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Coupling flash liquid chromatography with mass spectrometry for enrichment and isolation of milk oligosaccharides for functional studies

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ABSTRACT

Mass spectrometry has been coupled with flash liquid chromatography to yield new capabilities for isolating nonchromophoric material from complicated biological mixtures. A flash liquid chromatography/tandem mass spectrometry (LC/MS/MS) method enabled fraction collection of milk oligosaccharides from biological mixtures based on composition and structure. The method is compatible with traditional gas pressure-driven flow flash chromatography widely employed in organic chemistry laboratories. The online mass detector enabled real-time optimization of chromatographic parameters to favor separation of oligosaccharides that would otherwise be indistinguishable from coeluting components with a nonspecific detector. Unlike previously described preparative LC/MS techniques, we have employed a dynamic flow connection that permits any flow rate from the flash system to be delivered from 1 to 200 ml/min without affecting the ionization conditions of the mass spectrometer. A new way of packing large amounts of graphitized carbon allowed the enrichment and separation of milligram quantities of structurally heterogeneous mixtures of human milk oligosaccharides (HMOs) and bovine milk oligosaccharides (BMOs). Abundant saccharide components in milk, such as lactose and lacto-*N*-tetraose, were separated from the rarer and less abundant oligosaccharides that have greater structural diversity and biological functionality. Neutral and acidic HMOs and BMOs were largely separated and enriched with a dual binary solvent system.
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Low-pressure liquid chromatography (LPLC)[1] is a standard method for purifying substrates from complicated matrices, providing a crude but generally effective method for isolating relatively large amounts of material. In addition, the method is relatively simple and robust, allowing a wide range of applications. LPLC is often distinguished from medium-pressure liquid chromatography (MPLC) by the pressure typically used. LPLC is traditionally between 0 and 5 bar, whereas MPLC is between 5 and 20 bar. In contrast, high-performance liquid chromatography (HPLC) is operated between 20 and 400 bar, and ultra-performance liquid chromatography (UPLC) runs between 400 and 1200 bar. Each of these techniques offers unique strengths in terms of cost, compatible materials, speed, resolution, and loading capacity.

LPLC is unique in that it often does not require special pumps but can still be automated. Several manufacturers have integrated many functions of LPLC with some MPLC and HPLC and have marketed them as flash liquid chromatography (flash LC), making flash LC a critical technique for many preparative laboratories. Unlike the higher pressure techniques, LPLC is compatible with the same variety of glass columns and pressure-limited media as traditional flash LC [1] that employs gas pressure-driven flow between 0 and 2 bar [2–4]. However, operation in the lowest pressure regime is accompanied by unique spray stability challenges for sample introduction into the mass spectrometer.

Online sample detection for preparative LPLC has included ultraviolet/visible (UV/Vis) absorbance [5], fluorescence [6], evaporative light scattering [7], and refractometry [5]; however, there have been no reports, to our best knowledge, of online detection with mass spectrometry (MS). MS coupled to flash LC provides significant advantages over photometric detectors such as mass and structural information for all coeluting compounds. Online flash LC/MS can also initiate fraction collection for specific masses and specific structural types, allowing the automated enrichment of desired components from a highly complicated mixture. Similar approaches have been previously implemented with MPLC, HPLC, and UPLC [8].

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[1] *Abbreviations used:* LPLC, low-pressure liquid chromatography; MPLC, medium-pressure liquid chromatography; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; flash LC, flash liquid chromatography; UV/Vis, ultraviolet/visible; MS, mass spectrometry; HMO, human milk oligosaccharide; MS/MS, tandem MS; BMO, bovine milk oligosaccharide; GCC, graphitized carbon chromatography; SEC, size exclusion chromatography; TOF, time-of-flight; PGC, porous graphitized carbon; EIC, extracted ion chromatogram; GOS, galactose oligosaccharide.

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Detection with MS is particularly important for the isolation of compounds that lack useful chromophores or are found only in complicated mixtures such as human milk oligosaccharides (HMOs) [9]. HMOs are structurally heterogeneous and perform a variety of important biological functions in infants, including the establishment of beneficial intestinal microbiota and the prevention of pathogen infection [10]. Oligosaccharides are synthesized in the mammary gland of all mammals by the action of enzymes that add the monosaccharides glucose, galactose, *N*-acetylhexosamine, fucose, and sialic acid to the core lactose structure. From 3 to 15 monosaccharides are linked through various glycosidic bonds to form a plethora of linkage-specific isomeric forms. This structural complexity of oligosaccharides appears to be key to their biological activity. Detection of HMOs and other nonchromophores can be enhanced by derivatization with a chromophore or hydrophobic substituent [11,12]; however, biological assays such as cell binding and in vitro bacterial growth studies require compounds to be in their native (underivatized) states. Work by Thanawiroon and coworkers gives a good comparison of a chromatogram of oligosaccharides obtained by HPLC/MS versus HPLC/UV absorption [13]. The MS-based chromatogram showed more peaks and greater sensitivity because nonderivatized oligosaccharides do not efficiently absorb UV light [14].

Online LC/MS (with and without tandem MS [MS/MS]) is already a standard method for analytical and preparative HPLC [15]; however, MPLC methods have infrequently employed online LC/MS. One exception is the report by Hou and coworkers, where MPLC/MS was used to separate flavonoids and obtain structural information [16]. In both MPLC and HPLC, the MS is coupled via flow splitter with direct connection to the MS. This method works well because the flow is constant. In contrast, LPLC often requires varying the flow both within runs and between runs to obtain optimal separation. Modulating the flow requires significant retuning of the electrospray ionization source and is a consequence of changing flow rates with a static flow. We have overcome this limitation by employing a positive displacement piston pump.

This research focuses on isolating oligosaccharides from mammalian milks to obtain standards for determining specific biological functions of individual structures [9,17,18]. To test the function of specific oligosaccharides, the simpler carbohydrates (e.g., lactose) must be removed because they are fermentable by ostensibly all kinds of bacteria. Many intestinal bacterial species are also able to hydrolyze galactose and hexosamine, but bacterial binding and catabolism of fucosylated and sialylated oligosaccharides is restricted to beneficial commensal bacteria.

