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Optimizing HILIC-based Analyses of *Rapi*Fluor-MS Labeled Sialylated N-Glycans

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APPLICATION BENEFITS

- Tuned source parameters and acquisition settings to improve the intensity and quality of MS information for *Rapi*Fluor-MS® labeled sialylated N-glycans

- Optimized mobile phases used along with a wide-pore ACQUITY UPLC® Glycoprotein BEH Amide Column to enhance HILIC chromatographic resolution

- Linkage-specific neuraminidase profiling of *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard to demonstrate a cursory sialic acid linkage analysis

WATERS SOLUTIONS

GlycoWorks® *Rapi*Fluor-MS N-Glycan Kit

*Rapi*Fluor-MS Sialylated Glycan Performance Test Standard

ACQUITY UPLC Glycoprotein BEH Amide Column

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof Mass Spectrometer

UNIFI® 1.8 Software

MassLynx® 4.1 Software

LockSpray™ Exact Mass Ionization Source

KEYWORDS

ACQUITY UPLC H-Class Bio System, ACQUITY UPLC Glycoprotein BEH Amide Column, glycans, glycoprotein, GlycoWorks, HILIC, N-linked glycans, *Rapi*Fluor-MS labeling, *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard

INTRODUCTION

Sialic acids, a family of acidic monosaccharides, are often found as terminal residues of glycan structures and they play important roles in many physiological and pathological processes.[1] With respect to therapeutics, sialic acid residues are of interest because they can impact serum half-lives[2,3] and they can serve as indicators of cell culture conditions.[4,5] N-Glycolylneuraminic acid, a non-human mammalian sialic acid with a structure similar to the human sialic acid N-acetylneuraminic acid, can contaminate biotherapeutic glycoproteins via production in non-human cell lines or from animal-derived media,[6,7] which will result in different levels of immune responses in humans.[8,9] Accordingly, sialylated glycan profiling is often needed to monitor a critical quality attribute related either to drug stability, activity, or immunogenicity.[10] Due to the lability of sialic acid residues, accurate mass spectrometric analysis of sialylated glycans has sometimes been elusive or required neutralization of the sialic acids. The recent development of kits for the rapid release and labeling of N-glycans via GlycoWorks *Rapi*Fluor-MS enables enhanced sensitivity for fluorescence (FLR) and mass spectrometry (MS) based detection of all types of N-glycan species, including those that are sialylated.[11]

In this work, we applied hydrophilic interaction chromatography (HILIC)-FLR-MS analyses to the study of *Rapi*Fluor-MS labeled sialylated N-glycans. A wide-pore (300Å) amide HILIC stationary phase was used with high ionic strength mobile phases to improve chromatographic resolution for sialylated N-glycans. Combined with optimized electrospray ionization (ESI) source parameters, a high resolution separation was achieved along with high MS sensitivity. This analysis produces data of unparalleled quality, yet, as we additionally show, it is also readily amenable to being paired with neuraminidase treatments as a means to perform a cursory level sialic acid linkage analysis.

EXPERIMENTAL

It is important to note that the HILIC conditions optimized in this study involving a wide-pore amide HILIC stationary phase and high ionic strength mobile phases are different from what have been used for generating the *Rapi*Fluor-MS Glycan GU Scientific Library, thus the retention time obtained under these conditions cannot be searched against the library for glycan identification. In addition, extended use of higher ionic strength mobile phases can quicken the fouling of the source/ion optics of a mass spectrometer, so system sensitivity monitoring and routine maintenance is recommended to ensure optimal results.

Sample description

*Rapi*Fluor-MS Sialylated Glycan Performance Test Standard (p/n 186008660) was reconstituted in 50 µL of water.

α2–3 Neuraminidase S digestion

One vial of *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard was reconstituted in 9 µL of water, followed by sequential addition of 1 µL of GlycoBuffer 1 (10×) and 1 µL of α2–3 Neuraminidase S (New England BioLabs, p/n P0743). After overnight incubation at 37 °C, 39 µL of water was added to the solution.

α2–3,6,8,9 Neuraminidase A digestion

One vial of *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard was reconstituted in 9 µL of water, followed by sequential addition of 1 µL of GlycoBuffer 1 (10×) and 1 µL of α2–3,6,8,9 Neuraminidase A (New England BioLabs, p/n P0722). After overnight incubation at 37 °C, 39 µL of water was added to the solution.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Sample temp.:	10 °C
Column temp.:	60 °C
Flow rate:	0.4 mL/min
Injection volume:	1 µL
Columns:	ACQUITY UPLC Glycoprotein BEH Amide Column, 300Å, 1.7 µm, 2.1 × 150 mm (p/n 176003702, includes Glycoprotein Performance Test Standard); ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 × 50 mm (p/n 186004740)
Fluorescence detection:	Ex 265 nm/Em 425 nm, 2 Hz

Gradient used with 2.1 × 50 mm columns (Figures 1 and 2):

