Supporting information

Site-specific mapping of sialic acid linkage isomers by ion mobility spectrometry

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Table S1: Pattern of sialic acid linkages in α1AGP (top) and Fetuin (bottom) glycopeptides

Peptide	Glycan	α2-6	α2-3
NEEYNK	$Hex_5HexNAc_4NeuAc_2$	94.9%	5.1%
(34-39)	$Hex_6HexNAc_5NeuAc_3$	77.7%	22.3%
ENGTISR	$Hex_5HexNAc_4NeuAc_2$	86.6%	13.4%
(84-90)	$Hex_6HexNAc_5NeuAc_3$	65.9%	34.1%
	$Hex_7HexNAc_6NeuAc_4$	39.5%	60.5%
QDQCIYNTTYLNVQR	$Hex_5HexNAc_4NeuAc_2$	76.4%	23.6%
(69-83)	$Hex_6HexNAc_5NeuAc_3$	79.8%	20.2%
	$Hex_7HexNAc_6NeuAc_4$	42.9%	57.1%
LVPVPITNATLDR	$Hex_5HexNAc_4NeuAc_2$	86.3%	13.7%
(8-20)	$Hex_6HexNAc_5NeuAc_3$	73.6%	26.4%
YFTPNKTEDTIFLR	$Hex_5HexNAc_4NeuAc_2$	95.7%	4.3%
(50-63)	$Hex_6HexNAc_5NeuAc_3$	76.8%	23.2%
	$Hex_7HexNAc_6NeuAc_4$	40.6%	59.4%
LVPVPITNATLDQITGK	$Hex_5HexNAc_4NeuAc_2$	89.6%	10.4%
(8-24)	$Hex_6HexNAc_5NeuAc_3$	82.0%	18.0%

Peptide	Glycan	α2-6	α2-3	α2-6-GlcNAc
LCPDCPLLAPLNDSR	$Hex_5HexNAc_4NeuAc_2$	71.1%	28.9%	0.0%
(127-141)	$Hex_6HexNAc_5NeuAc_3$	54.5%	45.5%	0.0%
	$Hex_6HexNAc_5NeuAc_4$	78.4%	18.1%	3.5%
VVHAVEVALATFNAE…	$Hex_5HexNAc_4NeuAc_2$	58.7%	41.3%	0.0%
…SNGSYLQLVEISR	$Hex_6HexNAc_5NeuAc_3$	54.2%	45.2%	0.6%
(142-169)	$Hex_6HexNAc_5NeuAc_4$	72.8%	22.0%	5.2%
RPTGEVYDIEIDTLETT…	$Hex_5HexNAc_4NeuAc_2$	76.8%	23.2%	0.0%
…CHVLDPTPLANCSVR	$Hex_6HexNAc_5NeuAc_3$	55.5%	44.5%	0.0%
(54-85)	$Hex_6HexNAc_5NeuAc_4$	76.6%	18.7%	4.6%

