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Quantitative Analysis of Gangliosides in Bovine Milk and Colostrum-Based Dairy Products by Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry

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Supporting Information

ABSTRACT: Milk gangliosides have gained considerable attention because they participate in diverse biological processes, including neural development, pathogen binding, and activation of the immune system. Herein, we present a quantitative measurement of the gangliosides present in bovine milk and other dairy products and byproducts. Ultrahigh performance liquid chromatography separation was used for high-throughput analysis and achieved a short running time without sacrificing chromatographic resolution. Dynamic multiple reaction monitoring was conducted for 12 transitions for GM3 and 12 transitions for GD3. Transitions to sialic acid fragments (m/z 290.1) were chosen for the quantitation. There was a considerable amount of gangliosides in day 2 milk (GM3, 0.98 mg/L; GD3, 15.2 mg/L) which dramatically decreased at day 15 and day 90. GM3 and GD3 were also analyzed in pooled colostrum, colostrum cream, colostrum butter, and colostrum buttermilk. The separation and analytical approaches here proposed could be integrated into the dairy industry processing adding value to side-streams.

KEYWORDS: *gangliosides, dairy products, bovine colostrum, ultrahigh performance liquid chromatography, multiple reaction monitoring*

INTRODUCTION

Gangliosides are anionic glycosphingolipids that consist of a carbohydrate moiety and a ceramide lipid portion. The carbohydrate moiety is composed of monosaccharides that include sialic acids, e.g., *N*-acetylneuraminic acid (Neu5Ac), glucose, galactose, and other monosaccharides. The possible combinations of monosaccharides give rise to countless types of glycans, varying in composition, structure, and linkages. The ceramide is formed by attachment of a fatty acid by an amide linkage to the long-chain amino alcohol sphingoid base.[1]

Gangliosides are naturally found in biological tissues and fluids, including mammalian milks.[2] In milk, gangliosides are found exclusively in the milk fat globule membrane. The ganglioside composition of bovine milk is dominated by the disialoganglioside GD3 (Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcβCer) and the monosialoganglioside GM3 (Neu5Acα2-3Galβ1-4GlcβCer).[3,4] These two species are the most abundant, accounting for more than 80% of the total ganglioside content in milk.[5,6]

Gangliosides are also a distinct class of biomolecules present on the surface of cells. They are major lipid components in the apical membrane of the epithelial cells of the intestinal and urinary tracts.[7] The gangliosides come in contact with nutrients, pathogens, and other elements of the diet. Milk glycans have recently gained increasing attention as a major class of anti-infective agents.[8] Among them, gangliosides are regarded as pathogen decoys that compete for pathogen binding sites, thereby blocking pathogens from binding to human cell receptors in the intestinal mucosa.[9] Milk gangliosides are involved in the inhibition of enterotoxins.[10] Milk gangliosides appears to modify the intestinal ecology of newborns, stimulating growth of *Bifidobacterium* species and lowering the content of *Escherichia coli*.[11] Dietary gangliosides have also been related to the development of the intestinal immune system.[12,13] Although the potential use of gangliosides for improving health is extensive and highly promising, the lack of technologies for their synthesis has limited their practical application.

Traditional methods of ganglioside enrichment and analysis require several extraction and preparation steps and a combination of analytical methods. Several techniques have been used for purification, including solvent partition, column chromatography, and solid-phase extraction.[14−16] Extraction often requires defined chloroform−methanol−water mixtures.[17,18] Due to the amphiphilic nature of gangliosides, thin-layer chromatography is often used for separation and quantitative determination. The gangliosides are then visualized through the use of either resorcinol-HCl reagent or immunostaining.[19]

More recently, mass spectrometry (MS) has been used for the mass profiling and structural analysis of milk gangliosides.[3,20] Mass spectrometry can provide simultaneously qualitative information on both the glycan and the ceramide lipid portions of the heterogeneous gangliosides. With the qualitative identification of the gangliosides in hand,

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quantitative methods must be developed to measure changes in gangliosides under various physiological and processing conditions. Moreover, because bovine milk gangliosides are consumed in a staple food, it is also necessary to quantitate more accurately the exact amounts in dairy products and side streams that could become a valuable extraction source. Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis with a triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode has rapidly become an effective technique for highly specific and accurate quantitation of many complex bioactive molecules. Recent advances in liquid chromatography, such as ultrahigh performance liquid chromatography (UHPLC), expedite the use of these MS-based approaches, especially when the time-to-result must be short for quality control purposes. This approach may further provide the sensitive and selective determination of individual compounds in complicated mixtures, making it applicable for low-abundant species with complicated chemical structures, such as gangliosides. However, the LC-MS/MS method was not applied to the measurement of gangliosides in dairy products until Sørensen recently demonstrated its feasibility be measuring gangliosides GM3 and GD3 in bovine milk and infant formulas.[21] More recently, Zhang et al. reported a high throughput UHPLC-MS/MS quantification method using bovine brain GM1 as an internal standard.[22]

In this study, we report a method for quantitation of gangliosides GM3 and GD3 in bovine milk and other dairy products and side-stream, using MRM UHPLC MS/MS. This is the first application of advanced UHPLC coupled with triple quadrupole mass spectrometry for the quantitation of gangliosides with standard addition method. The quantitative changes undergone by gangliosides were observed over the different lactation periods. In addition, this measurement aids the identification of the processing streams that are richer in bioactive gangliosides, enabling the dairy industry to strategically capture a previously unavailable bovine milk component that could be rapidly adapted into novel functional foods and therapies.

