APPLICATION NOTE 22060

Understanding the effects of column temperature on high resolution glycan separations

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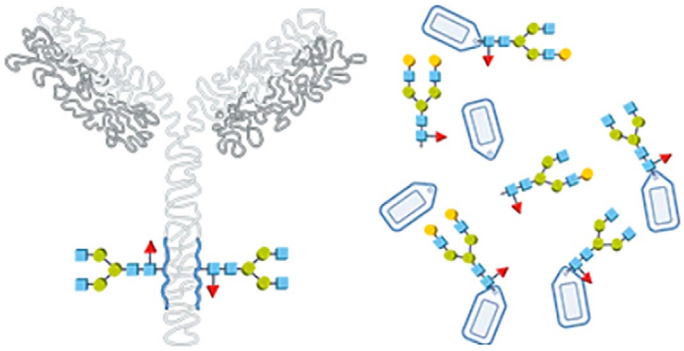
Goal

To demonstrate the resolving power of the Thermo Scientific™ Accucore™ 150 Amide HILIC column for 2-AB labeled glycans released from therapeutic monoclonal antibodies, and to determine the advantages of using this column compared to a market leader in glycan analyses.

Introduction

N-linked glycosylation of therapeutic monoclonal antibodies has a profound effect on the efficacy and biological activity of the drug. Therefore, monitoring the consistency of glycan profiles across different lots of the products or during cell line development is required by regulatory agencies.



Approaches most frequently used for glycan analyses are based on labeling the released glycans with certain fluorophores, such as 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (anthranilic acid; 2-AA), followed by clean-up of the excess dyes. Following the clean-up step, labeled glycans are separated using hydrophilic interaction liquid chromatography (HILIC) with fluorescence and mass spectrometric (MS) detection. MS detection of derivatized glycans is performed in negative mode ionization, except for a few dyes where the tag attached provides good ionization efficiency in positive ionization mode. While positive mode dyes show increased sensitivity, MS fragmentation of glycans is poor in positive mode making structural interpretation a challenge, and potentially leading to artifact formation such as fucose migration. Many companies perform routinely 2-AB (or 2-AA) based labeling, which is well-established for glycan analyses.

The columns used for separating labeled glycans are packed with particle with sizes ranging between 1.7 and 3 µm, making the column amenable to ultra-high-performance liquid chromatography (UHPLC). The surface chemistry of packing material (mostly based on amide functionality) is bonded on particles synthesized using different chemistries (e.g. bridged hybrid, non-porous core or silica).

Accucore 150 Amide HILIC LC columns have been designed to separate a wide variety of hydrophilic molecules and are an excellent tool for glycan analyses. Separation takes place in the relatively thin porous amide bonded phase surrounding the non-porous core, enabling fast analyses and excellent mass transfer. The particle size of this solid core packing material is 2.6 µm, allowing separation to be performed at lower back pressure compared to columns packed with sub-2 µm particles. Consequently, the temperature used can be lower, positively impacting both the selectivity and lifetime of the column.

Owing to the wider range of accessible operating temperatures, this study was performed to understand the effect of column temperature on glycan separation. Once the optimum temperature was defined, separations found to be the best were compared to a competitor column.

Experimental

Instrumentation

- Thermo Scientific™ Vanquish™ Flex UHPLC system (IQLAAAGABHFAPUMBJC) consisting of
  - Binary pump H (P/N VH-P10-A)
  - Split sampler HT (P/N VH-A10-A)
  - Fluorescence detector F, single PMT (P/N VF-D51-A)
  - Column compartment H (P/N VH-C10-A)
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (IQLAAEGAAPFALGMBDK)

Consumables

- Accucore 150 Amide HILIC HPLC column, 2.6 µm, 2.1 x 150 mm (P/N 16726-152130)
- Applied Biosystems™ MicroAmp™ reaction tube with cap, 0.2 mL, autoclaved (P/N N8010612)
- Invitrogen™ DynaMag™-2 magnet (P/N 12321D)
- Fisher Scientific™, Isotemp™ digital dry baths/block heater, four blocks (P/N 88860023)
- Invitrogen™, nonstick, RNase-free microfuge tubes, 2.0 mL (P/N AM12475)
- Thermo Scientific™ 9 mm Non-Assembled Plastic Screw Thread Wide Opening Autosampler Vial Kit (P/N C5000-97)

Reagents

- Applied Biosystems™, GlycanAssure™ HyPerfomance APTS Kit (P/N A33953, A33952)
- Fisher Scientific™ UHPLC-MS grade water (P/N W8-1)
- Fisher Scientific™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ Optima™ UHPLC-MS grade ammonium formate (P/N A115-50)
- Fisher Scientific™ Optima™ UHPLC-MS grade formic acid (P/N A117-50)
- Sigma-Aldrich dimethyl sulfoxide (DMSO), anhydrous, ≥99.9% (P/N 276855)
- Sigma-Aldrich anthranilamide (2-AB), ≥98%, (P/N A89804)
- Sigma-Aldrich acetic acid, glacial, ≥99.7%, (P/N 695092)
- Sigma-Aldrich triethylamine, ≥99% (P/N T0886)

