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Bovine Milk Glycome

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ABSTRACT

Bovine milk oligosaccharides have several potentially important biological activities including the prevention of pathogen binding to the intestinal epithelial and as nutrients for beneficial bacteria. It has been suggested that milk oligosaccharides are an important source of complex carbohydrates as supplements for the food and the pharmaceutical industries. However, only a small number of structures of bovine milk oligosaccharides (bMO) are known. There have been no systematic studies on bMO. High-performance mass spectrometry and separation methods are used to evaluate bMO, and nearly 40 oligosaccharides are present in bovine milk. Bovine milk oligosaccharides are composed of shorter oligomeric chains than are those in human milk. They are significantly more anionic with nearly 70%, measured abundances, being sialylated. Additionally, bMO are built not only on the lactose core (as are nearly all human milk oligosaccharides), but also on lactose amines. Sialic acid residues include both *N*-acetyl and *N*-glycolylneuraminic acid, although the former is significantly more abundant.
Key words: oligosaccharide, bovine milk, high-performance liquid chromatography, mass spectrometry

INTRODUCTION

Oligosaccharides are a class of bioactive molecules that are receiving increasing commercial attention from a health perspective for their roles in stimulating the growth of beneficial bacteria in the intestine and acting as decoy receptors to inhibit specific pathogen binding. Together, these functions act to create a protective intestinal environment that contributes to health. The foundation for these benefits is attributed to the effects of breast milk on the health of infants whose microflora are dominated by bifidobacterium species; these infants have fewer incidents of diarrhea, allergy, and other health problems (Morrow et al., 2005; Newburg et al., 2005; Stepans et al., 2006). Human milk contains oligosaccharides that are not digestible to the infant and remain largely intact until they reach the large intestine, where they can be used by beneficial intestinal bacteria. Recent evidence suggests that the structure of the oligosaccharide plays a role on how it is fermented (Ward et al., 2006; LoCascio, 2007; Ninonuevo et al., 2007). Commercial oligosaccharide products include inulin from chicory root, short- and long-chain fructo-oligosaccharides, and galacto-oligosaccharides; however, these structures are less complex than milk oligosaccharides and may not have all the functions of more complex oligosaccharides that are found in mammalian milks. Determining the variation and types of oligosaccharides found in bovine milk is an important step toward the development of commercial sources for complex oligosaccharides found to be important in human health.

Oligosaccharides (**OS**) are defined as carbohydrates that contain 3 to 10 monosaccharides covalently linked through glycosidic bonds. The monomers of milk oligosaccharides are D-glucose (**Glc**), D-galactose (**Gal**), *N*-acetylglucosamine (**GlcNAc**), L-fucose (**Fuc**), *N*-acetyl neuraminic acid (**NeuAc**), and *N*-glycolylneuraminic acid (**NeuGc**) (Rivero-Urgell and Santamaria-Orleans, 2001; Boehm and Stahl, 2007).

It has been suggested that, because of their biological activity, sialylated oligosaccharides from bovine milk may be a suitable source of additives for the food and pharmaceutical industries (Nakamura et al., 2003). For this to happen, better knowledge of the composition of bovine milk oligosaccharides (**bMO**), their biological activities, and their structural similarities to human milk oligosaccharides (**hMO**) is needed. Human milk oligosaccharides are significantly more abundant than bMO. Human milk oligosaccharide concentrations range between 20 and 23 g/L for colostrum (Coppa et al., 1999), whereas the bMO concentration in colostrum is approximately 0.7 to 1.2 g/L (Veh et al., 1981). Human milk oligosaccharides commonly have a lactose core at the reducing end and are extended by various combinations of glycosyltransferases. Although lactose may

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also be the major core structure of bMO, there has been no definitive study to demonstrate this. Research from our laboratory showed that there are more than 200 hMO (Ninonuevo et al., 2006), with at least 93 structures elucidated (Dai et al., 2000). In contrast, there are, to the best of our knowledge, fewer than 20 OS structures reported in the literature for bovine milk, consisting of 10 sialylated (Gopal and Gill, 2000) and 8 neutral (Finke et al., 2000; Urashima et al., 2001) OS. Among the 10 acidic OS, several contain NeuGc such as NeuGc(α2–3/6)Gal(β1–4)Glc and NeuGc(α2–6)Gal(β1–4)GlcNAc (Gopal and Gill, 2000), although these species are in relatively low abundance. Oligosaccharides containing NeuGc are not found in human milk. However, hMO are highly fucosylated, whereas fucosylation in bMO is either absent or in very low abundance. Finke et al. (2000) reported that there is no fucosylation in the milk of domestic animals including cows, sheep, and goats, whereas Saito et al. (1987) reported the presence of Fuc(α1–3)Gal(β1–4)GlcNAc in bovine colostrum.

Analysis of bMO has been hindered by the lack of effective analytical tools for OS analysis. Previous methods included nuclear magnetic resonance spectroscopy (Guerardel et al., 1999), high pH anion-exchange chromatography with pulsed amperometric detection (Kunz and Rudloff, 1996), capillary electrophoresis, and MS (Pfenninger et al., 2001, 2002). Recently, we proposed new methods employing high-performance MS and nanoflow HPLC on chip-based devices (Ninonuevo et al., 2006). In addition, new tandem MS methods such as infrared multiphoton dissociation (**IRMPD**) yield critical structural information.

In this report, we systematically examined the oligosaccharides in bovine employing microchip liquid chromatography separation and high-performance MS including Fourier transform ion cyclotron resonance (**FTICR**) and time-of-flight (**TOF**) analyzer. We obtained structures on both neutral and ionic oligosaccharides, as well as relevant information to identify specific oligosaccharides in all mixtures.

MATERIALS AND METHODS

Materials

Nonporous graphitized carbon cartridges (150 mg of bed weight, 4 mL tube size) were obtained from Alltech (Deerfield, IL). Sodium borohydride (98%) and 2,5-dihydroxybenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO). Lacto-*N*-tetraose (**LNT**), lacto-*N*-neotetraose (**LNnT**), lacto-*N*-hexaose (**LNH**), and lacto-*N*-neohexaose (**LNnH**) were obtained from Oxford Glycosystems (Oxford, UK). All reagents were of analytical or HPLC grade.

