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Rapid Screening of Bovine Milk Oligosaccharides in a Whey Permeate Product and Domestic Animal Milks by Accurate Mass Database and Tandem Mass Spectral Library

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Abstract

A bovine milk oligosaccharide (BMO) library, prepared from cow colostrum, with 34 structures was generated and used to rapidly screen oligosaccharides in domestic animal milks and a whey permeate powder. The novel library was entered into a custom Personal Compound Database and Library (PCDL) and included accurate mass, retention time, and tandem mass spectra. Oligosaccharides in minute-sized samples were separated using nanoliquid chromatography (nanoLC) coupled to a high resolution and sensitive quadrupole-Time of Flight (Q-ToF) MS system. Using the PCDL, 18 oligosaccharides were found in a BMO-enriched product obtained from whey permeate processing. The usefulness of the analytical system and BMO library was further validated using milks from domestic sheep and buffaloes. Through BMO PCDL searching, 15 and 13 oligosaccharides in the BMO library were assigned in sheep and buffalo milks, respectively, thus demonstrating significant overlap between oligosaccharides in bovine (cow and buffalo) and ovine (sheep) milks. This method was shown to be an efficient, reliable, and rapid tool to identify oligosaccharide structures using automated spectral matching.

Keywords

bovine milk oligosaccharides; tandem mass spectral library; whey permeate; cow milk; sheep milk; buffalo milk

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02039. Tables of sheep and buffalo milk oligosaccharides (PDF)

Notes

The authors declare no competing financial interest.

INTRODUCTION

Milk oligosaccharides are a class of nondigestible carbohydrates consisting of at least three monosaccharides linked by various glycosidic bonds. The compositions of human and bovine milk oligosaccharides have been extensively studied because of a widespread interest in their bioactive properties. Recent studies reported their biological efficacy as prebiotics and anti-infectives.[1,2] The biosynthesis of oligosaccharides in mammalian cells follows complex pathways where monosaccharide units are sequentially added by the activity of specific glycosyltransferases. They are structurally complicated molecules made of either lactose or lactosamine cores decorated with galactose, *N*-acetylhexosamine, sialic acid, and fucose.[3,4] Because monosaccharides are attached to the core via different glycosidic bonds, oligosaccharides are diverse in terms of chemical structure. Theoretically, because oligosaccharides can form glycosidic linkages at any hydroxyl site on a given monosaccharide, an incredible diversity of oligosaccharides are formed, based on the combinations of possible monosaccharides. This diversity arises from complex, nontemplate-based biosynthesis, involving several enzymes.[5] The length and branching of an oligosaccharide depends on the monosaccharides and enzymes available at the time of their assembly. In certain biological samples, such as bovine milk, a limited number of oligosaccharides are produced, due to limited glycosyl-transferase expression.

In glycomics, novel resources and technologies have been developed to investigate glycan structure–function relationships.[6–9] Among them, mass spectrometry (MS) has become the method of choice because of its sensitivity and speed of characterization of oligosaccharide structures.[10,11] Additionally, high-performance liquid chromatography (HPLC) is a technique used to separate oligosaccharides in a complex mixture based on their retentive properties.[12] A combination of HPLC and MS technologies provides accurate mass, isotope pattern, and retention time information. However, a more-detailed linkage analysis of each oligosaccharide is necessary for rigorous assignment of the structures. One approach involves the use of exoglycosidase enzymes that specifically cleave individual monosaccharides from the nonreducing end of the oligosaccharide. The shifts in the chromatographic retention time upon treatment with each glycosidase are used to assign the structure based on the specificity of cleavage.[13–15] However, this approach is rather expensive (requiring the use of all the possible hydrolases) and extremely time-consuming, requiring a prefractionation of the sample and multiple MS analyses for each fraction after the sequential enzymatic treatments. A more rapid throughput approach involves the use of tandem mass spectrometry (MS/MS), which enables assignment of fine structures with fragmentation pattern arising from a unique mass signature.[16,17] Recent studies show that some bovine milk oligosaccharide (BMO) structures in colostrums and mature milks were elucidated with the combination of the techniques mentioned above.[15,18,19] However, the techniques for the fine structure characterization of oligosaccharides have limitations when considering the highly heterogeneous oligosaccharide samples. The requirement of large sample amounts, multiple enzymatic steps, and MS/MS fragmentation methods complicates the use of these techniques for high-throughput analysis. There is still a need for a large amount of manual data analysis, which requires extensive knowledge of carbohydrate chemistry and mass spectrometry. To facilitate rapid and improved identification of an

ensemble of oligosaccharide structures, an alternative approach uses a reference library for oligosaccharides in certain biological matrices together with their analytical characteristics (e.g., accurate mass, isotope pattern, retention time, and MS/MS spectra). Recently, a human milk oligosaccharide (HMO) library was constructed and used to rapidly identify unknown oligosaccharides by comparing the fragmentation spectra.[20]

The present study details the use of a nanoliquid chromatographic (nanoLC) separation coupled to a high resolution and sensitive quadrupole-Time of Flight (Q-ToF) MS system for the detection of over 30 BMO that were previously elucidated.[15] Each BMO with known formulas was individually characterized to determine retention time, observed spectral molecular weight, and characteristic fragmentation pattern. The data generated were used to build a custom database and library that were used to screen unknown samples. The library was applied to a commercial whey permeate product and different milks from domestic animals.

MATERIALS AND METHODS

Materials and Chemicals

Bovine colostrum samples were collected from Jersey and Holstein cows within 12 h of calving. The samples were pooled and frozen at −80°C. A BMO-enriched bovine whey permeate powder was supplied by Hilmar Ingredients (Hilmar, CA). Sheep and buffalo milk samples were provided by AgResearch (New Zealand). The milks were collected from the animals at the end of their first month of lactation. Porous graphite carbon cartridges were purchased from Alltech Associated (Deerfield, IL). Sodium borohydride and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents used were either of analytical or HPLC grade.

