Electronic Supplementary Material (ESI) for Analyst.

N-glycosylation profiling of selected intact proteins by high-resolution mass spectrometry (MS) and glycan analysis using ion mobility-MS/MS

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Instrumental parameters and procedure overview

Table S1. Full scan ionization and MaxEnt1 deconvolution parameters for the analysis of intact TFN and TRA.

Protein	ToF mode	Capillary (kV)	Cone (V)	Source Offset (V)	Source T (°C)	Desolvation T (°C)	Acquired scan (scan)	Mass range selection (m/z)	Charge states selection	Dec. interval (kDa)	Dec. resolution (Da/channel)
TFN	Res	3	120	80	120	450	5-70	1500-3800	+24/+49	77-83	0.1
TRA	Res	3	120	80	120	450	5-70	2200-4500	+37/+68	147-152	0.1

Table S2. Full scan ionization and acquisition parameters for released neutral and sialylated glycans.

Glycan class	Acquisition mode	Capillary (kV)	Cone (V)	Source Offset (V)	Source T (°C)	Desolvation T (°C)	Cone gas (l/h)	Desolvation gas (l/h)	Nebulizer (bar)	Acquisition rate (scan/sec)
Sialylated	Negative	3	120	80	120	450	100	700	5	0.5
Neutral	Positive	3	80	80	120	450	100	800	5	0.5

Table S3. IMS separation and MS-MS fragmentation parameters for released neutral and sialylated glycans.

Glycan class	IMS Wave velocity (m/s)	IMS Wave height (V)	Transfer Wave velocity (m/s)	Transfer Wave height (V)	Trap gas (mL/min)	Helium cell gas (mL/min)	IMS cell gas (mL/min)	Collision energy (V)
Sialylated	800	35	250	10	2.5	150	110	53-73
Neutral	700	40	250	10	2.5	180	90	58-60 112-140*

