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Comprehensive Profiles of Human Milk Oligosaccharides Yield Highly Sensitive and Specific Markers for Determining Secretor Status in Lactating Mothers

Sarah M. Totten,[†] Angela M. Zivkovic,[‡,§] Shuai Wu,[†] UyenThao Ngyuen,[‡] Samara L. Freeman,[‡] L. Renee Ruhaak,[†] Momodou K. Darboe,[∥] J. Bruce German,[‡,§] Andrew M. Prentice,[∥] and Carlito B. Lebrilla*,[†]

[†]Department of Chemistry, University of California, Davis, California 95616, United States

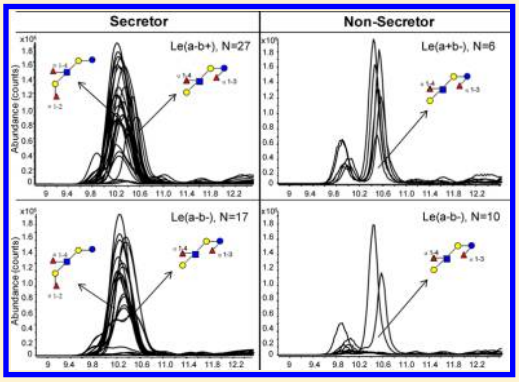
[‡]Foods for Health Institute, University of California, Davis, California 95616, United States

[§]Department of Food Science & Technology, University of California, Davis, California 95616, United States

[∥]MRC International Nutrition Group, London School of Hygiene & Tropical Medicine, London & MRC Keneba, The Gambia

S *Supporting Information*

ABSTRACT: Human milk oligosaccharides (HMOs), as an abundant and bioactive component of breast milk, work in many ways to promote the health of breast fed infants. The expression of HMOs has been shown to vary in accordance with Lewis blood type and secretor status, as women of different blood types differ in the expression of α1,2 fucosyltransferase (FUT2) and α1,3/4 fucosyltransferase (FUT3). In this study, HMOs were extracted from the milk of 60 women from The Gambia, Africa with various Lewis and secretor blood types. The HMOs were profiled using high resolution HPLC-Chip/TOF mass spectrometry. Notably, the amounts of fucosylation varied significantly between Le(a+b-) nonsecretors, Le(a-b+) and Le(a-b-) secretors, and Le(a-b-) nonsecretors. With higher frequency of expression of the recessive Lewis negative and nonsecretor phenotypes in West African populations, the HMO profiles of several milks from women of these phenotypes were examined, demonstrating decreased amounts of total oligosaccharide abundance and lower relative amounts of fucosylation. Also in this study, four specific fucosylated structures (2′FL, LNFP I, LDFT, and LNDFH I) were determined to be specific and sensitive glycan markers for rapidly determining secretor status without the need for serological testing.



KEYWORDS: *Lewis blood type, secretor status, human milk oligosaccharides, nano LC-TOF/MS*

INTRODUCTION

Free oligosaccharides are found in abundance in human milk, ranging between 20 and 24 g/L in colostrum and 7−14 g/L in mature milk.[1−4] Human milk oligosaccharides (HMOs) exhibit great structural diversity, and serve several important biological functions that promote infant health, including the prevention of pathogen binding to epithelial cell surfaces and functioning as prebiotics for beneficial bacteria in the intestine of breast fed infants.[3−5] In our previous studies, microfluidic Chip-TOF mass spectrometry was used to separate and assign monosaccharide compositions to 200 oligosaccharide structures.[6] Additionally, an annotated HMO library was developed, consisting of accurate mass, retention time, and CID MS/MS spectra of 75 of the most abundant neutral and acidic HMO structures and was used in this study for rapid HMO identification.[7,8] Most oligosaccharides contain a lactose core at the reducing end, and are further elongated with the addition of a β(1−3) or β(1−6)-linked *N*-acetylglucosamine, followed by the addition of β(1−3) or β(1−4)-linked galactose. These residues are added to several degrees of polymerization. Neutral oligosaccharides are further decorated with fucose or sialic acid residues. A series of fucosyltransferases are responsible for the pattern of fucosylation expressed in HMOs, and have been shown to be highly dependent on Secretor (Se) and Lewis blood group (Le) loci.[9−13] An individual is described as a secretor when ABH antigens are present in their bodily fluids and secretions. The secretor locus encodes for the α1,2 fucosyltransferase (FUT2), which transfers a fucose residue to a Galβ(1→3)GlcNAc unit (type 1 chain), producing the H-antigen in secretory epithelial cells.[11,14] Independent of the secretor gene is the Lewis blood group locus, which encodes for an α1,3/4 fucosyltransferase (FUT3) that transfers a fucose in an α(1−4)-linkage to a subterminal *N*-acetylglucosamine residue of a type 1 chain to produce the Lewis B antigen in secretors, and the Lewis A antigen in nonsecretors. As a result

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of the two genotypes described above, there are four possible phenotypes expressed in HMOs (Table 1): Lewis B secretors

Table 1. Secretor and Lewis Genotype and Phenotype Summary

Secretor Genotype (FUT2)	Lewis Genotype (FUT3)	Phenotype	Glycan Epitope, Fucose Linkage
se/se non-sec	le/le	Le (a-b-), Se-	Type 1 Chain
se/se non-sec	Le/Le or Le/le	Le (a+b-), Se-	Le^a Fuc α1-4
Se/Se or Se/se secretor	Le/Le or Le/le	Le (a-b+), Se+	Le^b Fuc α1-4 Fuc α1-2
Se/Se or Se/se secretor	le/le	Le (a-b-), Se+	H(O) Fuc α1-2

Galactose N-Acetylglucosamine Fucose

(denoted here as Le(a-b+),Se+), Lewis A nonsecretors (Le(a+b-),Se-), Lewis negative secretors (Le(a-b-),Se+), and Lewis negative nonsecretors (Le(a-b-),Se-).[11,13,14] Lewis negative and nonsecretor individuals make up a small part of European and American populations (nonsecretor frequency is around 20%), whereas these genotypes are found in higher abundance in West African populations (between 32 and 38%) including the Gambian population represented in this study.[15−18] This has afforded us a unique opportunity to describe the glycan expression in milk from women of these less abundant, recessive phenotypes that are typically underrepresented in previous studies. Thurl et al. previously showed that milk glycosylation is affected by the mother's secretor status and Lewis blood type .[13,19] A diagnostic test for rapidly assigning secretor status and Lewis blood groups based solely on milk oligosaccharides would not only expedite analysis, but it would also provide a less intrusive method for blood typing, eliminating the need for blood, serum, and/or saliva samples for serological agglutination assays. Recently, Blank et al. described a high-throughput MALDI MS method for rapidly assigning Lewis blood group based on characteristic fragment ion patterns of milk oligosaccharides.[20] Although this method provides a reproducible and highly accurate way for discriminating between Lewis blood types, it does not incorporate a separation component, yielding little information concerning specific isomeric structures that will be important for future functional studies.

In this study, a sensitive, rapid-throughput HPLC-Chip-TOF/MS-based method was used for profiling milk oligosaccharides and assigning secretor status from a cohort of 60 Gambian women of various Lewis blood types. Using this method, we are able to rapidly screen hundreds of milk oligosaccharides, providing a comprehensive representation of the oligosaccharide content in human milk. With the application of our previously published HMO libraries, we are able to assign specific structures to over 75 neutral and acidic oligosaccharides, allowing for a sensitive method for the discovery of biomarkers that can be systematically quantitated and used to rapidly assign secretor status.

