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1 **Quantitative Analysis of Gangliosides in Bovine Milk and Colostrum-based**

2 **Dairy Products by Ultra-high Performance Liquid Chromatography-Tandem**

3 **Mass Spectrometry**

4

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

29 **KEYWORDS:** gangliosides, dairy products, bovine colostrum, ultra-high performance liquid
30 chromatography, multiple reaction monitoring

31

32 **INTRODUCTION**

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

39 Gangliosides are naturally found in biological tissues and fluids, including mammalian
40 milks.[2] In milk, gangliosides are found exclusively in the milk fat globule membrane. The
41 ganglioside composition of bovine milk is dominated by the disialoganglioside GD3
42 (Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcβCer) and the monosialoganglioside GM3 (Neu5Acα2-
43 3Galβ1-4GlcβCer).[3, 4] These two species are the most abundant, accounting for more than 80%
44 of the total ganglioside content in milk.[5, 6]

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

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55 gangliosides for improving health is extensive and highly promising, the lack of technologies for
56 their synthesis has limited their practical application.

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

64 More recently, mass spectrometry (MS) has been used for the mass profiling and structural
65 analysis of milk gangliosides.[3, 20] Mass spectrometry can provide simultaneously qualitative
66 information on both the glycan and the ceramide lipid portions of the heterogeneous gangliosides.
67 With the qualitative identification of the gangliosides in hand, quantitative methods must be
68 developed to measure changes in gangliosides under various physiological and processing
69 conditions. Moreover, because bovine milk gangliosides are consumed in a staple food, it is also
70 necessary to quantitate more accurately the exact amounts in dairy products and side streams that
71 could become a valuable extraction source. Liquid chromatography (LC)-tandem mass
72 spectrometry (MS/MS) analysis with a triple quadrupole mass spectrometer in multiple reaction
73 monitoring (MRM) mode has rapidly become an effective technique for highly specific and
74 accurate quantitation of many complex bioactive molecules. Recent advances in liquid
75 chromatography, such as ultra-high performance liquid chromatography (UHPLC), expedite the
76 use of these MS-based approaches, especially when the time-to-result must be short for quality
77 control purposes. This approach may further provide the sensitive and selective determination of

78 individual compounds in complicated mixtures, making it applicable for low-abundant species
79 with complicated chemical structures, such as gangliosides. However, the LC-MS/MS method
80 was not applied to the measurement of gangliosides in dairy products until Sørensen recently
81 demonstrated its feasibility be measuring gangliosides GM3 and GD3 in bovine milk and infant
82 formulas.[21] More recently, Zhang et al. reported a high throughput UHPLC-MS/MS
83 quantification method using bovine brain GM1 as an internal standard.[22]
84 In this study, we report a method for quantitation of gangliosides GM3 and GD3 in bovine
85 milk and other dairy products and side-stream, using MRM UHPLC MS/MS. This is the first
86 application of advanced UHPLC coupled with triple quadrupole mass spectrometry for the
87 quantitation of gangliosides with standard addition method. The quantitative changes undergone
88 by gangliosides were observed over the different lactation periods. In addition, this measurement
89 aids the identification of the processing streams that are richer in bioactive gangliosides enabling
90 the dairy industry to strategically capture a previously unavailable bovine milk component that
91 could be rapidly adapted into novel functional foods and therapies.
92
93 **MATERIALS AND METHODS**
94 **Materials and Chemicals.** HPLC grade methanol and isopropyl alcohol were purchased
95 from Sigma (St. Louis, MO), and certified ACS grade chloroform was from Fisher (Fair Lawn,
96 NJ). Ammonium acetate and acetic acid were of analytical reagent grade from Merck (Darmstadt,
97 Germany). Gangliosides GM3 and GD3 from bovine buttermilk were purchased from Matreya
98 (Pleasant Gap, PA). Stock solutions of GM3 and GD3 standards were prepared separately in
99 methanol to obtain a concentration of 1 mg/mL. Bovine milk samples were collected from one
100 Holstein cow at the UC Davis dairy farm (day 2, 15, and 90 of lactation). Bovine colostrum and