Collection of Milk Samples

The postpartum colostrum samples from individual Jersey and Holstein cows (n = 20) were collected within 12 h of calving. Milk samples were obtained and frozen at −80°C after collection. Milk samples were pooled for MS analysis.

Sample Preparation

Milk samples were completely thawed, and 0.5 mL was centrifuged at 4,000 × *g* in a microfuge for 30 min at 4°C. After the top fat layer was removed, 4 volumes of chloroform/methanol (2:1 vol/vol) were added to the defatted milk samples. After centrifugation at 4,000 × *g* for 30 min at 4°C, the upper layer was carefully transferred. Two volumes of ethanol were added to the mixture overnight at 4°C, followed by a 30-min centrifugation at 4,000 × *g* to remove denatured protein. The supernatant (milk oligosaccharide-rich fraction) was freeze-dried using a speed vacuum. Native milk OS were reduced to alditol forms by using 1.0 *M* sodium borohydride and incubated at 42°C overnight. The bMO were purified by solid-phase extraction using a nonporous graphitized carbon cartridge and elution with 20% acetonitrile in water (vol/vol) before MS analysis.

Electrospray Ionization MS and IRMPD

Mass spectra were obtained using a nanoelectrospray FTICR MS (IonSpec Corp., Irvine, CA) equipped with a 9.4 T superconducting magnet in both positive and negative ion modes. Cone voltages were maintained at 2,000 V to obtain signals. Ions were accumulated in the hexapole, and transferred by quadrupole ion guide before the ion cyclotron resonance (**ICR**) cell for detection. Tandem MS was performed with IRMPD with a 10.6-μm CO_2 laser (Parallax Laser Inc., Waltham, MA) by transmitting the laser beam into the ICR cell through a BaF_2 window to fragment isolated ions.

HPLC-Chip/TOF MS

Milk OS fractions collected after solid-phase extraction were analyzed using a microfluidic 6200 Series HPLC-chip/TOF MS instrument (Agilent Technologies, Santa Clara, CA). The microfluidic HPLC-chip consists of an enrichment column, a liquid chromatography separation column packed with porous graphited carbon,

and a nanoelectrospray tip. Separation was performed by a binary gradient A: 3% acetonitrile in 0.1% formic acid solution and B: 90% acetonitrile in 0.1% formic acid solution. The column was initially equilibrated and eluted with a flow rate of 0.3 μL for nanopump and 4 μL for capillary pump. The 65-min gradient was programmed as follows: 2.5 to 20 min, 0 to 16% B; 20 to 30 min, 16 to 44% B; 30 to 35 min, B increased to 100%, continued with 100% B to 45 min, and finally, 0% B for 20 min to equilibrate the chip column before the next sample injection. Each composition of milk oligosaccharide was identified with an in-home program "Glycan Finder." Distinct compositions were identified based on accurate mass and retention times.

RESULTS

Mass Profiles of Mixtures of bMO

A typical mass spectrum for an OS mixture from bovine milk analyzed by electrospray ionization FTICR-MS is shown in Figure 1. The sample was obtained from the pooled colostrum of 3 cows. The positive mass spectrum showed primarily neutral OS (Figure 1A) with minor abundances corresponding to anionic components. Compositions were obtained based on accurate mass and tandem MS (see table insert in Figure 1A). All ions in the positive mode corresponded to sodium-coordinated species. Two prominent ions are present corresponding to the compositions 3 *N*-acetylhexosamines (**HexNAc**) + 2 hexoses (**Hex**) (m/z 935.333) and 4Hex+1HexNAc (m/z 894.306). The composition was determined based on mass accuracies of less than 10 ppm. The negative spectra provide complementary information (Figure 1B). In the negative mode, the most abundant peak (m/z 634.220) corresponds to sialyllactose with the composition 2Hex+1NeuAc. The other signals correspond primarily to other sialylated OS and some neutral OS (see table insert in Figure 1B). That sialyllactose and sialyllactosamine (1Hex+1HexNAc+1NeuAc, m/z 675.247) were highly abundant is consistent with earlier reports that 3′-sialyllactose and 6′-sialyllactose amines were the most abundant and constituted at least 50% of the total (Martin-Sosa et al., 2003). Oligosaccharides containing NeuGc made up only a very small proportion of the total milk OS. Only one OS (m/z 650.215) contained NeuGc, although others are present as shown below. We estimate, based on these data and in the profiling below, that NeuGc corresponds to about 1 to 5% of the total OS. Furthermore, we find no evidence in this type of analysis or in the LC profiling below for the presence of fucose-containing oligosaccharides.