■ REAGENTS AND MATERIALS

Milk Samples

Samples were collected from 100 Gambian women, once a month for the first sixth months of lactation. Donors were from six villages within The Gambia, and were of different ABO and Lewis blood types. Breast milk samples were collected by manual expression monthly between September 2001 and October 2004 according to methods and study design described previously.[21] The samples were shipped on ice to UC Davis after having been stored at −80 °C. All samples were stored at −80 °C prior to oligosaccharide extraction. The initial study and permission for the additional analysis of HMOs were approved by the Gambian Government/MRC Gambia Ethics Committee and all mothers provided signed, informed consent.

Serological Testing

Lewis blood types were determined by a hemagglutination tube test assay. Anti-Le^a and anti-Le^b monoclonal antibodies were purchased from BAG Health Care (Lich, Germany). Red blood cells were washed in isotonic saline (0.9% NaCl) and suspended in phosphate-buffered saline (2−3% erythrocytes in pH 6.8−7.2 PBS). Antibodies were mixed in suspension and incubated for 15 min at room temperature. Samples were monitored for agglutination and assigned as Le(a+b-), Le(a-b+), or Le(a-b-) blood type.

Oligosaccharide Extraction and Purification

Free oligosaccharides were extracted from breast milk samples and reduced according to our previously described methods.[6−8] Briefly, 200 μL of milk was defatted via centrifugation, followed by a Folch extraction and ethanol precipitation to remove lipids and proteins, respectively. Extracted HMOs were reduced to their alditol form with 1.0 M $NaBH_4$ in a 65 °C water bath for 1.5 h. Solid phase extraction (SPE) was used for desalting and enrichment. HMO samples were loaded onto a nonporous graphitized carbon cartridge, desalted with 24 mL of deionized water, and eluted with 20% acetonitrile in water and 40% acetonitrile in 0.05% trifluoroacetic acid (v/v). The eluents from both fractions were combined and evaporated. The samples were then reconstituted to appropriate concentrations for mass spectrometric analysis.

HPLC−CHIP/TOF Mass Spectrometry

The analysis of free oligosaccharides extracted from breast milk samples was performed on an HPLC−CHIP/TOF MS system equipped with both a capillary pump for sample loading and a nanopump for sample separation. The Agilent 1200 series LC unit was coupled to an Agilent 6210 series time-of-flight mass spectrometer via a chip−cube interface. The microfluidic chip consisted of a 40 nL enrichment column and a 75 μm × 43 mm analytical column, both packed with porous graphitized carbon (PGC). Data were collected in the positive mode and calibrated by a dual nebulizer electrospray source with internal calibrant ions ranging from m/z 118.086 to 2721.895.

HMO Chromatographic Separation

HMO samples were loaded onto the enrichment column using the capillary pump at a flow rate of 4.0 μL/min with a 1 μL injection volume. HMO separation was performed using a binary gradient consisting of an aqueous solvent A (3% acetonitrile/water (v/v) in 0.1% formic acid solution) and an organic solvent B (90% acetonitrile/water (v/v) in 0.1% formic acid solution). A 45 min gradient optimized for separating HMO mixtures developed by Wu et al. was used in this analysis.[7,8]

HPLC−CHIP/TOF MS Data Analysis

Free oligosaccharide identification and quantitation was performed using Agilent Mass Hunter Qualitative Analysis software, version B.03.01. Oligosaccharide peaks were identified using the *Find By Molecular Feature* function. Individual

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oligosaccharide species were rapidly identified by matching retention times and exact oligosaccharide masses (within <20 ppm mass error) obtained experimentally to theoretically calculated masses compiled in an annotated HMO library. A relative quantitation of oligosaccharide species was performed by directly correlating the abundance of free oligosaccharide to the absolute peak intensity (in ion counts) of the corresponding peak. The relative amount of each oligosaccharide species was calculated by normalizing the absolute abundance of the individual species to the total oligosaccharide ion abundance in each sample, yielding a relative abundance expressed as a percentage of the total. Additional structural information was acquired using collision-induced dissociation (CID) on an Agilent 6500 series HPLC-Chip/QTOF MS when further confirmation of specific structure was needed. Characteristic CID spectra of specific precursor ions were compared against an in-house HMO library using Agilent's Personal Compound Database Library software.

Statistical Analysis

For each secretor and Lewis group, mean and standard deviation of oligosaccharide intensities were reported. Bartlett test was used to test for homoscedasticity between groups in each oligosaccharide. The intensities were then $\log_2$ transformed to meet homogeneity of variances assumptions and to reduce the influence of large values for oligosaccharides that did not pass the test at an alpha level of 0.05. Analysis of variance (ANOVA) was used to test whether the mean intensities of several oligosaccharides were equal in each Lewis and secretor group.

Quantitation of HMO Markers

The absolute abundance of the HMO structures selected for use as secretor markers was normalized to another consistent peak in the spectrum to yield a relative abundance that could be compared between samples. HMO structures lacto-*N*-fucopentaose I (LNFP I), lactodifucotetraose (LDFT), and lacto-*N*-difucohexaose I (LNDFH I) were normalized to the abundance of lacto-*N*-tetraose (LNT), the most abundant peak present in every sample. The absolute abundance of 2′fucosyllactose (2′FL) was normalized to the abundance of its isomer 3′fucosyllactose (3′FL), which is also present in every sample. Receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity of HMO ratios used as diagnostic markers for determining secretor status. ROC curves and corresponding area under the curve (AUC) were calculated in MedCalc version 11.6.1. Optimal cutoff values for the abundance of oligosaccharide markers were also determined using ROC analysis.

RESULTS AND DISCUSSION

Lewis Blood Type and Secretor Status Distribution

Lewis blood types were determined by an in vitro hemagglutination assay using Lewis[a] and Lewis[b] antibodies. Of 60 Gambian women, 27 mothers were found to be Le(a-b+) secretors (45%), six were Le(a+b-) nonsecretors (10%), and 27 were Le(a-b-) (45%). Of the Le(a-b-) individuals, 17 were secretors and 10 were nonsecretors. In total, 73% of the samples were determined to be secretors. This statistic is comparable to Lewis blood type and secretor status distributions of West African populations previously reported in literature.[17,18]

Profiling Human Milk Oligosaccharides Using HPLC Chip-TOF/MS

HMOs were separated and quantitated by HPLC Chip-TOF/MS, which provides the sensitivity and mass accuracy needed for oligosaccharide identification, as has been demonstrated in our previous studies.[6−8] The majority of ionized oligosaccharides were detected in positive ion mode as singly protonated $[M + H]^+$ or doubly protonated $[M + 2H]^{2+}$ molecular ions. The mass accuracy of TOF-MS was between 2 and 15 ppm, allowing for the use of accurate mass to rapidly determine monosaccharide composition. Typically, between 100 and 200 different oligosaccharides are identified in a single milk sample using this method, with masses ranging from 490 to 3700 Da. With such a multitude of oligosaccharides, a wide distribution of masses as well as variation in abundances was observed, which can be seen in Supplemental Figure 1—a 2D plot of deconvoluted mass versus retention time. The method used for HMO analysis in this study provides a comprehensive representation of the oligosaccharide content in milk, and is not restrained by the number of HMO standards that are commercially available. Many peaks among sets of isomers coelute over a range of masses; however, mass spectrometric detection makes it possible to readily separate and quantitate compounds with similar retention times by extracting the chromatographic peaks associated with a particular *m/z* value (extracted ion chromatogram) (Supplemental Figure 1). Extracted ion chromatograms (EIC) are shown for *m/z* 1075.4, 1221.4, and 1367.5 (Figure 1), for which three, four, and six individual isomers were detected, respectively. Compounds with *m/z* 1367.5 are difucosylated oligosaccharides, typically with a lacto-*N*-hexaose (LNH) or lacto-*N*-neohexaose (LNnH) core. Difucosyllacto-*N*-hexaose a (DFLNHa), the most abundant isomer of *m/z* 1367.5 (Figure 1C), carries the H(O) epitope, an $\alpha(1-2)$-linked fucose at the terminal galactose, and was found in higher average relative abundance in secretors (1.24%) than in nonsecretors (0.07%). The *m/z* 1367.5 isomer at 19.9 min was separated and identified as DFLNHc, which contains the Lewis b epitope. The ability to separate isomers such as these that carry Lewis and H(O) is essential, as they may potentially be used as markers for Lewis blood type and secretor status.