101 colostrum products were kindly provided by Sterling Technology Co. (Brookings, SD). All
102 samples were kept frozen at –80°C.
103 **Sample Preparation.** Gangliosides were separated from neutral lipids in all samples by two
104 consecutive chloroform and methanol extractions described as follows. Two mL of liquid milk
105 sample was mixed with methanol:chloroform:water (8 mL:4 mL:1 mL). A 0.2-g sample of cream
106 and butter and a 0.02-g sample of buttermilk were also dissolved in the solvent solutions. The
107 solutions were sonicated for 5 min (Branson Ultrasonic Co., Danbury, CT) and centrifuged at
108 8,800 x g for 5 min in an Effendorf centrifuge (Hamburg, Germany). Subsequently, 2 mL of
109 water was added for phase separation, and the aqueous upper phase was collected. A methanol,
110 chloroform, and water (6 mL:3 mL:2 mL) mixture was added to the bottom layer for a second
111 extraction, and the mixture was shaken and centrifuged. The supernatant was collected and
112 pooled with that from the first extraction. The combined solution was dried by a SpeedVac rotor
113 concentrator (Savant Instruments, Inc., Holbrook, NY), and the lyophilized sample was re-
114 suspended in 1mL of methanol-water solution (1:1, v/v). The gangliosides were enriched with
115 C8 solid-phase extraction (SPE) cartridges (3mL, Supelco, Bellefonte, PA) with slight
116 modifications of previously published methods.[16, 21] The SPE cartridge was conditioned with
117 5mL of a methanol-water (1:1, v/v) solution, and the gangliosides were eluted with 10mL of
118 isopropyl alcohol-methanol (1:1, v/v). The addition of isopropyl alcohol increased the recovery
119 of ganglioside GM3, which is more hydrophobic than other ganglioside species. The eluant was
120 divided into five aliquots. For the quantification, ganglioside GM3 and GD3 standards were
121 added in five concentration levels (0.00, 0.05, 0.10, 0.15, and 0.20 mg/mL for GM3; 0.00, 0.50,
122 1.00, 1.50, and 2.00 mg/mL for GD3). The samples were dried by a SpeedVac rotor concentrator

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123 and the residues dissolved in 50 μL of the LC starting solvent. One microliter of the sample was
124 injected for MRM analysis.
125 For recovery studies, the samples were spiked with the GM3 and GD3 standards before the
126 extraction procedure. A representative portion of sample was collected and fortified with
127 appropriate volume of working standard solutions to reach 1 mg/L GM3 and 10 mg/L GD3 in the
128 spiked sample. Next, the extractions from the spiked sample were prepared following the
129 procedure described above.
130 **Ultra-high Performance Liquid Chromatography-Tandem Mass Spectrometry**
131 **(UHPLC-MS/MS).** Reversed-phase analysis was performed on an Agilent 1290 Infinity LC
132 (Agilent, Santa Clara, CA). An Agilent Eclipse Plus C18 Rapid Resolution High Definition
133 (RRHD) analytical column (i.d. 2.1 x 100mm, 1.8 μm, 80 Å) was used for the UHPLC
134 separation. The column was maintained at 50°C and eluted using a gradient of 80–90% of
135 solvent B from 0–0.2 min, then 90–100% over 2 min; 100% of B was maintained for 1 min, and
136 the column was re-equilibrated during a 1-min post run. Solvent A consisted of water and solvent
137 B was 15% isopropyl alcohol in methanol (v/v). Both solvents contained 20 mM ammonium
138 acetate and 0.1% acetic acid. The flow rate was 600 μL/min. Analytes were detected and
139 quantified using an Agilent 6490 triple quadrupole mass spectrometer operating in negative
140 electrospray ionization mode. The Agilent Jet Stream electrospray ionization source was set for
141 high sensitivity. The drying gas temperature and flow were 250°C and 12 L/min, respectively.
142 Sheath gas temperature and flow were 300°C and 12 L/min, respectively. The nebulizer gas
143 pressure was set at 35 psi. The capillary voltage was set at 4000 V. The collision cell accelerator
144 voltage was set to 5 V, and the collision energy was optimized on a compound-dependent basis.
145 Nitrogen was used as the collision gas. Resolution of the Q1 and Q3 quadrupoles was set at unit

146 resolution. Agilent Data Acquisition software was used for method development and data
147 acquisition. Agilent MassHunter Qualitative Analysis and Quantitative Analysis Software
148 (v.B.03.01) were used for data processing.

