JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Comparison of Milk Oligosaccharides Pattern in Colostrum of Different Horse Breeds

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Supporting Information

ABSTRACT: Colostrum oligosaccharides are known to exhibit prebiotic and immunomodulatory properties. Oligosaccharide composition is species-specific, and equine colostrum has been reported to contain unique oligosaccharides. Therefore, equine oligosaccharides (EMOS) from colostrum from different horse breeds were analyzed by CE-LIF, CE-MS^n, HILIC-MS^n, and exoglycosidase degradation. Sixteen EMOS were characterized and quantified, of which half were neutral and half were acidic. EMOS showed about 63% structural overlap with human milk oligosaccharides, known for their bioactivity. Seven EMOS were not reported before in equine oligosaccharides literature: neutral Gal(β1−4)HexNAc, Gal(β1−4)Hex-Hex, β4′-galactosyllactose, and lactose-*N*-hexaose, as well as acidic 6′-Sialyl-Hex-Ac-HexNAc, sialyllacto-*N*-tetraose-a, and disialylacto-*N*-tetraose (isomer not further specified). In all colostrum samples, the average oligosaccharide concentration ranged from 2.12 to 4.63 g/L; with β 6′and 3′- galactosyllactose, 3′-sialyllactose, and disialyllactose as the most abundant of all oligosaccharides (27−59, 16−37, 1−8, and 1−6%, respectively). Differences in presence and in abundance of specific EMOS were evident not only between the four breeds but also within the breed.

KEYWORDS: *milk, CE-MS, HILIC-MS, exoglycosidases, mass analysis, chromatography*

INTRODUCTION

Mammalian milk and colostrum contain many structures, which are essential for the nutrition and health of the suckling neonate. Throughout the past decades, specific oligosaccharides, which have been identified in milk and colostrum, have been shown to beneficially affect health.[1] For example, human milk oligosaccharides (HMOS) carrying the so-called bifidogenic factor (Gal(β1−3)GlcNAc) have been illustrated to enhance the growth of bifidobacteria in the intestine of neonates.[2] When bifidobacteria selectively ferment HMOS, they produce short-chain fatty acids (SCFA), leading to a pH decrease in the intestine. The lowered pH in the intestine creates a less favorable environment for the growth of the pathogens and stimulates the production of mucin, which in turn, enhances the host's resistance to infections.[3] The production of SCFA also leads to a stimulation of the immune system through a direct interaction with the immune cells of the intestine.[3,4] Moreover, increasing evidence has come forward to demonstrate direct immunomodulatory properties of oligosaccharides derived from milk and colostrum. Some properties could derive from interaction with specific receptors on intestinal epithelial cells and immune cells, such as carbohydrate receptors and Toll-like receptors.[4−6] In addition, milk oligosaccharides serve as soluble ligands for pathogens, preventing viral and bacterial attachment to the intestinal mucosa.[5]

The core structure of mammalian milk oligosaccharides (MMOs) is, in most cases, a lactose unit (Gal(β1−4)Glc) at the reducing end. However, in cow's, horse's, and goat's milk, *N*-acetyllactosamine (Gal(β1−4)GlcNAc) is also found at the reducing end instead of the lactose unit.[7,8] Starting from these disaccharides, glycosyltransferases can elongate the core structure with neutral and charged sugars. The neutral sugars that can be attached to the core structure are D-galactose (Gal), L-fucose (Fuc), *N*-acetylglucosamine (GlcNAc), and with the exception of HMOS, also *N*-acetylgalactosamine (GalNAc).[7]

The charged sugars that can decorate the core structure are sialic acids, both *N*-acetyl- and *N*-glycolyl-neuraminic acids, and glucose or *N*-acetylglucosamine substituted with one phosphoric group (e.g., in milk oligosaccharides of horses and cattle).[7−9] Depending on the linkage present in their chains, the MMOs are divided into two groups: Type I group contains Gal(β1−3)GlcNAc, and Type II group contains Gal(β1−4)GlcNAc.[10]

The reported mature-milk oligosaccharide concentration is 12−13 g/L for human milk and approximately 0.05 g/L for cow's milk.[8,11] The oligosaccharide concentration in colostrum is higher than the concentration in mature milk: 24 g/L in human colostrum and more than 1 g/L in cattle's colostrum.[7,12] So far, 43 equine milk oligosaccharides (EMOS) have been reported, of which 31 are neutral and 12 are acidic.[7,8,13] Compared to the HMOS, the EMOS have a higher degree of sialylation (20−30% and 60%, respectively)[11] and a lower degree of fucosylation (70% and 5%, respectively).[11] The previously documented EMOS showed a 17% overlap with oligosaccharides present in human milk. Although not reported so far, the presence and relative abundance of individual EMOS

Received: March 3, 2015
Revised: April 29, 2015
Accepted: April 30, 2015
Published: April 30, 2015

DOI: 10.1021/acs.jafc.5b01127
J. Agric. Food Chem. 2015, 63, 4805−4814

may vary between individual animal breeds, comparable to the variations in HMOS in different human populations.[14−16]

This study focuses on the analysis of EMOS in colostrum samples of four different horse breeds: Dutch Warmblood horse, Shetland pony, Crossbred Arabian/New Forest pony, and Friesian horse. After isolation from the colostrum samples, the EMOS were identified and quantified, highlighting the interbreed (EMOS for the different horse breeds) and the intrabreed (EMOS for the same breed) variations.

■ MATERIALS AND METHODS

Materials. Colostrum samples of horses belonging to four different breeds were obtained from private Dutch owners with consent. Five colostrum samples were available for the Crossbred Arabian/New Forest pony (Nederlands Rijpaarden and Pony Stamboek), four colostrum samples were available for the Dutch Warmblood horse (Royal Warmblood Studbook of The Netherlands), two colostrum samples were available for the Friesian horse, and one colostrum sample was available for the Shetland pony. Each colostrum was collected within 12 h postpartum and frozen (−20 °C) until use.

