Structural insights on oligosaccharides in commercial infant formula products using ion chromatography-mass spectrometry (IC-MS)

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ABSTRACT

Purpose: Develop a derivatization-free analytical solution for the qualitative assessment of functional oligosaccharides in commercial infant formula products

Methods: Functional oligosaccharides were extracted and purified for subsequent analysis with ion chromatography coupled to mass spectrometry (IC-MS). Oligosaccharides were separated on a Thermo Scientific™ Dionex™ CarboPac™ PA300-4µm Analytical column with a gradient of hydroxide and acetate eluents and detected sequentially by an electrochemical detector and a Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer.

Results: IC-MS takes advantage of the supreme resolving power of high-performance anion exchange chromatography (HPAE) to separate a variety of oligosaccharides in infant formula products, including fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), malto-oligosaccharides (MOS) and human milk oligosaccharides (HMOs), varying in molecular weights and structures. The Orbitrap MS facilitates the detailed structural identification and characterization of functional oligosaccharides.

INTRODUCTION

Interest in functional oligosaccharides has grown substantially because of their profound impact on the gut microbiome. As prebiotics, the health benefits of functional oligosaccharides often lead to their supplementation in infant formulas. The structural features of oligosaccharides are closely associated with their health benefits, and therefore, they need to be characterized in detail. We present a useful tool combining ion chromatography and high-resolution mass spectrometry for derivatization-free, high-resolution characterization of functional oligosaccharides in several infant formula products. Importantly, this strategy features easy sample preparation, premium isomer separations, and high-quality MS data for structural elucidation of oligosaccharides, thus positioning it as a beneficial tool for the qualitative assessment for formula products and other products supplemented with prebiotic oligosaccharides.

MATERIALS AND METHODS

Sample Preparation

Two malto-oligosaccharide standards, i.e., Maltohexaose and 6-α-D-maltotriosyl-maltotriose, were purchased from Biosynth Carbosynth (San Diego, CA). The standard solutions were prepared at 1000 mg/L concentrations for the IC-MS analysis.

Three commercial infant formula products fortified with functional oligosaccharides were purchased from grocery stores. Among three products, two of the products came in liquid form, and the last was a powdered formula. The powdered formula product was dissolved in water following manufacturer instructions. Samples were diluted with equal volumes of water and centrifuged to defat. The middle aqueous layer was collected, and proteins were precipitated with ethanol at -30 °C overnight. Precipitated proteins were separated by centrifugation. The supernatant was dried in a SpeedVac concentrator and subsequently redissolved in water. The resulting solutions containing functional oligosaccharides were purified with a Thermo Scientific™ HyperSep™ Hypercarb™ filter plate.

Test Method

Oligosaccharides were analyzed using a Dionex™ ICS-6000 HPIC™ system outfitted with an electrochemical detector operated in the pulsed amperometric detection (PAD) mode and a Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer in a sequential configuration. The system configuration is shown in scheme 1. Oligosaccharides were separated on a Dionex™ CarboPac™ PA300-4µm Analytical and Guard columns using sodium hydroxide and sodium acetate. Prior to MS injection, the column effluent was passed through a Thermo Scientific™ Dionex™ ERD 500-2 mm electrolytically regenerated desalter for salt removal. The ERD500-2 mm desalter was operated at 380 mA and regenerated with water at 4 mL/min.

The desalter effluent was introduced by a Thermo Scientific™ Heated Electrospray Ionization (HESI-II). Probe was operated in negative ionization mode. The spray voltage was 3.2 kV, and the capillary temperature was 320 °C. The sheath and auxiliary gas flow rate were set to 40 and 20 arbitrary units, respectively. For MS experiment, the data were acquired across the scan range of m/z 400-2000. The AGC target was set to 10^6 with a minimum injection time of 60 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.

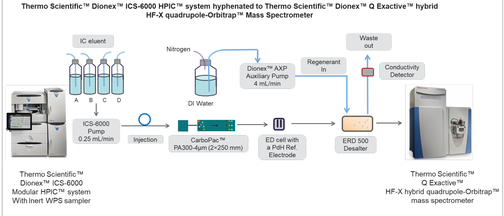
Data Analysis

Data were processed with Thermo Scientific™ Chromeleon™ Chromatography Data System and Thermo Scientific™ Xcalibur™ software. Precise oligosaccharide structures and MS/MS fragment ions were generated using GlycoWorkbench software [1]. All spectra were manually verified. Oligosaccharide structures were reported in accordance with Domon and Costello nomenclature [2].