Mobile phase A:	200 mM ammonium formate, pH 4.4 (LC-MS grade water)
Mobile phase B:	ACN (LC-MS grade)
Mobile phase C:	water (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	%C	%D	Curve
0.0	0.4	6.3	75.0	18.7	0	6
11.66	0.4	11.5	54.0	34.5	0	6
12.16	0.2	25.0	0	75.0	0	6
13.16	0.2	25.0	0	75.0	0	6
14.36	0.2	6.3	75.0	18.7	0	6
15.86	0.4	6.3	75.0	18.7	0	6
18.30	0.4	6.3	75.0	18.7	0	6

Gradient used with 2.1 × 150 mm columns:

Mobile phase A:	200 mM ammonium formate, pH 4.4 (LC-MS grade water)
Mobile phase B:	ACN (LC-MS grade)
Mobile phase C:	water (LC-MS grade)

To achieve 50 mM ammonium formate in aqueous mobile phases A and C (Figure 3A and 4A):

Time	Flow rate (mL/min)	%A	%B	%C	%D	Curve
0.0	0.4	6.3	75.0	18.7	0	6
35.0	0.4	11.5	54.0	34.5	0	6
36.5	0.2	25.0	0	75.0	0	6
39.5	0.2	25.0	0	75.0	0	6
43.1	0.2	6.3	75.0	18.7	0	6
47.6	0.4	6.3	75.0	18.7	0	6
55.0	0.4	6.3	75.0	18.7	0	6

To achieve 100 mM ammonium formate in aqueous mobile phases A and C (Figure 3B):

Time	Flow rate (mL/min)	%A	%B	%C	%D	Curve
0.0	0.4	12.5	75.0	12.5	0	6
35.0	0.4	23.0	54.0	23.0	0	6
36.5	0.2	50.0	0	50.0	0	6
39.5	0.2	50.0	0	50.0	0	6
43.1	0.2	12.5	75	12.5	0	6
47.6	0.4	12.5	75	12.5	0	6
55.0	0.4	12.5	75	12.5	0	6

To achieve 200 mM ammonium formate in aqueous mobile phases A and C (Figure 3C, 4B, 5, and 6):

Time	Flow rate (mL/min)	%A	%B	%C	%D	Curve
0.0	0.4	25.0	75.0	0	0	6
35.0	0.4	46.0	54.0	0	0	6
36.5	0.2	100.0	0	0	0	6
39.5	0.2	100.0	0	0	0	6
43.1	0.2	25.0	75.0	0	0	6
47.6	0.4	25.0	75.0	0	0	6
55.0	0.4	25.0	75.0	0	0	6

MS conditions (unless otherwise noted)

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~40 K)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Source offset:	50 V
Desolvation gas flow:	600 L/Hr
Calibration:	NaI, 0.1 µg/µL from 100-2000 *m/z*
Acquisition:	700–2000 *m/z*, 0.5 sec scan rate
LockSpray:	100 fmol/µL human Glu-fibrinopeptide B prepared in a solution composed of 70:30:0.1% water/acetonitrile/formic acid, sampled every 90 seconds
Data management:	MassLynx 4.1 Software UNIFI 1.8 Software

RESULTS AND DISCUSSION

OPTIMIZATION OF ESI-MS SOURCE PARAMETERS TO IMPROVE THE ION INTENSITIES OF PROTONATED *RAPI*FLUOR-MS LABELED SIALYLATED N-GLYCANS

In a positive ion mode analysis, multiple cation adducts of glycans can be readily observed if poorly optimized conditions are used. This, in turn, can dilute the overall ion intensity across several species within the same charge state and makes interpretation of spectra challenging. Proton, ammonium, and sodium adducts are commonly encountered, as is the case with the mass spectra of *Rapi*Fluor-MS labeled sialylated N-glycans following hydrophilic interaction chromatography. Moreover, in-source fragments (ISFs) can occur during ionization, again to the detriment of sensitivity and data quality. In order to maximize the signal intensities of protonated ions of *Rapi*Fluor-MS labeled sialylated N-glycans, the effects of numerous ESI source parameters were investigated using LC-MS with an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 × 50 mm Column coupled to a Xevo G2-XS QTof Mass Spectrometer. To facilitate this method development, we utilized a new standard, the *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard (p/n 186008660) containing upward of 20 unique *Rapi*Fluor-MS labeled mono- to pentasialylated N-glycans released from bovine fetuin. We focused on manipulating the MS response for one of the most abundant glycans from this profile, an A3G3S3 species (Oxford notation).[12,13] This is a structure comprised of a triantennary core (A3) modified with three galactose (G3) and three sialic acid (S3) residue extensions.

