

Profiling of Sialylated Oligosaccharides in Mammalian Milk Using Online Solid Phase Extraction-Hydrophilic Interaction Chromatography Coupled with Negative-Ion Electrospray Mass Spectrometry

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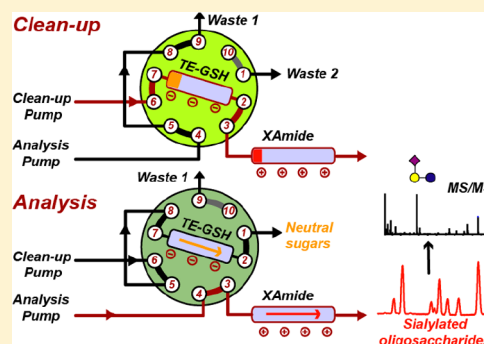
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Supporting Information

ABSTRACT: Sialylated oligosaccharides are important components in mammalian milk. They play a key role in the health and growth of infants by helping to shape up infant's gastrointestinal microbiota and defense against infection by various pathogenic agents. A detailed knowledge of the structures, compositions, and quantities of the sialylated milk oligosaccharides (SMOs) is a prerequisite for understanding their biological roles. However, because of the presence of very large amounts of lactose and neutral oligosaccharides, accurate analysis of SMOs is difficult. A pretreatment step is required to remove lactose and neutral oligosaccharides but conventional off-line pretreatment methods are time-consuming and of poor reproducibility. In this presentation, we linked solid-phase extraction (SPE) with hydrophilic interaction chromatography (HILIC) followed by mass spectrometry (MS) identification for the analysis of SMOs. A SPE column with electrostatic repulsion function was used for removal of lactose and neutral oligosaccharides, a HILIC analytical column for separation of the SMOs, and negative-ion electrospray ionization tandem MS was used for their identification and sequencing. The success of the established online SPE-HILIC-MS method was demonstrated by profiling of SMOs in human to investigate detailed SMO changes during lactation period and in animals to compare the difference in SMO contents among the different species.



Human milk is the primary source of nutrition for newborns and contains, in addition to proteins and fats, a large number of diverse oligosaccharides. Although human milk oligosaccharides (HMOs) are indigestible by infants, they play an important role in the health and growth of infants by helping to shape up the gastrointestinal microbiota¹ and defense against infection by various pathogenic agents.^{2–6}

Oligosaccharides are the most abundant component in human milk. As the disaccharide lactose is the dominant one (70 g/L), milk “oligosaccharides” are often referred as such without the “disaccharide” lactose. Even with this definition, HMOs (5–15 g/L) are still more abundant than proteins (8 g/L).⁷ Nonhuman mammalian milk contains much less oligosaccharides than human milk. HMOs commonly have a lactose core (Gal β 1-4Glc) at the reducing end and are elongated with N-acetylglucosamine (Gal β 1-3/4GlcNAc) units. The HMO backbones are frequently modified by fucose and sialic acid to form various recognition motifs, such as

blood-group and Lewis antigens. Sialic acid often includes N-acetylneuraminic acid (NeuAc) present in all mammals and N-glycolylneuraminic acid (NeuGc) in nonhuman mammals only.

Sialylated milk oligosaccharides (SMOs) have been recently shown to be particularly important due to their ability to act as soluble decoys preventing pathogen and toxin invasion.^{8–11} Infants are susceptible to influenza viruses and SMOs, e.g., 6'-sialyllactose (6'-SL), 3'-SL, and 6'-sialyl-N-acetylglucosamine (6'-SLN), may act as receptor mimics,¹² helping to protect infants from influenza infection. Other examples include the significant inhibition of *H. pylori*¹⁰ and *E. coli*¹³ adhesion by 3'-SL but not by the isomeric 6'-SL, two of the predominant SMOs. Similar effect of 3'-SL, but not 6'-SL, was found to influence colitis in mice through selective intestinal bacterial

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colonization.¹⁴ Recent *in vitro* experiments showed that SMOs could significantly inhibit leukocyte rolling and adhesion to endothelial cells while neutral HMOs had no such effects.¹¹ SMOs are also important for postnatal brain development¹⁵ and for promoting microbiota-dependent growth in models of infant undernutrition.¹⁶ A recent report showed that in a neonatal rat model, a specific disialylated milk sugar DSLNT had a protection effect from necrotising enterocolitis, a fatal intestinal disorder in premature infants.¹⁷

Detailed analysis of milk oligosaccharides is important in understanding their roles in infant's gastrointestinal microbiota development and defense against viral infection. Various methods for profiling of milk oligosaccharides have been described, including chromatographic^{18–21} and mass spectrometric analysis^{22–24} and online liquid chromatography–mass spectrometry (LC–MS).^{25–27} However, SMOs are of low concentrations in human and the dominant lactose and the large amounts of neutral oligosaccharides can interfere with the detection. In nonhuman mammals, SMOs are of very low abundance, e.g., bovine contains less than 0.06–0.09 g/L SMOs.²⁸ The low abundances of SMOs and/or the dominant neutral sugars are the major problem for analysis of mammalian SMOs.

There have been different ways for quantitative analysis of the SMOs. HPLC was used for analysis of SMOs in human and bovine milk and in infant formulas. Capillary electrophoresis was used for quantitation of major SMOs in human milk.^{29,30} Lebrilla and co-workers developed a HPLC-chip/MS method to characterize some 30 SMOs in humans.³¹ However, because of the lack of a robust profiling method, some of the data presented so far have not always been consistent, and this can cause confusion and lead to incorrect conclusions, for example, 3-fucosyl-3'-SL (3F-3'-SL) was detected as the most abundant sialylated oligosaccharide in both human colostrum and mature milk,³² but this was detected as a very minor component by others.^{21,29–31} A reliable and convenient profiling method is important for analysis of SMOs and defining their chemical structures and biological functions.

