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Role of pH in the recovery of bovine milk oligosaccharides from colostrum whey permeate by nanofiltration

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Abstract

Milk oligosaccharides are associated with improved health outcomes in infants. Nanofiltration (NF) is used for isolation of bovine milk oligosaccharides (BMO). The study aim was to improve the recovery of BMO from lactose-hydrolyzed colostrum whey permeate. The retention factors of carbohydrates at various pH and transmembrane pressures were determined for a nanofiltration membrane, which was used at pilot scale to purify BMO. Carbohydrates were quantified by liquid chromatography and characterized using nano-LC-Chip-QToF mass spectrometry. BMO purity was improved from an initial 4% in colostrum whey permeate to 98%, with 99.8% permeation of monosaccharides and 96% recovery of oligosaccharides, represented by 23 unique BMO compounds identified in the final retentate. The pH during NF was a determining factor in the selectivity of carbohydrate separation. This NF method can be applied to conventional cheese-whey permeate and other milk types for extraction of bioactive oligosaccharides providing new options for the dairy industry.

1. Introduction

Dysbiotic microbiota and chronic diarrhea are leading causes of infant mortality (Lin, Nasr, & Stoll, 2008). Breast-fed infants are generally protected against these afflictions, and recent research suggests that the gut microbiota and the way it interacts with milk components are determining factors. Complex carbohydrates with a degree of polymerization between three and ten called oligosaccharides are present in human milk and contribute to these protective properties (Morrow et al., 2004). In human milk, oligosaccharides can be as abundant as or even more so than protein depending on the stage of lactation (Thurl et al., 2010). Milk oligosaccharides exhibit prebiotic, anti-adhesive, immunomodulatory, and cognitive development roles in humans (Kunz, Rudloff, Baier, Klein, & Strobel, 2000; Wang, 2009). Considering the limited supply of human milk, a source of bioactive oligosaccharides,

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finding alternative sources of oligosaccharides is a crucial step towards the production of sufficient quantities of pure oligosaccharides for clinical trials, elucidation of the biological roles and mechanisms of those compounds in vivo, and potential translation to commercial production (Zivkovic & Barile, 2011).

Whey permeate, the lactose-rich co-product of whey protein isolation obtained using ultrafiltration, was traditionally considered a waste. However, it was recently discovered to be a source of bioactive compounds such as peptides and oligosaccharides, with bovine colostrum whey permeate being a particularly rich source compared with mature bovine milk (Dallas et al., 2014; Martín-Sosa, Martín, García-Pardo, & Hueso, 2003; Nakamura et al., 2003). The current major industrial utilization of colostrum is to produce high-value, immunoglobulin-rich concentrates, but this isolation process yields a low-value colostrum whey permeate (Stelwagen, Carpenter, Haigh, Hodgkinson, & Wheeler, 2008), from which valuable oligosaccharides could be recovered.

Sialylated oligosaccharides represent major components of bovine milk oligosaccharides (BMO) in mature milk, while neutral BMO (containing the prebiotic monosaccharide *N*-acetylhexosamine and possibly fucose, but lacking sialic acids) create a more diverse mixture of oligosaccharides that more closely resembles that of human milk oligosaccharides. Our group has demonstrated that colostrum whey permeate within three days post-parturition is a source of both neutral and acidic BMO (Barile et al., 2010). The exclusive sialic acid residue manifested in human milk oligosaccharides is *N*-acetylneuraminic acid (NeuAc), whereas bovine milk contains primarily NeuAc with trace amounts of *N*-glycolylneuraminic acid (NeuGc) (Tao et al., 2008).

Although lab-scale isolation and fractionation of BMO has been accomplished using chromatography, process costs and scalability of isolation remain a challenge (Thum, Cookson, McNabb, Roy, & Otter, 2015; Williams, Packer, Redmond, & Gooley, 2002). Membrane processes such as nanofiltration (NF) have been investigated for the enrichment or isolation of milk oligosaccharides (Sarney, Hale, Frankel, & Vulfson, 2000). However, substantial retention of lactose and monosaccharides typically accompanies oligosaccharide enrichment by membrane filtration. Simple sugars such as lactose and monosaccharides do not exert desired selective prebiotic or protective functions in the lower intestine and could subsequently overshadow, or otherwise confound, the biological activity of the BMO of interest in in vitro tests assessing bioactive functions.

Achieving highly selective isolation of oligosaccharides (separation from simple sugars) using membrane filtration alone has hindered the isolation of oligosaccharides at large scale (Sarney et al., 2000). Enzymatic hydrolysis of lactose into glucose and galactose prior to NF can improve efficiency and selectivity of membrane-based separations (Goulas, Kapasakalidis, Sinclair, Rastall, & Grandison, 2002). Recent investigations focused on improving the separation of oligosaccharides from monosaccharides at a variety of scales reported low selectivity using membrane filtration (Altmann et al., 2015; Martinez-Ferez et al., 2006; Nordvang et al., 2014). Generally, it has been found that critical operational parameters affecting membrane selectivity and performance include membrane chemistry,

molecular-weight cutoff, transmembrane pressure (TMP), pH and solute concentration of the feed, and the use of diafiltration.

The objective of this work was to develop a pilot-scale fractionation process to recover high purity BMO from colostrum whey permeate for future prebiotic evaluation, and identify the best operational parameters that favor high selective isolation of target oligosaccharides using NF. Upstream lactose hydrolysis and tangential flow NF with diafiltration steps were employed to recover BMO. Carbohydrates were quantified by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and BMO were also characterized using nano-LC Chip quadrupole time of flight mass spectrometry (Q-ToF MS).

2. Material and methods

2.1. Bovine colostrum whey permeate

Bovine colostrum whey permeate was kindly supplied by La Belle Colostrum (Bellingham, WA, USA). Colostrum liquid was initially defatted via cream separators and de-caseinated by enzymatic casein precipitation. The whey obtained by removal of caseins was pasteurized at 63 °C for 30 min. Whey proteins were concentrated by ultrafiltration (10 kDa membrane) and continuous diafiltration to produce whey permeate (de Moura Bell et al., 2016).