The standards 3′-, and 6′-sialyllactose, 3′-, and 6′-sialyl-*N*-acetyllactosamine, lacto-*N*-tetraose, sialyllacto-*N*-tetraose-a, and lacto-*N*-hexaose were purchased from Dextra Laboratories (Reading, U.K.). The standards β 3′-, 4′- , and 6′-galactosyllactose, lacto-*N*-tetraose, and lacto-*N*-neotetraose were bought from Carbosynth (Compton, U.K.). The labeling of the oligosaccharides was accomplished using the Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, CA, U.S.A.). All other chemicals used were of analytical grade. Millipore water (Millipore, Darmstadt, Germany) was referred to as water in the text.

Extraction of Equine Colostral Sugars. The Dutch Warmblood colostrum samples were available in larger quantities than those of other breeds. Therefore, a mixture of the Dutch Warmblood colostrum samples was used to optimize the extraction method and to obtain enough material to be loaded in the preparative Size Exclusion Chromatography. Specifically, 1.25 mL of each colostrum sample was pooled together and mixed with a vortex for 2 min. After method optimization, EMOS were extracted from 1 mL of the 12 individual colostrum samples. The optimized sugar extraction from the colostrum samples was performed by modification of the method of Nakamura et al.[13] Briefly, colostrum (5 mL) was treated with 4 volumes of chloroform. After 2 h of head over tail mixing, the sample was centrifuged (5000*g*, 30 min, 5 °C), and the supernatant was treated with 2 volumes of methanol. After the same mixing and centrifugation procedure, the methanol−water solution was concentrated by vacuum evaporation. The remaining water solution was freeze-dried.

Purification and Fractionation of EMOS. Size Exclusion Chromatography (SEC) was used to remove lactose from the extracted carbohydrates of the pooled Dutch Warmblood colostrum, in order to obtain pools containing EMOS with difference degrees of polymerization (DP). In total, four pools were obtained, and they were analyzed for the characterization of the EMOS. The fractionation was carried out at 35 °C on three Superdex 30 Hiload 26/60 preparative grade columns (GE Healthcare, Pittsburgh, PA, U.S.A.) connected in series on a AKTA Purifier (GE Healthcare). After manual injection, the EMOS (200 mg) were eluted with water containing 0.5% (v/v) EtOH and collected in 9 mL fractions. The fractions were pooled as follows: pool 1: 290−492 mL, pool 2: 493−688 mL, pool 3: 689−730 mL, and pool 4: 731−758 mL. Using water solution as eluent, charged oligosaccharides were excluded from the column material and were eluted first. Neutral oligosaccharides were instead fractionated based on their size. Lactose and monomers were the last to be eluted and were excluded from further analysis. The neutral oligosaccharides were pooled according to the retention times shown by a mixture of neutral galacto-oligosaccharides (DP from 1 to 6) (Vivinal GOS syrup) (FrieslandCampina Domo, Borculo, The Netherlands), which was considered a reference for its retention time. After freeze-drying, the resulting powders were rehydrated with 1 mL of water prior to analysis.

Solid Phase Extraction (SPE) was used to reduce the lactose content from the 12 individual colostrum samples. The extracted carbohydrates (1 mg) were solubilized in 1 mL of water and loaded onto activated graphitized carbon cartridge (150 mg bed weight, 4 mL tube size; Grace, Deerfield, IL, U.S.A.). Elution with water (1.5 mL) was used to remove salts, and 2% (v/v) acetonitrile (ACN) (1.5 mL) was used in order to elute monomers and lactose. The EMOS, still bound to the graphitized carbon cartridge, were recovered with 1.5 mL of 40/60% (v/v) ACN/water solution containing 0.05% (v/v) trifluoroacetic acid. The obtained EMOS were dried overnight under a stream of nitrogen and subsequently solubilized in 0.5 mL of water.

Capillary Electrophoresis with Laser-Induced Fluorescent and Mass Spectrometry Detection. The EMOS fractionated either by SEC or SPE were labeled with a fluorescent label (9-aminopyrene-1,4,6-trisulfonate, APTS) for their analysis by capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection as reported previously with minor modifications.[17] Briefly, 100 μL of sample containing 5 nmol of xylose, as internal standard, was dried and labeled with APTS. Each oligosaccharide standard (10 nmol), containing 5 nmol of xylose, was labeled with the APTS and run together with the EMOS samples. The labeled samples were diluted 40 times before the analysis. The experiment was performed using a ProteomeLab PA 800 system (Beckman Coulter), equipped with a LIF detector (Beckman Coulter). During the run, the sample was loaded hydrodynamically (7 s at 0.5 psi) into a polyvinyl alcohol (NCHO) coated capillary (50 μm × 50.2 cm (Beckman Coulter). Resultant peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA, U.S.A.). In the CE technique, the oligosaccharides are linked in a molar ratio of 1:1 with the fluorescent, negatively charged label (APTS). The resulting migration times of the linked oligosaccharides depend on their size and on their net charge.

The SEC-fractionated, labeled EMOS were also analyzed for their masses by the PA 800 plus system (Beckman Coulter) coupled to a Velos Pro mass spectrometer (LTQ Velos Pro ion trap MS, Thermo Scientific, Waltham, MA, U.S.A.), after 20 times dilution. The CE-MS^n experiment conditions were performed as reported by Albrecht et al.[18]

HILIC-ESI-MS^n. The EMOS pools, derived from SEC, were also analyzed through hydrophilic interaction liquid chromatography (HILIC) with mass spectrometry detection (MS^n) as described by Remoroza et al. with some minor modifications.[19]

Briefly, an Accela UHPLC system (Thermo Scientific) coupled to a mass spectometer (LTQ Velos Pro ion trap MS, Thermo Scientific) was used. The chromatographic separation was performed on an Acquity HILIC BEH Amide column (1.7 μm, 2.1 mm × 150 mm) combined with a Van Guard precolumn (1.7 μm, 2.1 mm × 5 mm; Waters Corporation, Milford, MA, U.S.A.). The acquisition time was 72 min, and the eluents had a flow rate of 600 μL/min. The injection volume was 5 μL. The composition of the three mobile phases were (A) water with 1% (v/v) ACN, (B) 100% (v/v) ACN and (C) 200 mM ammonium formate (pH 3.0). The elution program was performed as follows: 2 min isocratic 80% B; 58 min linear gradient from 80% to 30% B; followed by 12 min of column washing with a linear gradient from 30% to 20% B and column re-equilibration form 20 to 80% B. The eluent C was kept at 5% during the elution.