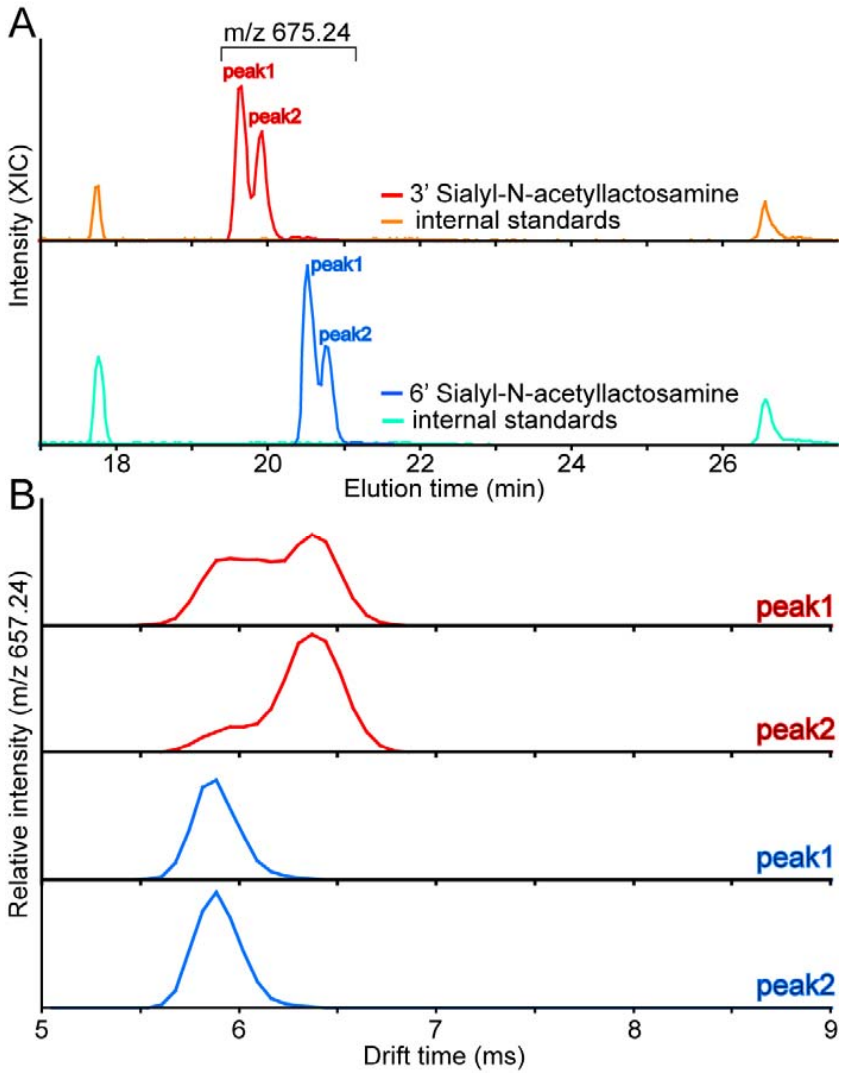

Figure S1: HILIC purification of 3' and 6' sialyl-N-acetyllactosamine (NeuAc-Gal-GlcNAc). **A)** The elution of each species was monitored for the protonated precursor ion at m/z 675.24. The doublet peaks for each species is due to separation of the α/β anomers. Internal standards (cyan and orange traces) were used to ensure that elution conditions were reproducible between the two LC runs. **B)** The ion mobility arrival time distributions for each eluting peak of the $[M\text{-}H_2O+H]+$ in source decay fragment (m/z 657.24) are shown for 3' (red) and 6' (blue) sialyl-N-acetyllactosamine.

