KIMIKA Volume 32, Number 2, pp. 11-25 (2021)
 ISSN 2508-0911
https://doi.org/10.26534/kimika.v32i2.11-25

Rapid-Throughput Analysis of Human Milk Oligosaccharides from Filipino Breastmilk

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ABSTRACT

Human milk oligosaccharides (HMOs), although considered indigestible to the infant, are the third most abundant solid component of breastmilk. They serve many health-related functions benefitting the infant, such as nourishment of gut bacteria, inhibition of pathogenic infection, and aiding in brain development. In this study, a rapid-throughput analytical method is applied for Filipino breastmilk HMOs. The method entails lipid extraction by centrifugation, protein precipitation using ethanol, alditol sugar reduction using sodium borohydride, and solid phase extraction purification using porous graphitized carbon, all in a 96-well plate format. Nano-HPLC Chip/TOF-MS, coupled with the use of Agilent MassHunter programs for filtering datasets using an in-house library, is then used to analyze purified HMOs. The results evidently show that the rapid-throughput method minimizes sample loss, ensures reproducible MS signals, and considerably shortens sample handling time. Furthermore, the method significantly reduced analysis time 5-fold and eliminated the individual sample preparation, which enabled simultaneous analysis of 386 samples (four 96-well plates) in a single run. Thus, the rapid-throughput analysis is highly efficient and effective for the profiling and structural determination of Filipino breastmilk HMOs even in large sample population sets.

Keywords: *Human milk oligosaccharides; Rapid-throughput; Nano-HPLC chip/TOF-MS*

INTRODUCTION

Human milk is the conclusion of 200 million years of Darwinian pressure on mammalian lactation (Bao et al., 2013). It is considered as nature's perfect food that nourishes neonates from their first day of life. Breastmilk has evolved to nourish infants and protect them against diseases whilst their immune system is still fragile and starting to mature. Human milk is composed of lactose, lipids, proteins, oligosaccharides, and other trace components such as vitamins and minerals (Andreas et al., 2015).

HMOs are the third most abundant macronutrient in breastmilk after lactose and lipids. They are highly abundant in breastmilk but not in formula milk. These oligosaccharides account for about 5-15 g per liter of breastmilk, which is much greater than proteins (Niñonuevo et al., 2006). It contains countless bioactive compounds that nourish infants throughout the neonatal stage (Coppa et al., 1993). Each is a complex mixture of polar, highly branched isomeric structures of 5 monosaccharide units (Figure 1), namely, D-glucose, D-galactose, L-fucose, N-acetyl-glucosamine and the sialic acid derivative N-acetyl-neuraminic acid (Zivkovic et al., 2011). Each HMO contains between 3 to 22 monosaccharide units with various sequences, linkages, and orientations, making structure elucidation challenging (Andreas et al., 2015).

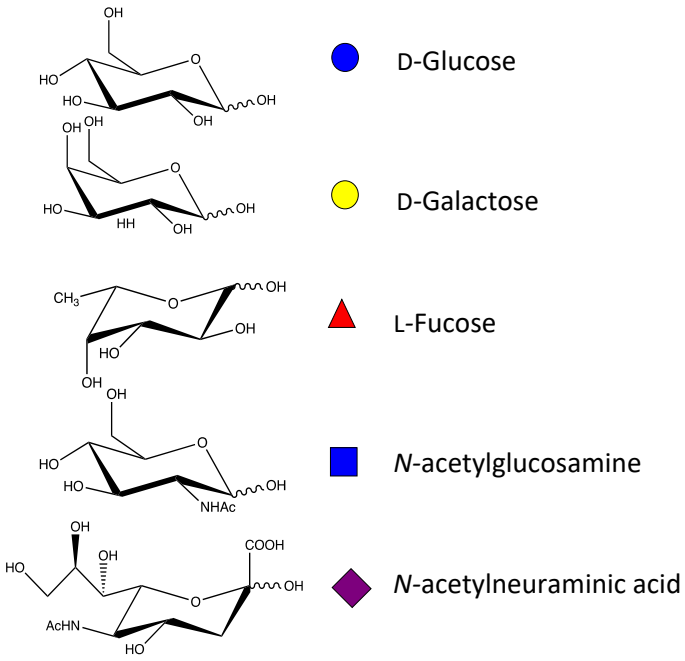


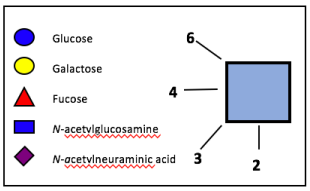
Figure 1. Five monosaccharide units of HMOs. Each sugar is represented by a shape with a corresponding color, as recommended by the Consortium of Functional Glycomics.

