ANALYSIS OF SIALYLATED N-GLYCANS USING AN OPTIMIZED HILIC-FLUORESCENCE-MS METHOD

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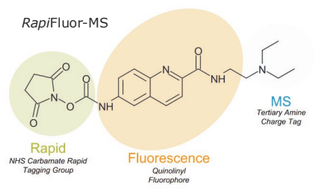
INTRODUCTION

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. With respect to therapeutics, sialic acid residues are of interest because they can impact serum half-lives and they can serve as indicators of cell culture conditions. *N*-Glycolylneuraminic acid (Neu5Gc), a non-human mammalian sialic acid with a structure similar to the human sialic acid *N*-acetylneuraminic acid (Neu5Ac), can contaminate biotherapeutic glycoproteins via production in non-human cell lines or from animal-derived media, which will result in different levels of immune responses in humans. Accordingly, sialylated glycan profiling is often needed to monitor a critical quality attribute related either to drug stability, activity, or immunogenicity. 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 a new approach for rapid release and labeling of N-glycans via *Rapi*Fluor-MS labeling enables enhanced sensitivity for fluorescence (FLR) and mass spectrometry (MS) based detection of all types of N-glycan species, including those that are sialylated.

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 (300 Å) 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), 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.

METHODS

SAMPLE DESCRIPTION:
*Rapi*Fluor-MS Sialylated Glycan Performance Test Standard (p/n 186008660), each vial contains 400 pmoles of *Rapi*Fluor-MS labeled released N-glycans from bovine fetuin, was reconstituted in 50 µL of water.



Neuraminidase 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 or α2-3,6,8,9 Neuraminidase A (New England BioLabs). After overnight incubation at 37 °C, 39 µL of water was added to the solution.

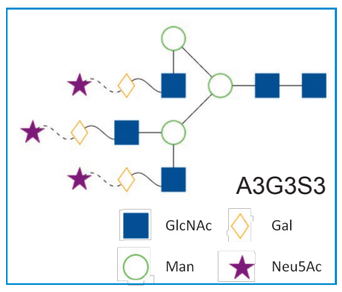
METHOD CONDITIONS:

LC Conditions:

LC system:	ACQUITY UPLC H-Class Bio System
Column Temp.:	60 °C
Flow Rate:	0.4 mL/min
Fluorescence Detection:	ACQUITY UPLC FLR; Excitation 265 / Emission 425 nm (2 Hz, Gain =1)
Columns:	ACQUITY UPLC Glycoprotein BEH Amide 300 Å 1.7 µm Column, 2.1 x 150 mm (p/n 176003702) ACQUITY UPLC Glycan BEH Amide 130 Å 1.7 µm, 2.1 × 50 mm (p/n 186004740)
Mobile phase A:	200 mM ammonium formate, pH 4.4 (LC-MS grade water)
Mobile phase B:	acetonitrile (LC-MS grade)
Mobile phase C:	water (LC-MS grade)
Gradient:	Initial 75% B, linear gradient from 75% to 54% B in 35 min. The ratios of aqueous mobile phase A and C were adjusted to the ionic strength of 50, 100, or 200 mM ammonium formate.

MS Conditions

MS system:	Xevo G2-XS QTof
Analyzer mode:	Resolution Mode (~40 K)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	600 L/Hr
Acquisition:	700–2000 m/z, 2 Hz



RESULTS

Optimization of ESI-MS Source Parameters to Improve the Ion Intensities of Protonated *Rapi*Fluor-MS Labeled Sialylated N-Glycans

