JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Quantification of Bovine Milk Oligosaccharides Using Liquid Chromatography−Selected Reaction Monitoring−Mass Spectrometry

Bertram Fong,* Kevin Ma, and Paul McJarrow

Fonterra Research Centre, Dairy Farm Road, Private Bag 11029, Palmerston North 4412, New Zealand

ABSTRACT: Oligosaccharides are important components of milk with bioefficacy as prebiotics, anti-infectives, and immune system modulators and as a possible source of sialic acid for neural function. Bovine milk oligosaccharides are lower in concentration and lack the diversity of human milk oligosaccharides but could be a commercial source of milk oligosaccharides for pediatric foods. For this development, an ability to quantify the oligosaccharides is required. This study validated a hydrophilic interaction chromatography high-performance liquid chromatography−high-resolution selected reaction monitoring−mass spectrometry (HILIC HPLC−HRSRM−MS) method for measuring six different oligosaccharides in bovine milk, bovine colostrum, and infant formulas. The extraction resulted in a high recovery (90−103%) with a repeatability coefficient of variation ranging from 2 to 9% for the two dominant oligosaccharides, 3′-sialyllactose and 6′-sialyllactose, and ranging from 1 to 17% for the much lower concentration oligosaccharides, 6′-sialyllactosamine, disialyllactose, and *N*-acetylgalactosaminyllactose. The sixth oligosaccharide, 3′-sialyllactosamine, was not detected in any of the samples.

KEYWORDS: sialyllactose, colostrum, hydrophilic interaction chromatography, infant formula

INTRODUCTION

Milk oligosaccharides are a complex family of glycans and are generally composed of 3−10 covalently linked monosaccharides. They represent a significant proportion of the components present in mature human milk (5−10 g/L)[1,2] after lactose (55−70 g/L) and fat (30−60 g/L). To date, although more than 200 different oligosaccharide compositions have been reported in human milk, the structures of only approximately 115 have been determined.[2−5] The number of oligosaccharides reported in mature bovine milk is significantly less, with approximately 25 oligosaccharide structures identified of the 39 oligosaccharide compositions reported.[3,5,6] The concentrations of the dominant bovine milk oligosaccharides (3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), 6′-sialyllactosamine (6′SLN), and disialyllactose (DSL)) range from 60−94 μg/mL[7,8] to 347−460 μ/mL[9] (Table 1). These dominant bovine milk oligosaccharides are sialylated oligosaccharides, with a lactose core structure, as opposed to the fucosylated oligosaccharides, with a lacto-*N*-tetraose core structure, which are the dominant human milk oligosaccharide species.

Both human milk oligosaccharides and bovine milk oligosaccharides have received a lot of interest, mainly for their biological efficacy as prebiotics, anti-infectives, and immune modulators and because they are a possible source of sialic acid for the nervous system. Their bioefficacies, monosaccharide compositions, structures, and concentrations of representative oligosaccharides in milk/colostrum have been reviewed over the past decade,[1−3,10−21] but additional work is required to further understand their activities in more detail. To gain this understanding, the oligosaccharides must be available for trials (in vitro, in vivo, and clinical) in larger (and purer) amounts. To support such trials, it is essential that robust methods are developed to quantify each oligosaccharide of interest. Currently, the commercially available oligosaccharides that are used for pediatric formulation fortification purposes are generally derived from the hydrolysis of chicory inulin (fructo-oligosaccharide) or are enzymatically synthesized from lactose (galacto-oligosaccharide); they are less diverse than milk oligosaccharides and are used to imitate the prebiotic activity found in human milk. Increasing interest is being shown in sourcing oligosaccharides with structures the same as or similar to those present in human milk for pediatric formulation fortification purposes.

For any future benefit claims for bovine milk oligosaccharides to be allowed, accurate quantitative data for the relevant oligosaccharides will be required, again emphasizing that robust quantitative analysis is essential. Over the past two to three decades, focus has been on the discovery, characterization, and profiling of human and bovine oligosaccharides,[4,6,22−30] but few studies on the absolute quantification of bovine oligosaccharides have been reported.[7−9]

The earliest direct quantification of bovine oligosaccharide species was carried out by indirectly measuring the sialic acid from bovine oligosaccharide fractions after hydrolysis.[31−33] This technique measured only sialic acid containing oligosaccharides and did not distinguish between the different oligosaccharide forms. All subsequent quantitative data published on bovine oligosaccharide concentrations were based on high-performance liquid chromatography−ultraviolet (HPLC-UV)[7,9] or HPLC−amperometric[8] detection: Martin-Sosa et al.[9] reported the concentrations of five oligosaccharides (3′SL, 6′SL, 3′-sialyllactosamine (3′SLN), 6′SLN, and DSL) through the progression of lactation; Nakamura et al.[7] reported the levels of three

Received: May 23, 2011
Revised: July 26, 2011
Accepted: July 27, 2011
Published: July 27, 2011

Table 1. Oligosaccharides Measured in Different Bovine Milk, Colostrum, and Infant Formula Samples, with Number of Samples and Coefficient of Variation in Parentheses

	3′SL (μg/mL)	6′SL (μg/mL)	6′SLN (μg/mL)	DSL (μg/mL)	GNL (μg/mL)
skim milk 1	51 ± 4 (30, 9%)	6.3 ± 0.4 (8, 6%)	0.13 ± 0.02 (2, 14%)[a]	1.5 ± 0.1 (2, 6%)	2.6 ± 0.3 (2, 10%)[a]
skim milk 2	55 ± 4 (2, 6%)	9 ± 0.2 (2, 2%)	0.10 ± 0.02 (2, 17%)[a]	2.1 ± 0.3 (2, 14%)	3.4 ± 0.4 (2, 13%)[a]
homogenized milk	48 ± 4 (6, 8%)	9.6 ± 0.8 (3, 8%)	0.1 ± 0.03 (3, 14%)[a]	3.1 ± 0.2 (3, 6%)	2.4 ± 0.1 (3, 2%)[a]
unpasteurized milk	47 ± 4 (6, 9%)	3.6 ± 0.3 (2, 9%)	<LOD (2)	0.54 ± 0.01 (2, 14%)[a]	<LOD (2%)
mature milk (Martin-Sosa et al.[9])[b]	94−119	67−88	145−176	41−77	
mature milk (μg/mL, McJarrow and van Amelsfort-Schoonbeek[8])[c]	35−50	14−25	9−12	2−7	3−4
mature milk (7 days postpartum, Namakura et al.[7])[d]	30	25	12	ND	
colostrum (second milking)	1245 ± 82 (7, 7%)	85 ± 6 (7, 7%)	119 ± 7 (7, 6%)	126 ± 8 (2, 8%)	1 ± 0.1 (2, 12%)[a]
colostrum (fourth milking)	739 ± 53 (5, 7%)	73 ± 2 (5, 2%)	117 ± 10 (5, 8%)	80 ± 7 (2, 9%)	1 ± 0.1 (2, 10%)[a]
colostrum (Martin-Sosa et al.[9])[b]	354	147	210	135	
colostrum (μg/mL, McJarrow and van Amelsfort-Schoonbeek[8])[c]	261−867	92−243	97−239	166−283	20−65
colostrum (Namakura et al.[7])[d]	850	141	117	ND	
infant formula 1[e]	17 ± 4 (2, 8%)	3.8 ± 0.2 (2, 10%)	<LOD (2)	0.8 ± 0.1 (2, 13%)[a]	<LOD (2)
infant formula 2[f]	19 ± 1 (2, 6%)	4.6 ± 0.5 (2, 10%)	<LOD (2)	0.5 ± 0.02 (2, 5%)[a]	<LOD (2)