MATERIALS AND METHODS

Materials and Chemicals. HPLC grade methanol and isopropyl alcohol were purchased from Sigma (St. Louis, MO), and certified ACS grade chloroform was from Fisher (Fair Lawn, NJ). Ammonium acetate and acetic acid were of analytical reagent grade from Merck (Darmstadt, Germany). Gangliosides GM3 and GD3 from bovine buttermilk were purchased from Matreya (Pleasant Gap, PA). Stock solutions of GM3 and GD3 standards were prepared separately in methanol to obtain a concentration of 1 mg/mL. Bovine milk samples were collected from one Holstein cow at the UC Davis dairy farm (day 2, 15, and 90 of lactation). Bovine colostrum and colostrum products were kindly provided by Sterling Technology Co. (Brookings, SD). All samples were kept frozen at −80 °C.

Sample Preparation. Gangliosides were separated from neutral lipids in all samples by two consecutive chloroform and methanol extractions described as follows. Two mL of liquid milk sample was mixed with methanol/chloroform/water (8 mL:4 mL:1 mL). A 0.2-g sample of cream and butter and a 0.02-g sample of buttermilk were also dissolved in the solvent solutions. The solutions were sonicated for 5 min (Branson Ultrasonic Co., Danbury, CT) and centrifuged at 8800*g* for 5 min in an Effendorf centrifuge (Hamburg, Germany). Subsequently, 2 mL of water was added for phase separation, and the aqueous upper phase was collected. A methanol, chloroform, and water (6 mL:3 mL:2 mL) mixture was added to the bottom layer for a second extraction, and the mixture was shaken and centrifuged. The supernatant was collected and pooled with that from the first extraction. The combined solution was dried by a SpeedVac rotor concentrator (Savant Instruments, Inc., Holbrook, NY), and the lyophilized sample was resuspended in 1 mL of methanol−water solution (1:1, v/v). The gangliosides were enriched with C8 solid-phase extraction (SPE) cartridges (3 mL, Supelco, Bellefonte, PA) with slight modifications of previously published methods.[16,21] The SPE cartridge was conditioned with 5 mL of a methanol−water (1:1, v/v) solution, and the gangliosides were eluted with 10 mL of isopropyl alcohol−methanol (1:1, v/v). The addition of isopropyl alcohol increased the recovery of ganglioside GM3, which is more hydrophobic than other ganglioside species. The eluant was divided into five aliquots. For the quantification, ganglioside GM3 and GD3 standards were added in five concentration levels (0.00, 0.05, 0.10, 0.15, and 0.20 mg/mL for GM3; 0.00, 0.50, 1.00, 1.50, and 2.00 mg/mL for GD3). The samples were dried by a SpeedVac rotor concentrator and the residues dissolved in 50 μL of the LC starting solvent. One microliter of the sample was injected for MRM analysis.

For recovery studies, the samples were spiked with the GM3 and GD3 standards before the extraction procedure. A representative portion of sample was collected and fortified with appropriate volume of working standard solutions to reach 1 mg/L GM3 and 10 mg/L GD3 in the spiked sample. Next, the extractions from the spiked sample were prepared following the procedure described above.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS). Reversed-phase analysis was performed on an Agilent 1290 Infinity LC (Agilent, Santa Clara, CA). An Agilent Eclipse Plus C18 Rapid Resolution High Definition (RRHD) analytical column (i.d. 2.1 × 100 mm, 1.8 μm, 80 Å) was used for the UHPLC separation. The column was maintained at 50 °C and eluted using a gradient of 80−90% of solvent B from 0−0.2 min, then 90−100% over 2 min; 100% of B was maintained for 1 min, and the column was re-equilibrated during a 1-min post run. Solvent A consisted of water and solvent B was 15% isopropyl alcohol in methanol (v/v). Both solvents contained 20 mM ammonium acetate and 0.1% acetic acid. The flow rate was 600 μL/min. Analytes were detected and quantified using an Agilent 6490 triple quadrupole mass spectrometer operating in negative electrospray ionization mode. The Agilent Jet Stream electrospray ionization source was set for high sensitivity. The drying gas temperature and flow were 250 °C and 12 L/min, respectively. Sheath gas temperature and flow were 300 °C and 12 L/min, respectively. The nebulizer gas pressure was set at 35 psi. The capillary voltage was set at 4000 V. The collision cell accelerator voltage was set to 5 V, and the collision energy was optimized on a compound-dependent basis. Nitrogen was used as the collision gas. Resolution of the Q1 and Q3 quadrupoles was set at unit resolution. Agilent Data Acquisition software was used for method development and data acquisition. Agilent MassHunter Qualitative Analysis and Quantitative Analysis Software (v.B.03.01) were used for data processing.

Quantitation. Gangliosides were quantified by a standard addition method. Volumes of 0, 2, 4, 6, and 8 μL of a standard solution containing 10 μg/mL GM3 and 100 μg/mL GD3 were added to each 400 μL extract. Ganglioside concentrations were calculated using the signal intensities of the transitions to the m/z 290.1 listed in Table 1.

