Absolute Quantitation of Human Milk Oligosaccharides Reveals Phenotypic Variations during Lactation[1–3]

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Abstract

Background: The quantitation of human milk oligosaccharides (HMOs) is challenging because of the structural complexity and lack of standards.

Objective: The objective of our study was to rapidly measure the absolute concentrations of HMOs in milk using LC-mass spectrometry (MS) and to determine the phenotypic secretor status of the mothers.

Methods: This quantitative method for measuring HMO concentration was developed by using ultraperformance LC multiple reaction monitoring MS. It was validated and applied to milk samples from Malawi (88 individuals; 88 samples from postnatal month 6) and the United States (Davis, California; 45 individuals, mean age: 32 y; 103 samples collected on postnatal days 10, 26, 71, or 120, repeated measures included). The concentrations of α(1,2)-fucosylated HMOs were used to determine the mothers' phenotypic secretor status with high sensitivity and specificity. We used Friedman's test and Wilcoxon's signed rank test to evaluate the change in HMO concentration during the course of lactation, and Student's t test was used to compare secretors and nonsecretors.

Results: A decrease ($P < 0.05$) in HMO concentration was observed during the course of lactation for the US mothers, corresponding to 19.3 ± 2.9 g/L for milk collected on postnatal day 10, decreasing to 8.53 ± 1.18 g/L on day 120 (repeated measures; $n = 14$). On postnatal day 180, the total concentration of HMOs in Malawi milk samples from secretors (6.46 ± 1.74 mg/mL) was higher ($P < 0.05$) than that in samples from nonsecretors (5.25 ± 2.55 mg/mL). The same trend was observed for fucosylated species; the concentration was higher in Malawi milk samples from secretors (4.91 ± 1.22 mg/mL) than from nonsecretors (3.42 ± 2.27 mg/mL) ($P < 0.05$).

Conclusions: HMOs significantly decrease during the course of lactation. Secretor milk contains higher concentrations of total and fucosylated HMOs than does nonsecretor milk. These HMO concentrations can be correlated to the health of breastfed infants in order to investigate the protective effects of milk components. The trials were registered at clinicaltrials.gov as NCT01817127 and NCT00524446.   *J Nutr* 2017;147:117–24.

Keywords: human milk oligosaccharides, mass spectrometry, secretor, lactation, fucosylation

Introduction

The quantitation of heterogeneous mixtures of biologically distinct but structurally similar compounds typically is limited by the small number of available standards. For this reason, mixtures are difficult to quantitate for total concentrations without strict isolation of the group of compounds. Human milk oligosaccharides (HMOs)[7] are composed of a large number of structures that in total are the third most abundant group of compounds in human milk, after lactose and lipids (1, 2). Although infants possess no enzymes to catabolize HMOs (3), their concentrations and compositions are found to be closely related to many

[7] Abbreviations used: HILIC, hydrophilic interaction liquid chromatography; HMO, human milk oligosaccharide; LDFT, lacto-difucotetraose; LNT, lacto-*N*-tetraose; LNFP I, lacto-*N*-fucopentaose I; LOD, limit of detection; LOQ, limit of quantitation; MRM, multiple reaction monitoring; PGC, porous graphitized carbon; QqQ, triple quadrupole; UPLC, ultraperformance liquid chromatography; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

aspects of infant health. HMOs are known to promote the growth of beneficial bacteria such as bifidobacteria (4–6). Strains of bifidobacteria exhibit unique consumption patterns that favor specific groups of HMOs over others (7). HMOs as a whole also are effective in inhibiting the adhesion of various pathogenic agents to receptors on epithelial cells (8). Higher HMO concentrations in human milk can protect infants against postnatal HIV transmission by reducing the binding of HIV-1 surface glycoprotein gp120 to dendritic cell-specific intercellular adhesion molecule-3–grabbing nonintegrin (9–11). In addition, sialylated HMOs may help to inhibit allergies and aid in brain development (12, 13). To gain a better understanding of how HMOs affect the health of infants, large human clinical studies are needed, which necessitate a simplified, rapid, and high-throughput method for quantitating total HMO content in milk or any biofluid such as feces and urine.

The measurement of total absolute concentrations (in grams per liter of milk) of HMOs has been challenging because of their structural complexity and the lack of standards for the hundreds of potential HMOs. Total HMO concentrations were reported to be 20–25 g/L in colostrum and 5–20 g/L in mature milk (14–16). The wide ranges are the result of biological variations and the lack of specific and sensitive methods for total HMO quantitation (17). Reported methods include chromatographically separating HMOs from lactose and detecting all of the HMOs in one or more fractions using refractive index detection or the phenol-sulfuric acid method (14, 16). Because these detection methods provide little structural information, quantitation may be affected greatly by interference from the matrix. Another method for estimating the total HMO concentration is to assess 10 or 20 of the most abundant compounds (1, 15). High-performance anion-exchange chromatography with pulsed amperometric detection (18–20) and HPLC separation with fluorescence or UV detection (21–23) are common techniques for separating and quantitating individual HMOs. These methods use the sum of the most abundant structures to estimate the total HMO concentrations, which means that the accuracy of quantitation is highly dependent on variations in the concentrations of the unknown compounds.