[a] Measured values at or above the LOD but less than the LOQ. The LOD and LOQ values for the respective oligosaccharides are provided in Table 5. [b] Normal phase HPLC-UV, data derived from their figure expressed as mg/kg but converted to μg/mL for comparison purposes using milk density of 1.033 g/mL[49] and average colostrum milk density (1.041 g/mL, second milking[50]). The 3′SLN levels reported in mature milk and colostrum at approximately 25 μg/mg each. [c] HPAEC, across different cow breeds. [d] Normal phase HPLC-UV, data derived from their figure expressed as mg/L. Colostrum was from the first milking. [e] Infant formula 1 reconstituted at 3.0 g/20 mL, as specified by the manufacturer. [f] Infant formula 2 reconstituted at 2.9 g/20 mL, as specified by the manufacturer.

oligosaccharides (3′SL, 6′SL, and 6′SLN) in colostrum and milk; and McJarrow and van Amelsfort-Schoonbeek[8] reported the seasonal variation of five oligosaccharides (3′SL, 6′SL, 6′SLN, *N*-acetylgalactosaminyl lactose (GNL), and DSL) in mature milk and the daily variation of these oligosaccharides in colostrum across different cow breeds.

Although 3′SL is consistently reported to be the most abundant bovine milk oligosaccharide, there is large variation in the reported absolute concentrations of this and other bovine milk oligosaccharides (Table 1). Although cow breed may explain some of these variations in the data, it is also very likely that the different extraction protocols with different extraction efficiencies and the analytical methods could also contribute to this variation, particularly when methods based on thin layer chromatography spraying (e.g., resorcinol), UV detection, and amperometric detection rely heavily on retention time identification and peak purity. Co-eluting components could lead to overestimation of oligosaccharide concentrations.

Although capillary electrophoresis (CE) with UV or laser-induced fluorescence detection has also been used successfully for the quantification of human milk oligosaccharides,[24,34] we could not find any papers relating to bovine milk. This technique has been reviewed by Boa and Newburg.[16]

Mass spectrometry (MS) analysis coupled to HPLC has been used extensively for the elucidation and characterization of milk oligosaccharides from various species.[4,6,22,23,25,26] There have been no reported applications of HPLC-MS for the absolute quantification of bovine oligosaccharides, although use of the relative intensities of extracted mass peaks and peak areas for comparative purposes has been reported.[6,35] MS as a means of detection offers significant advantages over UV detection because of its specificity, which is derived first from oligosaccharide mass selection and second from the fragmentation specific to linkage and monosaccharide sequence.

In this study, we report a validated method for the simultaneous quantification of six bovine milk oligosaccharides using liquid chromatography−high-resolution selected reaction monitoring−mass spectrometry (LC-HRSRM-MS). These six oligosaccharides were selected on the basis of their reported abundance in bovine milk and their availability as commercially pure standards.

MATERIALS AND METHODS

Standards and Chemicals. Oligosaccharide standards (3′SL, 6′SL, 3′SLN, 6′SLN, GNL, all >95% purity) were obtained from Dextra Laboratories Ltd. (Reading, U.K.). DSL (75 and >99% purity) was obtained from Sigma-Aldrich (St. Louis, MO). All solvents used were of liquid chromatography grade (Merck, Darmstadt, Germany), except for chloroform, which was of analytical grade (ethanol stabilized).

Samples. The skim milk (0.1% fat) and the homogenized milk (3.5% fat) were commercial samples. Unpasteurized whole milk was obtained from dairy factory supply (August 2010). The colostrum samples (second and fourth milkings) were obtained from a single Friesian cow. The two infant formulas were commercial, whey-protein-dominant, premium infant formulas (0−6 months), purchased from a supermarket.

Sample Preparation. Milk and colostrum samples were defatted by centrifugation at 5000*g* at 5 °C for 15 min and then diluted (6-fold) in water prior to extraction. The diluted defatted samples were extracted according to the method of Tao et al.[6] with modifications. Briefly, 2 mL of chloroform/methanol (2:1 v/v) was added to 0.5 mL of diluted milk sample and mixed. After centrifugation (2000*g* for 1 h), the upper phase was transferred to a 2 mL volumetric flask. The lower phase was re-extracted with 0.5 mL of methanol (50% v/v). After centrifugation

(2000*g* for 30 min), the upper phase was poured into the 2 mL volumetric flask and made up to the mark with 95% acetonitrile. Further dilutions were made in 95% acetonitrile so the oligosaccharide concentrations were within the calibration points. For milk-derived samples, a further dilution in 95% acetonitrile of either 8- or 2-fold (for the analysis of the lower abundance oligosaccharides (6′SL, 6′SLN, DSL, and GNL) was made. For colostrum, a further dilution of 80- or 160-fold or 8-fold (for the analysis of the lower abundance oligosaccharides (6′SL, 6′SLN, DSL, and GNL) was made. The infant formulas were reconstituted according to the manufacturer's instructions and were extracted as for milk.

The diluted sample extract was allowed to cool to 4 °C in a cool room (30 min) before centrifugation (20000*g* for 10 min) to remove any precipitated proteins. An aliquot of the supernatant was transferred to an HPLC vial for LC-MS/MS analysis.

Standard Preparation. Oligosaccharide stock standards were prepared at 1 mg/mL in water. The stock solution was diluted in 95% acetonitrile to give a seven-point calibration curve with the concentration ranging from 0.01 to 0.78 μg/mL for 3′SL, 6′SL, 3′SLN, 6′SLN, and DSL. The concentration range for GNL was from 0.39 to 6.25 μg/mL. Typical calibration curves are given in Figure 1. All stock solutions were stored at −30 °C.