Many important applications await the isolation and characterization of the nondigestible oligosaccharides in milk. Human clinical studies with purified oligosaccharides are lacking because large amounts of milk oligosaccharides are not available. Therefore, there is great interest in evaluating sources of complex oligosaccharides other than human milk. Several oligosaccharides have been isolated and characterized in bovine colostrums, indicating that they possess similar structures and functions to human milk glycans. However, the absolute abundance declines rapidly during the first week of lactation through to maturity, when cows' milk oligosaccharide content is several-fold lower than that of human milk [19]. Because bovine milk contains only low amounts of these valuable oligosaccharides, it has not been considered as a viable source of oligosaccharides. Cheese whey has been viewed as a secondary product from cheese production for centuries, yet we recently discovered in whey permeate the presence of previously unknown complex fucosylated and sialyated oligosaccharides, analogous in composition and structure to HMOs [20,21].

This discovery has led to collaborations with the dairy industry to advance the research and development of these specific oligosaccharides as ingredients. Results from this research suggest that concentrating certain oligosaccharides from whey permeate can be a cost-effective process for the valorization of whey permeate into high-quality, profitable novel dairy ingredients. Implementing a systematic recovery of milk oligosaccharides from whey permeate would enable cheese makers to capture the value from this by-product, generating high-value ingredients and direct revenue.

The basic goal of separating oligosaccharides as a class of biomolecules from complex matrices such as milk has been previously developed in the microscale. A major challenge in isolating significant quantities of oligosaccharides from milk and dairy streams is to enrich the desired oligosaccharides while simultaneously reducing the content of lactose and other simple but more abundant sugars that do not possess the desired prebiotic/protective functions. In this work, we demonstrate the chromatographic and mass spectrometric performance of online flash LC/MS with a simple mixture of maltooligosaccharide standards as well as a complex mixture of oligosaccharides from biological extracts of human milk and cheese whey. Separation of compositional and isomeric forms of HMOs and bovine milk oligosaccharides (BMOs) was achieved with self-packed columns of pressure-limited graphitized carbon and size exclusion media [22,23]. In both large-scale and small-scale carbohydrate work, graphitized carbon and porous graphitized carbon are widely used to separate heterogeneous glycan mixtures as well as glycan positional isomers [9,24,25]. Graphitized carbon is ideal for separating oligosaccharide isomers because of the polar retention effect that is particularly sensitive to small structural differences [26].

Materials and methods

Materials and chemicals

HMOs and BMOs were extracted from donated samples. Pooled human milk samples were kindly donated by Prolacta Bioscience (Monrovia, CA, USA), and whey permeate samples were donated by Hilmar Ingredients (Hilmar, CA, USA). Oligosaccharides were isolated from milk/whey with 4 volumes of a chloroform–methanol (2:1, v/v) solution. The emulsion was centrifuged at 3500 rpm for 30 min. The lower chloroform layer and denatured proteins were discarded. The upper layer was collected, and the fraction was freeze-dried. The resulting powder (freeze-dried oligosaccharide-rich fraction) was used for oligosaccharide analysis. Between 2 mg and 2 g of the carbohydrate mixture (oligosaccharides and lactose) was dissolved in water and injected onto the flash column in volumes of 200 μl to 20 ml.

Standards, including lactose, isomaltotreose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose, were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in deionized water, and 200 μg of each standard was combined into a water-based cocktail that was injected onto the flash column in a volume of 110 μl (1.4 mg).

Graphitized carbon was purchased under the name Carbograph 120/400 Mesh from Grace Davison (Deerfield, IL, USA). Prepacked 4-g amine cartridges (8% amine content, 60 Å, mesh 230–400) were purchased from Luknova (Mansfield, MA, USA). Empty 4-g and 40-g cartridges were also purchased from Luknova. Size exclusion gel was purchased under the name Ultra Fine Bio-Gel P-2 from Bio-Rad (Hercules, CA, USA).

Flash chromatography

Separation was performed with a Teledyne–Isco CombiFlash Rf 200 (Lincoln, NE, USA). Columns were self-packed either dry for graphitized carbon chromatography (GCC) with either 4 g or 40 g of graphitized carbon (3.2 × 1.8 cm or 12 × 2.8 cm) or wet for size exclusion chromatography (SEC) with 20 g (three serial columns of 12 × 2.8 cm each). Graphite particles were 60 to 90 μm. Separation

was also attempted with a 4-g amino stationary phase. All experiments presented here were run at 5 ml/min except for the 40-g graphitized carbon columns that were run at 40 ml/min. The graphitized carbon columns operated at 1.7 bar. The stacked SEC columns operated at 0.9 bar, just below the maximum sustainable pressure for the gel (1.0 bar). The eluate was split and directed to the mass spectrometer (Fig. 1) by positive displacement with an FMI "Q" pump (Syosset, NY, USA) and by pressure differentials of varying tube sizes connected with HPLC splitters and mixers from Upchurch Scientific (Oak Harbor, WA, USA). The flow rate into the mass spectrometer was determined from the difference of flow rate to waste measured with the HPLC splitter valve open and the flow rate with the valve closed.

The gradient program combined two binary solvent systems to accomplish elution of neutral HMOs with neutral solvents followed by elution of acidic HMOs with acidic solvents. Solvent A was pure water for the first binary system and 0.1% formic acid in water for the second binary system. Solvent B had a similar formulation as solvent A except that it was based on acetonitrile. Methanol experiments were also performed by substituting methanol for acetonitrile as solvent B. The mobile phase used for SEC was deionized water.

GCC experiments were performed with one of two gradient programs, depending on whether the mobile phase contained acetonitrile or methanol. Most experiments were performed with acetonitrile as the organic phase. The dual binary solvent system consisted of the following solvents: pure water (A1), pure acetonitrile (B1), 0.1% formic acid in water (A2), and 0.1% formic acid in acetonitrile (B2). The following gradient program was employed: solvent A1 and B1 were used first with solvent B1 isocratic at 0% for 20 min, then increased from 0% to 15% between 20 and 40 min, isocratic for 10 min followed by a switch to the acidic binary solvent system (A2 and B2), then isocratic for 10 min, after which solvent B2 increased from 15 to 40% between 60 and 80 min, 40% to 100% between 80 and 100 min, isocratic at 100% between 100 and 115 min, 100% to 0% between 115 and 120 min, and isocratic at 0% between 120 and 140 min, and then returned to the nonacidic system (A1 and B1). The SEC column was run with pure water as the mobile phase. The amino column was run with acetonitrile and water mobile phases in 0.1% formic acid, with a constant slope gradient from 0 to 100% water over 60 min.

