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Authors

Lee, Hyeyoung
de MeloSilva, Vitor Luiz
Liu, Yan
et al.

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Short communication: Quantification of carbohydrates in whey permeate products using high-performance anion-exchange chromatography with pulsed amperometric detection

Hyeyoung Lee[*,†,1], **Vitor Luiz de MeloSilva**[‡], **Yan Liu**[*], and **Daniela Barile**[*,†]

Daniela Barile: dbarile@ucdavis.edu

[*]Department of Food Science and Technology, University of California, Davis 95616

[†]Foods for Health Institute, University of California, Davis 95616

[‡]Departamento de Tecnologia de Alimentos, Faculdade de Veterinaria, Universidade Federal Fluminense, Niteroi 24230-340, Rio de Janeiro, Brazil

Abstract

A method was developed for the characterization and quantification of the disaccharide lactose and 3 major bovine milk oligosaccharides (BMO) in dairy streams. Based on high-performance anion-exchange chromatography-pulsed amperometric detection (HPAE-PAD), this method is advantageous because it requires minimal sample preparation and achieves good chromatographic separation of oligosaccharide isomers within 30 min. The linear dynamic range and limit of detection were 0.1 to 10 mg/L and 0.03 to 0.22 mg/L, respectively. Mean recoveries of the BMO were excellent and ranged from 98.4 to 100.4%. Without complicated sample preparation procedures, this HPAE-PAD method measured BMO [3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), and 6′-sialyllactosamine (6′SLN)] and lactose using a single instrument, therefore increasing the accuracy of the measurement and applicability for the dairy industry. In colostrum whey permeate, 3′SL, 6′SL, and 6′SLN were 94, 29, and 46 mg/L, respectively. This work is the first to demonstrate that some commercial products, currently marketed for supporting a healthy immune system, contain significant amounts of bioactive BMO and therefore, carry additional bioactivities.

Keywords

whey permeate; lactose; bovine milk oligosaccharide; high-performance anion-exchange chromatography-pulsed amperometric detection

Short Communication

The intestinal microbiota is made up of tens of trillions of gut bacteria and represents a recent target for research. Dysbiotic microbiota and compromised intestinal barrier function

Correspondence to: Daniela Barile, dbarile@ucdavis.edu.

[1]Current affiliation: Division of Metrology for Quality of Life, Korea Research Institute of Standards and Science, Yuseong, Daejon 305-600, Republic of Korea.

cause long-term morbidity and even infant mortality in developing countries. Recent evidence shows that intestinal barrier function is also compromised in several disparate Western diseases such as obesity, diabetes, allergies, and autism (Bischoff et al., 2014). Therapies that help repair epithelial barrier and gut microbiota have the potential to influence many health outcomes. Milk oligosaccharides have received a lot of interest for their biological activities as prebiotics (promoting the growth of beneficial microbiota), anti-infective agents, and immune modulators (Smilowitz et al., 2014). They are structurally complicated molecules that contain a lactose core decorated with galactose, *N*-acetylhexosamine, sialic acid, and fucose. Because monosaccharides are attached to the lactose core via different glycosidic bonds, a wide variety of structures is possible. Recently, a high-resolution mass spectrometry-based method was used to annotate the molecular and structural complexity of human milk oligosaccharides (**HMO**). Almost 200 HMO were identified, of which 45 neutral and 30 sialylated oligosaccharides were fully characterized (Wu et al., 2010, 2011). Unfortunately, not all infants are breastfed and donor milk availability is limited; therefore, commercial demand is strong for oligosaccharides that are similar to those in human milk for potential infant formula supplementation and other applications. However, commercial plant-derived prebiotics lack the structural complexity of HMO that correlate with protective microbiota effects, and current methods for their synthesis cannot deliver the needed HMO amounts. These combined situations generated an unmet need in the marketplace for the next generation of prebiotics that are able to guide the development of a desirable intestinal microbiota and immunological functions. Our group has pioneered investigation of abundant dairy streams (>200 million t/yr) as alternative sources of bioactive oligosaccharides.