RESULTS

IC-MS was utilized for the analysis of both dairy-based and soy-based formulas fortified with different functional oligosaccharides. A wide variety of oligosaccharides were characterized, including HMOs and other common prebiotic oligosaccharides such as GOS, FOS, and MOS ranging in various sizes and structures.

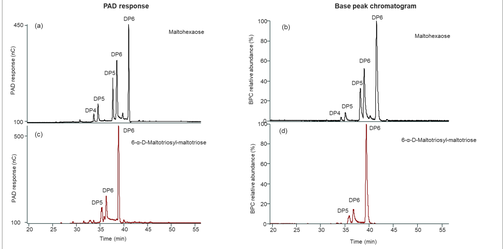
Scheme 1. System configuration of IC-MS



Analysis of Malto-oligosaccharides

The system performance was first demonstrated with an isomeric pair of MOS standards, Maltohexaose and 6-α-D-maltotriosyl-maltotriose. Maltohexaose is composed of glucose linked through α-1,4 linkages while 6-α-D-Maltotriosyl-maltotriose contains an α-1,6 linkage between the 3rd and 4th glucose units and α-1,4 linkages between the rest of adjacent glucose units. Figure 1 shows the oligosaccharide profiles of Maltohexaose and 6-α-D-maltotriosyl-maltotriose. The PAD response and base peak chromatogram were acquired sequentially from a single injection for each sample. The degree of polymerization (DP) for each detected peak was assigned according to the mass-to-charge (m/z) values acquired through Full MS-ddMS^2 experiment.

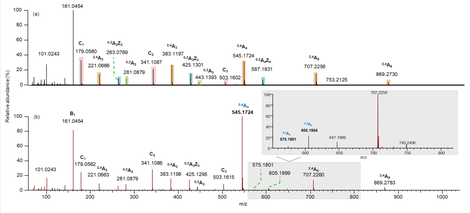
Figure 1. Oligosaccharide profiles of a pair of MOS isomers at 1000 ppm: (a) PAD response of Maltohexaose; (b) base peak chromatogram of maltohexaose; (c) PAD response of 6-α-D-Maltotriosyl-maltotriose; (d) base peak chromatogram of 6-α-D-maltotriosyl-maltotriose. For each sample, the PAD and BPC were acquired sequentially from a single injection. DP: degree of polymerization



The Q Exactive HF-X Orbitrap mass spectrometer has the capacity to generate higher-energy collisional dissociation (HCD) fragment ions with high mass accuracy. This allows for identification and differentiation of fragments ions with close m/z and is useful for branching and linkage assignment.

Figure 2 shows and compares the MS^2 spectra for Maltohexaose and 6-α-D-maltotriosyl-maltotriose. Both spectra show extensive A-type cross-ring fragmentation accompanied by C-type glycosidic linkage fragmentation. Maltohexaose is a homo α-1,4-linked oligosaccharide, and Figure 2(a) shows its MS^2 spectra. The spectrum contains clusters of peaks corresponding to $^{0,2}A_n$, $^{0,2}A_n$, Z_n, and $^{2,4}A_n$ ($n \leq 6$) cross-ring fragment ions, similar to previous findings [3]. The linkages of 6-α-D-maltotriosyl-maltotriose are hetero α-1,4/α-1,6. While many MS^2 spectral peaks are shared between two isomers, we can distinguish an α-1,6 linkage from an α-1,4 linkage with the help of unique fragment ions. Figure 2(b) shows the MS^2 data with a series of these unique diagnostic ions, including $^{0,2}A_4$ (m/z 605.1954), $^{0,3}A_4$ (m/z 575.1801), and $^{0,4}A_4$ (m/z 545.1724). These ions provide evidence of an α-1,6 linkage.

Figure 2. (a) MS^2 spectra of Maltohexaose and (b) 6-α-D-maltotriosyl-maltotriose. The shaded insert spectra is the zoomed-in view of the m/z ± 550-800.



Analysis of functional oligosaccharides supplemented to infant formula

The oligosaccharide content of three commercial formula products was characterized by IC-MS. One is a bovine-derived liquid formula product supplemented with 2'-FL HMO together with GOS. Another is a bovine-derived powder product supplemented only with non-HMO functional oligosaccharides GOS and FOS. The third product is a soy-based liquid formula fortified with short chain FOS. The product characteristics obtained from the manufacturers' websites and product labels are summarized in table 1.