Oligosaccharide Profiling by HPLC-Chip/TOF MS

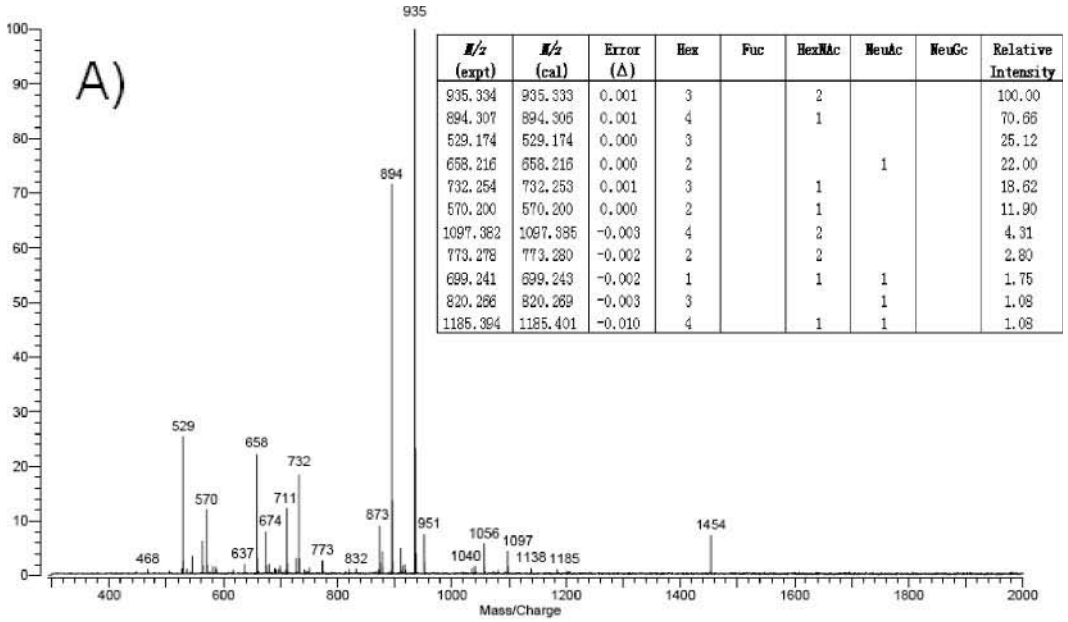
The mass profiles above provide a rapid albeit crude analysis of OS compositions of complicated mixtures. A more accurate method for profiling with quantification is HPLC separation. Mixtures of OS when combined tend to suppress each other. Neutral OS suppress sialylated OS in the positive spectra, whereas sialylated OS suppress neutral species in the negative mode. However, when separated, both neutral and anionic OS have similar ionization efficiencies in the positive mode. With nano-LC MS, we have shown previously that hMO could be profiled, yielding nearly 200 oligosaccharides with masses ranging from m/z 500 to >4,000 (Ninonuevo et al., 2006).

Nano-LC-chip/MS of the bMO sample described above yielded a chromatogram significantly different from that of hMO (Figure 2). The bMO contained significantly more sialylated oligosaccharides than hMO. The most abundant peak in the chromatogram is sialyllactose, with a retention time of 21.5 min, which is more than 50% of all bMO. There were OS that were common to both bMO and hMO; however, the majority of the bMO elute at a significantly later time, consistent with the elution times of sialylated OS.

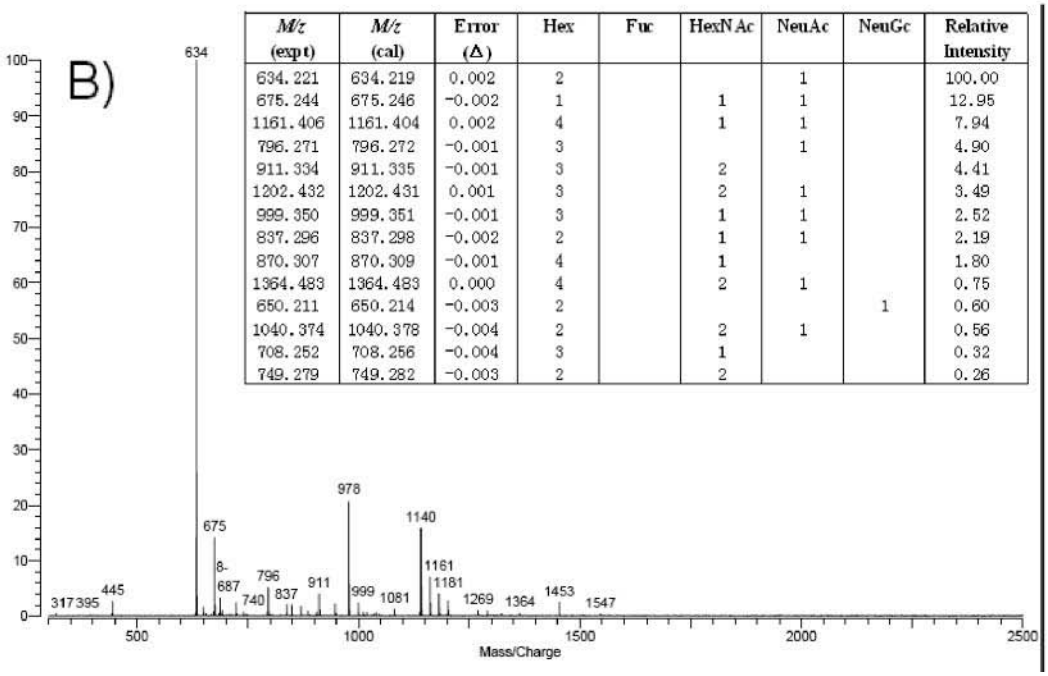
Table 1 summarizes the retention time, abundances, and compositions of each oligosaccharide separated by graphitized carbon cartridge HPLC. Based on the masses, there are major differences between humans and bovine. Of the masses listed, only 5 correspond to those in humans. The most abundant OS in bovine, sialyllactose, is only a minor component in human (Kunz et al., 2000). There are other notable differences between human and bovine milk OS. There are significantly fewer isomers for each composition in bovine compared with humans. There are 3 masses corresponding to 3 isomers, approximately 9 with 2 isomers, and several with only a single isomer. This is in contrast to humans, in which between 3 and 7 isomers can be present for a single composition, and many compositions have several isomers. As with the mass mapping results, the majority of the OS is sialylated (70%), with approximately 5% containing NeuGc. Little evidence for fucose was found; if present, the amount of fucose was estimated to be less than 0.01%.

