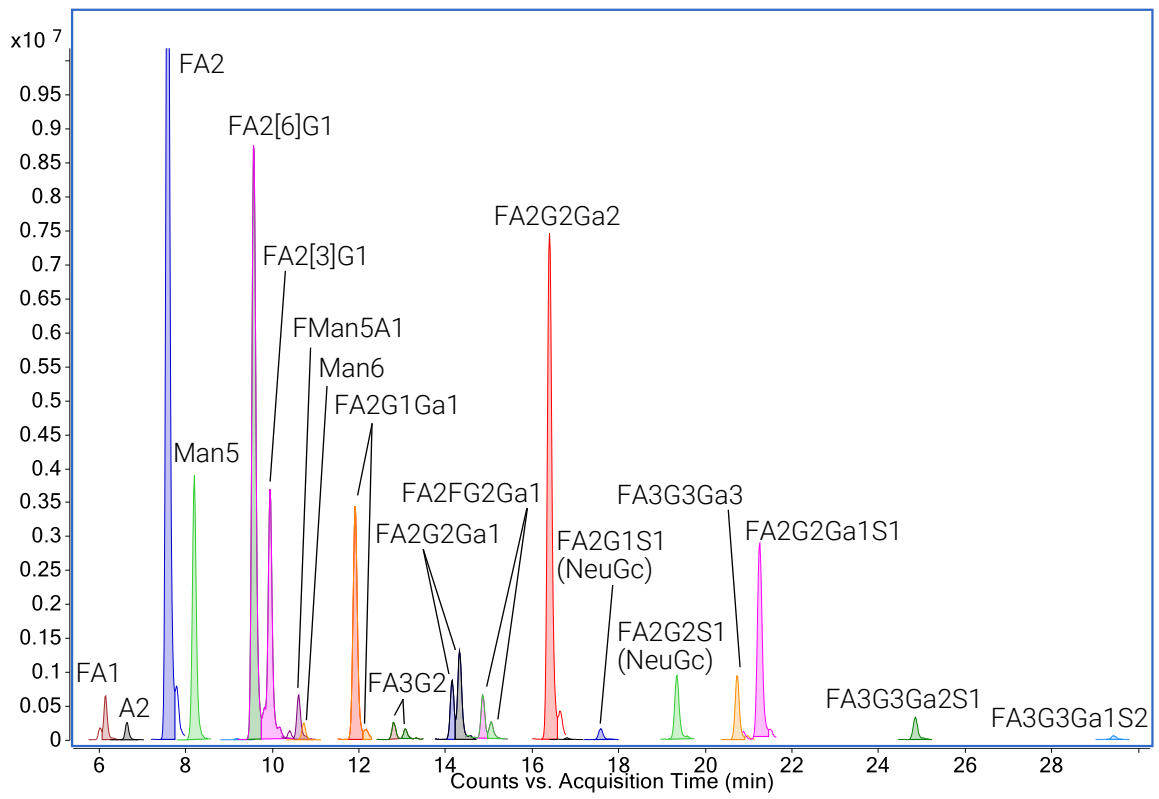


Figure 2. Extracted compound chromatograms of InstantPC-labeled cetuximab N-glycans analyzed with the prototype amide HILIC stationary phase.

Mitigating metal-glycan interactions in LC-MS analysis with the mobile phase deactivator.

