Chapter 10

Analysis of Milk Oligosaccharides by Mass Spectrometry

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Abstract

Human milk oligosaccharides (HMOs) are a highly abundant constituent in human milk, and its protective and prebiotic properties have attracted considerable attention. HMOs have been shown to directly and indirectly benefit the overall health of the infant due to a number of functions including serving as a beneficial food for gut bacteria, block to pathogens, and aiding in brain development. Researchers are currently exploring whether these structures may act as possible disease and nutrition biomarkers. Because of this, rapid-throughput methods are desired to investigate biological activity in large patient sets. We have optimized a rapid-throughput protocol to analyze human milk oligosaccharides using micro-volumes of human breast milk for nutritional biomarkers. This method may additionally be applied to other biological fluid substrates such as plasma, urine, and feces. The protocol involves lipid separation via centrifugation, protein precipitation using ethanol, alditol reduction with sodium borohydride, and a final solid-phase extraction purification step using graphitized carbon cartridges. Samples are analyzed using HPLC-Chip/TOF-MS and data filtered on Agilent MassHunter using an in-house library. Individual structural identification is matched against a previously developed HMO library using accurate mass and retention time. Using this method will allow in-depth characterization and profiling of HMOs in large patient sets, and will ease the process of discovering significant nutritional biomarkers in human milk.

Key words Oligosaccharide, Human milk, Mass spectrometry, Glycan, Carbohydrate, Free oligosaccharide, HPLC, TOF

1 Introduction

HMOs are free sugars commonly found with a lactose (Galβ1-4Glc) core at the reducing end, and can further be elongated linearly or branched by N-acetyllactosamine units with fucose and sialic acid that can be present at the terminal regions [1, 2]. They are the third or fourth most abundant component in milk, which is dependent on the mother and lactation time point, but do not pose a direct nutritional benefit to the infant. Because of their biological importance in gut floral development and in pathogen blocking [3–5], their presence in the infant gut is vital. Recent experiments have shown that oligosaccharides are not only excreted into the urine, but also upregulated into the bloodstream of the

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infant [6, 7], supporting the previous findings that sialic acid is important in brain development and memory [8].

Further investigating these properties and future nutritional biomarker studies will require large patient sample sets, where high-throughput sample preparation and analysis are favored. Because of their heterogeneity and structural diversity, there has been difficulty in creating a high throughput method to investigate their biological activity in different patient sets. There are a number of current separation methods to analyze free oligosaccharides in milk, involving anionic exchange chromatography [9–11], capillary electrophoresis [12–14], reverse phase chromatography [15, 16], and hydrophilic interaction chromatography [17]. However, many of these methods require derivatization tags and have partial or limited isomer separation. Ruhaak et al. give a detailed comparison of the various protocols [18]. Using our method involving graphitized carbon coupled to nano-LC mass spec, we have been able to roughly monitor 250 free oligosaccharides, with 100 structurally identified isomers in human milk [19, 20].

The traditional human milk oligosaccharide protocol has been optimized into a rapid-throughput manner, taking into consideration each separation and extraction step to avoid sample loss, ensure reproducible MS signal, and to decrease sample handling time. Here we have developed a rapid-throughput 96-well plate method using separation and purification steps to isolate free oligosaccharides in milk. Method validation and reproducibility is explained in detail in Totten et al. [21].

This method involves an initial 1:1 dilution of sample and deionized water, proceeding with plate centrifugation at 3220 × *g* at 4 °C for 30 min, in order to separate lipids. The aqueous portion is carefully extracted using a multichannel pipette, and aliquoted onto a new plate. Two volumes of ethanol is then added to the samples and placed in −80 °C for 1.5 h to fully precipitate proteins. Samples are then thawed and centrifuged using the same parameters to separate aqueous from solids. The aqueous portion is collected and dried prior to alditol reduction. Sample is reconstituted using deionized water to its original volume, and an equivalent volume of 2 M sodium borohydride is added to make a final concentration of 1 M. Sample is incubated for 1.5 h in 65 °C. Sample is then applied onto a 96-well plate packed with graphitized carbon after column conditioning, and centrifuged at room temperature at 340 × *g* in order for compounds to have enough interaction time with the column. Deionized water is used to desalt, and 20% acetonitrile and 40% acetonitrile in 0.05% TFA in water is used to elute and collect oligosaccharides using the same centrifugal conditions. Eluate is dried to completion prior to a 50-fold dilution, using a 1 μL injection onto Agilent HPLC-Chip/TOF-MS. Because HMO standards are limited and costly, a pooled HMO sample collected from a number of mothers is analyzed in parallel in order to closely match retention times with the already built library and