Peak areas of each GM3 and GD3 in the samples were summed, and the sums of the areas were used to draw regression lines to estimate the amount of gangliosides in the sample. For each ganglioside species, a plot of the spiked concentration (x-axis) against the peak area (y-axis) was generated with five concentrations: For GD3, 0 mg/mL, sum of areas from blank samples; 0.5 mg/mL, sum of areas from the samples with the 2 μL standard added; 1.0 mg/mL, sum of areas from the samples with the 4 μL standard added; 1.5 mg/mL, sum of areas from the samples with the 6 μL standard added; and 2.0 mg/mL, sum of areas from the samples with the 8 μL standard added. The concentrations of gangliosides in the sample extract were determined by the x-intercept of the regression line. The concentration in the original sample was calculated by dividing by dilution factor.

Recoveries were assessed by spiking a known amount of gangliosides (1 mg/L GM3 and 10 mg/L GD3) into the samples (2

Table 1. dMRM Table for Bovine Milk Gangliosides[a]

(A)

transition	ganglioside	collision energy (V)	retention time (min)
1123.7 → 290.1	GM3(d32:1)	50	1.53
1137.7 → 290.1	GM3(d33:1)	50	1.64
1151.7 → 290.1	GM3(d34:1)	51	1.75
1165.7 → 290.1	GM3(d35:1)	52	1.85
1179.7 → 290.1	GM3(d36:1)	53	1.96
1193.7 → 290.1	GM3(d37:1)	54	2.07
1207.7 → 290.1	GM3(d38:1)	54	2.25
1221.7 → 290.1	GM3(d39:1)	55	2.35
1235.7 → 290.1	GM3(d40:1)	56	2.38
1249.7 → 290.1	GM3(d41:1)	57	2.48
1263.7 → 290.1	GM3(d42:1)	57	2.63
1277.7 → 290.1	GM3(d43:1)	58	2.71

(B)

transition	ganglioside	collision energy (V)	retention time (min)
706.9 → 290.1	GD3(d32:1)	28	1.42
713.9 → 290.1	GD3(d33:1)	28	1.53
720.9 → 290.1	GD3(d34:1)	29	1.62
727.9 → 290.1	GD3(d35:1)	30	1.73
734.9 → 290.1	GD3(d36:1)	31	1.85
741.9 → 290.1	GD3(d37:1)	31	1.94
748.9 → 290.1	GD3(d38:1)	32	2.06
755.9 → 290.1	GD3(d39:1)	33	2.16
762.9 → 290.1	GD3(d40:1)	33	2.26
769.9 → 290.1	GD3(d41:1)	33	2.35
776.9 → 290.1	GD3(d42:1)	34	2.44
783.9 → 290.1	GD3(d43:1)	35	2.52

[a](A) ganglioside GM3 (B) gangliosde GD3. Delta retention was set at 0.3 min. It can be observed that molecular species are assigned as GM3(1123.7 + 14n) → 290.1 and GD3(706.9 + 7n) → 290.1.

mL) prior to sample extraction. The experiment was performed in triplicate. The percent recoveries were determined by subtracting the amount of gangliosides measured in the nonspiked samples from the amount measured in the spiked samples, divided by the spiked amounts.

RESULTS AND DISCUSSION

Fragmentation of Gangliosides GM3 and GD3 in the Negative Mode Triple Quadrupole MS. The electrospray ionization (ESI) was used in negative mode because of the intrinsic anionic characteristics of gangliosides. We previously reported that the tandem MS of gangliosides in the negative mode with a quadrupole time-of-flight instrument yielded intense signals due to sialic acid fragments.[23] The MS/MS spectra acquired by triple quadrupole instrumentation yielded essentially the same fragment ions obtained with the quadrupole time-of-flight. The tandem MS spectra of commercial GM3 and GD3 samples are shown in Figure 1. For GM3, the major product ion corresponded to a sialic acid fragment (Figure 1A), whereas for GD3 there were two major fragments that corresponded to the mono and the disialic acid fragments at m/z 290.1 and 581.2, respectively. For GM3, the best ion product for MRM was m/z 290.1, the monosialic acid fragment. For the GD3, the two options were further examined by determining the behavior of the two fragments over a range of collision energies (20 to 50 V). As shown in S-Figure 1A of the Supporting Information, SI, the ion intensities corresponding to transitions GD3(d43:1), 776.9 → 290.1 were higher than those of GD3(d43:1), 776.9 → 581.2 over the range of collision energies. Similar trends were observed for GD3 with different ceramide components, as shown in S-Figure 1B,C of the SI). On the basis of these results, the transitions to m/z 290.1 were selected for the MRM for both the GM3 and GD3 gangliosides corresponding to the glycan structures depicted in Figure 2.

Optimization of Chromatographic Separation of Gangliosides using Ultrahigh Performance Liquid Chromatography (UHPLC) and Mass Spectrometer Parameters. To assess the performance of the UHPLC separation, mixtures of bovine buttermilk gangliosides GM3 and GD3 obtained as commercial standards were examined. The gradient programs providing optimum separation of gangliosides with varying ceramide moieties were obtained (see Materials and Methods). The gangliosides eluted between 1.4 and 2.7 min with peak widths as narrow as 4.5 to 9 s at the base (Figure 3). The total time between injections was 4 min with a column flow rate of 600 μL/min, illustrating the high duty cycle of the method. The UHPLC separations achieved short running times without apparent sacrifice in the chromatographic separation.