Pretreatment for removal of the dominant lactose and the large amounts of neutral oligosaccharides is required before detailed analysis of SMOs. Off-line gel filtration^{22,24} and solid-phase extraction (SPE) using graphitized carbon^{23,25,31} have been successfully used for enrichment of SMOs. However, off-line pretreatment is not ideal for high-throughput and high sensitivity profiling. LC–MS with online SPE has been widely used in the fields of food safety and drug metabolism analysis and has shown advantages of potential high throughput, automation, and robustness.^{33–35} However, there has been no report of a similar method in analysis of milk oligosaccharides due to the challenge of incompatibility between the matrixes used for SPE and the analytical column.

The present work intended to develop such an online SPE-HILIC-MS platform for profiling of SMOs in human and animals. We used some typical HMO standards to establish the system, optimized the operation conditions, and validated the method before application to profiling of human and animal SMOs.

■ EXPERIMENTAL SECTION

Materials. Oligosaccharide standards, 3'-SL, 6'-SL, 2'-fucosyllactose (2'-FL), Lacto-N-neotetraose (LNnT), lacto-N-fucopentaose (LNFP I), and lacto-N-neohexaose (LNnH) were purchased from Elicityl (Grenoble, France). HPLC-grade ACN

was obtained from Merck (Darmstadt, Germany). Ammonium formate and formic acid were from J&K Scientific (Beijing, China). All other reagents used were of analytical grade or higher. Water was purified by a Milli-Q water purification system (Billerica, MA).

Preparation of Milk Oligosaccharides. Human milk samples were collected from a healthy mother at different periods of lactation (8 days, 88 days, and 140 days) and human colostrums were collected from further five individuals. Mature milk samples from cow, yak, buffalo, camel, donkey, swine, and sheep were kindly provided by China Agricultural University (Beijing, China). Mature milk from goat was purchased from a local farm. All milk samples were stored at –40 °C before use.

HMOs were isolated from milk, collected at different lactation times, essentially as described.²⁷ Briefly, 200 μ L of milk was centrifuged at 8 000 rpm for 10 min at 4 °C to remove lipid. Ethanol (400 μ L) was then added to the skim milk before centrifugation at 8 000 rpm for 10 min at 4 °C to remove protein. The obtained supernatant was used for analysis.

Animal milk oligosaccharides were isolated from the milk of sheep, goat, cow, yak, buffalo, camel, donkey, and swine using a modified procedure. Briefly, the milk sample (~2 mL) was centrifuged at 8 000 rpm for 10 min at 4 °C. After the removal of the top lipid layer, the defatted milk sample solution was freeze-dried. To the dried powder was added 1 mL ethanol/water (2:1) and the mixture was then centrifuged at 8 000 rpm for 10 min at 4 °C to remove the majority of proteins. The supernatant was used for analysis.

Online SPE-HILIC. Online SPE-HILIC platform was established using an Ultimate 3000 UHPLC system (Thermo-Fisher Scientific, Milan, Italy), consisting of a column compartment, an autosampler, and a 10-port valve, a charged aerosol detector (CAD), and a dual gradient pump system, one used as “cleanup pump” for purification and the other as “analysis pump” for analysis. The CAD was used for the initial development and validation of online SPE-HILIC system.

The configuration of the online SPE-HILIC platform and analytical process is shown in Figure 3. The mobile phase of the cleanup pump consisted of ACN (A) and H₂O (B) with a flow rate of 0.2 mL/min. The mobile phase of the analysis pump consisted of ACN (A), H₂O (B), and 100 mM ammonium formate buffer, pH 3.2 (C), with a flow rate of 1.0 mL/min. A homemade “Click TE-GSH” column (5 μ m, 50 mm $\times$ 2.1 mm)³² was used as the SPE cleanup column. The amide column, XAmide (5 μ m, 150 mm $\times$ 4.6 mm, Acchrom, Beijing, China), was used as the HILIC analytical column.

For operation, the sample solution was injected by autosampler when the 10-port valve was on the 1–2 position. The valve was then switched to 10–1 position after 0.1 min, linking the SPE cleanup column to the analytical column, in order for the sample passing through the two columns (Figure 3a) by using a gradient of ACN/H₂O (Table S1). At 5.1 min, the 10-port valve was switched to a position at which the two columns were at a “parallel” configuration (Figure 3b). The SMOs were separated on the analytical XAmide column and detected by CAD or MS (see below). The SPE and analytical columns were then equilibrated for the analysis of the next sample. The time programming of the valve and chromatographic gradient were listed in detail in Table S1.

Online SPE-HILIC-MS. For concomitant structural analysis of the complex SMOs, an Agilent Q-TOF mass spectrometer (Agilent Technologies 6540 UHD) was connected to the UHPLC system to form an online SPE-HILIC-MS system. The

mobile phase conditions were essentially the same as those used in the SPE-HILIC system described above, except the size of the XAmide column ($5\ \mu\text{m}$, $150\ \text{mm} \times 2.1\ \text{mm}$) and the flow rate ($0.2\ \text{mL/min}$). The drying gas temperature was at $350\ ^\circ\text{C}$ with a flow rate of $8.0\ \text{L/min}$. Both MS and MS/MS spectra were acquired in the negative-ion mode with an acquisition rate of $1\ \text{s}$ per spectrum over a mass range of $m/z\ 450\text{--}2000$ (for MS) and $m/z\ 100\text{--}2000$ (for MS/MS). Precursor-ion selection was made automatically by the data system based on ion abundance. Three precursors were selected from each MS spectrum to carry out product-ion scanning. Collision energy of $40\ \text{V}$ was used for collision-induced dissociation (CID).