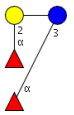
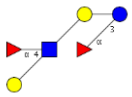
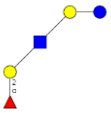
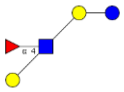
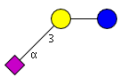
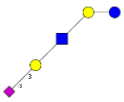
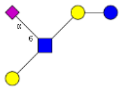
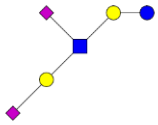
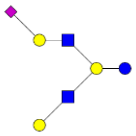
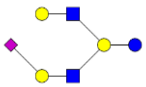
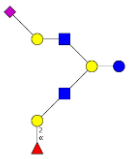
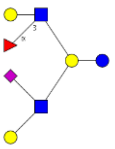
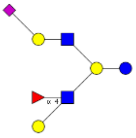
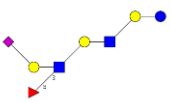
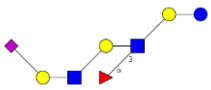
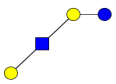
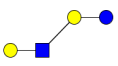
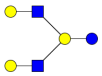
To date, more than 200 HMOs (some examples in Table 1) have been identified, all of which have lactose as the reducing end (Wu et al., 2010). All HMOs carry a lactose core that can be elongated in a β(1-3) or β(1-6) linkage by two different disaccharides, either Galβ(1-3)GlcNAc (type 1 chain) or Galβ(1-4)GlcNAc (type 2 chain). HMOs with more than 15 disaccharide units have been studied to form complex structural backbones that can be further modified with the addition of fucoses and/or sialic acids. It can be further diversified by adding fucose (fucosylated OS) and/or sialic acid (sialylated OS). In terms of overall composition, about 60-70% of HMOs are fucosylated and 5-15% are sialylated (Bode, 2015). This diversity of HMO structures poses difficulty in their elucidation and characterization.

HMOs serve different functions that impact neonates' gut and immune health. They serve as milk prebiotics for some bacteria like *Bifidobacteria* during the early stages of development. They serve as metabolic substrates that promote gut growth advantage (Bode, 2015; Marcobal et al., 2010; Davis et al., 2017). They also function like soluble decoys resembling the glycocalyx structure and block the binding of pathogenic microorganisms. These unbound microorganisms cannot attach to the gut cell surface and are simply eliminated, preventing infection (Ruiz-Palacios et al., 2013). Lastly, they serve as antimicrobials and reprogram epithelial cell surface receptors (Newburg, 2000;

Angeloni et al., 2005; Hua et al., 2011). Unlike breast-fed infants, formula-fed infants develop a more adult-like intestinal microflora, characterized by a lower percentage of beneficial bacteria. This adult-like microflora in the gut has been linked to reduced health of the infant's immune system and is thought to be due to the lack of HMOs in most infant formulas (Newburg et al., 2005; Newburg and Walker, 2007; Savino et al., 2013).

Table 1. Examples of HMOs found in human breastmilk.



Fucosylated HMOs	2'FL[a]	3'FL[a]	LDFT
			
	LNDFH II[b]	LNFP I[c]	LNFP II[c]
			
	3'SL[a]	LSTa[b]	LSTb[b]
			
Sialylated HMOs	DSLNT	S-LNH[c]	S-LNNH II[c]
			
	F-LSTc	FS-LNH[a]	FS-LNH I[a]
			
Fucosylated + Sialylated HMOs	FS-LNH III[a]	4121a[b]	4121b[b]
			
	LNT[a]	LNnT[a]	LNH[b]
			
Non-fucosylated neutral HMOs	LNnH[b]	P-LNH	
			

*Similar superscript letters denote isomers.

Milk oligosaccharides were first characterized in the early 1960s, but the elucidation of their structures has been very challenging. Unlike template-driven proteins and nucleic acids, free oligosaccharides have complex branching statuses and form multiple isomers with various linkages resulting in highly diverse structures. Several methods have been developed to uncover the complexity of these oligosaccharides. For instance, high-performance liquid chromatography (HPLC), high-pH anion-exchange chromatography (HPAEC), capillary electrophoresis (CE), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy using standard compounds have been used in combination or individually for structural quantitation and elucidation. However, these methods require tagging and derivatization (except NMR), which heavily rely on the availability of HMO standards; unfortunately, only standards for simple HMOs are available at present (Erney et al., 2000; Costello et al., 2007; Bode et al., 2012). A combination of HPLC-chip/orthogonal time of flight mass spectrometry (HPLC-Chip/oa-TOF MS) that uses a porous graphitized carbon column (PGC), and matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI FTICR-MS) IRMPD & CID have also been used for HMO structural elucidation (Wu et al., 2010). Moreover, the use of various exoglycosidases has enabled accurate determination of specific sugar linkage positions (Niñonuevo et al., 2006; Wu et al., 2010). However, these require longer processing time and use large amounts of samples and standards. With these limitations, more advanced methods have been employed in oligosaccharide analysis with the end results still requiring longer processing time and increased amounts of samples and standards (Ruhaak et al., 2012).