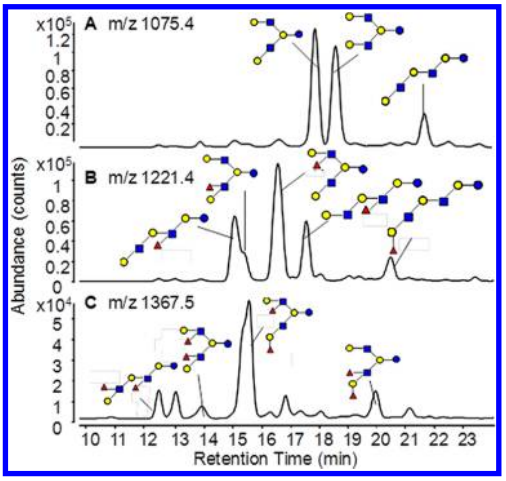

Figure 1. LC/MS Extracted Ion Chromatogram (EIC) of *m/z* 1075.4 (A), *m/z* 1221.4 (B), and 1367.5 (C).

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With the application of our previously published annotated libraries for both neutral and acidic HMOs, it is possible to rapidly identify up to 75 specific structures in a pool of milk oligosaccharides based on accurate mass and reproducible retention times. The robustness of this method for profiling oligosaccharides is dependent upon the reproducibility of the separation. HMO retention time reproducibility for this Chip-TOF/MS-based method was reported to be within 0.5% when run on the same day, and was within 5 s of the retention times of commercial standards.[6,7] In addition to accurate mass and retention time, CID fragmentation was used to confirm the structure of closely eluting compounds (Supplemental Figure 2).

Variation in HMO Abundance with Regard to Blood Type and Secretor Status

Between 100 and 130 oligosaccharide structures were separated in each sample, with no significant variation in the number of oligosaccharide structures found between the four different phenotypes. Total oligosaccharide concentrations, however, showed significant variation between blood groups (Figure 2A).

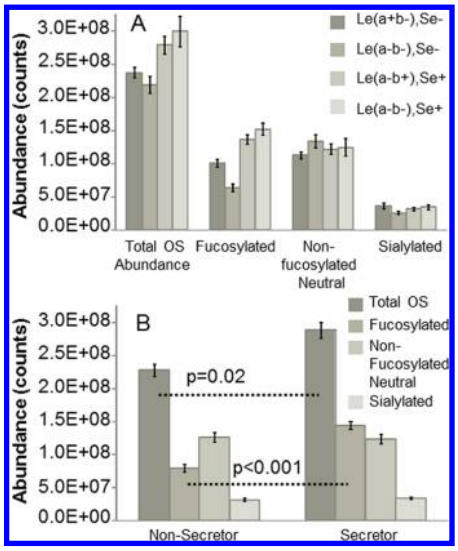

Figure 2. Absolute abundance of human milk oligosaccharide types are plotted in terms of ion counts as determined by HPLC Chip-TOF/MS. Comparison of oligosaccharide composition among Lewis blood types (A) and secretor status (B). Error bars are reported as standard deviation of the mean.

Milk from Le(a-b-),Se+ and Le(a-b+),Se+ mothers yielded a higher mean total oligosaccharide ion abundance (2.99×10^8 counts and 2.789×10^8 counts, respectively) than the two nonsecretor types, Le(a+b),Se- and Le(a-b-),Se- (2.37×10^8 counts and 2.18×10^8 counts, respectively). These data suggest that milk from secretor mothers is richer in oligosaccharides than that of nonsecretors, which may have an effect on the antipathogenic and prebiotic capacity of the milk. Significant differences in levels of fucosylation were also found between blood groups. Le(a-b-),Se+ and Le(a-b+),Se+ milk yielded the highest total ion count of fucosylated HMO structures. Notably, the absolute abundance of fucosylated oligosaccharides was lower by an order of magnitude in the Le(a-b-),Se- phenotype (see Table 2 and Supplemental Table 1 for summarized statistical analysis). Lower abundances of fucosylated oligosaccharides are a consistent characteristic in milk from nonsecretor women from a variety of geographical populations.[19,22] With a higher abundance of Lewis negative individuals in the Gambian population, we were able to profile several mothers with this phenotype. It should be noted that Le(a-b-) secretor profiles were quite different from Le(a-b-) nonsecretor profiles, demonstrating significant differences in the abundance of both fucosylated and nonfucosylated neutral oligosaccharides (Figure 2A), making evident that the HMO expression is more influenced based on the mother's secretor status than on the Lewis blood type.

To further evaluate the effects of secretor status, the 60 samples were grouped only as secretors or nonsecretors, regardless of Lewis blood type (secretor group, Le(a-b+),Se+ and Le(a-b-),Se+ individuals ($N = 44$); nonsecretor group, Le(a+b-),Se- and Le(a-b-),Se- individuals ($N = 16$)). Significant differences were found in the total oligosaccharide ion count ($p = 0.02$) and in the absolute amount of fucosylated compounds ($p < 0.001$) (Figure 2B). Previous studies have shown that increased fucosylation is characteristic of secretor milk.[13,19,22,23] To account for the biological variation in total oligosaccharide count between individuals, relative abundances (i.e., percentage of the total oligosaccharide count) of each oligosaccharide type were also determined (Figure 3), again confirming that secretor milk and nonsecretor milk differed most in the relative abundance of neutral oligosaccharides, both fucosylated ($p < 0.001$) and nonfucosylated ($p < 0.001$) (Figure 3B,C). Elevated concentrations of neutral HMOs found in nonsecretor individuals reflect the lack of fucose transfer expressed in these individuals, suggesting the lack or modified activity of the FUT2 and FUT3 fucosyltransferases. The extent of fucosylation found in Gambian secretor milk oligosaccharides (around 50% on average) was similar to that found in the milk from women in the United States in earlier studies,[6] in which fucosylated oligosaccharides accounted for 44−46% of the total oligosaccharide abundance. With a larger sampling of nonsecretor mothers, this study was able to show statistically significant lower amounts of fucosylation in nonsecreting individuals (only 35%) (Figure 3C). The relative abundance of sialylated oligosaccharides was slightly higher in nonsecretor milk (average of 14%) than in secretor milk (average of 11.9%), although the difference was found to be nonstatistically significant ($p = 0.176$) (Figure 3D). Oligosaccharides that are both fucosylated and sialylated were found in low abundance in secretors and nonsecretors, ranging from 1 to 9% in both groups.