Sample Preparation of Oligosaccharides from Bovine Colostrum, a Whey Permeate Product, and Sheep/Buffalo Milks

Identification of free oligosaccharides by MS requires their separation from the other components in the mixture. The oligosaccharides in the samples were extracted as described previously, with slight modifications.[15,21] A 500-μL aliquot of sample was centrifuged for 30 min at 15,000*g* and 4°C. The top fat layer was removed, leaving the oligosaccharide-rich bottom layer. The powdered whey permeate sample was dissolved in water 1% (w/v). Water (500 μL) and 4 mL of Folch solution (chloroform/methanol 2:1, v/v) were added to the defatted samples. The mixture was centrifuged for 30 min at 4,000*g* and 4°C. The aqueous top layer containing oligosaccharides was collected, and ethanol was added at a 2:1 ratio. The mixture was kept at 4°C overnight to precipitate the remaining protein and then centrifuged. The oligosaccharide-rich fraction was dried in a vacuum centrifuge (miVac, Genevac, Ipswich, UK). The dried oligosaccharides were resolubilized in 2 mL of 1.0 M sodium borohydride and kept at 60°C for 1 h to chemically reduce the oligosaccharides from aldehydes to alditols. The samples were further purified using graphitized carbon cartridges (4 mL, Alltech, Deerfield, IL). Prior to use, the cartridges were conditioned with three column volumes of 80% acetonitrile in 0.05% aqueous trifluoroacetic acid and another three column volumes of deionized water. The oligosaccharide-containing samples were loaded

onto the column and washed with five column volumes of deionized water. The oligosaccharides retained by the solid-phase extraction were eluted with five column volumes of 40% acetonitrile in 0.1% aqueous trifluoroacetic acid. The samples were dried in vacuo.

nanoHPLC-Chip/Q-ToF MS Analysis

Oligosaccharides were analyzed using an Agilent 6520 HPLC-Chip/quadrupole Time-of-Flight (Chip/Q-ToF) MS system (Agilent Technologies, Inc., Santa Clara, CA) equipped with a microwell-plate autosampler (maintained at 4°C), capillary sample loading pump, nano pump, HPLC-Chip/MS interface, and the ToF MS detector. The chip used consisted of a 9 × 0.075 mm i.d. enrichment column and a 43 × 0.075 mm i.d. analytical column, both packed with 5 μm porous graphitized carbon as the stationary phase. Chromatographic separation was performed by a binary gradient consisting of mobile phase A, 3% acetonitrile in water (v/v) with 0.1% formic acid, and mobile phase B, 90% acetonitrile in water (v/v) with 0.1% formic acid. The gradient conditions were as previously reported.[15] The dried samples were dissolved in 1 mL of water and diluted 10-fold for sheep milk and 100-fold for bovine colostrum and the whey permeate product, and 1 μL aliquots were injected. The mass acquisition ranges were m/z 450–2500 in MS mode and m/z 50–1500 in MS/MS mode. The instrument was operated in 2 GHz, extended dynamic range with a dual nebulizer ESI source. Sample data were collected in the positive ion mode using the HPLC-Chip interface. Mass accuracies are typically <5 ppm for MS and <20 ppm for MS/MS analyses. To ensure adequate resolution and mass accuracy, reference mass 922.0098 was monitored.

The collision energy applied was based on the following equation:

$$\text{Collision energy} = \frac{m \times z}{100} \times 1.3 - 3.5$$

Data Analysis

Oligosaccharide identification was performed using Agilent MassHunter Qualitative Analysis software (version B.06.00). Individual oligosaccharides were identified by two approaches, and the results were compared. First, only accurate mass and retention time were used for the assignment of oligosaccharide. Second, an accurate mass database with a spectral library in personal compound database and library format (PCDL) (Agilent Technologies, Inc., Santa Clara, CA) was compared with experimentally obtained data. Oligosaccharide structures were identified based on accurate masses, retention times, and MS/MS spectra.

In the first approach, MS scans provided accurate masses, which were used to assign composition, using a search algorithm, such as the glycan finder program (in-house). Briefly, as the experimental masses were inserted, the search algorithm examined a list of experimentally measured masses and searched for all possible monosaccharide combinations matching the experimental mass within <10 ppm mass errors. Relative quantitation was performed by measuring the peak area of the corresponding extracted ion chromatogram.

For the second method, BMO were identified by screening the accurate mass compound database and MS/MS spectral library. We built the PCDL format database and spectral library to contain BMO names, molecular formula, retention times, structures, and MS/MS spectra. Using a "Find by Molecular Features" algorithm, the Qualitative Analysis software extracted all of the possible compounds from each analysis based on *m*/*z* and retention time. Then, the database and library were applied, and they reported those BMOs whose retention time difference and mass errors were within restrictive set criteria (mass error <10 ppm; retention time window <2 min). The experimental MS/MS spectra were compared with the spectral data in the library. A match score was calculated for MS/MS spectra, as well as mass error, isotope spacing, and isotope abundances. More information is presented in a previous publication.[20]

RESULTS AND DISCUSSION

Building a BMO Personal Compound Database and Library (PCDL)

