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Coupling Flash LC with MS 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 non-chromophoric material from complicated biological mixtures. A flash 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 on-line mass detector enabled real-time optimization of chromatographic parameters to favor separation of oligosaccharides that would otherwise be indistinguishable from co-eluting components with a non-specific 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 1–200 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.

Keywords

flash chromatography; low pressure liquid chromatography; mass spectrometry; on-line detection; fraction collection; human milk oligosaccharides; bovine milk oligosaccharides; graphitized carbon; size-exclusion

Introduction

Low-pressure liquid chromatography (LPLC) 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

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between 0–5 bar, whereas MPLC is between 5–20 bar. In contrast, high performance liquid chromatography (HPLC) is operated between 20–400 bar and ultra high performance liquid chromatography (UPLC) runs between 400–1200 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 0–2 bar.[2, 3, 4] However, operation in the lowest pressure regime is accompanied by unique spray stability challenges for sample introduction into the mass spectrometer.

On-line sample detection for preparative LPLC has included UV/Vis absorbance[5], fluorescence[6], evaporative light scattering[7] and refractometry[5] however, there have been no reports, to our best knowledge, of on-line detection with mass spectrometry (MS). MS coupled to flash LC provides significant advantages over photometric detectors, such as mass and structural information for all co-eluting compounds. On-line 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]

Detection with MS is particularly important for the isolation of compounds that lack useful chromophores or are only found 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 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 non-chromophores 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 et al. 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 non-derivatized oligosaccharides do not efficiently absorb UV light.[14]

On-line LC/MS (with and without tandem MS) is already a standard method for analytical and preparative HPLC, [15]; however, MPLC methods have infrequently employed on-line LC/MS. One exception is the report by Hou et al., 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] In order to test the function of specific oligosaccharides, the simpler carbohydrates (e.g. lactose) must be removed, because they are fermentable by ostensibly all kind 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 non- digestible oligosaccharides in milk. Human clinical studies with purified oligosaccharides are lacking, as large amounts of milk oligosaccharides are not available. Therefore, there is great interest evaluating sources of complex oligosaccharides other than human milk. Several oligosaccharides have been isolated and characterized in bovine colostrum indicating that they possess similar structures and functions to human milk glycans. However, the absolute abundance declines rapidly in 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 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 sialylated 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 on-line flash LC/MS with a simple mixture of malto-oligosaccharide 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 BMOs was achieved with self-packed columns of pressure-limited graphitized carbon and size-exclusion media.[22, 23] In both large 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]

Experimental Section

Materials and Chemicals

Human milk oligosaccharides (HMOs) and bovine milk oligosaccharides (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 solution 2:1 (v/v). 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–2 g of the carbohydrate mixture (oligosaccharides and lactose) were dissolved in water and injected onto the flash column in volumes of 200 μ L–20 mL.

Standards including lactose, iso-maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were purchased from Sigma-Aldrich and dissolved in deionized water. 200 μ g of each standard was combined into a water-based cocktail that was injected onto the flash column in a volume of 110 μ L (1.4 mg).

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

Flash Chromatography

Separation was performed with a Teledyne-Isco CombiFlash Rf 200 (Lincoln, NE). Columns were self-packed either dry for graphitized carbon chromatography (GCC) with either 4 grams or 40 grams of graphitized carbon (3.2×1.8 cm or 12×2.8 cm) or wet for size-exclusion chromatography (SEC) with 20 grams (3 serial columns of 12×2.8 cm each). Graphite particles were 60–90 μ m. 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 (Figure 1) by positive displacement with an FMI “Q” pump (Syosset, NY) and by pressure differentials of varying tube sizes connected with HPLC splitters and mixers from Upchurch Scientific (Oak Harbor, WA). 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 size-exclusion chromatography was deionized water.

Graphitized carbon chromatography 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 minutes, then increased from 0% to 15% between 20–40 minutes, isocratic for 10 minutes followed by a switch to the acidic binary solvent system (A2 and B2), then isocratic for 10 minutes after which solvent B2 increased from 15% to 40% between 60–80 minutes, 40% to 100% between 80–100 minutes, isocratic at 100% between 100–115 minutes, 100% to 0% between 115–120 minutes, and isocratic at 0% between 120–140 minutes and then returned to the non-acidic system (A1 and B1).

