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A Strategy for Annotating the Human Milk Glycome

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Oligosaccharides in human milk represent a group of bioactive molecules that have evolved to be an abundant and diverse component of human milk, even though they have no direct nutritive value to the infant. A recent hypothesis proposes that they could be substrates for the development of the intestinal microflora and the mucosal immune system. The inability to determine the exact composition of these oligosaccharides limits research and the ability to understand their biological functions. Oligosaccharides isolated from the lipids and proteins of individual human milk samples were analyzed by a combination of techniques including microchip liquid chromatography mass spectrometry (HPLC-Chip/MS) and matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FT ICR MS). Accurate mass measurements obtained using an orthogonal time-of-flight (*o*-TOF) mass spectrometry provided oligosaccharide composition for approximately 200 individual molecular species. Comparison of HPLC-Chip/MS profiles from five different women revealed variations in milk oligosaccharide compositions. HPLC-Chip/MS profiling provides a method for routinely identifying milk oligosaccharides. Tandem MS in combination with exoglycosidase digestion provides unambiguous differentiation of structural isomers.

KEYWORDS: Human milk oligosaccharides; milk glycome; HPLC-Chip/MS technology; infrared multiphoton dissociation; exoglycosidases; matrix-assisted laser desorption/ionization Fourier transform mass spectrometry

INTRODUCTION

Recent studies suggest that nutrition is only one of the many benefits that infants obtain from their mother's milk. Human milk components have been proposed to offer additional benefits such as stimulation of development and regulation of the newborn digestive system (*1*), enhanced absorption of certain minerals, stimulation and function of the immune system (*2*), and promotion of the development of the nervous system and brain. Some studies have also linked newborn milk consumption with adult health outcomes (*3*).

Human milk is a complex biofluid containing mainly lactose, lipids, and protein. However, it is not widely recognized that oligosaccharides are the third largest solid component following lactose and lipids. It is a genuinely remarkable observation that under the constant evolutionary selective pressure of lactation, in which nourishment of the infant must be constantly reconciled to the metabolic and nutritional demands of the mother, human milk contains such an investment in a biomolecule class that infants cannot digest. It is not known precisely what benefits could arise to the infant to justify such an investment by the lactating mother. The current explanation for the predominant advantage to oligosaccharides in human milk is that they encourage the growth of beneficial bifidobacteria in the colon (*4*). This in turn is thought to inhibit the growth of pathogens by mechanisms including lowering intestinal pH (*5*, *6*). Oligosaccharides also bind competitively to cell adhesion receptors on pathogens, as some are analogous in structure to the cognate ligands present on epithelial cell surfaces. This binding is believed to prevent pathogens from binding to intestinal epithelial cells and initiating pathogenesis. Unfortunately, the research needed not only to validate that these effects occur in vivo but to identify various other functions and subsequently to link specific actions to specific oligosaccharide structures is limited by the analytical techniques necessary to measure them. The amount of oligosaccharides has been shown to vary in

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human milk during the lactation period (*7*), yet the details of this variation remain unclear due to lack of analytical sensitivity. Human milk represents a highly complex mixture of oligosaccharides, which differ in size, charge, sequence, and abundance. At present, milk oligosaccharides are thought to be mainly composed of two groups: (1) neutral oligosaccharides containing galactose, *N*-acetylglucosamine, fucose, and lactose core; (2) anionic oligosaccharides containing the same oligosaccharide compositions with *N*-acetylneuraminic acid. Interestingly, structures of sulfated oligosaccharides (two sulfated octasaccharides and one sulfated nonasaccharide) from human milk have been reported by using ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy and electrospray mass spectrometry (ESMS) (*8*). These three oligosaccharides have the same core, *p*-lacto-*N*-hexaose, with different numbers and positions of fucose.

Characterization of human milk oligosaccharides (HMO) started in the 1960s, yet even now oligosaccharide analysis of human milk remains a challenging task due to the number of structures and their overall complexity. To date, more than 200 neutral and anionic oligosaccharides have been identified including high molecular weights up to mass 6000 Da (*8*−*12*). The structural characterization and quantification of human milk oligosaccharides has been accomplished using HPLC, high pH anion-exchange chromatography (HPAEC), capillary electrophoresis (CE), and mass spectrometry (MS) (*13*−*19*).

The introduction of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and carbohydrate compatible matrices greatly expanded the utility of MS analysis of human milk oligosaccharides (*20*). A new MALDI preparation technique using combination of matrices such as 5-chloro-2-mercaptobenzothiazole (CMBT) in the first layer and 2,5-dihydroxybenzoic acid (DHB) above allowed rapid screening of oligosaccharides. More structural information was obtained using CMBT as the first layer for 3-aminoquinoline. Nanoelectrospray ionization (nanoESI) using a quadrupole ion trap mass spectrometer (QIT-MS) in the negative mode was also applied for the structural analysis of underivatized neutral oligosaccharides. This tested specific fragmentation in the deprotonated form $[M - H]^-$ using MS/MS and MS^n (consecutive collision-induced dissociation (CID) to see the position of fucoses and linkage type (*21*). The same group identified isomeric structures of fucosylated lacto-*N*-hexaose from pooled human milk with the same consecutive CID experiments approach (*22*). Schmid et al. have described nanoHPLC−mass spectrometry and micellar electrokinetic chromatography (MEKC) for human milk oligosaccharides which were derivatized with various esters of aminobenzoic acid (*23*). However, the current analytical techniques to profile milk oligosaccharides require large amount of sample, suffer from reproducibility and are relatively long. Therefore, a rapid, a more robust and a highly sensitive method to profile human milk oligosaccharides is desirable to aid in understanding their biological functions.

For this study, a strategy for profiling oligosaccharides was developed using a new HPLC-Chip/MS technology employing an integrated microfluidic device and an orthogonal time-of-flight (*o*-TOF) mass analyzer. Structural elucidation was performed by MALDI-FT ICR MS of HPLC separated compounds. Infrared multiphoton dissociation (IRMPD) was used to obtain structural information along with controlled exoglycosidase reactions.

MATERIALS AND METHODS

Materials and Reagents. Nonporous graphitized carbon cartridges (150 mg of bed weight, 4 mL tube size) for desalting were purchased from Alltech (Deerfield, IL). Evaporation of solvents was performed using a Centrivap Concentrator (Labconco Corp., Kansas City, MO). Sodium borohydride (98%) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant β(1-3)-galactosidase and α(1-2)-fucosidase were from New England Biolab (Beverly, MA). All other reagents used were of analytical grade.

Milk Oligosaccharide Isolation and Purification. Human milk samples were obtained from the milk banks of San Jose, CA, and Austin, TX. Each milk sample (5 mL) was extracted with 4 volumes (2:1) of a chloroform−methanol solution (v/v). The emulsion was centrifuged at 3500 rpm for 30 min, and the lower chloroform layer and denatured protein were discarded. The upper layer was collected, and the fraction was freeze-dried. The resulting powder (freeze-dried oligosaccharide rich fraction) was used for oligosaccharide analysis.