A database of tandem MS composed of 34 BMO structures was constructed (Table 1). The structures included in this study were characterized and elucidated in an earlier study by our group.[15] Among 50 BMO reported in the literature, the detailed structures, including linkage, monosaccharide type, and composition, were identified in 34 BMO structures. We also referred to other publications by Urashima et al. (21 BMO), Marino et al. (37 BMO), and Albrecht et al. (33 BMO).[19,22–24] Some structures reported were aided by nuclear magnetic resonance.[22,25,26] The tandem MS database and library are composed of accurate mass, chromatographic retention time, and tandem mass spectra of each BMO, along with the collision energy applied. All criteria correspond to unique characteristics. Regarding the precursor ion identification, due to the high mass resolution of Time-of-Flight (ToF) instruments, accurate mass provides the complete monosaccharide composition. In positive ionization mode, most BMOs are observed as the protonated molecular ions, $[M + H]^+$ or $[M + 2H]^{2+}$, and the list of BMO accompanied by neutral masses is detailed in the database. A graphitized carbon LC stationary phase was employed for the baseline HPLC separation for isomers.[27,28] A representative chromatogram of bovine colostrum BMO used in this study is presented in Figure 1A. Acidic oligosaccharides, including sialyllactose (SL) and sialyllactosamine (SLN), are predominant, and also a large portion of neutral tri- and tetra-saccharides is observed. This is consistent with previous studies.[18,29] It is worth noting that the chromatographic column enabled sufficient separation of sets of isomeric BMO, including 6′SL (retention time = 13.7 min) and 3′SL (retention time = 20.2 min). The separations of other milk oligosaccharides were demonstrated in previous publications.[15,20,28] An additional level of discrimination can be achieved through the fragmentation of the parent ions. A detailed list of the proposed product ions along with the relative abundance is listed in the library (Table 1). BMOs underwent fragmentation resulting in common fragment ions of monosaccharides such as hexose (*m*/*z* 183.09), hexosamine (*m*/*z* 204.09), and *N*-acetylneuraminic acid (Neu5Ac, *m*/*z* 292.10). Additional glycosidic and internal cleavages were generated, providing information related to oligosaccharide sequence and branching, therefore generating characteristic tandem MS spectra for each oligosaccharide. The ability to distinguish structures by tandem MS is demonstrated with two sialyllactose (SL) isomers: 3′SL and 6′SL (Figure 2). Both SL spectra showed B_1, B_2,

Y_1, and Y_2 fragment ions; however, their relative intensities differed. B type ions were major fragment ions in 3′SL, whereas Y_1 ion was the most intense in 6′SL spectra. The intensities of the fragment ions in tandem MS experiments are the result of a complex combination of factors such as the relative stability of precursors and products, bond strengths, activation barriers, or position of the charge.[30] All these factors are in turn the consequence of a specific chemical structure. Hence, even when isomers can yield the same products in tandem MS experiments, they can still be resolved based on the relative abundances of characteristic fragment ions.[31] To determine whether the corresponding structures could be identified using this method in a straightforward fashion, the MS/MS spectra in the library were compared with those found in bovine milk via the library search. In both comparisons, the correct isomer yielded the highest scores; the scores for 6′SL and 3′SL were 94% and 98%, respectively (Table 2a,b).

Application of the BMO Database and Spectral Library to a Whey Permeate Product

Whey permeate is a coproduct of cheese-making obtained after the removal of protein from whey. When cheese whey is passed through an ultrafiltration membrane to concentrate protein, smaller molecules cross through the membrane to yield the whey permeate. Recently, we demonstrated that whey permeate is a good source of prebiotic milk oligosaccharides.[21,32,33] The massive industrial quantities of bovine dairy streams can make large-scale isolation of oligosaccharides possible. Therefore, whey permeate is considered to be a source of functional compounds for novel product development. The database and library were tested and validated using an industrial BMO product from whey permeate. Several well-known BMO were observed in the product; however, the relative intensities of the major BMO appeared different from those in bovine colostrum (Figure 1). This can be explained by the fact that mature milk, not colostrum, is used to produce cheese, and it is well-known that oligosaccharide composition evolves during lactation.[29,34,35] Tentative assignment of oligosaccharides (with monosaccharide composition) showed that, in the whey permeate product, there were 38 potential oligosaccharide compositions including isomers (Table 3a). For comparative purpose, the tandem MS library was adopted as an additional identification tool. An extracted ion chromatogram for each compound in the database (within a specified mass window, e.g., 538.206 ± 10 ppm for $[LNnH + 2H]^{2+}$) was used to integrate the chromatogram to position compounds and to compare the isotope ratio of the mass spectra for each peak with the theoretical isotope ratio (Figure 3A–C). A further level of analyte discrimination can be achieved through the fragmentation of the compound position in the sample chromatogram, comparing the resultant product spectra with that stored in the spectral library (Figure 3D–E). A score was assigned from each of the search criteria, and the combination of the scores produced a match score. For example, the database score was 96%, and the library score was 85%. Therefore, the combined match score for LNnH (lacto-*N*-neohexaose) in the whey permeate product was 91%. The PCDL searching results produced 18 BMO that were assigned with their structures to their match scores (Table 3b).

Barile et al.[20] reported 15 oligosaccharide compositions in whey permeate in 2009, and in a following publication, Dallas et al.[21,32] assigned 24 oligosaccahrides, not including isomers. To the best of our knowledge, the present study is the first to show the many isomeric forms

of BMO in whey permeate with their structural details. 3′SL was the most abundant oligosaccharide in bovine whey permeate, followed by 3′-sialyl-galactosyl-lactose, 6′SL(Neu5Ac), 6′SLN(Neu5Ac), triose B, *N*-acetylglucosaminyl-lactose, and sialyllacto-*N*-tetraose (LST) in order of descending abundance (Table 3b). By employing the program-assisted spectral matching, the entire identification process can be done quickly with high confidence.