Mass spectrometry

Flash separated oligosaccharides were detected online with a Thermo Finnigan LCQ Duo quadrupole ion trap mass spectrometer (San Jose, CA, USA). Mass spectra were obtained approximately every 1 s (six microscans) between m/z 300 and 2000. Ions were generated in positive mode with an on-axis electrospray source at 4.2 kV.

Fractions were collected in 2-min intervals and dried down. Analyses of the fractions were performed with the Agilent 6210 HPLC–Chip/TOF–MS (Santa Clara, CA, USA). Time-of-flight (TOF) mass spectra were collected every 1.6 s with 16,000 transient scans per spectrum. The Chip LC contained a preconcentration column (40 nl, 4 mm) and an analytical column (43 × 0.075 mm), both made from porous graphitized carbon (PGC). For the gradient, solvent A was 0.1% formic acid in 3% acetonitrile and solvent B was 0.1% formic acid in 90% acetonitrile. The gradient program (in reference to solvent B) was 0% between 0 and 2.5 min, 0% to 16% between 2.5 and 20 min, 16% to 44% between 20 and 30 min, 44% to 100% between 30 and 35 min, isocratic at 100% between 35 and 45 min, and isocratic at 0% between 45 and 60 min. The preconcentration column was run on 100% A at 4 μl/min except when the column was in-line with the analytical column, which flowed on the described gradient program at 0.3 μl/min.

Data analysis

Data collected online with the quadrupole ion trap were processed into extracted ion chromatograms (EICs) with Thermo Scientific's Xcalibur 1.3 software (San Jose, CA, USA). EICs were generated with a 0.5-Da window around each theoretical oligosaccharide mass-to-charge ratio. The chromatograms were smoothed by the software with a 15-point boxcar moving average. Validation data collected with the HPLC–CHIP/TOF–MS were processed into total, base peak, and extracted ion chromatograms with Agilent's MassHunter version B 03.01 software. EICs were generated with a 20-ppm mass window around each theoretical mass-to-charge ratio. Each EIC was a composite of all the charge states detected for the given oligosaccharide. Chromatograms were smoothed with one iteration according to the software's built-in smoothing feature.

Results and discussion

Instrument integration

The most common mode of detection for traditional flash LC is to manually add a drop from each collected fraction onto a thin-layer chromatography (TLC) plate. In contrast, more sophisticated

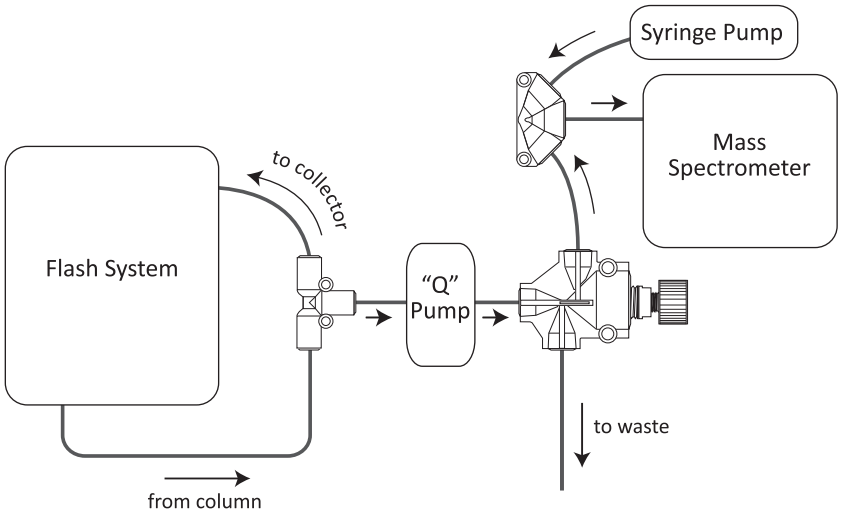

Fig.1. Diagram showing plumbing connections between the flash system and the mass spectrometer. The eluate is split twice to reduce the flow rate. Additional organic solvent and charge carriers are added through a mixing-T just before the eluate reaches the mass spectrometer.

flash LC devices typically provide online detection by UV/Vis absorbance or evaporative light scattering. Mass detection with flash LC is less common, probably due to cost or need for operator expertise. Mass detection was the only viable solution for our sample, so we set up a dedicated mass detector for flash LC/MS with reasonable economy and simplicity by salvaging a low-cost quadrupole ion trap [27] mass spectrometer equipped with an electrospray ionization source.

The mass of material commonly separated with flash LC ranges over multiple orders of magnitude, requiring the user to optimize flow rates between 1 and 200 ml/min and pressures between 0.5 and 5.0 bar. Mass detectors can be quite sensitive to these method changes, particularly for mid-run changes, resulting in singularities and poor signal. Previously published examples of flash LC/MS used a static split-flow connector to divert a small portion of the eluate to the mass spectrometer. Proper flow to the MS is typically accomplished by finding the correct combination of tubing lengths and inner diameters that direct the desired portion toward the mass spectrometer. Such connectors do not automatically accommodate real-time method changes. We overcame this issue by employing a commercially available positive displacement piston pump, the so-called "Q"-pump, to remove eluate from the postcolumn flow at a rate of 0.5 ml/min regardless of the flow rate from the flash system (Fig. 1). The Q-pump guaranteed accurate delivery and allowed the ionization conditions to be tuned only once regardless of method changes with the flash LC. One of the important characteristics of this pump in comparison with the other pumps available for this application is the steady flow rate with varying pressure and viscosity.

Following the Q-pump, the flow was split again with a valved HPLC T-splitter to reduce the portion that was sent to the mass spectrometer down to 70 μl/min. The flow was then combined in a mixing-T with a 20-μl/min flow of 0.1 mM sodium acetate in 30% acetonitrile (Fig. 1). The sodium acetate eliminated virtually all in-source fragmentation (Fig. 2) and permitted the use of pure (nonbuffered) solvents. The addition of solvent at the source also dampened the spray oscillation caused by the piston in the Q-pump and avoided contaminating the fractions with salt. The 30% acetonitrile solution improved ionization by lowering the viscosity of high aqueous mobile phases and increasing the viscosity for the high organic mobile phases. The added solvent diluted the eluate and improved the signal, presumably because the electrospray ionization process is dependent on concentration and is optimal at approximately 10^{-6} M [28].