2.2. Lactose hydrolysis and pasteurization at pilot scale

Lactose hydrolysis at pilot scale was performed according to an optimization procedure developed at laboratory scale (de Moura Bell et al., 2016). A food grade fungal lactase (Bio-Cat Inc, Troy, VA, USA) derived from *Aspergillus oryzae* was used to hydrolyze lactose into β-D-galactose and α-D-glucose. Approximately 100 L of colostrum whey permeate was treated with 0.2% enzyme (weight of enzyme/weight of whey) for 60 min at 50 °C under constant stirring at 36 rpm. Hydrolyzed whey permeate was pasteurized prior to NF using a MicroThermics UHT/HTST Lab 25 EHV Hybrid w/PLC Touchscreen Control (Raleigh, NC, USA) continuous pasteurizer at 72 °C for 15 s at a flow rate of 2 L min^{-1}.

2.3. Pilot-scale tangential membrane filtration for oligosaccharides recovery

The recovery of oligosaccharides from whey permeate was carried out in a pilot scale tangential-flow membrane filtration system (Model L, GEA Filtration, Hudson, WI, USA). The system was composed of a 2.5 inch diameter spiral membrane housing (1–2 m^2 area), a 95 L jacketed stainless steel reactor, a flowmeter for mass flow rate and density (Proline Promass 80E, Endress+Hauser, Reinach, Switzerland), a heat exchanger, inlet and outlet manometers, and a 7.0 HP feed pump (Hydra-Cell, Minneapolis, MN, USA).

2.3.1. Effects of TMP and pH on permeate flux and carbohydrate retention—To investigate the effects of TMP and pH on permeate flux and carbohydrate retention, whey permeate at three selected pH values (4.5, 7.0, 8.5) and TMP values from 4 to 35 bar was nanofiltered at a negligible concentration factor (CF; volume of feed/volume of retentate), less than 1.1 by recycling the permeate to the feed tank. TMP values were chosen within the accepted TMP range for both the filtration module and the nanofiltration membrane. The

effects of pH ranging from acid to alkaline were evaluated regarding the retention of charged and uncharged molecules. Citric acid and 1 M NaOH were used to adjust the initial whey permeate (pH 6.7) to pH 4.5, 7.0 and 8.5. TMP values varied from 4 to 35 bars with permeate and retentate samples withdrawn for each evaluated pressure at each respective pH. Experiments were conducted in duplicate.

2.3.2. Nanofiltration and diafiltration of whey permeate—Following lactose hydrolysis and pasteurization, 60 L of whey permeate was concentrated using a 500–700 Da spiral-wound sulfonated polyethersulfone NF membrane with an effective area of 1.86 m^2 (HYDRACoRe70pHT, Hydranautics, Oceanside, CA, USA). Performance parameters (pH and TMP values) were selected based on high oligosaccharide retention factors and low monosaccharide retention factors. After a concentration factor of ten was achieved, the NF retentate was diafiltered with 50 °C water at pH 8.5, at volumes equal to the retentate, to improve the removal of monosaccharides from the NF retentate. Ten discontinuous diafiltrations were performed. NF and diafiltration experiments were performed in duplicate.

2.4. Evaluating membrane performance

The performance of the membrane process was evaluated in regard to permeate flux and retention factors of carbohydrates (oligosaccharides, lactose, glucose and galactose). Permeate samples were collected and weighed in regular time intervals to determine the permeate flux (J_p) given in units of liters per meter squared per hour (L m^{-2} h^{-1}) (Equation 1):

$$J_p = \frac{V_p}{A * t} \quad (1)$$

where V_p is the permeate volume, A is the membrane area (m^2), t is the time (h) for collecting the permeate volume. The retention factor of simple sugars (lactose, glucose and galactose) and major acidic BMO (3'-SL, 6'-SL, and 6'-SLN) in recirculation mode experiments (CF <1.1), was determined according to the following equation:

$$R = \left(1 - \frac{C_p}{C_f}\right) \quad (2)$$

where C_p and C_f are the carbohydrate concentrations in the permeate and feed, respectively. TMP and pH were chosen according to the permeate flux versus TMP curve and retention of simple sugars and acidic oligosaccharides obtained from the recirculation mode experiment (CF<1.1).

During NF of hydrolyzed colostrum whey permeate to a CF of 10, permeates were collected at various concentration factors to determine the overall retention coefficient R according to the following equation (Cheryan, 1998):

$$\log\frac{C_R}{C_f} = R\ \log(\mathrm{CF}) \quad (3)$$

where C_R and C_f are the component concentration in the retentate and feed, respectively. The purity of the target oligosaccharides in relation to monosaccharides in the retentates before and after diafiltration was calculated according to the following equation:

$$\%\mathrm{Purity} = \left(\frac{C_{\mathrm{BMO}}}{C_{\mathrm{BMO}} + C_{\mathrm{Mono}}}\right) * 100 \quad (4)$$

where C_{BMO} is the summed concentration of all the major acidic BMO (3'-SL, 6'-SL, and 6'-SLN) quantified, and C_{Mono} is the summed concentration of all quantified monosaccharides (glucose and galactose).

The yield of oligosaccharides and monosaccharides in the retentates, before and after diafiltration, was calculated based on the following equation:

$$\%\mathrm{Yield} = \left(\frac{C_{i,r} * V_R}{C_{i,f} * V_f}\right) * 100 \quad (5)$$

where $C_{i,r}$ is the concentration of a specific component in the retentate, V_R is the volume of liquid in the retentate) $C_{i,f}$ is the concentration of a specific component in the feed, and V_f is the feed volume.

2.5. Carbohydrate profiling and quantification in permeates and retentates

2.5.1. Quantification of major bovine colostrum whey permeate oligosaccharides—

Major BMO 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), and 6'-sialyllactosamine (6'-SLN) as well as the simple sugars lactose, glucose, and galactose were quantified by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Thermo Scientific HPAEC-PAD ICS-5000, Sunnyvale, CA, USA) (Lee, de MeloSilva, Liu, & Barile, 2015). Samples were diluted between 10 and 1000 times with nanopure water as appropriate and filtered through a 0.2 μm syringe filter (Acrodisc 13mm PES, Pall Life Sciences, Port Washington, NY, USA). Calibration curves (coefficient of determination > 0.999) were prepared using the highest purity available commercial standards for 3'-SL, 6'-SL, 6'-SLN (V-Labs, Covington, LA, USA) and lactose, glucose, and galactose (Sigma, St. Louis, MO, USA). Twenty-five microliters of diluted, filtered samples were injected into a Carbo-Pac PA200 (Dionex, Sunnyvale, CA, USA) column at 0.5 mL min^{-1} flow rate using 100 mM NaOH and 10 mM NaOAc isocratically for BMO quantification. Lactose, glucose, and galactose quantification was carried out on a Carbo-Pac PA10 (Dionex, Sunnyvale, CA, USA) column with a flow rate of 1.2 mL min^{-1} and 10 mM NaOH for the first 12 min and a gradient from 10 to 100 mM NaOH for 12.5 min.