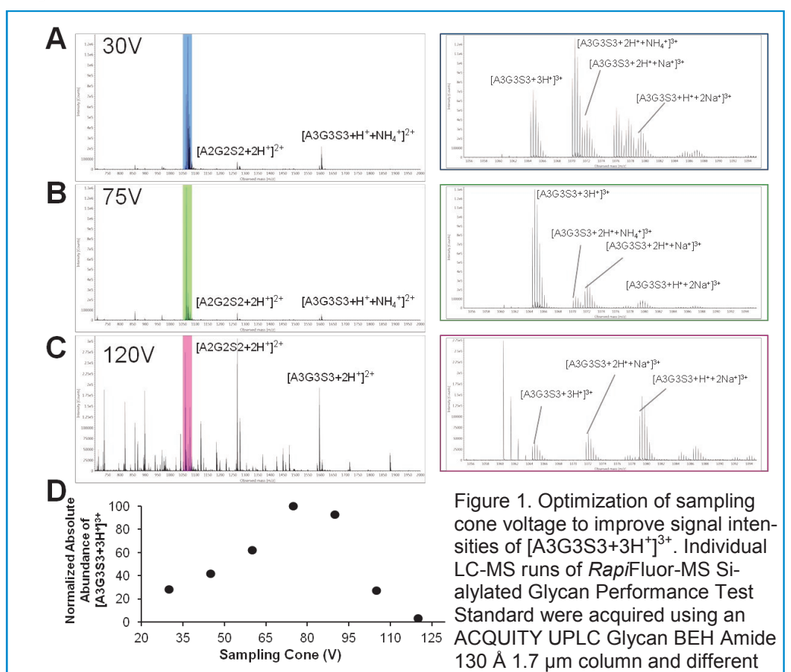


Figure 1. Optimization of sampling cone voltage to improve signal intensities of $[A3G3S3+3H^+]^{3+}$. Individual LC-MS runs of *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycan BEH Amide 130 Å 1.7 µm column and different sampling cone voltages. The mass spectra were obtained by summing a 0.1 min retention time window from the apex of the most abundant chromatographic peak from the LC-MS runs with different sampling cone voltage settings, e.g. 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 75V and graphed versus the sampling cone voltages (D).

RESULTS AND DISCUSSION

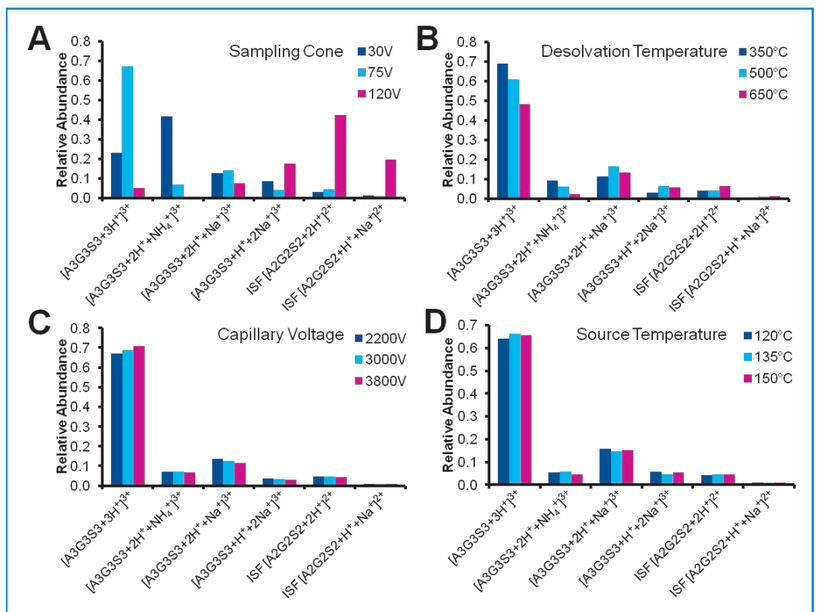
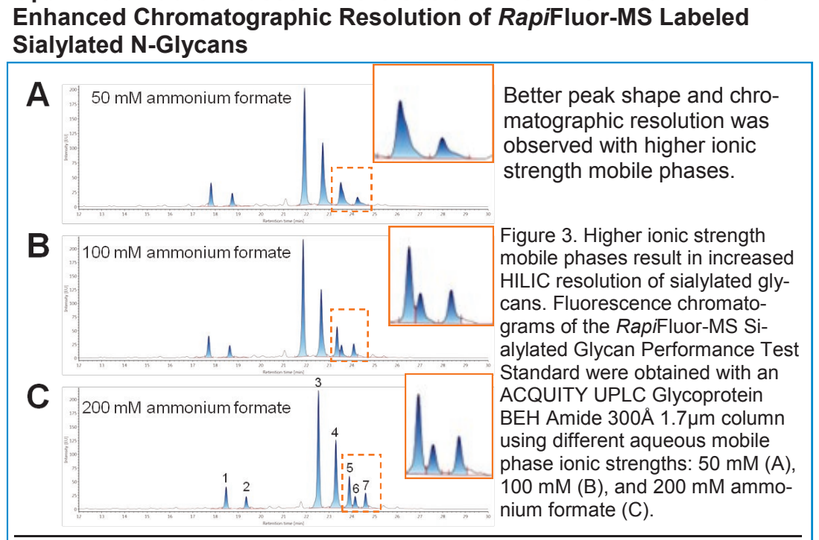