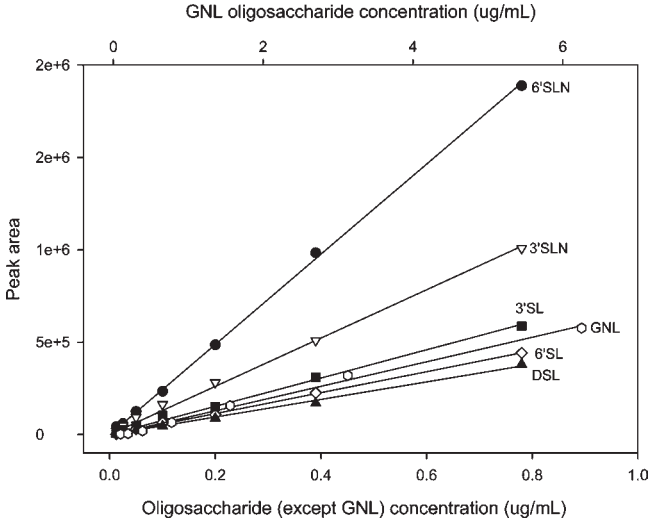

Figure 1. Typical calibration curves of oligosaccharide standards obtained using the HPLC-HRSRM method. All of the regression coefficients were in the range of 0.9−1.0.

Hydrophilic Interaction Chromatography (HILIC) LC-HRSRM-MS. Oligosaccharide separation was performed on a Waters ACQUITY ultraperformance liquid chromatography system (Waters Corp., Milford, MA), equipped with a column heater set to 60 °C, an autosampler set at 15 °C, and a Kintex HILIC column (50 mm × 2.1 mm, 1.7 μm, Phenomenex, Torrance, CA). The aqueous mobile phase (A) contained 50 mM ammonium acetate (pH 4.5), and the organic mobile phase (B) contained acetonitrile (100%). Standards and samples (5 μL) were loaded onto the column with 5% mobile phase A for the first 2 min. The oligosaccharides were eluted from the column with a gradient of 5−30% A over 18 min. The gradient was maintained at 30% A for 2 min before increasing to 50% over 1 min. The column was re-equilibrated to the initial conditions after 24 min for 11 min. The flow rate was set at 400 μL/min.

The LC system was coupled to a Thermo Scientific TSQ Quantum Ultra EMR mass spectrometer (Thermo Fisher Scientific, San Jose, CA), using a heated electrospray ionization (ESI) interface. The first 5 min and the last 15 min of the LC eluant were diverted to waste. Ions were generated and focused using a negative ion spray voltage of −4000 V, a vaporizer temperature of 400 °C, a sheath gas of 50 arbitrary units, an auxiliary gas of 20 arbitrary units, and a capillary temperature of 300 °C. The HRSRM-MS conditions were as follows: scan width, 0.2 *m*/*z*; scan time, 0.1 s; collision gas pressure, 1.5 mTorr. The collision energies were optimized by infusion of each of the oligosaccharide standards into the mass spectrometer (at 0.1 mg/mL in 25% ammonium acetate buffer/75% acetonitrile at 0.4 mL/min) to give a complete fragmentation of the parent ion and maximize the intensities of the selected daughter ions. The optimized collision energies for the two selected daughter ions are given in Table 2. The sums of the intensities of these two daughter ions were used to quantify the respective oligosaccharides.

A typical HRSRM total ion count trace and the respective extracted ion chromatograms acquired under these conditions are shown in Figure 2.

Standard Addition. A standard addition technique was used to access the presence of matrix effect such as ion suppression during ionization of the sample eluate during MS. For standard addition, oligosaccharide standards were spiked into the milk samples at four different levels. A plot of the spiked concentration (*x*-axis) against the peak area (*y*-axis) was generated. The *y*-intercept calculated from this plot represents the oligosaccharide concentration of the unspiked sample. A typical example of a standard addition plot for 3′SL is given in Figure 3.

Recovery. Recovery of the extraction protocol was assessed by spiking a known amount of oligosaccharide into the milk sample prior to sample extraction. The percent recovery was determined by subtracting the amount of each oligosaccharide measured in the nonspiked sample from the amount measured in the spiked sample, divided by the spiked amount.

Table 2. Daughter Ions and Collision Energies Used in the HRSRM-MS Method in Negative Ion Mode

parent (*m*/*z*) [oligosaccharide unit structure][a]	daughter ions (*m*/*z*) [proposed structures][b]	collision energy (V)
544 (*N*-acetylgalactosaminyllactose)	220.08 [C_1]	25
[GalNAcα1−3Galβ1−4Glu]	466.12 [$^{0,4}X_{Gal}$, $^{1,3}X_{Glc}$, $^{0,4}X_{Glc}$, $^{0,2}A_{Glc}$, $^{2,4}X_{GalNAc}$, $^{0,4}X_{GalNAc}$][c] − H_2O	
632 (3′ and 6′-sialyllactose)	290.09 [B_1][d]	35
[NeuAcα2−3/6Galβ1−4Glu]	408.11 [$B^{1,3}X_{NeuAc}$][d]	
673 (3′ and 6′-sialyllactosamine)	290.09 [B_1][d]	35
[NeuAcα2−3/6Galβ1−4GlcNAc]	572.18 [$^{0,2}A_{GlcNAc}$][d]	
923 (disialyllactose)	581.18 [B_2][d]	35
[NeuAcα2−8NeuAcα2−3Galβ1−4Glu]	632.20 [Y_1]	

[a] GalNAc, *N*-acetylgalactosamine; Gal, galactose; Glu, glucose; NeuAc, *N*-acetylneuraminic acid (sialic acid). [b] Nomenclature of fragment ions from ref 51. [c] Several possible daughter ions with the same *m*/*z* (484.12) followed by loss of water to give *m*/*z* 466.12. [d] Most likely daughter ions.