2.5.2. Profiling using nano-LC Chip quadrupole time of flight mass spectrometry (Q-ToF MS)

2.5.2.1. Sample preparation: Samples were purified by solid-phase extraction prior to analysis by mass spectrometry. Salts, monosaccharides and lactose were removed using 96 well plates with porous graphitized carbon solid phase extraction columns (PGC-SPE) (40 μL media bed volume, 2000 μg binding capacity, Glygen Corp. Columbia, MD, USA) according to the method of Meyrand et al (2013). The micro plate PGC-SPE cartridge was activated by adding 100 μL of 80% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA, %v/v) followed by centrifugation (Beckman Coulter Allegra X-30) for 1 min at 2150 × *g*. This step was repeated 3 times. Column equilibration was achieved by adding 200 μL nano pure water at same centrifugation conditions 3 times. After loading 150 μL of the samples onto the plates, the salts were thoroughly removed by washing with 200 μL nanopure water 6 times, and centrifuging for 3 min at 2150 × *g*. Total oligosaccharides were eluted by 600 μL of 40% (v/v) ACN with 0.1% (v/v) TFA in water by centrifuging for 3 min at 1650 × *g*. Solid-phase extractions occurred at 20 °C. Purified oligosaccharides were dried using miVac Quattro speed vacuum centrifugation (Genevac, Ipswich, Suffolk, England) at 30 °C for 18 h. Dried pellets were rehydrated in 100 μL of nanopure water. Rehydrated samples were diluted 100-fold for further analysis.

2.5.2.2. Nano-LC-Chip Q-ToF MS Analysis: The purified BMO fractions were analyzed by nano-LC-Chip-Q-TOF mass spectrometry. Diluted samples were injected on the Agilent 6520 (Santa Clara, CA, USA) nano-LC-chip-Q-TOF MS with a porous graphitized carbon (PGC) microchip according to a well-established protocol (Aldredge et al., 2013). The following modifications were applied: 350 °C drying gas with flow rate of 3 L min^{-1} with a spectral acquisition rate of 1 spectrum per second, and a mass/charge (m/z) range from 400 to 2000. Internal calibration was performed using calibrant ions of 922.010 and 1221.991 *m/z* (ESI-L, Low concentration tuning mix, Agilent Technology, Santa Clara, CA, USA).

2.5.2.3. Data analysis: Data analysis was performed on Agilent Mass Hunter ProFinder version 6.0 software. The “Batch Targeted Feature Extraction” algorithm was employed to extract peaks that matched a BMO database assembled in house. The peaks were matched with a mass accuracy filter of less than 20 ppm error and typical isotopic distribution. The compounds extracted had an exact mass of 400–3000 *m/z* and retention time between 5 and 40 min. The extraction was performed with the following parameters: absolute peak height cut-off of 250 ion counts, quality score over 50, ion species limited to H+, and charge state 1–3. The isotope type used was “glycan.” Isotopes were grouped by peak spacing 0.025 *m/z* + 7.0 ppm. Each extracted ion chromatogram was individually examined, and potentially incorrect assignments and integrations were manually verified.

2.6. Statistical analysis

Replicates of each measurement, for each filtration run, were analyzed by using Analysis of Variance (ANOVA) with generalized linear models from the SAS system (version 9.4, SAS Institute Inc., Cary, NC, USA). Two-way ANOVAs were used for evaluating the effects of transmembrane pressure (TMP) and pH on permeate flux of nanofiltered colostrum whey permeate and on the retention of monosaccharides and oligosaccharides. One-way ANOVA

was used for evaluating the effects of concentration factor on colostrum whey permeate flux, monosaccharides and oligosaccharide yields in the nanofiltration of hydrolyzed colostrum whey permeate. Multiple comparisons of least-square means were made by Tukey's adjustment with the level of significance set at $P < 0.05$. Due to limited space in the figures and a high number of letters indicating statistically significant differences within the data, letters are not displayed in the figures. Instead, only statistically significant differences of practical relevance for this work are discussed throughout the text.

3. Results and discussion

3.1. Effects of TMP and pH on permeate flux and carbohydrate retention

As determined by HPAEC-PAD, the proximate composition of the starting colostrum whey permeate was 18 g L^{-1} lactose, 0.082 g L^{-1} of 6'-SLN, 0.072 g L^{-1} 6'-SL, and 0.28 g L^{-1} of 3'-SL. Following treatment of the colostrum whey permeate with fungal lactase, approximately 99.6% of the lactose was converted into glucose and galactose with concentrations of 9.6 g L^{-1} and 9.2 g L^{-1} respectively.

The effects of pH and TMP on the retention of monosaccharides (glucose and galactose) and of oligosaccharides (3'-SL, 6'-SL, and 6'-SLN) are shown in Fig. 1A-B and Fig. 2A-C, respectively. A simultaneous increase in the retention of both glucose and galactose was observed when TMP pressure increased from 4 to 35 bar (CF < 1.1) at the same pH. Increased retention of monosaccharides at the same pH is likely due to the effect of higher TMP on membrane pore size reduction, which in turn can lead to increased retention (Goulas et al., 2002). In general, higher monosaccharide retention factors were observed when TMP increased from 4 to 12 bar (statistically different at $P < 0.05$), with no statistically significant increment being observed at the TMP range of 16 to 35 bar for each given pH. Our results are in agreement with those of Goulas et al (2002), where increased TMP was accompanied by increased retention of sugar in model solutions. However, both pH and TMP have been shown to affect monosaccharide retention. Increasing the pH of the whey permeate from 4.5 to 8.5 significantly reduced the retention of both glucose and galactose. Although at pH 4.5, the retention factor was nearly 0.9 for both monosaccharides at 35 bar, the retention factor at pH 8.5 was significantly lower ($P < 0.05$) at ~0.3 (Fig. 1A-B). Taking into account that whey permeate is a source of mineral salts, it is worth considering the possible effects of those salts on the membrane structure which could in turn affect permeate flux and solute retention. Nilsson, Trägårdh, and Östergren (2008) reported that upon the addition of salts, the retention of glucose decreased with increased pH, and our findings are in agreement. This trend was more pronounced at higher salt concentrations and higher pHs, being attributed to an increase in the effective membrane pore size (membrane swelling), which would reduce the retention of neutral compounds, with a similar trend reported in previous reports (Mänttäri, Pihlajamäki, & Nyström, 2006). The possibility of membrane swelling at higher pH could explain the higher permeate flux observed at higher pH, although not this was not statistically different at $P < 0.05$ (Fig. 3). When evaluating the effects of pH on the retention of ions and glucose, a decrease in the retention of uncharged molecules when permeate flux increased at higher pH was observed in a model solution for some membranes (Mänttäri et al., 2006).