Structural Analysis of OS

To annotate the nano-LC chromatogram, a combination of standards, tandem MS, and previously published structures were used. The relative lack of diversity in bMO made structural elucidation more tenable. For most compositions, only a single isomer was present,



M/z (expt)	M/z (cal)	Error (Δ)	Hex	Fuc	HexNAc	NeuAc	NeuGc	Relative Intensity
935.334	935.333	0.001	3		2			100.00
894.307	894.306	0.001	4		1			70.66
529.174	529.174	0.000	3					25.12
658.216	658.216	0.000	2			1		22.00
732.254	732.253	0.001	3		1			18.62
570.200	570.200	0.000	2		1			11.90
1097.382	1097.385	-0.003	4		2			4.31
773.278	773.280	-0.002	2		2			2.80
699.241	699.243	-0.002	1		1	1		1.75
820.266	820.269	-0.003	3			1		1.08
1185.394	1185.401	-0.010	4		1	1		1.08



M/z (expt)	M/z (cal)	Error (Δ)	Hex	Fuc	HexNAc	NeuAc	NeuGc	Relative Intensity
634.221	634.219	0.002	2			1		100.00
675.244	675.246	-0.002	1		1	1		12.95
1161.406	1161.404	0.002	4		1	1		7.94
796.271	796.272	-0.001	3			1		4.90
911.334	911.335	-0.001	3		2			4.41
1202.432	1202.431	0.001	3		2	1		3.49
999.350	999.351	-0.001	3		1	1		2.52
837.296	837.298	-0.002	2		1	1		2.19
870.307	870.309	-0.001	4		1			1.80
1364.483	1364.483	0.000	4		2	1		0.75
650.211	650.214	-0.003	2				1	0.60
1040.374	1040.378	-0.004	2		2	1		0.56
708.252	708.256	-0.004	3		1			0.32
749.279	749.282	-0.003	2		2			0.26

Figure 1. Nanoelectrospray ionization Fourier transform ion cyclotron resonance mass spectra obtained from pooled bovine milk oligosaccharides (A) in positive mode and (B) in negative mode. Table inserts correspond to the composition of the masses.

which means that tandem MS could be performed on a specific m/z isolated from the entire mixture.

To annotate the bMO nano-LC chromatogram with standards, hMO were used. There are a limited number of commercial hMO, and very few with masses that corresponded to bMO. By matching the masses, 2 pairs of isomers were found to be potentially common between bMO and hMO; namely, LNT and its isomer (LNnT) and lacto-*N*-hexaose (**LNH**) and its isomer lacto-*N*-neohexaose (**LNnH**).

Lacto-*N*-tetraose and LNnT are major components of hMO, with LNT having typically greater abundance (Kunz et al., 2000). Neither LNT nor LNnT has been reported previously in bovine milk. The composition

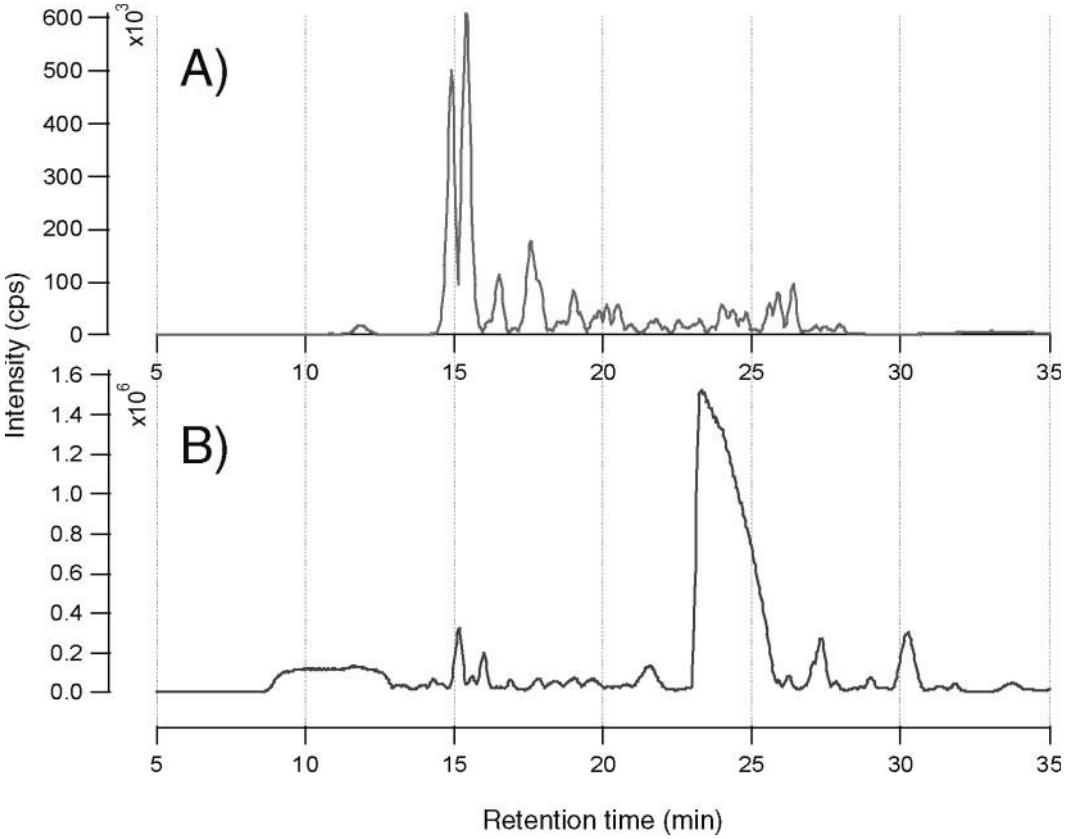


Figure 2. High-performance liquid chromatography-chip time-of-flight chromatograms of pooled human milk oligosaccharide (A) and bovine milk oligosaccharide (B) samples in the positive mode. The most abundant signal in the bovine sample corresponded to sialyllactose.

of the bovine analog was verified by tandem MS using IRMPD in an FTICR MS (Figure 3A). The sequence, without linkage information, based on the tandem MS was consistent with both structures (LNT and LNnT). The only difference between the 2 structures is the linkage of a terminal Gal: LNT contains a terminal Galß1–3 and LNnT contains a terminal Galß1–4. In the nano-LC chromatogram of bovine milk, *m/z* 710.264 $[M+H]^+$ was dominated by a single isomer. Retention times for oligosaccharide standards LNT and LNnT were 15.1 and 15.6 min, respectively. The difference of 0.5 min is large considering that the structures vary by a single terminal linkage. The OS in bovine milk showed a retention time at 15.6 min, confirming the structure of LNnT. This same structure was described previously by Urashima et al. (1991a), who isolated the compound from horse colostrum.