Application of BMO Database and Spectral Library to Domestic Animal Milks

Our custom database and library were further validated using milks from two additional domestic animals: sheep and buffalo. These species were chosen because they are ruminants, herbivores, and have digestive systems similar to those of cows. These milk samples were prepared and analyzed in the same way as the bovine milk samples were. Because of the low oligosaccharide concentration in the sheep and buffalo milk samples, the analyses were carried out using limited cleanup steps to limit oligosaccharides losses, which led to a more complex chromatogram that included non-oligosaccharide peaks (Figure 4).[36] A MS scan was conducted to obtain accurate masses that were used to assign oligosaccharide composition. Seventeen oligosaccharide compositions were identified in sheep milk samples (Supplemental Table 1a). Ovine milk oligosaccharides were composed of short oligomeric chains. Sialic acid residues included both Neu5Ac and *N*-glycolylneuraminic acid (Neu5Gc), and interestingly, the latter was significantly more abundant with the proportion of 24/76 (Neu5Ac/Neu5Gc), whereas bovine milk contained more Neu5Ac than Neu5Gc.[18] An automated tandem MS library search comparing the MS/MS spectra from the ovine milk sample was conducted. The library search produced 15 BMO with match scores above 70% (Supplemental Table 1b). The prominent peaks at 10.4 and 10.8 min corresponded to the triose B(Gal(β1–3)Gal(β1–4)Glc) and triose C(Gal(β1–6)Gal(β1–4)Glc) with the scores 95% and 97%, respectively (neutral mass: 506.18). The structures and relative abundances of the two isomers were consistent with reports in earlier papers that trioses were the most abundant neutral oligosaccharides in sheep milk and that Gal(β1–3)Gal(β1–4)Glc and Gal(β1–6)Gal(β1–4)Glc constituted the predominant isomeric form.[24,37] We also confirmed the presence of a fucosylated oligosaccharide (2′FL) with a confident match score of 83%.[24] In addition, triose A, triose D, LNnT, LNnH, and LNnP1 were found as minor neutral oligosaccharides.[24] In regard to anionic oligosaccharides, five Neu5Gc-containing oligosaccharides were found in MS only mode (Supplemental Table 1a); nonetheless, two of them, 3′SL(Neu5Gc) and 6′SLN(Neu5Gc), were assigned by a library search (Supplemental Table 1b).[24,38] Another three Neu5Gc-containing oligosaccharides may have unique structures that were not detected in bovine milk; therefore, the structures were not searchable using the BMO library.[38] Four Neu5Ac-containing oligosaccharides were found and assigned: 6′SL(Neu5Ac), 3′SL(Neu5Ac), 6′SLN(Neu5Ac), and 3′-sialyl-galactosyl-lactose with the match scores of 93%, 94%, 77%, and 70%, respectively. They were also reported to be present in sheep milk.[24]

A buffalo milk sample was also analyzed in the same way as the ovine milk sample. Accurate mass analysis revealed 18 oligosaccharide compositions (Supplemental Table 2a). Neither fucosylated nor Neu5Gc-containing oligosaccharides were found in the buffalo milk. All anionic oligosaccharides contained only Neu5Ac. This finding could be explained by the

lack of the activity of CMP-*N*-acetylneuraminic acid hydroxylase in buffalo mammary glands.[39] In Supplemental Table 2b, 13 oligosaccharides were assigned with detailed structures using the database and spectral library search. To the best of our knowledge, this is the first report of a buffalo milk oligosaccharide profile. 6′SL(Neu5Ac) was the major anionic oligosaccharide in buffalo milk with a match score of 94%, and 3′SL(Neu5Ac), 6′SLN(Neu5Ac), and 3′-sialyl-galactosyl-lactose were also present. Regarding neutral oligosaccharides, four trisaccharides (triose B, triose C, triose D, and *N*-acetylglucosaminyl-lactose) were found with their structural details. In addition, more complex neutral oligosaccharides such as LNnH, 40210, LNnP1, 20110, and LNnT were identified with confident match scores of 70%, 71%, 95%, 74%, and 72%, respectively. The composition of 3Hex+2HexNAc was determined based on mass accuracies of less than 10 ppm in MS scan mode; however, it was not assigned by the library search, which means that the structure was not present in cow's milk. The structure can be GlcNAc(β1–3)Gal(β1–4)GlcNAc-(β1–3)Gal(β1–4)Glc, the connectivity of which was elucidated in a previous publication.[40]

In this study, the accurate mass database and tandem mass spectra library were generated and used for the identification of BMO in several milk samples. The combination of highly reproducible LC, high mass accuracy MS, and high quality tandem MS allowed confident compound identification. The library contains 34 BMO with details of mass and retention time values. The identities of the oligosaccharides were further confirmed by accurate mass MS/MS analysis on the Q-ToF MS. This program assisted structural identification in a rapid and simple way by matching to a MS/MS spectral library. Comparing the results from accurate mass only versus the spectral library search, we found that the automatic spectral library search provided accurate assignment of oligosaccharide structures without false positive results. A great degree of the homology of milk oligosaccharides was found among the cow, ovine, and buffalo milks.