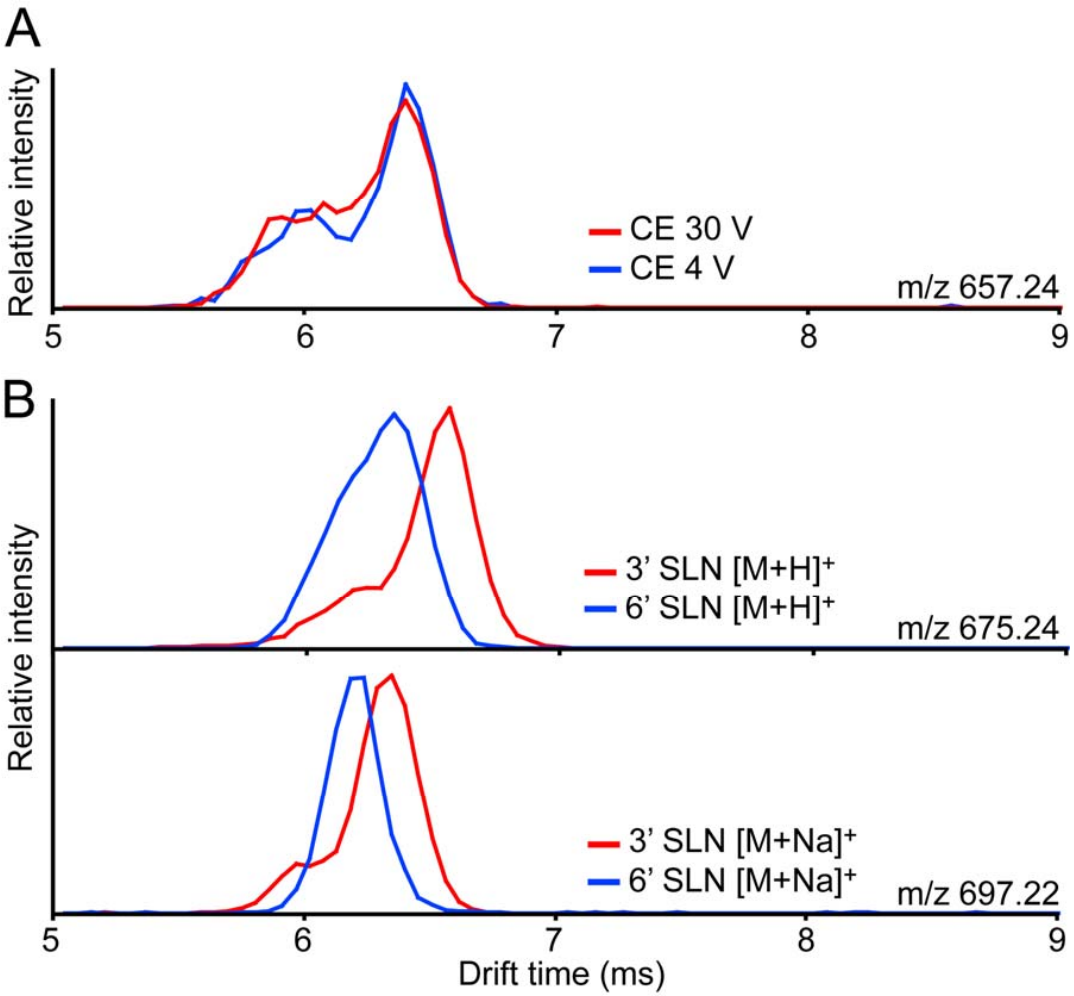

Figure S2: A) ATD of 3' SLN $[M\text{-}H_2O+H]^+$ fragment at a collision energy of 4 V (blue) or 30 V (red). **B)** ATD of the intact protonated (top) and sodium adduct $[M+Na]^+$ (bottom) of 3' SLN (red) and 6' SLN (blue). The broadened peaks for the protonated species likely arise from both the α/β anomers that are present in solution.