149 **Quantitation.** Gangliosides were quantified by a standard addition method. Volumes of 0, 2,
150 4, 6, and 8 μL of a standard solution containing 10 μg/mL GM3 and 100 μg/mL GD3 were
151 added to each 400 μL extract. Ganglioside concentrations were calculated using the signal
152 intensities of the transitions to the *m/z* 290.1 listed in Table 1.

153 Peak areas of each GM3 and GD3 in the samples were summed and the sums of the areas
154 were used to draw regression lines to estimate the amount of gangliosides in the sample. For
155 each ganglioside species, a plot of the spiked concentration (x-axis) against the peak area (y-axis)
156 was generated with five concentrations: For GD3, 0 mg/mL, sum of areas from blank samples;
157 0.5 mg/mL, sum of areas from the samples with the 2 μL standard added; 1.0 mg/mL, sum of
158 areas from the samples with the 4 μL standard added; 1.5 mg/mL, sum of areas from the samples
159 with the 6 μL standard added; and 2.0 mg/mL, sum of areas from the samples with the 8 μL
160 standard added. The concentrations of gangliosides in the sample extract were determined by the
161 x-intercept of the regression line. The concentration in the original sample was calculated by
162 dividing by dilution factor.

163 Recoveries were assessed by spiking a known amount of gangliosides (1 mg/L GM3 and 10
164 mg/L GD3) into the samples (2 mL) prior to sample extraction. The experiment was performed
165 in triplicate. The percent recoveries were determined by subtracting the amount of gangliosides
166 measured in the nonspiked samples from the amount measured in the spiked samples, divided by
167 the spiked amounts.

168

169 **RESULTS AND DISCUSSION**

170 **Fragmentation of Gangliosides GM3 and GD3 in the Negative Mode Triple Quadrupole**
171 **MS.** The electrospray ionization (ESI) was used in negative mode because of the intrinsic
172 anionic characteristics of gangliosides. We previously reported that the tandem MS of
173 gangliosides in the negative mode with a quadrupole time-of-flight instrument yielded intense
174 signals due to sialic acid fragments.[23] The MS/MS spectra acquired by triple quadrupole
175 instrumentation yielded essentially the same fragment ions obtained with the quadrupole time-of-
176 flight. The tandem MS spectra of commercial GM3 and GD3 samples are shown in Figure 1. For
177 GM3, the major product ion corresponded to a sialic acid fragment (Figure 1A), whereas for
178 GD3 there were two major fragments that corresponded to the mono and the disialic acid
179 fragments at *m/z* 290.1 and 581.2, respectively. For GM3, the best ion product for MRM was *m/z*
180 290.1, the monosialic acid fragment. For the GD3, the two options were further examined by
181 determining the behavior of the two fragments over a range of collision energies (20 to 50V). As
182 shown in S-Figure 1A, the ion intensities corresponding to transitions GD3(d43:1), 776.9 →
183 290.1 were higher than those of GD3(d43:1), 776.9 → 581.2 over the range of collision energies.
184 Similar trends were observed for GD3 with different ceramide components as shown in S-Figure
185 1B and 1C). Based on these results, the transitions to *m/z* 290.1 were selected for the MRM for
186 both the GM3 and GD3 gangliosides corresponding to the glycan structures depicted in Figure 2.

187 **Optimization of Chromatographic Separation of Gangliosides using Ultra-high**
188 **Performance Liquid Chromatography (UHPLC) and Mass Spectrometer Parameters.** To
189 assess the performance of the UHPLC separation, mixtures of bovine buttermilk gangliosides
190 GM3 and GD3 obtained as commercial standards were examined. The gradient programs
191 providing optimum separation of gangliosides with varying ceramide moieties were obtained

192 (see Materials and Methods). The gangliosides eluted between 1.4 and 2.7 min with peak widths
193 as narrow as 4.5 to 9 s at the base (Figure 3). The total time between injections was 4 min with a
194 column flow rate of 600 μL/min, illustrating the high duty cycle of the method. The UHPLC
195 separations achieved short running times without apparent sacrifice in the chromatographic
196 separation.

