Elucidating the Complexities of the Human Milk Glycome with Ion Chromatography-Mass Spectrometry (IC-MS)

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ABSTRACT

Purpose: Develop a novel analytical solution that is derivatization-free for the enhanced separation and analysis of the human milk glycome.

Methods: Free and released glycans from human milk samples were extracted and purified for subsequent analysis via ion chromatography and mass spectrometry. Glycans were separated with a Thermo Scientific™ Dionex™ CarboPac™ PA300-4µm column and detected with a pulsed amperometry detector (PAD) in sequence with a Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ mass spectrometer. Mass spectra were annotated for structural characterization of glycans.

Results: We introduced a novel instrument configuration with PAD and MS detection in sequence for the application of structurally characterizing the human milk glycome. Using this configuration, we identified 102 unique glycan structures of wide diversity, including isomers as well as neutral, fucosylated, and charged glycans.

INTRODUCTION

Glycans are major components of human milk, occurring both in free forms as lactose and oligosaccharides (HMOs) and in conjugation to glycoproteins primarily through N- and O-linked glycosylation. Glycosylation of milk proteins is known to be important for myriad biological processes, such as protein folding, ligand recognition, enabling resistance to proteolytic digestion, and promoting the growth of gut probiotics. The characterization of human milk glycosylation is challenging due to the large dynamic range of human milk proteins and to the complexity of protein glycosylation. Few tools are currently available to analyze the human milk glycome without derivatization. We developed a novel workflow featuring straightforward, IC-friendly sample preparation. The workflow enhances separation and characterization of milk-derived free oligosaccharides as well as both N- and O-linked glycans.

MATERIALS AND METHODS

Sample Preparation

Human milk samples were collected from three mothers and were stored at -20 ºC until analysis. Milk samples were diluted with water and centrifuged. The middle aqueous layer was collected and subjected to cold ethanol precipitation at -30 ºC overnight. Milk proteins were separated from free oligosaccharides by centrifugation. Both free oligosaccharides and milk protein fractions were concentrated in a speed vacuum concentrator and dissolved in water. N-linked glycans were released from collected proteins by PNGase F digestion. The remaining proteins were precipitated by cold ethanol, concentrated, and resuspended in water. O-Glycans were released from the remaining proteins by beta-elimination using sodium borohydride. Released and free glycans were purified with Thermo Scientific™ HyperSep™ Hypercarb™ filter plates.

Test Method(s)

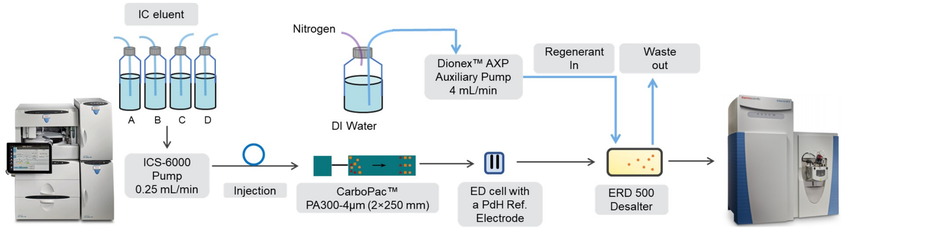
Glycans were separated using a CarboPac PA300-4µm column with a Dionex™ ICS-6000 HPIC™ system outfitted with both a pulsed amperometric detector and a Q Exactive HF-X Orbitrap mass spectrometer. Prior to MS, the column effluent was passed through a Dionex™ ERD 500 electrolytically regenerated desalter for salt removal. Briefly, the ERD 500 was operated under the consistent current mode at 220 mA and regenerated with deionized water at 4 mL/min.