Recently, we demonstrated a significant overlap between HMO and bovine milk oligosaccharides (**BMO**), a discovery that has great implications for the future use of dairy products and by-products as sources of oligosaccharides (Tao et al., 2008; Aldredge et al., 2013). We demonstrated that whey permeate, a by-product of cheese making, is a good source of milk oligosaccharides. Initial studies using high-resolution mass spectrometry showed that cheese whey from mature milk contains 15 oligosaccharides, half of which have a composition identical to those present in human milk (Barile et al., 2009). In addition to their structural similarities, the massive industrial quantities of bovine dairy streams make large-scale production of oligosaccharides possible. Utilizing whey by-products in food and health industries may be a way to increase their economic value.

More recent investigations of whey permeate from bovine colostrum produced at the pilot scale by membrane filtration revealed almost 50 individual oligosaccharide compositions (Dallas et al., 2014). Out of the 50 oligosaccharides, 3′-sialyllactose (**3′SL**), 6′-sialyllactose (**6′SL**), and 6′-sialyllactosamine (**6′SLN**) were the most abundant. Despite significant advancement in isolating glycosylated bioactive molecules from complex matrices at laboratory scale, progress in translating this science to practice has been limited. For this reason, we began collaborations with dairy and colostrum companies who have large-scale processing in place for protein concentration and isolation, and started annotating available dairy supplements—mostly marketed as immune system boosters—for their oligosaccharide content.

To assist the dairy industry in monitoring the efficiency of their oligosaccharide isolation processes and quality control, we developed a robust analytical method to quantify oligosaccharides of interest and simultaneously monitor the removal of lactose, which does not possess the desirable bioactivities of oligosaccharides. Simple carbohydrates such as lactose and trihexoses remain an obstacle for in vitro demonstration of many oligosaccharide bioactivities. Traditional chemical methods, such as the phenol-sulfuric acid assay, only provide a measure of total carbohydrates and cannot discriminate between simple lactose and complex oligosaccharides. High-performance liquid chromatography is able to separate the molecular species; however, derivatization is often required due to the lack of a chromophore in the oligosaccharide structure. Derivatization adds several preparation steps and ultimately causes sample losses; therefore, it may result in inaccurate quantification (Martin-Sosa et al., 2003; Nakamura et al., 2003). Recently, HPLC tandem mass spectrometry methods were developed (Fong et al., 2011; Bao et al., 2013; Hong et al., 2014; Liu et al., 2014). However, careful consideration must be given to evaluating and eliminating matrix effects that may affect quantification results (Matuszewski et al., 2003; Niessen et al., 2006). High-performance anion exchange chromatography-pulsed amperometric detection (**HPAE-PAD**) is used to separate a wider variety of milk carbohydrates because of its high-resolution separation, sensitive detection, simplicity of use, and reduced-cost compared with mass-spectrophotometric separation and detection (Kunz et al., 1996; Thurl et al., 1996; McJarrow and van Amelsfort-Schoonbeek, 2004).

In this study, we developed and validated an HPAE-PAD-based method for determination of the major bioactive BMO and lactose in whey permeate and commercial whey permeate-based products. To the best of our knowledge, this is the first report of the content of BMO in whey permeate products.

Whey permeate and whey permeate products were obtained from several companies. Liquid colostrum whey permeate was supplied by LaBelle Inc. (Bellingham, WA); 2 colostrum whey permeate-based commercial products (product A and product B) were provided by Sterling Technology Inc. (Brookings, SD), in powder and liquid form, respectively. Product C, a developmental product made from mature bovine milk using a processing method designed to increase the BMO naturally present, was supplied by Hilmar Ingredients (Hilmar, CA). Products A and B were generated solely using membrane filtration, whereas product C was obtained by combining membrane filtration with large-scale ion-exchange chromatography. The oligosaccharide standards (3′SL, 6′SL, and 6′SLN) were purchased from V-Labs Inc. (Covington, LA).