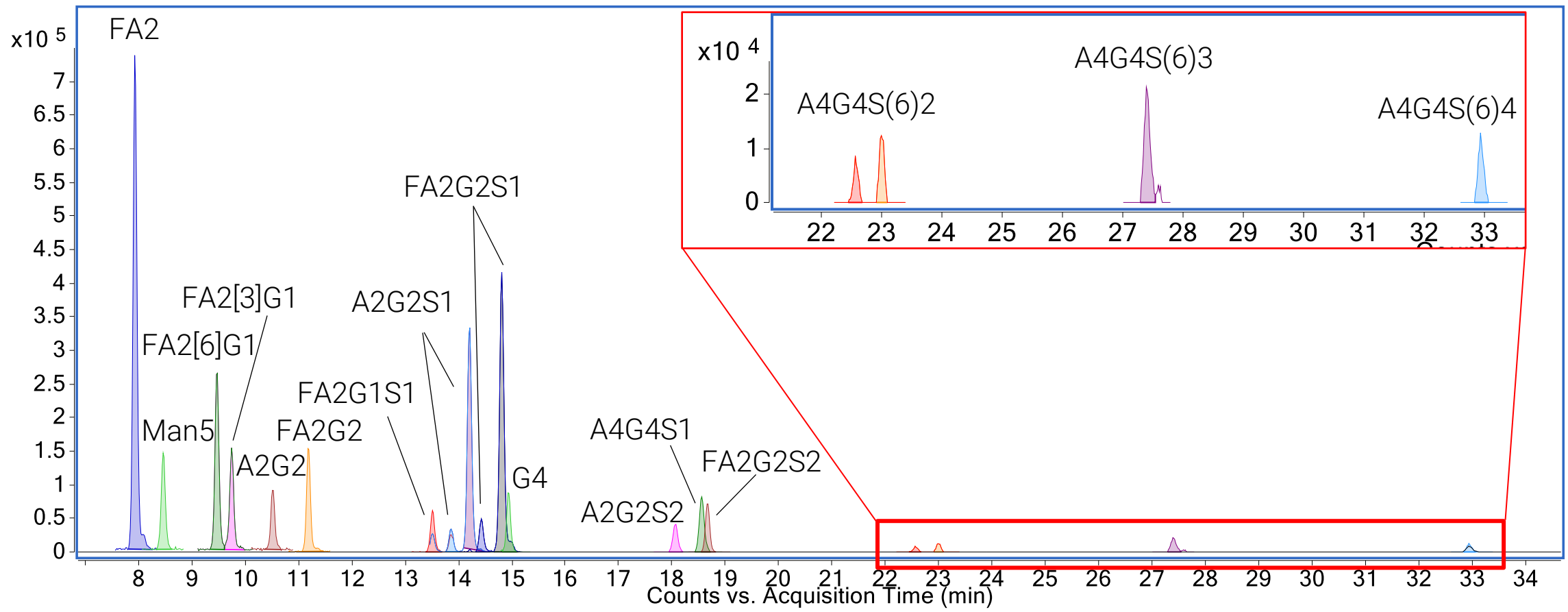


Figure 3. Extracted compound chromatograms of InstantPC-labeled glycans analyzed on a steel LC system with the InfinityLab Deactivator Additive in mobile phase bottle A.

Glycan-metal interactions are undesirable due to their tendency to produce poor chromatographic peak shape and reduced sensitivity. The severity of this problem increases with increasing glycan charge and can be addressed through either the use of a bio-compatible LC system, or through passivation and/or deactivation of problematic metal sites. We have shown that a low concentration (5 µM) of a potent metal chelator in the aqueous mobile phase bottle is both sufficient to ensure sharp chromatographic peaks for sialylated glycans and is compatible with MS detectors (Figure 3). Before-and-after comparisons of acidic glycan peak shapes on a metal-contaminated column are shown in Figure 4.

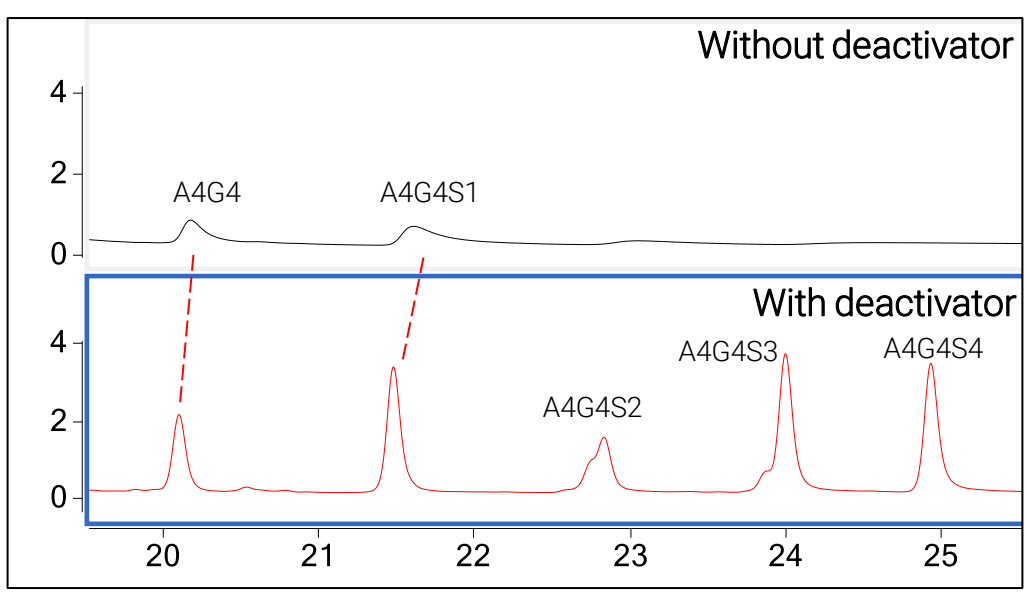


Figure 4. HPLC-fluorescence chromatograms of InstantPC-labeled glycans on a competitor amide HILIC column.

Conclusions

- Glycan charge group selectivity is modulated by varying the HILIC buffer strength within an MS-compatible range
- The column has a high peak capacity and can profile common antibody glycans within a 30-min elution window
- When profiling glycans on a steel LC system, unwanted glycan-metal interactions are easily mitigated by incorporation of an MS-compatible mobile phase deactivator

References

[1]Yang, X. and Bartlett, M. *J. Chromatogr. B.* **2019,** *1120,* 29-40.

[2]Reusch, D. and Tejada, M.L. *Glycobiology.* **2015,** *25,* 1325-1334.

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