Sample was introduced by a heated electrospray ionization (HESI-II) probe operating in negative ionization mode. The spray voltage was 3.2 kV, and the capillary temperature was 320 ºC. The sheath and auxiliary gas flow rate were set to 40 and 20 arbitrary units, respectively. For full scans, the data were acquired across the scan range of 400-2000 *m/z*. The AGC target was set to 3×10^6, with a maximum injection time of 120 ms and a resolution of 60,000 (FWHM at *m/z* 200). For MS^2, the AGC target was set to 10^5 with a maximum injection time of 300 ms and a resolution of 15,000. Five scans were performed at a normalized collisional energy of 28.

Data Analysis

Data were processed with Thermo Scientific™ Chromeleon™ Chromatography Data System and Thermo Scientific™ Xcalibur™ software. Mass spectra were annotated using SimGlycan® software and the UniCarb-DB database. An error tolerance of 10 ppm for precursor ions and 20 ppm for fragment ions was used as a cutoff for database searching. The resulting glycan library was validated using previous publications [1-2].

Precise glycan structures and MS/MS fragment ions were generated using GlycoWorkbench software [3]. Glycan structures were reported in accordance with Domon and Costello nomenclature [4].



Scheme 1. System configuration of HPAE-PAD with a PdH reference electrode hyphenated to a Q Exactive HF-X Orbitrap Mass Spectrometer.

RESULTS

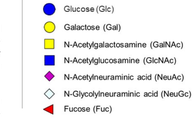
High Performance Anion Exchange Chromatography (HPAE) has long been used to separate complex carbohydrates. High resolution separation of chemically similar but structurally heterogenous glycans is important for mass spectrometric characterization. Negative ESI and subsequent high mass accuracy and high resolution Orbitrap analysis facilitate both the sequence and linkage characterization of glycans from human milk. Diagnostic fragments from tandem MS enable the assignment of glycan epitopes.

Novel IC-MS instrument configuration

The Dionex ICS-6000 HPIC system was configured for the hyphenation of electrochemical detection to MS detection. Scheme 1 shows a schematic of the HPAE-PAD-MS configuration used to characterize milk glycan samples. Two pumps were used—one for the IC eluent system, and another for water regeneration of the downstream desalter. Column effluent was first passed through the PAD detector with a PdH reference electrode. The oxyanions of carbohydrates were detected by measuring the electrical current generated by their oxidation at the surface of a gold working electrode. The cell effluent was then passed through the Dionex ERD 500 electrolytically regenerated desalter, where sodium ions were exchanged for hydronium and eluting glycans were in water (hydroxide-only eluent) or weak acetic acid. This effluent, containing glycans, was injected into the MS with a HESI-II probe.

Structural characterization of free human milk oligosaccharides

Figure 1 shows the separation of free human milk oligosaccharides (HMO) on a CarboPac PA300-4µm column. Overall, 75 free oligosaccharides were detected with the IC-MS platform. Small neutral and fucosylated structures eluted in the first half of the chromatogram, whereas monosialylated oligosaccharides eluted afterwards, followed by disialylated structures. Ten known HMO structures were annotated in Figure 1.



Legend 1. Graphical representation of a variety of common monosaccharide building blocks of glycans. The symbols are used routinely for the structural annotation of MS data in the following figures.

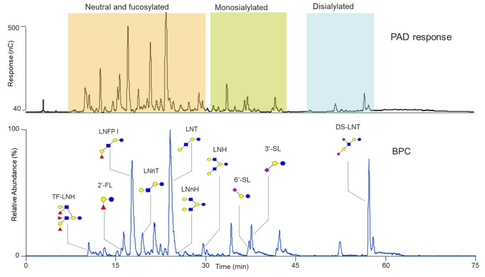


Figure 1. Separation of free human milk oligosaccharides on a CarboPac PA300–4µm column. The top trace was acquired by pulsed amperometric detection, and the bottom trace was obtained by HF-X Orbitrap MS (BPC; base peak chromatogram).