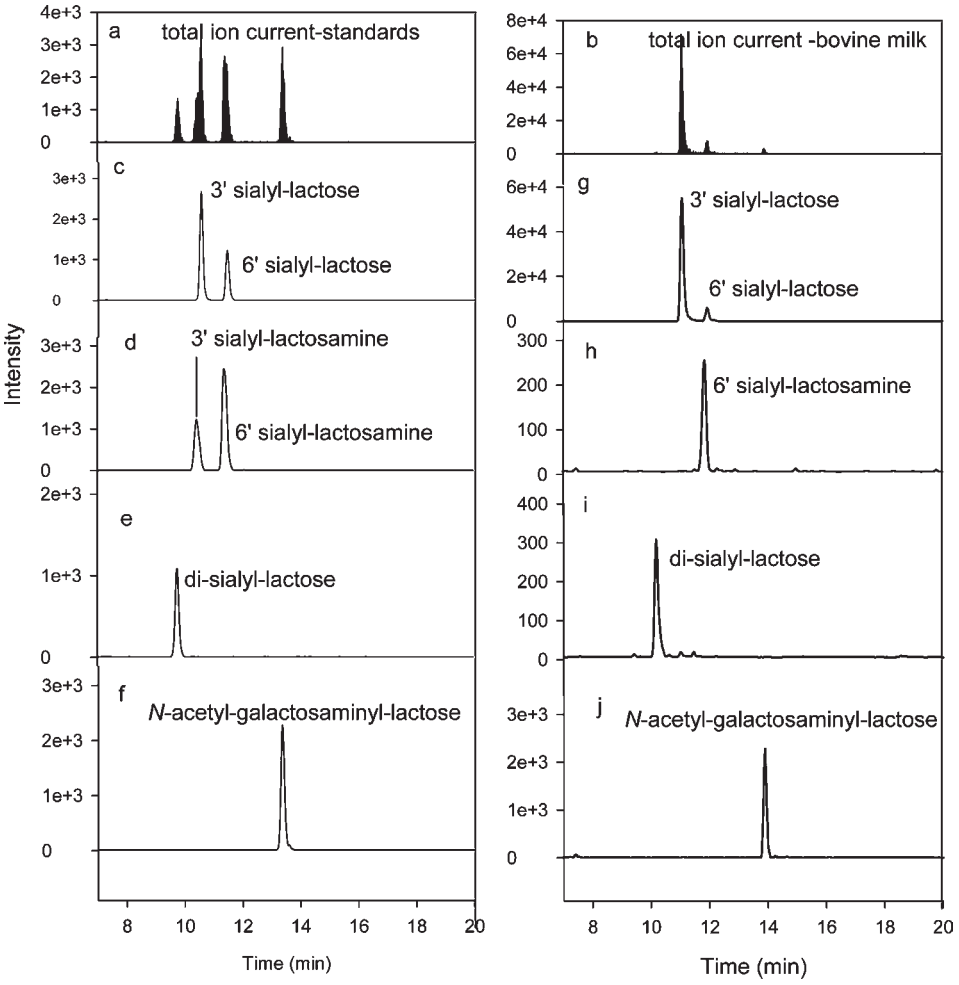

Figure 2. Bovine milk oligosaccharide analysis by HPLC-HRSRM: typical total ion current trace of oligosaccharide standards (a) followed by their extracted ion chromatograms (c–f) and total ion current trace of bovine milk oligosaccharide (b) followed by the extracted ion chromatograms (g–j).

RESULTS AND DISCUSSION

Optimization of Oligosaccharide Extraction. A number of methods for the extraction of oligosaccharides from milk have been reported. Typically, the milk samples are first defatted by centrifugation, followed by protein precipitation. The protein precipitation methods reported for milk include acetonitrile,[28] barium hydroxide/zinc sulfate,[8] ethanol (overnight at 4 °C),[36] a chloroform/methanol mixture,[29] and a chloroform/methanol mixture followed by overnight (4 °C) ethanol precipitation of residual protein.[4,6,37−39]

In this study, we adopted the chloroform/methanol/ethanol extraction protocol, but replaced the ethanol with acetonitrile. Although ethanol was effective in removing the residual protein from the aqueous extract, we observed lactose crystallization and significant losses of oligosaccharides (30−50%). It is not unusual to observe lactose precipitation in an ethanol-rich mixture because of poor solubility. We postulate that the oligosaccharides were cocrystallizing with the lactose, leading to reduced recovery. This was confirmed when the standard oligosaccharide solutions were spiked with lactose at levels similar to those of the samples and were put through the same extraction protocol (i.e., we observed a loss of oligosaccharide standard; data not shown). Furthermore, ethanol-containing extracts were incompatible with the HILIC mobile phase and resulted in peak splitting, so that the sample extracts had to be dried and reconstituted in 95% acetonitrile.

To circumvent this problem, initial samples were diluted 6-fold prior to extraction, and ethanol was replaced with acetonitrile. All sample dilutions were carried out in 95% acetonitrile, and an aliquot was centrifuged before it was directly analyzed by LC-HRSRM-MS. Acetonitrile gave a much cleaner sample extract that was compatible with the HILIC mobile phase used, eliminating the need for subsequent evaporation of sample extract followed by reconstitution or a further cleanup step such as solid phase extraction, as used by some researchers.[6,7,9,35] Residual proteins precipitated quickly in acetonitrile, and at these lower concentrations lactose appears to remain in solution; hence, we did not observe any lactose crystallization. Furthermore, there was no need to stand the sample at 4 °C overnight to aid protein precipitation, as in the ethanol method.

To ensure maximum recovery, we included an extra extraction step in which the bottom chloroform and interface layer was re-extracted with 50% methanol.

Oligosaccharide Separation Using HILIC. Human and bovine oligosaccharides have generally been separated by methods based on reversed-phase,[17,40,41] anion exchange,[8,28,42] or graphitized carbon[4,6,43] column chromatography; the last method is

most commonly used because of its ability to resolve many of the more complex glycans and its use of a solvent system that is compatible with subsequent MS detection. The separation of oligosaccharides using a graphitized carbon HPLC column has been well reviewed by Ruhaak et al.[44]

We attempted to use a Hypercarb graphitized carbon column (Alltech, Grace Division, Deerfield, IL) using conditions reported by Ninonuevo et al.[43] for bovine milk oligosaccharide separation. Unfortunately, we found that the bovine milk oligosaccharides standard exhibited very poor chromatographic separation. The peaks were very broad (3−4 min) and exhibited significant tailing. Furthermore, peaks corresponding to the isobaric oligosaccharides were poorly resolved. Melmer et al.[45] recently reported that the redox state of the porous graphitized carbon column greatly affects the chromatographic separation of oligosaccharides and recommended that porous graphitized carbon columns be chemically reduced and oxidized to achieve satisfactory oligosaccharide separation. Similarly, Pabst and Altmann[46] reported that the redox state for graphitized carbon columns was critical for good chromatographic separation of nucleotide sugars. A poorly reduced graphitized carbon column resulted in unreproducible peak retention times, peak broadening, and peak tailing, to the extent of near loss of peak detectability. It is possible that our lack of success with the graphitized carbon column prior to the HILIC column separation was due to its redox state.