Discovery of Markers for Specific Secretor and Lewis Epitopes

For marker discovery, 33 of the most abundant oligosaccharide structures were monitored in each sample, accounting for approximately 75−80% of the total oligosaccharide abundance (Table 2). Nonfucosylated neutral structures, including isomers LNT (m/z 710.3) and lacto-*N*-neotetraose (LNnT, m/z 710.3), as well as m/z 1075.4 isomers LNH, LNnH and p-LNH (para-lacto-*N*-hexaose) were common to all samples and were not found in statistically different amounts in secretors versus nonsecretors (Table 2). Consistent with reports from previous literature, milk from nonsecretors had a significantly lower abundance of oligosaccharide structures containing fucα(1→2)Gal residues, if not lacking them completely. The variation in abundance in accordance with blood type makes these structures ideal for quantitative markers.[13,19,22−24] 3′FL, Galβ(1→4)[Fucα(1→3)]Glc, was present in both secretor and nonsecretor milk, but was in higher abundance on average in

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Table 2. Statistical Analysis[a]

feature	mean abundance (counts)		SD		missing count		ANOVA
	nonsecretor (n = 16)	secretor (n = 44)	nonsecretor (n = 16)	secretor (n = 44)	nonsecretor (n = 16)	secretor (n = 44)	P val (ANOVA)
Fucosylation	7.94×10^{7}	1.44×10^{8}	2.51×10^{7}	3.71×10^{7}	0	0	0
% Fucosylation	3.47×10^{1}	5.05×10^{1}	8.99×10^{0}	6.85×10^{0}	0	0	0
Nonfucosylated neutral	1.26×10^{8}	1.23×10^{8}	2.85×10^{7}	4.73×10^{7}	0	0	0.494
% Nonfucosylated neutral	5.56×10^{1}	4.20×10^{1}	9.53×10^{0}	7.46×10^{0}	0	0	0
Sialylation	3.15×10^{7}	3.40×10^{7}	1.14×10^{7}	1.22×10^{7}	0	0	0.473
%Sialylation	1.35×10^{1}	1.19×10^{1}	3.90×10^{0}	3.75×10^{0}	0	0	0.176
Total OS Intensity	2.28×10^{8}	2.89×10^{8}	3.57×10^{7}	7.95×10^{7}	0	0	0.017
3′FL	2.42×10^{5}	1.49×10^{5}	1.57×10^{5}	1.12×10^{5}	0	0	0.014
2′FL	2.26×10^{5}	1.84×10^{7}	2.51×10^{5}	7.79×10^{6}	2	0	0
LDFT	4.09×10^{3}	7.08×10^{6}	0.00×10^{0}	4.76×10^{6}	2	0	0
LNT + LNnT	9.78×10^{7}	8.96×10^{7}	2.93×10^{7}	3.19×10^{7}	0	0	0.374
LNFP II	1.56×10^{7}	9.71×10^{6}	1.26×10^{7}	5.81×10^{6}	0	0	0.425
LNFP I	8.76×10^{5}	3.88×10^{7}	2.38×10^{6}	2.09×10^{7}	0	0	0
LNFP III	9.04×10^{6}	1.57×10^{6}	5.60×10^{6}	4.98×10^{6}	0	0	0
LNDFH I	1.20×10^{5}	4.32×10^{6}	2.85×10^{5}	3.18×10^{6}	0	0	0
LNDFH II	1.46×10^{6}	3.45×10^{5}	1.62×10^{6}	1.07×10^{6}	0	0	0
LNH	4.41×10^{6}	4.68×10^{6}	2.62×10^{6}	4.90×10^{6}	0	0	0.633
LNnH	4.22×10^{6}	5.93×10^{6}	2.78×10^{6}	3.87×10^{6}	0	0	0.111
pLNH	1.08×10^{6}	2.91×10^{6}	1.04×10^{6}	4.48×10^{6}	0	0	0.197
MFpLNH.IV	2.97×10^{6}	2.56×10^{6}	1.93×10^{6}	1.78×10^{6}	0	0	0.439
4120a	1.60×10^{6}	1.59×10^{6}	3.08×10^{6}	2.07×10^{6}	0	0	0.181
MFLNH III	5.52×10^{6}	4.42×10^{6}	2.55×10^{6}	2.42×10^{6}	0	0	0.129
IFLNH III	2.54×10^{6}	2.05×10^{6}	1.78×10^{6}	1.29×10^{6}	0	0	0.248
IFLNH I	1.85×10^{5}	1.22×10^{6}	1.65×10^{5}	7.49×10^{5}	0	1	0
DFpLNH II	1.46×10^{6}	8.50×10^{5}	7.20×10^{5}	7.58×10^{5}	0	0	0.007
m/z 1367.5 @13 min	1.25×10^{6}	6.79×10^{5}	1.13×10^{6}	6.15×10^{5}	2	1	0.766
DFLNHb	2.07×10^{6}	8.16×10^{5}	1.42×10^{6}	9.36×10^{5}	0	4	0.016
DFLNHa	1.52×10^{5}	3.59×10^{6}	5.09×10^{5}	2.71×10^{6}	1	0	0
m/z 1367.5 @16.7 min	1.80×10^{5}	3.69×10^{5}	5.82×10^{5}	3.67×10^{5}	0	2	0
m/z 1367.5 @17.4 min	9.45×10^{4}	2.17×10^{5}	1.19×10^{5}	2.51×10^{5}	2	0	0.125
DFLNHc	1.27×10^{5}	5.55×10^{5}	1.30×10^{5}	3.47×10^{5}	1	1	0
6′SL	8.99×10^{5}	8.61×10^{4}	7.42×10^{5}	3.70×10^{5}	1	3	0
3′SL	3.24×10^{6}	2.91×10^{6}	1.09×10^{6}	1.30×10^{6}	0	0	0.36
LSTc	5.95×10^{6}	6.16×10^{6}	2.72×10^{6}	3.24×10^{6}	0	0	0.817
LSTb	2.52×10^{6}	2.09×10^{6}	1.92×10^{6}	1.68×10^{6}	0	0	0.41
LSTa	1.55×10^{6}	1.12×10^{6}	8.00×10^{5}	9.52×10^{5}	0	0	0.118
FLSTc	1.19×10^{6}	8.41×10^{5}	1.15×10^{6}	6.30×10^{5}	0	0	0.659
sLNH	1.64×10^{6}	1.14×10^{6}	1.40×10^{6}	9.70×10^{5}	0	0	0.125
4012a	2.52×10^{5}	4.99×10^{5}	2.97×10^{5}	4.12×10^{5}	0	1	0.032
sLNnH II	2.33×10^{6}	4.13×10^{6}	1.30×10^{6}	3.18×10^{6}	0	0	0.008
2′FL:3′FL	1.62×10^{0}	2.54×10^{2}	2.01×10^{0}	2.99×10^{2}	2	1	0
LNFP I:LNT	6.00×10^{-3}	4.21×10^{-1}	1.60×10^{-2}	2.33×10^{-1}	0	1	0
LDFT:LNT	0.00×10^{0}	8.00×10^{-2}	0.00×10^{0}	5.30×10^{-2}	2	0	0
LNDFH I:LNT	1.00×10^{-3}	4.70×10^{-2}	2.00×10^{-3}	3.40×10^{-2}	0	1	0
LNDFH II:LNT	1.60×10^{-2}	5.00×10^{-3}	1.90×10^{-2}	1.70×10^{-2}	0	1	0.03
6′SL:3′SL	3.45×10^{-1}	4.80×10^{-2}	3.12×10^{-1}	1.95×10^{-1}	0	4	0

[a]Mean and standard deviation (SD) are reported in ion counts as determined by TOF/MS. Percentages were calculated by normalizing the ion count of that group to the total oligosaccharide count. ANOVA p-value of "0" indicates a value determined to be <0.001.

the milk of nonsecretors ($\bar{x}_{sec}$= 1.49×10^{5} counts, $\bar{x}_{nonsec}$= 2.42×10^{5} counts, p = 0.01). 2′FL was produced in significantly higher abundance by secretor mothers by 2 orders of magnitude ($\bar{x}_{sec}$= 1.84×10^{7} counts, $\bar{x}_{nonsec}$ = 2.26×10^{5} counts, $p < 0.001$). It should be noted that the 2′FL isomer was not completely absent in nonsecretors, but rather found in much lower abundance by comparison, as shown in the 60 overlaid EIC of m/z 491.2 in Figure 4. Lactodifucotetraose (LDFT, m/z 636.2), a difucosylated tetrasaccharide containing an fucα(1→2)Gal residue, was also found in much higher abundance in secretor milk ($\bar{x}_{sec}$ = 7.08×10^{6} counts, $\bar{x}_{nonsec}$ = 4.09×10^{9} counts, $p < 0.001$), but again was not totally absent from nonsecretor milk. Because of the increased sensitivity of TOF/MS detection, oligosaccharides such as 2′FL and LDFT can be detected in nonsecretor milk in low abundances, making it useful to calculate relative abundances normalized to another consistent peak in the spectrum, making the markers more widely applicable. Several other less abundant fucosylated