Separation was performed on a self-packed column of graphitized carbon. The packing procedure is simple yet vital for success. Unlike size exclusion gel and certain other chromatographic media, graphitized carbon should not be added to the column as a slurry. The dry powder should be added to the column directly and without compression. Similar to dry-packed silica gel columns, the graphitized carbon powder should be settled by gently tapping the column with a flexible rubber hose. The column should be wetted at 5 ml/min, avoiding irreversible compression. After being fully wetted, the column can be operated at any flow rate or pressure that does not compress the packing material. The columns were operated at flow rates up to 60 ml/min and pressures up to 50 psi with only minor reversible compression of the graphitized carbon. The maximum pressure and flow rate that could have been employed by the flash chromatograph were 200 psi and 200 ml/min.

Graphitized carbon chromatography

The application of a positive displacement piston pump and a preparative scale graphitized carbon cartridge is a novel approach for flash LC/MS. This technical article demonstrates the feasibility of the technique for the fractionation of carbohydrates extracted from human and bovine milk and whey. The crucial requirement for meaningful fractionation of these samples is removal of lactose, an abundant carbohydrate in milk that tends to interfere with mass detection of lower abundant oligosaccharides and subsequent functional testing. Abundant carbohydrates also interfere with bacterial growth studies on the low-abundant nondigestible oligosaccharides. The capabilities of the method include isolation of oligosaccharides by monosaccharide composition and class [29] (e.g., neutral vs. acidic). Some isomer and size-based separation was also achieved.

Bovine milk oligosaccharides

Separation of oligosaccharides by class with GCC was demonstrated with a 200-mg sample of extracted carbohydrates from bovine whey. Prior to flash LC/MS, the abundant galactose oligosaccharides (GOSs) and neutral BMOs suppressed the signal of less abundant neutral and acidic BMOs. The abundant GOSs were mostly removed, and the BMOs were separated into several simpler fractions that included the separation of sialylated and nonsialylated BMOs. Prior to fractionation with flash LC, the base peak was the GOS 3Hex (Fig. 3A). The second most abundant compound had a 10% relative base peak intensity and was the BMO 2Hex 1GlcNAc. Four additional GOSs and three additional BMOs were identified at a 5% base peak height. After flash LC/MS, several fractions were collected that lacked the most abundant GOSs and had enriched for several BMOs. A representative fraction that was largely enriched with two BMOs, 4Hex 1GlcNAc and 3Hex 3GlcNAc, is shown in Fig. 3B. The fraction shown in Fig. 3C was enriched primarily for the acidic BMO 2Hex 1NeuAc with an additional acidic BMO detected below 5% of the base peak height. Additional fractions that were collected will be described in another publication. Representative neutral and acidic BMO structures are provided in Supplemental Fig. 1 of the supplementary material.

The observed BMOs and GOSs are annotated in Supplemental Table 1 of the supplementary material. Although only a few acidic BMOs were observed, we were able to completely isolate them from the neutral BMOs. The most abundant carbohydrate that was primarily responsible for the ion suppression in the original sample was virtually absent from the later fractions, including the fractions not shown here. Neutral and acidic BMOs were separated within the same run by employing two different solvent systems (two solvent compositions per system) that were automatically integrated into the gradient program. The neutral BMOs were eluted with neutral solvent, and the acidic BMOs were eluted with acidic solvent. Acidic BMOs were clearly identified by a pair of oxonium mass-to-charge ratios that almost always accompany sialylated BMOs: m/z 274 and 292. Separation of large amounts of oligosaccharides by class (e.g., neutral and acidic) will enable functionality studies of oligosaccharides that would otherwise be prohibitively expensive or entirely unavailable.

Human milk oligosaccharides

The online mass detection of a 12-mg HMO and lactose sample is shown in Fig. 4. Lactose eluted between 0 and 37 min. The neutral HMOs eluted between 37 and 70 min, and the acidic HMOs eluted between 80 and 105 min. The neutral HMOs were separated into 14 fractions (Table 1). Each fraction was characterized in real time by MS/MS. The precursor and product ion scans for one of the fractions are shown in Fig. 5. Online MS/MS was vital for isolation of HMOs with flash LC. The fragmentation data confirmed the carbohydrate composition that was frequently ambiguous for ions with multiple charge states due to low-mass accuracy. The fragmentation also allowed efficient fractionation by class, including neutral, fucosylated, and sialylated HMOs. The fractions were