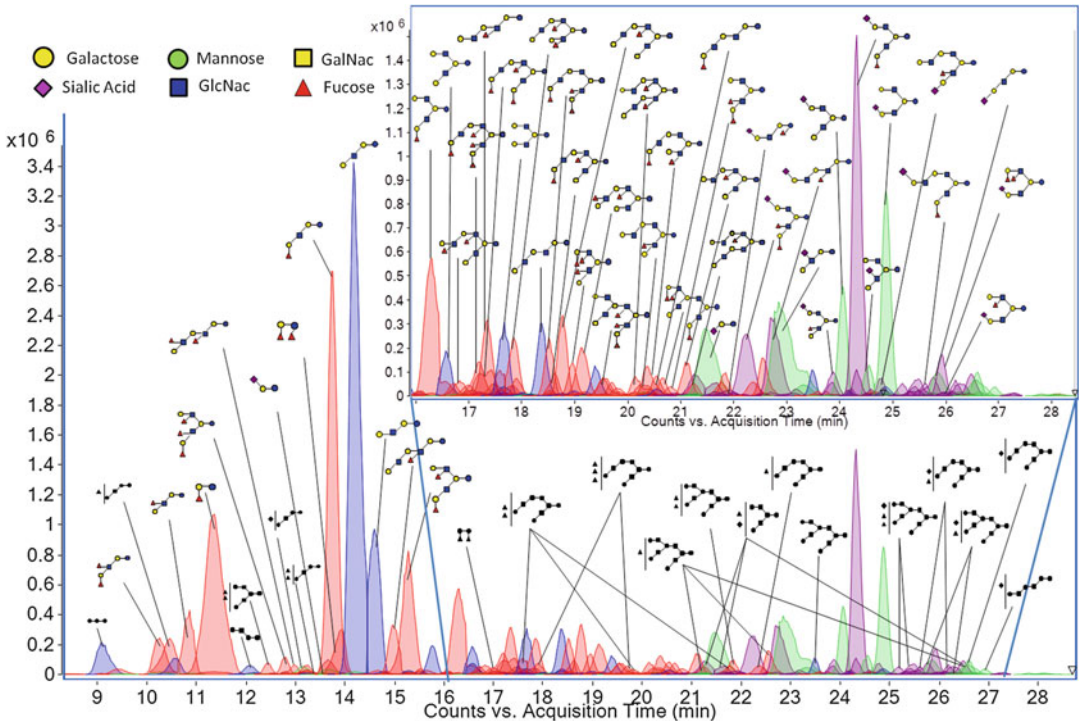

Fig. 1 An HMO pool sample was analyzed using HPLC Chip/TOF-MS to determine glycan signal. Sample representing a 50-fold dilution of milk was injected into the nano-LC instrument. All known compounds were extracted, overlaid, and were structurally identified and annotated using an in-house library. *Each color* indicates a different glycan class—*blue* (non-fucosylated neutrals), *red* (fucosylated), *green* (sialylated), and *purple* (fucosylated and sialylated)

also to monitor retention time shift and signal intensity throughout a large run. Figure 1 is an example of an extracted compound chromatogram of an HMO pool sample.

This rapid-throughput approach has already been applied to global lactation and nutritional studies as large as 2000 samples, and has already given promising results between the different groups. Not only can this process be applied to human milk, but can be applied to other mammalian milks as well, and other biological matrices such as feces, serum, and urine [6, 22], with minimal alternations to the protocol. With this method we can compare the overall HMO profiles in different patients and additionally monitor the individual isomers in human milk at the same time.

