Human milk oligosaccharide (HMO) screening in term and preterm human milk (HM) samples

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INTRODUCTION & OBJECTIVES

HMOs are the third most abundant solid component naturally present in HM. These multifunctional glycans shape the infants' gut-microbiota and modulate neonatal immunity. Up to 250 structurally different HMOs are known, but clinical studies so far only focused on a small number of HMOs.

The **objectives** of this study were to:

- Develop a method for the simultaneous annotation and quantification covering a wide range of HMOs
- Explore differences between the HMO pattern of milk from mothers of preterm and term infants
- Explore differences caused by pasteurization

HM PREPARATION

Human milk 50 µL

1. HMO isolation
   - Centrifugation (14000 g, 30 min, 4 °C)
   - Addition of 4 x 2:1 v/v cloroform-MeOH
   - Centrifugation
   - Addition of 2 x of EtOH
   - 4 °C overnight
   - Centrifugation
   - Evaporation
   - Reconstitution in 200 µL H_2O

2. Purification with porous graphitic carbon **solid phase extraction**

HMO ANALYSIS

HILIC - Q-Exactive Orbitrap MS

Automated MSMS-based **annotation**

Absolute **quantification** with standards

Multivariate analysis

HILIC-Orbitrap-MS Method

- ✓ Q-exactive Orbitrap spectrometer in polarity switching ionization mode
- ✓ Column: ACQUITY Glycoprotein BEH Amide column, 300 Å (1.7 µm, 2.1 mm × 150 mm)
- ✓ Mobile phase: (A) 10 mmol/L ammonium formate with 0.1% (v/v) formic acid; (B) 99.9% (v/v) ACN with 0.1% (v/v) formic acid
- ✓ Acquisition time: 32 minutes
- ✓ Inclusion list for MSMS acquisition of HMOs

Data Processing

HMO Screening

- ✓ Peak table generation with XCMS online
- ✓ Automated MSMS-based annotation of metabolites using the NIST milk oligosaccharide MS library[1] and in-house developed MATLAB scripts[2]

QUANTIFICATION

- ✓ External calibration lines for 2FL, 3FL, 3SL, 6SL, LNT, LNFP I, LNDFH I, DSLNT (authentic standards)
- ✓ Isomaltotriose used as ISTD

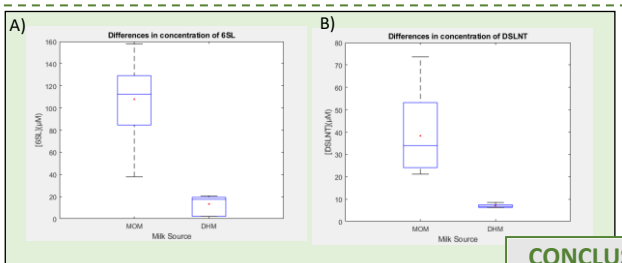
[1]Remoroza, C. A. *et al.* "Increasing the Coverage of a Mass Spectral Library of Milk Oligosaccharides Using a Hybrid-Search-Based Bootstrapping Method and Milks from a Wide Variety of Mammals." Analytical Chemistry vol. 92,15 (2020): 10316–10326.
[2]Ten-Doménech, I. *et al.* "Comparing Targeted vs. Untargeted MS^2 Data-Dependent Acquisition for Peak Annotation in LC–MS Metabolomics." Metabolites vol. 10, 4 (2020):10040126.

RESULTS

QUANTITATIVE ANALYSIS

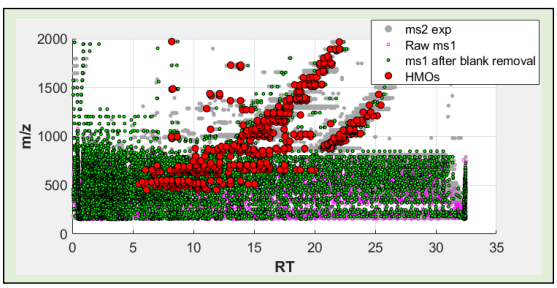
Concentrations and main characteristics of quantified HMOs in **mother's own milk (MOM)** (including term and preterm) (n=10) collected during **early stages of lactation.**

Name	Mol. Weight (g/mol)	Adduct	RT (min)	Median (µM)	IQR (µM)
2'-Fucosyllactose (2FL)	488.174	NH_4^+	8.43	1468	757-1573
3'-Fucosyllactose (3FL)	488.174	NH_4^+	9.04	7	4-24
3'-Sialyllactose (3SL)	633.211	H^+	10.64	26	23-37
6'-Sialyllactose (6SL)	633.211	H^+	11.48	112	85-129
Lacto-N-Tetraose (LNT)	707.248	H^+	11.64	1111	982-1265
Lacto-N-FucoPentaose I (LNFP I)	853.306	H^+	13.35	407	184-653
Lacto-N-DifucoHexaose I (DNDFH I)	999.364	H^+	15.78	585	376-717
Disialyllacto-N-Tetraose (DSLNT)	1289.439	H^+	17.64	34	24-53



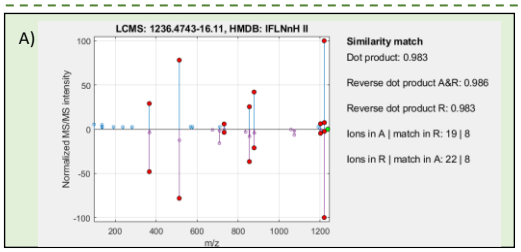
As an example, concentrations in MOM (n=10) and donor human milk (DHM) (n=6) of **A)** 6SL and **B)** DSLNT are shown.

QUALITATIVE ANALYSIS

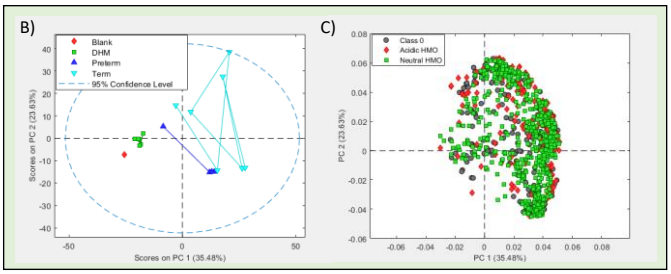


Retention time and *m/z* profile of features detected and annotated in ESI⁺.

In total (ESI+/-), **139 unique HMOs** were detected, including molecules with up to **14 monosaccharide units**.



A) Example of spectral match of the MS2 experimental spectra (up) and the library (down) for the HMO IFLNnH II.
B) Principal component analysis was used to reveal samples with similar HMO profiles (i.e., DHM, preterm and term milk).
C) PCA loadings of annotated HMO features.



CONCLUSIONS

- ✓ We developed a method suitable for the **qualitative and quantitative analysis of over 100 HMOs** which will be applied in ongoing clinical studies focused on the impact of nutrition on preterm health.
- ✓ **DHM** is **less rich** in HMOs than MOM, possibly because differences due to the stage of lactation; some HMOs are **more abundant** in milk from mothers of **term** than **preterm** infants

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