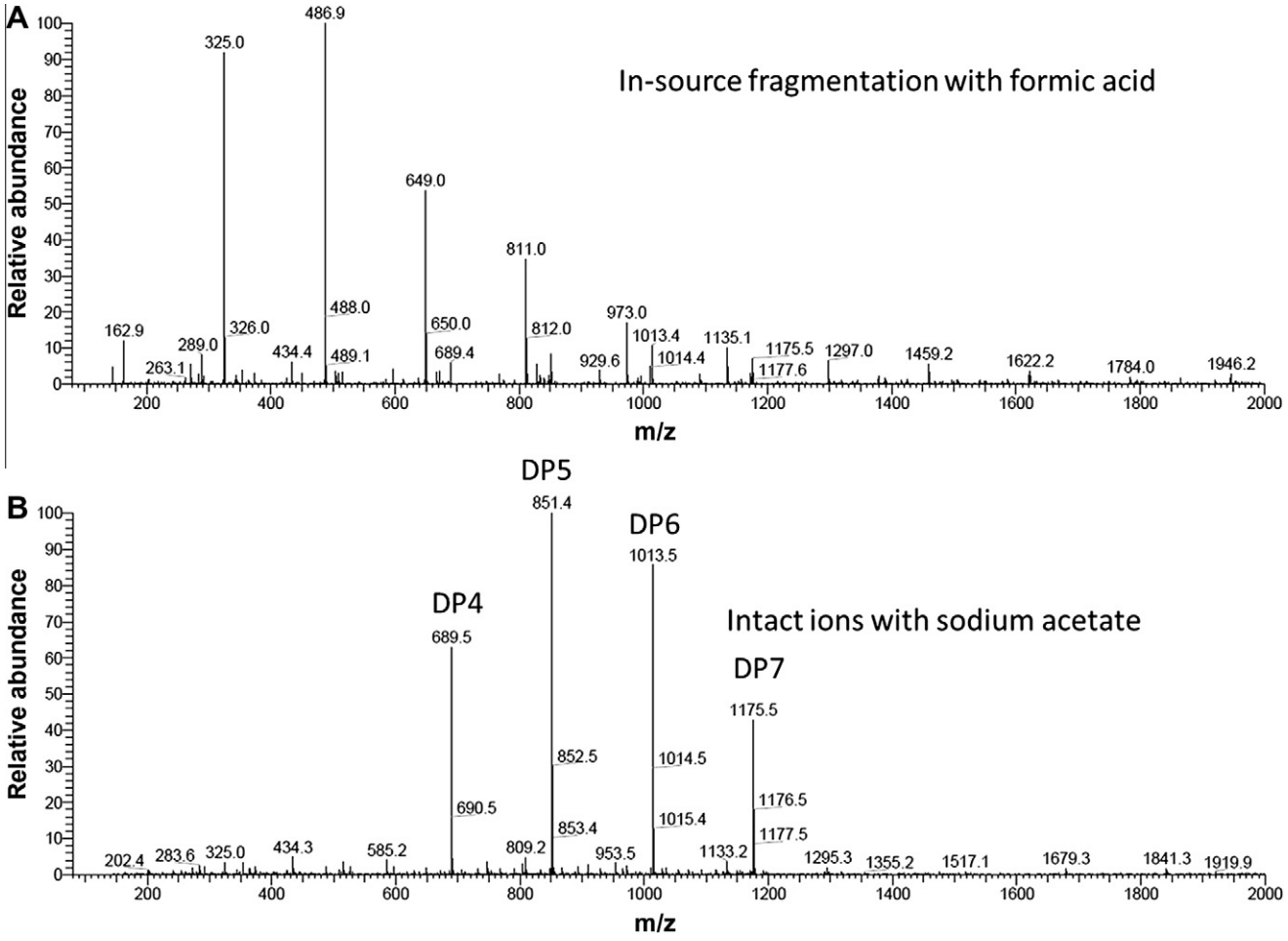

Fig.2. Comparison of electrospray ionization of maltooligosaccharides with degree of polymerization (DP) 4 through 7 in the presence of formic acid (A) versus sodium acetate (B). The sodiated oligosaccharides do not fragment appreciably in the source due to a higher energy activation barrier. The signals for DP8 and higher in panel A are a result of in-source rearrangements that are common for protonated oligosaccharides analyzed by electrospray ionization–MS.

collected constantly throughout the run, allowing us to use the elution profiles to determine which tubes to pool together. The fractions containing coeluting HMOs were further separated with HPLC/MS (data not shown here). The secondary separation afforded additional isolated compounds. The crude fractionation of these compounds with flash LC/MS was a time-saving step permitting the HPLC column to be loaded with a greater amount of fewer compounds and, therefore, fewer repetitive separations to obtain a sufficient amount of each compound.

Method characterization

The method for flash separation of oligosaccharides with GCC was characterized with several experiments. First, the performance of GCC was compared with two readily available stationary phases for flash LC columns: amine and size exclusion gel. Second, the day-to-day reproducibility of the elution profile was demonstrated. Third, a range of acceptable loading capacities was determined. Fourth, the separation performance of partially resolved peaks was explored. Fifth, acetonitrile was compared against methanol as an organic mobile phase (solvent B). Finally, fractionation of nearly coeluting isomers was accomplished.

Comparison with common stationary phases

Separation of HMOs was compared among three stationary phases: graphitized carbon, amine, and size exclusion gel. These media were compared in regard to the following three applications for human milk: removal of lactose, isolation of HMOs by monosaccharide composition, and separation of the neutral and acidic HMOs. Only graphitized carbon performed adequately for all three tasks, whereas the amine column failed at all three tasks. The size exclusion gel succeeded at separating lactose from the majority of HMOs; however, the size exclusion gel was unable to perform the other two tasks. The performance of the size exclusion gel with the novel instrument setup may still be useful for some applications and is shown in Fig. 6. Although the HMOs with masses greater than 800 Da were not significantly resolved from each other, several lower mass HMOs were sufficiently resolved from the higher masses, removing these highly concentrated compounds from the low-abundant high-mass HMOs that have greater bioactivity.

According to these results, size exclusion gel could be a valuable medium for flash removal of abundant low-mass carbohydrates such as lacto-*N*-tetraose (3Hex 1GlcNAc, protonated adduct *m*/*z* 708.0) and lactose (2Hex, sodiated adduct *m*/*z* 365.1). These abundant sugars limit the dynamic range for analysis of complex mixtures of HMOs. The separation of the higher mass HMOs could be further improved by lengthening the column; however, our experiments approached the 1.0-bar limit of the gel at a 36-cm column length and a 2.8-cm column diameter. Additional column length would require larger column diameters to reduce the pressure. Although inferior to GCC for most applications, the size exclusion gel has the benefit of allowing all high-mass HMOs to readily elute together for flash removal of the low-mass carbohydrates. Coelution of the high-mass HMOs significantly reduces subsequent sam-

