

Supplementary Material (ESI) for Analyst
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Supporting Information

Non-derivatized Glycan Analysis by Reverse Phase Liquid Chromatography and Ion Mobility-Mass Spectrometry

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Abstract: A simple method for the analysis of non-derivatized glycans using a reverse phase column on a liquid chromatography- ion mobility- mass spectrometry (LC-IM-MS) instrument. Methodology supports both glycomic and proteomic work without the necessity of switching columns.

Experimental Details

A 1 mg mL⁻¹ sample of bovine fetuin (Sigma Aldrich) was prepared in 10 mM sodium phosphate buffer solution to a final volume of 100 μ L. To denature the protein, the sample was heated to 90°C for 10 minutes and subsequently cooled to room temperature. While alkylation and reduction is recommended, it was not necessary as sufficient cleavage of the glycan was achieved for this experiment with only denaturing by heat. The sample was then treated with 10 μ L of 500 units/mL PNGaseF (Sigma Aldrich) followed by incubation at 37 °C for 24 hours. To quench PNGaseF activity, the sample was heated to 90°C for 5 minutes followed by 15 minutes in the -20°C freezer. The sample was dried down by a vacuum concentrator (speedvac) and reconstituted in approximately 250 μ L of 100% ACN with 0.1% TFA and remaining protein content was precipitated by centrifugation. The glycan containing solvent (~150-200 μ L) was transferred to an LC maximum recovery vial. It should be noted that removal of protein content is an optional step for IM-MS analysis as the protein will not occupy the same region of conformational space as the glycans.

Fetuin samples were analyzed using an Acquity UPLC system (Waters, Milford, MA) coupled to the ESI source of a Synapt G2 HDMS instrument using a Waters HSS C18 column (1.8 μ m, 1.0 x 100 mm) stored at 40°C. Samples are loaded into an autosampler which is held at 4°C. The LC solvents are prepared such that solvent A consists of 100% H₂O with 0.1% TFA and solvent B consists of 100% ACN with 0.1% TFA. Alternately, buffered solvents can be used to make this method compatible with the analysis of less stable samples. The maltose sample contained 10 μ g mL⁻¹ of each M1, M3-M7 in 10 mM ammonium acetate with 0.1% formic acid. The chromatographic separation used 100% H₂O with 0.1% formic acid and 100% ACN with 0.1% formic acid as solvents A and B, respectively. Sample can also be spiked with small amounts of NaI or NaCl to induce ionization preference of the sodiated species. The 20 minute method starts with 100% solvent B and linearly transitions to 100% solvent A over the course of 12 minutes and held for 3 minutes for at 60 μ L min⁻¹ with an injection volume of 5 μ L. The flow is then reversed to initial conditions for the remainder of the experiment. IM-MS data is collected in positive resolution mode over a mass range of 100-4000 Da using the following instrument conditions; 3.5 kV capillary, 80°C source temperature, 40 V sampling cone, 2 V extraction cone, and 150 °C desolvation temperature. The traveling wave velocity was set to 650 m s⁻¹ and wave height to 40 V for mobility separation. Fragmentation data was collected post-mobility in the transfer region with a collision energy ramp from 5-30 eV in a MS^E experiment. The incorporation of MS/MS to this method allows for the potential of carbohydrate identification by

fragmentation. When this capability is employed in a secondary function during the LC run, fragmentation spectra is automatically acquired and can be customized using a targeted precursor mass lists or various CID voltage ramps dependent on the type of analysis required. A key advantage to fragmentation post-mobility is related to the alignment of fragment peaks as they retain the mobility of their respective precursor.^{1,2} Lock mass correction was applied using Leucine-enkephalin to maintain high mass accuracy. Data is processed off line with Driftscope software (Waters, Milford, MA) which allows mobility selection of regions of the IM-MS data that pertain to carbohydrate signals.

It should be noted that signal abundances in Figure 2 and 3 describe peak intensities relative to the base peak in the spectrum. Figure 2 (c) and (d) display relative ion abundances from 300-1600 m/z such that the base peak of the spectrum represents a relative abundance of 100%. The relative abundance of the base peak in 2(c) was 2.93×10^4 and in 2(d) 3.78×10^4 . Figure 3(b) and (c) display relative ion abundances from 950-1650 m/z such that the base peak of the spectrum represents a relative abundance of 100%. The relative abundance of the base peak in 3(b) was 1.15×10^4 and 6.57×10^3 in 3(c).