Preparation of 2-AB labeled *N*-linked glycans

Releasing and labeling glycans

N-linked glycans of therapeutic monoclonal antibodies were released using the corresponding release protocol of the Applied Biosystems™ GlycanAssure™ HyPerfomance APTS Kit. Briefly, the monoclonal antibody (mAb) solutions were diluted to 5 mg/mL concentration with water. Ten microliters of diluted mAb solution (containing 50 µg of protein) were mixed with 6 µL denaturant, 1.2 µL denaturation buffer, and 8.3 µL UHPLC-MS grade water in a 0.2 mL reaction tube. These tubes were incubated in an equilibrated block heater at 80 °C for 5 min with the caps open. After denaturation, tubes were removed from the block heater, 3 µL of digestion buffer was added, and the solution containing the denatured glycoproteins was cooled down at ambient room temperature for 2 min. This was followed by the addition of 1.5 µL of PNGase F enzyme. The solution was thoroughly mixed by pipette action and the tubes with open caps were incubated for 10 min in another block heater equilibrated at 55 °C. During incubating, the labeling reagent was prepared by dissolving 2-AB at 120 mg/mL concentration in a solvent consisting of 20% (v/v) glacial acetic acid in anhydrous DMSO. Ten microliters of freshly prepared 2-AB labeling reagent and 4 µL of reductant solution were added to the deglycosylation mixture consisting of the released glycans, the reagents used during the denaturation/release, and the deglycosylated protein. The temperature was increased on the second block heater (from 55 °C to 60 °C), and the vials were incubated for 2 h at 60 °C. It is important for the wet labeling reactions here, that open cap vials are used. This allows a controlled evaporation of the reagent, which will improve the labeling efficiency.[1]

Magnetic bead-based clean-up of the labeled *N*-linked glycans

One milliliter of suspension of the magnetic beads is provided in the kit and was washed twice with 1 mL of UHPLC-MS water (after resuspension, the microfuge tube with the bead suspension was placed in DynaMag-2 Magnet stand, beads were captured for 30 s, and supernatant was removed with a pipette). The washed beads were resuspended in 450 µL water to obtain 2x beads working suspension. Fifty microliters of this working solution were added to the mixture after the labeling reaction went to completion, mixed by pipette action, and transferred into a microfuge tube. Afterwards, 700 µL of 143 mM triethylamine (TEA) in acetonitrile was added to the mixture, vortexed vigorously, briefly centrifuged and the tubes were positioned on the magnetic stand. After 30 s, the supernatant was carefully removed and discarded.[2] The tubes were then washed twice with 200 µL of the provided wash buffer. The washed beads were resuspended in 30 µL of UHPLC-MS water for 10 min, followed by collection of supernatant containing the 2-AB labeled glycans. Ten microliters of labeled glycan solution were diluted by 40 µL of UHPLC-MS grade acetonitrile prior to UHPLC-MS analyses.

Chromatographic conditions

Table 1 summarizes chromatographic conditions consistently used throughout this study. The gradient started at 80% B and was decreased to 73% in 3 min. Afterwards, mobile phase B was further decreased to 63% for an additional 32 min, resulting in a total gradient time of 35 min. Mobile phase B was then dropped to 0% in 1.5 min while the flow rate was decreased from 0.5 to 0.25 mL/min, and this condition was kept for additional 3 min, washing the column with aqueous mobile phase A. After the washing step, %B was slowly increased to 80% at 0.25 mL/min between 39.5 and 43.1 min. This was followed by an increase of flow rate from 0.25 to 0.5 mL/min between 43.1 and 47.6 min. Lastly, the column was equilibrated between 47.6 and 55 min. This gradient is similar to competitor column gradient recommendations.

Table 1. Chromatographic condition of the separation of 2-AB labeled *N*-linked glycans

Parameter	Value
Mobile phase A	50 mM ammonium formate buffer (pH 4.4)
Mobile phase B	Acetonitrile
Flow rate	0.5 mL/min during the gradient
Run time	55 min
Column temperature	Preheating, still air mode, 35 °C for Accucore 150 Amide HILIC and 60 °C for Competitor
Sample injected	10 µL

2-AB labeled glycans were detected by FLD-MS detection. The fluorescence detection parameter at channel 1 was set to 320 nm excitation and 420 nm emission, and at channel 2 at 250 nm excitation and 430 nm emission wavelengths. The sensitivity at both channels was set at 7.

Mass spectrometric conditions

2-AB labeled glycans were analyzed in negative ionization mode. The spray voltage was 3.3 kV, the capillary temperature was set to 300 °C, and the probe heater temperature was 200 °C. For the flow rates of sheath and auxiliary gas, 20 and 10 (arbitrary units) were employed, the sweep gas flow rate was 1. MS^1 spectra were collected at *m/z* 600–2000 over 55 min at 70,000 resolution, the AGC target for MS^1 data acquisition was set to 1 × 106 at 200 ms maximum injection time. The number of microscans in MS^1 was 1. MS data was acquired using Thermo Scientific™ Xcalibur™ software version 3.0 and chromatographic and MS^1 data were processed by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.0.

Results and discussion

Labeled *N*-linked glycan analysis is typically performed at 60 °C. This elevated temperature keeps the HPLC backpressure low, allowing a sub-2 µm particle column to operate higher flow rates (0.4-0.5 mL/min), for maximum method throughput. Changing the column to a solid core 2.6 µm Amide-HILIC, allows users to run at higher flow rates and realize high resolution separations, without elevated system backpressure. The lower operational pressure of the solid core column permitted experimentation with column temperature, to uncover the effect on chromatographic separation of released glycans from therapeutic antibodies.