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

210 Twenty-four MRM transitions corresponding to GM3 and GD3 subspecies for samples under
211 the dMRM mode were monitored. Within each dMRM table, the cycle time was maintained at
212 300 ms to ensure that all analytes in the same time window were sufficiently sampled. This
213 condition corresponded to 15–30 data points across chromatographic peaks. As a result, it was
214 possible to obtain a sufficient number of points across the narrow chromatographic peaks to

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215 obtain good ion statistics. Sensitivity down to the nano gram range and more than 10^3 dynamic
216 range were obtained through targeted analysis using UHPLC-MS/MS in the dMRM mode (S-
217 Figure 3).

218 **Construction of dMRM Transition Table.** The MRM mode was used initially to monitor
219 the elution of gangliosides from the UHPLC column, thus providing a measure of their
220 abundances for suitability to quantitation. In this mode, specific precursor-product ion pairs were
221 monitored with conditions optimized for each transition. The MRM mode required the
222 construction of detailed tables with parameters for each compound transition. Through the
223 examination of the MS/MS spectra and comparison of the MRM peak intensities for different
224 collision energies, initial experiments were conducted to find the optimal collision energy
225 conditions for each ceramide moiety in the ganglioside standards.

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

233 The MRM is usually used for the analysis of compounds with known precursor-product ion
234 pairs; however, in a survey, it was also used to scan for pairs of many theoretically expected *m/z*
235 values. Once the appropriate gangliosides of interest were selected, a MRM assay was set up to
236 perform mass screening on a bovine milk ganglioside extract so that the presence of these
237 gangliosides could be observed and the appropriate signal intensities (signal-to-noise ratio > 3)

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238 obtained. The observed gangliosides were included in the final dMRM table (Table 1). Twelve
239 transitions for each ganglioside species were monitored, and optimum collision energies were
240 chosen. It is worth noting that as the alkyl chain length increases, fragmentation of the compound
241 becomes increasingly difficult, hence there may be some response differences between short and
242 long lipid chain species.[25] The characteristic UHPLC retention times of the transition ions were
243 also determined for dMRM analysis. Samples were analyzed in dMRM mode, with 0.3-min
244 retention time windows.

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

255 The method elaborated above was used on different samples of bovine milk. The
256 concentrations of gangliosides GM3 and GD3 in bovine milk collected during three different
257 lactation periods were determined in this manner and are presented in Table 2. The mean
258 concentrations for days 2, 15, and 90 from three experimental replicates were GM3, 0.98 mg/L;
259 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,
260 respectively. The efficiency of the extraction procedure was also evaluated, and recovery studies

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261 were carried out at concentrations of 1 mg/L GM3 and 10 mg/L GD3. Samples were also used to
262 assess intra-sample precision, and the data are presented in Table 2. Recoveries in all three
263 samples were greater than 80%, and relative standard deviations were within 20%. The recovery
264 levels provide confidence in the extraction protocol.

265 Our observation is in line with the lactation trend reported by Puente et al., with a high
266 ganglioside levels in colostrums (7.5mg lipid-bound sialic acid/kg) and dropping to 2.3mg lipid-
267 bound sialic acid/kg in transitional milk.[6] It was reported that bovine milk contained 1.2 mg/L
268 GM3 and 8.8 mg/L GD3, which was measured by traditional high performance thin layer
269 chromatography combined with densitometric measurement after staining with resorcinol
270 reagent.[5] Iwamori et al. showed that pooled bovine milk contains GM3(1.5mg/L) and
271 GD3(5.5mg/L). Pan and Izumi reported GM3(0.4mg/L) and GD3(12.0mg/L) in cow's milk. The
272 levels of GD3 reported by using previous LC-MS/MS approach were 9.2mg/L and 13.5mg/L.[21,
273 26] These reported values are in the range of the results obtained in our study.