Table S1. LC Method Details

Time (min)	Flow rate ($\mu\text{L min}^{-1}$)	% Solvent A (Water)	% Solvent B (ACN)	Curve
1. Initial	60.000	0.0	100.0	
2. 1.00	60.000	0.0	100.0	6
3. 12.00	60.000	100.0	0.0	6
4. 15.00	60.000	100.0	0.0	6
5. 15.10	60.000	0.0	100.0	6

Figure S1. Charge Adduct Distribution for Bovine Fetuin Released Glycans

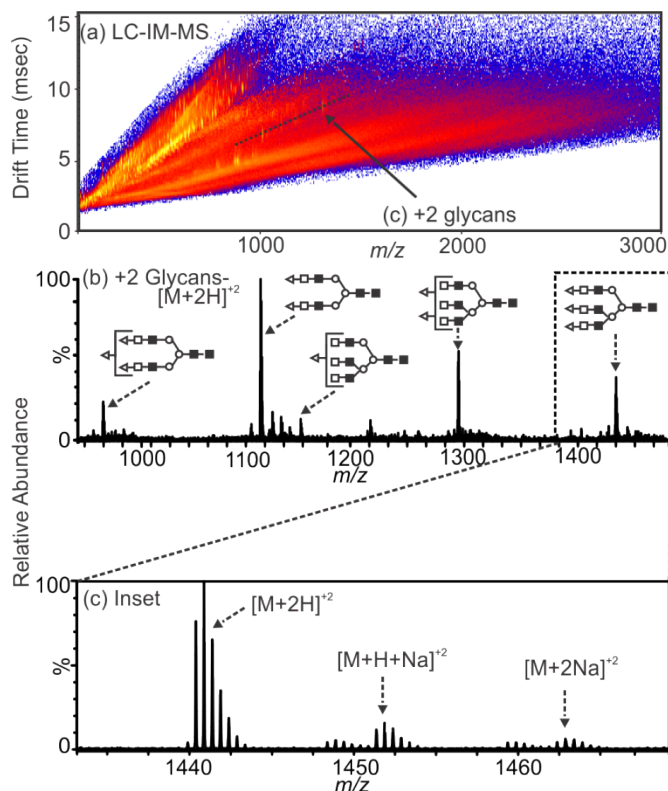


Figure S1. (a) A 2D IM-MS plot of glycans released from bovine fetuin after treatment with PNGaseF as discussed in the manuscript. Free non-derivatized glycans are separated by the method described above. Doubly charged glycans are annotated as (b). (b) A mobility selected mass spectrum of doubly charged free glycans from fetuin. A glycan segment of the mass spectrum was selected and magnified in the inset noted as (c). (c) A mass spectrum depicting the presence of both proton adducted and sodium adducted glycan species. As noted in the manuscript, the protonated species is more prominent than that of the sodium adducted species.

Figure S2. Mobility Separation of N-Linked Glycans Cleaved from Bovine Fetuin

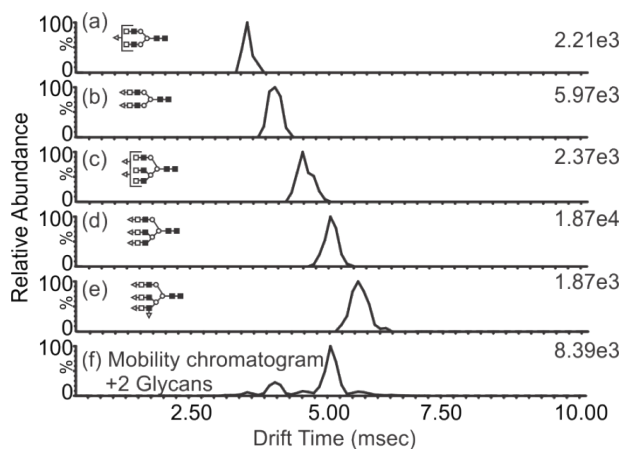


Figure S2. A series of drift time chromatograms for five of the glycans released from bovine fetuin. (a)- (e) Mobility chromatograms specific to the mass of the glycan depicted to the left of the peak. Relative abundances are noted to the right of the mobility peak. Some peaks do not fit a Gaussian profile, indicating the potential for multiple isomers. Further mobility and fragmentation studies to distinguish these overlapping isomeric peaks contributing to the mobility chromatogram are beyond the scope of this manuscript. (f) A total mobility chromatogram summed from the region annotated as the region of glycans in the 2D IM-MS plot in Figure S1 above.