Table 1. Product characteristics of the commercial infant formula products examined in the current study

Product	Type	Base	Functional oligosaccharides	HMO added?
A	Liquid	Bovine milk	2'-FL HMO, GOS	Yes
B	Powder	Bovine milk	GOS, and FOS (<1%)	No
C	Liquid	Soy	Short chain FOS	No

Figure 3(a)-(c) shows the oligosaccharide profiles of the three formula products tested. For each subfigure, the top trace is the BPC acquired from MS analysis, and the bottom trace is the PAD chromatogram acquired simultaneously from the same injection. The compositional identities of the detected peaks are tabulated next to the chromatograms. Oligosaccharides ranging in size from DP3 to 17 were characterized by IC-MS.

Both HMO and non-HMO structures were discovered. The HMO 2'-FL was identified in product A, as shown in Figure 3(a). We also detected a low level of the HMO 3'-SL in product A. Although the manufacturers did not specify the addition of 3'-SL to formula, it is likely coming from the bovine milk component. The CarboPac PA300-4µm Analytical column resolved target HMOs from other complex carbohydrates and other matrix components in the sample. The high quality MS^2 fragments provide diagnostic information for high confidence structural annotation.

The different forms of oligosaccharide isomers are relevant to their diverse biological functions, and thus it is crucial to characterize their structures. However, this is challenging due to the enormous amount of stereochemical information and heterogeneity of oligosaccharides, making them difficult to resolve. Therefore, it is essential to optimize the chromatographic separation of oligosaccharides and utilize the HRAM MS to achieve comprehensive characterization of complex samples that contain oligosaccharide isomers.

Figure 3. Oligosaccharide profiles in commercial infant formula products. The top trace is BPC acquired from MS detection and the bottom is the PAD trace acquired simultaneously from the same injection.

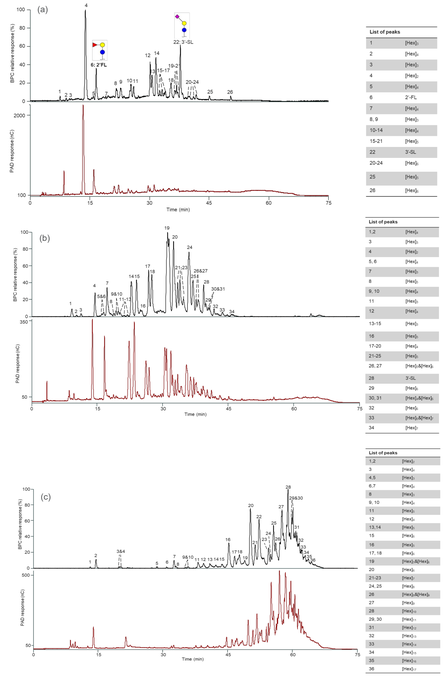


Figure 4(a) shows the separation of DP4 oligosaccharide isomers in Product B from Table 1 on a CarboPac PA300-4µm Analytical column. Table 2 summarizes their retention times and mass accuracies. Figure 4(b) shows the MS^2 spectra of four selected isomers. The glycosidic linkage fragments (m/zs 161.0457, 179.0562, 305.0885, 323.0984, 341.1099, and 485.1509), shaded in red, aid in compositional identification. A-type cross-ring fragments (m/zs 221.0666, 263.0783, 383.1995, 425.1307, and 545.1723), shaded in blue, are also present; however, without reference standards or enzymatic confirmation, it is difficult to assign the linkage configuration based on MS^2 spectra.

Figure 4. (a) XIC for DP4 GOS in Product B, (b) MS^2 spectra for four selected isomers.

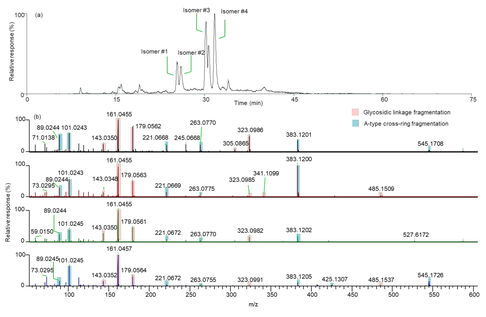


Table 2. The retention time, m/z, and mass accuracy of the selected isomers in Figure 5.

	RT (min)	m/z	Mass accuracy (ppm)
Isomer #1	25.32	665.2167	3.2
Isomer #2	25.98	665.2166	3.0
Isomer #3	30.20	665.2167	3.2
Isomer #4	31.65	665.2173	4.0

CONCLUSIONS

- IC-MS is a powerful tool for structural characterization of functional oligosaccharides
- The CarboPac PA300-4 µm column resolves a heterogeneous mixture of oligosaccharides structures in complex matrices
- IC-MS identifies structural isomers of functional oligosaccharides without the need for specific enrichment or derivatization prior to analysis

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TRADEMARKS/LICENSING

PO66154-EN0422S