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

279 Milk polar lipids, including phospholipids and glycosphingolipids, are mainly situated in the
280 milk fat globule membrane. When milk is processed at the dairy, this membrane is disrupted and,
281 as such, is no longer associated with the fat globules. It was reported that during processing,
282 polar lipids are preferentially distributed to aqueous phases such as buttermilk.[27, 28] It appears
283 that the churning process also enriches buttermilk in gangliosides GM3 and GD3 being GM3 and

284 GD3 19.2 mg/kg and 43.8 mg/kg, respectively, which are more than twice as high as the amounts
285 found in the colostrum cream (GM3, 8.2 mg/kg; GD3, 21.8 mg/kg) and in the colostrum butter
286 (GM3, 8.0 mg/kg; GD3, 17.0 mg/kg) (Table 2, Figure 4(B-D)). As such, colostrum buttermilk
287 which currently a side-stream, could be considered a suitable source for the isolation of milk fat
288 globule membrane gangliosides.

289 Gangliosides from dairy products ultimately derived from the milk fat globule have industrial
290 applications because of their contribution to dairy and other food products. In this study, a
291 UHPLC-MS/MS method was developed for the quantitation of gangliosides GM3 and GD3 in
292 bovine milk and colostrum-based dairy products. The dMRM method was made quantitative by
293 the use of a standard addition technique with standards purified from bovine milk showing
294 similar ganglioside profiles. This method is an efficient targeted approach due to the instrument's
295 high dynamic range and the selectivity of retention time in conjunction with the information
296 regarding precursor and product ion pairs. These features are especially important for the
297 analysis of gangliosides, which usually exist in trace amounts. This method can be used for
298 profiling the change of gangliosides and determining their physiological function, which was
299 conducted by traditional TLC.[29, 30] Moreover, for efficient utilization of bioactive milk fat
300 globule membrane (MFGM) materials, these separation principles and analytical approaches can
301 be integrated into the dairy industry. Data indicate that the gangliosides were abundant in
302 colostrum, and during the dairy process, they were preferentially concentrated in a side stream
303 like buttermilk.

304

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308

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

313

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Table 1. The dMRM table for bovine milk gangliosides. (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.

(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

Table 2. Absolute amount of GM3 and GD3 measured by the UHPLC-MS/MS method on bovine milk and colostrum dairy products. 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.

Sample	GM3 (RSD%)[1]	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)

[1]Relative standard deviation.

FIGURE CAPTIONS

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 = 50V. (B) MS/MS spectrum of GD3(d42:1) at *m/z* 776.9. collision energy = 30V. 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]

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.

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.

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.

Figure 1.