Figure 1 shows 30 chromatograms of 2-AB labeled glycans released from infliximab; the first 10 injections were performed at 45 °C. The temperature was then increased to 60 °C, which is widely recommended in literature and application notes. Additionally, 10 separations were carried out (second 10 injections) with the temperature set at 45 °C. After thorough equilibration of the column, 10 separations were performed to investigate the performance of the column, after and before using 60 °C (third set of 10 injections).

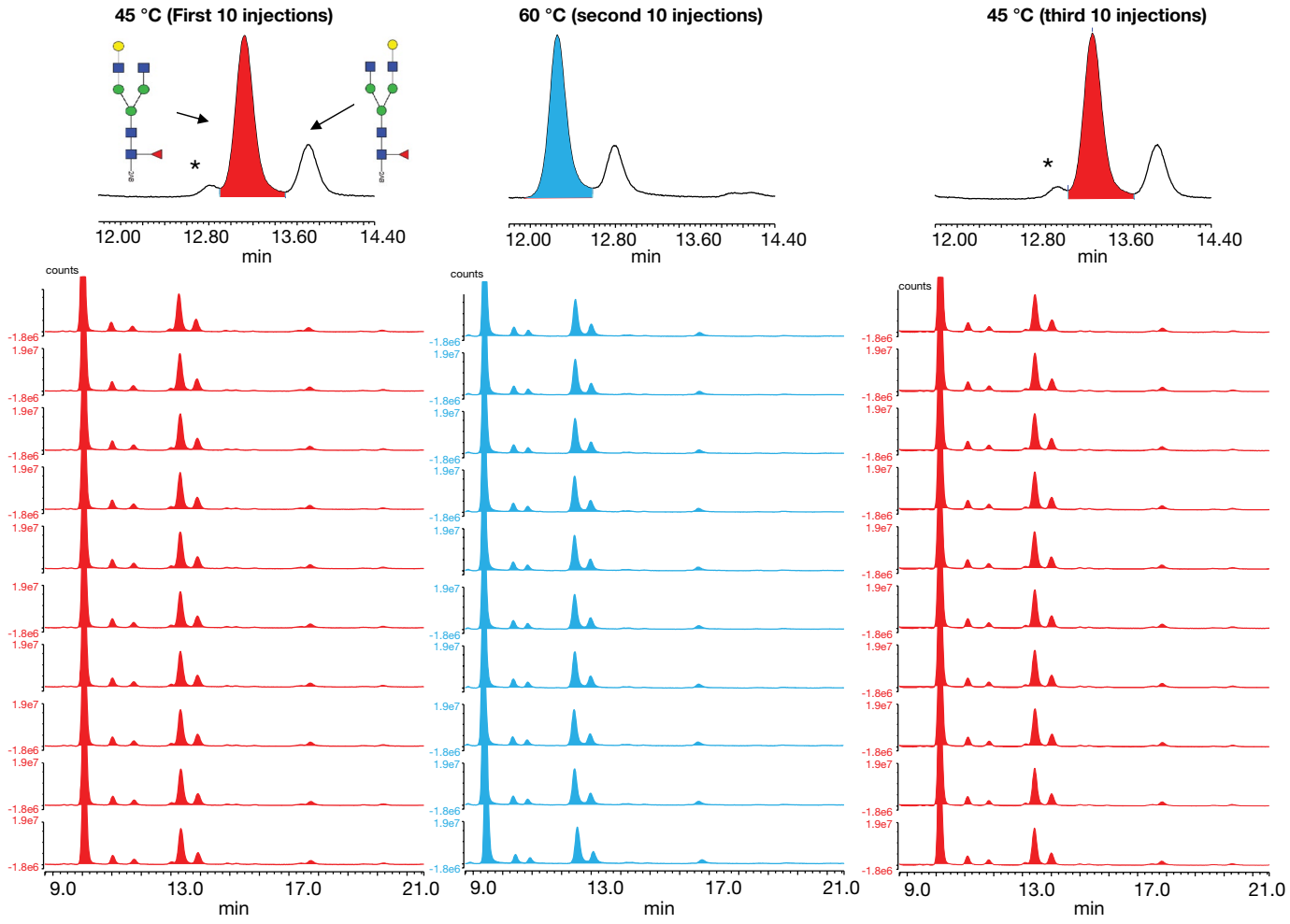


Figure 1. Separation of 2-AB labeled Infliximab glycans. Ten injections were first performed at 45 °C, followed by additional 10 injections at 60 °C. The third 10 injections, performed again at 45 °C, shows good reproducibility compared to the first 10 injections. The insets zoomed in provide further evidence of reproducibility, the peak denoted by asterisk appears at 45 °C.

The resolution of two positional isomers (FA2(6)G1 and FA2(3)G1), as well as the peak width at half height calculated for the two isomers, were compared as shown by Figure 1 insets and Table 2. As clearly shown, resolution was slightly higher at lower temperature for both sets of data generated at 45 °C from 10 injections. The resolution was found to be 1.92 with excellent reproducibility (for separations performed before the temperature was increased, the % CV was 1.25, and those of being performed after 60 °C the % CV was at 1.06). Furthermore, the asterisk shows the appearance of an additional peak at 45 °C, which was improved by decreasing the temperature to 35 °C. Figure 2 accounts for the effect of the different temperature values on the separation of infliximab 2-AB-labeled glycan pool, and highlights the advantages of using a lower temperature. With the better selectivity of this column at lower temperature, the increase in retention time is relatively small, hence the throughput of the analyses is not compromised.