Multiple reaction monitoring (MRM) is a highly sensitive technique that has been widely applied to the absolute quantitation of proteins (24–28) and metabolites (29–31). It also has been used for the analysis of free milk oligosaccharides in bovine milk (32) and rat serum (33); however, the application of MRM to HMOs remains limited. Recently, our laboratory developed an absolute quantitation method for measuring HMOs in milk samples using MRM (34). The individual and total concentrations of chemically reduced HMOs were estimated through the use of a universal response factor obtained from 7 commercial

HMO standards. This MRM-based method makes HMO quantitation possible when only a small number of standards are available. The sample preparation and analysis time of this method is still relatively long, requiring reduction and further purification of the analytes. The method is problematic in large human clinical studies. Furthermore, some of the HMO components are lost in the sample cleanup.

In the current study, we developed a streamlined method for the rapid absolute quantitation of HMOs using ultraperformance LC triple quadrupole-MS (UPLC/QqQ-MS) in MRM mode. The current method required minimal sample cleanup, no derivatization, and no enrichment with the use of a 96-well plate platform. The sample preparation and analysis time was minimized so that hundreds of samples could be processed (each LC-MS analysis lasts only 10 min). This method was validated and applied to milk samples from 2 human cohorts, thereby demonstrating its practical applicability to large-scale studies.

Methods

Samples and materials. Standards 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), lacto-N-tetraose (LNT), and lacto-N-fucopentaose I (LNFP I) were purchased from Carbosynth Ltd. 6'-Sialyllactose (6'SL) was purchased from Sigma-Aldrich. Acetonitrile (HPLC grade) was purchased from Honeywell. Formic acid was purchased from Fisher Scientific. A total of 103 breast milk samples were obtained from 45 healthy women who were enrolled in the Foods for Health Institute Lactation Study at the University of California, Davis. These milk samples were collected on postnatal days 10, 26, 71, and/or 120 from women who delivered healthy full-term infants (>37 wk of gestation). The University of California, Davis institutional review board approved all aspects of the US study, and informed consent was obtained from all of the subjects. This observational study was registered at clinicaltrials.gov as NCT01817127. Breast milk samples also were collected from Malawi mothers (88) at 6 mo postnatal who delivered healthy full-term infants (>37 wk of gestation). The Malawi study was conducted with the approval of institutional review boards from the University of Malawi, Pirkanmaa Hospital, and Washington University School of Medicine. The mothers and infants included in this analysis were enrolled in the Controlled Trial to Test the Efficacy of Lipid-Based Nutrient Supplements to Prevent Severe Stunting Among Infants (LCNI-5; clinicaltrials.gov identifier: NCT00524446), a randomized, controlled, single-blind, parallel-group clinical trial of micronutrient-fortified lipid-based nutrient supplements that was conducted in southern Malawi. The HMO data reported are secondary outcomes of this trial.

Sample preparation. The US and Malawi milk samples (10 μL each in 96-well plates) were mixed with 90 μL of Nanopure water (Thermo Scientific) and defatted by centrifugation at $3200 \times g$ for 30 min at room temperature. The aqueous layers were transferred into new plates. Then 2 vol of ethanol were added to precipitate the proteins at −80°C for

FIGURE 1 The extracted dynamic MRM chromatogram monitored for a pooled human milk sample. *Numbers stand for monosaccharide composition (e.g., 4_2_1_1: 4 Hex_2 HexNAc_1 Fucose_1 Neu5Ac). HexNAc, N-acetylhexosamine; LDFT, lacto-difucotetraose; LNFP I, lacto-N-fucopentaose I; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; MRM, multiple reaction monitoring; Neu5Ac, N-acetylneuraminic acid; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.
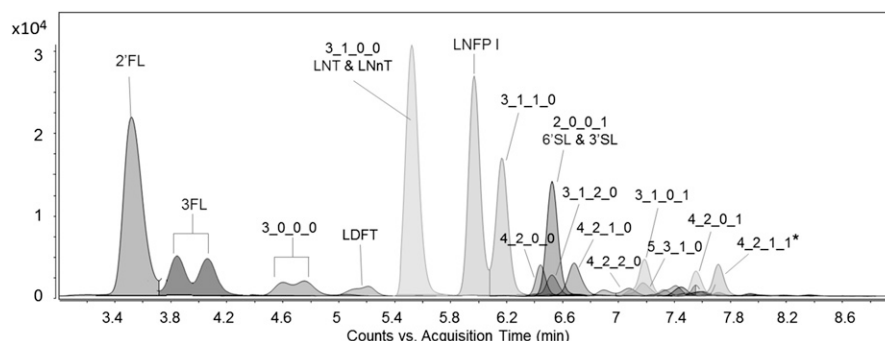
TABLE 1 Evaluation of linearity, sensitivity, and accuracy of MRM quantitation[1]