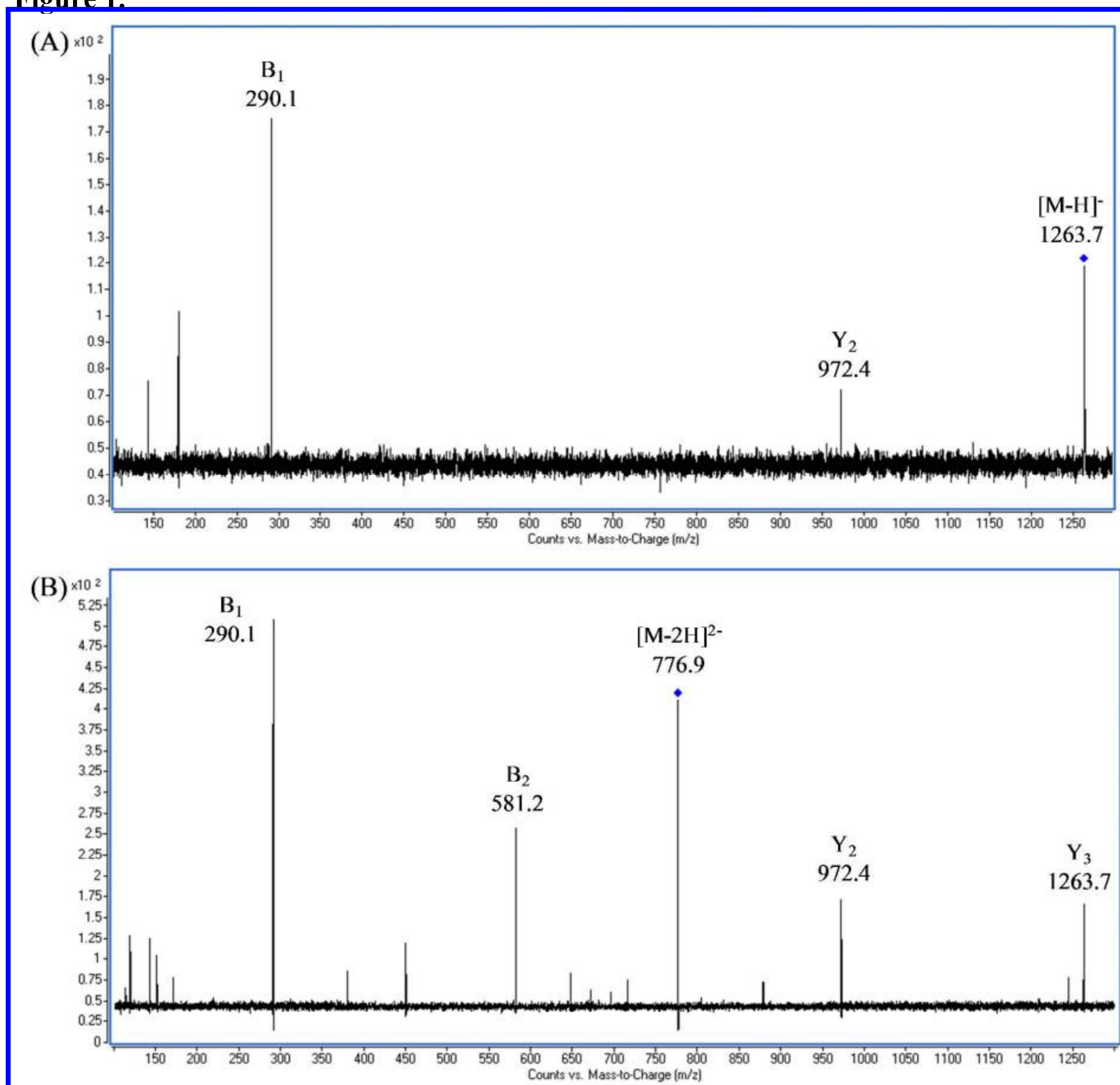


Figure 2.