*Collision energy used for sodiated neutral glycans

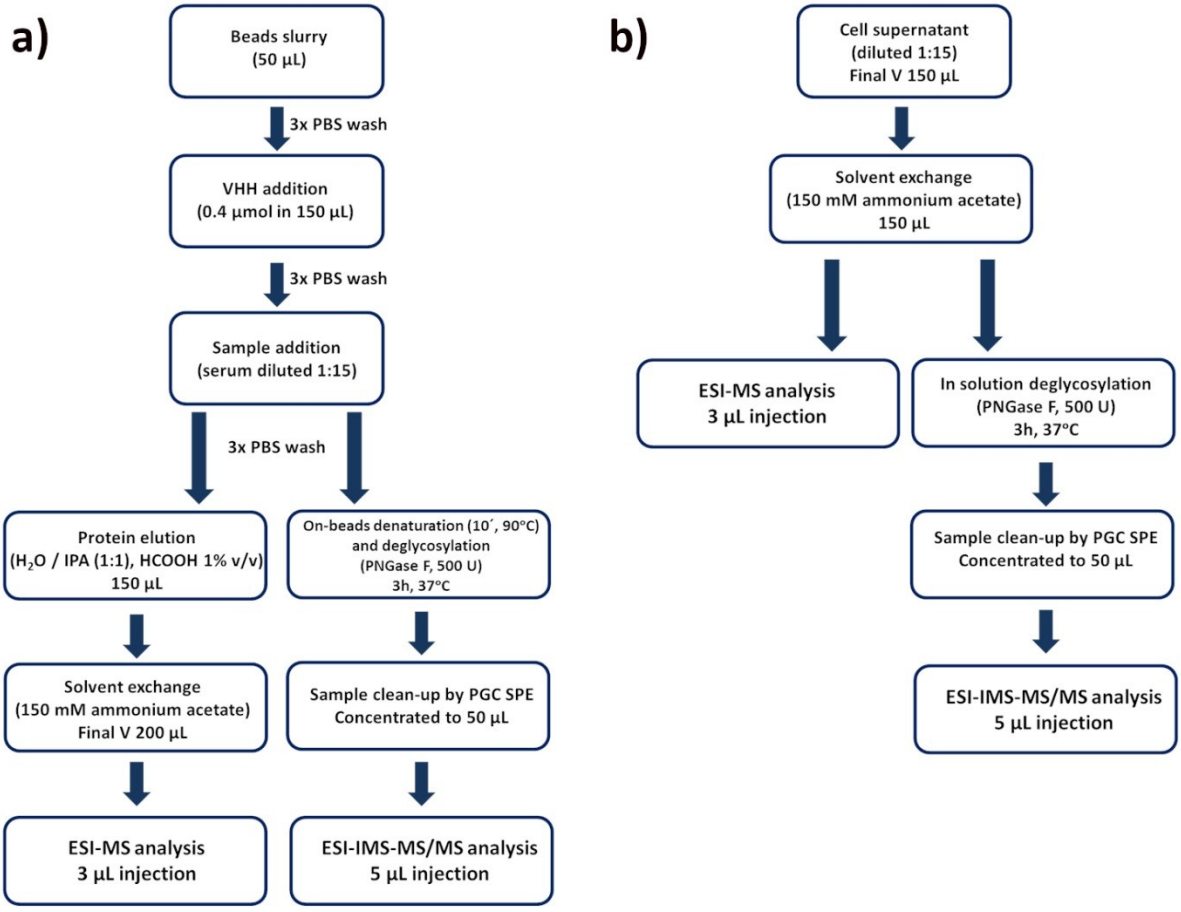


Figure S1. a) Schematic overview for the immunoaffinity purification of TFN from serum and the subsequent parallel analysis of intact proteins by ESI-MS and released glycans by ESI-IMS-MS/MS and **b)** schematic overview of the analytical approach for the analysis of TRA, at the intact protein level by ESI-MS, and of its released glycans by ESI-IMS-MS/MS.

Intact proteins linearity and negative controls

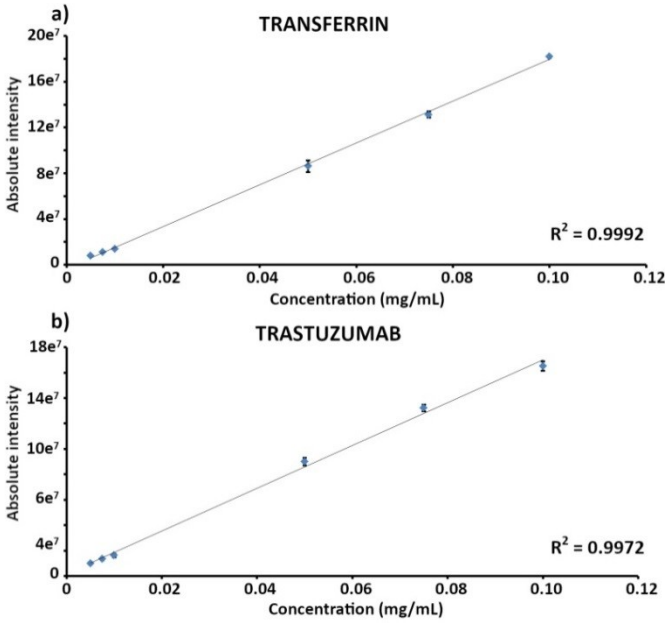


Figure S2. Calibration curves for **a)** TFN and **b)** TRA.