Each freeze-dried oligosaccharide rich fraction was redissolved in fixed volume of deionized water. Oligosaccharides were fractionated by solid-phase extraction using a nonporous graphitized carbon cartridge (GCC-SPE). Prior to use, the GCC-SPE cartridge was washed with 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) (v/v) followed by deionized water. After loading of the oligosaccharide mixture onto a cartridge, salts were removed by washing with several cartridge volumes of deionized water. The oligosaccharides were fractionated using 20% acetonitrile in water (v/v) and 40% acetonitrile in 0.05% TFA (v/v) as eluting solvents. Each fraction (~6 mL) was collected and evaporated in vacuo prior to MS analysis.

Reduction of Milk Oligosaccharides. Milk oligosaccharides were reduced to an alditol form using 1.0 M sodium borohydride in deionized water and incubated at 42 °C overnight. After the reducing reaction, the nonporous graphitized carbon-solid-phase extraction procedure (described above) was used to remove borate salts and fractionate the mixtures. After fractionation by GCC-SPE, the oligosaccharide fractions were further separated using HPLC.

HPLC Analysis. Separations using HPLC were performed on a Hewlett-Packard Series 1100 instrument with a hypercarb porous graphitzed carbon (PGC) column (100 × 2.1 mm, 5 μm particle size, Thermoquest, Hypersil Division) at 206 nm. Deionized water (A) and acetonitrile (B) were used as eluents at a flow rate of 0.25 mL/min with a gradient of 5% B to 16% B over 44 min, from 16% B to 28% B over 12 min, from 28% B to 32% B over 4 min, isocratic run for 11 min and then bringing up from 32% B to 82% B, and isocratic run for 5 min with 5% B. A total of 80 fractions (0.25 mL each) were collected and evaporated in vacuo prior to MALDI−FT ICR MS analysis.

Exoglycosidase Digestion. Detailed procedures for exoglycosidase digestions were reported in the previous publication (*24*). The digestion procedure was optimized for several exoglycosidases. Briefly, before use, 1−2 μL of enzyme solution was dialyzed against deionized water for 30 min. Buffer solutions for enzyme digestions were prepared by dissolving ammonium acetate in deionized water and adjusting to the required pH by the addition of glacial acetic acid. The dialyzed enzyme solution was added into the oligosaccharide solution, and the mixture was incubated at 37 °C.

MALDI-FT ICR MS Analysis. MALDI-FT ICR MS was performed on an HiResMALDI (IonSpec Corp., Irvine, CA) equipped with an external MALDI source, a 4.7 or 7.0 T superconducting magnet and a pulsed Nd:YAG laser (355 nm). 2,5-Dihydroxybenzoic acid (DHB) was used as a matrix (5 mg/100 μL in ethanol or in 50% acetonitrile/water (v/v)). The solution of oligosaccharide (1 μL) was applied to the MALDI probe followed by addition of 0.01 M NaCl (1 μL) and the matrix solution (1 μL). The sample was dried under a stream of air and subjected to mass spectrometric analysis.

For the IRMPD experiment, a continuous-wave Parallax CO_2 laser (Waltham, MA) with 20-W maximum power and 10.6 μm wavelength was installed at the reel of the magnet. The laser beam diameter was 6 mm and was expanded to ~12 mm by means of a 2× beam expander (Synard, Mukilteo, WA). Detailed procedures and modifications were previously published (*25*).

HPLC-Chip/MS Analysis. Oligosaccharide fractions from GCC-SPE were analyzed using a microfluidic HPLC-Chip/MS technology (Agilent). The microfluidic HPLC-Chip was made using laser ablated and laminated biocompatible polyimide film. The chip consists of an integrated sample loading structure, a packed LC separation column, and nanoelectrospray tip. It is hydrautically interfaced with LC pumps

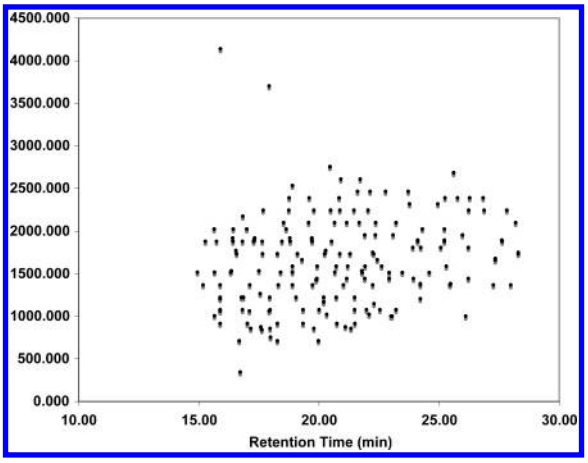

Figure 1. 2-Dimensional deconvoluted plot of a pooled human milk oligosaccharides sample (*x*-axis, LC retention time; *y*-axis, mass).

and an autosampler through a face-seal rotary valve. A nanoliter pump was used to deliver the LC gradient at 300 nL/min. The chip was interfaced to an *o*-TOF/MS for on-line nanoESI. Both on-chip enrichment column and on-chip LC separation column were packed with porous graphitized carbon media.

Separation was performed by a binary gradient consisting of two solutions A (water + 0.1% formic acid) and B (90% acetonitrile/water + 0.1% formic acid). A 40 min gradient was run from 2% B to 42% B and then bringing B to 90% at the completion of the run.

Milk oligosaccharides were identified using a Glycan Finder program developed in our laboratory. This program is written in Igor Pro version 5.04B software available from WaveMetrics, Inc. The algorithm was designed to examine a list of experimentally measured masses and search for all possible monosaccharide combinations matching the experimental mass within a specified tolerance level (mass error). In addition to providing information regarding the possible monosaccharide composition, the program output sorts each measured mass on the basis of its retention time and relative intensity.

RESULTS AND DISCUSSION

Three different methods have been used to remove lipids and proteins from milk serum: (1) centrifugation/precipitation; (2) ultrafiltration; (3) liquid/liquid extraction (Folch extraction) (*26*). Thurl et al. compared the extraction efficiency of centrifugation/precipitation method to ultrafiltration and found that the oligosaccharide concentrations by ultrafiltration process were significantly lower than the ethanol precipitation procedure (*27*). In this study, a combination of the centrifugation and liquid/liquid extraction was used, adapted from Sumiyoshi et al. (*16*), to remove lipids, proteins, and concentrate oligosaccharides from pure human milk samples. Once the oligosaccharides were isolated, a general desalting and concentrating cartridge for oligosaccharides using GCC-SPE was used to purify and fractionate oligosaccharides. Nonporous graphitized carbon cartridge (GCC) (a material similar to PGC) has been used generally to remove salts, monosaccharides, detergents (SDS and Triton X-100), proteins (including enzymes), and reagents for the release of oligosaccharides from glycoconjugates (such as hydrazine and sodium borohydride). Complete recovery was reported when using PGC-SPE (*28*); however, the mechanism of interaction between carbon and sugar is poorly understood.

HPLC-Chip/MS Separation of Pooled HMO Samples. Oligosaccharides were reduced to alditol forms for two main reasons. There have been reports that the anomeric isomers present at equilibrium in solution yielded split peaks (*29*) and preliminary results indicated less structural ambiguity when the reducing ends were converted to alditols. The alditols are two mass units larger than the aldehydes.