| Compound | Regression line | R^2 | Linear range, nM | LODs, fmol | LOQs, fmol | Concentration, g/L | | Difference, % |
						Standard calibration	Standard addition	
6'SL	y = 6150x + 5550	1.00	5.3–158,000	1.6	5.3	0.69	0.67	3.6
2'FL	y = 5150x + 2860	0.99	20–51,200	6.8	20	2.0	3.0	−33
3FL	y = 4650x + 3030	1.00	20–76,800	6.8	20	0.81	0.84	−4.3
LNT	y = 5170x + 5770	1.00	4.7–106,000	1.4	4.7	1.6	1.7	−1.8
LNFP I	y = 5100x + 5830	1.00	1.2–87,900	0.6	1.2	1.2	1.3	−2.6

[1] LNFP I, lacto-N-fucopentaose I; LNT, lacto-N-tetraose; LOD, limit of detection; LOQ, limit of quantitation; MRM, multiple reaction monitoring; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 6'SL, 6'-sialyllactose.

≥1.5 h. After 30 min of centrifugation, the supernatant fluids containing mainly lactose and oligosaccharides were dried, reconstituted in 500 µL of water (50-fold dilution), and injected directly for UPLC/QqQ-MS analysis in MRM mode.

Calibration curves. A reference US milk pool was made by mixing milk samples from 20 unique mothers (100 µL each). The milk samples were selected at random, with all 4 time points included. A reference Malawi milk sample pool was made by mixing milk from 80 mothers (20 µL each). The milk pools were extracted by the same technique as described above and reconstituted to their original volumes. The following steps were performed for each milk pool to build calibration curves for HMO

quantitation: *1*) Half of the pool was serially diluted and injected directly to UPLC/QqQ-MS. The peak areas of all of the transitions were summed to provide the total response of HMOs in each diluted pool. *2*) The other half was cleaned up by solid-phase extraction by use of porous graphitized carbon (PGC) cartridges, to remove lactose and other impurities. The eluent was lyophilized and the dry mass was weighed. *3*) The total mass of HMOs in the milk pool was calculated by adding the mass of 3FL (calculated from the standard calibration curve of 3FL) to the dry mass after PGC because 3FL was lost during PGC cleanup. *4*) This mass of HMOs was used to calculate the concentrations of total HMOs in each diluted pool. The calculated concentrations and their corresponding total responses were used to build a calibration curve.
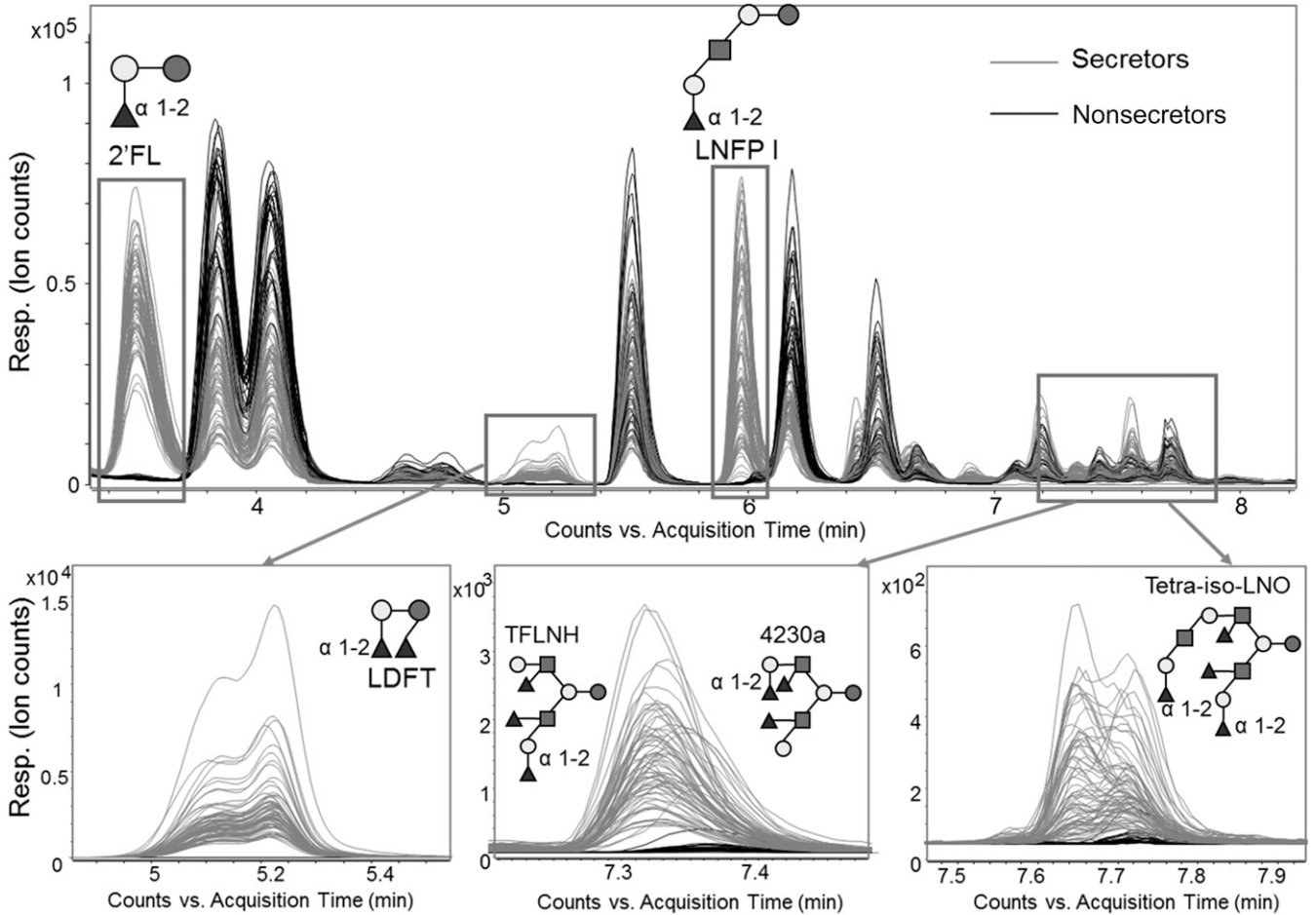
FIGURE 2 Comparison of total and MRM chromatograms of milk from secretor and nonsecretor mothers in the US study. Milk from secretors contained a much higher concentration of α(1,2)-fucosylated HMOs. Glucose (gray circles), galactose (white circles), N-acetylglucosamine (squares), fucose (triangles). HMO, human milk oligosaccharide; LDFT, lacto-difucotetraose; LNFP I, lacto-N-fucopentaose I; Resp., response; tetra-*iso*-LNO, tetrafucosyl-*iso*-lacto-N-octaose; TFLNH, trifucosyllacto-N-hexaose; 2'FL, 2'-fucosyllactose.