However, TMP and pH did not affect the retention of oligosaccharides to the same extent as it did for monosaccharides (Fig. 2A–C). Oligosaccharide (3'-SL, 6'-SL, 6'-SLN) retention was >94% at all evaluated pressures and pH values. At lowest TMP (4 bar), increased retention of oligosaccharides was observed at pH 4.5 compared with pH 7.0 and 8.5 (statistically different at $P < 0.05$). Slightly higher retention of all oligosaccharides at increased pressures for all pHs was observed, although this was not statistically different at $P < 0.05$. These results might be in part explained by molecular mass differences between the target oligosaccharides (633.2–674.2 Da) and monosaccharides (180 Da) as well as by the increase of the net negative charge of the membrane at higher pH (Mänttäri et al., 2006; Nilsson et al., 2008), which could lead to an increase in the retention of acidic oligosaccharides. Of note, NF at pH 4.5 had a lower retention of oligosaccharides than at pH 8.5, which suggests a pH-dependent interaction, particularly at lower TMP where its permeation would be favored by reduced membrane compaction (Fig. 2A–C). Other investigators observed similar trends, though without simultaneously examining the combined effects of sialylated oligosaccharide retention and monosaccharide retention at various pH values. That said, in the majority of investigations there was an improved selectivity of the membrane to separate neutral monosaccharides from sialylated oligosaccharides, which appeared to be due to a combination of pore swelling and other possible surface-chemistry mechanisms, wherein the combined effect of increased pH and salt concentration appeared to play an important role (Luo & Wan, 2013).

Permeate flux increased from 1.8 to 26.4 (L m^{-2} h^{-1}), 3.47 to 36.79 (L m^{-2} h^{-1}), and 3.19 to 34.86 (L m^{-2} h^{-1}) when TMP was increased from 4 to 35 bar at pH 4.5, 7.0, and 8.5, respectively (CF < 1.1) (Fig. 3). TMP affected the permeate flux, with increased permeate flux corresponding to significantly higher fluxes at higher TMP values (for example comparing 4 or 8 bar to 30 or 35 bar at a given pH). Minimal increments in permeate flux were observed at TMP higher than 20–25 bar for all pH conditions, being more pronounced at pH values of 7.0 and 8.5. Although higher permeate flux is expected at higher pressures, membrane compaction could reduce subsequent permeate flux increases due to pressure increase (Cheryan, 1998). Permeate fluxes at pH 7 and pH 8.5 were not significantly different ($P < 0.05$) throughout the range of TMP values evaluated, with slightly lower permeate fluxes being observed at pH 4.5. At 20 bar, the permeate flux at pH 8.5 was 60% higher than the permeate flux at pH 4.5, whereas at 35 bar, the permeate flux at pH 8.5 was 30% higher than the permeate flux at pH 4.5, which could be related with increased membrane compaction. Although pH appears to have some effect on the permeate flux, independently of the TMP chosen, the role of pH on permeate flux is still unclear. Conflicting trends demonstrating increased permeate flux as well as decreased flux with an increase in pH suggest complex mechanisms involving changes in concentration polarization and membrane permeability (Luo & Wan, 2013). The nature of the membrane surface and the material composition complexity has obvious implications as well.

Because high oligosaccharide and reduced monosaccharide retentions were observed at 20 bar, subsequent experiments concerning NF of whey permeate were performed at 20 bar. Discontinuous diafiltrations were conducted to increase the removal of monosaccharides from the final retentate and to increase the purity of the final oligosaccharide fraction.

3.2. Nanofiltration of whey permeate

Fig. 4 shows the permeate flux and carbohydrate yields during NF of the whey permeate using a 500–700 Da membrane at 20 bar. Permeate flux decreased from 40 to 3.3 L m^{-2} h^{-1} when CF increased from 2 to 10, likely a consequence of increased solute concentration, as evidenced by increased density from 1004.7 g mL^{-1} to 1075.5 g mL^{-1}, which would in turn increase the concentration polarization layer. A similar trend was observed by Goulas et al. (2002), who showed that increasing sugar concentration in the retentate, at constant pressure and cross-flow velocity, had a marked effect on permeate flux. The density of the retentate increased from 1.0046 g mL^{-1} in the starting material to 1.0738 g mL^{-1} at CF 10 in a linear fashion.

Average oligosaccharide and monosaccharide retention factors are presented in Supplementary Fig. S1. According to the given Equation 3, the retention factor is given as the slope of the line when the logarithm of C_R/C_f is plotted against the logarithm of the CF. The oligosaccharide retention factor was 0.9947, while monosaccharides had an overall retention coefficient of 0.4211. Monosaccharide retention was slightly higher than observed in recirculation mode (0.3–0.35), and oligosaccharide retention corresponded well with recirculation mode experiments. A glucose retention factor of 0.45 has been observed at pH 9 and 50 °C in a model solution with 7.5 mM KCl (Nilsson et al., 2008).

At a CF of 10, approximately 20% of the total monosaccharides and 99% of the oligosaccharides were still present in the retentate (Fig. 4). This corroborates the high selectivity of the membrane observed during the recirculation mode experiment.

3.4. Diafiltration of whey permeate

To improve the purity of the retentate with respect to BMO, discontinuous diafiltration was performed. The efficacy of diafiltration was assessed by monitoring the concentration of the monosaccharides and oligosaccharides in the permeates from each diafiltration step (Supplementary Fig. S2). The concentration of oligosaccharides in the permeates ranged from 0.01 to 0.03 g L^{-1} by virtue of the high retention factor. However, monosaccharide concentration in the permeates decreased rapidly from approximately 5 g L^{-1} to 0.03 g L^{-1} after eight discontinuous diafiltration steps, which suggests that eight diafiltration steps are sufficient to remove the majority of monosaccharides under the experimental conditions with minimum loss of oligosaccharides. Further amounts of diafiltration (up to 10 volumes) were conducted with minimal improvement. In a study investigating oligosaccharide purification, similar results were observed by other researchers in a small-scale, dead-end filtration experiment using a model solution of lactose and sialyllactose, where lactose recovery in the retentate was nearly zero after approximately seven rounds of diafiltration (Nordvang et al., 2014). The removal of monosaccharides and other dissolved solids with discontinuous diafiltration reduced the density of the retentate from 1.074 g mL^{-1} to 1.032 g mL^{-1}, being equivalent to a decrease in total solids from 17.2% in the retentate before diafiltration to 8.9% after ten discontinuous diafiltration steps.