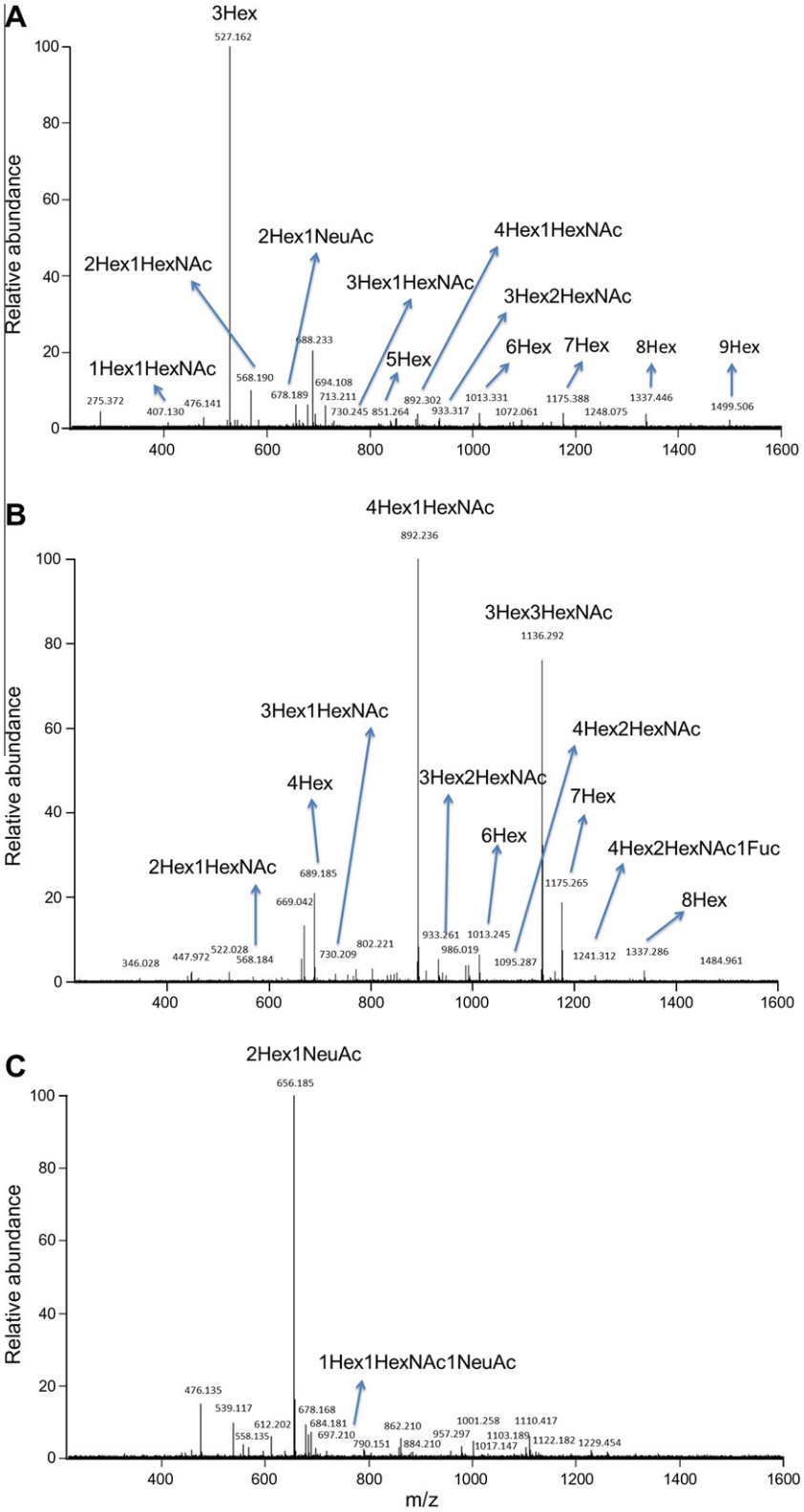

Fig.3. (A) Pooled BMO signal that is suppressed by GOSs. (B) Representative neutral BMO fraction that enriches for two BMOs. (C) Representative acidic BMO fraction that is highly enriched for 2Hex 1NeuAc.

ple storage and dry-down. The high-mass HMOs elute fairly rapidly, saving significant time. Another nontrivial benefit is the use of water as the mobile phase. In contrast, GCC elutes the moderate- and high-mass HMOs based on polarity rather than size; therefore, low- and high-mass HMOs might not always be separated. GCC also requires significantly more time and solvent.

Reproducibility

After packing several columns with graphitized carbon and reviewing the separation profiles, it is clear that the separation of HMOs is robust and can be repeated. The retention times are also quite reproducible from day to day for a given column, as shown by identical separations performed 24 h apart (Supplemental

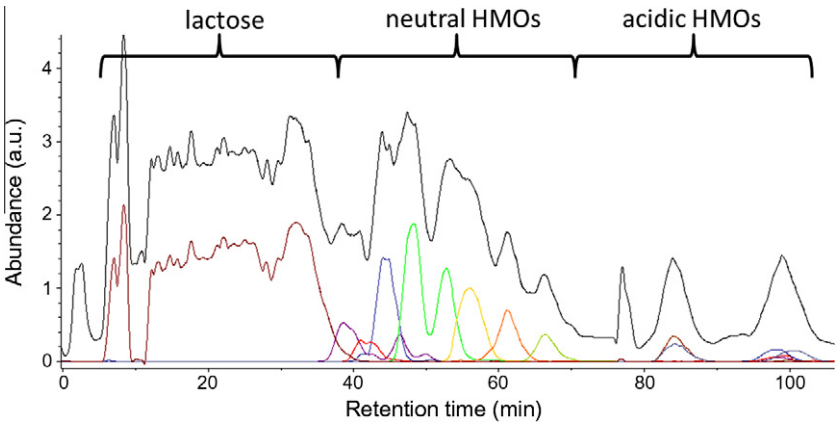

Fig.4. Separation of a 12-mg mixture of HMOs and lactose. Some of the base peaks eluted with greater than 90% purity. The EICs shown here are tabulated in Table 1. The first compound in Table 1 (3Hex 1GlcNAc 2dHex) is the purple line. The red line is 2Hex 2dHex. The blue line is 3Hex 1GlcNAc 2dHex. The green line is 3Hex 1GlcNAc 1dHex. The light orange line is 3Hex 1GlcNAc. The dark orange line is 4 Hex 2GlcNAc 1dHex. The off-green line is 4Hex 2GlcNAc. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.).

Table 1
Isolated HMOs collected.

m/z	Purity (%)	Retention time (min)	%B	Composition	Adduct	Class
1022.7	55	39	14.3	3Hex 1GlcNAc 2dHex	$[M + Na]^+$	Neutral
657.8	36	42	15	2Hex 2dHex	$[M + Na]^+$	Neutral
999.8	63	45	15	3Hex 1GlcNAc 2dHex	$[M + H]^+$	Neutral
876.8	87	48	15	3Hex 1GlcNAc 1dHex	$[M + Na]^+$	Neutral
876.8	85	53	15	3Hex 1GlcNAc 1dHex	$[M + Na]^+$	Neutral
730.8	69	56	15	3Hex 1GlcNAc	$[M + Na]^+$	Neutral
1241.8	95	62	17.5	4Hex 2GlcNAc 1dHex	$[M + Na]^+$	Neutral
1095.7	93	66	22.5	4Hex 2GlcNAc	$[M + Na]^+$	Neutral
634.5	99	84	52	2Hex 1NeuAc	$[M + H]^+$	Acidic
999.1	77	98	94	3Hex 1GlcNAc 2dHex	$[M + H]^+$	Neutral
656.7	86	103	100	2Hex 1NeuAc	$[M + Na]^+$	Acidic