Exoglycosidase Degradation. To determine the linkage type of the sialic acid decorating the EMOS, two types of sialidases were used: Sialidase S and Sialidase T (ProZyme, Hayward, CA, U.S.A.). Sialidase S is specific for the α2−3 linked neuraminic acid, while Sialidase T splits α 2−3 and α 2−6 linked neuraminic acid. For the structural analysis of the neutral EMOS Glyco(β1−3)galactosidase, Glyco(β1−4)galactosidase, Glyco(β1−4,6)galactosidase, and Glykoβ-*N*-acetylhexosaminidase (ProZyme) were used. All reactions were performed as suggested by the manufacturer. After the enzyme degradation at 37 °C overnight, the solutions were centrifuged (5000*g*, 5 min, 25 °C), and the supernatants were analyzed by HILIC-MS^n.

Quantification of the EMOS. In CE-LIF, the quantification of the oligosaccharides was enabled by the use of an internal standard and by a 1:1 stoichiometry between the oligosaccharide and the APTS

DOI: 10.1021/acs.jafc.5b01127
J. Agric. Food Chem. 2015, 63, 4805−4814

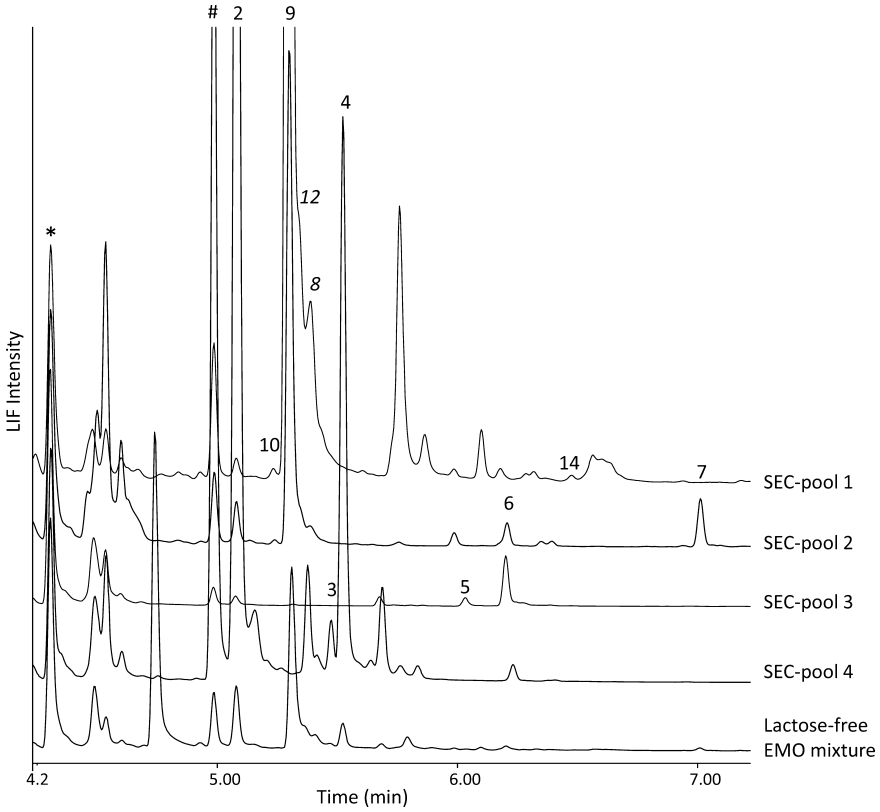

Figure 1. CE-LIF electropherograms of lactose-free EMO mixture and the EMO SEC pools 1−4. *: Internal standard xylose, #: Lactose, 1−14: identified peaks as named in Table 1

molecule. A linear correlation between concentration and CE-LIF peak area has been shown.[17] CE-LIF peak areas were converted to the corresponding EMOS nanomoles and their concentrations in the colostrum samples (g/L). The samples were run in duplicate, and the quantification was validated with the help of labeled neutral and acidic oligosaccharides standards. The quantification of 3′-sialyl-*N*-acetyllactosamine (3′-SLN) and Gal(β1−4)Hex-Hex, annotated by HILIC-MS^n and not assigned in the CE-LIF electropherograms, was performed with HILIC-MS^n. A calibration curve was made based on the peak areas in mass spectrometry of the 3′-SLN and β 4′-galactosyllactose (β 4′-GL) standards, respectively. The curves fitting the 3′-SLN and β 4′-GL standards had a linear correlation with R^2 of 0.997 and 0.998, respectively.

■ RESULTS AND DISCUSSION

Structural Characterization of the Equine Milk Oligosaccharides. In order to elucidate the structure of the EMOS, a colostrum mixture was prepared using Dutch Warmblood colostrum. After sugar extraction, part of the oligosaccharides was fractionated using Size Exclusion Chromatography (SEC). A lactose-free EMO mixture was obtained pooling part of the SEC fractions. Lactose-free EMO mixture and four SEC pools obtained were labeled with the fluorescent dye APTS and subsequently analyzed by CE-LIF and CE-MS^n.

In Figure 1, CE electropherograms of labeled lactose-free EMO mixture and SEC pools are shown. The migration times of labeled EMOS were compared with available standards and with HMOS elution patterns, which have been described in literature.[20] In total, 24 peaks were counted of which 11 peaks were annotated (numbers 2−14, Figure 1). Together with remaining lactose after SEC (symbol #, Figure 1), the annotation of five oligosaccharides was confirmed by CE-MS^n analysis. Acidic oligosaccharides were the first eluted from the SEC columns, and they were mainly pooled in pool 1. Pool 2 contained sialyllactose together with neutral pentamer and hexamer oligosaccharides. Pool 3 and pool 4 contained neutral tetramer and trimer, respectively.