In contrast, HILIC has commonly been used for the separation of complex glycans, glycoconjugates, glycopeptides, and plant-sourced oligosaccharides for structural and glycomics research.[47] To our knowledge, bovine oligosaccharide separation has not been reported. In this study, we used a HILIC column coupled to MS to separate and quantify bovine oligosaccharides.

Separation of the bovine milk oligosaccharides was carried out using a Kintex HILIC column (Phenomenex). The column provided excellent baseline separation of the two positional isomers (3′- and 6′-) of both SL and SLN (Figure 2). The peaks were sharp and symmetrical, compared with the broader peaks reported with the nanochip graphitized carbon column,[6,38] making it more suitable for quantification purposes. Similar separation was also achievable with the Grace Division Vision HT HILIC column (100 mm × 1 mm, 1.5 μm, Alltech, Grace Division, Deerfield, IL) but not on the ACQUITY UPLC BEH HILIC column (Waters Corp.) (data not shown). The latter column produced very broad tailing peaks that were 2 min wide.

To avoid peak splitting with the Kintex HILIC system described, the sample extract must be in an acetonitrile-rich solvent mixture (90−95% acetonitrile), similar to the initial solvent gradient composition. It is postulated that too much water in the sample extract affects the hydration layer surrounding the stationary phase and, combined with insufficient re-equilibration, causes the peaks to split. The advantages and pitfalls of HILIC have been well discussed by Wuhrer et al.[47]

SRMs for lactose were not monitored in this work but lactose elutes between 3 and 4 min under our conditions, and this was diverted to waste to reduce fouling of the MS ion transfer tube.

Calibration Curve. The oligosaccharides analyzed in this study showed different ionization efficiencies and, hence, MS responses on an equimolar basis. The SLNs had a much higher response than the SLs, with GNL having the lowest response; hence, a wider calibration concentration range was used for GNL (Figure 1). This observation of the difference in response highlights the difficulties of attempting a quantitative comparison of oligosaccharides using only the total ion count or area under peak without external standards.

Method Validation. *Assessment of Matrix Effects.* HPLC-MS methodologies employing external standard calibration techniques can be prone to matrix problems (mainly ion suppression). In this study, we looked for the presence of matrix effects by comparing results obtained using the described method with those obtained using a standard addition technique. The standard addition results (Table 3) were not significantly different from the external calibration standard method results, indicating the absence of any major matrix effects.

Recovery. The recovery experiments were conducted in a commercial skim milk sample. Standards were spiked into the skim milk samples at approximately 50 and 100% of the endogenous oligosaccharide level for 3′SL and 6′SL. The other oligosaccharides (3′SLN, 6′SLN, DSL, and GNL) were spiked at approximately 5 μg/mL. Recovery levels ranging from 90 to 103% (Table 4) were observed, providing confidence in the extraction protocol and also confirming the absence of or minimal interference from the sample matrix effects.

Limit of Detection (LOD). The LOD was determined by loading decreasing amounts of the standards onto the HPLC-MS, with the LOD being defined as the amount of oligosaccharide required to give a signal that was approximately 3−4 times the noise level. The LODs (ranging from 13 to 125 pg) for the oligosaccharides are listed in Table 5. The LODs reported in this study are comparable to those obtained with the CE method (32−43 pg) of Shen et al.[24] for human oligosaccharides, but much lower than those reported for the reversed-phase HPLC-UV

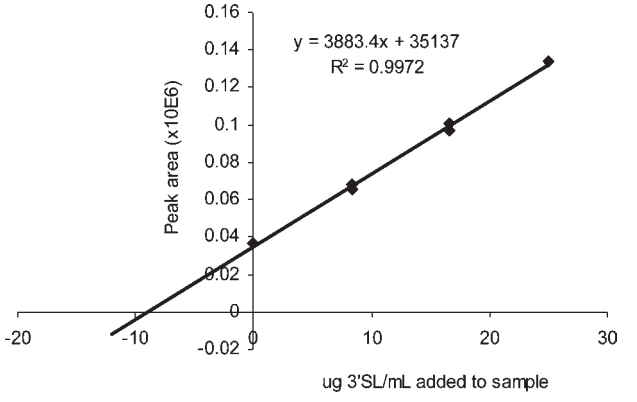

Figure 3. Typical standard addition experiment for 3′SL. A standard solution of 3′SL was spiked into the sample at 8.3, 16.7, and 25 μg/mL.

Table 3. Oligosaccharides Measured in Bovine Skim Milk Using the Current HPLC-HRSRM External Standard Method Compared with Standard Addition Method, with Number of Samples and Coefficient of Variation in Parentheses

	3′SL (μg/mL)	6′SL (μg/mL)	6′SLN (μg/mL)	DSL (μg/mL)	GNL (μg/mL)
skim milk 1 (from Table1)	50.9 ± 4.5 (30, 9%)	6.3 ± 0.4 (8, 6%)	0.13 ± 0.02 (2, 14%)[a]	1.5 ± 0.1 (2, 6%)	2.6 ± 0.3 (2, 10%)
standard addition (n = 2)	53 ± 2.8 (2, 5%)	6.2 ± 0.4 (2, 6%)	0.19 ± 0.01(2, 6%)[a]	1.7 ± 0.1 (2, 7%)	2.9 ± 0.3 (2, 9%)

[a] Measured values at or above the LOD but less than the LOQ. The LOD and LOQ values for the respective oligosaccharides are provided in Table 5.

Table 4. Recoveries of Bovine Milk Oligosaccharides Spiked into Skim Milk, with Number of Samples in Parentheses

spike level[a]	3′SL (%)	6′SL (%)	3′SLN (%)	6′SLN (%)	DSL (%)	GNL (%)
1	98 ± 9 (4)	97 ± 6 (4)				
2	100 ± 6 (4)	90 ± 4 (4)	97 ± 2 (2)	96 ± 6 (2)	94 ± 2 (2)	103 ± 4 (2)

[a] For 3′SL and 6′SL, spike level 1 was 50% endogenous level (see Table 1) and spike level 2 was 100% of endogenous level (see Table 1). All other oligosaccharides were spiked at 5 μg/mL.