High-resolution chromatographic separation is particularly important for distinguishing compositionally identical but structurally distinct species. The CarboPac PA300-4µm column afforded supreme resolution, enabling the structural characterizing of glycan isomers when coupling IC to MS analysis. Figure 2 shows the extracted ion chromatogram (XIC) of Lacto-N-neotetraose (LNnT) and Lacto-N-tetraose (LNT) alongside their corresponding MS^2 profiles. The high quality MS^2 data provided several diagnostic fragment ions, enabling the structural characterization of each isomer.

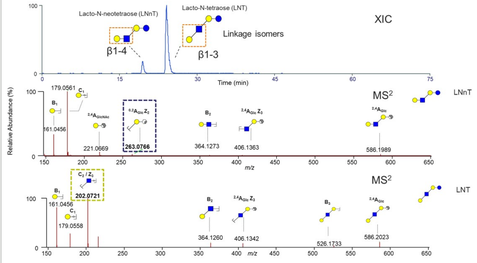


Figure 2. Extracted ion chromatogram and the MS^2 spectra of two isomeric structures: Lacto-N-neotetraose (LNnT) and Lacto-N-tetraose (LNT). The $^{0,2}A_{2\beta1,3}Z_2$ (*m/z* 263.0766) and C_2/Z_3 (*m/z* 202.0721) peaks in the MS^2 spectra were used as diagnostic ions for the LNnT and LNT isomers, respectively.

Identification of N-Glycans

The N-glycan structures of human milk glycoproteins have been investigated using various technologies; however, to our knowledge, this is the first demonstration of the use of IC-MS for this application. In total, 20 structures were identified, including multiple pairs of structural isomers. The 20 structures included 4 neutral, 5 fucosylated, 3 sialylated, and 8 both fucosylated and sialylated glycans, as shown in Figure 3. Putative glycan structures were determined based on their MS^2 profiles using SimGlycan software. The peaks eluting early within the first 20 mins are mainly remaining free HMOs in the sample. N-linked glycans elute following a known pattern; fucosylated glycans eluted earlier than their non-fucosylated counterparts, and sialylated glycans eluted later than neutral glycans. Peaks 11 a&b and 14 a&b are two pairs of isomers for which the linkage configuration of the terminal sialic acid moiety is unclear.

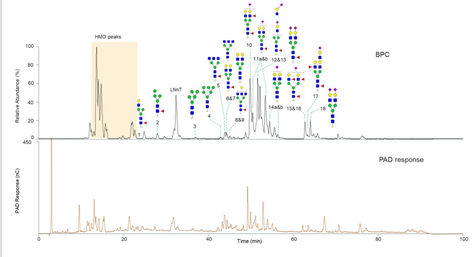


Figure 3. Separation of N-linked glycans released from human milk proteins on a CarboPac PA300–4µm column. The top trace shows the BPC from the Q Exactive HF-X Orbitrap MS, and the bottom trace was obtained by pulsed amperometric detection.

Identification of O-Glycans

In addition to annotating N-linked glycans, HMO samples were subjected to beta elimination for the characterization of O-linked glycans. In total, six O-linked glycans were identified, and their respective extracted ion chromatograms are shown in Figure 4.

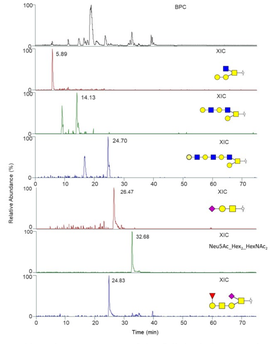


Figure 4. The extracted ion chromatograms of the O-linked glycans released from human milk proteins on a CarboPac PA300–4µm column.

CONCLUSIONS

We demonstrate the ability to resolve heterogeneous glycans in human milk and capitalize on the power of the hyphenated IC-MS platform to identify structural glycan isomers without the need for specific enrichment or derivatization prior to analysis. Understanding the complexities of human milk glycans is crucial, as the ability to separate and detect these structures is important to predict their biological functions. To our knowledge, this is the first time that the IC-MS platform was successfully applied to the structural characterization of human milk glycans.

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TRADEMARKS/LICENSING

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