Structural Characterization of Sialylated Oligosaccharides by Off-Line MS/MS. Five SMO fractions (LSTb, LSTc, 3'SLNFP II, 6'SLNFP VI, and DSLNT) were collected by an off-line method and identified by MS/MS using a Waters Q-TOF Premier mass spectrometer (Waters, Manchester, U.K.) equipped with nano UPLC used for sample introduction. The spray voltage was $2.3\ \text{kV}$ and the source temperature at $100\ ^\circ\text{C}$. The sampling cone was at $35\ \text{V}$ and collision energy at $50\ \text{V}$.

RESULTS AND DISCUSSION

Construction of an Online SPE-HILIC Platform. Selection of a suitable SPE matrix for removal of the neutral

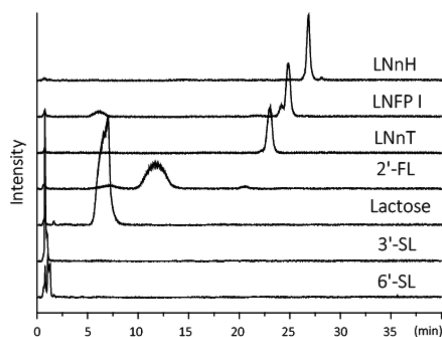


Figure 1. Separation of selected HMO standards and lactose on a Click TE-GSH column. Mobile phase: solvent A, ACN; solvent B, H_2O . Gradient: $0\text{--}10\ \text{min}$, A/B (80/20); $10\text{--}30\ \text{min}$, A/B (80/20) to A/B (50/50).

oligosaccharides/lactose and an analytical column for separation of SMOs are of particular importance. HILIC is a powerful tool for separation of oligosaccharides, and different HILIC matrix-materials can provide different selectivity for specific oligosaccharides. Neutral oligosaccharides (including lactose) and sialylated oligosaccharides are different in their electrostatic property. We used this feature to select matrix-materials to suit the purpose of an online SPE-HILIC system. Because of the lack of chromophore in SMOs, a CAD detector was used for the initial development to allow a wide choice of solvent system.

A zwitterionic matrix prepared from tripeptide glutathione ("Click TE-GSH") with mixed-mode action of hydrophilic interaction and cation-exchange was selected as the SPE matrix for the cleanup of SMOs. The ξ -potential of zwitterion "Click TE-GSH" material carries negative charge above $\text{pH}\ 2$,³⁶ enabling separation of SMOs from the neutral sugars. At an appropriate pH , both the column and the SMOs carry negative charge and therefore, the SMOs could flow through the column

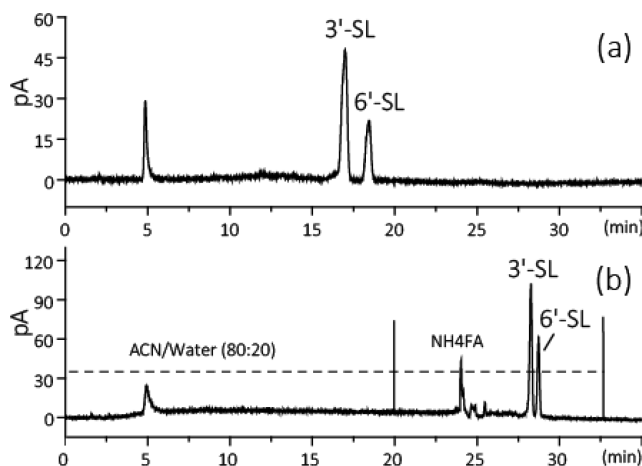


Figure 2. Separation of 3'-SL and 6'-SL on a XAmide column using different elution solvent. Mobile phase: solvent A, ACN; solvent B, NH_4FA ($100\ \text{mM}$, $\text{pH}\ 3.2$); solvent C, H_2O . Gradient in part a, $0\text{--}30\ \text{min}$, A/B/C (80/10/10) to A/B/C (50/10/40). Gradient in part b, $0\text{--}20\ \text{min}$, A/B/C (80/0/20); $20\text{--}20.1\ \text{min}$, A/B/C (80/0/20) to A/B/C (80/10/10); $20.1\text{--}30\ \text{min}$, A/B/C (80/10/10) to A/B/C (50/10/40).

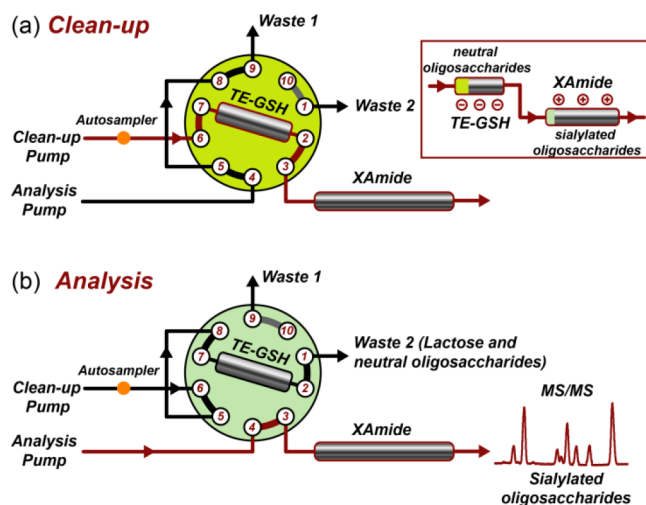


Figure 3. Schematic illustration of the online SPE-HILIC-MS system.