TABLE 2 Accuracy of total HMO quantitation[1]

Sample	Actual, g/L	Measured, g/L	Error, %
1	12.9	13.7	6.2
2	8.79	8.49	−3.4
3	5.00	4.76	−4.8
4	6.17	5.70	−7.6
5	5.31	6.02	13

[1] HMO, human milk oligosaccharide.

UPLC/QqQ-MS in MRM mode. Detection and quantitation of HMOs were performed using an Agilent 6490 QqQ-MS (Agilent Technologies) equipped with an Agilent 1290 infinity LC system and a Waters ACQUITY UPLC BEH Amide column (2.1 mm × 100 mm i.d., 1.7 μm particle size) (Waters Corporation). The aqueous mobile phase A was 3% acetonitrile:0.1% formic acid in water (vol:vol), and the organic mobile phase B was 95% acetonitrile in water (vol:vol). Separation of HMOs was performed by use of an optimized 10-min binary gradient of 0.00–4.00 min, 2–5% A; 4.01–8.50 min, 35–65% A; 8.50–8.70 min, 65–25% A; 8.70–10.00 min, 25% A. The flow rate was set at 0.3 mL/min.

Electrospray ionization was used as the ionization source and was operated in positive ion mode. The MS parameters optimized by Hong et al. (34) for oligosaccharide analysis were used in this study, with some modifications. The capillary voltage and the fragmentor voltage were set at 1800 and 280 V, respectively. At least 31 compositions were monitored, representing >100 HMO structures based on our previously published libraries (35, 36). For most compositions, one transition was chosen based on the transitions published by Hong et al. (34), with some modifications (**Supplemental Table 1**).

Data processing and statistical analysis. Linear regression was conducted in Microsoft Excel 2010 (Microsoft Corporation) to evaluate the sensitivity and linear range of this quantitation method. Limits of detection (LODs) and limits of quantitation (LOQs) were defined as the concentrations of analytes that yielded a signal-to-noise ratio of 3 or 6, respectively. The peak areas and signal-to-noise ratios were calculated by Agilent MassHunter Quantitative Analysis B.05.02.

To determine the effect of time during the course of lactation through use of all 4 time points (postnatal days 10, 26, 71, and/or 120) on HMOs, Friedman's test, a nonparametric test for repeated measures, was conducted on paired data for the US milk samples. To determine whether HMOs were substantially different between early (postnatal day 10) and mature milk (postnatal day 120), Wilcoxon's signed rank test, a nonparametric test used to compare 2 related samples, was conducted on paired data for the US milk samples. Friedman's test and Wilcoxon's signed rank test were conducted in SPSS version 22 (IBM SPSS Statistics). Student's *t* test was performed with JMP Pro version 11.0 (JMP) to compare the means of HMO concentrations from secretors and nonsecretors and to compare the Malawi and US (postnatal day 120) samples. All of the statistical analyses were considered significant at $P < 0.05$.