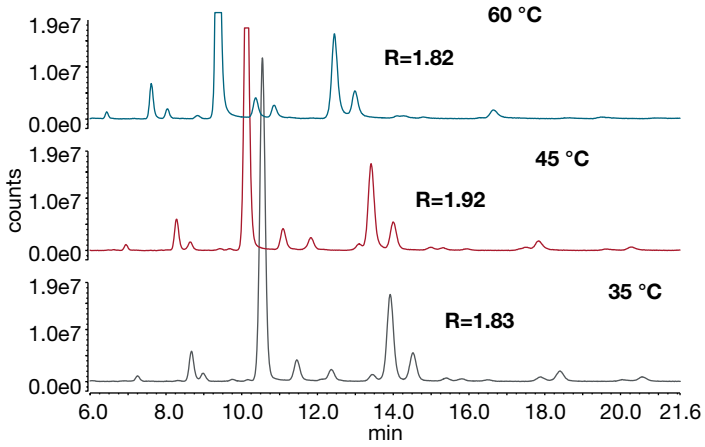


Figure 2. Separation of 2-AB labeled infliximab glycans carried out at different temperatures show the effect of temperature on the selectivity of column.

To better demonstrate the excellent resolving power of the Accucore 150 Amide HILIC column, UHPLC-MS analyses were performed on NIST mAb and three therapeutic antibody glycans. Glycan annotation was based on the monoisotopic *m/z* of the precursor ion, and retention behavior of glycans were governed by their hydrodynamic volumes. NIST mAb is known to contain immunogenic carbohydrate residues such as α-1,3 linked galactose and *N*-glycolylneuraminic acid (Neu5Gc). Both these motifs terminate glycans structures by capping β-1,4 galactoses. Furthermore, the presence of bisecting GlcNAc is also revealed on NIST mAb, making the glycosylation profile of this mAb quite challenging.

45 °C Samples	tR2	tR1	w/21	w/22	R
R1	13.93	13.34	0.18	0.18	1.95
R2	13.85	13.27	0.17	0.18	1.95
R3	13.88	13.30	0.17	0.18	1.94
R4	13.89	13.32	0.18	0.18	1.92
R5	13.89	13.32	0.17	0.18	1.95
R6	13.90	13.32	0.18	0.18	1.90
R7	13.90	13.32	0.18	0.19	1.89
R8	13.90	13.32	0.18	0.18	1.90
R9	13.91	13.33	0.18	0.18	1.91
R10	13.92	13.33	0.18	0.18	1.91
Average	13.90	13.32	0.18	0.18	**1.92**
STDEV			0.00	0.00	0.02
%CV			1.18	1.67	1.25

60 °C Samples	tR2	tR1	w/21	w/22	R
R1	13.01	12.47	0.17	0.18	1.86
R2	13.00	12.47	0.17	0.18	1.78
R3	12.99	12.45	0.17	0.18	1.78
R4	12.97	12.44	0.17	0.18	1.77
R5	12.98	12.43	0.17	0.18	1.85
R6	12.99	12.44	0.17	0.18	1.82
R7	12.98	12.44	0.17	0.18	1.84
R8	12.99	12.45	0.17	0.18	1.83
R9	12.98	12.44	0.17	0.18	1.83
R10	13.07	12.53	0.17	0.18	1.83
Average	13.00	12.46	0.17	0.18	**1.82**
STDEV			0.002	0.003	0.031
%CV			1.02	1.52	1.73

45 °C Samples	tR2	tR1	w/21	w/22	R
R1	14.02	13.44	0.18	0.18	1.91
R2	14.03	13.43	0.18	0.18	1.95
R3	14.01	13.42	0.18	0.18	1.90
R4	14.00	13.42	0.18	0.18	1.91
R5	14.01	13.43	0.18	0.18	1.93
R6	14.01	13.43	0.18	0.18	1.92
R7	14.02	13.42	0.18	0.19	1.93
R8	14.01	13.42	0.18	0.19	1.90
R9	13.99	13.41	0.18	0.18	1.88
R10	14.01	13.42	0.18	0.18	1.94
Average			0.18	0.18	**1.92**
STDEV			0.003	0.003	0.020
%CV			1.43	1.47	1.06

Table 2. Reproducibility of the separation of FA2(3)G1 and FA2(6)G1 glycans. Peak width at half height and resolution have been presented.

Figure 3 compares the glycan profile of this mAb using the Accucore 150 Amide HILIC column and a competitor 1.7 µm fully porous HILIC column. Both separations look very similar, although thorough observation sheds light on additional separations delivered by the Accucore 150 Amide HILIC column, but not noticeable in the case of the competitor. At first, two relatively minor glycans, FA2B(3) G1 and FA2G1aGal-N are partially separated, with baseline separation achieved for FA2G1aGal and FA2G2. The α-1,3 Gal terminated glycan structures are well-characterized on this mAb, and it is imperative to identify and quantify them in the glycan pool due to the anaphylactic potential of an IgE isotype of anti-α-Gal antibodies.[3]