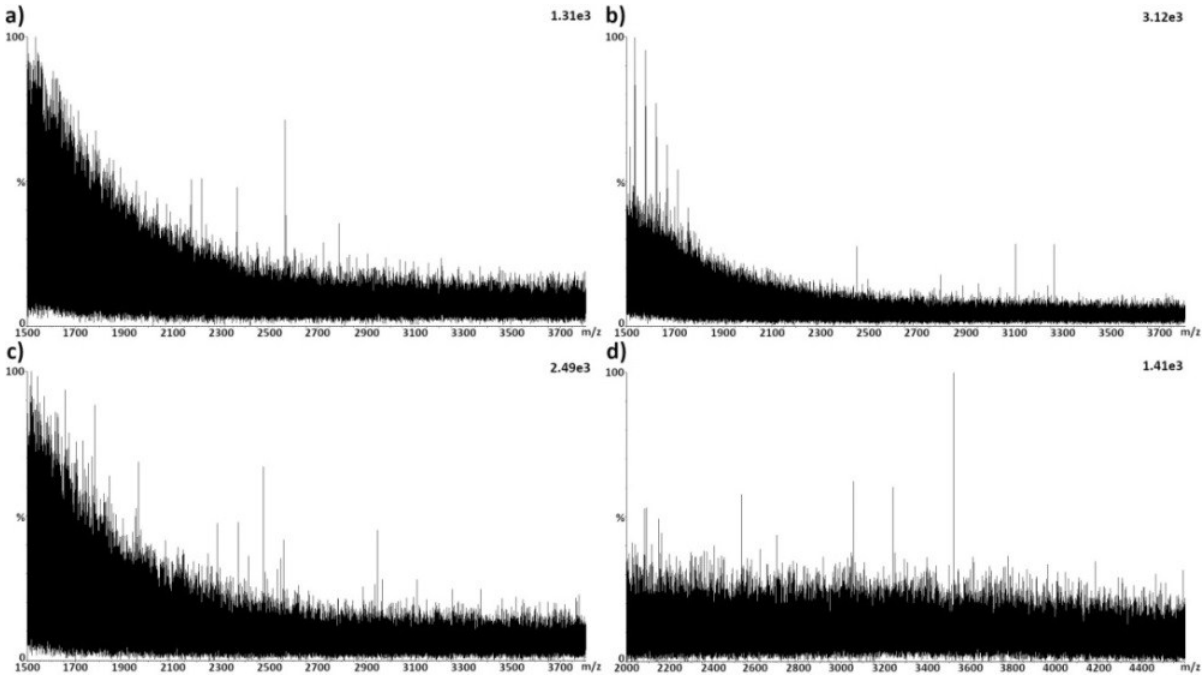


Figure S3. Representative spectra for the negative control experiments for intact TFN and TFN. **a)** Human serum subjected to the analytical procedure on non-derivatized magnetic beads, used as negative control for TFN; **b)** Mouse serum subjected to the analytical procedure on magnetic beads functionalized with the anti-TFN VHH, negative control for TFN. Peaks appearing in the 1500-1900 m/z region were singly-charged and showed a

regular spacing of 44 m/z, suggesting PEG origin (-CH_2CH_2O-); **c)** Mouse serum spiked with human serum albumin and TRA, subjected to the analytical procedure on magnetic beads coupled with the anti-TFN VHH, used as negative control for TFN; **d)** Non-spiked cell supernatant from non-transfected cells, used as negative control for TRA. All the spectra were acquired and the data treated as illustrated in table S1.

TFN: glycoforms identification and structure elucidation

Table S4. Sugar composition of the glycans associated with the target intact glycoproteins, theoretical mass, mass measured by deconvolution, and mass accuracy expressed as relative error in ppm. Theoretical mass values were obtained by the addition of glycan masses obtained with the Glycoworkbench software to the mass of the deglycosylated protein. Measured masses are average values of three standard replicates analyzed in triplicate. All the spectra were acquired and the data treated as illustrated in table S1. Mass accuracy is calculated as relative error between theoretical and measured masses and is expressed in ppm. Sugar composition abbreviations: H, Hexose; N, N-acetyl hexose; S, Sialic acid; F, Fucose.

Glycoform	Identified glycans	Theoretical mass (Da)	Measured mass (Da)	Mass accuracy (RE, ppm)
TFN 0	H5N4S2	77349.2	77348.3 ± 2.1	11.2
TFN 1	H5N4S2; H5N4S2	79555.0	79555.1 ± 1.3	-2.0
TFN 2	H5N4S2; H5N4S2F	79701.0	79701.6 ± 3.1	-7.5
TFN 3	H5N4S2; H6N5S3	80211.2	80211.3 ± 0.7	-0.9
TFN 4	H5N4S2; H6N5S3F	80357.2	80356.6 ± 1.0	7.7
TRA a	H3N3F; H3N4F	147852.2	147851.5 ± 0.9	4.5
TRA b	H3N4; H3N4F	147909.2	147907.4 ± 1.3	12.4
TRA c	H4N3F; H3N4F	148014.3	148011.8 ± 0.6	21.5
TRA 1	H3N4F; H3N4F	148055.3	148055.7 ± 0.4	-2.8
TRA 2	H4N4F; H3N4F	148217.3	148216.9 ± 0.5	2.5
TRA 3	H4N4F; H4N4F	148379.4	148378.5 ± 0.5	6.0
TRA d	H5N4F; H4N4F	148541.0	148539.6 ± 0.4	12.6
TRA e	H5N4F; H5N4F	148703.5	148700.8 ± 0.7	18.5