Results

UPLC/QqQ-MS analysis of HMOs in MRM mode

A typical MRM chromatogram for a pooled milk sample is shown in **Figure 1**, where the abundant MRM transitions are annotated. All of the HMOs were eluted within a 9-min period, for a total analysis time of 10 min. The 5 most abundant compounds—2′FL, 3FL, LNT, LNFP I, and 6′SL—were readily identified through the use of commercial standards. 3FL showed 2 peaks caused by the presence of anomers that were separated by hydrophilic interaction liquid chromatography (HILIC)-UPLC. For the analysis of this compound, the 2 peaks were summed. The separation of anomers at the reducing end is present in other separation media such as PGC (37). For this

reason, oligosaccharides often were reduced to produce a single alditol at the reducing end. Peak splitting did not appear to affect other compounds as strongly with HILIC; however, this also reflected the generally lower effectiveness of HILIC relative to PGC for separating oligosaccharide isomers. Nonetheless, for a selected group of compounds (2′FL, 3FL, and LNFP I), baseline separation was achieved.

Quantitation of individual HMO compounds

We examined whether unreduced HMOs can be quantitated individually when commercial standards are available. For this analysis, 5 commercial standards (2′FL, 3FL, LNT, LNFP I, and 6′SL) were used. **Table 1** lists the regression lines, linear regression coefficients (R^2), linear ranges, LODs, and LOQs for the HMO standards. The response factors (slopes of the regression lines) were similar for the 5 compounds, including neutral (LNT), fucosylated (2′FL, 3FL, LNFP I), and sialylated (6′SL) species. All of the compounds showed excellent linearity. For the 5 HMO standards, the linear ranges spanned 4–5 orders of magnitude, which was sufficient to cover the concentration ranges in biological samples. The LODs and LOQs for all 5 compounds were at the femtomole to subfemtomole level. With high sensitivity, even low-abundance HMOs could be detected. Concentrations of these 5 compounds in a pooled milk sample were determined by use of both the standard addition and standard calibration curves. As shown in Table 1, the differences in HMO concentrations obtained from these 2 methods were within 5% for 6′SL, 3FL, LNT, and LNFP I, but for 2′FL the difference was 33%. The larger variation with 2′FL may be caused by ion suppression from lactose because it is abundant in milk and elutes immediately before 2′FL.
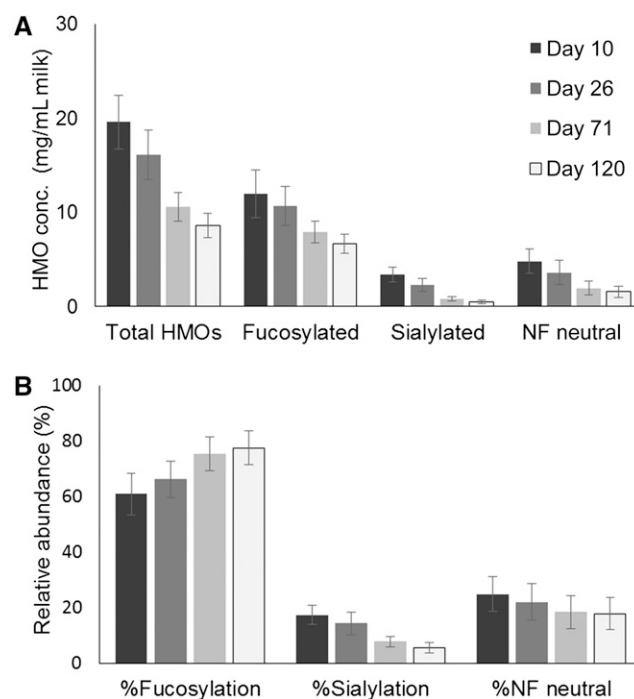

FIGURE 3 Changes in absolute (A) and relative (B) concentrations of HMOs in US milk samples during the course of lactation. Values are means ± SDs, *n* = 6 or 14. (A) Total, fucosylated, sialylated, and NF neutral HMO concentrations decreased over time ($P < 0.05$). (B) Percentage of fucosylation increased and percentage of sialylation and of NF neutrals decreased over time ($P < 0.05$). Conc., concentration; HMO, human milk oligosaccharide; NF, nonfucosylated.

Individual HMO profiles are largely influenced by mothers' genotypes (38). People with an active secretor (Se) gene that can encode α(1,2)-fucosyltransferase are classified as "secretors." The MRM chromatograms (**Figure 2**) illustrated the variations between secretor (gray) and nonsecretor (black) milk from the US lactation study. The mothers were genotyped using a saliva test (39). As shown, milk from secretor mothers contained much higher concentrations of α(1,2)-fucosylated HMOs such as 2′FL, LNFP I, lacto-difucotetraose (LDFT), trifucosyllacto-N-hexaose, and tetrafucosyl-iso-lacto-N-octaose than did milk from nonsecretors. The concentrations of these α(1,2)-fucosylated compounds were used to identify the phenotypic secretor status of the mothers.