High-performance anion-exchange chromatography with pulsed amperometric detection (Thermo Scientific HPAE-PAD ICS-5000, Sunnyvale, CA) was equipped with a detector/chromatography module including a pulsed amperometry electrochemical detector, an electrochemical cell with a disposable gold working electrode, a pH-Ag/AgCl reference electrode, an auto-sampler, and a single pump. Samples were diluted 100-fold in distilled-deionized water without pretreatment because whey permeate and the commercially available whey-based products contained only trace amounts of other milk components such as lipids and proteins (Barile et al., 2009). The samples were filtered through a 0.22-μm membrane (Pall, Port Washington, NY) before analysis. A 25-μL sample was injected into

the CarboPacPA200 analytical column (3 × 250 mm, Dionex, Sunnyvale, CA) and a CarboPacPA200 Guard Column (3 × 50 mm, Dionex) for oligosaccharide analysis. The isocratic eluant used was 10 m*M* sodium acetate in 100 m*M* sodium hydroxide (NaOH). After each run, the column was cleaned with 200 m*M* NaOH for 5 min and equilibrated under initial conditions. The flow rate was 0.5 mL/min, and run time was 30 min. A CarboPacPA10 analytical column (4 × 250 mm, Dionex) and Guard Column (3 × 50 mm, Dionex) were used for lactose analysis. Lactose was analyzed in gradient, and the elution conditions were 0.0 to 6.0 min, 10 m*M* NaOH; 6.0 to 12.0 min, 10 to 50 m*M* NaOH; and 12.0 to 17.0 min, 50 m*M* NaOH. Then the column was cleaned with 200 m*M* NaOH for 8 min and equilibrated with 10 m*M* NaOH for 10 min. The flow rate was 1.3 mL/min.

To optimize the separation of BMO, a mixture of standards was prepared in 10 mg/L in distilled-deionized water. Figure 1 shows a chromatogram of mixed BMO standards with an optimized isocratic separation. The retention times of 6′SLN, 6′SL, and 3′SL were 16.8, 20.4, and 22.8 min, respectively. All 3 BMO were well resolved within 30 min.

Standard solutions for making a calibration curve were prepared by diluting the stock solutions to yield final concentrations of 0.1, 0.3, 1.0, 3.0, and 10 mg/L for each of the 3 analytes (3′SL, 6′SL, 6′SLN). Calibration curves were obtained by plotting the analyte peak areas against the concentrations of each compound (n = 3 at each concentration). The calibration curve for all 3 compounds was linear, with a correlation coefficient (R^2) of 0.999.

For precision and accuracy, recovery tests were performed on the whey permeate samples of known BMO concentrations added (10 and 100 μg in 1 mL; n = 5). The accuracy was verified by determining recoveries of BMO in spiked samples. Five replicates of each of the samples were spiked with known amounts (10 and 100 μg/mL) of BMO before sample preparation. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The average recovery of 3′SL, 6′SL, and 6′SLN ranged from 98.45 to 100.44% with a relative standard deviation of 1.17 to 5.89% (Table 1). The results indicate that this method can accurately measure BMO in whey permeate.

The limit of detection (**LOD**) and limit of quantification (**LOQ**) are measures of the precision of preparing and analyzing low-level standards according to the method. Based on Environmental Protection Agency criteria, the LOD for 3 BMO were determined by making 7 injections of a low-level solution fortified with BMO at 3 to 5 times the estimated LOD. The LOD were calculated using the calibration curve. The calculated LOD obtained by this method were 0.10 mg/L for 6′SLN, 0.03 mg/L for 6′SL, and 0.22 mg/L for 3′SL. The LOQ were 0.33 mg/L for 6′SLN, 0.10 mg/L for 6′SL, and 0.70 mg/L for 3′SL.

A commercial colostrum whey permeate (derived from the production of immunoglobulin-rich products) and whey permeate products (derived from cheese making) were evaluated for their lactose and acidic BMO content (Table 2). Figure 2A shows the separation of BMO in whey permeate. Lactose was eluted in the void volume. This chromatogram showed that the 3 BMO were well separated from each other and from matrix-related peaks. In colostrum whey permeate, 3′SL, 6′SL, and 6′SLN were present at 94, 29, and 46 mg/L, respectively.

Previous studies showed that the concentration ranges of 3 oligosaccharides in bovine colostrum were 261 to 850, 92 to 147, and 97 to 210 mg/L (Martin-Sosa et al., 2003; Nakamura et al., 2003; McJarrow and van Amelsfort-Schoonbeek, 2004). Our values were lower than those reported in previous studies; indeed, the colostrum whey we analyzed was significantly diluted. This was also evidenced by the low lactose concentration: 7 g/L instead of 27 g/L (Tsioulpas et al., 2007). In the whey protein industry, it is common practice to maintain a constant volume during filtration by adding water (a process known as diafiltration) to achieve effective lactose wash-out and reach the desired protein concentration.