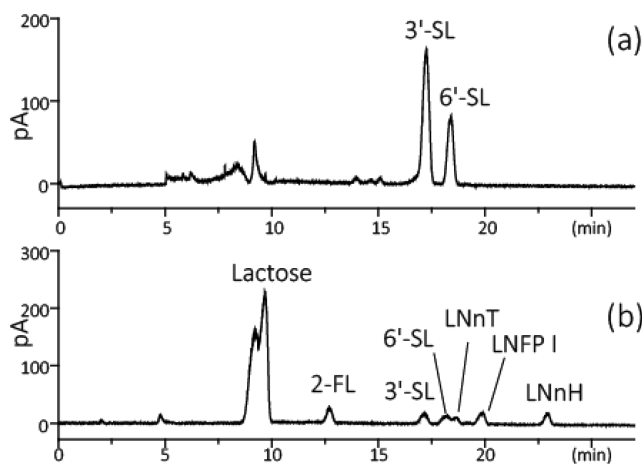


Figure 4. Comparison of online SPE-HILIC (a) and direct HILIC analysis of a HMO standard solution (b).

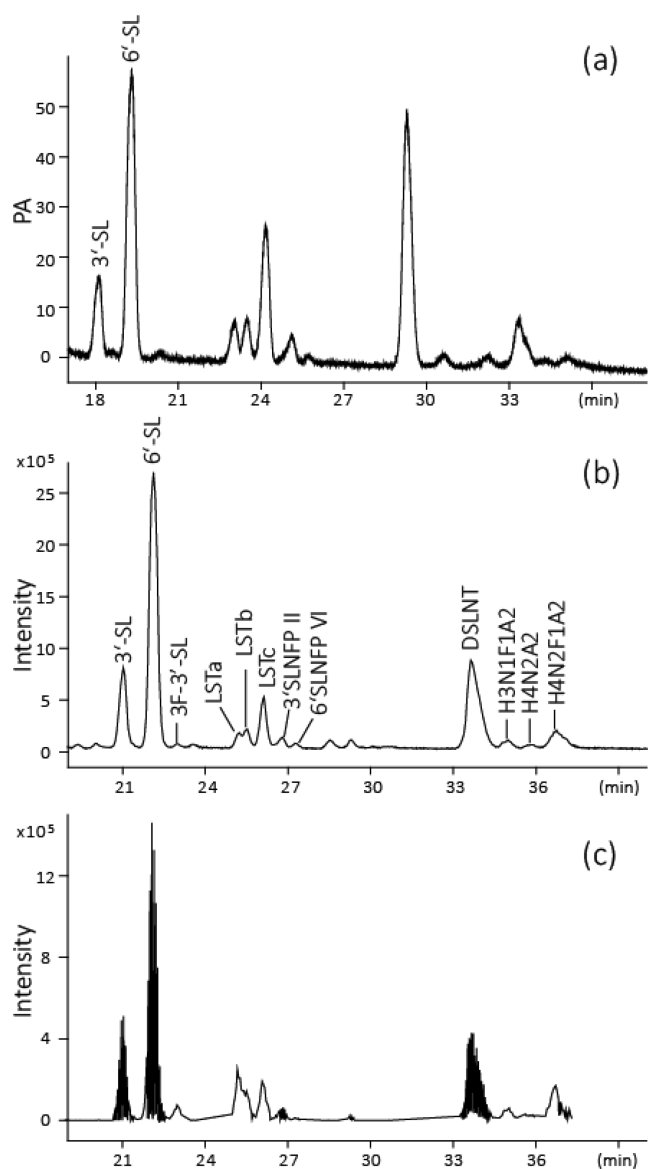


Figure 5. HPLC profiles of SMOs from a human sample with different detection system: (a) CAD detector, (b) total-ion chromatogram, and (c) single-ion (*m/z* 290) chromatogram.

quickly due to electrostatic repulsion, while neutral sugars would retain in the column by hydrophilic interaction.

To test the effect of electrostatic repulsion of the Click TE-GSH column, a panel of selected HMO standards were used, including sialylated 3'-SL and 6'-SL, neutral 2'-FL, LNnT, LNFP I and LNnH, and lactose. These oligosaccharides were separated on a short Click TE-GSH column with ACN/H₂O as the mobile phase. As shown in Figure 1, there was no retention for the two sialyllactoses (eluted at the void volume), due to electrostatic repulsion. All neutral sugars retained to certain degree on the column, due to hydrophilic interaction, with an elution order of lactose, 2'-FL, LNnT, LNFP I, and LNnH. This clearly indicated that separation of sialylated from the neutral sugars could be realized with the flow-through SPE.

Having selected a suitable matrix for separating the acidic and neutral fractions, we proceeded to evaluate a HILIC column for resolving SMOs, repelled and eluted from the Click TE-GSH. The amide column can simultaneously provide weak anion exchange and hydrophilic interactions for acidic oligosacchar-

ides.³⁷ The former action helps to capture sialylated oligosaccharides eluted from the SPE column, and the latter is used for their resolution. Again 3'-SL and 6'-SL were used as models to investigate the property of the XAmide column. Figure 2a shows the elution profile of the well-resolved isomeric 3'- and 6'-SL when ammonium formate was used as an additive in mobile phase ACN/H₂O. The good peak shape and selectivity indicated that the XAmide column has good separation ability for SMOs.

We next investigated the linking of the two LC systems: repulsion and elution of SMOs by SPE and their trapping and separation by XAmide. As shown in Figure 2b, the model sialylated oligosaccharides could not be eluted out within 20 min using 20% H₂O, whereas when ammonium formate was used, 3'-SL and 6'-SL were successfully resolved and eluted out. The result indicated that the SMOs could be captured by the XAmide column from the effluent of Click TE-GSH column with 20% H₂O and then released and fractionated by an alternative mobile phase. On the basis of these results, the XAmide column was selected as the analytical column.