Figure 2. Optimization of ESI-MS source parameters to improve signal intensities of $[A3G3S3+3H^+]^{3+}$. Individual LC-MS runs of *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycan BEH Amide 130 Å 1.7 µm column and different sampling cone voltages (A), 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.

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. 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.

Optimized Mobile Phases and the Use of Wide-Pore Amide HILIC for Enhanced Chromatographic Resolution of *Rapi*Fluor-MS Labeled Sialylated N-Glycans



Better peak shape and chromatographic resolution was observed with higher ionic strength mobile phases.

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

Peak	Name	*Rapi*Fluor-MS Labeled Glycan Composition	M_i (Da)	Monoisotopic 2+	Monoisotopic 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

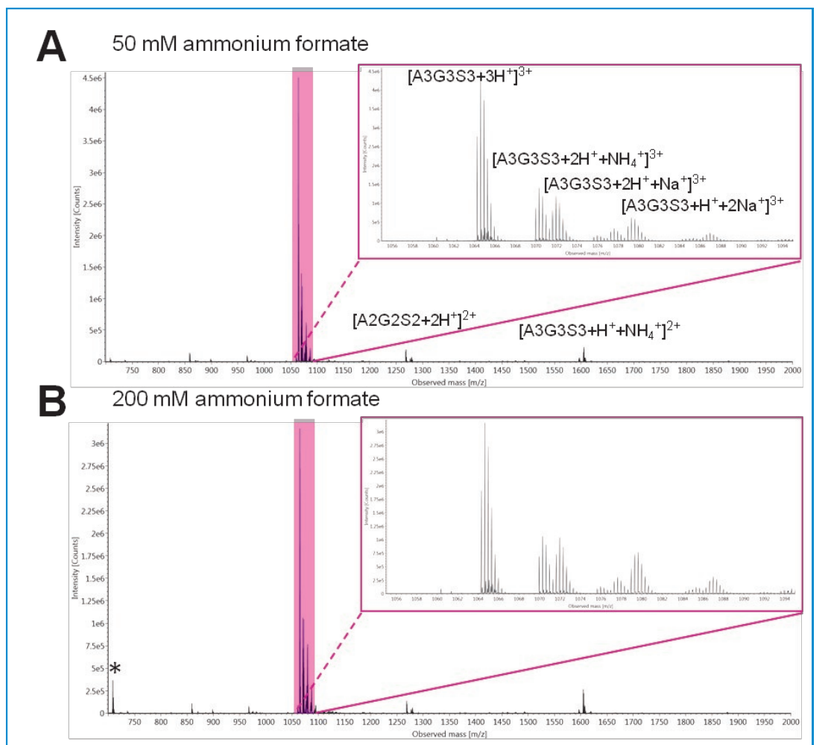


Figure 4. Comparing the quality of MS data as obtained with mobile phases of differing ionic strengths. LC-MS runs of *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycoprotein BEH Amide 300 Å 1.7 µm column. Mass spectra of A3G3S3 were obtained by summing a 0.1 min 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.

The increased background ion intensities from the higher ionic strength mobile phase obscured the glycan profile. To circumvent this, the acquisition window was narrowed to 715 to 2000 *m/z*.