Figure 2B–2D and Table 2 show the separation of BMO and their concentrations in the whey permeate products. Total sialylated BMO contents were 151 mg/g and 665 mg/L for product A and product B, respectively. Product A contained 107 mg/g of 3′SL, 18 mg/g of 6′SL, and 27 mg/g of 6′SLN. Product B had 453 mg/L of 3′SL, 93 mg/L of 6′SL, and 119 mg/L of 6′SLN. Christiansen et al. (2010) reported that product A contained 58.2% (wt/wt) lactose, which is higher than our finding (42.4%, wt/wt; Christiansen et al., 2010). They assumed that the oligosaccharide content in the product was only 3% (wt/wt); however, our measurement revealed that it was 15.1% (wt/wt). Product C was lactose-free and contained high levels of BMO, with 3′SL, 6′SL, and 6′SLN being 325, 37, and 5 mg/g, respectively.

With regard to the ratio of BMO to lactose, 100 times more lactose was found than BMO in colostrum whey permeate (BMO/lactose = 0.0097). The lactose was partially removed from product A (BMO/lactose = 0.3585) and product B (BMO/lactose = 0.0198). The undesirable lactose was partially eliminated during membrane filtration; therefore, much less lactose was found in products A and B. Product C did not contain detectable lactose. Ion-exchange chromatography may have separated neutral lactose and anionic sialylated BMO, therefore resulting in a lack of lactose in the product.

Christiansen et al. (2010) initially established the basic chemical composition of colostrum, colostrum whey permeate, and ultrafiltration permeate colostrum whey low-molecular weight fraction (**CLMWF**). The composition of CLMWF is similar to that of whey permeate and is quite different from that of bovine colostrum; however, no information on BMO content was reported. Our work demonstrated that whey permeate products contain significant amounts of BMO; therefore, these products may have biological efficacy as prebiotics and anti-infectives. Products enriched in BMO and impoverished in lactose will enable their use in specific functional tests with cell lines and probiotic bacteria that, in turn, will further advance knowledge about the functional properties of milk oligosaccharides.

This paper describes an accurate method to separate and quantify the major BMO (3′SL, 6′SL, and 6′SLN) in whey permeate and whey permeate products. The method uses a semi-direct injection into HPAE-PAD and separation is achieved in just 30 min. The sample preparation is simplified compared with that used in other methods and does not necessitate multiple vial transfers, which are associated with sample losses. In the present study, several whey permeate products were measured accurately and rapidly. Once these oligosaccharides are available in large scale, their functionality beyond infant formula supplementation can be

expanded to explore their activity on intestinal disorders associated with HIV infection, premature birth, and chemotherapy (Underwood et al., 2014).