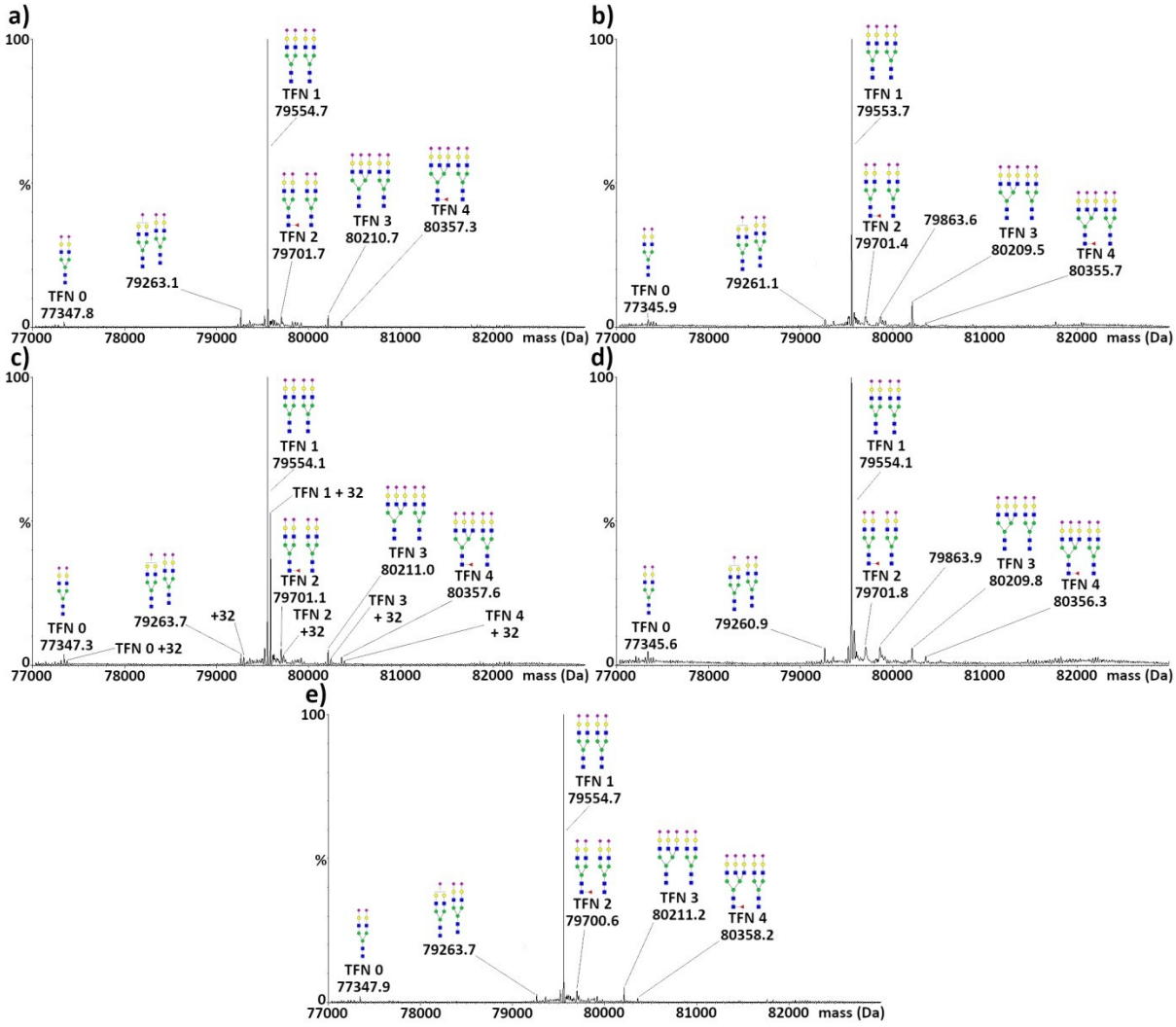


Fig.S4. Representative deconvoluted MS spectra for TFN purified from the 5 target serum samples, showing differences in the levels of expressed glycoforms, and identification of other forms not found in standard TFN. **a)** serum S1; **b)** serum S2; **c)** serum S3, with stress on the +32 protein peaks; **d)** serum S4; **e)** serum S5.