Another pair of neutral milk OS, LNH and LNnH from human milk, were examined in a similar manner. Tandem MS confirmed the composition and the sequence (spectrum not shown). The extracted ion chromatogram (Figure 4) showed only a single isomer for *m/z* 1074.396 with a retention time of 20.00 min, consistent with the LNnH; LNnH was also reported to be present in horse colostrum (Urashima et al., 1991a).

Several structures were previously reported for bMO. Tandem MS can be used to identify those structures in the mixtures. To illustrate this process, the IRMPD of a molecular ion (*m/z* 894.306) corresponding to a single isomer is shown in Figure 3B. The fragment ions are consistent with the composition corresponding to $[4Hex+1HexNAc+Na]^+$. The fragment ions ranged in mass from 203.054 and 205.068, the terminal monosaccharide residues corresponding to $[1Hex+Na]^+$ and $[1Hex\text{-}ol+Na]^+$, respectively, to *m/z* 732.253. The peaks are labeled according with their structures (Figure 3B). The latter mass suggests that the nonreducing ends are hexoses. The characteristic loss of hexose, generating *m/z* 732.253, shows that HexNAc is not on the reducing end or the nonreducing end. The hexose loss is followed by the further loss of a HexNAc to yield the ion *m/z* 529.174, a trisaccharide corresponding to $[3Hex+Na]^+$. The ions *m/z* 570.201 and *m/z* 550.176 correspond to $[1Hex+1HexNAc+1Hex\text{-}ol+Na]^+$ and $[2Hex+1HexNAc\text{-}H_2O+Na]^+$, respectively, which are products from the loss of terminal hexose from *m/z* 732.253. The connectivity and the mass correspond

Table 1. Common oligosaccharides found in bovine milk with composition[1]

m/z (expt)	*m/z* (cal)	Hex	HexNAc	NeuAc	NeuGc	Retention time (min)	Abundance
506.183	506.185	3				14.762	10,289,782
506.184	506.185	3				10.160	1,303,070
506.186	506.185	3				8.995	109,094
547.210	547.211	2	1			15.221	11,142,751
547.211	547.211	2	1			9.287	1,086,602
635.226	635.227	2		1		20.545	311,431,904
651.221	651.222	2			1	20.007	30,130,712
676.253	676.254	1	1	1		15.143	15,870,276
676.252	676.254	1	1	1		12.759	10,912,929
676.257	676.254	1	1	1		17.384	672,159
692.251	692.249	1	1		1	14.540	679,863
692.246	692.249	1	1		1	20.237	1,200,180
709.262	709.264	3	1			15.349	19,018,856
750.290	750.291	2	2			13.503	6,407,956
750.292	750.291	2	2			14.437	27,596,110
797.278	797.280	3		1		21.823	2,041,891
797.281	797.280	3		1		24.257	18,258,772
813.284	813.275	3			1	24.130	1,307,872
838.304	838.307	2	1	1		15.380	4,845,144
838.306	838.307	2	1	1		13.809	19,874,340
871.315	871.317	4	1			16.272	33,374,806
912.342	912.343	3	2			13.842	6,936,200
912.341	912.343	3	2			14.848	22,044,166
926.321	926.323	2		2		20.990	1,970,504
942.323	942.318	2		1	1	21.314	427,510
967.350	967.349	1	1	2		25.144	5,103,399
967.350	967.349	1	1	2		17.466	246,649
1,000.355	1,000.359	3	1	1		23.717	3,736,359
1,000.356	1,000.359	3	1	1		20.134	1,148,196
1,016.360	1,016.354	3	1		1	19.830	117,576
1,041.386	1,041.386	2	2	1		21.835	14,704,525
1,074.398	1,074.396	4	2			19.580	10,433,410
1,162.422	1,162.412	4	1	1		23.165	4,808,720
1,162.411	1,162.412	4	1	1		27.174	15,932,500
1,178.406	1,178.407	4	1		1	23.082	944,698
1,203.438	1,203.439	3	2	1		26.494	4,306,686
1,203.438	1,203.439	3	2	1		24.790	4,756,937
1,203.441	1,203.439	3	2	1		20.785	3,956,023
1,365.490	1,365.491	4	2	1		26.203	3,697,598

[1]expt = experimental; cal = calculated; Hex = hexose; HexNAc = *N*-acetylhexosamine; NeuAc = *N*-acetyl neuraminic acid; NeuGc = *N*-glycolylneuraminic acid.

precisely to the structure of lacto-*N*-novopentaose I (**LNnP I**), which is a previously published structure (Urashima et al., 1991a).

For structures that have not been previously reported, it is possible to obtain sequence using tandem MS that can later be refined to complete structures. Figure 3C shows the IRMPD, in the negative mode, of a compound with *m/z* 1,161.405. The mass corresponds to the composition $[4Hex+1HexNAc+1NeuAc-H]^-$. The most abundant peak in the tandem MS spectrum corresponds to the sialic acid fragment $[NeuAc–H]^-$ *m/z* 290.088. The peak at *m/z* 634.220 corresponds to $[1NeuAc+2Hex-H]^-$, which suggests that the NeuAc is not on the last hexose in the chain. The desialylated species is consistent with LNnP I, suggesting that this OS is simply a sialylated LNnP I.

Several neutral-anionic OS pairs differ by only a single sialic acid. One such pair is the composition 4Hex+2HexNAc and 4Hex+2HexNAc+1NeuAc. The neutral OS corresponds to LNnH, suggesting that the compound is LNnH with an additional sialic acid.