In the CE-MS^2, the APTS-labeled oligosaccharides showed a specific fragmentation behavior due to the fluorescent molecule attached at their reducing end and a multiple charge state in their fragments spectrum. One example of CE-MS^n identification is given for lacto-*N*-novo-pentaose I, previously identified in equine milk, and shown in Figure 2.[8,21] APTS-lacto-*N*-novo-pentaose I loses one terminal galactose yielding a APTS-tetramer fragment, with m/z 573 and m/z 382, having charge states −2 and −3, respectively. The APTS-tetramer fragment loses a terminal galactose producing the fragment GlcNAc-Gal-Glc-APTS with m/z 492 and 328, having charge states −2 and −3, respectively. The GlcNAc-Gal-Glc-APTS subsequently forms APTS-lactose, with m/z 391, having charge state −2, via the loss of the terminal *N*-acetyl-glucosamine. From the fragmentation of the APTS-lactose, a molecule of APTS-glucose was formed, with m/z 310, having charge state −2.

In order to enhance the characterization of the EMOS, the mass analysis was also performed with HILIC-MS^n with non-labeled oligosaccharides obtained after SEC fractionations. With this technique, five more EMOS were annotated. All found molecules had a charge state of −1. One example for HILIC-MS^n identification of 3′-sialyl-*N*-acetyllactosamine (3′-SLN) is shown in Figure 3. Two peaks are highlighted for the sialyl-*N*-acetyllactosamine (Figure 3a). The highest peak refers to 3′-SLN, while the lowest peak refers to 6′-SLN. Each of the molecules were found as a double peak. It is hypothesized that

DOI: 10.1021/acs.jafc.5b01127
J. Agric. Food Chem. 2015, 63, 4805−4814

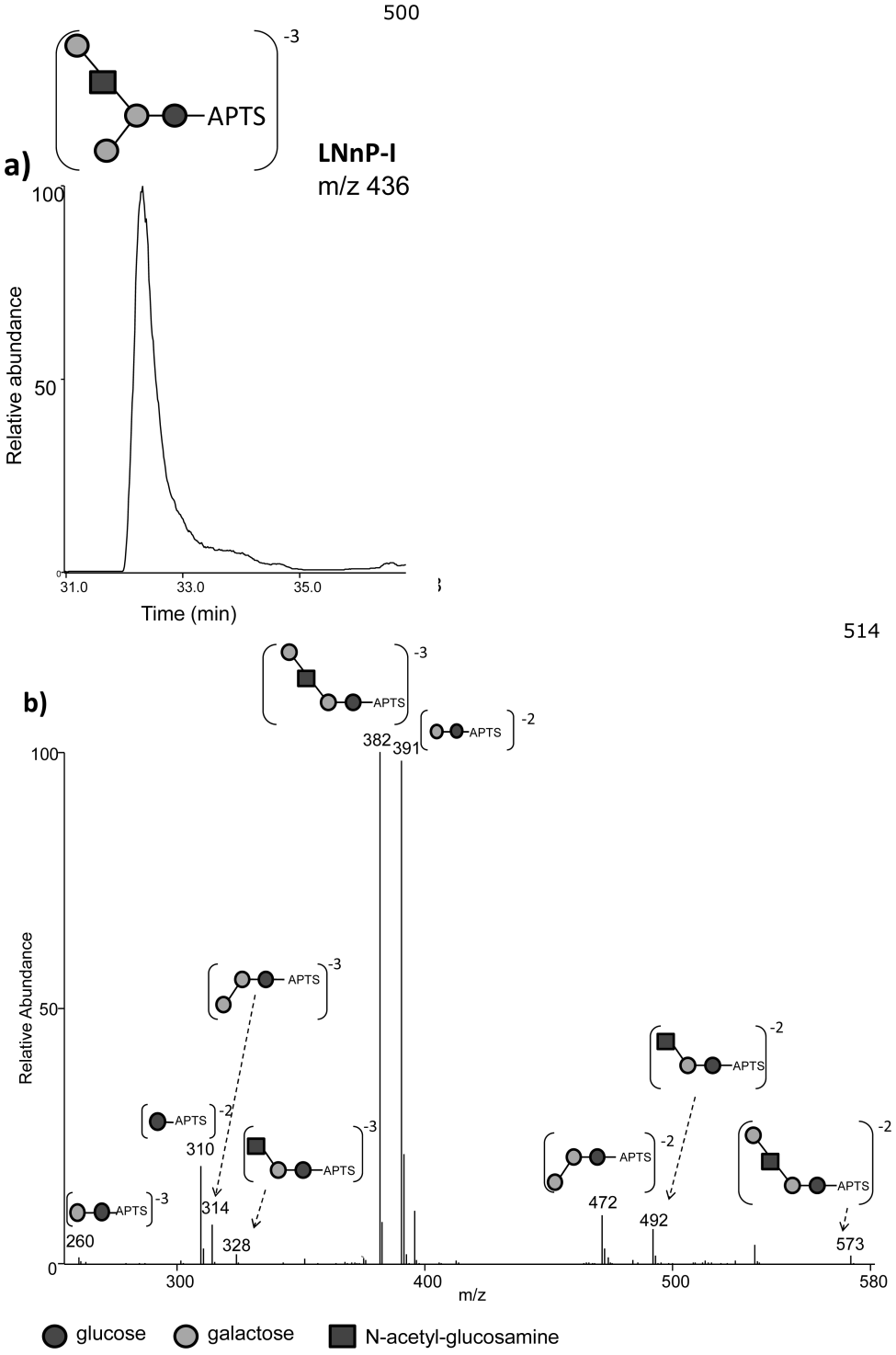

Figure 2. Selected base peak for the lacto-*N*-novo-pentaose-I (LNnP-I) in CE-MSn as present in lactose-free EMO mixture (a). Corresponding MS2 fragmentation patterns and structural composition of LNnP-I (b). *m/z* 436 precursor ion in CE-MS^n. [−2], [−3]: charge state of the molecules formed after fragmentation.

the double peaks correspond to the α/β conformation of the reducing end.[22] The MS^2 fragmentation of the 3′-SLN was compared with existing literature and with MS^2 fragmentation of available standard.[23] The 3′-SLN loses water (Figure 3b), forming thereby the fragment with *m/z* 655. A characteristic intraring fragmentation of the molecule leads to the formation of the fragment with *m/z* 572. The fragment with the highest intensity refers to the *N*-acetylneuraminic acid, with *m/z* 290.