2 Materials

2.1 Separation Reagents

1. Nanopure water.
2. 100% ethanol (200 Proof).
3. 2 M sodium borohydride ($NaBH_4$) in deionized H_2O.
4. Folch solution (2:1 chloroform–methanol (v/v))—optional.

2.2 Solid-Phase Extraction Solvents

1. 20% ACN in H_2O (v/v).
2. 40% ACN in H_2O (0.05% TFA) (v/v).
3. 80% ACN in H_2O (0.1% TFA) (v/v).

2.3 Specialized Supplies and Equipment

1. 96-well carbon plate for solid-phase extraction (Glygen, Cat #FNSCAR).
2. 0.2 mL 96-well plate (GeneMate, Cat #T-3183-1) with compatible 96-well plate lid (Phenix Research Products, Cat #SMX-PCR96).
3. 0.8 mL 96-well polypropylene plate (Thermo Scientific, Cat #AB0765) with compatible 96-well plate lid (Thermo Scientific, Cat #AB0566).
4. 1.3 mL 96-well polypropylene plate (Thermo Scientific, Cat #260251) with compatible 96-well plate lid (Thermo Scientific Nunc, Cat #12565560).
5. Refrigerated bucket centrifuge for plate centrifugation and for solid-phase extraction.

2.4 MALDI-MS Analysis of Oligosaccharides

1. Matrix: 2,5-dihydroxybenzoic acid (DHB) (5 mg/100 μL in 50% ACN in H_2O).
2. Positive mode dopant: 0.1 M NaCl in 50:50 H_2O–ACN.

2.5 Nano LC/MS Analysis of Oligosaccharides

1. 3% ACN in 0.1% FA (v/v) (A1 Solvent).
2. 90% ACN in 0.1% FA (v/v) (B1 Solvent).
3. Compatible 96-well autosampler plate (Eppendorf, Cat #951020401).
4. Compatible pierceable 96-well plate lid (Phenix Research Products, Cat #SMX-PCR96).
5. PGC Chip (40 nL with 43 × 0.075 mm analytical column, Agilent, Cat#G4240-64010).

3 Methods

3.1 Separation and Purification of Free Oligosaccharides

1. Aliquot 25 μL of milk onto a 0.2 mL 96-well plate (*See* **Note 1**).
2. Add 25 μL water using a multichannel pipette and vortex lightly with a sealed plate lid (*See* **Note 2**).
3. Centrifuge samples at 4 °C for 30 min at maximum speed (3220 × *g*).
4. Extract aqueous layer between the top lipid layer and the bottom protein/cell detritus layer and transfer to 0.8 mL plate for reduction (*See* **Note 3**).

5. Protein precipitation (*See* **Note 4**).
   (a) Add 2 sample volumes of ethanol, and vortex completely. Spin down briefly.
   (b) Place samples in −80 °C for a minimum of 1.5 h. Samples may be stored overnight.
6. Thaw and centrifuge at 4 °C for 30 min at 3220 × *g*.
7. Carefully collect supernatant without disturbing the protein pellet, and dry to completion on low heat using a centrifugal evaporator. Samples may be stored in −80 °C overnight.
8. Reduction from aldehyde to alditol.
   (a) Add 25 μL of water to dried sample, and place on plate vortex for 30 min with a sealed plate on highest speed setting (*See* **Note 5**). Add an equivalent volume of 2 M $NaBH_4$, vortex, and briefly centrifuge. Final concentration of $NaBH_4$ buffer is 1 M.
   (b) Incubate for 1.5 h at 65 °C in a dry incubator (*See* **Note 6**).