Of all the parameters studied herein, sampling cone voltage was found to produce the most significant effects, not only in the relative abundance of the protonated molecular ions, but also the intensities of ISFs. At lower sampling cone voltages, eg, 30 V, the signal intensities of the 3+ charge states of A3G3S3 were much higher than those of the 2+ charge states or ISFs (Figure 1A). Within the 3+ charge state window, multiple cation adducts of A3G3S3 were observed, and $[A3G3S3+2H^++NH_4^+]^{3+}$ gave the most abundant ion intensity (Figure 1A inset). When the sampling cone voltage was increased to 75 V, A3G3S3 ions in a 3+ charge state were still the most abundant, while the intensities of ions from in-source fragmentation slightly increased (Figure 1B). Within the 3+ charge state window, $[A3G3S3+3H^+]^{3+}$ gave the most abundant ion intensity (Figure 1B inset). When the sampling cone voltage was increased to 120 V, the intensities of molecular ions of A3G3S3 decreased significantly, meanwhile the mass spectrum became dominated by ions from in-source fragmentation (Figure 1C and inset). A graph of the correlation between the normalized absolute abundance of $[A3G3S3+3H^+]^{3+}$ and sampling cone voltages revealed a trend with a bell-shaped distribution: as the sampling cone voltages increased from 30 V, the signal intensities of $[A3G3S3+3H^+]^{3+}$ increased, reached a maximum at a sampling cone voltage of 75 V, and decreased thereafter because of increasing levels of in-source fragmentation (Figure 1D). In terms of relative abundance, a similar trend was observed: the relative abundance of $[A3G3S3+3H^+]^{3+}$ was higher when the sampling cone voltage was set at 75 V versus either 30 or 120 V; when the sampling cone voltage was changed from 75 to 120 V, significantly higher relative abundances of ISFs were encountered (Figure 2A). In consideration of this, a sampling cone voltage of 75 V has been selected for the analysis of *Rapi*Fluor-MS labeled sialylated N-glycans.
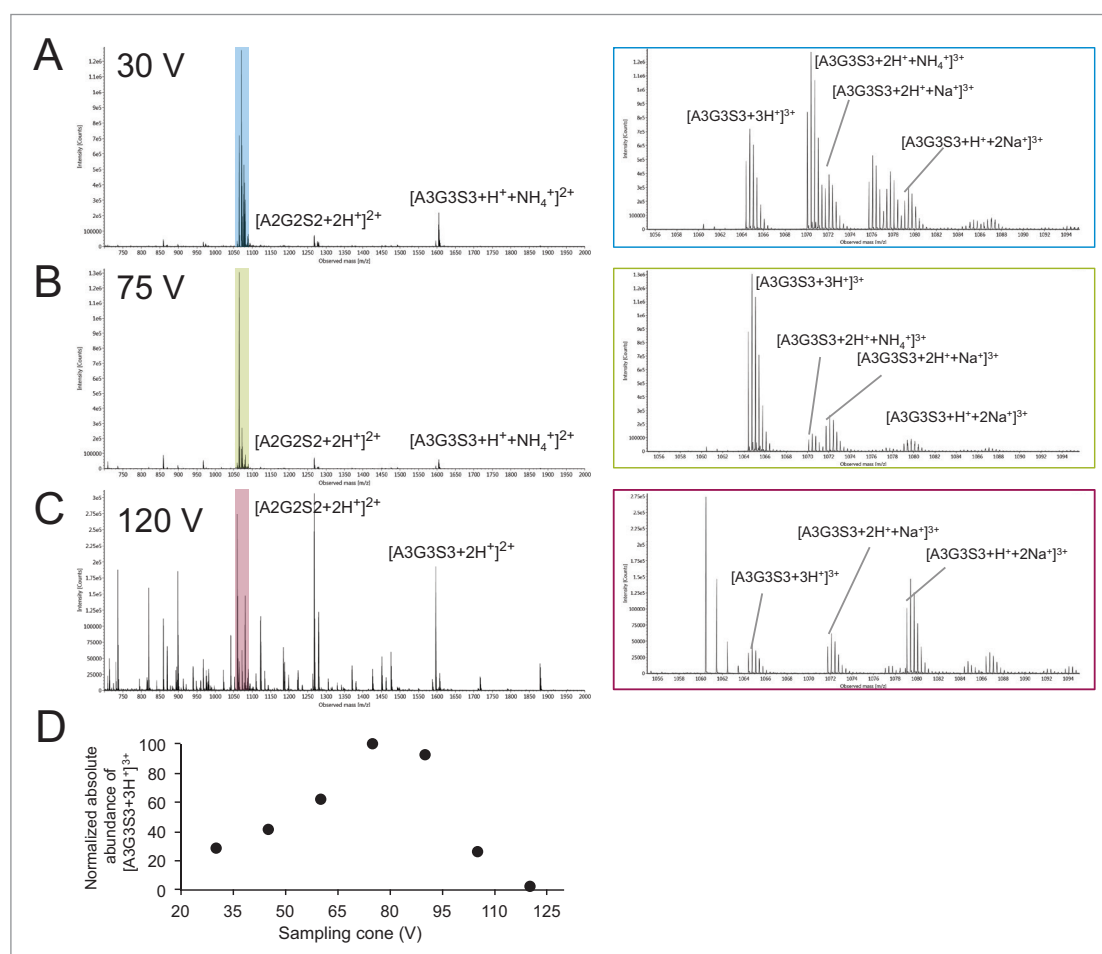

Figure 1. Optimization of sampling cone voltage to improve signal intensities of $[A3G3S3+3H^+]^{3+}$. Individual LC-MS runs of RapiFluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Column and different sampling cone voltages. The mass spectra were obtained by summing a 0.1 minute retention time window from the apex of the most abundant chromatographic peak from the LC-MS runs with different sampling cone voltage settings, eg, 30 V (A), 75 V (B), and 120 V (C), respectively. The intensities of the monoisotopic peak of $[A3G3S3+3H^+]^{3+}$ with different sampling cone voltages were normalized to the one obtained at 75 V and graphed as shown in panel (D).