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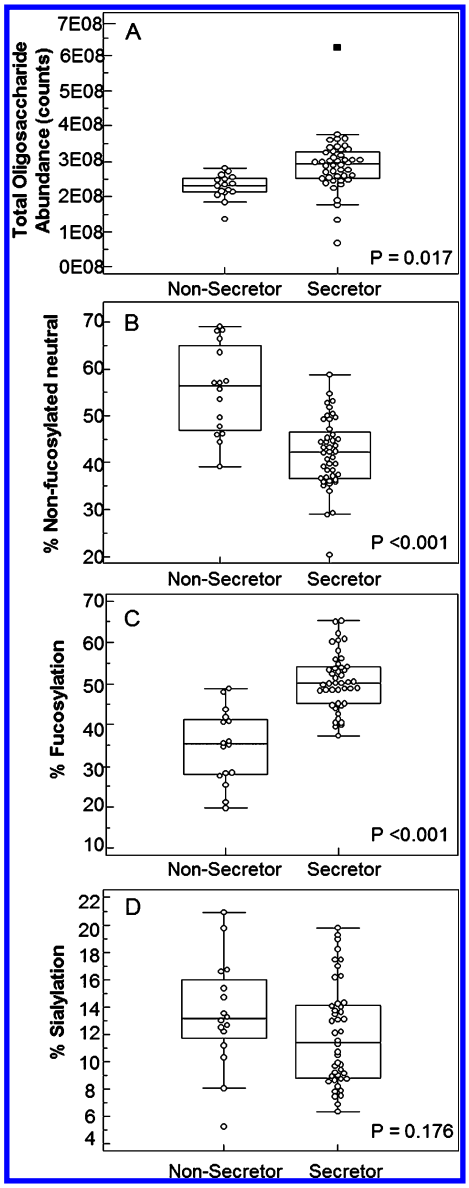

Figure 3. Total HMO abundance (A) and relative abundance of nonfucosylated neutral oligosaccharides (B), fucosylated oligosaccharides (C), and sialylated oligosaccharides (D). ANOVA *p*-values are inset.

oligosaccharides with larger degrees of polymerization were also determined to be secretor marker candidates. These include lacto-*N*-fucopentaose II (LNFP II, *m*/*z* 856.3), lacto-*N*-difucohexaose I (LNDFH I, *m*/*z* 1002.4, Figure 5), isomeric fucosyl-lacto-*N*-hexaose (IFLNH I, *m*/*z* 1220.5), and isomers DFLNHa and DFLNHc (*m*/*z* 1367.5), all of which contain $\alpha(1-2)$-linked fucose residues and were produced in higher amounts in secretor mothers ($p < 0.001$ for all structures, see Table 2 for means and SD).

The proposed markers described above can be used to rapidly distinguish between secretor and nonsecretor mothers, but cannot necessarily be used to distinguish between Lewis blood types. To further differentiate secretors and nonsecretors into their respective Lewis blood types, HMOs containing Le^a and Le^b epitopes were quantified and compared between the various milks. Among nonsecretors, Le(a+b-) differ from Le(a-b-) in their ability to produce HMOs containing the Le^a epitope—an $\alpha(1-4)$-linked fucose to a subterminal GlcNAc, as determined by serological agglutination assays, and observed in HMO expression.[13,19] In this study, the difucosylated oligosaccharide LNDFH II (*m*/*z* 1002.4), which contains the Le^a epitope, was produced in significantly lower abundances in Le(a-b-) milk compared with Le(a+b-),Se- and Le(a-b+),Se+ ($p < 0.001$ in both, see Supplemental Table 1 for means and SD) and was largely absent from Le(a-b-),Se- milk, with the exception of two samples, demonstrating moderate consistency with the serological data (Figure 5D). Figure 5C demonstrates the preference of Le(a+b-) mothers to produce LNDFH II in considerably larger amounts, making this glycan an ideal marker for distinguishing Le(a+b-) and Le(a-b+) from Le(a-b-). The Le^b-containing isomer LNDFH I (*m*/*z* 1002.4) was also absent or found in lower abundances in Le(a-b-) nonsecretors, as was found in previous studies. Although a clear preference was observed for LNDFH II in nonsecretors and LNDFH I in secretors, it was not suitable for distinguishing between Le(a-b+) secretors from Le(a-b-) secretors, as shown in the overlaid extracted ion chromatograms in Figure 5A,B. From the overlaid EICs of *m*/*z* 1002.4, it is made clear that Lewis sugars, namely, Le^b, were present in several Lewis negative secretors, contradictory to the serological blood type assignment. LNDFH I and LNDFH II structures are reported in previous studies to be absent in Le(a-b-) individuals, but in this study were found in nearly 70% of Le(a-b-) milks in abundances similar to those of Le(a+b-) and Le(a-b+) milk. This phenomenon was also observed in the expression of other HMOs carrying the Le^a and Le^b determinants, such as the LNFP isomers (*m*/*z* 856.3). LNFP III, an LNT core with an $\alpha(1-3)$-linked fucose to a subterminal GlcNAc, was detected in all samples and was found at significantly higher abundance in Le(a+b-),Se- milks ($p < 0.001$). It is consistently reported in previous literature that oligosaccharides containing the Lewis a and b antigens are typically absent in the milk of Le(a-b-) individuals.[19,22,24] However, in this study, LNFP II, although it contains a Le^a epitope, was found in appreciable amounts in almost 90% of milk from Lewis negative mothers. LNFP II was found in significantly higher absolute abundance in Le(a+b-) milk ($2.72 \times 10^7 \pm 3.89 \times 10^6$ counts) than in Le(a-b-) milk ($8.34 \times 10^6 \pm 7.63 \times 10^6$) ($p < 0.001$, Supplemental Table 1); however, it could not be used as a marker to distinguish between Le(a-b+) secretors and Le(a-b-) secretors, as the abundance of LNFP II in these groups was not significantly different ($1.07 \times 10^7 \pm 5.87 \times 10^6$ counts and $8.34 \times 10^6 \pm 7.63 \times 106$, respectively). The detection of Lewis antigens in Lewis negative individuals suggests modified fucosyltransferase activity in the expression of milk sugars compared to the antigenic determinants present in serum samples from the same mother. This observation has been previously investigated. Studies have shown that Lewis antigens found on glycolipids from plasma and serum samples can be detected in the erythrocytes of Lewis negative individuals.[25,26] Studies have also shown that blood group antigens may be expressed differently in various types of tissues (antigenic determinants found on erythrocyte versus epithelial expression), and may be dependent on factors such as recent pregnancy, race, and health status (cancer versus healthy).[9−11,26−28] As a result of the expression of Lewis blood group epitopes in Le(a-b-) secretor milks, markers were not identified to distinguish between milk from Le(a-b+) secretors and Le(a-b-) secretors.