Given the expandable nature of the database, additional compounds can be included in the database by the simple addition of a molecular formula and MS/MS spectra. This would enable newly acquired sample chromatograms to be interrogated in the event of milk oligosaccharide metabolites, which are not currently part of the database, being identified using a complementary technique. Therefore, future application would comprise analyzing body fluids derived from clinical studies. A growing number of clinical studies are being carried out with BMO supplements: the novel tool described here would be useful for tracking the metabolic fate of oligosaccharides in blood, urine, and stool, and would be of pivotal importance to validate the observed activities.[41,42] The ability to automate the database makes it an attractive tool for milk oligosaccharide research as it is not possible to predict the biological activity of oligosaccharides a priori without knowing their structural characteristics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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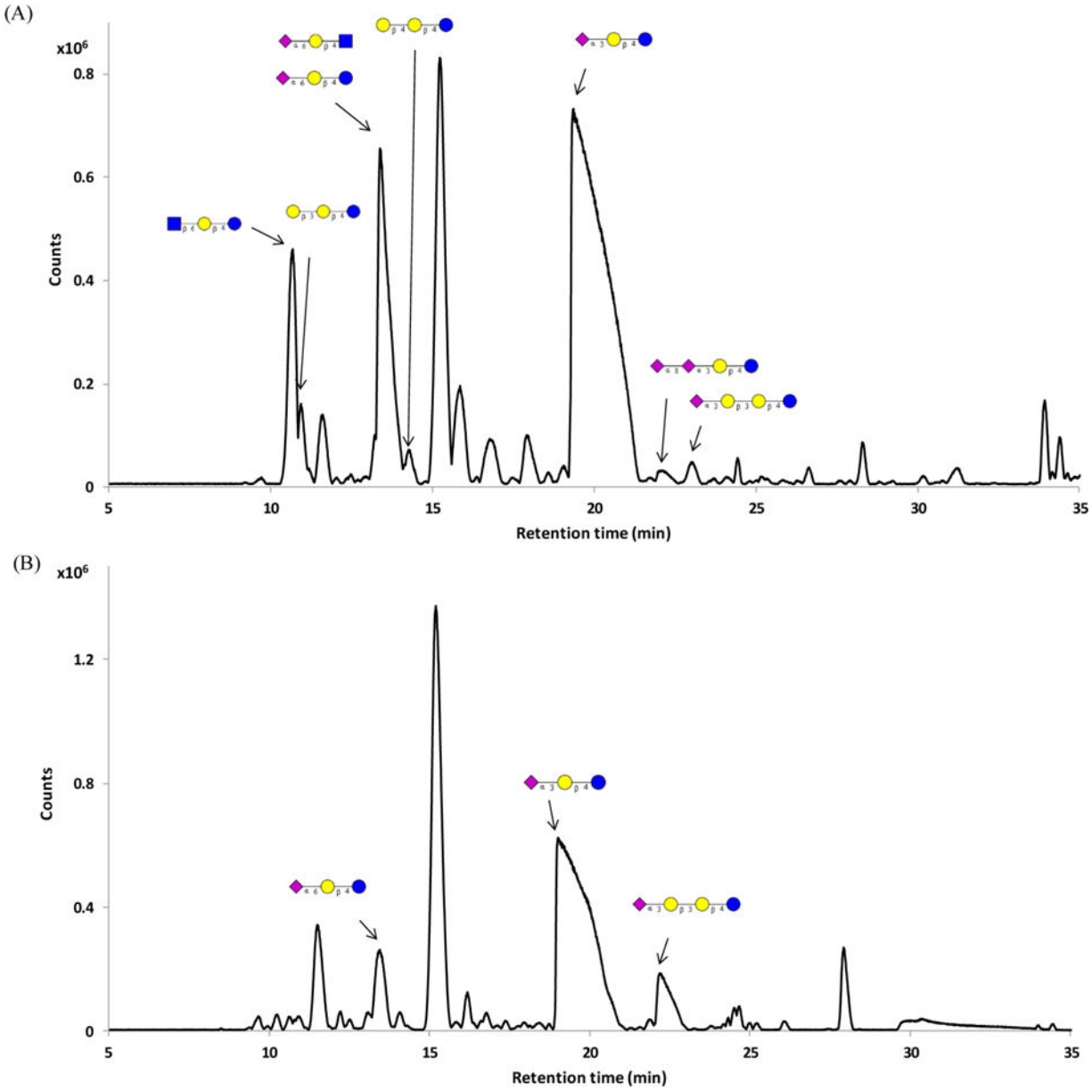


Figure 1.

Representative base peak chromatograms of oligosaccharides from (A) bovine colostrum and (B) a whey permeate product with some structures annotated.