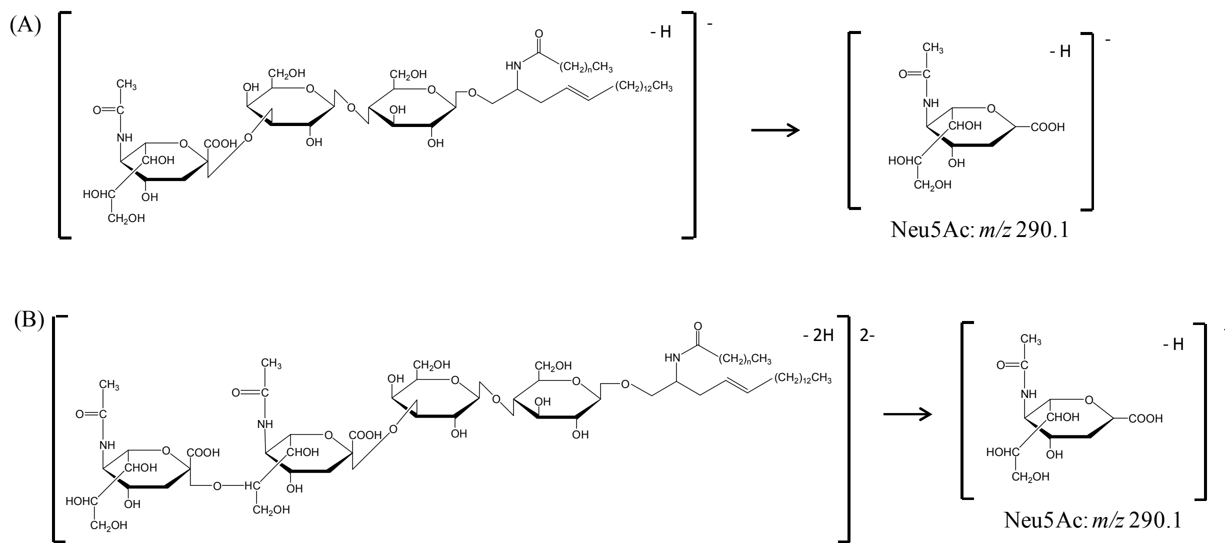


Figure 3.