Table 5. LOD and LOQ of the HPLC-HRSRM-MS Method for Bovine Milk Oligosaccharides

	amount (pg) of oligosaccharide loaded on column (and as μg/mL milk)					
	3′SL	6′SL	3′SLN	6′SLN	DSL	GNL
LOD	25 (0.2)	25 (0.2)	13 (0.1)	13 (0.1)	50 (0.48)	125 (1.2)
LOQ	75 (0.6)	75 (0.6)	39 (0.3)	39 (0.3)	150 (1.44)	375 (4.8)

method (low nanogram level).[48] The LOD (pg oligosaccharide loaded on column) was also calculated back to product equivalent (μg oligosaccharide/mL sample) in Table 5. The limit of quantification (LOQ) was estimated as 3 times the LOD.

To measure some of the lower level oligosaccharides such as 6′SL, 6′SLN, DSL, and NGL, we had to either increase the amount of sample loaded into the HPLC-MS system or reduce the sample dilution factor.

Assay Variation. The repeatability coefficient of variation determined from duplicate measurements across different days ranged from 2 to 10% for the two dominant oligosaccharides 3′SL and 6′SL (Table 1). Much larger coefficients of variation values (1−17%) were observed for the lower concentration oligosaccharides (Table 1) as they were present at levels close to their respective LOQs.

Oligosaccharides Levels in Bovine Milk. In this study, 3′SL was found to be the most abundant oligosaccharide in bovine milk (Table 1), which is consistent with literature reports.[7−9,29] However, the published concentrations of 3′SL vary from 94−119 μg/mL[9] to about 30−50 μg/mL[7,8] (Table 1). The levels measured in this study for mature milk were in the range of 47−55 μg/mL, which is consistent with the lower levels reported by the latter researchers.[7,8]

The level of 6′SL reported in our study ranged from 3.6 μg/mL in raw milk to 9.6 μg/mL in homogenized milk. It is possible that seasonal variation may have accounted for some of this difference. A seasonal range for 6′SL of 14−25 μg/mL was reported by McJarrow and van Amelsfort-Schoonbeek.[8] Although our finding that 6′SL is the second most abundant oligosaccharide is consistent with Nakamura et al.[7] and McJarrow and van Amelsfort-Schoonbeek,[8] the actual concentrations that we found were much lower than those reported by these authors (25 and 14−25 μg/mL, respectively) and those reported by Martin-Sosa et al. (67−88 μg/mL).[9] However, Martin-Sosa et al.[9] reported that 6′SLN was the second most abundant bovine milk oligosaccharide (145−176 μg/mL), compared with levels of 12 and 9−12 μg/mL reported by Nakamura et al.[7] and McJarrow and van Amelsfort-Schoonbeek,[8] respectively. Our HPLC-MS method measured significantly less 6′SLN in bovine milk (from <LOD in unpasteurized milk to 0.13 μg/mL in skim milk, Table 1).

In this study the measured levels of DSL ranged from 0.54 to 3.1 μg/mL, whereas the measured levels of GNL ranged from <LOD to 3.4 μg/mL. These ranges were reasonably similar to those reported previously (2−7 and 3−4 μg/mL for DSL and GNL, respectively) by McJarrow and van Amelsfort-Schoonbeek.[8] However, the levels of DSL in our study and those reported by McJarrow and van Amelsfort-Schoonbeek[8] were significantly less than the 41−77 μg/mL reported by Martin-Sosa et al.[9]

We did not detect any 3′SLN in any of the dairy-based samples measured in this study, in contrast to Martin-Sosa et al.,[9] who reported levels up to 25 μg/mg in mature milk using UV detection. Given the results from this study and that of Tao et al.,[38] this peak identification must be considered to be in doubt because of the greater specificity of MS detection over UV detection. The mass-based detection system used in our study provides highly specific detection of individual oligosaccharide species in a background of coeluting or overlapping peaks, irrespective of the separation technique. Such specificity of detection is not provided by methods that use either UV or amperometric detection. Indeed, we have previously been unable to consistently quantify *N*-acetyllactosamine in bovine milk and bovine colostrum by high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection due to interference caused by partial coelution of *N*-acetyllactosamine with lactose, which is usually present at high levels in these samples.[8]

Given the wide range of human milk oligosaccharide standards available, this MS-based methodology could be applied to human milk. This would provide more accurate quantification of human milk oligosaccharides compared with the historical data currently available, which are based on levels measured following purification of the individual oligosaccharides. The inevitable losses resulting from various purification strategies are a source of error that cannot be fully corrected for by the use of internal standards.

The range of oligosaccharides quantifiable by this MS-based methodology is limited to those for which external standards are available due to the significant response factor differences obtained with different oligosaccharide species (Figure 1).

Oligosaccharide Levels in Bovine Colostrum. We analyzed two colostrum samples (second and fourth milkings from a Friesian cow). The second milking sample had almost twice the 3′SL level compared with the fourth milking sample. This observation is in line with the lactation trend reported by Nakamura et al.,[7] with a high 3′SL concentration immediately after parturition (850 μg/mL) and dropping rapidly to 98 μg/mL by 3 days postpartum. Similar decreases have also been reported for 6′SL and 6′SLN,[7] although the decrease we measured in our fourth milking sample was less prominent. We also observed more 6′SLN than 6′SL in colostrum, which was the opposite of our findings in mature milk. This trend appears to be consistent with the findings reported by Nakamura et al.[7] and McJarrow and van Amelsfort-Schoonbeek.[8] The level of GNL was approximately 1 μg/mL, which was a third lower than the levels measured in mature milk (Table 1). This differs from the data reported in our previous study,[8] which showed much higher levels of GNL in colostrum. This may have been due

to the presence of coeluting peaks, which could have led to an overestimation of the GNL level previously.[8] Indeed, Tao et al.[38] suggested an increase in neutral oligosaccharides in mature milk compared with colostrum; however, as their data were not quantitative, this needs to be investigated further. It would be possible to quantify other neutral milk oligosaccharides using the current method provided the standards are available.

Oligosaccharides in Infant Formulas. We were able to extend the scope of the current HPLC-MS method to include oligosaccharide measurement in two commercial whey-protein-dominant infant formulas. In both infant formula samples, the oligosaccharide distribution was similar to that in mature milk, but at levels 30−50% lower than those measured in mature bovine milk. These lower levels compared with bovine milk are likely to result from the manner in which infant formulas are manufactured from their ingredients. Given that formulations and individual ingredient processing can vary significantly, it can be expected that the variation in oligosaccharides of different infant formula manufacturers will be high but that the oligosaccharide profile will resemble that of the bovine milk from which it is derived. Thermal losses, particularly of sialyl oligosaccharides, are also expected to contribute to variations in the oligosaccharide concentrations in infant formulas.