Mass Spectrometry

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

Fractions were collected in two-minute intervals and dried down. Analyses of the fractions were performed with the Agilent 6210 HPLC-Chip/TOF-MS (Santa Clara, CA). Time-of-flight (TOF) mass spectra were collected every 1.6 seconds with 16,000 transient scans per spectrum. The Chip LC contained a pre-concentration 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 0–2.5 min, 0 to 16% between 2.5–20 min, 16–44% between 20–30 min, 44–100% between 30–35 min, isocratic at 100% between 35–45 min, and isocratic at 0% between 45 and 60 min. The pre-concentration column was run on 100% A at 4 μ L/min, except when the column was in-line with the analytical column, which flowed on the described gradient program at 0.3 μ L/min.

Data Analysis

Data collected on-line with the quadrupole ion trap were processed into extracted ion chromatograms (EIC) with Thermo Scientific's Xcalibur 1.3 software (San Jose, CA). EIC's 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 chromatogram's with Agilent's MassHunter version B 03.01 software (Santa Clara, CA). EIC's 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 TLC plate. In contrast, more sophisticated flash LC devices typically provide on-line 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 1–200 mL/min and pressures between 0.5–5.0 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 post-column flow at a rate of 0.5 mL/min regardless of the flow rate from the flash system (Figure 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 μ L/min. The flow was then combined in a mixing-t with a 20 μ L/min flow of 0.1 mM sodium acetate in 30% ACN (Figure 1). The sodium acetate eliminated virtually all in-source fragmentation (Figure 2) and permitted the use of pure (non-buffered) 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 eluted. 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 note 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 non-digestible oligosaccharides. The capabilities of the method include isolation of oligosaccharides by monosaccharide composition and class[29] (e.g. neutral versus 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 (GOS) and neutral BMOs suppressed the signal of less abundant neutral and acidic BMOs. The abundant GOS were mostly removed and the BMOs were separated into several simpler fractions that included the separation of sialylated and non-sialylated BMOs. Prior to fractionation with flash LC, the base peak was the GOS 3 Hex (Figure 3A). The second most abundant compound had a 10% relative base peak intensity and was the

BMO 2Hex 1GlcNAc. Four additional GOS 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 GOS and had enriched for several BMOs. A representative fraction is shown in Figure 3B that was largely enriched with two BMOs: 4Hex 1GlcNAc and 3Hex 3GlcNAc. The fraction in shown in Figure 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 were collected that will be described in another publication. Representative neutral and acidic BMO structures are provided in Supplemental Figure 1.

The observed BMOs and GOS are annotated in Supplemental Table 1. 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 m/z 292. Separation of large amounts of oligosaccharides by class (such as neutral and acidic) will enable functionality studies of oligosaccharides that would otherwise be prohibitively expensive or entirely unavailable.

Human Milk Oligosaccharides

The on-line mass detection of a 12 mg HMO and lactose sample is shown in Figure 4. Lactose eluted between 0 and 37 minutes. The neutral HMOs eluted between 37 and 70 minutes and the acidic HMOs eluted between 80 and 105 minutes. The neutral HMOs were separated into 14 fractions (Table 1). Each fraction was characterized in real-time by tandem MS. The precursor and product ion scans for one of the fractions are shown in Figure 5. On-line tandem 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 low mass accuracy. The fragmentation also allowed efficient fractionation by class, including neutral, fucosylated and sialylated HMOs. The fractions were collected constantly throughout the run, allowing us to use the elution profiles to determine which tubes to pool together. The fractions containing co-eluting 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 to 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 co-eluting isomers was accomplished.

Comparison with common stationary phases—Separation of HMOs was compared between three stationary phases: graphitized carbon, amine and size exclusion gel. These media were compared in regards 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. 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 with the novel instrument set-up may still be useful for some applications and is shown in Figure 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 media 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 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 has the benefit of allowing all high mass HMOs to readily elute together for flash removal of the low mass carbohydrates. Co-elution of the high mass HMOs significantly reduces subsequent sample 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 may 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 hours apart (Supplemental Figure 2); however, we have observed some variation of peak intensities that have not been explained.