Figure 5 lists all the OS with complete and partial structures found in bovine milk. In all, 39 species of free OS were found in bovine milk; complete or partial structures are presented for 25. Ten sialylated OS (Gopal and Gill, 2000) and 8 neutral OS (Finke et al., 2000; Urashima et al., 2001) were reported in the literature. Of the 8 neutral OS, 5 were confirmed in this study. One fucosylated bMO was reported in the literature (Saito et al., 1987). However, we found no fucosylated free OS in bovine milk. The other previously reported neutral OS were disaccharides that could not be detected in this study. Of the 10 known sialylated OS, 7 were found corresponding to 2 isomers of NeuAc-linked lactose, and 2 isomers of NeuGc-linked lactose, NeuAc(α2–6)-lactosamine, NeuGc(α2–6)-lactosamine,

and 1 disialyllactose (NeuAc-NeuAc-lactose) were found in this study. Other anionic OS such as sulfated OS (Gopal and Gill, 2000) were not found in this study. Five new anionic structures were characterized with connectivities but without linkage information. For a few more anionic OS, the abundances were too small for tandem MS; however, compositions are provided. Included in Figure 5 are retention times, relative abundances, and references (Kuhn and Gauhe, 1965; Veh et al., 1981; Chaturvedi and Sharma, 1988; Urashima et al., 1989a,b, 1991a,b, 1994, 1997), when present, for each individual component.

In Figure 5, we also include several proposed structures, with or without linkage information, but whose structures are estimated based on structural homology. These structures are labeled "P" (proposed). For these compounds, neither tandem MS analysis nor a previous report in literature is available. Structures are proposed by building on known structures. For example, the composition corresponding to 2Hex+2NeuAc corresponds to a previously reported structure NeuAc-NeuAc-Gal-Glc (Kuhn and Gauhe, 1965). A composition corresponding to 1Hex+1HexNAc+2NeuAc was obtained but with abundances too low for tandem MS. The core structure is likely a lactose amine (1Hex+1HexNAc). We can predict with some confidence that the structure corresponds to NeuAc-NeuAc-Gal-GlcNAc, Gal-GlcNAc being the lactose amine core. Similarly, the oligosaccharide with the composition 4Hex+1HexNAc+1Na corresponded to the previously reported structure LNnP I (Urashima et al., 1989b, 1991a,b). A composition corresponding to a sialylated version was found containing both NeuAc and NeuGc. We can conclude that these compounds are merely LNnP I with NeuAc or NeuGc on the nonreducing end. The NeuAc-containing compound was verified with tandem MS, whereas the NeuGc-containing compound was too small in abundance for further analysis.

DISCUSSION

The data presented here indicate that there are large differences between human milk and bovine milk. We have verified that the concentration of OS in bovine milk was lower than that of human milk (Veh et al., 1981). In bovine colostrum, where OS are most abundant, about 40 are observed compared with about 200 in human. Bovine milk also has less diversity with fewer structures per composition. Bovine milk was, however, more highly sialylated with over 70% containing at least one sialic acid. In human milk, less than 20% of the oligosaccharides are sialylated. In contrast, fucosylated oligosaccharides make up about 70% of hMO but are absent in bMO. The lack of fucosylated oligosaccharides observed in this study is supported by the findings of Finke et al. (2000).

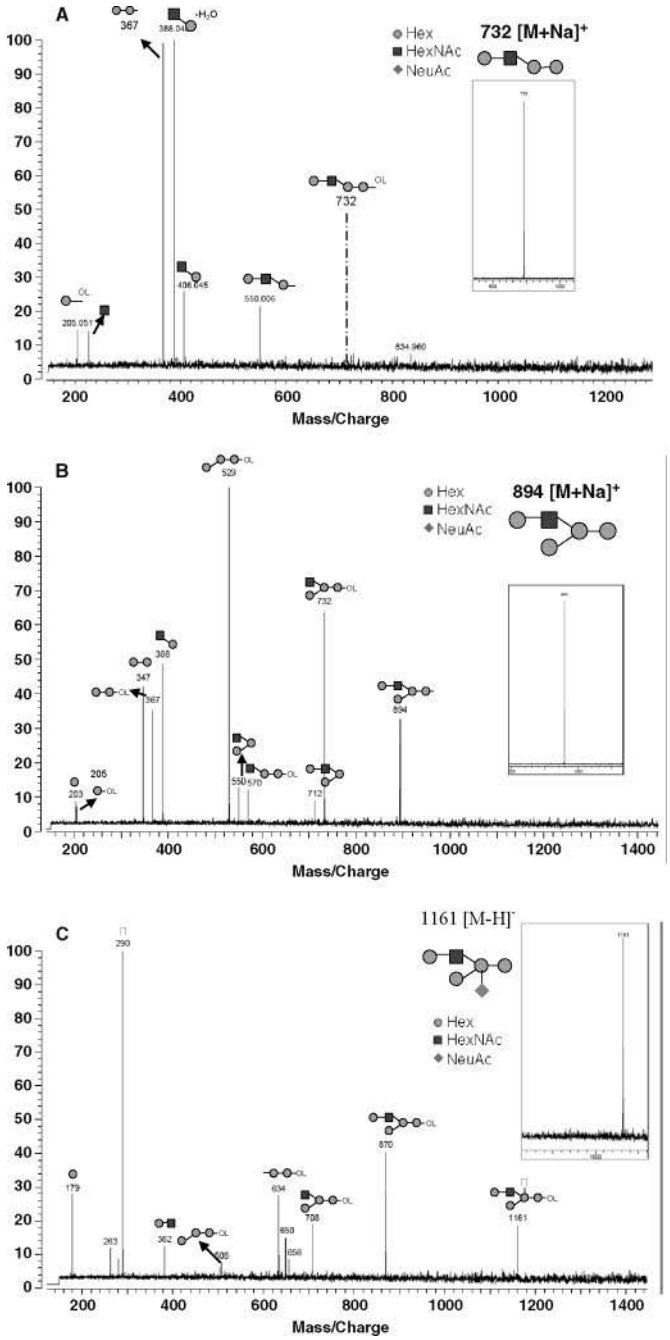


Figure 3. Infrared multiphoton dissociation spectra of ions with composition corresponding to A) m/z 732.253 $[3Hex+1HexNAc+Na]^+$; B) m/z 894.306 $[4Hex+1HexNAc+Na]^+$; and C) m/z 1161.405 $[4Hex+1HexNAc+1NeuAc-H]^-$. The peaks are labeled with the corresponding fragment structures. Hex = hexose; HexNAc = *N*-acetylhexosamine; NeuAc = *N*-acetyl neuraminic acid.