As shown in Figure 3, an online SPE-HILIC platform was established using a HPLC system consisting of dual-gradient pumps (left pump for "cleanup" and right pump for "analysis"), a SPE (Click TE-GSH column, 5 μ m, 50 mm $\times$ 2.1 mm), an analytical column (XAmide column, 5 μ m, 150 mm $\times$ 4.6 mm or 2.1 mm), a 10-port valve, and a CAD detector. At the initial stage, when the valve was at the "cleanup" position (Figure 3a), the SPE and the analytical columns are in "series", i.e., the two columns are linked together. The deproteinized skim milk samples were injected onto the SPE column by an autosampler. The flow-through SPE column was used to capture lactose and neutral oligosaccharides, and the targeted SMO fraction was eluted out from the SPE and trapped by the analytical column. The 10-port valve was then switched to "analysis" position, at which the SPE and analytical columns were in "parallel" configuration, i.e., the two columns are disconnected and work independently (Figure 3b). The SMOs were separated on the analytical column by mobile phase delivered by the analysis pump. The unwanted neutral sugars on the SPE column were washed out by solvent delivered by the cleanup pump.

Establishment of Detailed Experimental Conditions for SPE-HILIC. Having established the platform, we proceeded to optimize the detailed conditions for the SPE-HILIC using a mixture of standard oligosaccharides, selected to simulate HMOs in both composition and concentration. A carefully selected elution solvent system for the SPE is important. It should be compatible with the subsequent analytical column while maintaining the selectivity and not to allow the undesirable components to be leaked out to the analytical column. As described above, Click TE-GSH column exhibited electrostatic repulsion of SMOs when ACN/H₂O was used. As low water content can lead to longer elution time of SMOs and high water content may deteriorate the resolution between SMOs and lactose, a detail composition of ACN/H₂O needed to be established. Figure S1 shows the chromatographic profiles of the standard mixture eluted from the Click TE-GSH under different elution conditions. With increased water content, the time span between sialyllactoses (3'-SL and 6'-SL) and lactose became shorter. The time span needs to be carefully selected. On the one hand, it should be long enough to allow sialylated oligosaccharides with strong retentions on SPE column to be eluted out without loss of any highly polar SMOs, but on the other it should not be overly long to compromise the resolution

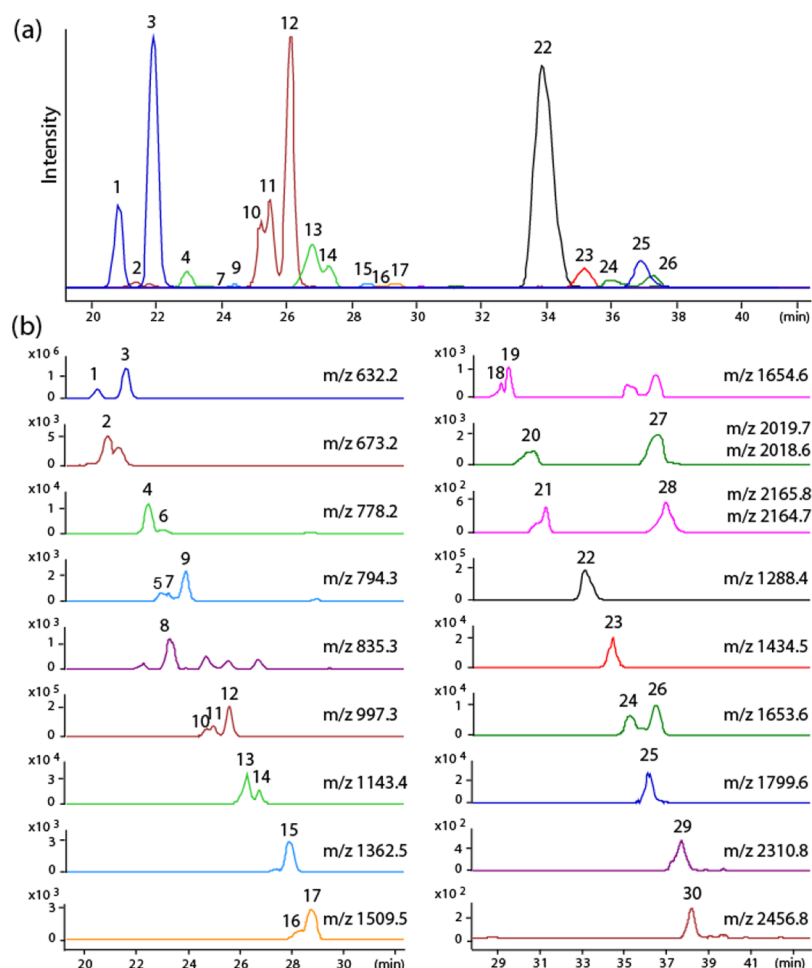


Figure 6. Elution profiles of SMOs from a human milk sample. (a) Overlaid extracted single-ion chromatogram. All peaks except 1 (3'-SL) and 3 (6'-SL) were magnified by a factor of 5; (b) extracted single-ion chromatograms.

of the analytical column and extend analysis time. The elution by 20% H₂O (Figure S1b) was thus selected and the switching time of valve was set at 5.1 min, 1 min earlier than the retention time of lactose, in order to maintain the optimum extraction efficiency and resolution. The total volume collected from the SPE column at the flow rate of 0.2 mL/min was 1 mL.

The same standard oligosaccharide mixture was then used to assess the online SPE-HILIC method. As shown in Figure 4a, only sialylated 3'-SL and 6'-SL were detected with good resolution, while lactose and neutral oligosaccharides were largely removed. For comparison, the oligosaccharide mixture was also injected directly on to the XAmide column without the SPE (Figure 4b). In this case both acidic and neutral oligosaccharides, in addition to lactose, were eluted out from the XAmide column and the elution positions of the sialylated 3'-SL and 6'-SL were between the neutral tri- and tetrasaccharides 2'-FL and LNT. Clearly with the efficient online SPE cleanup, SMOs can be readily detected with good resolution.

The results were reproducible as shown by repeated injection of the standard SMO mixture (Figure S2). The reproducibility was further assessed with human colostrum samples from five mothers. Three repeated injections were made for each sample and representative chromatograms were shown in Figure S3. Using 3'-SL and 6'-SL within the samples, the retention times and peak areas were measured and relative standard deviations

calculated (Table S2). These results indicated a satisfactory reproducibility.