Loading—The method can be scaled up from 0.5 to 200 milligrams 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 Figure 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 (such as evaporative light scattering or refractometry). This problem is particularly relevant to flash LC in the low pressure regime. We demonstrated the ability of this technique to fractionate unresolved oligosaccharides in real time. A mixture of malto-oligosaccharides, DP2 (two degrees of polymerization) through DP7, was fractionated with size-exclusion gel flash LC/MS as shown in Supplemental Figure 4. The on-line detection of

the poorly resolved EIC peaks enabled collection at the appropriate times for enrichment of each malto-oligosaccharide relative to the original mixture.

Although not well resolved, detection of the EIC's in Supplemental Figure 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 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 demonstrated previously with flash chromatography in other pressure regimes.

Acetonitrile vs. 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 EIC's in Supplemental Figure 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 only separated HMOs 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 (less than 40 minutes). While 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/TOF-MS, a technique that resolves isomers and provides high mass accuracy (Supplemental Figure 6).[9, 30] This technique was used to determine 1) the quality of the collected fractions, 2) validate the signal from the on-line detection with the quadrupole ion trap, and 3) 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 1GlcNAc2dHex that was detected on-line with flash LC/MS (Figure 7). Isomer separation was further verified by off-line 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 EIC's from the HPLC-CHIP/TOF-MS in Supplemental Figure 7A–C. The overlaid EIC's 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 homogenous HMOs and BMOs will be used for functional testing to advance our knowledge of these key milk constituents that provide selective health benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 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 acid are added through a mixing-t just before the eluate reaches the mass spectrometer.

Figure 2.

Comparison of electrospray ionization of malto-oligosaccharides with degree of polymerization (DP) 4 through 7 in the presence of 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 A are a result of in-source rearrangements that are common for protonated oligosaccharides analyzed by ESI-MS.

Figure 3.

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

Figure 4.

Separation of a 12 mg mixture of HMOs and lactose. Some of the base peaks eluted with greater than 90% purity. The EIC's 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.

Figure 5.

Representative mass spectrum and tandem mass spectrum (MS²) of an HMO separated from a pool of Human milk oligosaccharides. The tandem data was vital for recognizing sugars due to the low mass accuracy of the instrument. All mass-to-charge ratios are singly protonated species.

Figure 6.

Abundant HMOs less than 800 Da were significantly separated from HMOs greater than 1200 Da with size-exclusion chromatography. A. Extracted ion chromatograms for five HMOs obtained with a quadrupole ion trap on-line with flash LC. Extracted ion chromatograms 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), 1387.5 (4Hex 2GlcNAc 2dHex), 1095.4 (4Hex 2GlcNAc) and 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 on-line with flash LC.

Figure 7.

Extracted ion chromatogram for HMO isomers with m/z 1022.7 and a composition of 3Hex 1GlcNAc2dHex. 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 porous-graphitized carbon columns used in analytical scale separations.

Eleven isolated HMOs were collected with detailed information included here. Retention time is indicated with ‘rt.’

Table 1

m/z	purity (%)	rt	%B	composition	adduct	class
1022.7	55	39	14.3	3 Hex 1 GlcNAc2 dHex	[M+Na] ⁺	neutral
657.8	36	42	15	2 Hex 2dHex	[M+Na] ⁺	neutral
999.8	63	45	15	3 Hex 1 GlcNAc2 dHex	[M+H] ⁺	neutral
876.8	87	48	15	3 Hex 1 GlcNAc1 dHex	[M+Na] ⁺	neutral
876.8	85	53	15	3 Hex 1 GlcNAc1 dHex	[M+Na] ⁺	neutral
730.8	69	56	15	3 Hex 1 GlcNAc	[M+Na] ⁺	neutral
1241.8	95	62	17.5	4 Hex 2GlcNAc1 dHex	[M+Na] ⁺	neutral
1095.7	93	66	22.5	4 Hex 2 GlcNAc	[M+Na] ⁺	neutral
634.5	99	84	52	2 Hex 1 NeuAc	[M+H] ⁺	acidic
999.1	77	98	94	3 Hex 1 GlcNAc2 dHex	[M+H] ⁺	neutral
656.7	86	103	100	2 Hex 1 NeuAc	[M+Na] ⁺	acidic