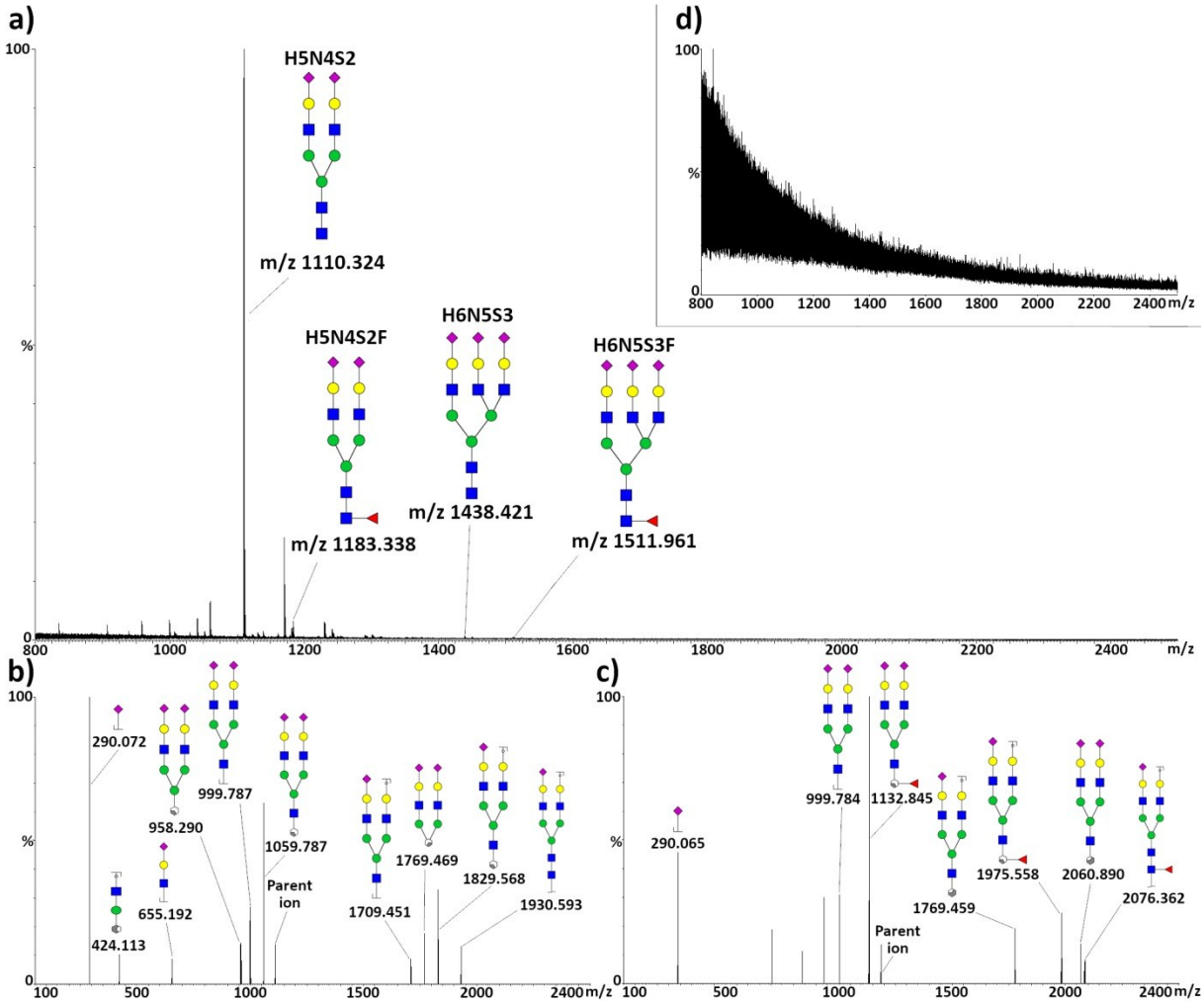
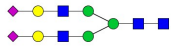
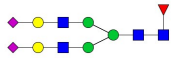
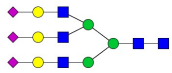
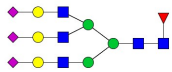
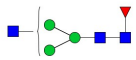
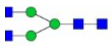
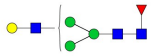
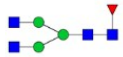
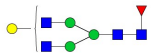
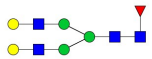