For the MS analysis, dynamic MRM (dMRM) was used, which required obtaining specific retention time ranges for each specific analyte. In triple quadrupole MS analysis, the dwell time and cycle time are optimized to achieve high sensitivity and reliable quantitation. However, when UHPLC separation is used, the peak widths significantly narrow, thus requiring increased duty cycle. Increased duty cycle is accomplished either by reducing the dwell times for the transitions or increasing the cycle time for each MS scan. Reducing dwell times can decrease the sensitivity while maintaining the dwell time, but increasing the overall MS cycle time may mean that insufficient data points are collected during the elution of very narrow LC peaks to allow reliable quantitation. These limitations were overcome with the use of dMRM tables using retention times and detection windows (delta RT). This time segmented MRM enabled the instrument to acquire specific MRM data only during specified retention time window minimizing wasted duty cycles.[24] In our investigations, we observed substantial sensitivity enhancement with dMRM for the ganglioside samples (S-Figure 2 of the SI).

Twenty-four MRM transitions corresponding to GM3 and GD3 subspecies for samples under the dMRM mode were monitored. Within each dMRM table, the cycle time was maintained at 300 ms to ensure that all analytes in the same time window were sufficiently sampled. This condition corresponded to 15−30 data points across chromatographic peaks. As a result, it was possible to obtain a sufficient number of points across the narrow chromatographic peaks to obtain good ion statistics. Sensitivity down to the nano gram range and more than 10^3 dynamic range were obtained through targeted analysis using UHPLC-MS/MS in the dMRM mode (S-Figure 3 of the SI).

Construction of dMRM Transition Table. The MRM mode was used initially to monitor the elution of gangliosides from the UHPLC column, thus providing a measure of their abundances for suitability to quantitation. In this mode, specific precursor-product ion pairs were monitored with conditions optimized for each transition. The MRM mode required the construction of detailed tables with parameters for each compound transition. Through the examination of the MS/MS spectra and comparison of the MRM peak intensities for