Following 10 discontinuous diafiltration steps, 95% of oligosaccharides and 1% of the monosaccharides initially present in the whey permeate were retained by the membrane. The

membrane concentration step to a concentration factor of ten (before diafiltration) increased the initial purity of the colostrum whey permeate from 4% to 20%, whereas the addition of ten discontinuous diafiltration volumes increased the final product purity to 98% as calculated from Equation 4 (Table 1). An overall permeation of 99.8% of monosaccharides and 5.3% of BMO demonstrates how a high oligosaccharide yield was attained herein with minimal losses while removing nearly all monosaccharides.

A total BMO concentration of 6.95 g L^{-1} (represented by 5.07 g L^{-1} 3'-SL, 0.89 g L^{-1} 6'-SL, and 0.99 g L^{-1} 6'-SLN) and monosaccharide concentration of 0.08 g L^{-1} (0.04 g L^{-1} each of glucose and galactose) were obtained in the retentate following diafiltration. A previous attempt to isolate BMO from whey permeate using NF by another group reported a final concentration of 0.83 g L^{-1} (Altmann et al., 2015). A higher final concentration of BMO will facilitate further processing to concentrate the retentate for integration into food products, which may include drying, evaporation, or reverse osmosis.

3.4. Oligosaccharide profile

Although quantification of the major BMOs in colostrum whey permeate is a prerequisite for monitoring process parameters such as mass balance and retention coefficients during membrane filtration, only a limited number of highly pure commercial oligosaccharide standards are available for accurate measurement, which is a persistent issue in oligosaccharide quantification (Corradini, Cavazza, & Bignardi, 2012; Martinez-Ferez et al., 2006). For that reason, the entire pool of BMO cannot be quantified. To understand the diversity as well as relative amounts of BMO, employing sensitive and accurate mass spectrometers and chromatography systems with high resolving power is required. By utilizing Chip-Q-ToF-MS and a BMO library generated in house (Aldredge et al., 2013), we identified 23 oligosaccharides in the starting material and final retentate after diafiltration (Fig. 5). Extracted compound chromatogram peak areas were summed for identical compositions, wherein isomeric structures were combined. All but 4 compounds in the colostrum whey permeate increased in concentration. The BMO are discussed and presented based on the glycan composition notation with the monosaccharide residue order of Hex-HexNAc-Fuc-NeuAc-NeuGc, which corresponds with the number of hexose, *N*-acetylhexosamine, fucose, *N*-acetylneuraminic acid, and *N*-glycolylneuraminic acid moieties, respectively. Therefore, an oligosaccharide with composition 1_1_0_0_0 will have one hexose and one HexNAc. Oligosaccharides 1_1_0_1_0 (SLN), 2_0_0_1_0, (SL), 2_1_0_0_0, as well as what were putatively identified as galactooligosaccharides with a degree of polymerization from 4 to 8 were the primary BMO in the colostrum whey permeate and final retentate. The oligosaccharides 3_2_0_0_0 and 3_1_1_0_0 were detected in the hydrolyzed colostrum whey permeate and final retentate in trace amounts.

The greatest observed increase in BMO area was for 7 Hex, which increased 4 fold, with 6 Hex, 8 Hex, and 4 Hex-1 HexNAc increasing 3 fold. Due to different ionization energies and inherent variability in the instrument's electrospray ionization, quantification and relative comparisons within and between samples is considered a major challenge. This impediment highlights the need for accurate and complete quantitative methods to understand the minute differences in separation of diverse oligosaccharides at pilot scale using NF.

Although the major sialylated oligosaccharides identified with the Chip-Q-ToF-MS contained NeuAc as its sialic acid moiety, small amounts of NeuGc were detected as well, which is consistent with previous findings of other investigations (Tao et al., 2008). Structures that include *N*-acetylhexosamine may be particularly bifidogenic, due to the presence of a *N*-acetyl-β-hexosaminidase in *B. longum* subsp. *infantis*, which allows for the utilization of *N*-acetylhexosamine as both a carbon and nitrogen source in the gut (Sela et al., 2008). This process, yielding a rich oligosaccharide pool, represents a major advancement in utilizing NF to recover pure BMO from dairy co-products.