The HPLC-Chip/MS instrument provided the high mass accuracy necessary to identify oligosaccharides and determine their compositions. Oligosaccharide mixtures were separated on on-chip LC separation column packed with porous graphitized carbon media (*30*). **Figure 1** shows the deconvoluted 2-dimensional plot of mass vs retention time of HMO from a pooled

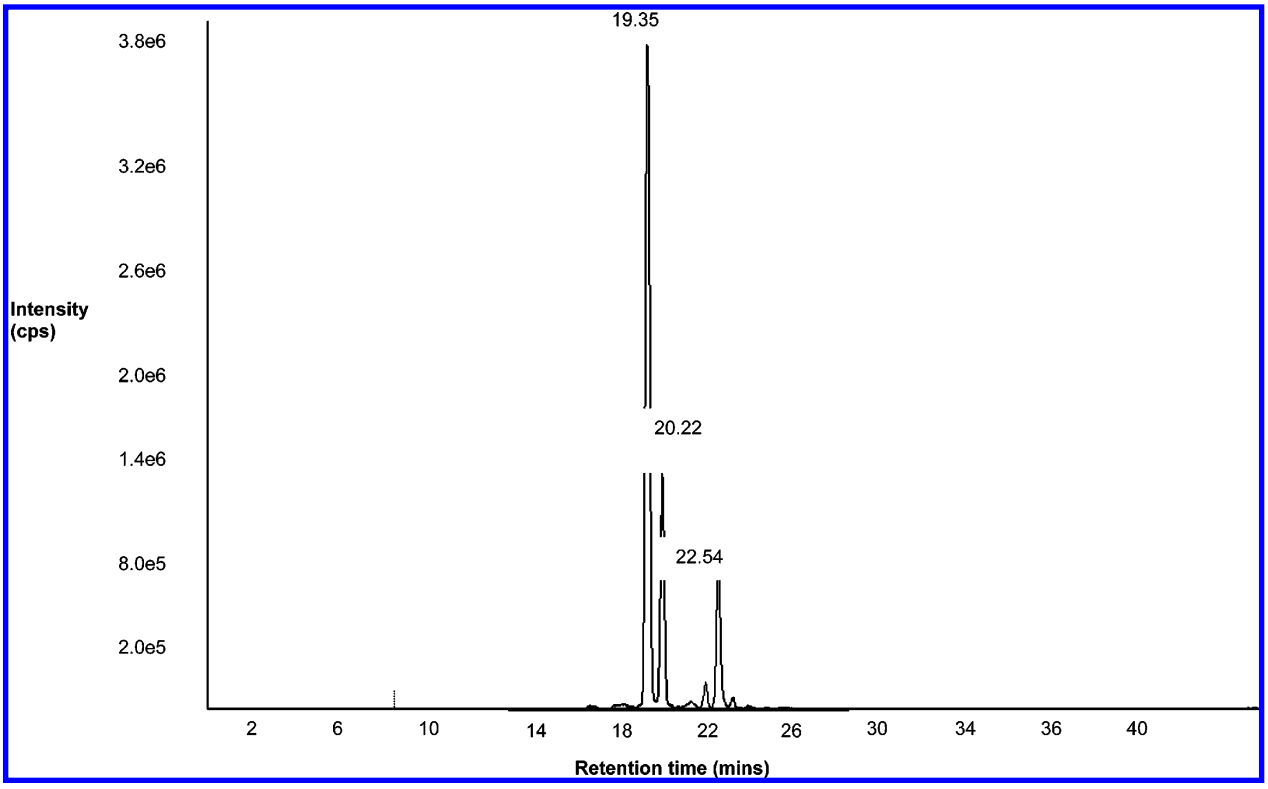

Figure 2. Extracted ion chromatogram of *m*/*z* 1075.4.

Table 1. LC Retention Times, Masses, and Mass Error for Oligosaccharide Isomers at 1074.4 Da

		mass		
RT (min)	intensity	exptl	calcd	error (ppm)
15.90	9.50	1074.394	1074.396	2.048
16.83	30.38	1074.396	1074.396	0.186
17.98	46.40	1074.396	1074.396	0.186
18.10	6.37	1074.394	1074.396	2.048
19.35	509.41	1074.396	1074.396	0.186
20.02	259.57	1074.394	1074.396	2.048
21.49	7.48	1074.397	1074.396	–0.745
21.98	21.70	1074.394	1074.396	2.048
22.54	107.14	1074.395	1074.396	1.117
23.21	5.65	1074.392	1074.396	3.909

human milk sample and illustrates the number of oligosaccharides and the spread in the distribution of masses. The mass accuracy of orthogonal time-of-flight was typically less than 2 ppm and provides rapid and precise compositional analysis. In this experiment, calibration was performed using at least two reference compounds (lock masses), which were sprayed simultaneously into the mass analyzer. The analysis of the masses from **Figure 1** yielded nearly 200 oligosaccharides ranging in size from disaccharides up to approximately 22 residues (10 ppm mass error tolerance). The oligosaccharides are listed in Table S1 (Supporting Information) from the most to the least abundant along with their possible compositions. Less abundant masses observed with intensities of less than 5.00 units were rejected, which was approximately 0.3% of the most abundant species. The intensities were not corrected but were expected to be representative of actual abundances. While there was overlap in the base peak chromatogram, isomers were obtainable and baselined resolved when monitoring a single mass. The overlapping peaks, where present, often corresponded to oligomers with similar base structure and elongated by a single residue. These compounds have different masses and were readily separated. Isomers with the same masses were, however, readily separated chromatographically as they often differed by linkage position, e.g., the position of a fucose residue. An example of such structural diversity is the isomers of *m/z* 1075.4 whose extracted ion chromatogram is shown in **Figure 2**. There were at least three major isomers observed (**Table 1**); however, as many as seven were observed with abundances significantly less than the three major components. We have previously reported suppression effects of sialylated species by sulfated oligosaccharides in the negative mode (*31*). However, this effect was eliminated when the compounds were separated and similar ionization efficiencies were observed. The sialylated and neutral oligosaccharides were therefore expected to have very similar ionization efficiencies. Sulfated oligosaccharides have been reported as a minor constituent of milk but were not observed in this analysis. This was possibly due to their very low abundances, i.e., less than 0.1% of the total amount of milk oligosaccharides. Guerardel and co-workers isolated three sulfated oligosaccharides from 20 L of pooled human milk samples, as it is necessary to obtain milligram quantities to perform NMR experiments (*8*). There were approximately 50 abundant species that accounted for 83% of the total intensity, while the least abundant half of the total (i.e., 92) made up only 8% of the entire intensities.