A human milk sample was also used to check if all neutral oligosaccharides can be retained efficiently by the SPE. The trapped neutral oligosaccharides were gradient-eluted out from the SPE and a chromatogram recorded (Figure S4a). This is compared with the same sample analyzed using the SPE column alone in the conventional way (Figure S4b). The similarity of these two chromatograms indicated an efficient capture of the neutral oligosaccharides and column resolution retained after cleaning.

A recovery experiment for the sialylated oligosaccharides was also carried out using 3'-SL and 6'-SL as examples. Within the linear range of 20–300 $\mu\text{g/mL}$ ($R^2 > 0.99$, Figure S5), the average recovery was 90% for 3'-SL and 106% for 6'-SL with 5 repeated injections and a spike concentration at 50 $\mu\text{g/mL}$ (Table S3).

SPE-HILIC-MS for Analysis of SMOs. As electrospray ionization (ESI) MS, including tandem MS with collision-induced dissociation (CID-MS/MS), is a powerful for identification of oligosaccharides,^{38–41} a SPE-HILIC-MS system was then assessed. The solvent system ACN/H₂O containing low concentration (10 mM) volatile ammonium formate for chromatography established above is compatible with negative-ion ESI-MS. The total ion chromatogram obtained by MS detection (Figure 5b) is very similar to that

Table 1. Detected SMOs and Their Corresponding Peak Numbers

peak ^a	RT ^b	molecular mass	composition ^c	assignment ^d	human	goat	sheep	cow	buffalo	yak	donkey	swine	camel
1	20.50	633.2	H2A1	3'-SL	+	+	+	+	+	+	+	+	+
2	20.94/21.33	674.2	H1N1A1	6'-SLN	+	+	-	+	+	+	-	+	+
3	21.60	633.2	H2A1	6'-SL	+	+	+	+	+	+	+	+	+
4	22.31	779.2	H2F1A1	3F-3'-SL	+	-	-	-	-	-	-	-	-
5	22.93	795.3	H3A1	3''-S-β3'-GL	+	+	+	+	+	+	-	+	+
6	22.98	779.2	H2F1A1	?	+	-	-	-	-	-	-	-	-
7	23.23	795.3	H3A1	6'-S-β3'-GL	+	+	-	+	-	-	-	+	-
8	23.27	836.3	H2N1A1	6'-S-LNTRI-II	+	-	-	-	-	-	-	-	-
9	23.90	795.3	H3A1	6''-S-β3'-GL	+	-	-	-	-	-	-	-	-
10	24.72	998.3	H3N1A1	LSTa	+	-	-	-	-	-	-	+	-
11	25.00	998.3	H3N1A1	LSTb	+	-	-	-	-	-	-	-	-
12	25.61	998.3	H3N1A1	LSTc	+	-	-	-	-	-	-	+	-
13	26.28	1144.4	H3N1F1A1	3'SLNFP II	+	-	-	-	-	-	-	-	-
14	26.73	1144.4	H3N1F1A1	6'SLNFP VI	+	-	-	-	-	-	-	-	-
15	27.94	1363.5	H4N2A1	MSLNnH	+	-	-	-	-	-	-	-	-
16	28.36	1509.5	H4N2F1A1	MSMFLNH	+	-	-	-	-	-	-	-	-
17	28.77	1509.5	H4N2F1A1	MSMFLNnH	+	-	-	-	-	-	-	-	-
18	29.18	1655.6	H4N2F2A1	DFS-LNH	+	-	-	-	-	-	-	-	-
19	29.50	1655.6	H4N2F2A1	DFS-LNnH	+	-	-	-	-	-	-	-	-
20	30.72	2020.7	H5N3F2A1	?	+	-	-	-	-	-	-	-	-
21	31.29	2166.8	H5N3F3A1	?	+	-	-	-	-	-	-	-	-
22	33.17	1289.4	H3N1A2	DSLNT	+	-	-	-	-	-	-	-	-
23	34.43	1435.5	H3N1F1A2	DS-SLNFP II	+	-	-	-	-	-	-	-	-
24	35.25	1654.6	H4N2A2	?	+	-	-	-	-	-	-	-	-
25	36.17	1800.6	H4N2F1A2	Structure 25	+	-	-	-	-	-	-	-	-
26	36.53	1654.6	H4N2A2	?	+	-	-	-	-	-	-	-	-
27	36.60	2019.6	H5N3A2	?	+	-	-	-	-	-	-	-	-
28	36.97	2165.7	H5N3F1A2	?	+	-	-	-	-	-	-	-	-
29	37.70	2311.8	H5N3F2A2	?	+	-	-	-	-	-	-	-	-
30	38.19	2457.8	H5N3F3A2	?	+	-	-	-	-	-	-	-	-
31	19.77/21.17	674.2	H1N1A1	3'-SLN	-	-	-	-	-	-	-	+	-
32	21.20	649.2	H2G1	3'-Neu5GcL	-	+	+	-	-	-	-	-	-
33	21.52/22.10	689.2	H1N1G1	6'-GLN	-	+	+	-	-	-	-	-	-
34	22.16	649.2	H2G1	6'-Neu5GcL	-	+	+	-	-	-	-	-	-
35	22.24	836.3	H2N1A1	3'-S-LNTRI-II	-	-	-	-	-	-	-	+	-
36	23.44	811.2	H3G1	3''-Neu5Gc-β3'-GL	-	+	+	-	-	-	-	-	-
37	25.35	924.3	H2A2	DSL	-	+	+	+	+	+	-	-	-
38	26.15	1160.4	H4N1A1	SLNPa	-	-	-	-	-	-	-	+	+
39	26.31	940.3	H2A1G1	Structure 39	-	+	+	-	+	-	-	-	-
40	26.87	956.3	H2G2	DGL	-	+	+	-	+	-	-	-	-
41	31.40	1086.3	H3A2	?	-	-	-	+	+	+	-	+	+
42	33.49	1451.5	H4N1A2	?	-	-	-	-	-	-	-	+	-
43	34.29	1451.5	H4N1A2	?	-	-	-	-	-	-	-	+	-