4. Conclusion

The findings offer a new processing strategy to isolate BMO from colostrum whey permeate using lactose hydrolysis and NF at a pH of 8.5 and 20 bar. Using these improved conditions will yield a high-purity oligosaccharide fraction and high total recovery with minimal loss of oligosaccharides throughout processing. Additional profiling of the purified oligosaccharide fraction reveals a wide variety of enriched neutral and acidic oligosaccharides, which could be used as a supplement for infant formula. The development of a large-scale process to recover BMO is critical to enable use as a food supplement and utilization in clinical trials. Scalable and translatable technologies as described in this study could be applied to conventional cheese whey permeate and permeates from milk sources such as caprine or sufficient quantities of donor human milk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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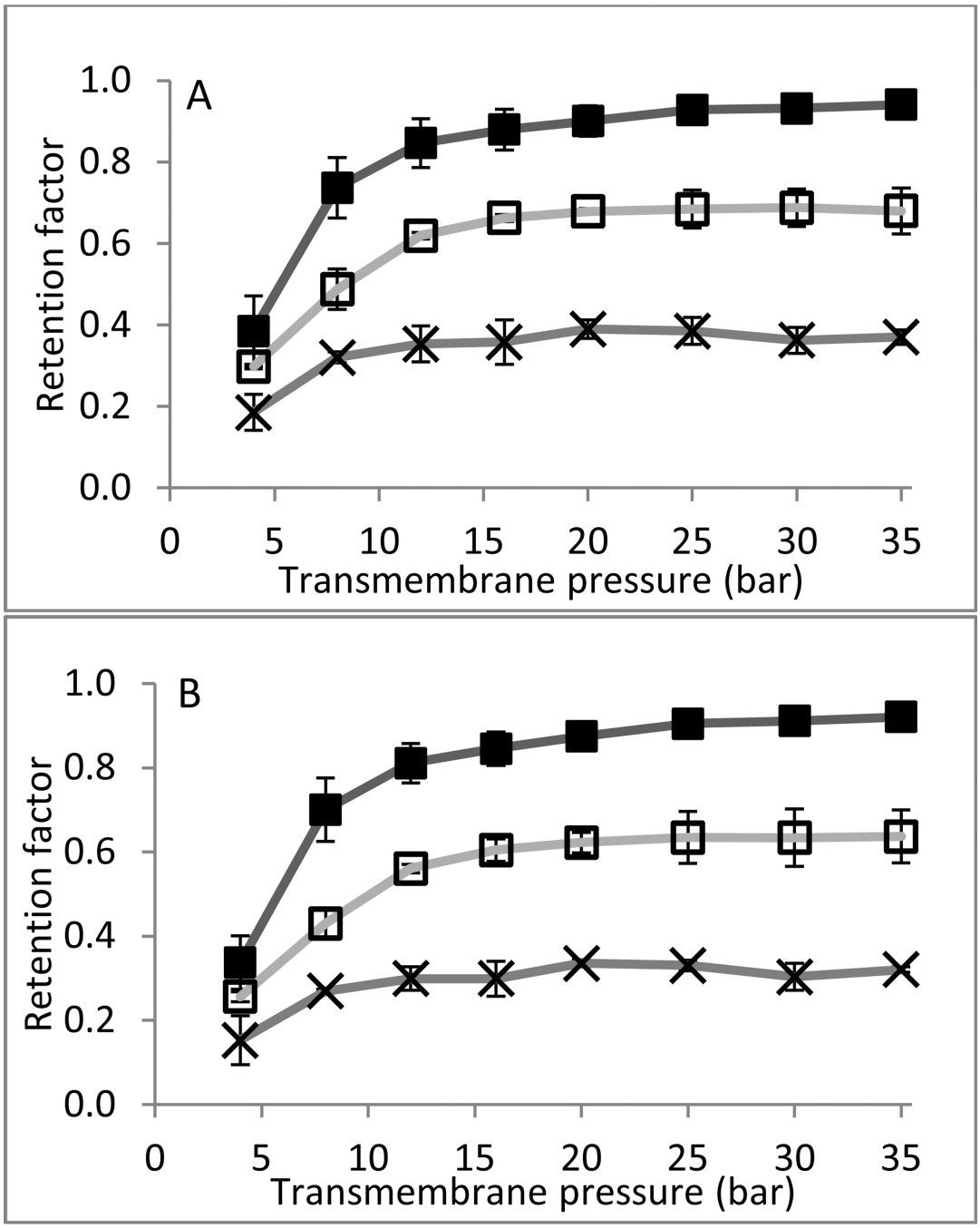

Fig. 1.

Effects of transmembrane pressure (TMP) and pH (■, pH 4.5; □, pH 7; ×, pH 8.5) on the retention of the hydrolyzed colostrum whey permeate monosaccharides (A) glucose and (B) galactose (500–700 Da membrane). Error bars represent one standard deviation.

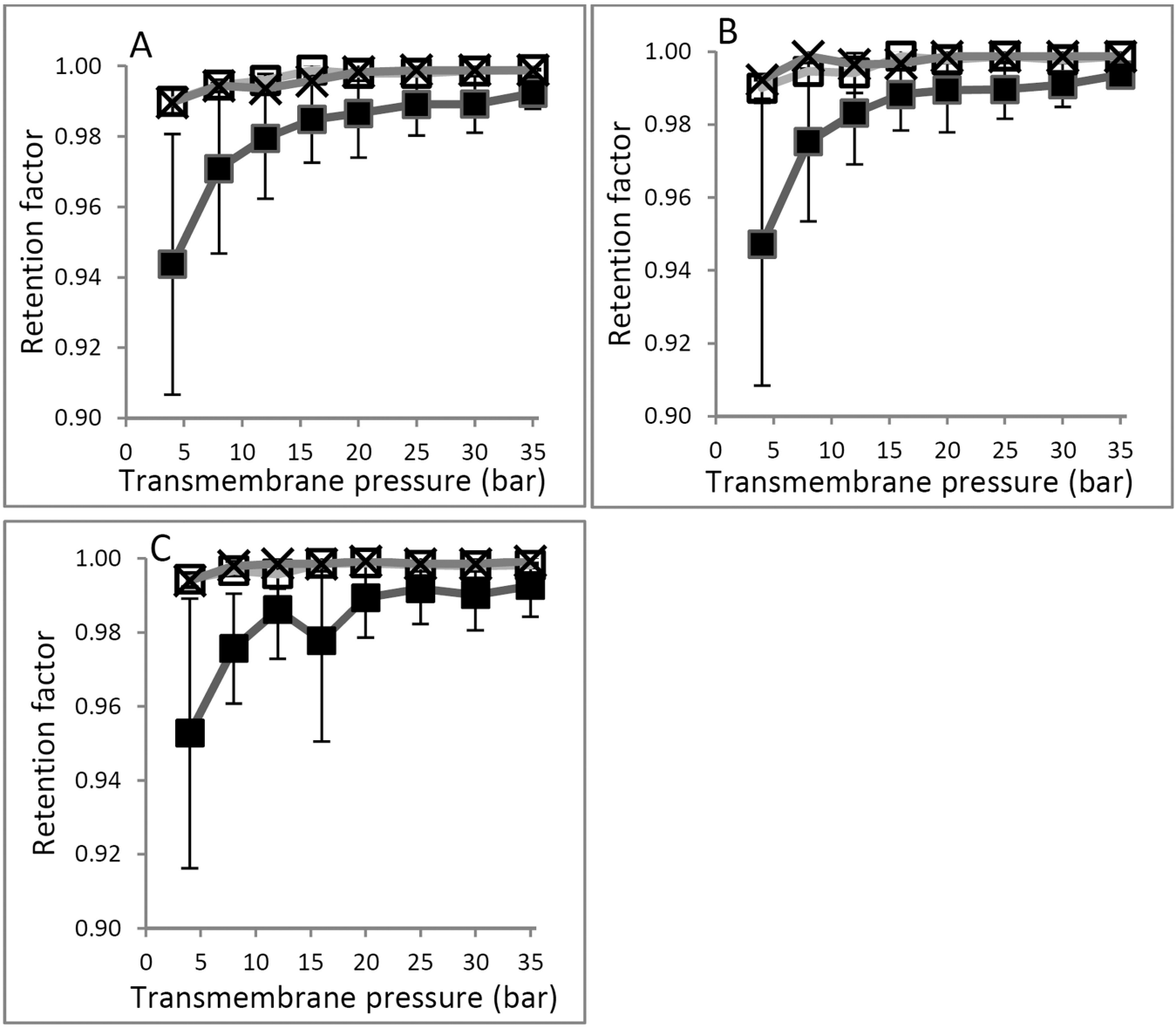

Fig. 2.