With respect to four milk oligosaccharides, enzymatic degradation was necessary to determine their linkages. The neutral dimer Gal(β1−4) HexNAc and the trimer Gal(β1−4)Hex-Hex could be characterized only by using Glycogalactosidases specific for β1−3, 1−4, and 1−4, 6 linkages, in addition to using the Glykoβ-*N*-acetylhexosaminidase. The acidic 6′-sialyl-Hex[Ac]-HexNAc and 3′-sialyllacto-*N*-tetraose a (LSTa), could be characterized only by using Sialidase S

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J. Agric. Food Chem. 2015, 63, 4805−4814

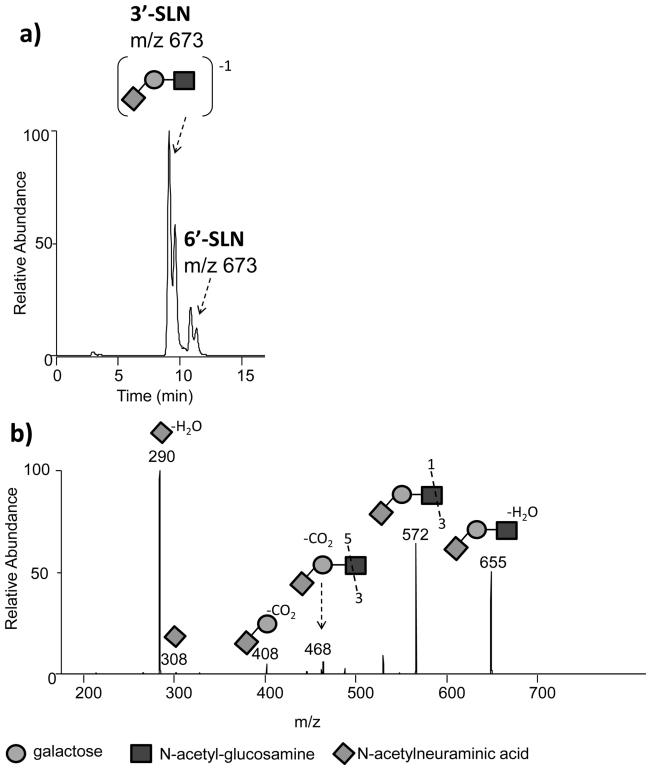

Figure 3. Selected base peak for the 3′-sialyl-*N*-acetyllactosamine (3′-SLN) in HILIC-MS^n as present in lactose-free EMO mixture (a). Corresponding MS^2 fragmentation patterns and structural composition of 3′-SLN as found in the lactose-free EMO mixture (b). *m/z* 673 precursor ion in HILIC-MS^n. [−1]: charge state of molecules.

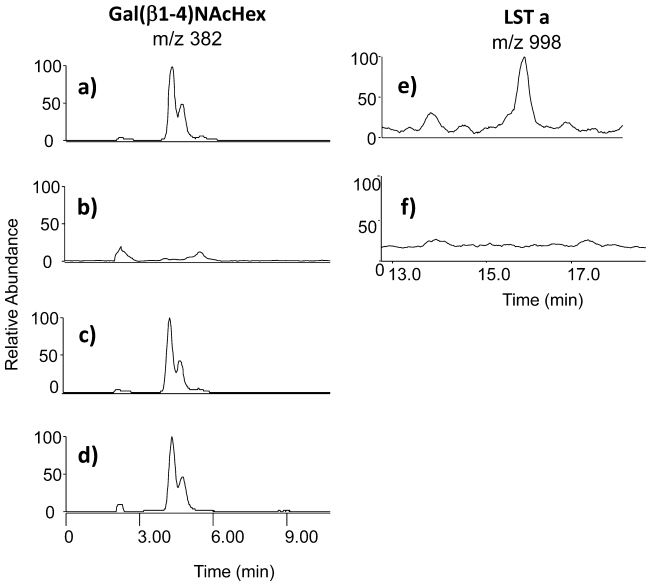

Figure 4. Selected base peak for the Gal(β1−4)HexNAc in HILIC-MS^n (a) as present in lactose-free EMO mixture. Corresponding base peak after degradation by Glyco-galactosidases specific for β1−4 linkage (b), by Glyco-galactosidases specific for β1−3 linkage (c), by Glykoβ−*N*-acetylhexosaminidase (d). Selected base peak for the 3′-sialyllacto-*N*-tetraose-a (LSTa) in HILIC- MS^n as present in lactose-free EMO mixture (e). Corresponding base peak after degradation by Sialidase S specific for the α2−3 linkage (f). *m/z* 382 precursor ion for Gal(β1−4)HexNAc in HILIC-MS^n; *m/z* 998 precursor ion for LSTa in HILIC-MS^n.

(specific for the α2−3 linked *N*-acetylneuraminic acid), Sialidase T (specific for α2−3 and 6 linked *N*-acetylneuraminic acid), and Glykoβ-*N*-acetylhexosaminidase. After their enzyme degradation, the samples were analyzed by HILIC-MS^n. As an example, Figure 4 illustrates the enzymatic degradation of Gal(β1−4)HexNAc and LSTa prior to analysis by HILIC-MS^n. The Gal(β1−4)HexNAc was found as double peak (Figure 4a), as consequence of α/β conformation of the reducing end. Using the enzymes tested, Gal(β1−4)HexNAc was digested by Glyco(β1−4)galactosidase (Figure 4b), while neither Glyco-(β1−3)galactosidase nor Glyko-*N*-acetylhexosaminidase showed any activity, indicating the presence of a β1−4 linked, terminal galactose. The LST molecule, as found in the mare colostrum samples, was digested by Sialidase S (Figure 4f), proving the presence of a terminal *N*-acetylneuraminic acid α 2−3 linked. Moreover, the identification of LSTa was confirmed by its migration time in CE, similar to commercially available LSTa standard (Table S1, Supporting Information).