Validation of total HMO quantitation with pooled HMO standards

To verify the accuracy of this method for the absolute quantitation of total HMOs in biological fluids, HMOs from 5 milk samples were extracted, lyophilized, weighed, and analyzed. An HMO standard pool from the US lactation study was used to build a calibration curve, $y = 2840x + 41700$ (range of x: 11.7–493 μg/mL, $R^2 = 1.00$), where y was the total peak area of all of the compositions and x was the total concentration of HMOs in micrograms per milliliter. This calibration curve was used to calculate the total HMO concentration in each sample. The measured HMO concentrations as well as their actual concentrations in each sample are shown in **Table 2**. The mean relative error was <10%.

Quantitation of HMOs in human milk samples

The quantitation method was applied to milk samples from 2 sets of clinical studies. One was the US lactation study, in which 103 samples collected at 4 different time points throughout the course of lactation were analyzed. The other 88 milk samples analyzed were collected from Malawi mothers. The absolute concentrations of total, fucosylated, sialylated, and nonfucosylated neutral HMOs were estimated through use of the universal calibration curves made from pooled standard samples. The relative (percentage of total) and absolute concentrations (in grams per liter of milk) of HMOs were compared according to the mothers' secretor status and lactation time points.

US milk samples. The US study sample set comprised 103 milk samples from 45 mothers, including 23, 26, 31, and 23 samples from postnatal days 10, 26, 71, and 120, respectively. The total concentrations of HMOs in these samples were found
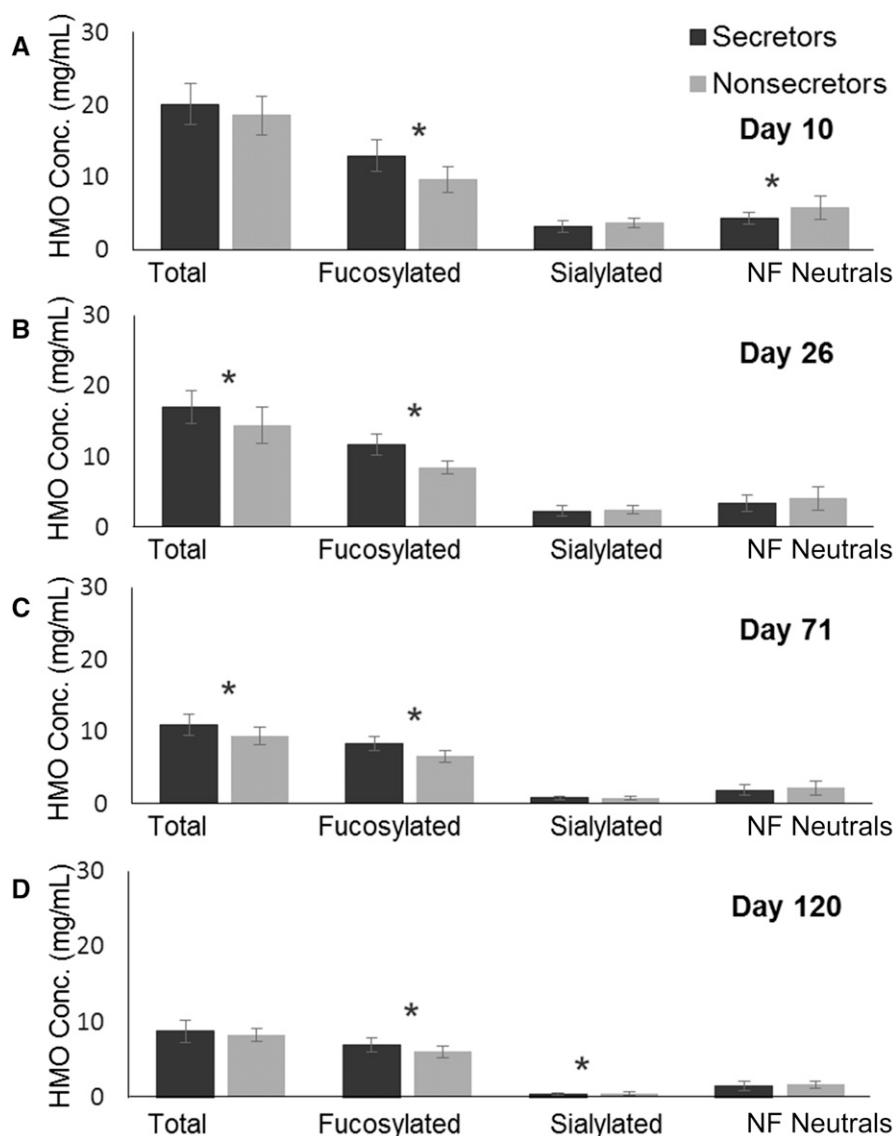
FIGURE 4 Differences in HMO concentrations between secretor and nonsecretor mothers in the US study. Values are means ± SDs. (A) Day 10: secretors, $n = 15$; nonsecretors, $n = 8$. (B) Day 26: secretors, $n = 18$; nonsecretors, $n = 8$. (C) Day 71: secretors, $n = 24$; nonsecretors, $n = 7$. (D) Day 120: secretors, $n = 15$; nonsecretors, $n = 8$. *Means differ, $P < 0.05$. Conc., concentration; HMO, human milk oligosaccharide; NF, nonfucosylated.
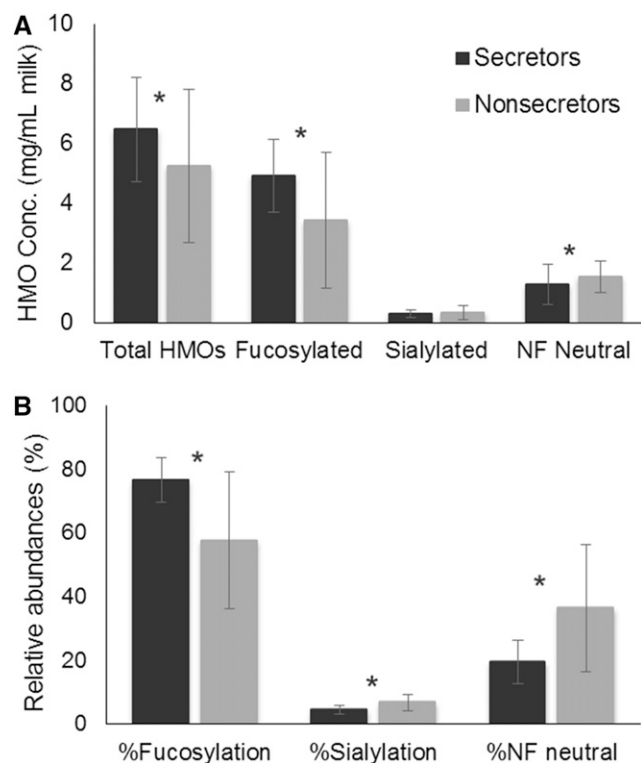
FIGURE 5 Differences in absolute (A) and relative (B) concentrations of HMOs between secretor and nonsecretor mothers in the Malawi study. Values are means ± SDs, $n = 69$ (secretors) or 19 (nonsecretors). *Means differ, $P < 0.05$. Conc., concentration; HMO, human milk oligosaccharide; NF, nonfucosylated.