Effects of transmembrane pressure (TMP) and pH (■, pH 4.5; □, pH 7; ×, pH 8.5) on the retention of the hydrolyzed colostrum whey permeate oligosaccharides (A) 3'-sialyllactose (3'-SL), (B) 6'-sialyllactose (6'-SL) and (C) 6'-sialyllactosamine (6'-SLN).

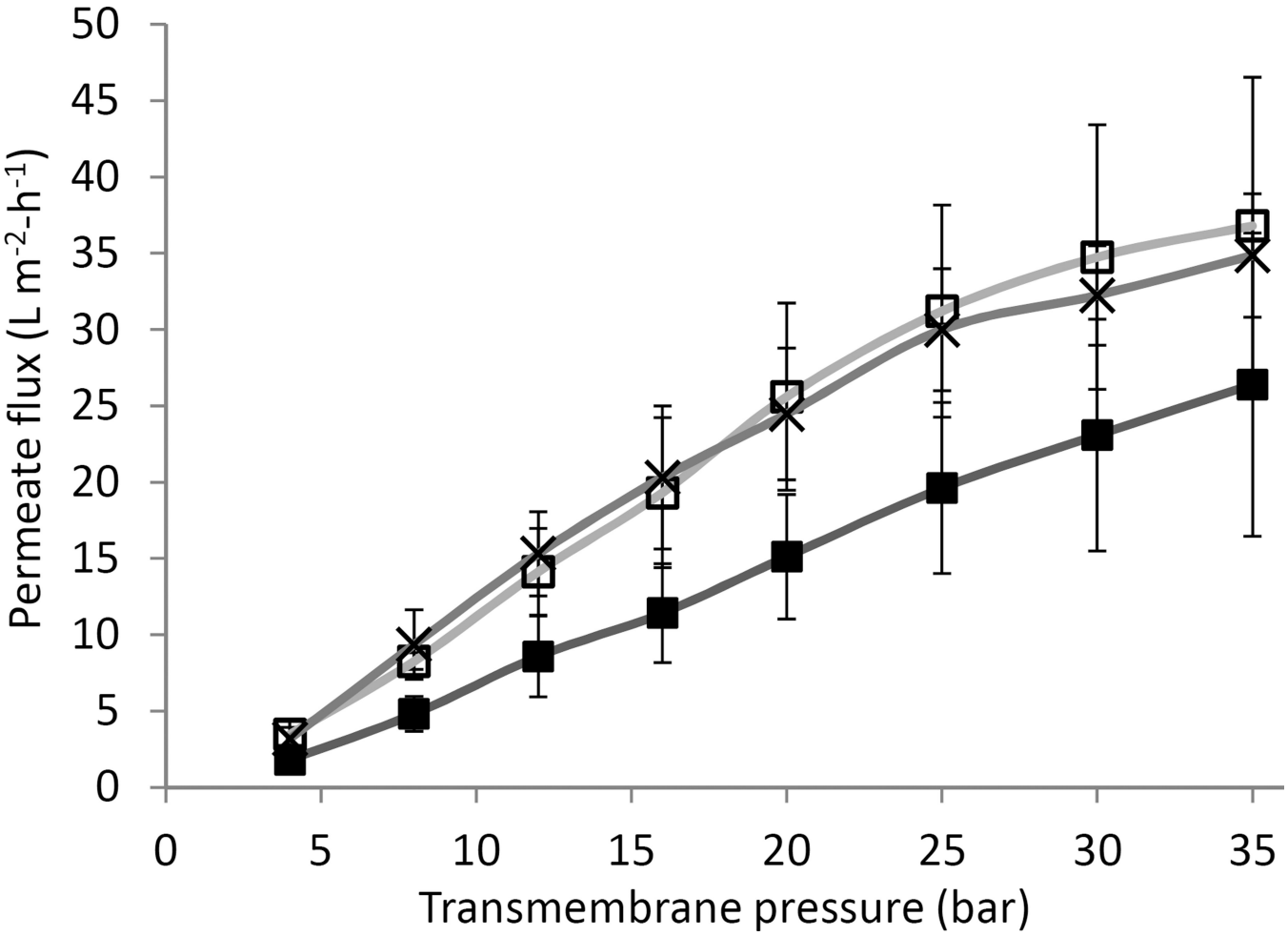

Fig. 3.

Effects of transmembrane pressure (TMP) and pH (■, pH 4.5; □, pH 7; ×, pH 8.5) on permeate flux of nanofiltered colostrum whey permeate (500–700 Da membrane). Error bars represent one standard deviation.