Figure S3. Mobility Separation of N-Linked Glycans Cleaved from Bovine Fetuin

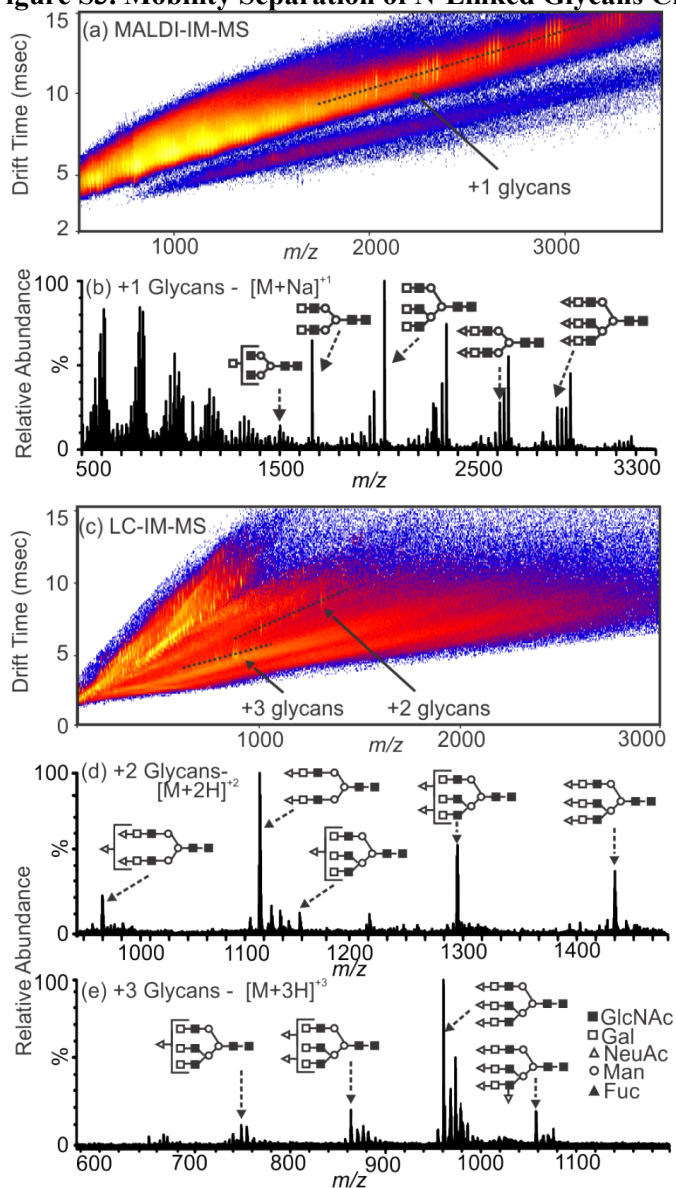


Figure S3. Experiments in this study utilize Scheme 3 for the separation and analysis of carbohydrates from the model glycoprotein, bovine fetuin. MALDI-IM-MS serves as additional confirmation of LC-ESI-IM-MS results. (a) A 2-D MALDI-IM-MS plot of conformation space for the analysis of fetuin carbohydrates. Selected regions for singly charged glycans represent the extracted mass spectrum (b). (b) Mass spectrum of free N-linked glycans from fetuin. (c) A 2D LC-ESI-IM-MS plot of conformation space for the analysis of fetuin carbohydrates with annotated regions representing the mass spectra, (d) and (e), of the doubly- and triply-charged glycans, respectively. It should be noted that analysis of carbohydrate by MALDI results in primarily singly charged glycans, $[M+Na]^+$, whereas LC-ESI-IM-MS preferentially creates doubly- and triply- protonated ions, $[M+2H]^{+2}$ and $[M+3H]^{+3}$ respectively. In LC-ESI-IM-MS, the sodiated glycan ions are presents as minor ionization products. The protocol for most MALDI-MS studies of glycans involves doping the sample-matrix solution with trace levels of salt to promote ionization. This gives rise to the sodiated glycans observed in Figure S3 (b). The LC-IM-MS analysis of fetuin glycans compares favourably (in terms of both the appearance of representative ion signals and their corresponding signal-to-noise) to that of the previous MALDI-IM-MS experiments. This is demonstrated in the spectra in Figure S3 (d) and (e), which show well-resolved doubly and triply-charged glycan signals.

Figure S4. LC TIC Chromatogram of a Mixture of Maltoses

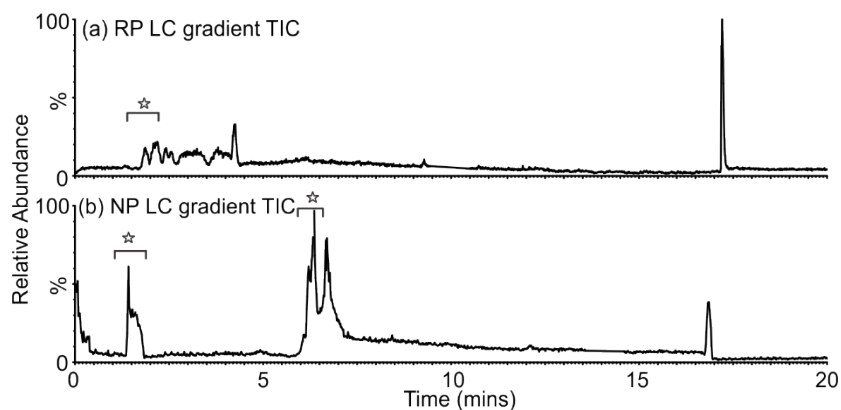


Figure S4. Liquid chromatography total ion chromatograms of a series of maltose standards (M3-M7). S4(a) is an LC chromatogram for the reversed phase gradient annotated with a star for the region of sugars. S4(b) illustrates an LC chromatogram using the proposed method of a normal phase gradient and annotates the regions which contain maltose sugars with stars.

References

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