An analysis of the masses and the compositions was also useful from the point of view of biologically important residues. The analysis of the nearly 200 components from the pooled human milk sample yielded several noticeable trends with respect to fucosylated and sialylated species, which were both readily identified by mass. As fucoses and sialic acids are typically found in terminal positions in HMO, they represent the residues that most strongly influence the interaction of HMO with other molecules or cells. Thus, comprehensive analysis of both is of interest from the biosynthetic perspective, i.e., how their synthesis is regulated, and also for their putative functions as microbe binding antagonists. Since human milk oligosaccharides are similar in structures with the epithelial cell surface glycoconjugates, they have been proposed to inhibit competitively the binding of pathogenic bacteria and viruses to epithelial ligands (*5*). In particular, fucosylated human milk oligosaccharides have been found to inhibit binding in vitro or in vivo of

Table 2. All Oligosaccharide Masses and Compositions Found in Pooled Human Milk

no.	intensity	M(calcd)	Hex	HexNAc	Fuc	NeuAc
1	2562.66	1220.454	4	2	1	
2	2127.79	709.264	3	1		
3	1378.41	1366.512	4	2	2	
4	997.23	1074.396	4	2		
5	735.45	1731.644	5	3	2	
6	603.75	1585.586	5	3	1	
7	505.39	1877.702	5	3	3	
8	385.71	1511.549	4	2	1	1
9	369.28	2096.776	6	4	2	
10	314.30	1000.359	3	1		1
11	296.27	1950.718	6	4	1	
12	273.07	1365.492	4	2		1
13	202.62	1439.528	5	3		
14	190.50	1512.570	4	2	3	
15	181.02	2242.834	6	4	3	
16	145.75	1058.401	3	2	1	
17	141.09	1804.661	6	4		
18	135.94	912.343	3	2		
19	96.98	871.317	4	1		
20	80.60	2023.760	5	3	4	
21	72.15	855.322	3	1	1	
22	67.56	1876.682	5	3	1	1
23	52.48	1382.507	5	2	1	
24	51.36	1915.655	9		1	1
25	43.09	1528.565	5	2	2	
26	36.89	1163.433	4	1	2	
27	32.90	2241.814	6	4	1	1
28	32.77	1893.697	6	3	2	
29	30.88	2388.892	6	4	4	
30	29.83	2461.909	7	5	2	
31	28.93	1769.597	9			1
32	28.87	1146.417	3	1	1	1
33	28.19	1017.375	4	1	1	
34	26.35	1657.607	4	2	2	1
35	24.33	2387.872	6	4	2	1
36	20.52	838.307	2	1		1
37	20.39	1747.639	6	3	1	
38	17.66	1899.660	8		2	1
39	17.59	2022.740	5	3	2	1
40	17.37	1658.628	4	2	4	
41	17.37	2607.966	7	5	3	
42	16.00	2315.851	7	5	1	
43	15.00	2169.793	7	5		
44	14.47	1261.481	3	3	1	
45	13.05	1730.624	5	3		1
46	12.20	344.132	2			
47	11.87	3700.327	8	6	2	3
48	11.59	1674.623	5	2	3	
49	10.55	1203.439	3	2		1
50	9.96	1204.459	3	2	2	
51	9.09	4138.501	8	6	5	3
52	8.71	750.291	2	2		
53	8.15	2095.756	6	4		1
54	6.42	1001.380	3	1	2	
55	6.07	2754.024	7	5	4	
56	6.02	2534.950	6	4	5	
57	5.83	2680.983	8	6	1	
58	5.56	1423.533	4	3	1	

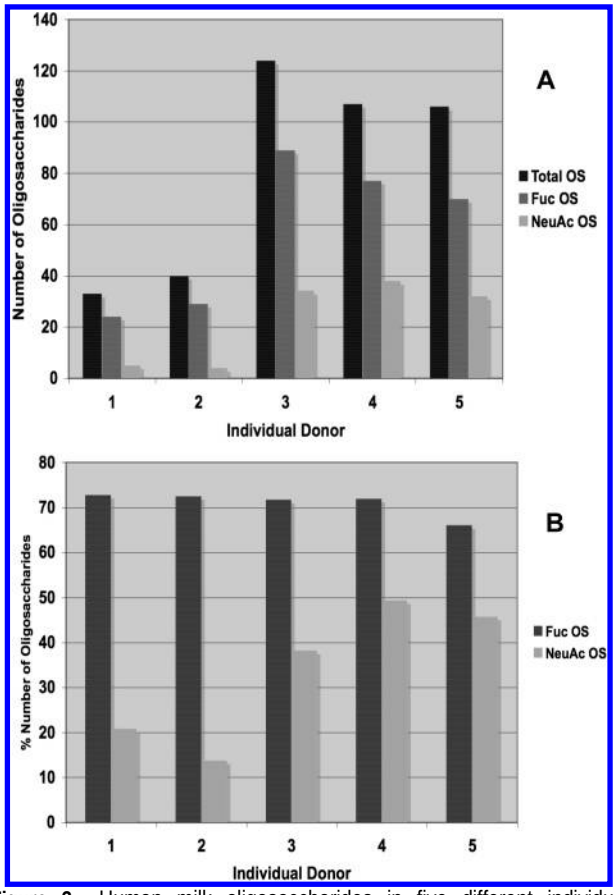

Figure 3. Human milk oligosaccharides in five different individual women: (**A**) total number of HMOl (**B**) total intensity of HMO.

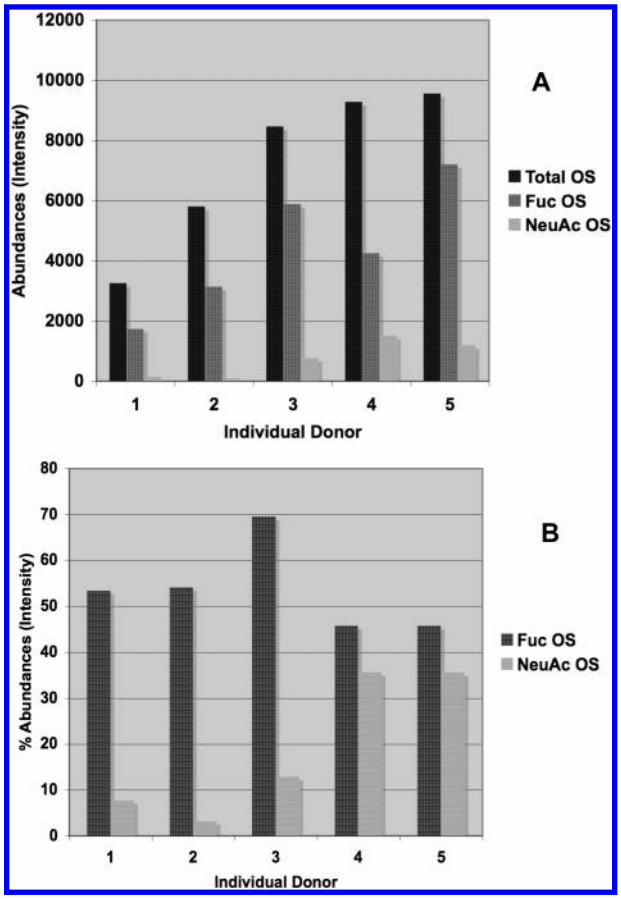

Figure 4. Percentage of fucosylated and sialylated HMO: (**A**) number of HMO; (**B**) intensity of HMO.

several pathogens, such as enteropathic *Escherichia coli* (*32*), *Campylobacter jejuni* (*33*) Norwald-like virus (*34*), and *Helicobacter pylori* (*35*). Alternatively, sialic acids are vital structural and functional components of brain gangliosides. They are thought to play an essential role in nerve cell transmission, memory formation, and cell−cell communication (*36*). Human milk contains a larger portion of sialylated oligosaccharides than is found in cow's milk or currently present in infant's formula (*37*). These sialylated oligosaccharides and glycoconjugates have been reported to inhibit binding of *E. coli* in the gut (*5*). **Table 2** lists all 58 oligosaccharide masses along with the total intensities of their isomers.