Figure S5. ESI-IMS-MS/MS structural elucidation of enzymatically released glycans from TFN, purified from individual S3. **a)** Fullscan showing the four identified glycan species; **b)** Tandem MS fragmentation of m/z 1110.324 (H5N4S2 glycan) showing the identity of fragments, using an acquisition threshold ca. 5% of the intensity of the base peak; **c)** Tandem MS fragmentation of m/z 1183.338 (H5N4S2F glycan) showing the identity of fragments, using an acquisition threshold of ca. 10% of the intensity of the base peak; **d)** Procedural blank: Serum of individual S3 after incubation on beads not derivatized with the anti-TFN VHH. Fullscan showing the total absence of glycans signals.

Monosaccharide symbols follow the SNFG (Symbol Nomenclature for Glycans): N-acetylglucosamine (GlcNAc): blue square; Mannose (Man): green circle; Galactose (Gal); yellow circle; Fucose (Fuc): red triangle; Sialic acid (Neu5Ac): purple diamond.

Table S5. Identification of the glycans released from the target proteins. Structure obtained from the composition and from MS/MS experiments; theoretical m/z value were calculated by using the Glycoworkbench software; mass accuracy is expressed as relative error in ppm between measured and theoretical mass value.

TFN	Sugar composition	Ionization form	Measured mass (m/z)	Theoretical mass (m/z)	Mass accuracy (RE, ppm)
	H5N4S2	$[M-2H]^{2-}$	1110.324	1110.384	54.2
	H5N4S2F	$[M-2H]^{2-}$	1183.324	1183.413	75.4
	H6N5S3	$[M-2H]^{2-}$	1438.421	1438.615	-81.3
	H6N5S3F	$[M-2H]^{2-}$	1511.654	1511.527	-84.0

TRA	Sugar composition	Ionization form	Measured mass (m/z)	Theoretical mass (m/z)	Mass accuracy (RE, ppm)
	H3N3F	$[M+Na]^+$	1282.454	1282.454	0.2
	H3N4	$[M+Na]^+$	1339.476	1339.476	-0.2
	H4N3F	$[M+Na]^+$	1444.407	1444.507	69.3
	H3N4F	$[M+Na]^+$	1485.534	1485.534	-0.2
	H4N4F	$[M+Na]^+$	1647.685	1647.587	-60.0
	H5N4F	$[M+H]^+$	1787.198	1786.755	-248.0