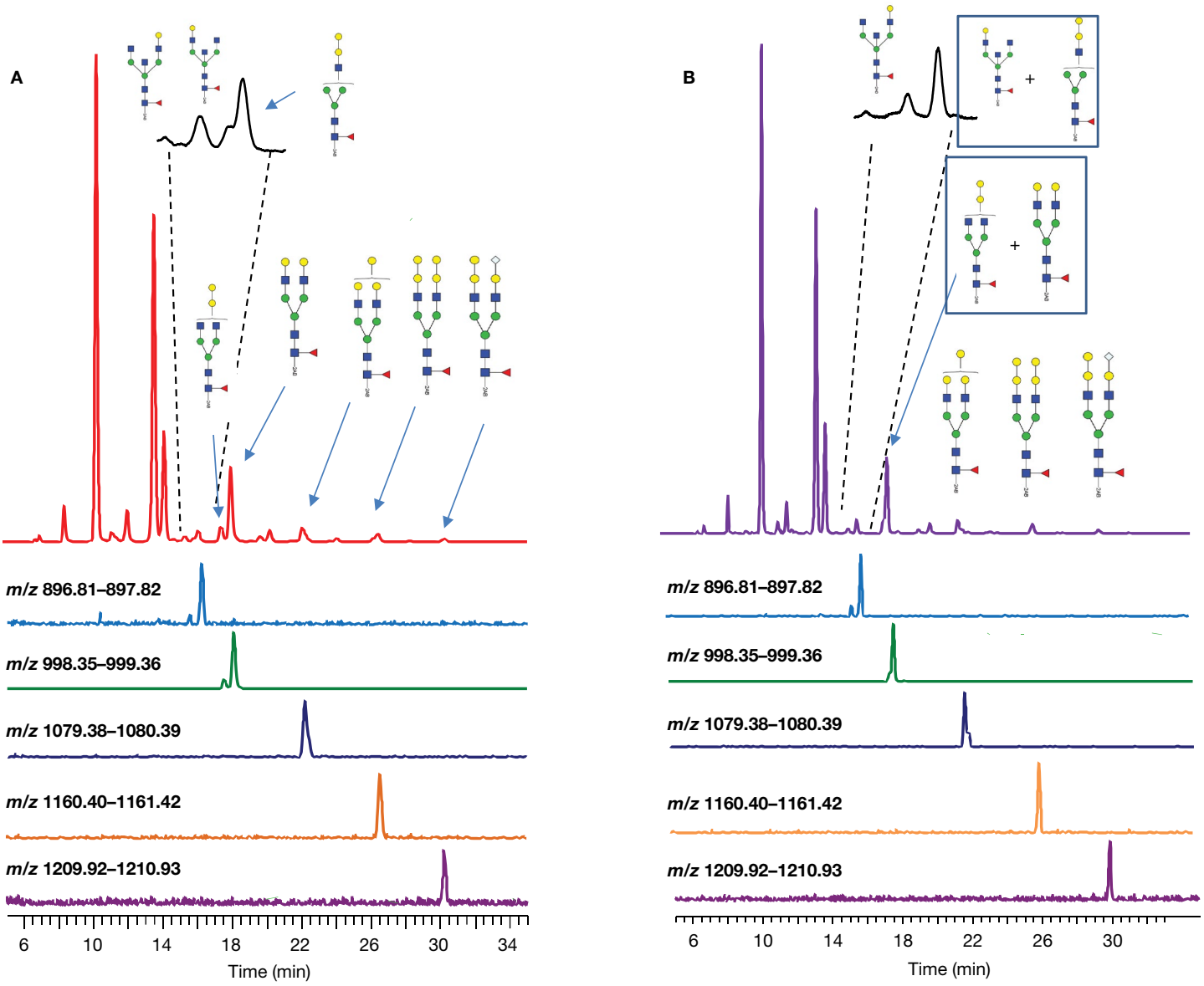


Figure 3. Separation of 2-AB labeled NIST mAb glycans. Separation A) was performed using an Accucore 150 Amide HILIC column at 35 °C; separation B) was carried out on a competitor column at 60 °C. Extracted ion chromatograms further evidence the structures annotated.

Figure 4 highlights three important separations of infliximab glycans that are possible to achieve using the Accucore 150 Amide HILIC column as opposed to the competitor.

A hybrid structure (hybMan5N) formed by deposition of a GlcNAc on the α-1,3 arm separates well from FA2(6)G1.

Furthermore, the Accucore 150 Amide HILIC column well resolves the core fucosylated hybrid Man5N (hybMan5FN) and FA2(6)G1aGal and afucosylated hybrid Man5(3)G1, and FA2G2 glycan pairs, whereas no separations were attained for these two biologically important glycan pairs on the competitor column.