The effects of desolvation temperature on signal intensities of protonated *Rapi*Fluor-MS labeled sialylated N-glycans was investigated in a similar way. The relative abundance of $[A3G3S3+3H^+]^{3+}$ slightly increased as the desolvation temperature was decreased from 650 to 350 °C, while no significant difference was observed regarding the relative intensities of ISFs (Figure 2B). A desolvation temperature between and including 350 and 500 °C appears optimal, although the absolute ion intensities near a 500 °C desolvation temperature were higher than those from a 350 °C desolvation temperature. Therefore, a desolvation temperature of 500 °C has been chosen for the analysis of *Rapi*Fluor-MS labeled sialylated N-glycans. Altering three other ESI source parameters, capillary voltage (Figure 2C), source temperature (Figure 2D), and desolvation gas flow (data not shown), did not result in any significant changes, neither in the relative abundance of $[A3G3S3+3H^+]^{3+}$ nor in the level of in-source fragmentation.

Based on the above observations, a sampling cone voltage of 75 V, desolvation temperature of 500 °C, capillary voltage of 2200 V, source temperature of 120 °C, and desolvation gas flow of 600 L/Hr have been chosen for the analysis of *Rapi*Fluor-MS labeled sialylated N-glycans, when using a Xevo G2-XS QTof Mass Spectrometer. Under these conditions, the protonated ions of A3G3S3 were optimized to be the most abundant molecular ions, and, at the same time, the formation of ISFs was minimized.
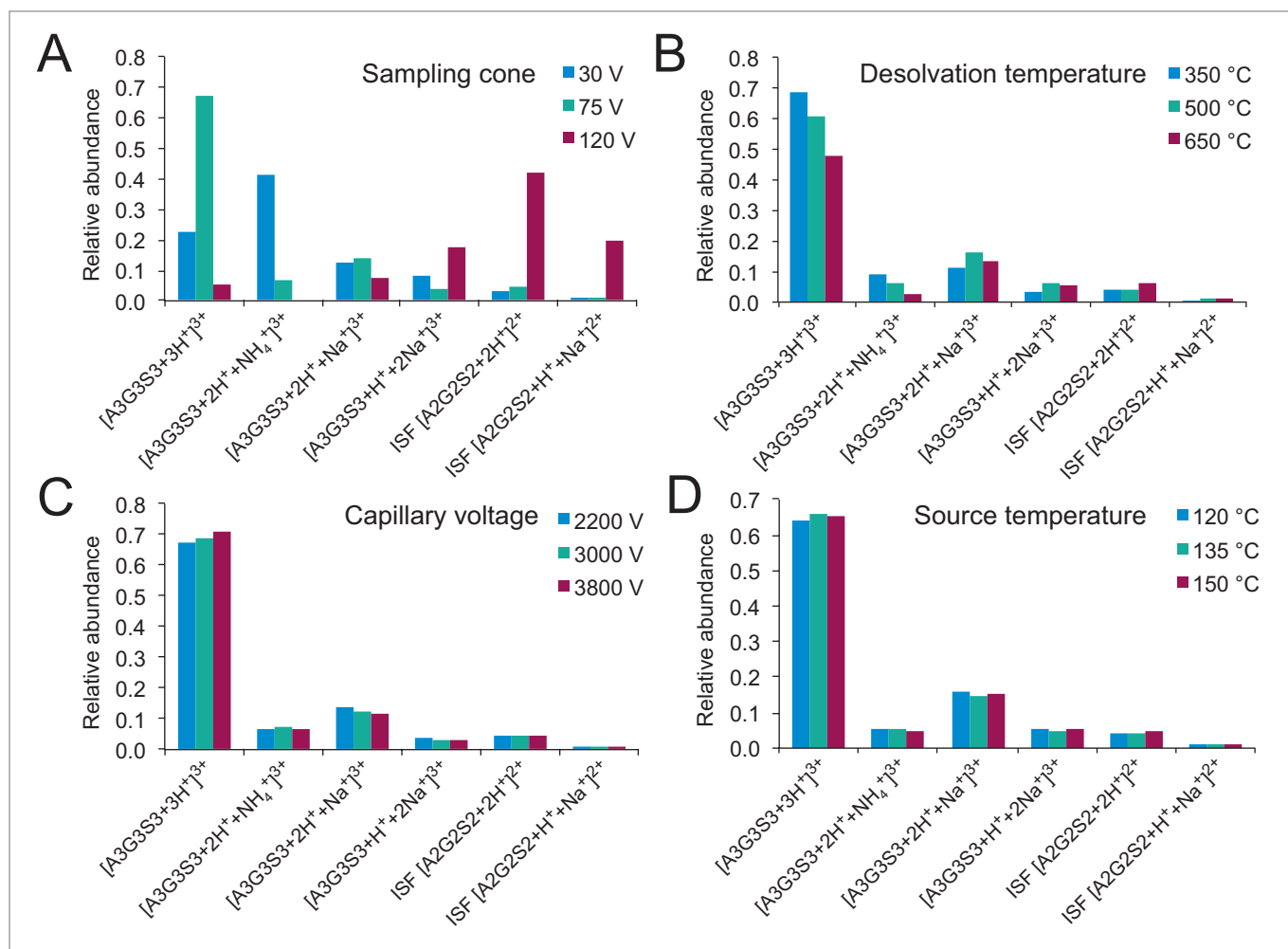