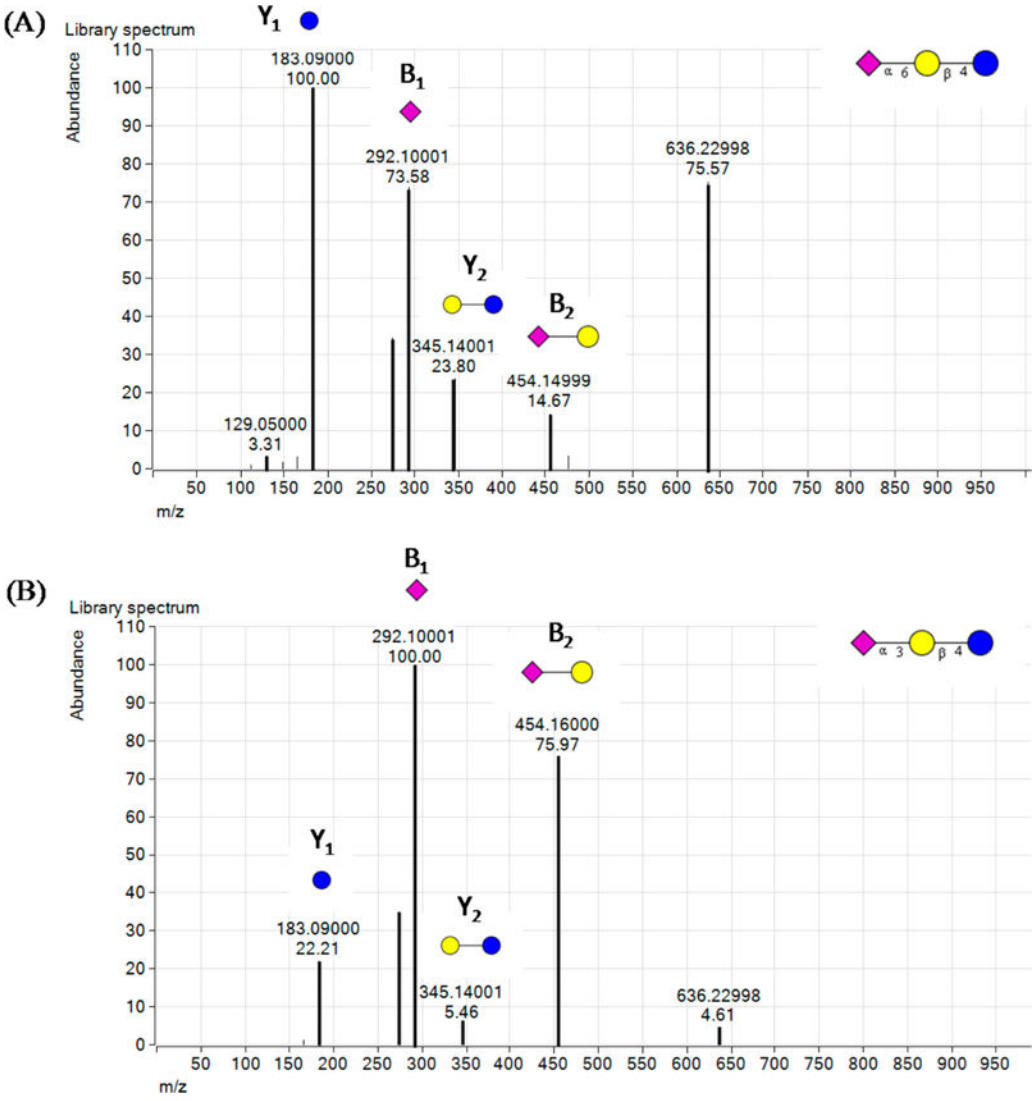


Figure 2.

MS/MS fragmentation patterns for two sialyllactose (SL) isomers: (A) 6′SL and (B) 3′SL with the precursor at *m*/*z* 636.236. The collision energy used was 4.8 eV.

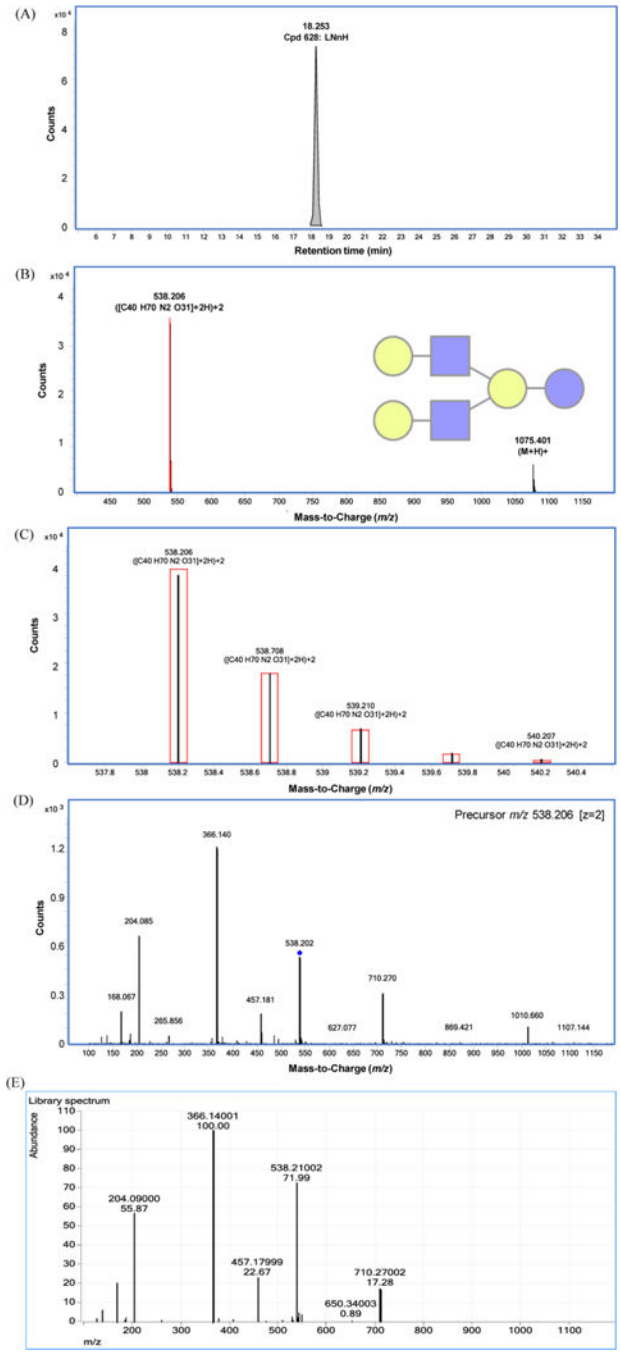


Figure 3.

Identification of LNnH in a whey permeate product using the custom PCDL. (A) Chromatogram identifying LNnH; (B) an extracted mass spectrum for LNnH with the structures inserted; (C) zoomed-in image for the LNnH (*m*/*z* 538.206) where the solid lines represent the observed mass spectra, and the overlaid boxes refer to the theoretical isotopic abundances; (D) MS/MS spectrum of a LNnH precursor ion (*m*/*z* 538.206); and (E) an MS/MS spectrum from the library.