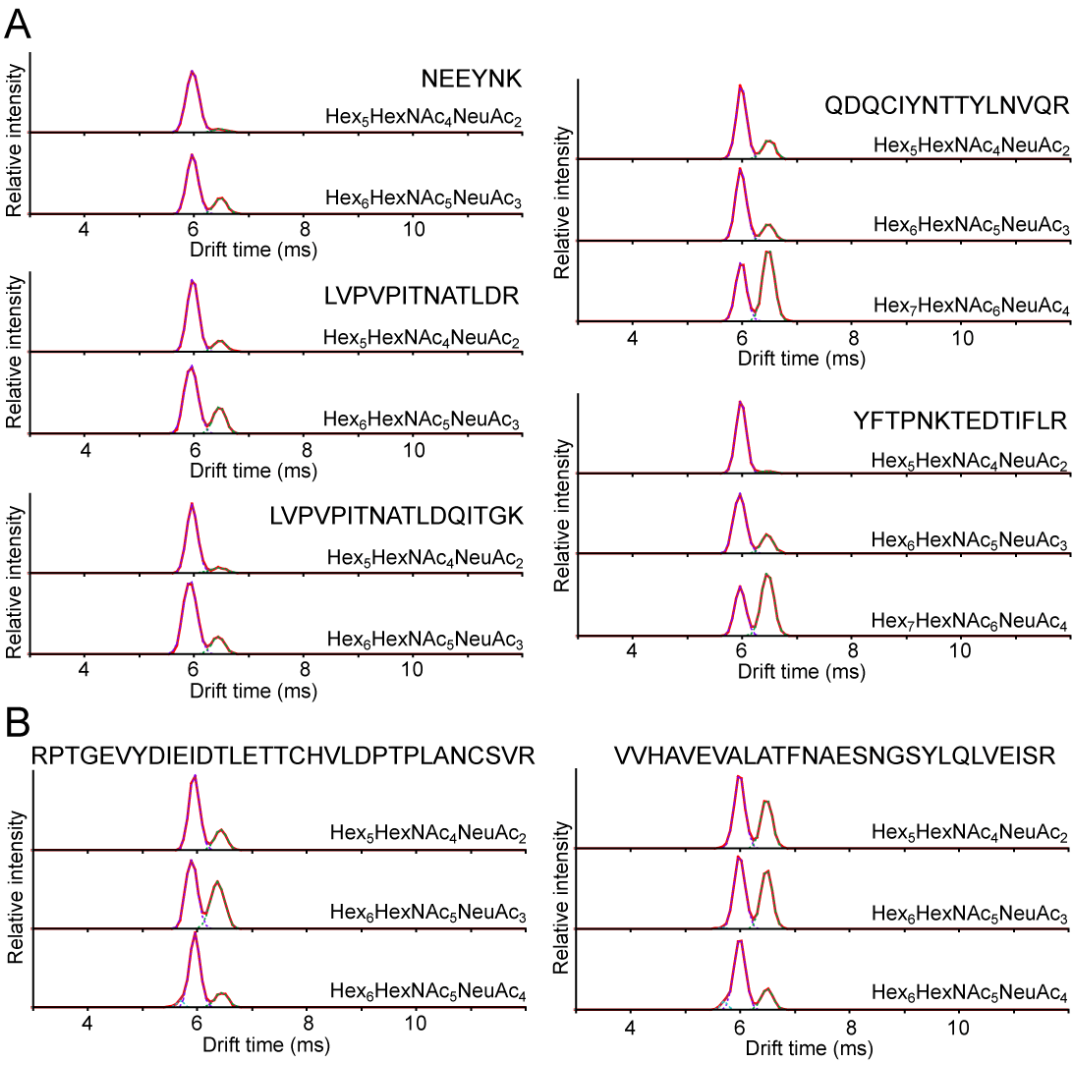

Figure S3: ATDs of siayl-N-acetyllactosamine fragments (m/z 657.24) from α1AGP **(A)** and Fetuin **(B)** glycopeptides. Dashed lines show Gaussian fits used to quantify the each peak, and the results are summarized in table S1. Glycan compositions are indicated in each panel.

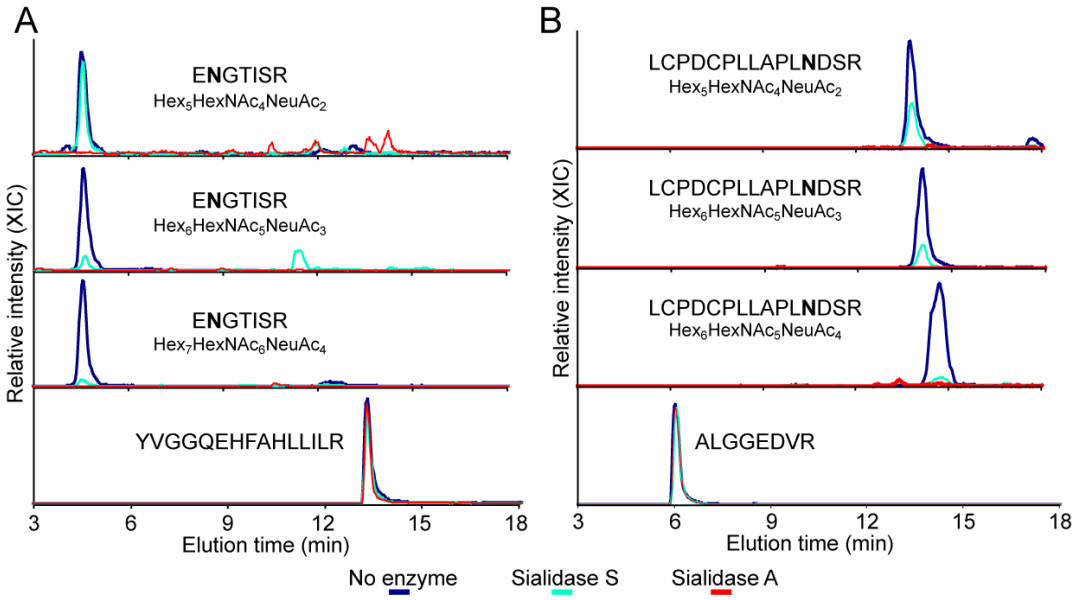

Figure S4: Extracted ion chromatograms of glycopeptides after treatment with sialidases. **A)** Ion chromatogram for α1AGP glycopeptide with a biantennary bisialo (1st panel), triantennary trisialo (2nd panel) and tetraantennary tetrasialo (3rd panel) glycan is shown before any sialidase treatment (blue), after sialidase S treatment (cyan) to cleave α2-3 sialic acids, and sialidase A treatment to cleave both α2-3 and α2-6 sialic acids (red). **B)** Ion chromatogram traces for Fetuin glycopeptide with a biantennary bisialo (1st panel), triantennary trisialo (2nd panel) or triantennary tetrasialo (3rd panel) glycan after sialidase treatments. The last panels show the intensity of a non glycosylated tryptic peptide, which indicate that the overall signals are comparable.