to be 19.6 ± 2.9 g/L on postnatal day 10, 16.3 ± 2.7 g/L on postnatal day 26, 10.4 ± 1.4 g/L on postnatal day 71, and 8.64 ± 1.30 g/L on postnatal day 120 (all samples included). The concentrations ranged from 6.56 to 24.3 g/L. Statistical analyses on the effect of time were conducted on paired samples across all 4 time points (Friedman's test; $n = 6$) and between early (postnatal day 10) and mature milk (postnatal day 120) (Wilcoxon's signed rank test; $n = 14$). A significant decrease ($P < 0.05$) was observed during the course of lactation (**Figure 3**A). The absolute concentrations and percentages of fucosylated, sialylated, and nonfucosylated neutral HMOs also were estimated (Figure 3A, B). The absolute concentrations (in grams per liter of milk) decreased substantially throughout lactation. For example, fucosylated compounds decreased from 12.0 ± 2.5 g/L on postnatal day 10 to 6.67 ± 0.98 g/L on postnatal day 120, and sialylated species decreased from 3.39 ± 0.80 to 0.48 ± 0.20 g/L. Similarly, the percentage of sialylation and percentage of nonfucosylated neutrals also decreased. The percentage of fucosylation, however, significantly increased from 60.9% to 77.4% ($P < 0.05$) during the lactation period.

The variations of the oligosaccharide compositions also were investigated (**Supplemental Figure 1**). When comparing early and mature milk using Wilcoxon's signed rank test (for repeated measures; $n = 14$), a substantial increase in 3FL and decreases in all of the other compositions were observed, except for LDFT (composition 2020), which remained unchanged. The same trends across all 4 time points also were found to be statistically significant ($P < 0.05$ from Friedman's test on paired samples; $n = 6$) for all of the compositions except 2020, 2FL, 3120, and 4200.

The phenotypic secretor status of the mothers was determined through use of the concentrations of $\alpha(1,2)$-fucosylated

HMOs in breast milk. In the US sample set, 11 of the 45 mothers (24%) were identified as phenotypic nonsecretors because their milk contained low concentrations of 2′FL, LNFP I, or LDFT. The percentage of nonsecretors is consistent with previous studies (40). Conversely, the remaining mothers were deemed to be secretors because their milk contained much higher concentrations of these compounds. The secretor phenotypes of all of the mothers in this study were 100% consistent with their genotypes, which were determined previously by saliva DNA tests (39). The total concentrations of these $\alpha(1,2)$-fucosylated HMOs (as shown in Figure 2) in the milk from secretor mothers were found to be >1.8 g/L (>28% of their total HMOs), and those for nonsecretor mothers were ≤0.28 g/L, corresponding to <3% of their total HMO concentrations. The differences were especially dramatic for 2′FL and LDFT. The receiver operating characteristic curves (**Supplemental Figure 2**) demonstrated that the concentrations of these individual $\alpha(1,2)$-fucosylated HMOs as well as their total concentrations could be used to determine the mothers' secretor status with high sensitivity and specificity. In addition, the concentrations of the $\alpha(1,3)/\alpha(1,4)$-fucosylated HMOs such as 3FL, and LNFP II/III/V could be used as markers for nonsecretors (**Supplemental Figure 3**).