The proportion of fucosylated oligosaccharides was approximately 77% (137 out of 183) (Table S1) with the three most abundant species being fucosylated. The 10 most abundant masses were nearly all (except for 3) fucosylated and approximately 46% of the entire quantity. Of the remaining oligosaccharides those that have been proposed to be particularly interesting biologically were those containing sialic acids. Less than 28% of the oligosaccharides contained sialic acid (39 out of 183). However, the sixth and seventh most abundant species contained sialic acids namely monofucosylmonosialyllacto-*N*-hexaose (4:2:1:1 Hex:HexNAc:Fuc:NeuAc) and sialyllacto-*N*-tetraose (3:1:1 Hex:HexNAc:NeuAc), respectively (Table S1). In all, nearly 16% of the total oligosaccharide abundances corresponded to sialylated oligosaccharides.

HPLC-Chip/MS Separation of Individuals HMO Samples. The HPLC-Chip LC/MS of samples from five randomly selected individuals were analyzed separately to determine person-to-person variability. Equal amounts (5 mL) of human milk were used for the study. The HPLC-Chip/MS analysis showed considerable diversity between individuals in terms of the total numbers and abundances of oligosaccharides (Tables S2−S6). The total number of oligosaccharides in one individual milk sample varied from 33 to 124 (**Figure 3A**). As expected, these values are less than the pooled sample from five different individuals, which totaled 183 oligosaccharides. There was no effort made in this study to obtain samples at specific intervals or at a specific age of the infant at this time. Although, the number of oligosaccharides has been reported to vary from beginning to end corresponding to the growth of the infant (*7*). The time variation of oligosaccharides as a function of the age of the infant will be the subject of future reports.

Table 3. Common Oligosaccharide Masses Found in Five Different Individual Donors

M(calcd)	Hex	HexNAc	Fuc	NeuAc	possible structure
636.248	2	0	2	0	LDFT series
709.264	3	1	0	0	LNT series
1074.396	4	2	0	0	LNH series
1220.454	4	2	1	0	FLNH series
1366.512	4	2	2	0	DFLNH series
1439.528	5	3	0	0	LNO series
1511.550	4	2	1	1	MFMSLNH series
2096.776	6	4	2	0	LNDFD series

The relative numbers of fucoses appeared to remain consistently high, despite the varying number of total oligosaccharides. Shown in **Figure 3** are the total numbers of oligosaccharides for each individual (**Figure 3A**) and the total relative intensities (**Figure 3B**). For the five individuals analyzed separately, the number of fucosylated oligosaccharides range between 66 and 73% (**Figure 3B**) of the total oligosaccharide number, while

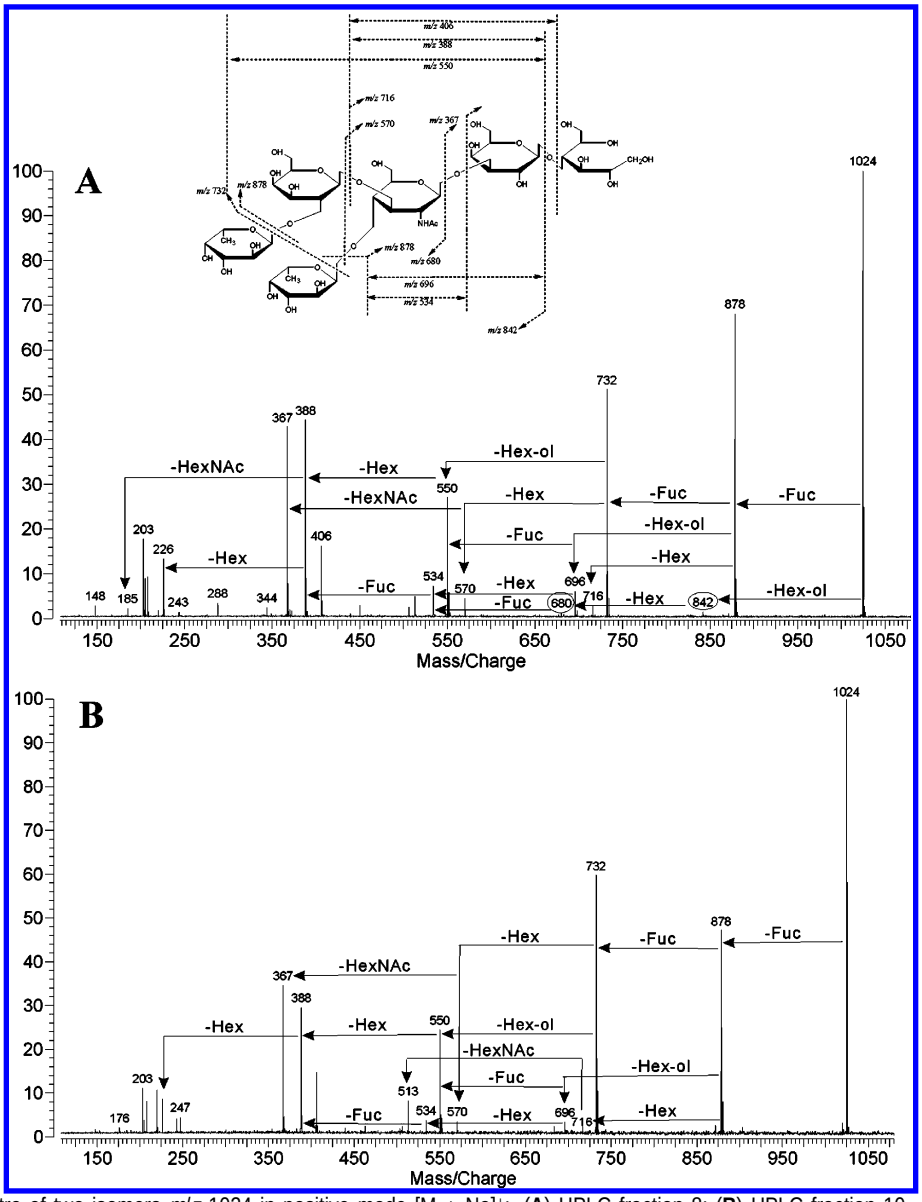

Figure 5. IRMPD spectra of two isomers *m*/*z* 1024 in positive mode $[M + Na]^+$: (**A**) HPLC fraction 8; (**B**) HPLC fraction 10.