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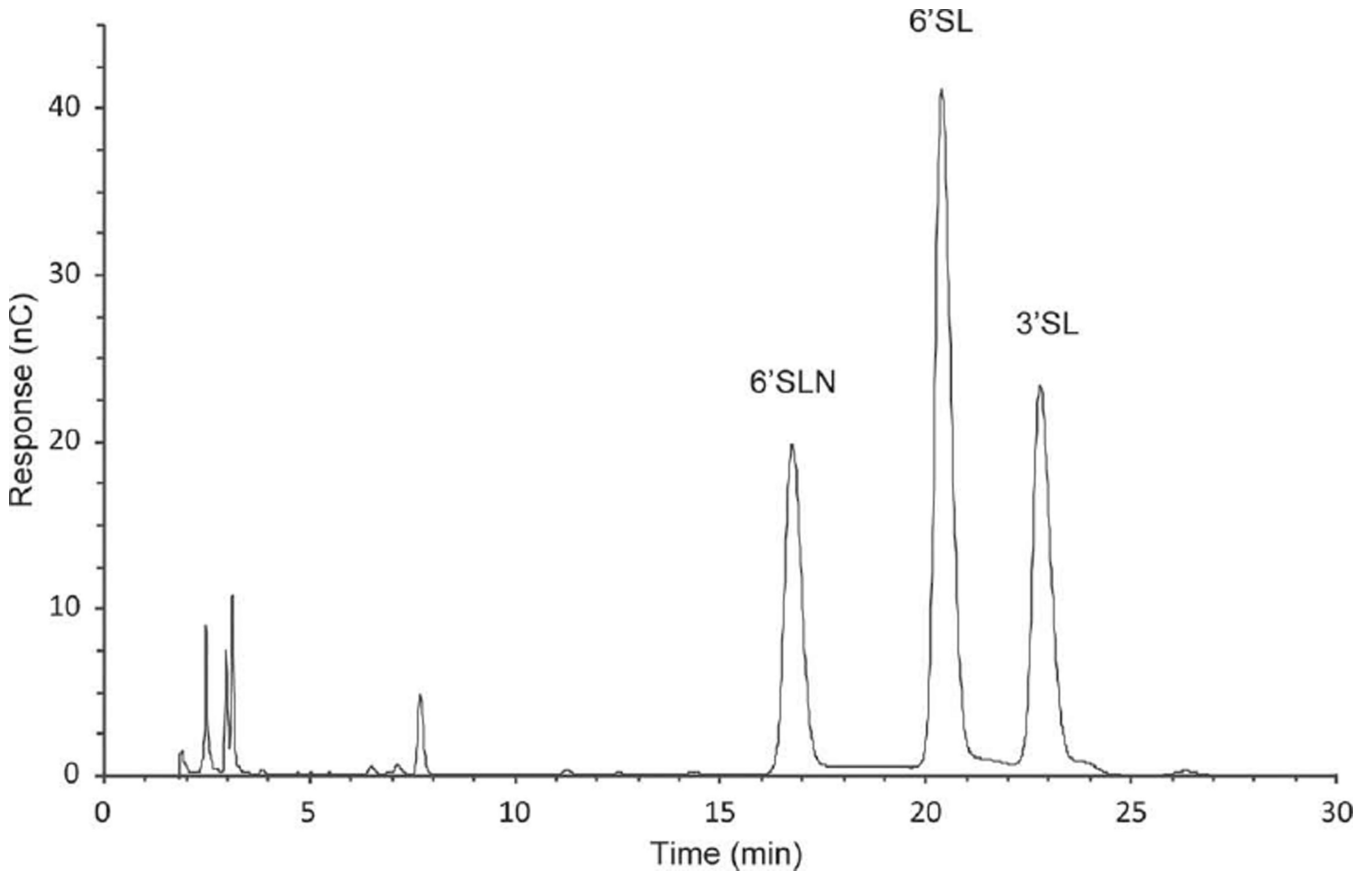

Figure 1.

Separation of a mixed-bovine milk oligosaccharide standard of 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL) and 6′-sialyllactosamine (6′SLN). The analytical column was Dionex CarboPac PA200 (3 × 250 mm; Dionex, Sunnyvale, CA) and the guard column was Dionex CarboPac PA200 (3 × 250 mm). The flow rate was 0.5 mL/min. The standards (10 mg/L) were injected in 25 μL. Retention times of 6′SLN, 6′SL, and 3′SL were 16.8, 20.4, and 22.8 min, respectively.