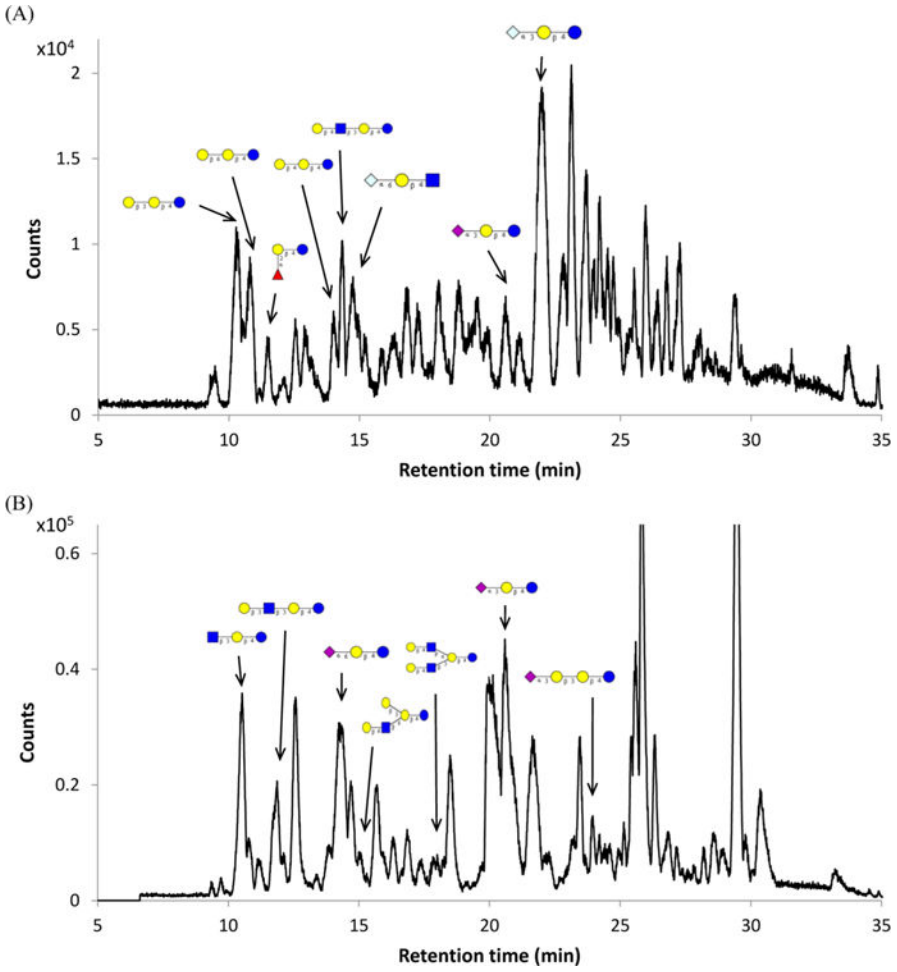


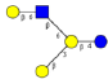
Figure 4.

Base peak chromatograms of oligosaccharides from (A) sheep milk and (B) buffalo milk with some structures annotated.

Table 1

Compound Database and Library Data, Including Collision Energy, Proposed Product Ion, and Relative Abundance for Each Bovine Milk Oligosaccharide (BMO)

BMO	Structure	Neutral mass (reduced)	Composition[a]	Retention time (min)	Collision energy (eV)	Proposed product ions generated (Relative abundance)
3′FL		490.190	21000	1.3	2.89	183.082 (100), 491.187 (23), 345.132 (18)
2′FL		490.190	21000	11.7	2.89	183.081 (100), 491.200 (16), 329.140 (11), 345.1400 (11), 165.070 (11)
triose A		506.185	30000	9.9	3.1	183.090 (100), 345.140 (16)
triose B		506.185	30000	10.8	3.1	183.090 (100), 345.140 (30), 507.190 (24), 163.060 (11), 165.080 (10)
triose C		506.185	30000	11.1	3.1	183.090 (100), 163.060 (29), 345.140 (24), 507.190 (16), 325.110 (14)
triose D		506.185	30000	14.4	3.1	183.090 (100), 507.190 (97), 325.120 (74), 163.060 (74), 345.140 (26), 145.050 (15)
galactosyl-lactosamine		547.211	20100	11.5	3.7	548.220 (100), 224.110 (69), 386.170 (19)
N-aetylgalactosaminyl-lactose		547.211	20100	12.0	3.7	204.090 (100), 548.230 (54), 366.140 (43)
N-acetylglucosaminyl-lactose		547.211	20100	10.4	3.7	204.090 (100), 548.220 (93), 366.140 (35), 183.090 (11)
6′SL (Neu5Ac)		635.227	20010	13.7	4.8	183.090 (100), 636.230 (76), 292.100 (74), 274.090 (35), 345.140 (24), 454.150 (15)
3′SL (Neu5Ac)		635.227	20010	20.2	4.8	292.100 (100), 454.160 (76), 274.090 (35), 183.090 (22)
3′SL (Neu5Gc)		651.222	20001	20.3	5.0	308.100 (100), 290.090 (57), 470.150 (46), 183.090 (17)
tetraose a		668.238	40000	12.4	5.2	183.080 (100), 325.110 (60), 345.140 (39), 507.190 (34), 669.250 (29), 163.060 (22), 287.160 (14)
tetraose b		668.238	40000	14.5	5.2	183.090 (100), 345.140 (60), 507.190 (52), 669.240 (36), 163.060 (18), 325.110 (16)
6′SLN (Neu5Ac)		676.254	10110	13.5	5.3	677.260 (100), 386.170 (65), 224.110 (33)
6′SLN (Neu5Gc)		692.249	10101	13.2	5.5	693.260 (100), 386.160 (77), 224.110 (43), 677.260 (36)