Figure 2. Optimization of ESI-MS source parameters to improve signal intensities of [A3G3S3+3H⁺]³⁺. Individual LC-MS runs of RapiFluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Column and different sampling cone voltages (A), desolvation temperatures (B), capillary voltages (C), and source temperatures (D). Mass spectra were obtained by summing 21 scans from the apex of the most abundant chromatographic peak from the LC-MS runs, and the relative abundance of different adducts were calculated from the ratio of the intensities of the monoisotopic peaks from individual species to all adducts. Note: ISF denotes in-source fragment.

OPTIMIZED MOBILE PHASES AND THE USE OF WIDE-PORE AMIDE HILIC FOR ENHANCED CHROMATOGRAPHIC RESOLUTION OF *RAPI*FLUOR-MS LABELED SIALYLATED N-GLYCANS

Just as it was used to optimize the quality of MS data, the *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard has also been used for the development of an improved chromatographic separation of sialylated N-glycans. To begin, we selected a wide-pore amide HILIC stationary phase, specifically an ACQUITY UPLC Glycoprotein BEH Amide 300Å, 1.7 µm phase, because of its demonstrated applicability to resolving large, released N-glycan structures.[14,15] The HILIC separation obtained with such a phase, in the form of a 2.1 x 150 mm column, is shown in Figure 3A. Here, a typical aqueous mobile phase comprised of 50 mM ammonium formate buffer was employed. As can be seen, the peak shape and resolution obtained with this mobile phase was not ideal, particularly for the most strongly retained, tetrasiaylated species. HILIC is intended to be a separation driven by the hydrophilicity of analytes, yet it can be complicated by ionic secondary interactions. With this in mind, we explored the use of higher mobile phase ionic strengths. As the ammonium formate concentration of the aqueous mobile phase was increased from 50 to 200 mM, the HILIC retentivity of the glycans increased, as shown in the higher retention time for all the components. A similar trend was observed in previous research.[16] Moreover, better peak shape and chromatographic resolution was observed with higher ionic strength mobile phases. For example, peaks 5 and 6, corresponding to isomers of A3S1G3S3 and A3G3S3, co-eluted with 50 mM ammonium formate, but were near baseline resolved when the ionic strength was increased to 200 mM ammonium formate (half-height resolution 1.32).
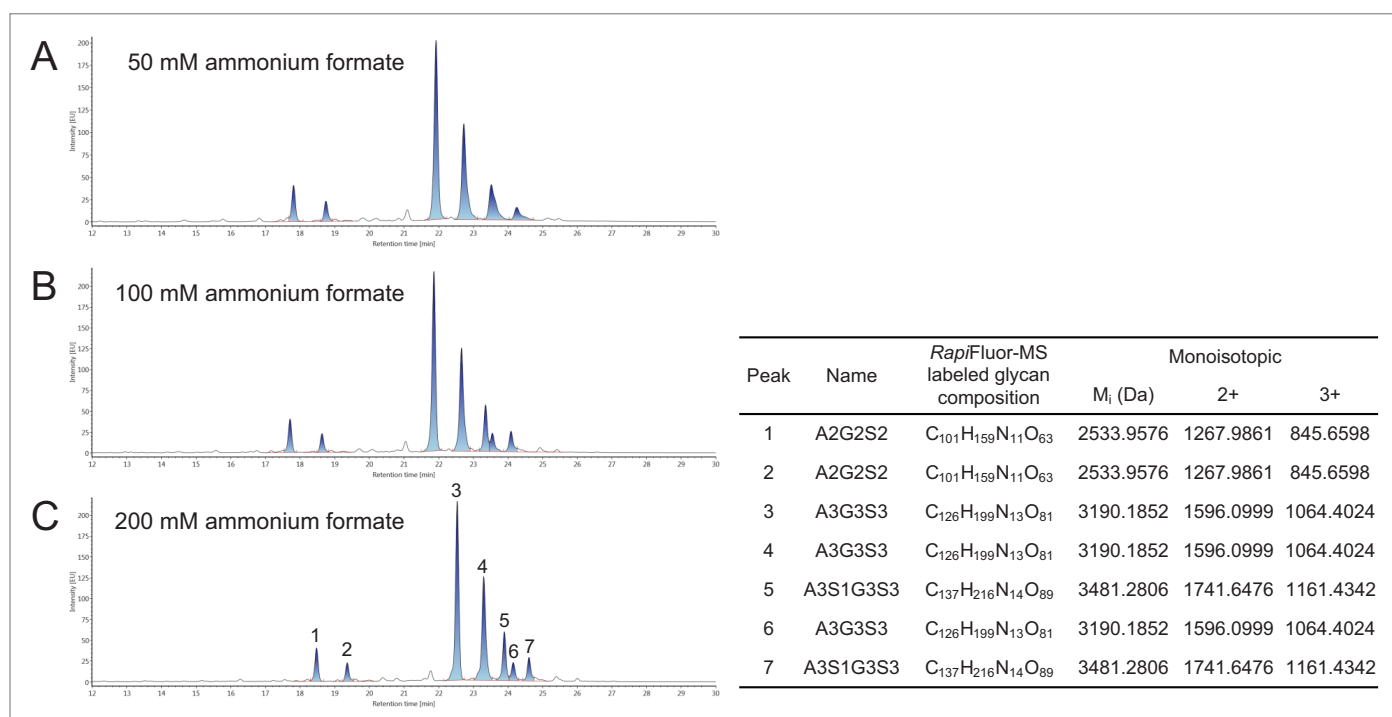