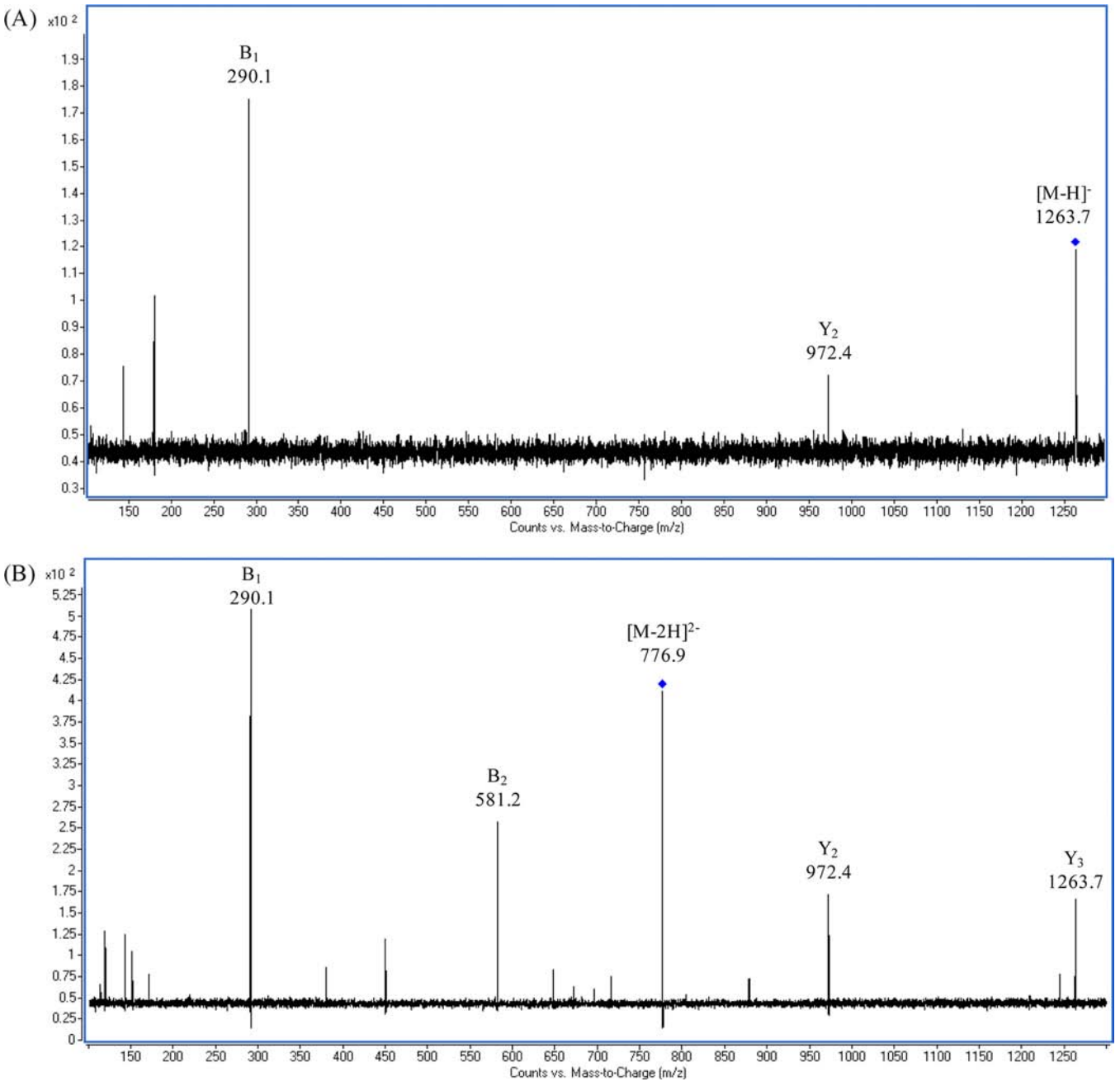

Figure 1. Representative product ion spectra of gangliosides GM3 and GD3. (A) MS/MS spectrum of GM3(d42:1) at m/z 1263.7, collision energy = 50 V. (B) MS/MS spectrum of GD3(d42:1) at m/z 776.9. collision energy = 30 V. Gangliosides (50 ng) were injected. MRM transitions chosen for quantitation were as follows: GM3(d42:1), 1263.7 → 290.1; and GD3(d42:1), 776.9 → 290.1. Fragmentations are assigned according to the nomenclature for carbohydrate fragmentation by Domon and Costello.[31]

different collision energies, initial experiments were conducted to find the optimal collision energy conditions for each ceramide moiety in the ganglioside standards.

The ceramide structures of the commercial standards and bovine milk ganglioside extracts were similar. Therefore, the commercial ganglioside standards were used for the preliminary experiment. Transitions were selected initially based on the preliminary data from bovine buttermilk ganglioside standards. The list of gangliosides previously obtained through high resolution Fourier transform ion cyclotron resonance mass spectrometry analysis showed that the major gangliosides in bovine milk are GM3 and GD3, which are composed of heterogeneous ceramide portions.[3]

The MRM is usually used for the analysis of compounds with known precursor-product ion pairs; however, in a survey, it was also used to scan for pairs of many theoretically expected m/z values. Once the appropriate gangliosides of interest were selected, an MRM assay was set up to perform mass screening on a bovine milk ganglioside extract so that the presence of these gangliosides could be observed and the appropriate signal intensities (signal-to-noise ratio >3) obtained. The observed gangliosides were included in the final dMRM table (Table 1). Twelve transitions for each ganglioside species were monitored, and optimum collision energies were chosen. It is worth noting that as the alkyl chain length increases, fragmentation of the compound becomes increasingly difficult, hence there may be

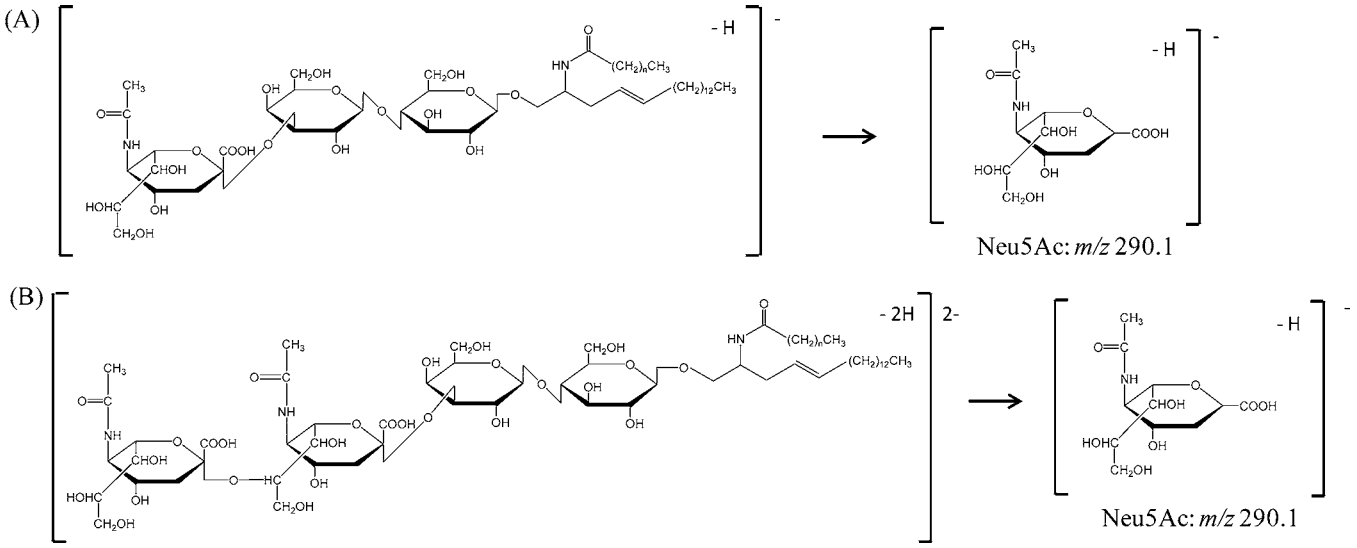

Figure 2. Structures of bovine milk ganglioside GM3 and GD3. The precursor and product ions used in this study are shown. Other isomeric structures may be plausible. (A) GM3 (B) GD3. n = 14−25 in fatty acid chains.

some response differences between short and long lipid chain species.[25] The characteristic UHPLC retention times of the transition ions were also determined for dMRM analysis. Samples were analyzed in dMRM mode, with 0.3-min retention time windows.