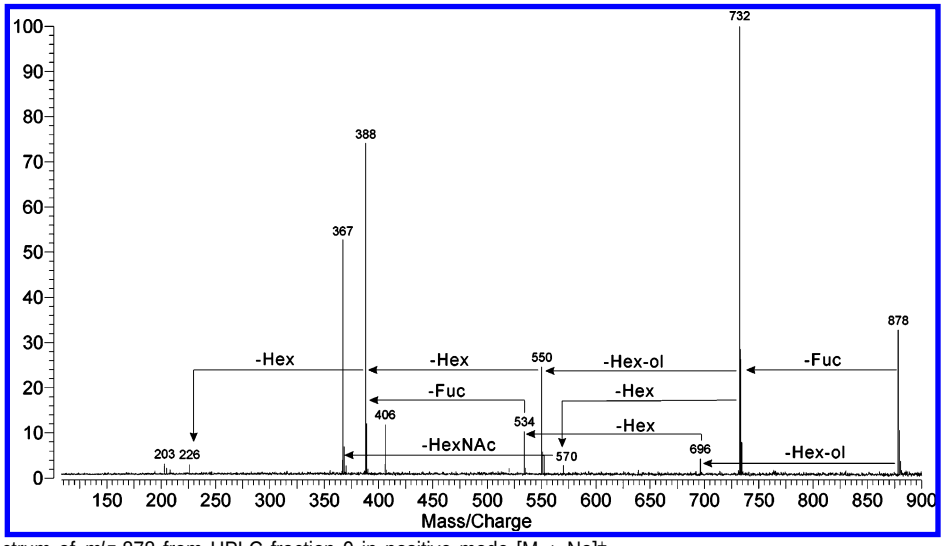

Figure 6. IRMPD spectrum of *m*/*z* 878 from HPLC fraction 9 in positive mode $[M + Na]^+$.

the analysis of total intensities found that the total content of fucosylated oligosaccharides varied from 46% to 70% (**Figure 4B**) of the total of all carbohydrates.

The sialylated oligosaccharides remained lower and varied widely from 4 to 38% (**Figure 3B**) of the total number of oligosaccharides. In terms of abundances, sialylated oligosac-

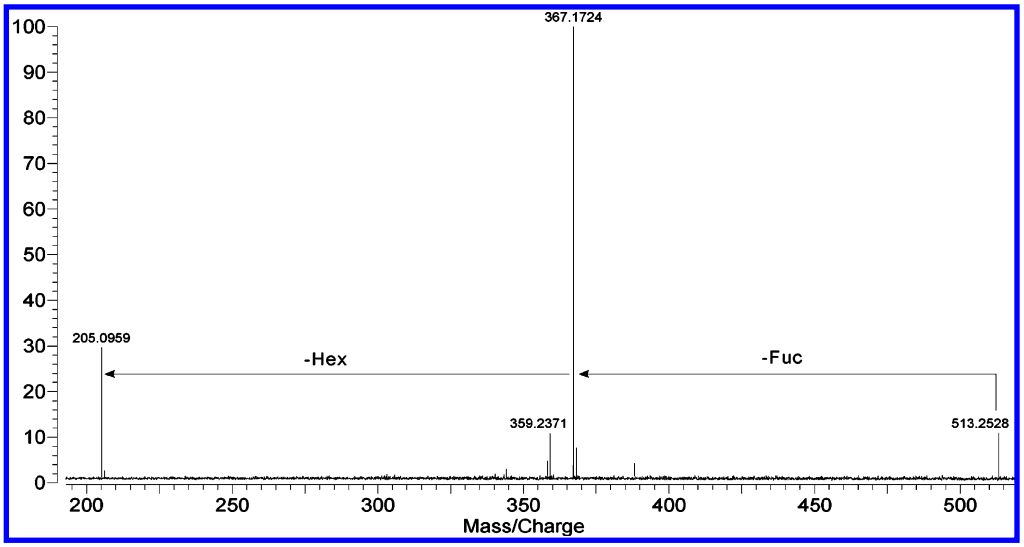

Figure 7. IRMPD spectrum of *m*/*z* 513 from HPLC fraction 13 in positive mode $[M + Na]^+$.

charides fall between 3 and 36% of the total amount of oligosaccharides (**Figure 4B**).

From the five individual donors, there were a relative small number of common oligosaccharides—LDFT, LNT, LNH, FLNH, DFLNH, LNO, MFMSLNH, and LNDFD series. These are listed in **Table 3** and corresponded to several well-known groups of oligosaccharides.

Methods for Structural Determination. Structural determination of human milk oligosaccharides as previously discussed is necessary to fully understand their biological functions. Our strategy to annotate the human milk glycome is to perform tandem mass spectrometry, specifically infrared multiphoton dissociation (IRMPD), on the oligosaccharides to obtain the sequence and connectivity of each residue and the position of the fucoses and the sialic acids. To differentiate between isomers and to determine ambiguous structures, exoglycosidases were used. An offline PGC-HPLC-MALDI FT ICR MS was performed to annotate the HPLC-Chip chromatogram. Each component was separated by PGC-HPLC to obtain sufficient quantities of the compounds for tandem MS and enzyme reaction. The HPLC chromatogram of the pooled milk sample contained features similar to the HPLC-Chip/MS. Thus, it was possible to match the peaks and the corresponding compounds. To obtain structural information, the oligosaccharide alditols in each PGC-HPLC fraction were analyzed by IRMPD in combination with exoglycosidase digestion. Oligosaccharides were reduced into their respective alditol forms to easily elucidate the structures from IRMPD data. This made the data interpretation less complicated because identifying the reducing end is more straightforward. From previous publications, we have developed optimized conditions for exoglycosidase digestion (buffer, temperature, and pH) on neutral *O*-linked oligosaccharide alditols that are compatible with MALDI-FT ICR MS (*24*). IRMPD of alkali metal-coordinated oligosaccharides provides fragments of the glycan components, which are essential to obtain key information of the structure (*25*).

To illustrate the structural elucidation strategy, several examples are provided that illustrate the IRMPD and the use of exoglycosidases for structural assignments. IRMPD of two fractions were performed with different retention times but corresponding to the same molecular ion peak of *m*/*z* 1024. In **Figure 5A**,**B**, both IRMPD spectra are compared. Fraction 8 (**Figure 5A**) has a characteristic loss of monosaccharides, generating *m*/*z* 842 and 680. These molecular ion peaks are

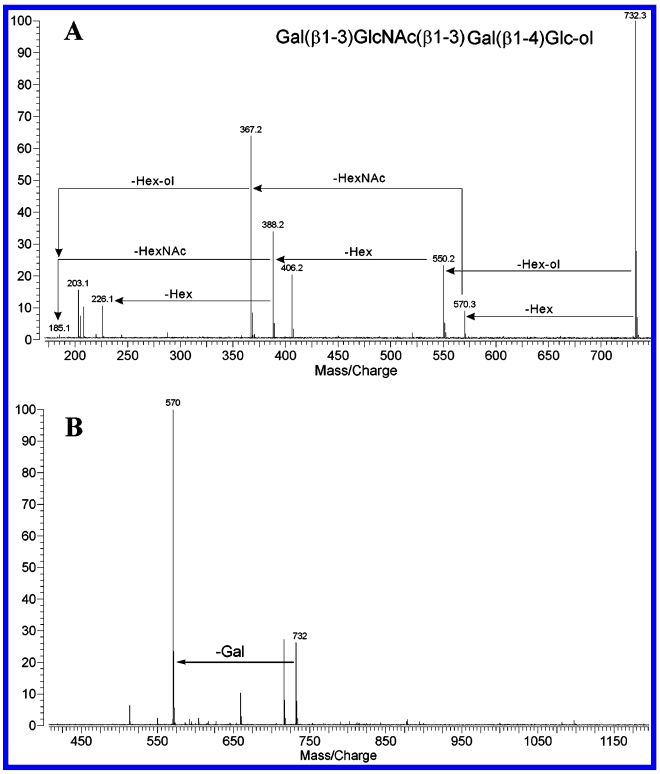

Figure 8. (**A**) IRMPD spectrum of *m*/*z* 732 from HPLC fraction 21 in positive mode $[M + Na]^+$ and (**B**) MALDI-FTMS spectrum of *m*/*z* 732 in digestion mixture with recombinant β(1–3)-galactosidase. The loss of a β(1–3)-galactose during digestion is consistent with the structure of LNT.