Several acidic structures were also monitored and are summarized in Table 2. Most of the sialylated structures were found in low abundance and showed no significant variation

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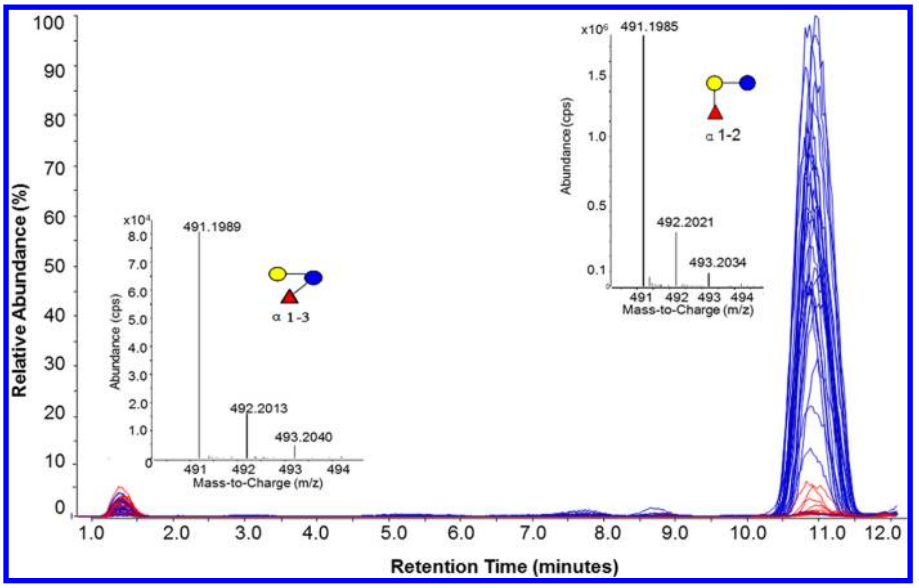

Figure 4. Overlaid secretor (blue chromatograms) and nonsecretor (red chromatograms) EIC of m/z 491.19.

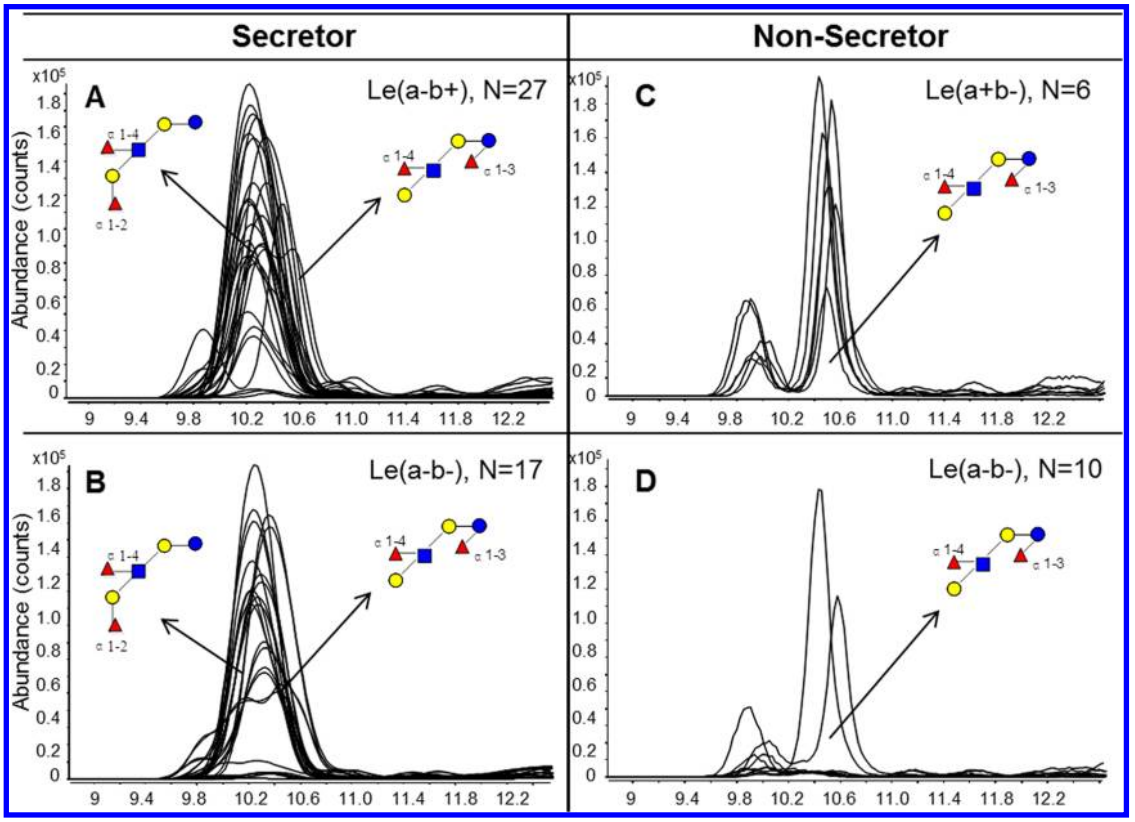

Figure 5. Overlaid Extracted Ion Chromatogram (EIC) of m/z 1002.4 isomers LNDFH I (10.3 min) and LNDFH II (10.5 min) in Le(a-b+) and Le(a-b-) secretor phenotypes (A and B, respectively), and Le(a+b-) and Le(a-b-) nonsecretor phenotypes (C and D, respectively).

between secretors and nonsecretors, as was also reported by Thurl et al.[19] One notable difference in this study, however, was the variation in the amounts of sialyllactose isomers found between secretors and nonsecretors. The amount of 6′-sialyllactose (6′SL, m/z 635.2) detected in nonsecretors was higher by an order of magnitude in comparison to secretors (8.99×10^5 vs 8.61×10^4 counts, respectively, $p < 0.001$), whereas 3′sialyllactose (3′SL, m/z 635.2) was produced in comparable amounts among all blood types. Comparing the isomeric ratio of 6′SL to 3′SL in secretors and nonsecretors revealed a clear preference in nonsecretors to produce the isomer containing an (α2−6)-linked *N*-acetylneuraminic acid (Neu5Ac) residue. There was no other evidence, however, to confirm higher levels of α2,6-sialylated HMOs. For instance, sialyllactose-*N*-tetraose (m/z 1000.4) isomers LSTa, LSTb, and LSTc, which differ by the placement and linkage of Neu5Ac on an LNT or LNnT (in the case of LSTc) core, were all found in similar abundances regardless of secretor status (see Table 2 for statistical analysis).

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Evaluation of Specific HMO Markers for Secretor Status

As reported in previous studies, HMO structures containing (α1−2)-linked fucose (the H-determinant) are present in significantly lower abundance if not entirely absent from nonsecretor milk.[12,13,19,22,28] In this study, we describe a sensitive and reproducible way to quantitate this phenotype, and to use these structures as diagnostic markers to systematically assign secretor status of a lactating mother without having to collect serum, blood, or saliva samples. From the statistical data, four fucosylated oligosaccharides and one sialylated oligosaccharide were chosen as prospective secretor markers based on abundance and statistical differences of the means as determined by ANOVA: 2′FL, LNFP I, LDFT, LNDFH I, and 6′SL.

ROC curves were used to determine the sensitivity and the specificity of the markers (Figure 6), and to determine appropriate criteria for the cutoff values of the isomeric ratios used to distinguish secretors form nonsecretors (Table 3). It was determined that the optimal cut off value for describing an individual as a secretor using the 2′FL/3′FL abundance ratio was >6.5, with a sensitivity and specificity of 95.5 and 100, respectively. The ROC curve for the 2′FL/3′FL marker yielded an AUC of 0.967, suggesting 2′FL to be one of the most effective secretor markers (Figure 6A). LDFT also yielded a highly sensitive and specific secretor marker by normalizing the absolute abundance of LDFT to the absolute abundance of LNT (AUC = 0.984). Sensitivity was not lost at the expense of specificity, or vice versa, due to the remarkable consistency of the oligosaccharides containing α1−2-linked fucose residues to be present in secretor milk in appreciable amounts. LNFP I and LNDFH I proved equally effective markers when normalized to the absolute abundance of LNT, yielding low false positive rates and high true positive rates, both with AUC ≥ 0.95. The ROC curve for LNDFH I yielded a higher false-negative rate than the other fucosylated markers (AUC = 0.924) and was not included in Figure 6, but the statistics of this marker are summarized in Table 2. Forty-two out of 44 secretors were correctly identified by 2′FL/3′FL, LDFT/LNT, and LNDFP I/LNT markers; however, the sensitivity was compromised by two samples that were assigned Le(a-b+) (secretor) type by the serological tests, but produce the markers in amounts comparable to that of nonsecretors.