^aLC peak numbers. ^bRetention time (in min). ^cH, Hex; N, HexNAc; F, dHex; A, NeuNAc; G, NeuNGc. ^dThe short form of the trivial name is shown if the SMO structure can be identified by MS/MS or by comparison with the literature data. For the identified structure without a trivial name the full structure is listed in Table S4. A question mark indicates the structure has not been identified but only composition is shown.

obtained from CAD detection (Figure 5a) of SMOs from a human sample, indicating no interference from the gradient of the formate-containing solvent system. For MS/MS, the characteristic fragment m/z 290 (NeuAc) and m/z 306 (NeuGc) can be used for detection of SMOs. The profile of single ion (m/z 290) chromatogram of the human sample shown in Figure 5c is identical to the total ion chromatogram (Figure 5b) and this suggested that all the components detected by MS were SMOs and no neutral oligosaccharides identified.

For those peaks with relatively high abundances, CID-MS/MS spectra were of sufficient quality and were used to assign the structure of the components,³⁸ and these include 3'-SL, 6'-

SL, 3F-3'-SL, LSTa, LSTb, LSTc, 3'-SLNFP II, 6'-SLNFP IV, and DSLNT. Many SMOs with lower abundance can also be detected. A total of 30 sialylated HMOs, including 21 mono- and 9 disialylated oligosaccharides with degree of polymerization (DP) from 3 to 13, were identified (Figure 6 and Table 1). Neutral sugars were not detected. Isomeric SMOs with low DPs, such as LSTa, LSTb, and LSTc ($[M - H]^-$ at m/z 997.30) can be well separated. However, the isomers of large-sized SMOs were difficult to resolve, as observed by others using alternative methods.³¹

Application to Analysis of SMOs in Human Milk. The established method was used for profiling of SMOs in humans.

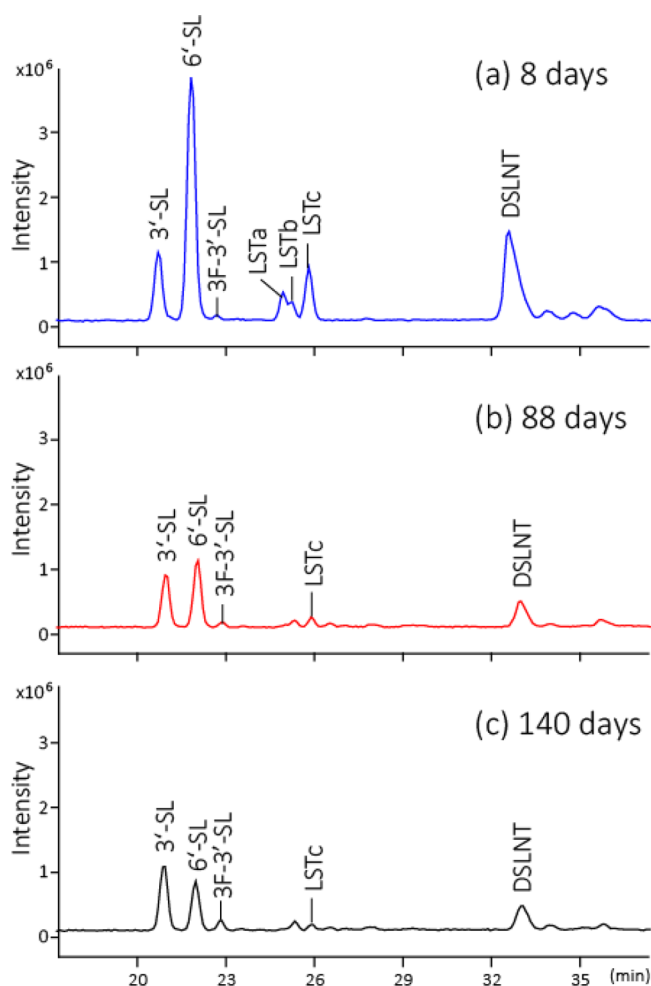


Figure 7. Changes of SMOs in human at different lactation time: (a) 8 days, (b) 88 days, and (c) 140 days. Identity of each component was assigned by negative-ion ESI-CID-MS/MS.

For analysis, the supernatant after ethanol precipitation of the defatted human milk was injected directly into the LC system.

For assessment of changes of SMOs during lactation, human milk samples from the same mother were collected at 8, 88, and 140 days after delivery (Figure 7). The most profound feature

was the significant reduction of 6'-SL (about 5 times reduction from day 8 to day 140) and the constant level of 3'-SL during lactation. LSTc contains α 2,6-linked NeuAc and also showed a major reduction. The disialylated DSLNT, containing both α 2,3- and α 2,6-linked NeuAc, showed a similar decrease in concentration, likely due to the same reason for reduction of α 2,6-sialylation. The decrease of the minor component LSTa is also apparent as shown in the clean chromatogram obtained by the method.

Profiling of SMOs from Mammalian Animal Milk.

Although SMOs are more abundant than the neutral sugars in milks of many mammalian animals, the total contents of oligosaccharides are much lower. Removal of the neutral sugars is still important to obtain good SMO profiles and to deduce the sequence of the main components by MS/MS (Table 1 and Figure S6).

Mature milk samples from eight animals, including sheep, goat, cow, yak, buffalo, camel, donkey and swine, were selected for analysis. The profiles (Figure 8), shown as overlapped single-ion chromatograms of detected SMOs, were compared with that of human milk at day 8 (Figure 8a). The apparent difference in SMOs between human and animals is the lack of mono- and disialylated lacto-N-tetraose (LST and DSLNT, respectively) in animals. Moreover, SMOs in animals have less diversity, are lacking in fucosylation, and are of short chain lengths (Figure 8 and Table 1).