Peak	Name	*Rapi*Fluor-MS labeled glycan composition	Monoisotopic		
			M_i (Da)	2+	3+
1	A2G2S2	$C_{101}H_{159}N_{11}O_{63}$	2533.9576	1267.9861	845.6598
2	A2G2S2	$C_{101}H_{159}N_{11}O_{63}$	2533.9576	1267.9861	845.6598
3	A3G3S3	$C_{126}H_{199}N_{13}O_{81}$	3190.1852	1596.0999	1064.4024
4	A3G3S3	$C_{126}H_{199}N_{13}O_{81}$	3190.1852	1596.0999	1064.4024
5	A3S1G3S3	$C_{137}H_{216}N_{14}O_{89}$	3481.2806	1741.6476	1161.4342
6	A3G3S3	$C_{126}H_{199}N_{13}O_{81}$	3190.1852	1596.0999	1064.4024
7	A3S1G3S3	$C_{137}H_{216}N_{14}O_{89}$	3481.2806	1741.6476	1161.4342

Figure 3. Higher ionic strength mobile phases result in increased HILIC resolution of sialylated glycans. Fluorescence chromatograms of the RapiFluor-MS Sialylated Glycan Performance Test Standard were obtained with an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column using different aqueous mobile phase ionic strengths: 50 mM (A), 100 mM (B), and 200 mM ammonium formate (C).

It is noteworthy that the benefit of improved chromatographic resolution coming from higher ionic strength mobile phases came at a price of a slight increase in the intensity of background ions. Analyzed under the same mass spectrometric parameters, the absolute signal intensities of $[A3G3S3+3H^+]^{3+}$ were marginally suppressed with the shift from 50 to 200 mM ammonium formate (Figure 4). More notably, the intensity of a 1+ ion at *m/z* 708 was higher in the mass spectrum acquired with 200 versus 50 mM ammonium formate. As such, the increased background ion intensities from the higher ionic strength mobile phase obscured the glycan profile, as shown in the observed base peak intensity (BPI) chromatogram (Figure 5A). To circumvent this, the acquisition window was narrowed to 715 to 2000 *m/z*. In turn, a HILIC-FLR-MS method for sialylated N-glycans was developed that provides effective chromatographic resolution along with high quality MS information, even for very low abundant glycans (Figure 5B).

USE OF NEURAMINIDASES FOR CURSORY LEVEL SIALIC ACID LINKAGE ELUCIDATION

The improvements in the HILIC-FLR-MS method outlined above are beneficial to interrogating the compositions of a glycan profile containing sialylated species. Linkage analysis of terminal sialic acid residues is of great importance due to their critical roles in binding specificities. Avian influenza preferentially recognizes α2–3-linked over α2–6-linked sialic acids. Interestingly, humans have innate protection against the virus since α2–6-linked sialic acids are enriched on the airway epithelium of humans.[17] Sialic acid linkages are also of interest in the development of therapeutics. Therapeutic glycoproteins produced in Chinese hamster ovary (CHO) cells only possess α2–3-linked sialic acid residues due to the lack of α2,6-sialyltransferases. Efforts have been made to engineer cell lines for the production of glycoproteins with both α2–3- and α2–6-linked sialic acid residues, as such is more similar to human glycosylation.[18]
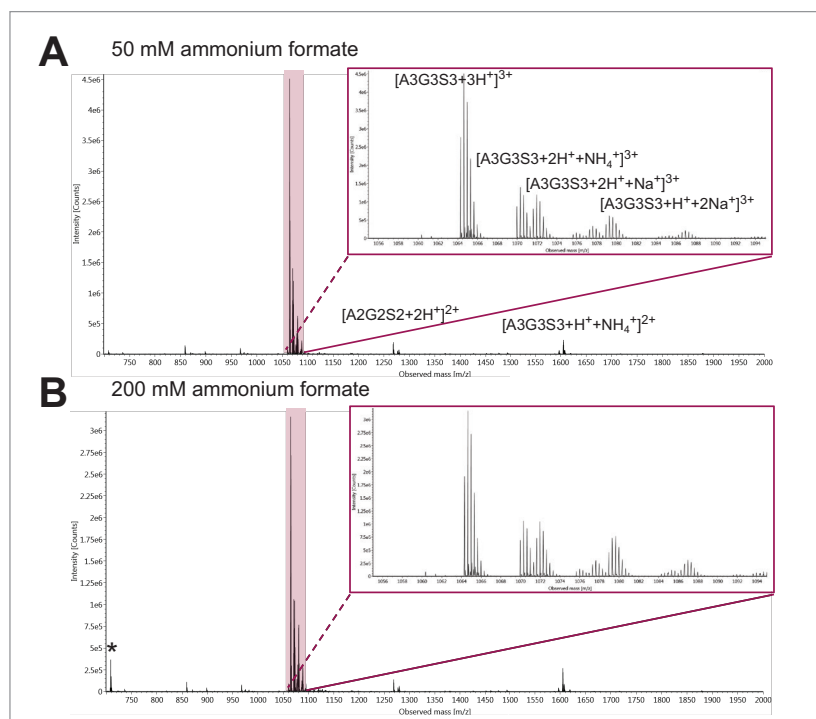