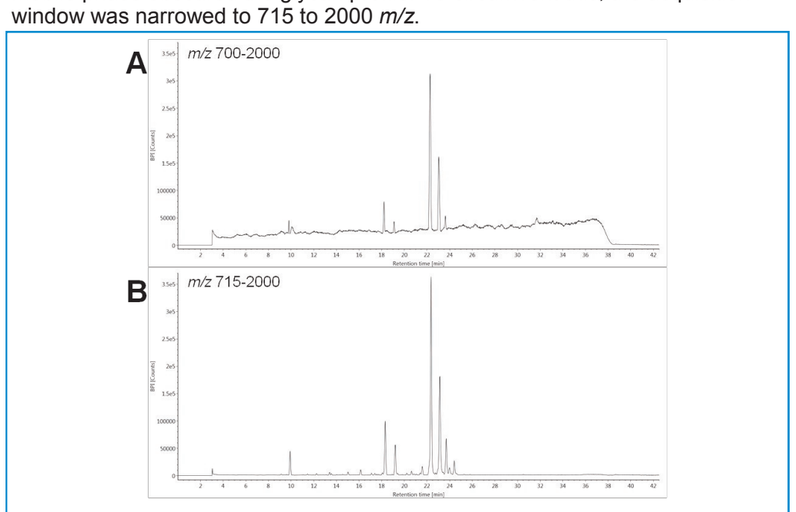
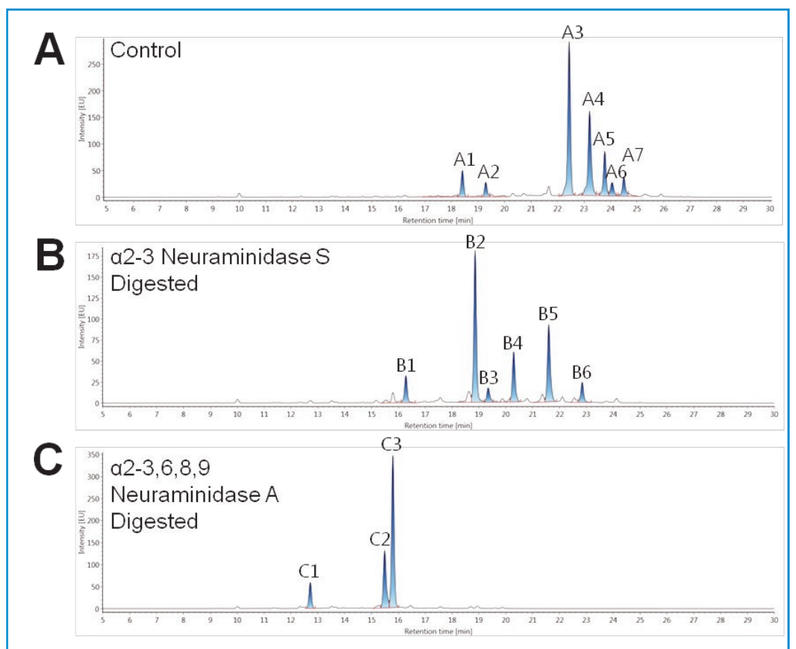


Figure 5. The effect of MS acquisition window on the signal-to-noise of a base peak intensity (BPI) chromatogram obtained with a high ionic strength mobile phase. HILIC-MS runs of *Rapi*Fluor-MS Sialylated Glycan Performance Test Standard were acquired using an ACQUITY UPLC Glycoprotein BEH Amide 300 Å 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).

Ultimately, 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 abundance glycans.

Use of Neuraminidases for Cursory Level Sialic Acid Linkage Elucidation



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 *Rapi*Fluor-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.

The overall ratio of α2-3- to α2-6-linked sialic acids observed herein for bovine fetuin N-glycans is in agreement with the range reported in the literature, and these chromatographic profiles provide a reference for future studies with this method.

CONCLUSIONS

Through detailed analyses of *Rapi*Fluor-MS labeled N-glycans derived from bovine fetuin, 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 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 demonstrated methods showcase a very useful collection of tools for characterizing sialylated N-glycans.

References

1. Lauber, M. A.; Yu, Y. Q.; Brousmiche, D. W.; Hua, Z.; Koza, S. M.; Magnelli, P.; Guthrie, E.; Taron, C. H.; Fountain, K. J., Anal Chem 2015, 87 (10), 5401-9.
2. Wang, Q.; Lauber, M. A., Optimizing HILIC-based Analyses of *Rapi*Fluor-MS Labeled Sialylated N-Glycans. Waters Application Note 720005850EN December 2016.

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