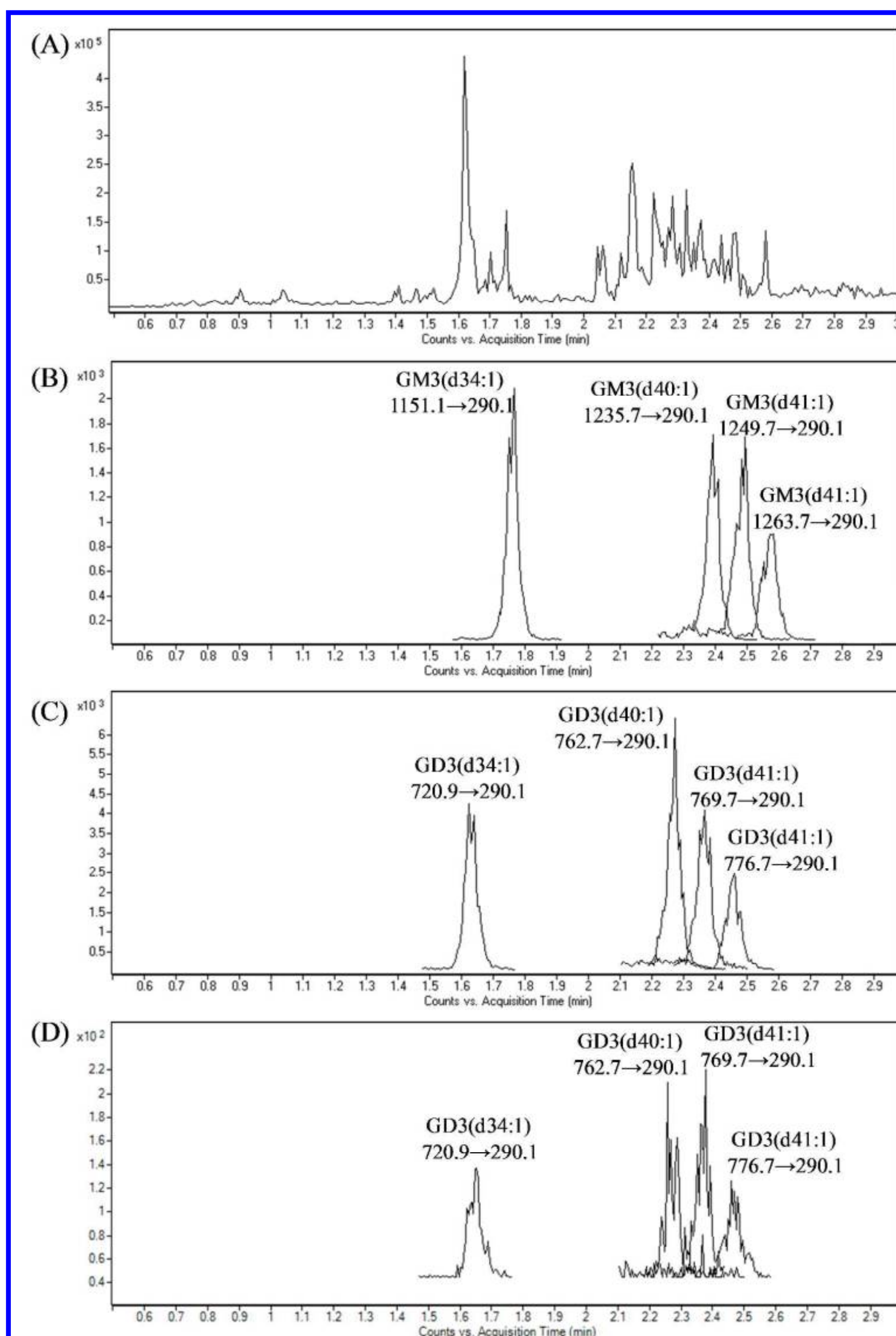
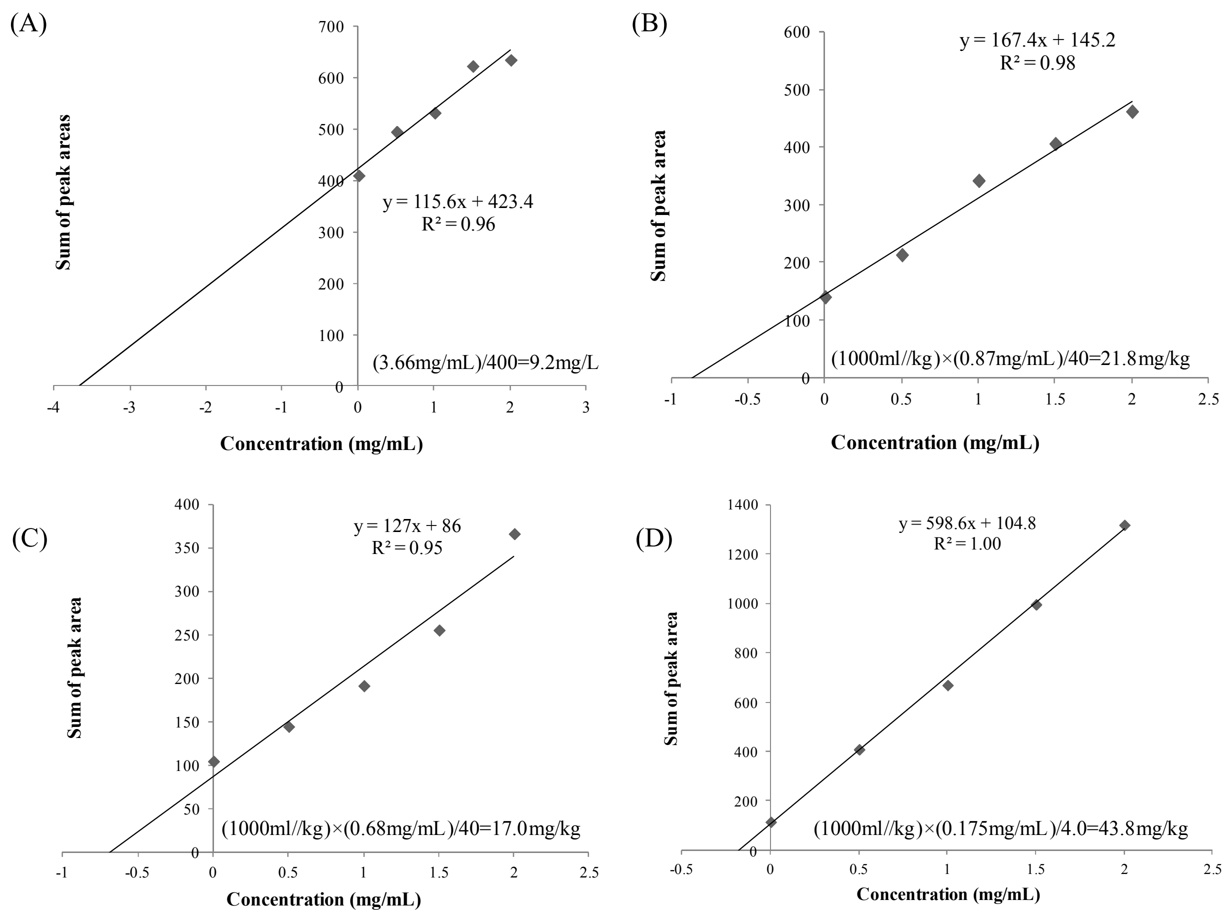


Figure 4.



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