In summary, the HILIC HPLC-HRSRM-MS method reported in this study allows the absolute quantification of five different bovine milk oligosaccharides simultaneously. The oligosaccharide profiles are similar to those in published papers, except for the absolute concentrations. This could be due, in part, to different extraction protocols and cleanup, or the fractionation steps, all of which can result in loss of oligosaccharides. Other natural variables to be considered when comparing published results include breed, feed, seasonal variations, and the small number of cows sampled in most studies. It is also likely that some differences are in part due to the nonspecific nature of the detection methods used, for which the identification of oligosaccharides is based purely on retention time, where it is difficult to rule out the presence of coeluting compounds. These variabilities in individual oligosaccharide concentrations reported suggest there is still further work to understand the influences on bovine milk oligosaccharide concentrations and the effect of processing.

In contrast, the current HILIC HPLC-HRSRM-MS method is able to resolve the individual isomeric oligosaccharide species even in the presence of coeluting compounds, and because sample extracts are analyzed without further sample cleanup/fractionation or derivatization, oligosaccharide losses are minimized.

In addition to validating the application of HILIC-HPLC-HRSRM MS methodology to bovine milk for some oligosaccharides, we have demonstrated extension of the method to infant formulas. Other oligosaccharide species could also be quantified by this methodology provided that suitable standards are available.

AUTHOR INFORMATION

Corresponding Author

*Phone: +64-6-350-4649. Fax: +64-6-356-1476. E-mail: bertram.fong@fonterra.com.

ACKNOWLEDGMENT

We thank Carmen Norris for mass spectrometry discussions and Julian Reid and Claire Woodhall for editing the manuscript.

REFERENCES

(1) 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.

(2) Kunz, C.; Rudloff, S. Health benefits of milk-derived carbohydrates. *Bull. Int. Dairy Fed.* **2002**, *375*, 72–79.

(3) Urashima, T.; Kitaoka, M.; Asakuma, S.; Messer, M. Milk oligosaccharides. In *Advanced Dairy Chemistry: Lactose, Water, Salts and Minor Constituents*, 3rd ed.; McSweeney, P. L. H., Fox, P. F., Eds.; Springer Science: New York, 2009; Vol. 3, pp 295−349.

(4) Ninonuevo, M. R.; Park, Y.; Yin, H. F.; Zhang, J. H.; 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.

(5) Urashima, T.; Kitaoka, M.; Terabayashi, T.; Fukuda, K.; Ohnishi, M.; Kobata, A. Milk oligosaccharides. In *Oligosaccharides: Sources, Properties and Application*; Gordon, N. S., Ed.; Nova Science Publishers: New York, 2011; pp 1−58.

(6) Tao, N.; Depeters, E. J.; Freeman, S.; German, J. B.; Grimm, R.; Lebrilla, C. B. Bovine milk glycome. *J. Dairy Sci.* **2008**, *91*, 3768–3778.

(7) Nakamura, T.; Kawase, H.; Kimura, K.; Watanabe, Y.; Ohtani, M.; Arai, I.; Urashima, T. Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation. *J. Dairy Sci.* **2003**, *86*, 1315–1320.

(8) McJarrow, P.; van Amelsfort-Schoonbeek, J. Bovine sialyl oligosaccharides: seasonal variations in their concentrations in milk, and a comparison of the colostrums of Jersey and Friesian cows. *Int. Dairy J.* **2004**, *14*, 571–579.

(9) Martin-Sosa, S.; Martin, M. J.; Garcia-Pardo, L. A.; Hueso, P. Sialyloligosaccharides in human and bovine milk and in infant formulas: variations with the progression of lactation. *J. Dairy Sci.* **2003**, *86*, 52–59.

(10) Kunz, C.; Rudloff, S. Health promoting aspects of milk oligosaccharides. *Int. Dairy J.* **2006**, *16*, 1341–1346.

(11) Casado, B.; Affolter, M.; Kussmann, M. OMICS-rooted studies of milk proteins, oligosaccharides and lipids. *J. Proteomics* **2009**, *73*, 196–208.

(12) Mehra, R.; Kelly, P. Milk oligosaccharides: structural and technological aspects. *Int. Dairy J.* **2006**, *16*, 1334–1340.

(13) Gopal, P. K.; Gill, H. S. Oligosaccharides and glycoconjugates in bovine milk and colostrum. *Br. J. Nutr.* **2000**, *84* (Suppl. 1), S69–S74.

(14) Kobata, A. Structures and application of oligosaccharides in human milk. *Proc. Jpn. Acad. Ser. B−Phys. Biol. Sci.* **2010**, *86*, 731–747.

(15) Bode, L. Recent advances on structure, metabolism, and function of human milk oligosaccharides. *J. Nutr.* **2006**, *136*, 2127–2130.

(16) Bao, Y. W.; Newburg, D. S. Capillary electrophoresis of acidic oligosaccharides from human milk. *Electrophoresis* **2008**, *29*, 2508–2515.

(17) Leo, F.; Asakuma, S.; Nakamura, T.; Fukuda, K.; Senda, A.; Urashima, T. Improved determination of milk oligosaccharides using a single derivatization with anthranilic acid and separation by reversed-phase high-performance liquid chromatography. *J. Chromatogr., A* **2009**, *1216*, 1520–1523.

(18) 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*, 365–372.

(19) Vandenplas, Y. Oligosaccharides in infant formula. *Br. J. Nutr.* **2002**, *87* (Suppl. 2), S293–S296.

(20) Boehm, G.; Stahl, B. Oligosaccharides. In *Functional Dairy Products*; Mattila-Sandholm, T., Saarela, M., Eds.; Woodhead Publishing: Cambridge, U.K., 2003; pp 203−243.

(21) Zivkovic, A. M.; Barile, D. Bovine milk as a source of functional oligosaccharides for improving human health. *Adv. Nutr.* **2011**, *2*, 284–289.

(22) Zaia, J. Mass spectrometry of oligosaccharides. *Mass Spectrom. Rev.* **2004**, *23*, 161–227.

(23) Ninonuevo, M. R.; Lebrilla, C. B. Mass spectrometric methods for analysis of oligosaccharides in human milk. *Nutr. Rev.* **2009**, *67*, S216–S226.

(24) Shen, Z. J.; Warren, C. D.; Newburg, D. S. High-performance capillary electrophoresis of sialylated oligosaccharides of human milk. *Anal. Biochem.* **2000**, *279*, 37–45.

(25) Broberg, A. High-performance liquid chromatography/electrospray ionization ion-trap mass spectrometry for analysis of oligosaccharides derivatized by reductive amination and N,N-dimethylation. *Carbohydr. Res.* **2007**, *342*, 1462–1469.