Bovine milk OS are generally smaller than hMO and consist primarily of tri- and tetrasaccharides. The largest we observed corresponded to the heptamer, which

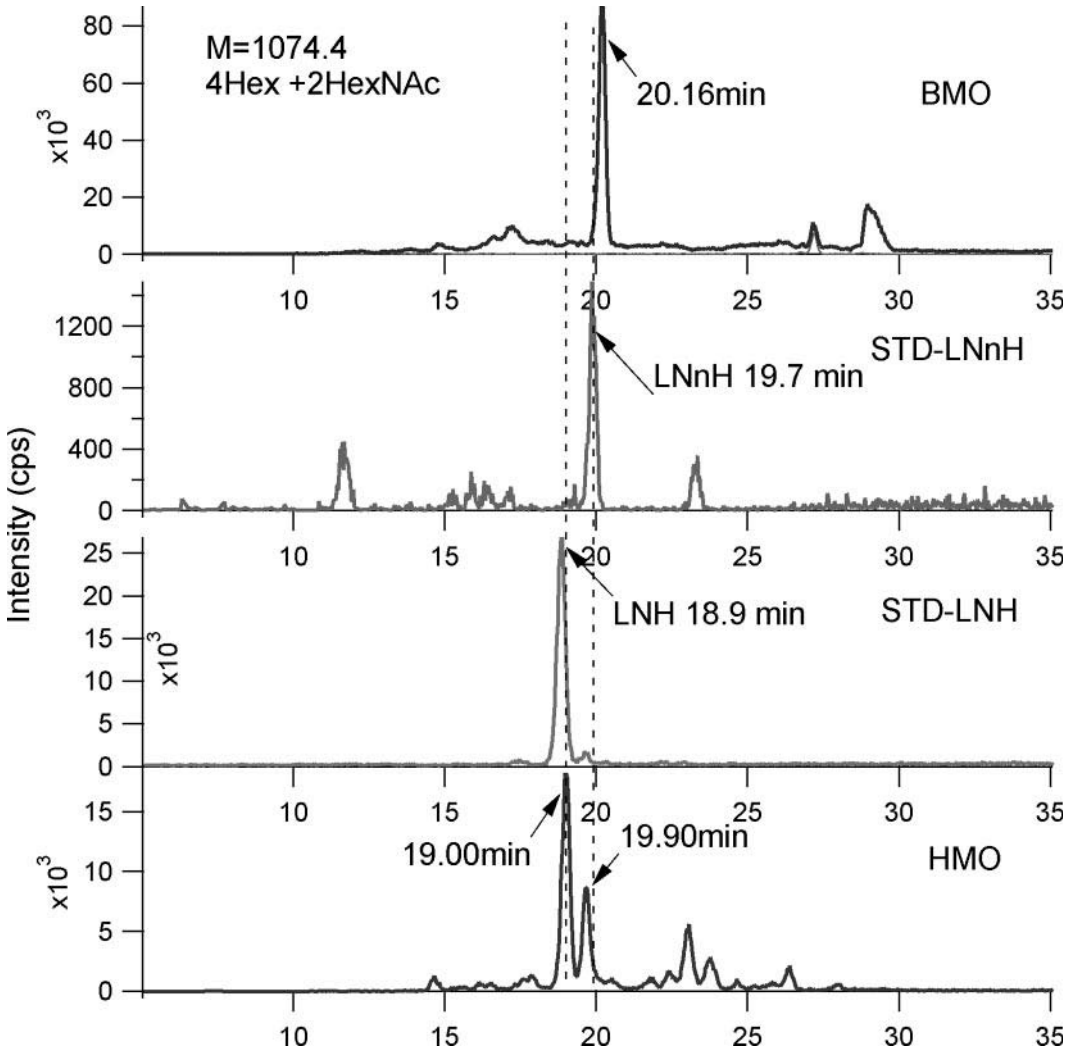


Figure 4. Extracted ion chromatograms for ions with *m/z* 1,074.396 in bovine milk (BMO), human milk (HMO), and standard mixtures [lacto-*N*-hexaose (LNH) and lacto-*N*-neohexaose (LNnH)]. M = *m/z*; Hex = hexose; HexNAc = *N*-acetylhexosamine; NeuAc = *N*-acetyl neuraminic acid.

contrasts to the 15-mer found in humans. Bovine milk OS are based on 2 core disaccharides: lactose and lactose amine; humans do not have lactose amine. In addition, a trisaccharide core is also present in bovine that is composed of a Galß(1–3) or ß(1–6) linked to a lactose. This core structure can be either sialylated or branched with an additional lactose amine (e.g., *m/z* 871.315; Figure 5). The trisaccharide core is either not present or is at very low abundance (Ninonuevo et al., 2006).

The large abundance of sialylated OS further contrasts to that of human milk. The most abundant OS found in the nano-LC profiling were sialyllactose (2Hex+1NeuAc) followed by sialyllactosamine (1Hex+1HexNAc+1NeuAc). These results are supported by additional experiments employing electrospray ionization FTICR MS (results not shown). Several possible functions have been suggested for sialylated oligosaccharides. They act as receptors to pathogens, thereby inhibiting pathogen binding to epithelial cell surfaces (Morrow et al., 2005; Newburg et al., 2005; Bao et al., 2007). Additionally, sialic acids increase the production of gangliosides, important components in membrane receptors and cell surfaces of the nervous system. All mammals have the capacity to synthesize sialic acids; however, newborn infants typically incorporate exogenous sialic acids because of their immaturity, and the rapid growth and development of the brain exceeds their capacity to produce it.