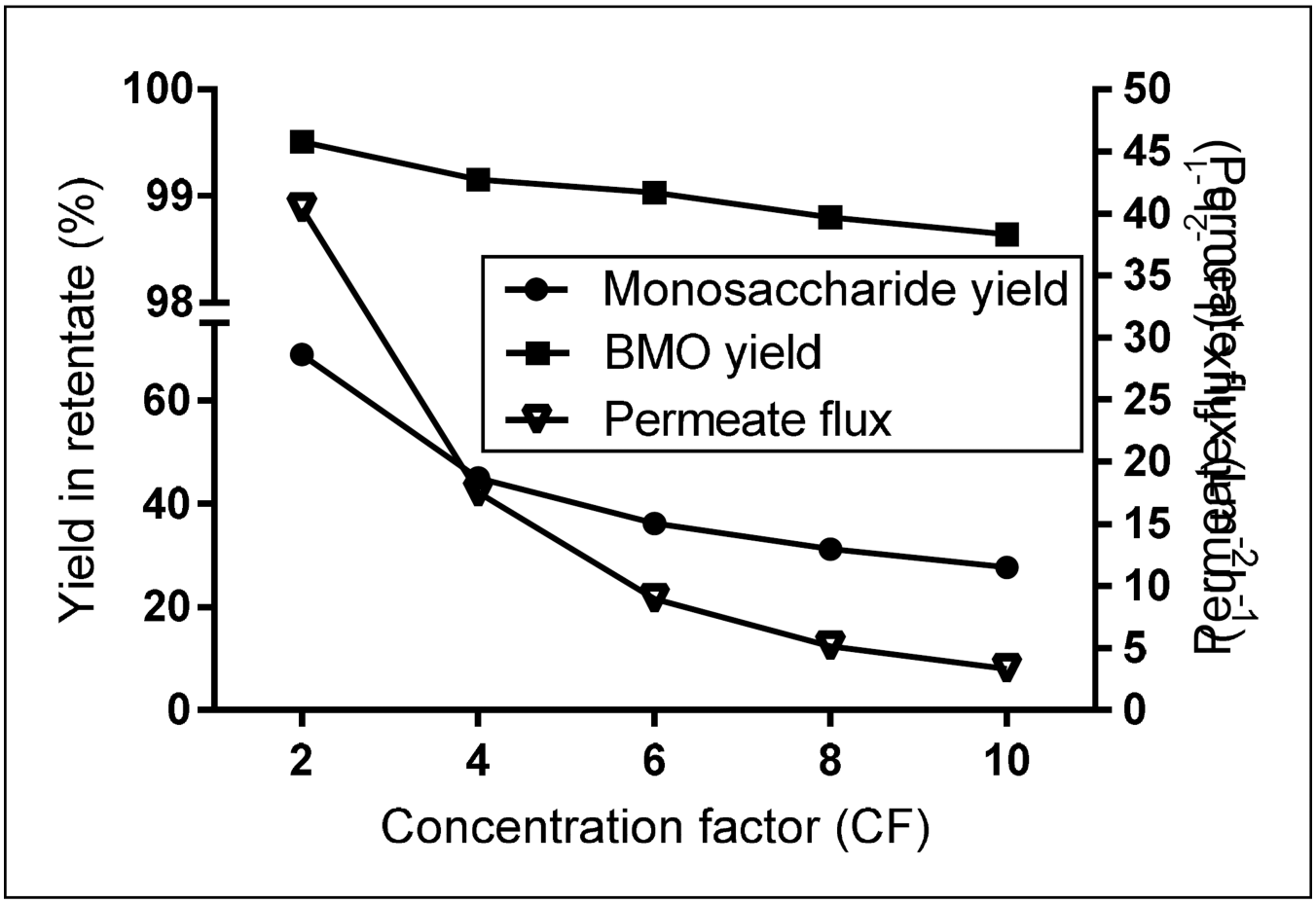

Fig. 4.

Effects of concentration factor on colostrum whey permeate flux (▽, monosaccharides (●) and oligosaccharide (■) yields in the nanofiltration of hydrolyzed colostrum whey permeate (500–700 Da membrane, at 20 bar, 50 °C). Error bars represent one standard deviation.

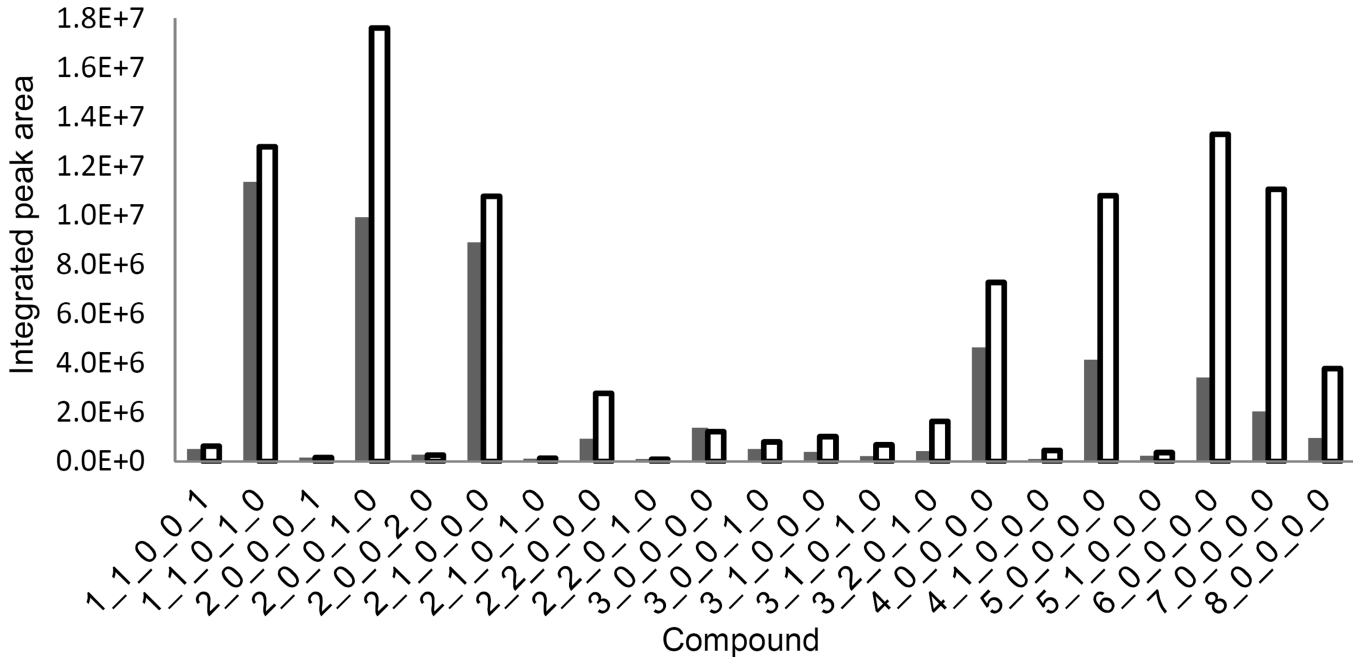

Fig. 5.

Nano-liquid chromatography Chip quadrupole time of flight mass spectrometry (Nano-LC-chip Q-ToF): ■, hydrolyzed colostrum whey permeate; □, final retentate. Integrated peak areas of extracted compound chromatograms identified by Mass Hunter Profinder's "Batch Targeted Feature Extraction" algorithm using an in-house bovine milk oligosaccharide bioformatic library. Component nomenclature is based upon the number of monosaccharide residues in the order of Hex- HexNAc-Fuc-NeuAc-NeuGc; where Hex, hexose; HexNAc, *N*-acetylhexosamine; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid.

Table 1

Oligosaccharide and monosaccharide yields (± one standard deviation) in hydrolyzed colostrum whey retentate before and after diafiltration at pilot scale.

Component	Yield before diafiltration (%)		Yield after diafiltration (%)		Total permeation (%)
	Retentate	Permeate	Retentate	Permeate	
Monosaccharides	19.78 ± 3.09	80.22 ± 3.09	1.06 ± 0.13	98.94 ± 0.13	99.79 ± 0.01
Oligosaccharides	99.01 ± 0.02	0.99 ± 0.02	95.60 ± 0.45	4.40 ± 0.45	5.34 ± 0.42