(26) Pfenninger, A.; Chan, S. Y.; Karas, M.; Finke, B.; Stahl, B.; Costello, C. E. Mass spectrometric detection of multiple extended series of neutral highly fucosylated *N*-acetyllactosamine oligosaccharides in human milk. *Int. J. Mass Spectrom.* **2008**, *278*, 129–136.

(27) Saito, T.; Itoh, T.; Adachi, S. Chemical-structure of 3 neutral trisaccharides isolated in free form from bovine colostrum. *Carbohydr. Res.* **1987**, *165*, 43–51.

(28) Coppa, G. V.; Pierani, P.; Zampini, L.; Carloni, I.; Carlucci, A.; Gabrielli, O. Oligosaccharides in human milk during different phases of lactation. *Acta Paediatr. Suppl.* **1999**, *430*, 89–94.

(29) Veh, R. W.; Michalski, J. C.; Corfield, A. P.; Sanderwewer, M.; Gies, D.; Schauer, R. New chromatographic system for the rapid analysis and preparation of colostrum sialyloligosaccharides. *J. Chromatogr.* **1981**, *212*, 313–322.

(30) Bull, S. M.; Miller, J. C. B. Variation in oligosaccharide composition of human milk: temporal and individual variations. *Proc. Nutr. Soc. Aust.* **1992**, *17*, 63.

(31) Neeser, J. R.; Golliard, M.; Delvedovo, S. Quantitative-determination of complex carbohydrates in bovine-milk and in milk-based infant formulas. *J. Dairy Sci.* **1991**, *74*, 2860–2871.

(32) Martin, M. J.; Martin-Sosa, S.; Garcia-Pardo, L. A.; Hueso, P. Distribution of bovine milk sialoglycoconjugates during lactation. *J. Dairy Sci.* **2001**, *84*, 995–1000.

(33) Carlson, S. E. *N*-Acetylneuraminic acid concentrations in human-milk oligosaccharides and glycoproteins during lactation *Am. J. Clin. Nutr.* **1985**, *41*, 720–726.

(34) Newburg, D. S.; Shen, Z. J.; Warren, C. D. Quantitative analysis of human milk oligosaccharides by capillary electrophoresis. *Adv. Exp. Med. Biol.* **2000**, *478*, 381–382.

(35) Barile, D.; Marotta, M.; Chu, C.; Mehra, R.; Grimm, R.; Lebrilla, C. B.; German, J. B. Neutral and acidic oligosaccharides in Holstein-Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry. *J. Dairy Sci.* **2010**, *93*, 3940–3949.

(36) Bao, Y. W.; Zhu, L. B.; Newburg, D. S. Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. *Anal. Biochem.* **2007**, *370*, 206–214.

(37) Kobata, A.; Yamashita, K.; Tachibana, Y. Oligosaccharides from human milk. *Methods Enzymol.* **1978**, *50*, 216–220.

(38) Tao, N.; DePeters, E. J.; German, J. B.; Grimm, R.; Lebrilla, C. B. Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. *J. Dairy Sci.* **2009**, *92*, 2991–3001.

(39) Tao, N.; Ochonicky, K. L.; German, J. B.; Donovan, S. M.; Lebrilla, C. B. Structural determination and daily variations of porcine milk oligosaccharides. *J. Agric. Food Chem.* **2010**, *58*, 4653–4659.

(40) Sumiyoshi, W.; Urashima, T.; Nakamura, T.; Arai, I.; Nagasawa, T.; Saito, T.; Tsumura, N.; Bing, W.; Brand-Miller, J.; Watanabe, Y.; Kimura, K. Sialyl oligosaccharides in the milk of Japanese women: changes in concentration during the course of lactation. *J. Appl. Glycosci.* **2003**, *50*, 461–467.

(41) Sumiyoshi, W.; Urashima, T.; Nakamura, T.; Arai, I.; Saito, T.; Tsumura, N.; Wang, B.; Brand-Miller, J.; Watanabe, Y.; Kimura, K. Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. *Br. J. Nutr.* **2003**, *89*, 61–69.

(42) Kunz, C.; Rudloff, S.; Hintelmann, A.; Pohlentz, G.; Egge, H. High-pH anion-exchange chromatography with pulsed amperometric detection and molar response factors of human milk oligosaccharides. *J. Chromatogr., B: Biomed. Sci. Appl.* **1996**, *685*, 211–221.

(43) Ninonuevo, M.; An, H. J.; Yin, H. F.; Killeen, K.; Grimm, R.; Ward, R.; German, B.; Lebrilla, C. Nanoliquid chromatography-mass spectrometry of oligosaccharides employing graphitized carbon chromatography on microchip with a high-accuracy mass analyzer. *Electrophoresis* **2005**, *26*, 3641–3649.

(44) Ruhaak, L. R.; Deelder, A. M.; Wuhrer, M. Oligosaccharide analysis by graphitized carbon liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.* **2009**, *394*, 163–174.

(45) Melmer, M.; Stangler, T.; Premstaller, A.; Lindner, W. Effects of the redox state of porous graphitic carbon on the retention of oligosaccharides. *J. Chromatogr., A* **2010**, *1217* (39), 6097–6101.

(46) Pabst, M.; Altmann, F. Influence of electrosorption, solvent, temperature, and ion polarity on the performance of LC-ESI-MS using graphitic carbon for acidic oligosaccharides. *Anal. Chem.* **2008**, *80*, 7534–7542.

(47) Wuhrer, M.; de Boer, A. R.; Deelder, A. M. Structural glycomics using hydrophilic interaction chromatography (HILIC) with mass spectrometry. *Mass Spectrom. Rev.* **2009**, *28*, 192–206.

(48) Chaturvedi, P.; Warren, C. D.; RuizPalacios, G. M.; Pickering, L. K.; Newburg, D. S. Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. *Anal. Biochem.* **1997**, *251*, 89–97.

(49) Walstra, P.; Geurts, T. J.; Noomen, A.; Jellema, A.; van Boekel, M. A. J. S. Milk components. In *Dairy Technology: Principles of Milk Properties and Processes*; CRC Press: Boca Raton, FL, 1999; pp 57.

(50) Wronski, M.; Sosnowska, W. Relationships between the density and composition of colostrum and the performance of calves. *Pol. J. Nat. Sci.* **2008**, *23*, 573–582.

(51) Domon, B.; Costello, C. E. A systematic nomenclature for carbohydrate fragmentations in FAB-MS MS spectra of glycoconjuates. *Glycoconjugate J.* **1988**, *5* (4), 397–409.