Quantitative Changes of Gangliosides GM3 and GD3 over the Different Lactation Periods. Quantitation of compounds by MS is best performed by comparison of the peak intensities with that of a stable isotope-labeled standard, thereby ensuring ionization of the same compounds under identical experimental conditions. However, such requirements can be achieved only for the quantitation of a limited number of known lipids. The use of an internal standard for each ganglioside subspecies is at the moment not practical. Quantitation by a standard mixture addition with the use of ganglioside reference materials extracted from bovine milk appeared the most feasible approach, because the ganglioside compositions of commercial standards are very close to those of extracted bovine milk samples. Therefore, in this study, the commercial standards were used for quantitation through the standard addition method.

The method elaborated above was used on different samples of bovine milk. The concentrations of gangliosides GM3 and GD3 in bovine milk collected during three different lactation periods were determined in this manner and are presented in Table 2. The mean concentrations for days 2, 15, and 90 from three experimental replicates were GM3, 0.98 mg/L; GD3, 15.2 mg/L; GM3, 0.15 mg/L; GD3; 3.3 mg/L, and GM3, 0.15 mg/L; GD3, 2.4 mg/L, respectively. The efficiency of the extraction procedure was also evaluated, and recovery studies were carried out at concentrations of 1 mg/L GM3 and 10 mg/L GD3. Samples were also used to assess intrasample precision, and the data are presented in Table 2. Recoveries in all three samples were greater than 80%, and relative standard deviations were within 20%. The recovery levels provide confidence in the extraction protocol.

Our observation is in line with the lactation trend reported by Puente et al., with a high ganglioside levels in colostrums (7.5 mg lipid-bound sialic acid/kg) and dropping to 2.3 mg lipid-bound sialic acid/kg in transitional milk.[6] It was reported that bovine milk contained 1.2 mg/L GM3 and 8.8 mg/L GD3, which was measured by traditional high performance thin layer chromatography combined with densitometric measurement after staining with resorcinol reagent.[5] Iwamori et al. showed that pooled bovine milk contains GM3(1.5 mg/L) and GD3(5.5 mg/L). Pan and Izumi reported GM3(0.4 mg/L) and GD3(12.0 mg/L) in cow's milk. The levels of GD3 reported by using previous LC-MS/MS approach were 9.2 mg/L and 13.5 mg/L.[21,26] These reported values are in the range of the results obtained in our study.

Amounts of GM3 and GD3 in Pooled Bovine Colostrum and Dairy Side Streams. Because the early lactation bovine milk resulted to have the greatest amount of gangliosides GM3 and GD3, colostrum and dairy products based on colostrum were chosen as the starting material for further analysis. The mean GM3 and GD3 results obtained in this study on pooled bovine colostrum were 0.50 mg/L and 9.2 mg/L, respectively (Table 2, Figure 4(A)).

Milk polar lipids, including phospholipids and glycosphingolipids, are mainly situated in the milk fat globule membrane. When milk is processed at the dairy, this membrane is disrupted and, as such, is no longer associated with the fat globules. It was reported that during processing, polar lipids are preferentially distributed to aqueous phases such as buttermilk.[27,28] It appears that the churning process also enriches buttermilk in gangliosides GM3 and GD3 being GM3 and GD3 19.2 mg/kg and 43.8 mg/kg, respectively, which are more than twice as high as the amounts found in the colostrum cream (GM3, 8.2 mg/kg; GD3, 21.8 mg/kg) and in the colostrum butter (GM3, 8.0 mg/kg; GD3, 17.0 mg/kg) (Table 2, Figure 4(B−D)). As such, colostrum buttermilk, which is currently a side-stream, could be considered a suitable source for the isolation of milk fat globule membrane gangliosides.

Gangliosides from dairy products ultimately derived from the milk fat globule have industrial applications because of their contribution to dairy and other food products. In this study, a UHPLC-MS/MS method was developed for the quantitation of gangliosides GM3 and GD3 in bovine milk and colostrum-based dairy products. The dMRM method was made quantitative by the use of a standard addition technique with standards purified from bovine milk showing similar ganglioside profiles. This method is an efficient targeted approach due to