marked in **Figure 5A**, in which the loss of Hex-ol (*m*/*z* 842) and subsequent loss of Hex (*m*/*z* 680) are observed. This confirms the structure of LNDFH-I, in which two fucoses are attached to terminal Gal and GlcNAc. Due to the existence of a terminal α(1-2)-fucose, this compound did not digest when subjected to β(1-3)-galactosidase (data not shown). On the basis of the IRMPD mass spectrum of fraction 11 (**Figure 5B**), the peak contained another major isomer of *m*/*z* 1024, LNDFH-II.

From another fraction, a molecular ion of *m*/*z* 878 was obtained as a major oligosaccharide. IRMPD of *m*/*z* 878 is shown in **Figure 6**. From the IRMPD fragmentation three structural isomers, LNFP I−III, are possible. Three different exoglycosidases β(1−3)-galactosidase, nonspecific galactosi-

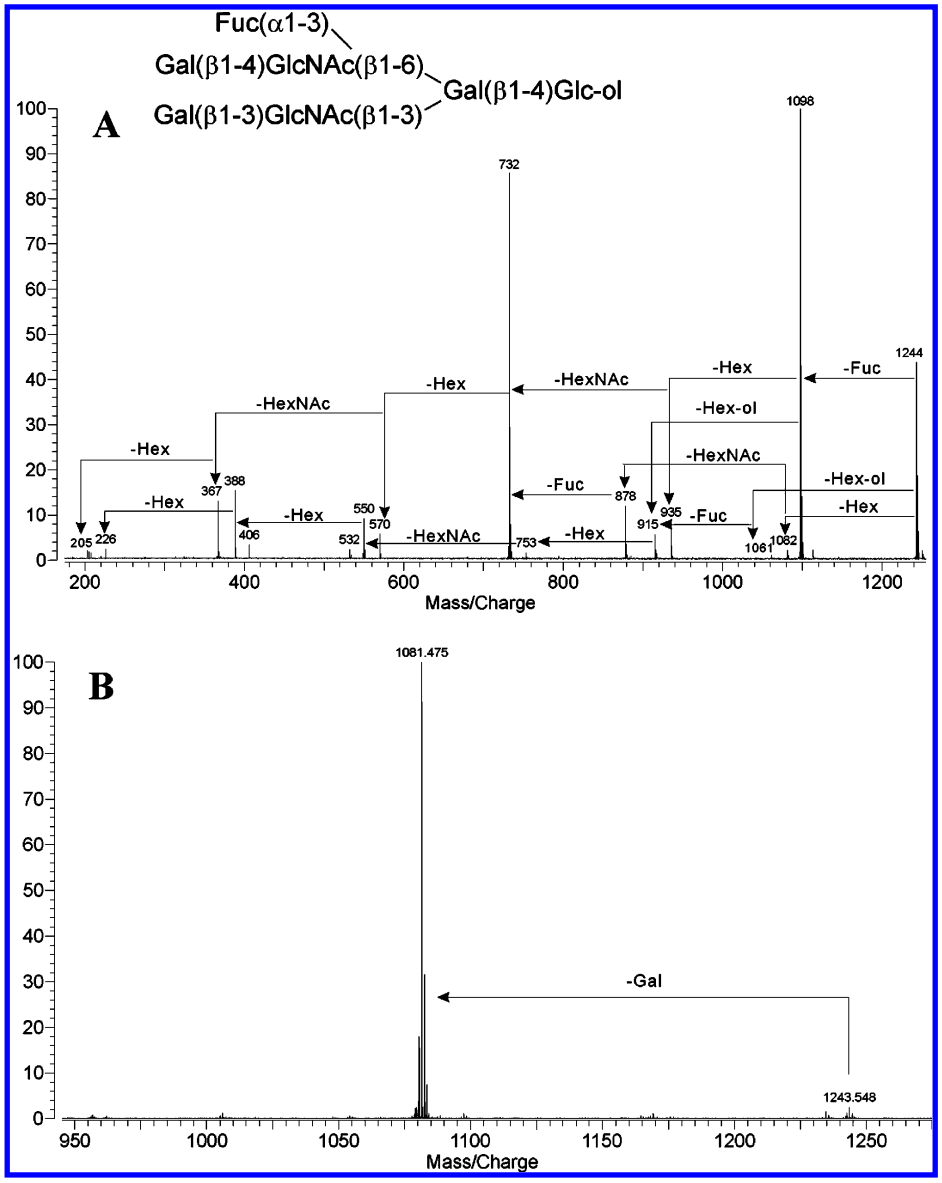

Figure 9. (**A**) IRMPD spectrum of *m*/*z* 1244 from HPLC fraction 26 in positive mode $[M + Na]^+$ and (**B**) MALDI-FTMS spectrum of *m*/*z* 1244 (HPLC 26) in digestion mixture with recombinant β(1–3)-galactosidase. The loss of a β(1–3)-galactose during digestion is consistent with the structure of 3′-FLNH.

dase, and α(1−2)-fucosidase were used to digest the oligosaccharide; however, none reacted with the compound. HPLC fraction 13 contained a major neutral oligosaccharide whose molecular ion is at *m*/*z* 513 that corresponds to fucosyllactose (2 Hex, 1 Fuc). The IRMPD data confirmed this monosaccharide composition (**Figure 7**).

Another fraction contained an oligosaccharide whose molecular ion is *m*/*z* 732. IRMPD of *m*/*z* 732 is shown in **Figure 8A**. From IRMPD fragmentations, this compound could be either of two isomers, lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT). The only difference between the two is the linkage type of the galactose at the nonreducing end. For this case, exoglycosidase digestion was used to validate the known structure components. Recombinant β(1-3)-galactosidase digestion was performed, producing a molecular ion peak at *m*/*z* 570 due to the loss of terminal galactose (**Figure 8B**). These data showed the galactose linkage at the nonreducing end was β(1-3), confirming the structure of LNT.