Through the combination of CE and HILIC mass analysis and enzyme degradation of the SEC pools, it was possible to identify 16 EMOS. All identified EMOS are listed in Table 1, and an overview of methods used in order to characterize them (CE migration time, mass over charge values, and retention times in CE-MS^n, and HILIC-MS^n, and enzymes) are described in Supporting Information (Table S1). Comparing the outcomes of this study with previous data, seven EMOS were not reported previously and were presented in Table 1 in **bold**: the neutral oligosaccharides Gal(β1−4)HexNAc, Gal(β1−4)Hex-Hex, β4′-galactosyllactose, and lactose-*N*-hexaose, as well as the acidic oligosaccharides 6′-sialyl-Hex-Ac-HexNAc, LSTa, and disialylacto-*N*-tetraose (isomer not further specified).[7,8] Full identification was possible for six of the novel EMOS, while partial identification was possible for three of the novel EMOS. For 6′-sialyl-Hex-Ac-HexNAc, the presence of an additional acetyl group (Ac) was confirmed by mass fragmentation. An additional acetyl group linked to acidic milk oligosaccharides has previously been reported in equine colostrum.[8] The additional acetyl group was reported to be linked to the sialic acid group, while in our study it was found to be linked to the hexose moiety.

Comparison of the Equine Milk Oligosaccharides per Breed. After annotation of the EMOS, peaks in the CE-LIF electropherogram of the lactose-free EMO mixture were assigned. The peaks assigned were used as a reference for the identification of EMOS in the CE profiles of the 12 colostrum samples of the four horse breeds. In Figure 5, the EMO profiles of the four breeds are shown, including the peak assignments. The CE-LIF electropherograms of the labeled EMOS showed high reproducibility and high peak resolution.

Interbreed EMOS Comparison. The comparison of the EMO profiles for the four breeds (A, B, C, and D in Figure 5) reveals that the presence and relative abundance of the different oligosaccharides varied depending on the breed. Quantification was carried out after manual integration of CE-LIF peaks area. For two compounds, Gal(β1−4)Hex-Hex and 3′-sialyl-*N*-acetyllactosamine, the assignment in the CE-LIF EMO profiles was not possible. For these two molecules, the quantification was obtained with HILIC-MS^n.

The total concentrations of EMOS varied between the different colostrum samples: 4.63 g/L for the Shetland pony, 1.81−6.71 g/L for the Crossbred Arabian/New Forest ponies, 1.42−2.75 for the Dutch Warmblood horses, and 1.76−2.48 g/

DOI: 10.1021/acs.jafc.5b01127
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Table 1. Presence and Concentration of Equine Milk Oligosaccharides for the Four Breeds' Colostrum Samples[a]

degree of polymerization	EMOS structures	abbreviation	MW	peak CE-LIF (Figure 1)	concentration of equine milk oligosaccharide (g/L)											
					Dutch Warmblood				crossbred Arabian/New Forest ponies					Shetland pony	Friesian	
					A1	A2	A3	A4	B1	B2	B3	B4	B5	C1	D1	D2
	neutral															
dimer	**Gal(β1−4)HexNAc**		383	1	0.11	0.14	0.05	0.08	0.14	0.11	0.38	0.09	0.51	0.22	0.29	0.13
trimer	**Gal(β1−4)Hex-Hex**		504	na	++	++	++	+	+	+	+	++	+	+	——	——
	β6′-galactosyllactose	β6′-GL		2	0.98	2.08	0.65	0.84	0.83	0.55	1.64	0.83	3.15	1.91	1.20	0.88
	β4′-galactosyllactose	β4′-GL		3	tr	tr	tr	tr	tr	tr	——	——	——	——	tr	tr
	β3′-galactosyllactose	β3′-GL		4	0.16	0.16	0.10	0.09	0.23	0.08	0.04	0.14	0.23	0.11	0.05	0.05
tetramer	lacto-*N*-neotetraose	LNnT	708	5	0.02	tr	0.13	0.02	0.39	0.21	0.13	0.26	0.09	0.10	0.07	0.03
pentamer	lacto-*N*-novo-pentaose I	LNnP I	870	6	0.03	0.08	0.09	tr	0.05	0.04	0.04	0.04	0.11	0.06	tr	tr
hexamer	**lacto-*N*-hexaose**	LNH	1073	7	0.03	0.05	0.02	tr	0.04	0.03	0.02	0.04	0.07	0.04	tr	0.01
	acidic															
trimer	6′-sialyl-*N*-acetyllactosamine	6′-SLN	675	8	0.07	——	——	0.01	0.12	0.02	0.06	0.01	0.02	0.02	0.07	0.19
	3′-sialyl-*N*-acetyllactosamine	3′-SLN		na	0.05	0.03	0.02	0.02	0.01	0.003	0.01	0.02	0.03	0.02	——	——
	3′-sialylactose	3′-SL	633	9	1.00	1.16	0.71	0.29	0.48	0.38	0.69	0.44	1.75	1.73	0.53	0.33
	6′-sialyllactose	6′-SL		10	0.10	0.15	0.07	0.03	0.12	0.06	0.12	0.03	0.23	0.09	0.09	0.02
	6′-sialyl-Hex-Ac-HexNAc		716	11	tr	tr	tr	tr	0.10	0.05	0.01	0.06	0.02	0.03	tr	0.02
tetramer	disialyllactose	DSL	925	12	0.16	0.13	0.04	0.02	0.09	0.06	0.21	0.02	0.40	0.23	0.13	0.07
pentamer	**sialyllacto-*N*-tetraose-a**	LSTa	999	13	0.02	0.03	0.06	0.02	0.27	0.16	0.83	0.16	0.06	0.07	0.05	tr
hexamer	**disialyllacto-*N*-tetraose***	DS-LNT*	1290	14	0.02	tr	tr	tr	0.15	0.06	0.03	0.05	0.04	tr	tr	0.03
total oligosaccharides concentration					2.75	4.01	1.94	1.42	3.02	1.81	4.21	2.19	6.71	4.63	2.48	1.76
average oligosaccharides concentration per breed					2.52				3.58					4.63	2.12	

[a]na = peak not assigned in CE-LIF and quantified by HILIC-MS^n; ++ = 0.001−0.002; + = 0.001−0.0002 g/L; tr = detectable but not quantifiable; —— = not detected; the symbol * = isomer not further specified. Structures in **bold**: oligosaccharides novel for the equine milk literature.[8,21,34]