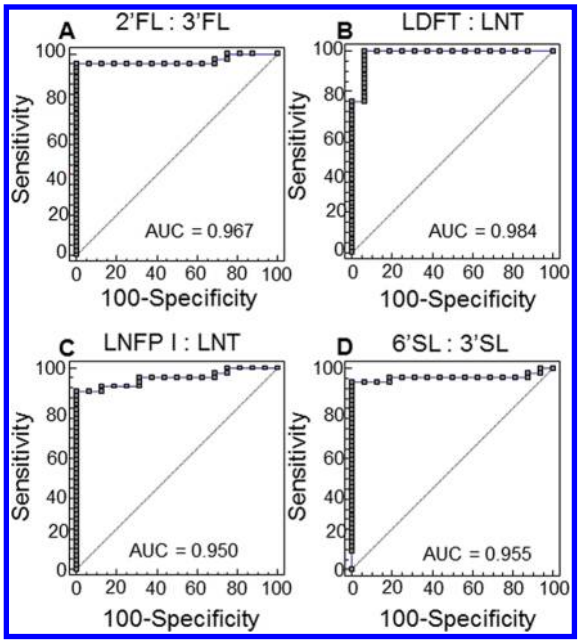

Figure 6. ROC curves for the assessment of secretor markers 2′FL/3′FL (A), LDFT/LNT (B), LNFP I/LNT (C), and 6′SL/3′SL (D). AUC values are inset.

CONCLUSION

The proposed method for determining secretor status based exclusively on the relative quantitation of milk oligosaccharides is a valuable tool for future studies seeking to correlate the specific structure of HMO isomers carrying blood group epitopes with their biological function. With the effects of secretor status on HMO composition now well characterized in the literature, it is important to investigate the biological implications of varying oligosaccharide content among secretors and nonsecretors, which poses the following question: does the specific milk oligosaccharide composition determined by the mother's secretor status have an effect on the health of the infant? It has already been shown that secretor oligosaccharide structures inhibit the binding of a number of pathogenic bacteria to gastric epithelial cells.[2,3,29−31] On the other hand, the nonsecretor phenotype occurs with higher frequency in African, Central Asian, Pacific, and Far East populations, and has been linked to providing protection against a number of viral infections including certain strains of norovirus, influenza, rhinovirus, and HIV-1.[32] HMOs also provide an abundant food source and encourage the growth of health-promoting microorganisms in the infant's gut, protecting the infant from the colonization of harmful pathogens.[2,3,33−35] The secretor genotype is associated with the bifidobacteria composition in the human intestine, with the diversity, richness, and abundance of bifidobacteria significantly reduced in nonsecretor individuals.[36] As oligosaccharide expression in breast milk is dependent on Lewis blood type and secretor status, it is important to correlate the HMO expression of secretor and nonsecretor mothers to the microbiota in breast-fed infants in order to investigate the mircobiota-mediated protective properties of secretor milk versus nonsecretor milk.

Table 3. Summary of Secretor Status Marker Analysis by ROC Curves

marker (*m/z*, RT min)	normalized to (*m/z*, RT)	criterion ratio	specificity	sensitivity
2′FL (491.19, 10.8)	3′FL (491.19, 1.56 min)	>6.5827	100.	95.5
LNFP I (856.33, 13.45)	LNT (710.27, 14.17 min)	>0.0567	100.	88.6
LDFT (637.25, 13.50)	LNT (710.27, 14.17 min)	>0.0001	93.8	100.
LNDFH I (1002.38, 10.25)	LNT (710.27, 14.17 min)	>0.0086	100.	89.2
6′SL (636.23, 13.61)	3′SL (636.23, 21.97 min)	<0.0235	100.	93.2

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■ ASSOCIATED CONTENT

Supporting Information

Supplemental Figure 1, a 2D plot of deconvoluted mass versus retention time; Supplemental Figure 2, acquired CID spectra; Supplemental Table 1, statistical analysis by Lewis blood type. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Emai: cblebrilla@ucdavis.edu; Tel: +1-530-752-6364; Fax: +1-530-752-8995.

Notes

The authors declare no competing financial interest.

HPLC-Chip/TOF MS, high performance liquid chromatography-chip time-of-flight mass spectrometry; CID, collusion-induced dissociation; MALDI MS, matrix-assisted laser desorption/ionization mass spectrometry; Hex, hexose; HexNAc, *N*-acetylhexosamine; GlcNAc, *N*-acetylglucosamine; Neu5Ac, *N*-acetylneuraminic acid; 3′FL, 3′-fucosyllactose; 2′FL, 2′-fucosyllactose; 6′SL, 6′-sialyllactose; 3′SL, 3′-sialyllactose; LDFT, lactodifucotetraose; LNT + LNnT, lacto-*N*-tetraose + lacto-*N*-neotetraose; LNFP II, lacto-*N*-fucopentaose II; LNFP I, lacto-*N*-fucopentaose I; LNFP III, lacto-*N*-fucopentaose III; LSTc, sialyllacto-*N*-tetraose c; LSTb, sialyllacto-*N*-tetraose b; LSTa, sialyllacto-*N*-tetraose a; LNDFH I, lacto-*N*-difucohexaose I; LNDFH II, lacto-*N*-difucohexaose II; LNH, lacto-*N*-hexaose; LNnH, lacto-*N*-neohexaose; p-LNH, *para*-lacto-*N*-hexaose; F-LSTc, monofucosylmonosialyllacto-*N*-tetraose c; MFpLNH IV, monofucosyl *para*-lacto-*N*-hexaose; MFLNH III, monofucosyllacto-*N*-hexaose III; IFLNH III, isomeric fucosylated lacto-*N*-hexaose III; IFLNH I, isomeric fucosylated lacto-*N*-hexaose I; s-LNH, sialyllacto-*N*-hexaose; s-LNnH II, sialyllacto-*N*-neohexaose II; DFpLNH II, difucosyl-*para*-lacto-*N*-hexaose; DFLNHb, difucosyllacto-*N*-hexaose b; DFLNHa, difucosyllacto-*N*-hexaose a; DFLNHc, difucosyllacto-*N*-hexaose c. 4120 a and 4021a are HMOs named systematically for their monosaccharide composition (Hex:HexNAc:Fucose:Neu5Ac), with letters designating the elution order of isomers.

■ REFERENCES

(1) Coppa, G. V.; Pierani, P.; Zampini, L.; Carloni, I.; Carlucci, A.; Gabrielli, O. Oligosaccharides in human milk during different phases of lactation. *Acta Pædiatr.* **1999**, *88*, 89−94.

(2) Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu. Rev. Nutr.* **2000**, *20*, 699−722.

(3) Urashima, T.; Kitaoka, M.; Asakuma, S.; Messer, M. Milk Oligosaccharides. In *Advanced Dairy Chemistry, Vol. 3: Lactose, Water, Salts and Minor Constinuents*; McSweeney, P. L. H., Fox, P. F., Eds.; Springer Science+Business Media LLC: New York, NY, 2009; Vol. 3, pp 295−348.

(4) Boehm, G.; Stahl, B. *Oligosaccharides in Functional Dairy Products*; Mattila-Sandhol, T., Scarela, M., Eds.; CRC Press: Cambridge, England, 2003; pp 203−243.

(5) Ruhaak, L. R.; Lebrilla, C. B. Advances in analysis of human milk oligosaccharides. *Adv. Nutr.* **2012**, *3* (3), 406S−414S.