Figure 4A–D illustrates the HMO concentrations between secretors and nonsecretors for different lactation days. The total concentration of HMOs in secretors was slightly (6.3–18%) higher than that in nonsecretors at all 4 time points, whereas the difference was statistically significant at postnatal days 26 and 71 ($P < 0.05$) but not postnatal days 10 ($P = 0.23$) or 120 ($P = 0.30$). Changes in absolute concentrations of fucosylated, sialylated, and nonfucosylated neutral HMOs also are shown in Figure 4A–D. Fucosylation was 14–39% higher at all times tested, whereas sialylation was 26% lower on postnatal day 120 and nonfucosylated neutrals were 25% lower on postnatal day 10 in secretor compared with nonsecretor mothers ($P < 0.05$).

Malawi milk samples. The Malawi milk samples were obtained only from a single point corresponding to 6 mo postnatal. The HMO concentrations in this cohort also were determined by use of the calibration curve built from a pooled milk sample. The total HMO concentration was 6.20 ± 2.00 g/L, which was lower ($P < 0.05$) than that in US samples from postnatal day 120 and confirmed the decrease in the HMO concentration during the course of lactation. The phenotypic secretor status for all 88 mothers was determined using $\alpha(1,2)$-fucosylated HMOs, and the HMO differences between secretors and nonsecretors in this sample set were obtained. Among the 88 Malawian mothers, a total of 19 were nonsecretors (22%), and the total concentrations of HMOs in their milk (5.25 ± 2.55 g/L) were significantly lower than those in secretors (6.46 ± 1.74 g/L) ($P < 0.05$). Absolute concentrations and percentages of fucosylated, sialylated, and nonfucosylated neutral HMOs in the Malawi samples are shown in **Figure 5**A, B. Fucosylation was higher (4.91 ± 1.22 compared with 3.42 ± 2.27 g/L; $P < 0.05$) and the other classes were lower in secretor milk in both absolute and relative terms. This was consistent with the results from the US study.

Discussion

Analysis of milk samples from the United States revealed that total HMO concentration decreased significantly during the course of lactation. The concentrations and trends are consistent with results from previous studies of US and European women (14, 16, 17, 41). Interestingly, not all of the HMO compositions

decreased during the lactation period. LDFT, an α(1,2)-fucosylated compound, remained unchanged, and 3′FL, an α(1,3)-fucosylated compound, increased substantially. This demonstrated the complexity of HMO production.

This method also provided rapid analyses of α(1,2)-fucosylated HMOs, which proved to be highly specific markers for the identification of phenotypic secretor status. Similarly unique variations of these α(1,2)-fucosylated HMOs have been shown by Totten et al. (42); however, the current study provides, to our knowledge, the first absolute quantitative analysis of total HMOs and these specific components in secretor and nonsecretor milk. Comparisons between secretor and nonsecretor milk showed that milk from secretor mothers contains substantially higher concentrations of total and fucosylated HMOs. Similar trends have been shown in previous studies of term and preterm milk samples (15, 41). The total and fucosylated HMO differences between secretors and nonsecretors reported in those studies, however, were greater than what we found in this cohort. This may be the result of population as well as method differences. Previous studies were conducted on only the 10–20 most abundant HMO compounds, which excluded the variations of the low-abundance compositions. The current method provided the concentrations of total HMOs as well as individual compositions. The results demonstrated that part of the reductions in α(1,2)-fucosylated HMOs (3.38 g/L for 2′FL and 2.02 g/L for LNFP I) were counterbalanced by increases in other components.

As part of this project, a rapid method for absolute quantitation of HMOs with minimal sample preparation was developed through use of UPLC/QqQ-MS in MRM mode. The quantitation was performed using pooled milk samples as standards instead of commercial standards because the number of available HMO standards is limited. Compared with other existing methods for HMO analysis, the sample preparation and analysis time was greatly minimized. The sensitivity and linearity of this method were tested using commercial HMO standards before it was applied to human studies. The LODs and LOQs for all 5 compounds were at the femtomole to subfemtomole level, and the linear ranges spanned 4–5 orders of magnitude. This validated method was applied to 2 human milk sample sets from the United States and Malawi. All of the statistical analyses and quantitation results supported our previous findings that total HMO concentration decreased during the course of lactation while the percentage of fucosylation increased, and that milk from secretor mothers contained substantially higher concentrations of fucosylated HMOs. In the future this method will be applied to large human studies for absolute quantitation and bioactivity analysis of HMOs.

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