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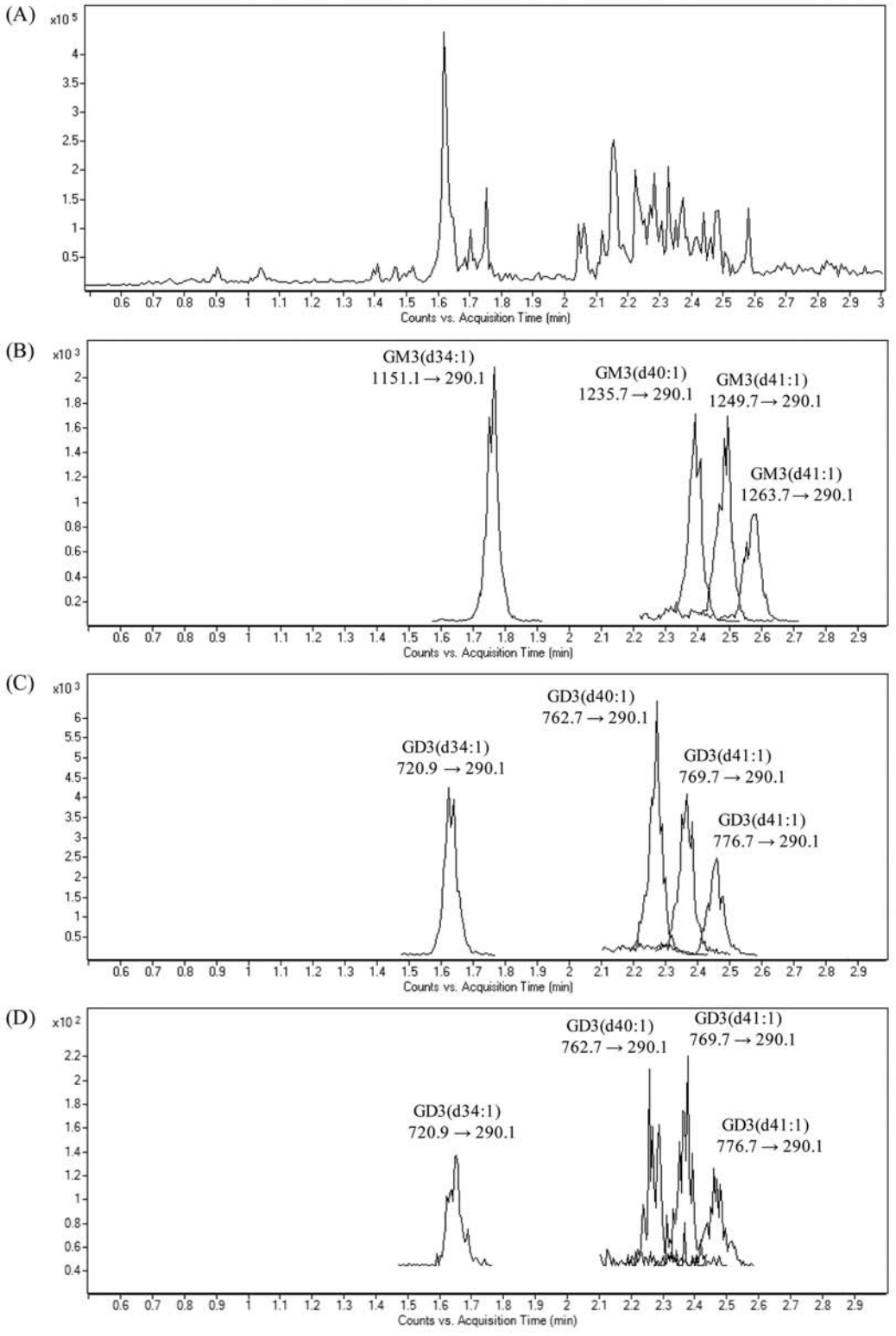

Figure 3. Negative mode full scan and selected MRM chromatograms of gangliosides GM3 and GD3 standard mixture (0.1 μg of each standard) and bovine colostrums GD3. (A) Base peak chromatogram. (B) Overlaid MRM chromatograms of GM3 subspecies: 1151.7 → 290.1; GM3 (d34:1), 1235.7 → 290.1; GM3 (d40:1), 1249.7 → 290.1; GM3 (d41:1), 1263.7 → 290.1; GM3 (d42:1). (C) MRM chromatograms of GD3 subspecies. 720.9 → 290.1; GD3 (d34:1), 762.9 → 290.1; GD3 (d40:1), 769.9 → 290.1; GD3 (d41:1), 776.9 → 290.1; GD3 (d42:1). (D) MRM chromatograms of bovine colostrums GD3 subspecies. Gangliosides with long ceramide chains eluted later than those with short chains.

the instrument's high dynamic range and the selectivity of retention time in conjunction with the information regarding precursor and product ion pairs. These features are especially important for the analysis of gangliosides, which usually exist in trace amounts. This method can be used for profiling the change of gangliosides and determining their physiological function, which was conducted by traditional TLC.[29,30] Moreover, for efficient utilization of bioactive milk fat globule

Table 2. Absolute Amount of GM3 and GD3 Measured by the UHPLC-MS/MS Method on Bovine Milk and Colostrum Dairy Products[a]

sample	GM3 (RSD%)[b]	GD3 (RSD%)
milk, day 2	0.98 mg/L(8.0)	15.2 mg/L (4.8)
recovery	82% (9.1)	93% (6.1)
milk, day 15	0.15 mg/L (12.0)	3.3 mg/L (10.2)
recovery	84% (13.3)	91% (8.0)
milk, day 90	0.15 mg/L (14.2)	2.4 mg/L (10.0)
recovery	89% (14.8)	92% (11.8)
bovine colostrum	0.50 mg/L (12.3)	9.2 mg/L (5.2)
recovery	90% (9.7)	94% (6.8)
colostrum cream	8.2 mg/kg (15.1)	21.8 mg/kg (6.2)
recovery	83% (10.6)	89% (7.1)
colostrum butter	8.0 mg/kg (16.2)	17.0 mg/kg (7.0)
recovery	88% (9.7)	87% (5.8)
colostrum buttermilk	19.2 mg/kg (13.5)	43.8 mg/kg (9.5)
recovery	89% (8.9)	92% (6.2)

[a]The average values of triplicate experiments (n = 3) are presented. For recovery studies, the samples were spiked with 1 mg/L GM3 and 10 mg/L GD3 and subjected to the sample preparation procedure explained in Materials and Methods. [b]Relative standard deviation.

membrane (MFGM) materials, these separation principles and analytical approaches can be integrated into the dairy industry. Data indicate that the gangliosides were abundant in colostrum, and during the dairy process, they were preferentially concentrated in a side stream like buttermilk.