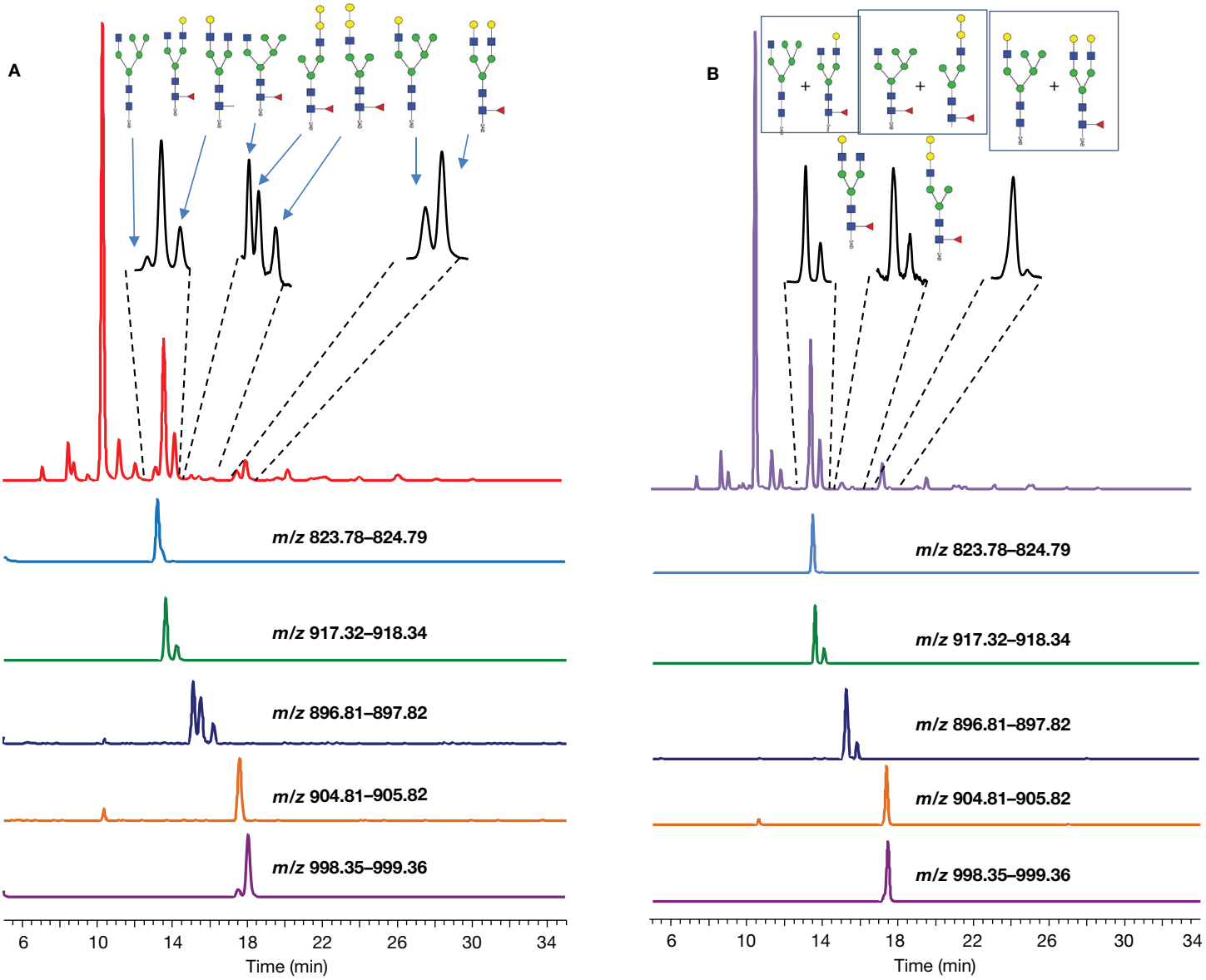


Figure 4. Separation of 2-AB labeled infliximab glycans. Separation A) was performed using an Accucore 150 Amide HILIC column; separation B) was carried out on a competitor column. Extracted ion chromatograms further evidence the structures annotated.

High mannose (mostly Man5) and afucosylated glycans located on the Fc portion of mAbs have been reported to enhance binding to Fcγ receptors, thus these mAbs exhibit increased ADCC function and faster clearance. Separation of Man5, as well as afucosylated glycans, is especially important during cell culture screening and downstream QC analyses. The competitor column fails to deliver this expectation, in so far as no separation was achieved between afucosylated A2(6)G1 and fucosylated FA2G1-N. In many reports, this co-elution is frequently overlooked, and identified inappropriately as if it was only A2(6)G1, as shown by Figure 5 and Figure 6. In contrast to this observation, separations of trastuzumab and a hyperglycosylated mAb glycans carried out on the Accucore 150 Amide HILIC column clearly show that FA2G1-N elutes between the two A2G1 isomers, as corroborated by MS. Although trastuzumab exhibits only low level of A2G2 glycan (Figure 5), some therapeutic glycoproteins such as eanercept (fusion protein) bear a high level of this glycan on the Fc portion. In the separation shown by Figure 5, A2G2 partially separates from FA2G1S1-N, and can be integrated based on the