DOI: 10.1021/acs.jafc.5b01127
J. Agric. Food Chem. 2015, 63, 4805−4814

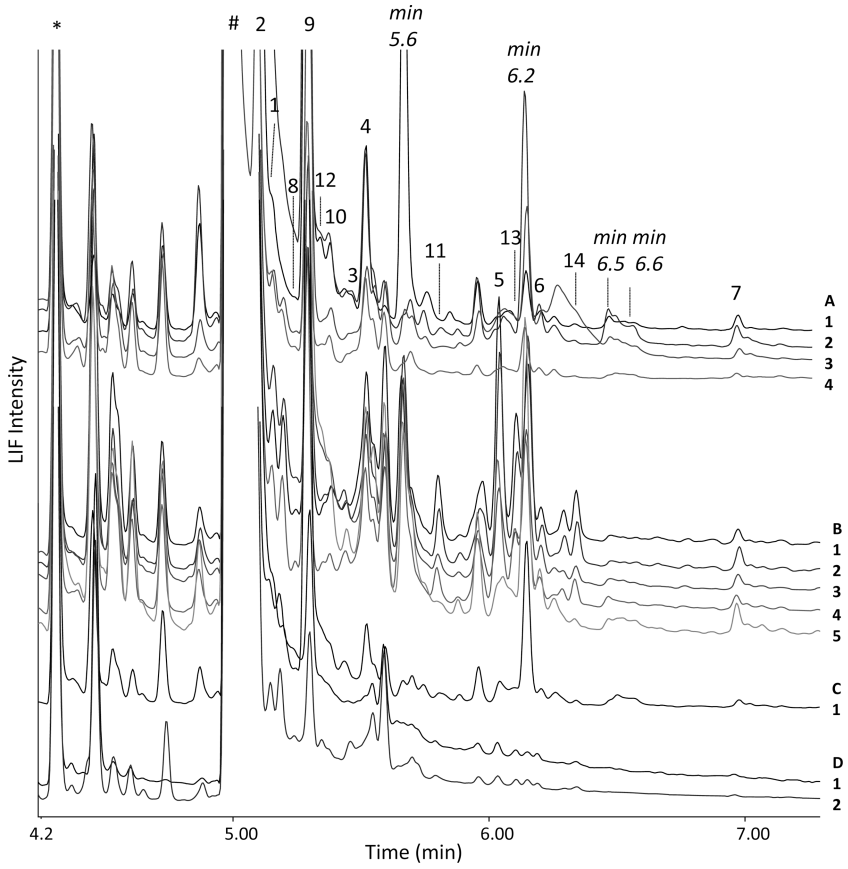

Figure 5. CE-LIF electropherograms of APTS-derivatized oligosaccharides for the four breeds. A: Dutch Warmblood horse; B: Crossbred Arabian/New Forest pony; C: Shetland Pony; D: Friesian horse. *: Internal standard xylose, #: Lactose, 1−14: identified peaks as named in Table 1

L for the Friesian horses (Table 1). Oligosaccharide concentrations found in this study are higher than concentration found in cow colostrum (about 1 g/L).[8] For other animals, the oligosaccharide concentration has only been reported for mature milks with levels (0.02 to 0.25 g/L), as expected, lower than the values obtained in this study for equine colostrum.[24] Although a large variation in the total concentration of EMOS was observed, it was noticed that the ponies' colostrum samples contained the highest concentration of EMOS, followed by the Dutch Warmblood horses' and the Friesian horses' colostrum samples.

Twelve out of the 16 EMOS were present in all colostrum samples. Gal(β1−4)Hex-Hex and 3′-sialyl-*N*-acetyllactosamine were absent in the Friesian horses' colostrum samples, β4′-galactosyllactose was absent in the Crossbred ponies' colostrum samples, and 6′-sialyl-*N*-acetyllactosamine was absent in the Dutch Warmblood horses' colostrum samples (Table 1).

In all breeds, the most abundant oligosaccharides were β3′-galactosyllactose, β6′-galactosyllactose, 3′-sialyllactose, and disialyllactose (numbers 2, 4, 9, and 12 in Table 1). The highest concentrations of β6′-galactosyllactose, β3′-sialyllactose, and disialyllactose were found in the colostrum sample of the Shetland pony (concentrations of 1.91 g/L, 1.73 g/L, and 0.23 g/L, respectively). These values for EMOS present in Shetland pony colostrum are only an indication, because only one colostrum sample was available for the analysis. The highest concentration of the β3′-galactosyllactose was found in the colostrum samples of the Crossedbred ponies, with an average concentration of 0.14 g/L.

In profiles A1 and B1−5 (Figure 5) high concentrations of non-characterized EMOS, migrating at 5.6 and 6.2 min, were observed. From the CE migration time, it could be suggested that these are neutral trimers and pentamers, respectively. The unidentified peaks with a retention time of 6.5 and 6.6 min (Figure 5), suggesting acidic or neutral hexamers, were present in all colostrum samples.

The oligosaccharide with the lowest observed concentration in all colostrum samples was 3′-sialyl-*N*-acetyllactosamine with an average concentration of 0.02 g/L.

Intrabreed EMOS Comparison. Evaluating the EMOS profiles within the same breed, differences were also noticed. Specifically, for the Dutch Warmblood profiles, β6′-galactosyllactose and 3′-sialylactose (numbers 2 and 9 in Table 1) were the most abundant oligosaccharides, and their concentrations varied by a factor 3.2 and 4, respectively. β6′-Galactosyllactose was present with a concentration of 0.65−2.08 g/L, while 3′-sialylactose with was present with a concentration of 0.29−1.16 g/L.