BMO	Structure	Neutral mass (reduced)	Composition[a]	Retention time (min)	Collision energy (eV)	Proposed product ions generated (Relative abundance)
LNnT		709.264	30100	14.0	5.8	366.139 (100), 710.270 (28)
30100		709.264	30100	15.6	5.8	710.270 (100), 204.090 (100), 366.140 (67), 528.190 (38)
20200a		750.291	20200	13.2	6.3	407.170 (100), 751.290 (30), 204.090 (18)
20200b		750.291	20200	14.1	6.3	751.300 (100), 528.190 (76), 589.240 (23), 427.190 (13), 366, 140 (11)
3′-sialyl-galactosyl-lactose		797.280	30010	23.3	6.9	454.160 (100), 292.100 (82), 274.090 (20)
pentaose		830.290	50000	14.6	7.3	831.300 (100), 507.190 (78), 345.140 (75), 669.240 (68), 183.090 (67), 487.160 (50), 325.110 (41), 163.060 (27)
20110		838.307	20110	14.5	7.4	495.180 (100), 839.310 (22)
31100		855.322	31100	10.9	7.7	512.200 (100), 366.140 (76), 856.340 (28), 204.080 (24)
LNnPl		871.317	40100	15.1	7.9	366.140 (100), 872.330 (45)
LNnP2		871.317	40100	17.7	7.9	528.190 (100)
30200a		912.343	30200	13.2	8.4	913.350 (100), 690.240 (54), 751.310 (33), 528.190 (23), 366.140 (23)
30200b		912.343	30200	13.9	8.4	407.165 (100), 913.350 (56)
DSL		926.323	20020	22.4	8.6	292.100 (100), 745.250 (97), 583.200 (74), 454.150 (49), 274.090 (35)
LST		1000.359	30110	22.4	9.5	657.230 (100), 1001.360 (40), 366.140 (15), 819.280 (12)
50100		1033.369	50100	17.5	10.0	528.190 (100), 1034.360 (17)
LNnH		1074.396	40200	18.2	3.5	366.140 (100), 538.210 (72), 204.090 (56), 457.180 (23), 168.060 (20), 710.270 (17)
30300		1115.423	30300	16.2	3.8	204.090 (100), 366.140 (83), 558.720 (55), 407.170 (37)

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BMO	Structure	Neutral mass (reduced)	Composition[a]	Retention time (min)	Collision energy (eV)	Proposed product ions generated (Relative abundance)
					11.0	1116.430 (100), 407.170 (55), 366.140 (43), 751.290 (15), 204.080 (12), 710.270 (11)
40210		1365.491	40210	24.6	5.4	366.140 (100), 683.750 (77), 657.240 (61), 204.080 (26), 710.270 (22), 274.089 (17), 685.380 (16), 538.200 (14), 292.110 (12)
					14.3	1366.500 (100), 657.230 (78), 366.140 (41), 1001.340 (12), 710.270 (11)

[a]Composition lists the number of hexose (Hex), fucose (Fuc), *N*-acetylhexosamine (HexNAc), *N*-acetylneuraminic acid (Neu5Ac), and *N*-glycolylneuraminic acid (Neu5Gc). Abbreviations: 3′FL, 3′-fucosyllactose; 2′FL, 2′-fucosyllactose; 6′SL (Neu5Ac), 6′-sialyllactose; 3′SL (Neu5Ac), 3′-sialyllactose; 3′SL (Neu5Gc), 3′-glycolylneuraminyl-lactose; 6′SLN (Neu5Ac), 6′-sialyllactosamine; LNnT, lacto-*N*-neotetraose; 6′SLN (Neu5Gc), 6′-glycolylneuraminyl-lactosamine; LNnP, lacto-*N*-novopentaose; DSL, disialyllactose; LST, sialyllacto-*N*-tetraose; LNnH, lacto-*N*-neohexaose. Symbols: Glc (blue circle), Gal (yellow circle), Fuc (red triangle), GlcNAc (blue square), GalNAc (yellow square), Neu5Ac (purple diamond), HexNAc (white square), and Neu5Gc (white diamond).

Table 2

(a) PCDL Search Results for 6′-Sialyllactose (6′SL), Including the *m/z* of the Precursor Ion and Matching Scores and (b) PCDL Search Results for 3′-Sialyllactose (3′SL), Including the *m/z* of the Precursor Ion and Matching Scores

(a)

name	precursor *m/z*	collision energy (eV)	mass	score (%)
6′SL (Neu5Ac)	636.236	4.8	635.229	94
3′SL (Neu5Ac)	636.236	4.8	635.229	33

(b)

name	precursor *m/z*	collision energy (eV)	mass	score (%)
3′SL (Neu5Ac)	636.236	4.8	635.229	98
6′SL (Neu5Ac)	636.236	4.8	635.229	35

Table 3

(a) Details of Oligosaccharide Tentatively Identified in a Whey Pearmeate Product Using Accurate Mass and (b) Details of Oligosaccharide Identified in a Whey Permeate Product Using the Automated BMO Library

(a)							
mass (exp)	**retention time (min)**	**Hex**	**Fuc**	**HexNAc**	**Neu5Ac**	**Neu5Gc**	**abundance**
506.186	11.0	3	0	0	0	0	1808414
506.186	20.3	3	0	0	0	0	171498
547.212	10.7	2	0	1	0	0	817819
547.213	14.6	2	0	1	0	0	71141
635.229	13.5	2	0	0	1	0	7848718
635.229	19.3	2	0	0	1	0	13953249
651.222	29.8	2	0	0	0	1	68416
668.239	12.5	4	0	0	0	0	192418
676.255	13.4	1	0	1	1	0	1815296
692.249	13.1	1	0	1	0	1	79043
692.249	17.3	1	0	1	0	1	68875
709.264	14.5	3	0	1	0	0	125862
709.265	25.3	3	0	1	0	0	386381
734.295	21.2	1	1	2	0	0	65899
797.281	20.3	3	0	0	1	0	1760230
797.282	22.4	3	0	0	1	0	8618667
813.275	22.0	3	0	0	0	1	58496
813.276	29.8	3	0	0	0	1	55775
822.313	11.0	1	1	1	1	0	127738
830.289	13.8	5	0	0	0	0	70608
830.291	14.6	5	0	0	0	0	44182
838.308	17.4	2	0	1	1	0	243767
838.308	14.1	2	0	1	1	0	1351702
871.318	25.3	4	0	1	0	0	374757
895.322	13.7	1	0	2	0	1	28535
912.344	24.7	3	0	2	0	0	16667

(b)

molecular formula	name	retention time (min)	*m/z*	mass	score	abundance
$C_{37}H_{64}N_2O_{29}$	LST	22.2	501.187	1000.360	96	1436320
$C_{40}H_{70}N_2O_{31}$	LNnH	18.3	538.206	1074.398	91	934061
$C_{42}H_{73}N_3O_{31}$	30300	16.5	558.718	1115.421	86	666946