Figure 4. Comparing the quality of MS data as obtained with mobile phases of differing ionic strengths. LC-MS runs of RapiFluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column. Mass spectra of A3G3S3 were obtained by summing a 0.1 minute retention time window from the apex of the most abundant chromatographic peak from the LC-MS runs using mobile phases of different ionic strengths: 50 mM (A) versus 200 mM ammonium formate (B). Note: * labels a background ion resulting from the use of a high ionic strength mobile phase.
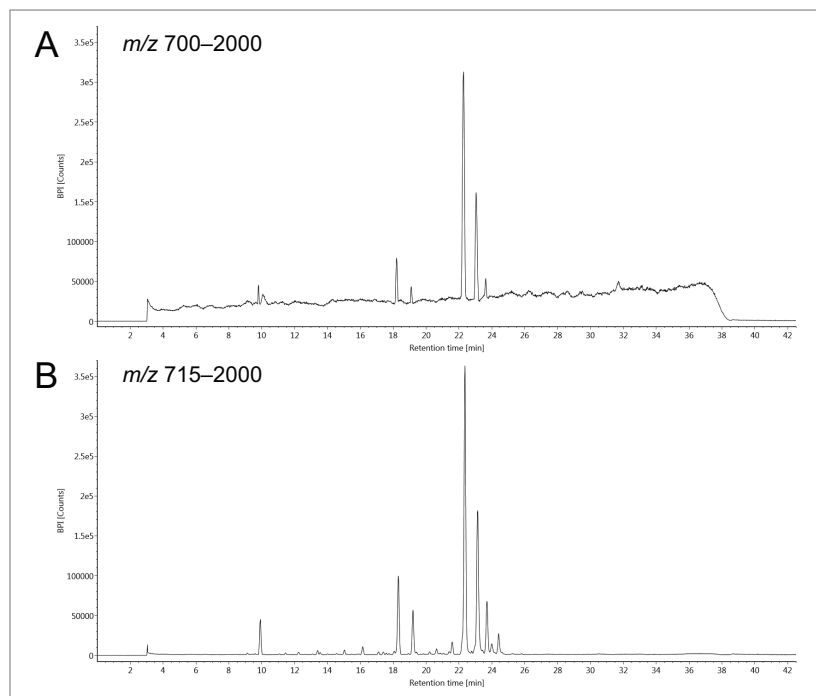

Figure 5. The effect of MS acquisition window on the signal-to-noise of a base peak intensity (BPI) chromatogram obtained with a high ionic strength mobile phase. HILIC-MS runs of RapiFluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column with a 200 mM ammonium formate aqueous mobile phase and MS acquisition windows of either m/z 700–2000 (A) or m/z 715–2000 (B).

As mentioned before, the *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard (p/n 186008660) is comprised of a series of *Rapi*Fluor-MS labeled sialylated N-glycans released from bovine fetuin. Though it contains various glycans at a wide range of relative abundances, it clearly has 7 predominant species from 3 glycan compositions, as labeled and displayed in Figure 6A. The glycan profile of bovine fetuin also contains a mix of both α2–3- and α2–6-linked sialic acids, which have been reported to be present at ratios ranging from 38:62 to 51:49, depending on the detection and sample preparation techniques used.[19-21] Exoglycosidases, enzymes that break the glycosidic bonds at the non-reducing ends of glycans, have been widely applied for structural elucidation of complex glycans. In this study, α2–3 Neuraminidase S and α2–3,6,8,9 Neuraminidase A were used as exoglycosidases to selectively release sialic acid residues from the glycans of the *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard. With this, a cursory level linkage elucidation assay can be demonstrated.