Looking at the overall electropherograms, the Crossbred ponies' colostrum samples contained the same EMOS structures, although they highly varied in concentration. β4′-Galactosyllactose, however, was not detected in three colostrum samples (B3−5, Table 1). In the Crossbred ponies' colostrum samples, similarly to the Dutch Warmblood horses' colostrum samples, the most abundant oligosaccharides were the β6′-galactosyllactose and the 3′-sialylactose. The β6′-galactosyllactose was present in a concentration of 0.55−3.15 g/L, while the 3′-sialylactose with was present in a concentration of 0.38−1.75 g/L, exhibiting a 5.7 and a 4.6 factor, respectively. Intrabreed

DOI: 10.1021/acs.jafc.5b01127
J. Agric. Food Chem. 2015, 63, 4805−4814

Table 2. EMOS Found in the Dutch Warmblood Horses' Colostrum and Their Presence in Other Mammalian Milk Samples[a]

degree of polymerization	EMOS structures (Dutch Warmblood milk)[8,21,34]	milk			
		cow[7,9]	sheep[7,27]	goat[7,27]	human[10,20]
	neutral equine milk oligosaccharides				
dimer	**Gal(β1−4)HexNAc**	——	——	——	——
trimer	**Gal(β1−4)Hex-Hex**	——	——	——	——
	β6′-galactosyllactose	X	X	X	X
	β4′-galactosyllactose	X	——	——	X
	β3′-galactosyllactose	X	X	X	X
tetramer	lacto-*N*-neotetraose	——	——	——	X
pentamer	lacto-*N*-novo-pentaose I	X	——	——	——
hexamer	**lacto-*N*-hexaose**	——	——	X	X
	acidic equine milk oligosaccharides				
trimer	6′-sialyl-*N*-acetyllactosamine	X	——	——	——
	3′-sialyl-*N*-acetyllactosamine	X	X	X	——
	3′-sialylactose	X	X	X	X
	6′-sialyllactose	X	——	X	X
	6′-sialyl-Hex-Ac-HexNAc	——	——	——	——
tetramer	disialyllactose	X	——	X	X
pentamer	**sialyllacto-*N*-tetraose-a**	——	——	——	X
hexamer	**disialyllacto-*N*-tetraose***	——	——	——	X

[a]X = present; —— = not present; the symbol * = isomer not further specified. Structures in **bold**: oligosaccharides novel for the equine milk literature.[8,21,34]

analysis showed relatively high variation in EMOS concentration (Table 1). Most probably the variation in EMOS concentration could be dependent on the intrinsic variation in composition and in quality of colostrum among individuals, as shown before for cow milk and colostrum.[25,26]

Presence of Equine Milk Oligosaccharides Structures in Comparison with the Profile in Milk of Other Mammals. Ten out of the 16 EMOS found in this study were also found in milk samples from other domestic animals (Table 2). So far, β6′- and 3′- galactosyllactose, 3′-sialylactose, and 3′-sialyl-*N*-acetyllactosamine have been found in bovine, caprine, and ovine milk, while 6′-sialyllactose and disialyllactose have been found both in bovine and caprine milk.[7,27] Lacto-*N*-hexaose was characteristic for caprine milk, while β4′-galactosyllactose, lacto-*N*-novo-pentaose I, and 6′-sialyl-*N*-acetyllactosamine were present in bovine milk.[7,9,27] Ten out of 16 milk oligosaccharide structures present in equine colostrum were previously reported to be present in human milk: the neutral trimers, tetramer, and hexamer, and the acidic 3′-, 6′-sialyllactose, tetramer, pentamer, and hexamer.[10,20]

The presence of milk oligosaccharides carrying the bifidogenic factor (Gal(β1−3)GlcNAc) (numbers 6, 7, and 13 in Table 1) and the presence of eight EMOS decorated with sialic acids suggest bioactivity of the EMOS, such as being bifidogenic and soluble ligands for intestinal pathogens.[2,28−31] In this study, EMOS decorated with *N*-glycolylneuraminic acid (Neu5Gc) were not detected, while they were reported to count for less than 1% in previous study.[8] The presence of Neu5Gc linked to milk oligosaccharides is not rare in oligosaccharides derived from domestic animals. For example, such oligosaccharides account for about 6% of the oligosaccharides in cows' colostrum.[8] On the contrary, milk oligosaccharides decorated with Neu5Gc are absent in human colostrum, which is considered as beneficial because blood antibodies against Neu5Gc are present in several diseases such as acute and chronic hepatitis, hyperacute transplant rejection, and cancer.[10,31−33]

In summary, oligosaccharide profile of colostrum from horses showed significant differences between breeds as well as per individual. EMOS identified showed quite some overlap with oligosaccharides present in milk from human and other mammals, although also horse specific structures were identified. The presence of EMOS carrying the bifidogenic factor and/or the *N*-acetylneuraminic acid, together with low concentrations of EMOS with *N*-glycolylneuraminic acid, could make equine colostrum a good source for further research into in vitro bioactive effects of milk oligosaccharides.

■ ASSOCIATED CONTENT

Supporting Information

Analytical techniques used for the identification of EMOS in the Dutch Warmblood horses' colostrum, and EMOS' liquid chromatography and mass spectrometry characteristics. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01127.

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Funding

This project is jointly financed by the European Union, European Regional Development Fund and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the Municipality of Groningen, the Provinces of Groningen, Fryslân and Drenthe, the Dutch Carbohydrate Competence Center (CCC WP25; www.cccresearch.nl), Danone Nutricia Research and FrieslandCampina.

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.jafc.5b01127
J. Agric. Food Chem. 2015, 63, 4805−4814

ACKNOWLEDGMENTS

We would like to thank Edwin Bakx (Laboratory of Food Chemistry, Wageningen University) for the fruitful discussions on the interpretation of the mass spectra.

ABBREVIATIONS

DSL,Disialyllactose; DS-LNnT,Disialyllacto-*N*-neotetraose; Fuc,Fucose; Gal,D-Galactose; GalNAc,*N*-Acetylgalactosamine; β3′-GL,β3′-Galactosyllactose; β4′-GL,β4′-Galactosyllactose; β6′-GL,β6′-Galactosyllactose; Glc,D-Glucose; GlcNAc,*N*-Acetylglucosamine; Hex,Hexaose; HexNAc,*N*-Acetyl-hexosamine; LNH,Lacto-*N*-hexaose; LNnT,Lacto-*N*-neo-tetraose; LNnP I,lacto-*N*-novo-pentaose I; LSTa,Sialyllacto-*N*-tetraose a; 3′-SL,3′-Sialylactose; 6′-SL,6′-Sialyllactose; 3′-SLN,3′-Sialyl-*N*-acetyllactosamine; 6′-SLN,6′-Sialyl-*N*-acetyllactosamine

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