(6) Ninonuevo, M. R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R. E.; Clowers, B. H.; German, J. B.; Freeman, S. L.; Killeen, K.; Grimm, R.; Lebrilla, C. B. A strategy for annotating the human milk glycome. *J. Agric. Food Chem.* **2006**, *54* (20), 7471−7480.

(7) Wu, S.; Grimm, R.; German, J. B.; Lebrilla, C. B. Annotation and structural analysis of sialylated human milk oligosaccharides. *J. Proteome Res.* **2010**, *10* (2), 856−868.

(8) Wu, S.; Tao, N.; German, J. B.; Grimm, R.; Lebrilla, C. B. Development of an annotated library of neutral human milk oligosaccharides. *J. Proteome Res.* **2010**, *9* (8), 4138−4151.

(9) Le Pendu, J.; Caillard, T.; Mollicone, R.; Couillin, P.; Oriol, R. Expression of ABH and X (Lex) antigens in various cells. *Biochimie* **1988**, *70* (11), 1613−1618.

(10) Watkins, W. M.; Greenwell, P.; Yates, A. D.; Johnson, P. H. Regulation of expression of carbohydrate blood group antigens. *Biochimie* **1988**, *70* (11), 1597−1611.

(11) Oriol, R.; Le Pendu, J.; Mollicone, R. Genetics of ABO, H, Lewis, X and related antigens. *Vox Sang.* **1986**, *51* (3), 161−171.

(12) Kobata, A. Structures and application of oligosaccharides in human milk. *Proc. Jpn. Acad., Ser. B* **2010**, *86* (7), 731−747.

(13) Thurl, S.; Henker, J.; Siegel, M.; Tovar, K.; Sawatzki, G. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate J.* **1997**, *14* (7), 795−799.

(14) Brooks, S. A.; Dwek, M. V.; Schumacher, U. *Functional and Molecular Glycobiology*; BIOS Scientific: Oxford, 2002.

(15) Camps, F. E.; Dodd, B. E.; Lincoln, P. J. Frequencies of secretors and non-secretors of ABH group substances among 1,000 alcoholic patients. *Br. Med. J.* **1969**, *4* (5681), 457−459.

(16) Kelly, R. J.; Rouquier, S.; Giorgi, D.; Lennon, G. G.; Lowe, J. B. Sequence and expression of a candidate for the human secretor blood group α(1,2)fucosyltransferase gene (FUT2). *J. Biol. Chem.* **1995**, *270* (9), 4640−4649.

(17) Barnicot, N. A.; Lawler, S. D. A study of the Lewis, Kell, Lutheran and P blood group systems and the ABH secretion in West African Negroes. *Am. J. Phys. Anthropol.* **1953**, *11* (1), 83−90.

(18) Stedman, R. Human population frequencies in twelve blood grouping systems. *J. Forensic Sci. Soc.* **1972**, *12* (2), 379−413.

(19) Thurl, S.; Munzert, M.; Henker, J.; Boehm, G.; Muller-Werner, B.; Jelinek, J.; Stahl, B. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br. J. Nutr.* **2010**, *104* (9), 1261−1271.

(20) Blank, D.; Gebhardt, S.; Maass, K.; Lochnit, G.; Dotz, V.; Blank, J.; Geyer, R.; Kunz, C. High-throughput mass finger printing and Lewis blood group assignment of human milk oligosaccharides. *Anal. Bioanal. Chem.* **2011**, *401* (8), 2495−2510.

(21) Darboe, M. K.; Thurnham, D. I.; Morgan, G.; Adegbola, R. A.; Secka, O.; Solon, J. A.; Jackson, S. J.; Northrop-Clewes, C.; Fulford, T. J.; Doherty, C. P.; Prentice, A. M. Effectiveness of an early supplementation scheme of high-dose vitamin A versus standard WHO protocol in Gambian mothers and infants: a randomised controlled trial. *Lancet* **2007**, *369* (9579), 2088−2096.

(22) Viverge, D.; Grimmonprez, L.; Cassanas, G.; Bardet, L.; Solere, M. Discriminant carbohydrate components of human milk according to donor secretor types. *J. Pediatr. Gastroenterol. Nutr.* **1990**, *11* (3), 365−370.

(23) Chaturvedi, P.; Warren, C. D.; Altaye, M.; Morrow, A. L.; Ruiz-Palacios, G.; Pickering, L. K.; Newburg, D. S. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* **2001**, *11* (5), 365−372.

(24) Albrecht, S.; Schols, H. A.; van den Heuvel, E. G. H. M.; Voragen, A. G. J.; Gruppen, H. Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. *Carbohydr. Res.* **2011**, *346* (16), 2540−2550.

(25) Hammar, L.; Månsson, S.; Rohr, T.; Chester, M. A.; Ginsburg, V.; Lundblad, A.; Zopf, D. Lewis phenotype of erythrocytes and lebactive glycolipid in serum of pregnant women1. *Vox Sang.* **1981**, *40* (1), 27−33.

(26) Henry, S. M.; Oriol, R.; Samuelsson, B. E. Detection and characterization of lewis antigens in plasma of lewis-negative individuals evidence of chain extension as a result of reduced fucosyltransferase competition. *Vox Sang.* **1994**, *67* (4), 387−396.

(27) Orntoft, T.; Holmes, E.; Johnson, P.; Hakomori, S.; Clausen, H. Differential tissue expression of the Lewis blood group antigens: enzymatic, immunohistologic, and immunochemical evidence for Lewis a and b antigen expression in Le(a-b-) individuals. *Blood* **1991**, *77* (6), 1389−1396.
(28) Blank, D.; Dotz, V.; Geyer, R.; Kunz, C. Human milk oligosaccharides and lewis blood group: individual high-throughput sample profiling to enhance conclusions from functional studies. *Adv. Nutr.* **2012**, *3* (3), 440S−449S.
(29) Moulds, J. M.; Nowicki, S.; Moulds, J. J.; Nowicki, B. J. Human blood groups: incidental receptors for viruses and bacteria. *Transfusion* **1996**, *36* (4), 362−374.
(30) Kunz, C.; Rudloff, S. Health promoting aspects of milk oligosaccharides. *Int.l Dairy J.* **2006**, *16* (11), 1341−1346.
(31) Newburg, D. S.; Ruiz-Palacios, G. M.; Morrow, A. L. Human milk glycans protect infants against enteric pathogens. *Annu. Rev.Nutr.* **2005**, *25*, 37−58.
(32) Anstee, D. J. The relationship between blood groups and disease. *Blood* **2010**, *115* (23), 4635−4643.
(33) Sela, D. A.; Chapman, J.; Adeuya, A.; Kim, J. H.; Chen, F.; Whitehead, T. R.; Lapidus, A.; Rokhsar, D. S.; Lebrilla, C. B.; German, J. B.; Price, N. P.; Richardson, P. M.; Mills, D. A. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (48), 18964−18969.
(34) Asakuma, S.; Hatakeyama, E.; Urashima, T.; Yoshida, E.; Katayama, T.; Yamamoto, K.; Kumagai, H.; Ashida, H.; Hirose, J.; Kitaoka, M. Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. *J. Biol. Chem.* **2011**, *286* (40), 34583−34592.
(35) Marcobal, A.; Barboza, M.; Froehlich, J. W.; Block, D. E.; German, J. B.; Lebrilla, C. B.; Mills, D. A. Consumption of human milk oligosaccharides by gut-related microbes. *J. Agric. Food Chem.* **2010**, *58* (9), 5334−5340.
(36) Wacklin, P.; Mäkivuokko, H.; Alakulppi, N.; Nikkilä, J.; Tenkanen, H.; Räbinä, J.; Partanen, J.; Aranko, K.; Mättö, J. Secretor genotype FUT2 is strongly associated with the composition of bifidobacteria in the human intestine. *PLoS One* **2011**, *6* (5), e20113.