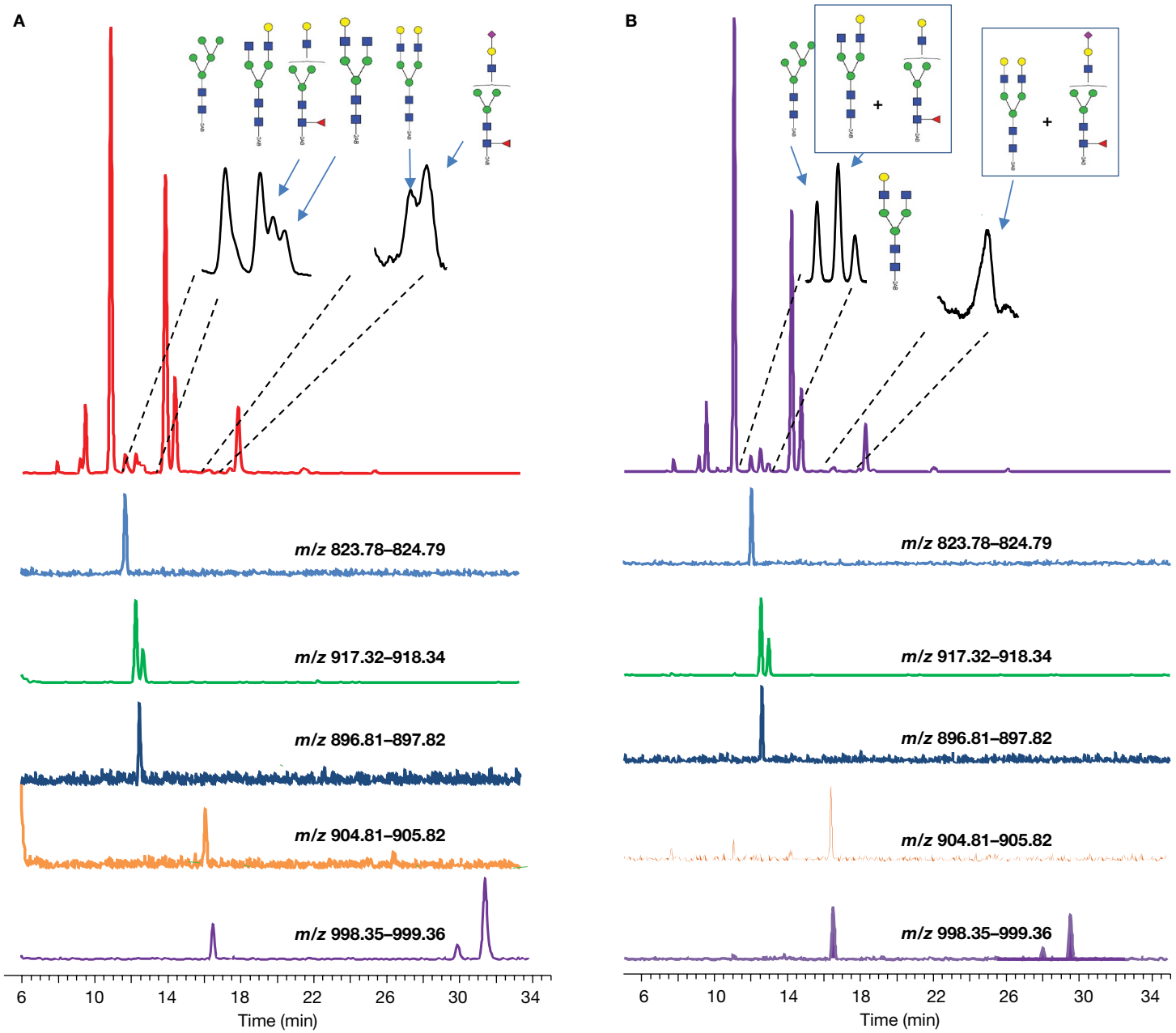


Figure 5. Separation of 2-AB labeled trastuzumab glycans. Separation A) was performed using an Accucore 150 Amide HILIC column; separation B) was carried out on a competitor column. Extracted ion chromatograms further evidence the structures annotated.

FLD chromatogram. On the competitor column, these two peaks are also frequently identified as one peak, co-elution is corroborated by MS, quantitation of these two glycans are not possible due to their different ionization efficiency. In addition, Figure 6 presents an interesting separation on the Accucore 150 Amide HILIC column between a hybrid glycan (HybMan4(6)G1S1) and FA2G2. These glycans appeared to show co-elution on the competitor column.

Table 3 summarizes glycans separated by the Accucore 150 Amide HILIC column and reports the m/z values of the precursor ions. High accuracy obtained for the mostly doubly charged precursor ions has proven to be helpful in annotating unique structures that could not have been identified based solely on only chromatographic behavior.