TRA: Glycoforms quantification in spiked cell supernatant

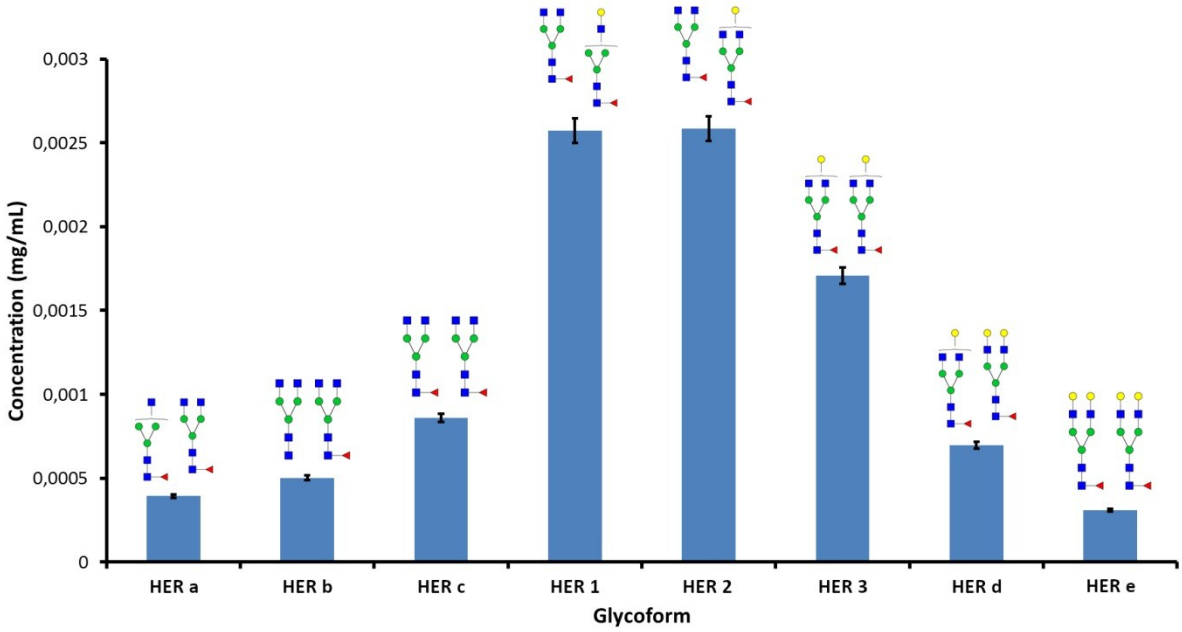


Figure S6. Different glycoforms identified on the two TRA glycosylation sites and related concentration values obtained after quantification of spiked standard protein in non-transfected matrix. Each value is the average of triplicate samples analyzed in triplicates (n=3). Error bars expressed as standard deviation of the average concentration values.

IMS: Effect of sodium coordination on TRA glycans

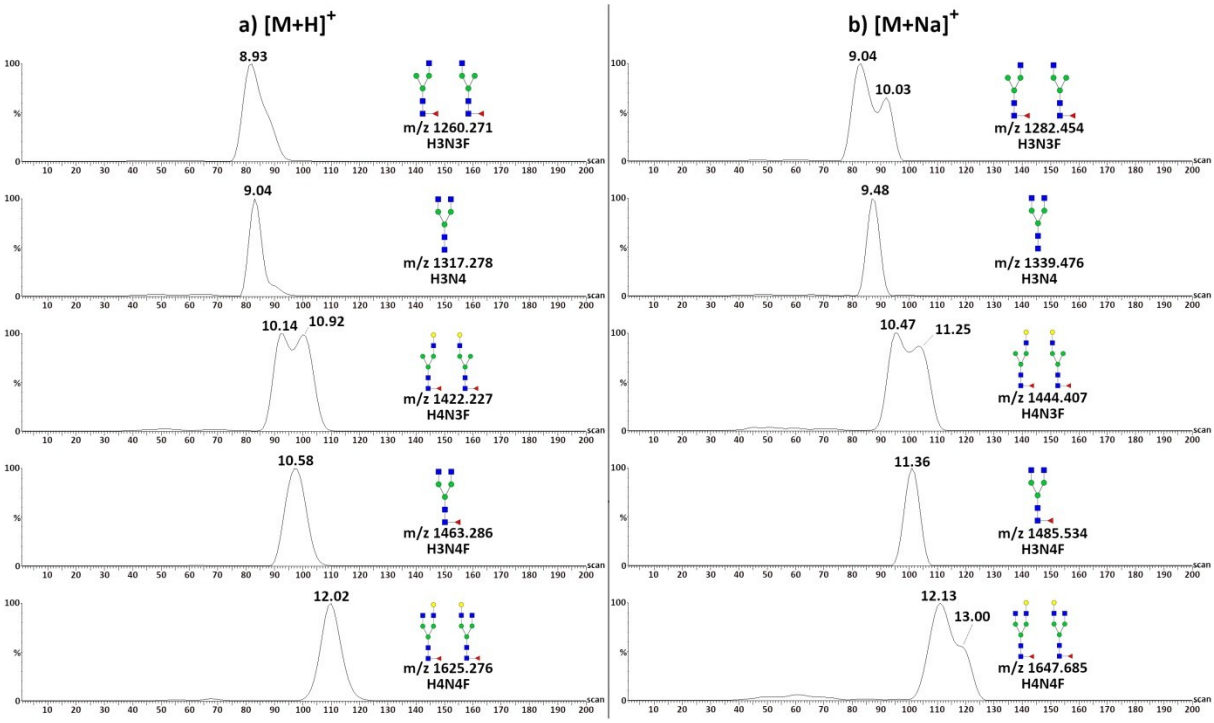


Figure S7. Effect of sodium coordination on the mobility of glycans released from standard TRA. **a)** Mobilograms of singly charged protonated species, ionized in positive mode; **b)** mobilograms of singly charged sodiated species, ionized in positive mode. Sodium coordination had the effect of slowing the glycans in the IMS cell under the present conditions, as well as increasing differences in the mobility of isomeric asymmetric species, such as H3N3F, H4N3F, and H4N4F. Data related to the H5N4F glycan are not shown as the related sodium adduct had too low intensity.