3.2 Solid-Phase Extraction Using 96-Well Carbon Packed Plates

1. Add 2 volumes of nanopure water to Glygen carbon plate (200 μL well capacity) to wet the column. Waste collection should be placed below cartridge plate and should be discarded for each run.
2. Centrifuge at 340 × *g* for 1 min at room temperature. Ensure water has passed through the column.
3. Add 2 volumes of 80% acetonitrile in 0.1% TFA to the SPE plate to remove contaminants. Centrifuge using same parameters.
4. Add 3 volumes of nanopure water to the SPE plate to equilibrate the column and centrifuge. (*See* **Note 7**)
5. Load sample to the SPE plate. Centrifuge at 340 × *g* for 2–3 min until sample has been completely loaded. Ensure that sample has been fully loaded onto column before continuing.
6. Desalt a total of 5 times with nanopure water (*see* **Note 8**), centrifuging and discarding waste between each water addition.
7. Replace waste plate with 1.3 mL collection plate. Be sure not to discard any of the collection eluate, as these fractions contain the analytes of interest.
8. Collect 0.4 mL of 20% ACN and 0.4 mL of 40% ACN in 0.05% TFA (2 volumes each) in the same 1 mL Thermo/Nunc plate with centrifugation steps in between. Final elution volume will be 0.8 mL. (*See* **Note 9**).
9. Dry to completion using a centrifugal evaporator on low to medium heat for 6–8 h (*See* **Note 10**).
10. Store between −80 °C to −20 °C with a sealed lid until ready for analysis. Wrap 96-well plate in foil to prevent ice forming around the lid seal. Recommended storage is up to 1 year.

3.3 Sample Reconstitution

1. To the dried samples, add the amount of water equivalent to original volume of milk aliquotted (in this case, 25 μL).
2. Vortex on high speed setting for 30 min with secure lid.
3. Spin down briefly.

3.4 MALDI-MS Analysis

1. Pipette mix glycan solution (1 μL), matrix solution (0.7 μL), and NaCl dopant (0.3 μL) onto the MALDI probe for positive mode analysis. In negative mode, NaCl dopant is excluded.
2. Dry sample in vacuum chamber prior to analysis.
3. Data was generated using an IonSpec HiResMALDI equipped with external MALDI source, a 7.0 T superconducting magnet, and a pulsed Nd:YAG laser (355 nm).

3.5 Nano LC/MS Analysis

1. To a 0.2 mL 96-well autosampler compatible plate, add a 50× dilution of sample using nanopure water. Vortex with a secured lid and spin down briefly. Secure an autosampler compatible lid to prevent sample evaporation.
2. Apply a 45 min gradient to each method (2.5–20 min, 0–16% B1; 20–30 min, 16–44% B1; 30–35 min, 100% B1; 20 min equilibration, 0% B1) with a flow rate of 0.3 μL/min.
3. Load 1 μL of sample onto a 40 nL enrichment column and analytical column (porous graphitized carbon) using a 4 μL/min flow rate (Solvent A).
4. Data was generated using an Agilent 1200 series HPLC coupled to an Agilent Technologies 6210 TOF mass spectrometer and Agilent 1200 series HPLC coupled to Agilent 6520 Q-TOF mass spectrometer, with both detectors having a chip-cube interface.

3.6 Human Milk Oligosaccharide Identification for LC/MS

1. Using an in-house created HMO library, we are able to filter all the detected compounds using accurate mass, and at this point can separate compounds by glycan class and composition.
2. Once all compositions have been identified, we then can accurately assign specific linkage structures to these compositions using the published HMO library (*See* **Note 11**).
3. We have further developed an in-house peak alignment software to correct for retention time shift. The description of this software is explained in Totten et al. [21].

4 Notes

1. 10–50 μL of original milk may be used as starting volume. Using more than 50 μL of milk may saturate the SPE plate cartridge [21]. Figure 2 depicts the complete sample preparation workflow for HMO extraction.