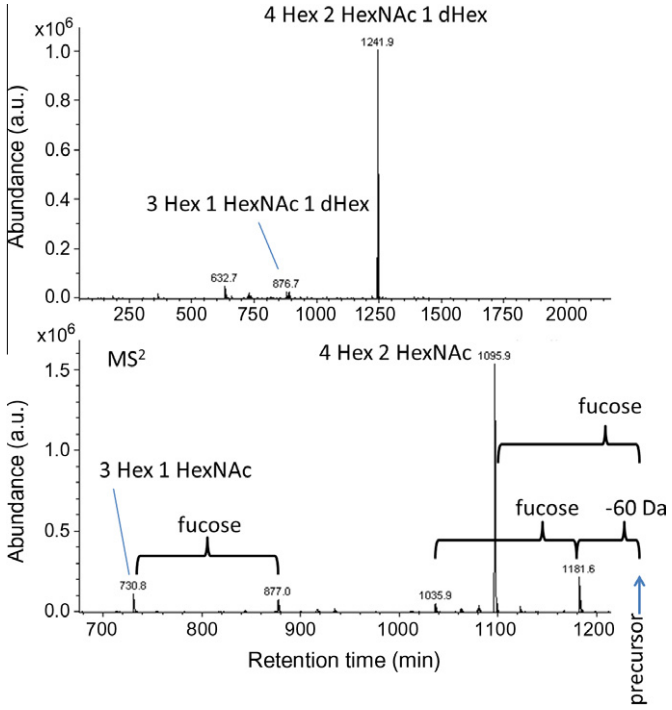

Fig.5. Representative mass spectrum and tandem mass spectrum (MS^2) of an HMO separated from a pool of HMOs. The tandem data were vital for recognizing sugars due to the low-mass accuracy of the instrument. All mass-to-charge ratios are singly protonated species.

Fig. 2); however, we have observed some variations of peak intensities that have not been explained.

Loading

The method can be scaled up from 0.5 to 200 mg with only minor changes to the elution profile and gradient program. Depending on the amount of graphitized carbon and sample, the user may see some flow-through within the first few minutes; however, this problem can generally be avoided by using more stationary phase material with a longer and slightly wider column. A 60-mg sample of HMOs and lactose was separated without flow-through on a 40-g column, as shown in Supplemental Fig. 3. Furthermore, the flow-through has not been associated with any significant effect on the chromatographic performance. The seemingly insignificant effect of the flow-through on the HMO peaks might be due to the lack of mass transport of the oligosaccharides during the isocratic water flow at the beginning of the gradient program, the time when the most abundant component, lactose, is removed. Indeed, HMOs remain indefinitely bound to the stationary phase with a mobile phase of 100% water.

Fractionation of unresolved peaks

Flash LC/MS is particularly useful for partially resolved compounds that would otherwise be detected under one large peak by less specific detection techniques (e.g., evaporative light scattering, refractometry). This problem is particularly relevant to flash LC in the low-pressure regime. We have demonstrated the ability of this technique to fractionate unresolved oligosaccharides in real time. A mixture of maltooligosaccharides, DP2 (two degrees of