Before neuraminidase digestion, the majority of glycans in the *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard were found to be trisialylated (72%) and tetrasialylated (17%) triantennary glycans or disialylated biantennary glycans (11%) (Figure 6A). Overnight digestion with a neuraminidase specific to α2–3-linked sialic acids (α2–3 Neuraminidase S) resulted in a chromatographic profile with six abundant peaks from five glycan compositions: two with biantennary structures (12%; A2G2S1 and A2G2S2), and the other three with triantennary structures (88%; A3G3S1, A3G3S2, and A3G3S3) (Figure 6B). No tetrasialylated glycan peaks were seen to remain after α2–3 Neuraminidase S digestion. Under the assumption that α2–3 Neuraminidase S performs specific and complete cleavage of α2–3-linked sialic acids, it can be proposed that about two thirds of A2G2S2 contain one α2–3- and one α2–6-linked sialic acid residue and that one third contains two α2–6-linked sialic acids. The amounts of A2G2S2 with two α2–3-linked sialic acids appeared to be minimal. For triantennary N-glycans, it seems that the majority contain one (43%) or two (40%) α2–6-linked sialic acids. After overnight digestion with the broad specificity α2–3,6,8,9 Neuraminidase A, three chromatographic peaks from two glycan compositions were observed, biantennary A2G2 (11%) and triantennary A3G3 (89%) (Figure 6C). In sum, the overall ratio of α2–3- to α2–6-linked sialic acids observed herein is in agreement with the range reported in the literature, and these chromatographic profiles provide a reference for future studies with this method.

Peaks	RT (min)	Glycan	RA (%)
A1	18.45	A2G2S2	7.1
A2	19.35	A2G2S2	3.9
A3	22.47	A3G3S3	42.5
A4	23.25	A3G3S3	26.7
A5	23.84	A3S1G3S3	11.3
A6	24.12	A3G3S3	3.3
A7	24.56	A3S1G3S3	5.4

Peaks	RT (min)	Glycan	RA (%)
B1	16.33	A2G2S1	7.2
B2	18.91	A3G3S1	42.7
B3	19.39	A2G2S2	4.4
B4	20.34	A3G3S2	15.5
B5	21.65	A3G3S2	24.4
B6	22.91	A3G3S3	5.9

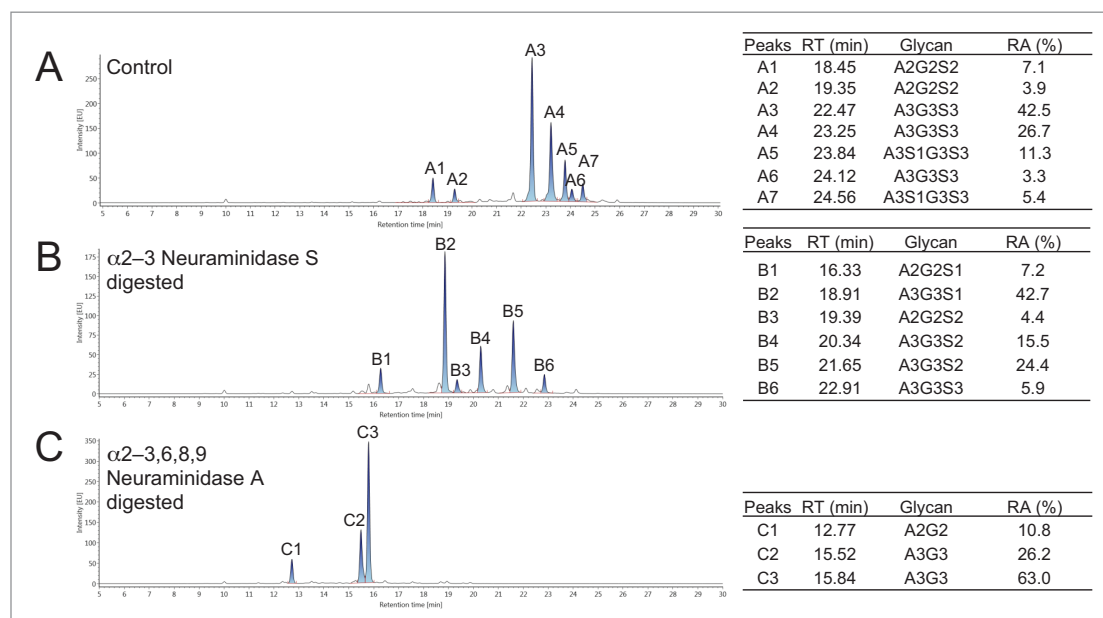| Peaks	RT (min)	Glycan	RA (%)
C1	12.77	A2G2	10.8
C2	15.52	A3G3	26.2
C3	15.84	A3G3	63.0

Figure 6. Use of neuraminidase digestions for cursory level sialic acid linkage elucidation. Fluorescence chromatograms were obtained for various preparations of the RapiFluor-MS Sialylated Glycan Performance Test Standard: without neuraminidase treatment (A), digested with α2-3 Neuraminidase S (B), and digested with α2-3,6,8,9 Neuraminidase A (C). Note: RA denotes relative abundance as calculated from the peak area for the component of interest divided by the summed peak area for all labeled components.

CONCLUSIONS

Using the *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard, we have optimized ESI-MS source parameters for improved ionization and detection of sialylated N-glycans. In addition, we have demonstrated an improvement in chromatographic resolution of sialylated N-glycans through the use of higher ionic strength mobile phases with a wide pore ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 μm Column. Moreover, it has been shown that these methods can be effectively combined with neuraminidase digestion to elucidate linkage specific information about the sialic acid content of a sample. Together, the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard, and wide-pore ACQUITY UPLC Glycoprotein BEH Amide Column establish a very useful collection of tools for characterizing sialylated N-glycans.

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