An HPLC fraction whose most abundant peak corresponded to the molecular ion *m*/*z* 1244 was analyzed. For *m*/*z* 1244, there could be as many as six possible isomers for this mass. The IRMPD spectrum of *m*/*z* 1244 is shown in **Figure 9A**. To confirm the structure, recombinant β(1-3)-galactosidase digestion was carried out, and the resulting spectrum is shown in **Figure 9B**. The loss of the terminal galactose generated a new molecular ion peak at *m*/*z* 1081, confirming the structure of 3′-FLNH where the Gal is connected via β(1-3).

In summary, HPLC-Chip/MS profiling of oligosaccharides provides a rapid and accurate method for determining the number of milk oligosaccharide components and those that contain fucosylated and sialylated residues. The microfluidic HPLC-Chip/MS device is both robust and reproducible. We have shown in an earlier study that the detection limit is in the low femtomoles range and that reproducibility between runs for the same sample varies by less than 0.5%. In the future, retention times and accurate masses will be sufficient to identify known human milk oligosaccharides. While these experiments employed IRMPD for tandem MS, collision-induced dissociation could also be used to provide similar results. The advantages of IRMPD are the information content obtained from a single tandem MS event compared to CID, MS^n, where $n = 3-5$, are often necessary to obtain the complete sequence. Furthermore, IRMPD is superior for analyzing high-mass oligosaccharides compared to CID (*25*).

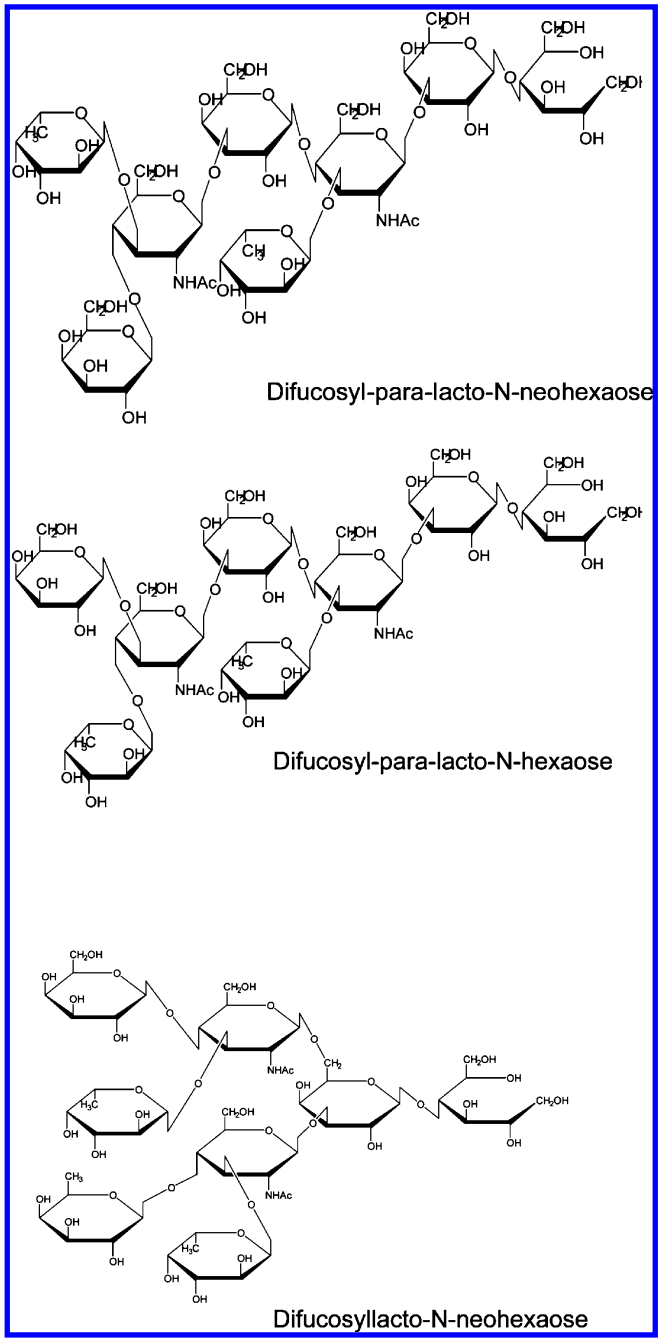

Figure 10. Known structural isomers of oligosaccharide with neutral mass 1366.5 Da. The species were reduced to alditols.

The use of exoglycosidase digestion provides the linkage and the identity of the residue. However, extensive use of glycosidases is both time-consuming and expensive. Fortunately, several of the milk oligosaccharide structures are known (over 80 at last count) and often identification of the oligosaccharides depends primarily on elucidating one or two residues from its isomers. For example, neutral mass of 1366.5 corresponds to the difucosyl-para-lacto-*N*-neohexoase (**Figure 10**) of which three isomers are known and vary by the relative position of the fucose. The use of specific glycosidases, one or two, is sufficient to identify either of these isomers. The major effort at this moment is to annotate the chromatogram to known structures. Once the structure has been determined, tandem MS will be sufficient to identify that structure again. Tandem MS of alditols has been shown to be distinct and provide unambiguous differentiation of isomers.

Once a new isomer is found, its sequence and composition can be compared by homology to a known oligosaccharide with a known sequence. These analytical advances provide for a standardized platform to discovering and annotating oligosaccharides from human milk and other sources. Assembling a centralized curated database of these oligosaccharides is necessary to harmonize the field of functional carbohydrates and propel the research and its development applications as prebiotics. The remarkable abundance of complex oligosaccharides in the breast milk of humans is evidence of the footprint of evolutionary selective pressure. With the exhaustive analytical capabilities now having resolved the chemical compositions of these molecules, it is now of considerable nutritional interest to determine the various specific functions and health benefits these undigestible molecules provide to the infant (*38*).

ABBREVIATIONS USED

CID, collision-induced dissociation; DFLNH, difucosyllacto-*N*-hexaose; DHB, 2,5-dihydroxybenzoic acid; FL, fucosyllactose; FLNH, fucosyllacto-*N*-hexaose; Fuc, L-fucose; GCC-SPE, graphitized carbon chromatography−solid phase extraction; Glc, D-glucose; Glc-ol, D-glucosaminitol; Gal, D-galactose; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; Hex-ol, hexosaminitol; HMO, human milk oligosaccharide; IRMPD, infrared multiphoton dissociation; LC/MS, liquid chromatography/mass spectrometry; LDFT, lactodifucotetraose; LNT, lacto-*N*-tetraose; LNneoT, lacto-*N*-neotetraose; LNFP, lacto-*N*-fucopentaose; LNDFH, lacto-*N*-difucohexaose; LNDFD, lacto-*N*-difucodecaose; LNH, lacto-*N*-hexaose; LNO, lacto-*N*-octaose; MFLNH, monofucosyllacto-*N*-hexaose; MFMSLNH, monofucosylmonosialyllacto-*N*-hexaose; MALDI-FT ICR MS, matrix-assisted laser desorption/ionization Fourier transform ion cyclotron mass spectrometry; NeuAc, *N*-acetyl-neuraminic acid; PGC-HPLC, porous-graphitized carbon-high performance liquid chromatography; TOF, time-of-flight.

Supporting Information Available: Full list of oligosaccharide masses, retention times, compositions, and mass errors from pooled human milk sample and from five individual women. This material is available free of charge via the Internet at http://pubs.acs.org.

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