■ ASSOCIATED CONTENT

Supporting Information

Figures of the relative MRM peak abundance of fragment ions, dMRM and MRM transitions of ganglioside GM3 and GD3, and linear dynamic range of ganglioside GD3(d42:1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

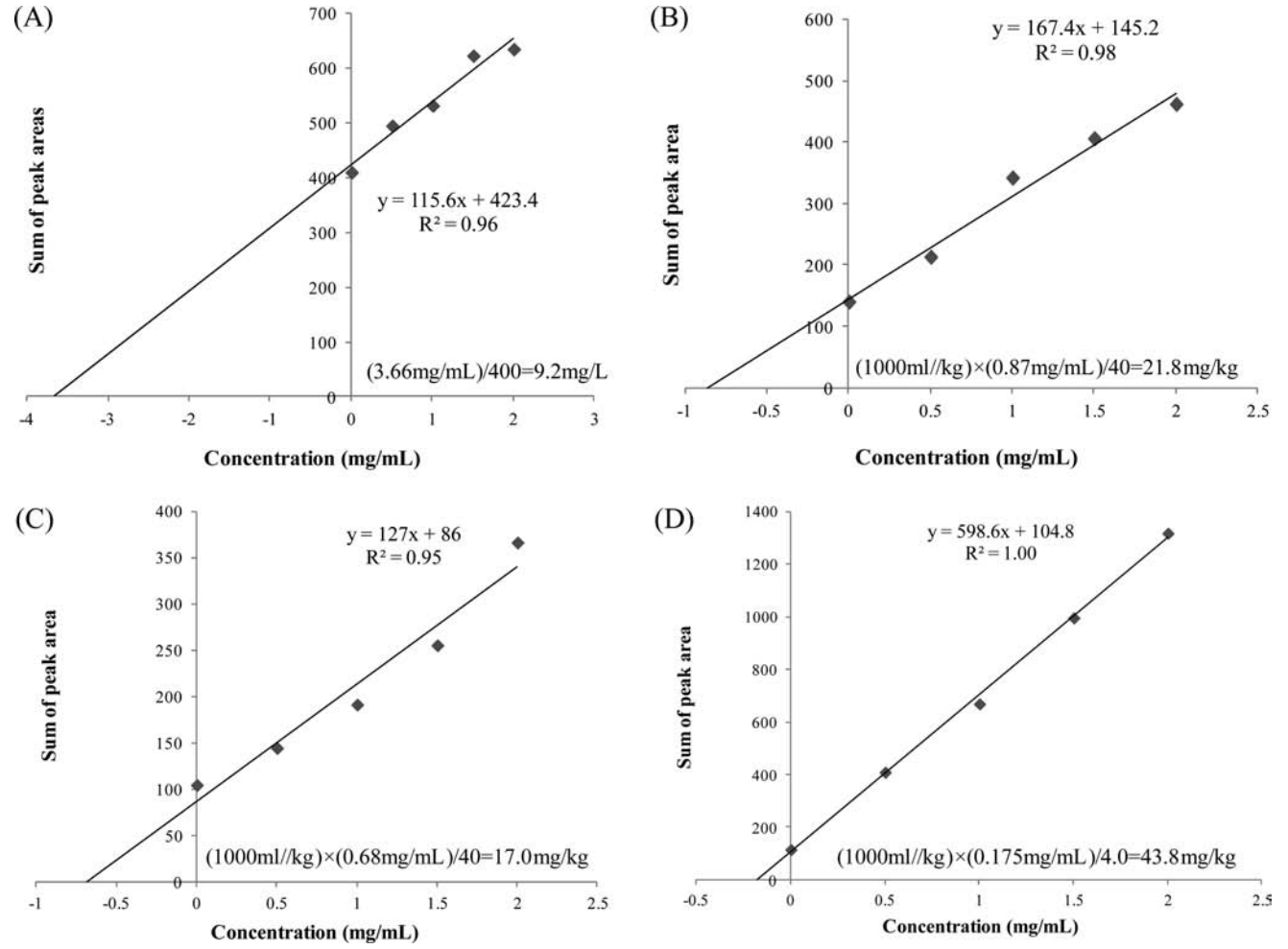

Figure 4. Regression curves of GD3 in the pooled colostrums and colostrums dairy products. (A) pooled colostrum, (B) colostrum cream, (C) colostrum butter, and (D) colostrum buttermilk. For the quantitation, five different levels of GD3 standards (0.0, 0.5, 1.0, 1.5, and 2.0 mg/mL) were added in the samples. Linear equations are inserted in the figures. The concentrations in the samples were estimated according to the equations shown. The concentrations of gangliosides in the sample were determined by the x-intercept of the regression line. The concentration in the original sample was calculated by dividing by dilution factor.

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