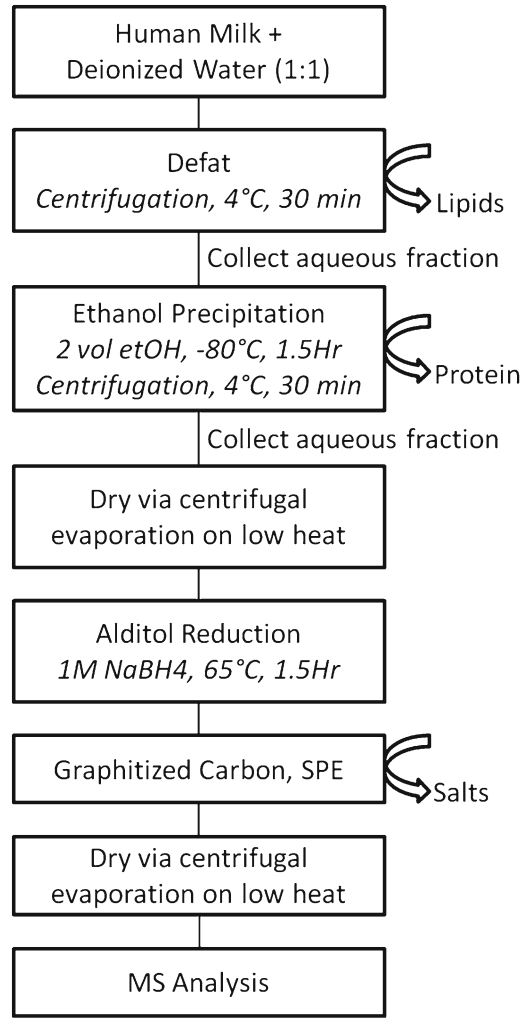

Fig. 2 Chart depicts the complete sample preparation workflow for rapid-throughput HMO analysis. The complete protocol for 96 samples prior to MS analysis can be completed in 2–3 work days with drying time incorporated

2. A 1:1 water to sample addition is typical. But because this method uses such small volumes, separation may be difficult. If this step is repeatedly becoming an issue, bring total volume to 100 μL using nanopure water prior to centrifugation. For large nutritional studies, our methods have optimized 25 μL of milk as the standard.
3. It is important to avoid collecting the upper layer of lipids. Trace amounts are unavoidable during the puncturing of the lipid layer. To ensure an ultraclean final mixture, a Folch step may be included in-between the initial defatting step and the protein removal step. Add 4 volumes of Folch solution (2:1 chloroform–methanol [v/v]) with glass pipette tips, vortex, and centrifuge at maximum speed at 4 °C. Carefully

collect the upper layer, and proceed with **step 5** for protein precipitation in Subheading 3. This step is currently omitted in the rapid-throughput method due to insignificance of its exclusion using starting volumes of 10–50 μL of milk [21].

4. An alternative method to ethanol precipitation is protein removal using C8 cartridges. Ethanol precipitation is published in the high-throughput protocol since it is a much cheaper and simpler alternative. The following steps are provided if protein removal using C8 is desired. Load sample onto C8 SPE plate and collect flow-through containing HMOs and salts. Add a one cartridge volume of nanopure water and combine with previous flow-through. Collection mixture would then be reduced and directly added to carbon cartridge in solid-phase extraction section under methods.
5. It is important to vortex microplate with nanopure water to ensure sample is homogenous prior to sodium borohydride addition. Vortex on a microplate vortexer for 30 min at maximum speed setting with a secured lid.
6. Before incubation, it is important to properly secure lid to microplate to avoid sample evaporation due to the chemical reaction. Apply lab tape to all sides of the lid creating a tight seal to completely prevent gases from escaping the wells. Incubating in a water bath causes condensation formation on the lid and wells and may introduce cross-contamination. For reduction purposes, our study has utilized a dry incubator to avoid this issue. SPE must be administered immediately after this step. Temporary sample storage should be avoided.
7. Because acetonitrile is an elution solvent, small analytes may wash off during the loading and desalting step if there are leftover traces of ACN prior to sample loading. Make sure ACN is completely washed off of the column by adding an extra column volume of water during the equilibration step.
8. Small compounds may also wash off during the desalting step if excess water has passed through the column. 7 column volumes of water should be the maximum amount to wash off salts and small peptides when using 50 μL of sample. The amount of desalting volume also depends on the original sample volume. 5 column volumes for desalting is enough for a starting volume of 25 μL of sample.
9. If using MALDI-MS, keep fractions separate in order to prevent ion suppression. If using HPLC, these elutions may be combined.
10. On higher heat settings, sialic acid on the terminal end may be unstable and will detach from the oligosaccharide during the drying process. It is important to dry on low to medium heat.
11. Refer to Wu et al. [19, 20] for the complete method on oligosaccharide identification.

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