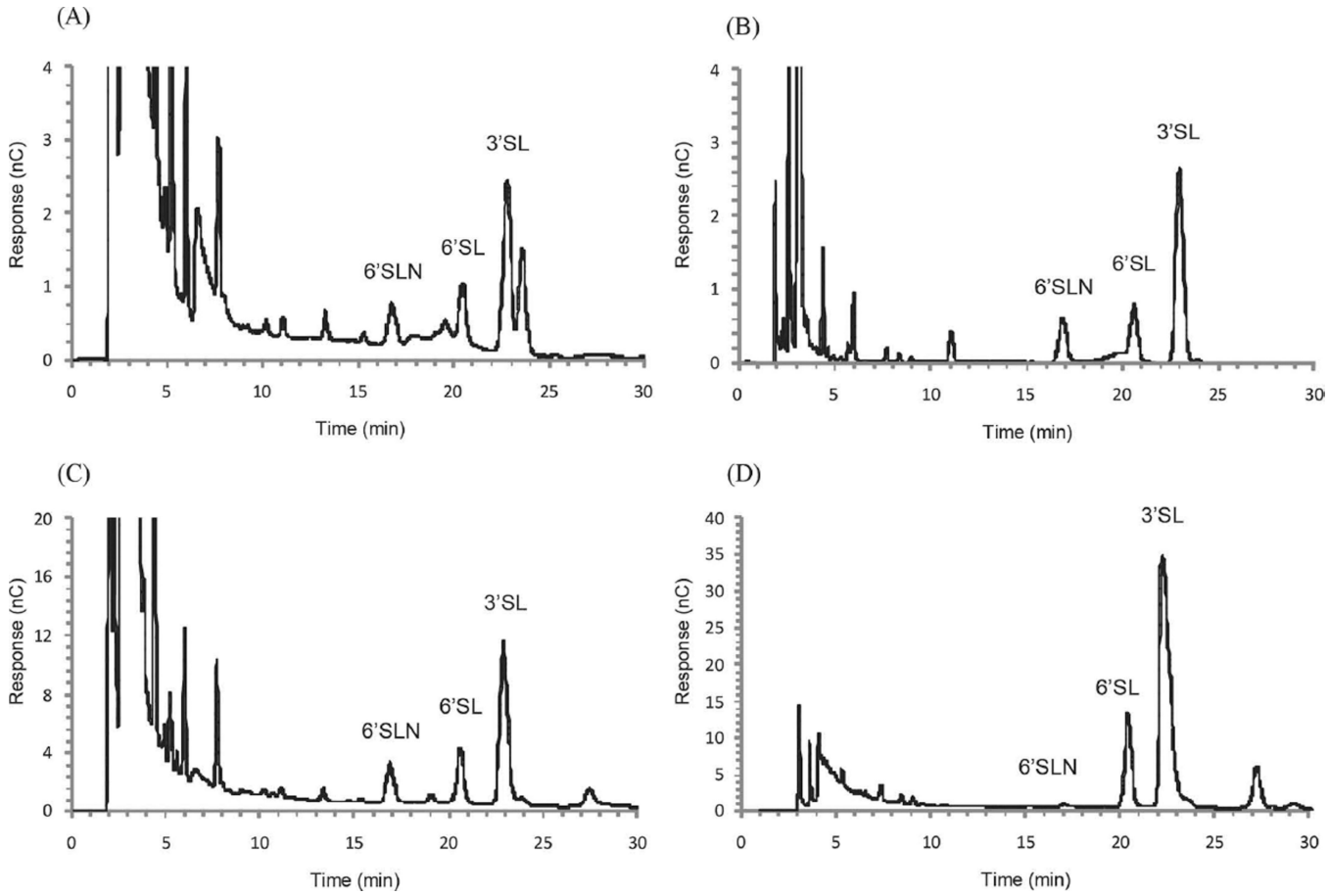

Figure 2.

Separation of bovine milk oligosaccharides (BMO) in whey permeate and whey permeate products. (A) Colostrum whey permeate, (B) product A, (C) product B, and (D) product C. 3′SL = 3′-sialyllactose; 6′SL = 6′-sialyllactose; 6′SLN = 6′-sialyllactosamine.

Table 1

Recovery of bovine milk oligosaccharides (BMO) from whey permeate samples (n = 5)

BMO	Added (μg)	Recovery (%)	RSD[1] (%)
3′-Sialyllactose	10	99.45	3.53
	100	100.11	1.80
6′-Sialyllactose	10	100.44	2.40
	100	99.96	1.17
6′-Sialyllactosamine	10	98.45	5.89
	100	100.08	4.12

[1] RSD = relative standard deviation.

Table 2

Lactose and bovine milk oligosaccharide (BMO) concentrations in a colostrum whey permeate and whey permeate products (n = 5)

Carbohydrate	Whey permeate (mg/L, RSD%[1])	Product A (mg/g, RSD%)	Product B (mg/L, RSD%)	Product C (mg/g, RSD%)
Lactose	17,417, 3.6	424, 2.0	33,624, 0.4	Below LOQ
3′-Sialyllactose	94, 7.5	107, 4.2	453, 6.3	325, 6.5
6′-Sialyllactose	29, 5.2	18, 3.8	93, 5.1	37, 4.2
6′-Sialyllactosamine	46, 7.1	27, 9.8	119, 6.1	5, 0.6
BMO/lactose	0.0097	0.3585	0.0198	

[1] RSD = relative standard deviation.