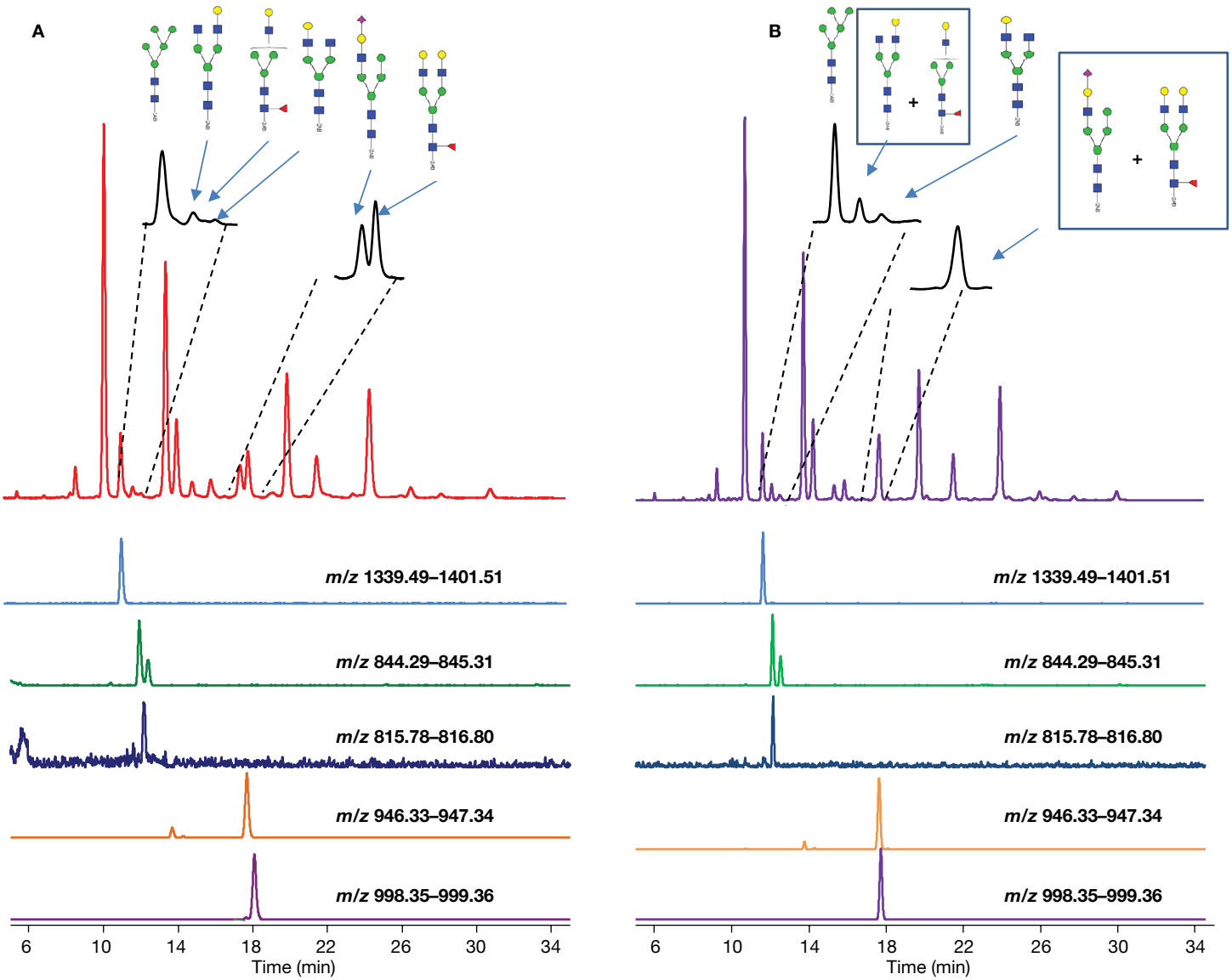
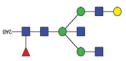
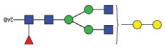
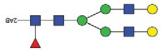
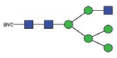
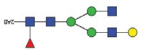
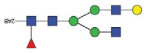
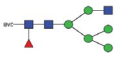
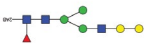
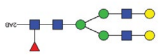
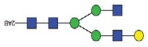
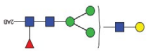
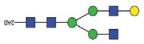
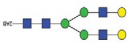
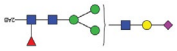
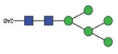
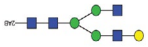
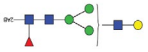
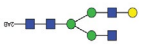
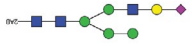
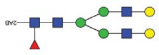


Figure 6. Separation of 2-AB labeled hyperglycosylated mAb glycans. Separation A) was performed using an Accucore 150 Amide HILIC column; separation B) was carried out on a competitor column. Extracted ion chromatograms further evidence the structures annotated.

Table 3. Additional glycan separations of the four mAbs using an Accucore 150 Amide HILIC column. Glycan compositions are corroborated by HRAM.

MAb	Glycan symbol	Glycan name	Experimental *m/z*	Theoretical *m/z*	Accuracy, ppm
NIST MAb		FA2B(3)G1	1018.8698	1018.8709	-1.08
		FA2G1aGal-N	896.8176	896.8179	-0.33
		FA2G1aGal	998.3571	998.3576	-0.50
		FA2G2	998.3561	998.3576	-1.50
Infliximab		hybMan5N	823.7839	823.7890	-6.19
		FA2(6)G1	917.3279	917.3312	-3.60
		FA2(3)G1	917.3275	917.3312	-4.03
		hybMan5FN	896.8161	896.8179	-2.01
		FA2G(6)1aGal	896.8168	896.8179	-1.23
		FA2(3)G1aGal	896.8171	896.8179	-0.89
		hybMan5(3)G1	904.8104	904.8154	-5.53
		FA2G2	998.3542	998.3576	-3.41
Trasutsumab		Man5	1399.5006	1399.5003	0.21
		A2(6)G1	844.2970	844.3023	-6.28
		FA2G1-N	815.7915	815.7915	0.00
		A2(3)G1	844.2967	844.3023	-6.63
		A2G2	925.3239	925.3287	-5.19
		FA2G1S1-N	938.3339	938.3365	-2.77
Hyperglycosylated MAb		Man5	1399.4985	1399.5003	-1.29
		A2(6)G1	844.3001	844.3023	-2.61
		FA2G1-N	815.7892	815.7915	-2.82
		A2(3)G1	844.3002	844.3023	-2.49
		hybMan4(3)G1S1	946.3292	946.3339	-4.97
		FA2G2	998.3562	998.3576	-1.40

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Conclusion

A solid core 2.6 µm column affords lower backpressures than a fully porous sub-2 µm column. This lower backpressure allows users to experiment with column temperature to understand its impact on chromatographic separation.

In this study, it was found that lower column temperatures showed higher resolution chromatograms, with more glycan variant forms identified versus higher temperature experiments on four therapeutic monoclonal antibodies. The Accucore 150-Amide-HILIC column has demonstrated outstanding separation compared to a fully porous 1.7 µm competitor HILIC column.

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