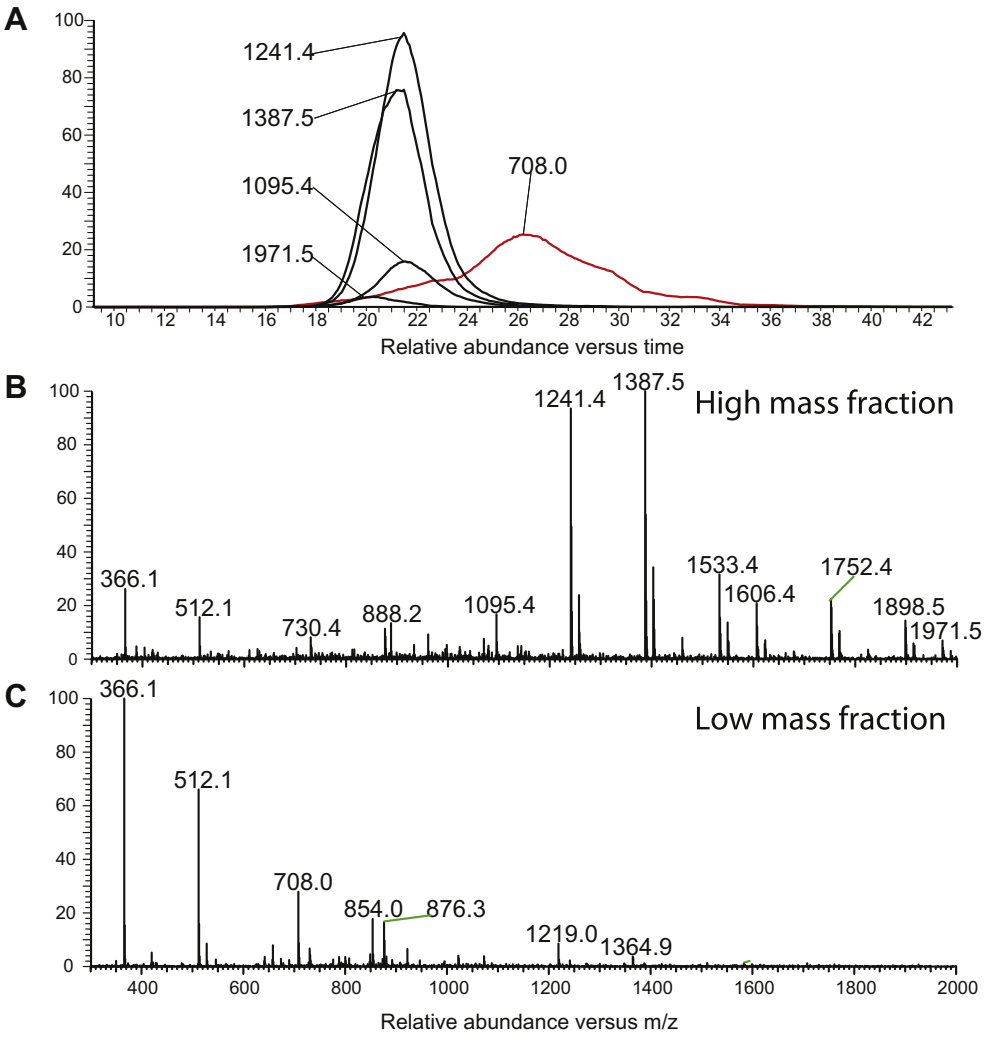

Fig.6. Abundant HMOs less than 800 Da were significantly separated from HMOs greater than 1200 Da with SEC. (A) EICs for five HMOs obtained with a quadrupole ion trap online with flash LC. EICs for four HMOs larger than 1200 Da are shown as black lines: (in descending order from the most abundant chromatographic peak): *m/z* 1241.4 (4Hex 2GlcNAc 1dHex), *m/z* 1387.5 (4Hex 2GlcNAc 2dHex), *m/z* 1095.4 (4Hex 2GlcNAc), and *m/z* 1971.5 (6Hex 4GlcNAc 1dHex). The red extracted ion chromatogram is *m/z* 708.0 (3Hex 1GlcNAc, predominantly the protonated form of LNT, typically the most abundant HMO). (B and C) Representative mass spectra of high- and low-mass fractions collected online with flash LC. (For interpretation of the reference to color in this figure legend, the reader is referred to the Web version of this article.).

polymerization) through DP7, was fractionated with size exclusion gel flash LC/MS, as shown in Supplemental Fig. 4. The online detection of the poorly resolved EIC peaks enabled collection at the appropriate times for enrichment of each maltooligosaccharide relative to the original mixture.

Although not well resolved, detection of the EICs in Supplemental Fig. 5 allowed an informed selection of the amount of peak overlap of the desired components. Furthermore, a common feature for commercial mass spectrometers is a programmable contact closure between the mass spectrometer and fraction collector to indicate when a peak is eluting and should be collected into a new tube. Unlike less-specific detection techniques, these compounds need not be resolved in the total ion chromatogram (TIC) in order to trigger collection into different test tubes. As long as a minimum intensity threshold is reached for any monitored ion, the software can activate a relay switch connected to any external fraction collector to advance the collection to a new tube and enrich the eluting compound. This strategy has been previously demonstrated with flash chromatography in other pressure regimes.

Acetonitrile versus methanol

Additional experiments were performed with methanol rather than acetonitrile. The more polar methanol solvent effected greater separation of the neutral HMOs, as shown by the EICs in Supplemental Fig. 5 and the chromatographic performances in Supplemental Table 1. The weaker methanol solvent retarded the elution time of all the HMOs. However, the acidic HMOs never eluted with methanol, and the resulting signal-to-noise ratio was lower with methanol, even though partial methanol solvent is ideal for electrospray ionization. The reason may be due to the significantly broader peaks, resulting in lower concentrations during each scan in the mass spectrometer. The observation of broader peaks corresponds with the greater polarity of methanol relative to acetonitrile.

The experiments comparing the acetonitrile and methanol solvent systems were not performed with the optimized dual binary solvent system and, therefore, separated HMOs only by monosaccharide composition and not by class (neutral vs. acidic). The benefit of this type of fractionation was that separation was much faster than the standard gradient we used before (<40 min). Although each collected fraction was significantly less complicated than the original HMO pool and, therefore, amenable for additional separations with preparative HPLC, the fractions were much more complex than the fractions collected with the dual binary solvent system.

We further determined the separation of HMOs with the two solvents by analyzing the collected fractions with HPLC–CHIP/

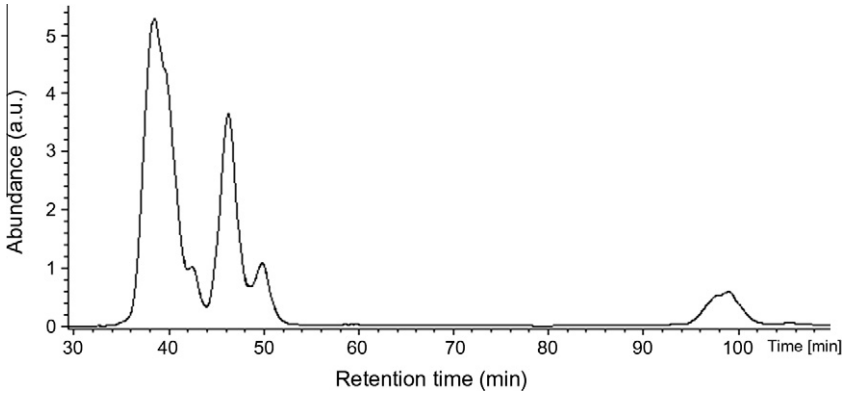

Fig.7. EIC for HMO isomers with *m*/*z* 1022.7 and a composition of 3Hex 1GlcNAc 2dHex. Isomer separation was achieved on a 12-mg mixture of HMOs and lactose mixture with a 4-g self-packed graphitized carbon column. The smaller peaks eluting next to each major peak are probably anomers, a common observation with the high-performance PGC columns used in analytical scale separations.

TOF–MS, a technique that resolves isomers and provides high-mass accuracy (Supplemental Fig. 6) [9,30]. This technique was used to (i) determine the quality of the collected fractions, (ii) validate the signal from the online detection with the quadrupole ion trap, and (iii) determine additional information about the fractions, including isomer separation between fractions and the presence of HMOs greater than *m*/*z* 2000.

Isomer separation

HMO isomers were separated by the graphitized carbon, as shown by the EIC profile for 3Hex 1GlcNAc 2dHex that was detected online with flash LC/MS (Fig. 7). Isomer separation was further verified by offline analysis of two sequential fractions with HPLC–CHIP/TOF–MS. Indeed, some of the HMO isomers were either completely or partially separated between fractions, as shown by the overlaid EICs from the HPLC–CHIP/TOF–MS in Supplemental Figs. 7A–7C. The overlaid EICs for each monosaccharide composition have different relative abundances for each of the isomers, showing partial isomer separation between the two characterized fractions.

This innovative approach yields new capabilities for eliminating lactose and simultaneously isolating gram amounts of pure oligosaccharides from human milk and bovine dairy streams such as whey permeate. Milligram to gram quantities of structurally homogeneous HMOs and BMOs will be used for functional testing to advance our knowledge of these key milk constituents that provide selective health benefits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2012.02.012.

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