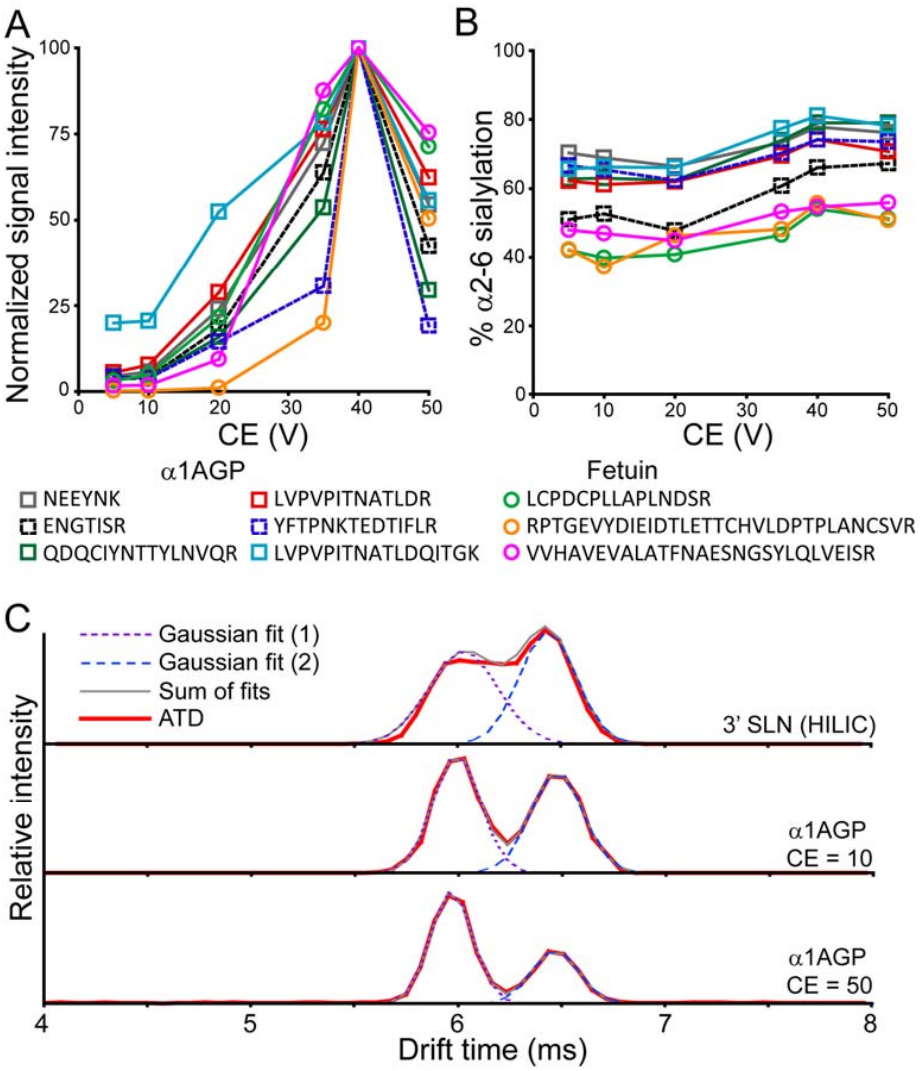

Figure S5: Effect of the collision energy (CE) on the observed sialylation linkage by IMS. **A)** Normalized signal intensity for the SLN fragment (m/z 657.24) as a function of collision energy from 5 V up to 50 V from α1AGP (squares) and Fetuin (circles) peptides with triantennary trisialo glycans. **B)** The percentage of α2-6 sialylation based on the relative intensities of the 3' and 6' SLN fragments at different collision energies. **C)** Gaussian fits to the ATD (m/z 657.24) of the first peak of 3' SLN standard during HILIC purification (top), and from the triantennary trisialo α1AGP glycopeptide (residues 84-90) at a collision energy of 10 V (middle) or 50 V (bottom).