As all SMOs in animals are dominated by the pair of 3'/6'-SL, the 9 profiles can be categorized into four groups based on the patterns of sialyllactose. Donkey (Figure 8b) is the only one showed similar 3'/6'-SL patterns as human with more of 6'-SL, while all the rest contain more 3'-SL. Buffalo (Figure 8e) and camel (Figure 8h) gave similar 3'/6'-SL patterns with a ratio of 2:1. Cow (Figure 8c), yak (Figure 8f), and swine (Figure 8i) contains less 6'-SL (3'/6'-SL ratio 6:1) but there was more of a variety of SMOs with longer chains in swine milk. The most striking difference is the presence of NeuGc-containing sialyllactoses 3'/6'-GL in goat (Figure 8d) and sheep (Figure 8g). Between the milks from the closely related goat and sheep, there was also some major difference; goat contains similar amounts of SL and GL, and also with similar ratio between 3'- and 6'-linked sialic acid (1.5:1), whereas sheep contains almost exclusively NeuGc-modified.

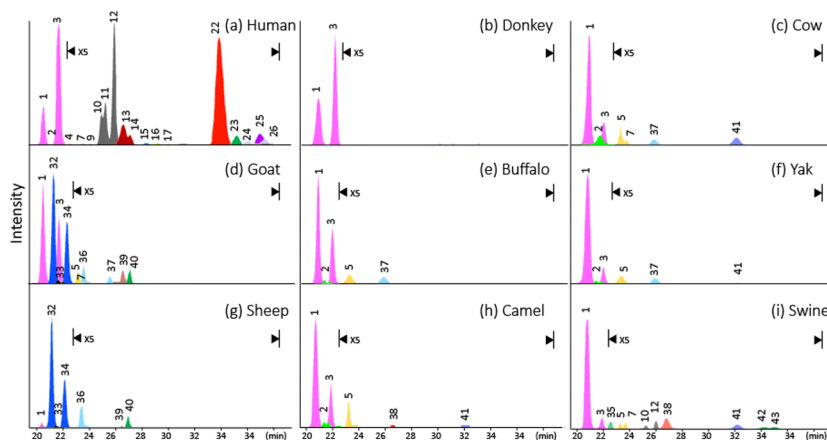


Figure 8. Overlapped extracted single-ion chromatograms of SMOs from samples of mammals in comparison with human: (a) human, (b) donkey, (c) cow, (d) goat, (e) buffalo, (f) yak, (g) sheep, (h) camel, and (i) swine. For better comparison of the minor components, intensities of peaks eluted after 23 min are increased by 5 times as marked in the chromatograms.

Apart from sialyllactose, sialyllactosamine 6'-SLN was also found in all mammalian milks, except in donkey and sheep. As with 3'/6'-GL, the NeuGc form of sialyllactosamine, 3'-GLN and 6'-GLN, were only found in goat and sheep.

CONCLUSIONS

An online SPE-HILIC-MS method was developed for analysis of mammalian SMOs. Defatted and deproteinized milk samples can be injected directly into the system without preremoval of the dominant lactose and neutral oligosaccharides. Under the conditions established, lactose and neutral oligosaccharides are trapped in the cleanup column, and the SMOs are eluted out and flown into the analytical column for separation. The fractionated SMOs are then detected by MS and sequenced by MS/MS.

Using the system, 30 mono- and disialylated oligosaccharides were detected in humans. Changes of SMOs during lactations can be readily compared. The content of 3'-SL was constant during the entire lactation period monitored (1 week to 4 months). A major reduction of 6'-SL was observed in agreement of other reports.^{42,43} The amount of 6-linked sialic acid-containing SMOs were all reduced.

The method was also used to compare SMO profiles of nonhuman mammals. The different patterns and the major features of SMOs can be readily identified. In previous reports,^{44–46} NeuGc was detected in many animal milk, such as cow and camel. The absence of NeuGc in samples of our study could be due to mature milk collected but not colostrum and, therefore, the numbers and concentrations of SMOs can change over the course of lactation, as previously described by Urashima and colleagues.²⁸ In our study, it is not clear if the concentration of any of the major sialyllactoses in nonhuman mammals remains constant during the lactation period, as in the case of human milk. Further detailed work is required to establish this.

To the best of our knowledge, this is the first report on online SPE-LC-MS analysis of SMOs. Compared with conventional off-line methods, online SPE was convenient and faster due to minimal sample preparation. With improved detection sensitivity of the modern mass spectrometers and added capability of isomeric resolution by ion mobility,⁴⁷ this robust method could hold great potential for analysis of large numbers of milk samples. The detailed knowledge of SMOs will certainly help to gain new insights into the complex biochemical pathways underlying their potential health-promoting properties.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04468.

Parameters for HPLC operation; reproducibility demonstrated with 3'-SL and 6'-SL in five human colostrum samples from three repeated injections; recoveries of two SMO standards spiked in a human milk sample; selected SMO structures determined by ESI-CID-MS/MS; separations of selected HMO standards and lactose on a Click TE-GSH column at different chromatographic conditions; reproducibility of the online SPE-HILIC method with chromatograms of three consecutive injections shown; HPLC profiles of SMOs from different

human samples; chromatograms of neutral oligosaccharides eluted from the SPE column of online system and HMOs analyzed by TE-GSH column alone; linearity range of standard 3'-SL and 6'-SL; product-ion spectra of SMOs in humans: 6'-SLN, LSTb, 6'SLNFP VI, DSLNT, DS-SLNFP II, DFS-LNnH, and Structure 25 (PDF)

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Notes

The authors declare no competing financial interest.

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