There were 2 types of sialic acids in the free OS of bovine, NeuAc and NeuGc. Cytidine monophosphate-

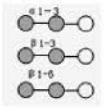
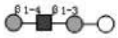
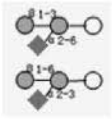
m/z (Exptl)	m/z (Cal)	m/z (Delta)	Hex	Hex NAc	Neu Ac	Neu Gc	Retention time (min)	Abundances	Possible structures	Reference Source
506.1833	506.1846	-0.0013	3				14.762	10289782		Chaturvedi et al., 1988; Urashima et al., 1994;
506.1839	506.1846	-0.0007	3				10.16	1303070		
506.1856	506.1846	0.0010	3				8.995	109094		
547.2098	547.2112	-0.0014	2	1			15.221	11142751		Urashima et al., 1989; Urashima et al., 1991; T;
547.211	547.2112	-0.0002	2	1			9.287	1086602		
635.2263	635.2272	-0.0009	2		1		20.545	311431904		Urashima et al., 1989; T;
651.2211	651.2221	-0.001	2			1	20.007	30130712		Urashima et al., 1989; Veh et al., 1981;
676.2533	676.2538	-0.0005	1	1	1		15.143	15870276		Urashima et al., 1989; Urashima et al., 1997;
676.2523	676.2538	-0.0015	1	1	1		12.759	10912929		
676.257	676.2538	0.0032	1	1	1		17.384	672159		
692.2513	692.24	0.0026	1	1		1	14.54	679863		Kuhn et al., 1965;
692.2464	692.2487	-0.0023	1	1		1	20.237	1200180		
709.2621	709.264	-0.0019	3	1			15.349	19018856		Urashima et al., 1989; Urashima et al., 1991;
750.2902	750.2906	-0.0004	2	2			13.503	6407956		
750.2924	750.2906	0.0018	2	2			14.437	27596110		
797.2782	797.28	-0.0018	3		1		21.823	2041891		T;
797.2809	797.28	0.0009	3		1		24.257	18258772		
813.2835	813.2749	0.0086	3			1	24.13	1307872		T
838.3043	838.3066	-0.0023	2	1	1		15.38	4845144		T
838.3056	838.3068	-0.001	2	1	1		13.809	19874340		

Figure 5. Possible structures of bovine milk oligosaccharides with relative abundance and retention times. Numbers marked shows references source; T = tandem MS available; P = new proposed structures (i.e., not found in reference or tandem MS, but proposed based on other known similar structures); ○ and ● indicate glucose (Glc) and galactose (Gal), respectively; ■ and □ represent *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), respectively; and ◊ and ♦ indicate *N*-acetyl neuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc), respectively. Exptl = experimental; cal = calculated; Hex = hexose; HexNAc = *N*-acetylhexosamines; NeuAc = *N*-acetylneuraminic acid; and NeuGc = *N*-glycolylneuraminic acid.

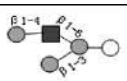
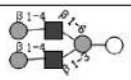
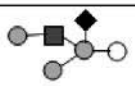
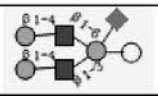
871.3153	871.3168	-0.0015	4	1			16.272	33374806		Urashima et al., 1989; Urashima et al., 1991; T
912.3419	912.3434	-0.0015	3	2			13.842	6936200		
912.3409	912.3434	-0.0025	3	2			14.848	22044166		
926.3209	926.3226	-0.0017	2		2		20.99	1970504		Kuhn et al., 1965;
942.3234	942.3175	0.0059	2		1	1	21.314	427510		P
967.3499	967.3492	0.0007	1	1	2		25.144	5103399		P
967.3502	967.3492	0.001	1	1	2		17.466	246649		
1000.355	1000.359	-0.004	3	1	1		23.717	3736359		
1000.356	1000.359	-0.003	3	1	1		20.134	1148196		
1016.36	1016.354	0.0061	3	1		1	19.83	117576		
1041.386	1041.386	0.0002	2	2	1		21.835	14704525		
1074.398	1074.396	0.0015	4	2			19.58	10433410		Urashima et al., 1989; T;
1162.422	1162.412	0.0101	4	1	1		23.165	4808720		T
1162.411	1162.412	-0.0013	4	1	1		27.174	15932500		
1178.406	1178.407	-0.0012	4	1		1	23.082	944698		P
1203.438	1203.439	-0.0012	3	2	1		26.494	4306686		
1203.438	1203.439	-0.0008	3	2	1		24.79	4756937		
1203.441	1203.439	0.0019	3	2	1		20.785	3956023		
1365.49	1365.491	-0.0014	4	2	1		26.203	3697598		T

Figure 5 (Continued). Possible structures of bovine milk oligosaccharides with relative abundance and retention times. Numbers marked shows references source; T = tandem MS available; P = new proposed structures (i.e., not found in reference or tandem MS, but proposed based on other known similar structures); ○ and ● indicate glucose (Glc) and galactose (Gal), respectively; ■ and □ represent *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), respectively; and ◊ and ♦ indicate *N*-acetyl neuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc), respectively. Exptl = experimental; cal = calculated; Hex = hexose; HexNAc = *N*-acetylhexosamines; NeuAc = *N*-acetylneuraminic acid; and NeuGc = *N*-glycolylneuraminic acid.

NeuAc (CMP-NeuAc) is the substrate for sialyltransferases, but is converted to CMP-NeuGc by active CMP-NeuAc hydroxylase. Then, NeuGc is added, using existing sialyl transferases, to the nascent glycoconjugates. Humans are reported to have only NeuAc (Varki, 2001). The transforming enzyme was either inactivated or removed from humans during our evolutionary split from the great apes. Three NeuGc-linked

oligosaccharides in bMO were previously reported corresponding to trisaccharides; namely, 3′- and 6′-*N*-glycolylneuraminyllactose (2Hex+1NeuGc) (Urashima et al., 2001) and 6′-*N*-glycolylneuraminyl-lactosamine (1Hex+1HexNAc+1NeuGc) (Veh et al., 1981). In this study, we found at least 7 structures containing NeuGc ranging in size from trisaccharides to hexasaccharides in bovine milk.

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