Previously, we performed unconjugated oligosaccharide analysis using individual samples. Such an analysis is without problem for a few samples but tends to be very time-consuming for large sample population sets since extraction, reduction, and purification require considerable time (Wu et al., 2010). Hence, in this study, for efficient analysis, we tried a rapid-throughput extraction method for the analysis of HMOs from Filipino breastmilk obtained between colostrum, transition, and late stages of lactation, using a 96-well format method of free-oligosaccharide extraction, reduction, and purification. We then applied an automated approach to HMO structure identification.

METHODS

Materials and Equipment. PGC 96-well packed plates (40 µL capacity) for purification were purchased from Glygen (Columbia, MD). 0.2-, 0.8-, and 1.3-mL 96-well polypropylene plates were purchased from Sigma-Aldrich (St. Louis, MO). Solvent evaporation was done using a Minivac centrifugal evaporator (Gardiner, NY). Absolute ethanol and sodium borohydride (98%) were purchased from Sigma-Aldrich (St. Louis, MO) and Medline Industries (San Ramon, CA), respectively. Sterile Falcon tubes (15 mL, Biologix) used for storing breastmilk samples were purchased from Belman Laboratories (Makati, Philippines).

Sample Collection. Human breastmilk samples were collected in the morning (after infant feeding) from 155 healthy volunteer Filipino mothers of Brgy. Putho-Tuntungin, San Antonio, Maahas, Lalakay, Anos and Bambang of Los Banos, Laguna, Philippines (all located within a 5 km radius from the UPLB). Inclusion and exclusion criteria were used in determining potential breastmilk donors. Informed consent (in both English and Tagalog) was obtained from the mothers prior to breastmilk collection. Mature milk samples were collected from 150 donors at different stages of lactation, while colostrum samples were collected from 5 donors at 1-7 days postpartum. A total of 225 breastmilk samples were collected. Milk samples were placed in an ice box during transport to the research laboratory of Dr. Carmina Villaruel of Institute of Biological Science, UPLB and temporarily stored in a -80°C biofreezer. The breastmilk samples were then shipped to University of California Davis under Dr. Carlito Lebrilla's research

laboratory prior to HMO analysis. The University of San Agustin Iloilo Health Ethics Review Board approved the study protocol.

HMO Isolation, Reduction and Purification. A rapid-throughput analytical method was used to analyze HMOs using a 96-well plate format optimized for Nano-HPLC chip/TOF-MS (Agilent technologies, Santa Clara, California). Each breastmilk sample was centrifuged briefly, then homogenized by vortexing at a low speed. 25 μL each of the sample and nanopore water was dispensed using a pipettor into a 96-well (0.2 mL) plate and pipette-mixed. The plate was centrifuged for 30 min. The aqueous layer was collected and transferred into a 96-well (0.8 mL) plate. Two volumes of ethanol were added to the collected solution for protein precipitation. The plate was vortexed, then stored at -80°C, thawed, and centrifuged for 30 min. The supernatant was collected and transferred into a 96-well (1.3 mL) plate for oligosaccharide reduction, to which an equal volume of 2.0 M $NaBH_4$ was added. The resulting solution was incubated at 65°C for 1.5 h to reduce aldose sugars to their alditol form.

Purification was done by solid phase extraction (SPE) using a PGC 96-well packed plate. The PGC plate was first conditioned with two volumes (100 μL) of nanopure water and centrifuged for 1 min. Two volumes of 80% acetonitrile (ACN) with 0.1% triflouroacetic acid (TFA) was added to remove contaminants, followed by three volumes of nanopure water before centrifugation at the same settings. The samples were loaded into the plate and centrifuged again for 1 min. The waste plate was replaced with a Thermo Nunc 96-well (1 mL) plate for oligosaccharide collection. Four volumes each of 20% and 40% ACN was used to elute the oligosaccharides with centrifugation in between at the same settings. The final sample was dried in a vacuum centrifuge for 5-8 h and stored at -80°C prior to Nano-HPLC chip/TOF-MS.

Nano-HPLC Chip/TOF-MS. Each sample was reconstituted by adding 25 μL of nanopure water followed by complete vortexing for 30 min. Then 2 μL of the reconstituted sample was then diluted 50-fold with nanopure water (98 μL) and transferred into a 96-well TOF plate. Free oligosaccharides were analyzed in three runs using a Nano-HPLC chip/TOF-MS system (Agilent Technologies, Santa Clara, CA) equipped with a capillary pump (maintained at 60-80 mBar pressure for sample loading and enrichment), a nanopump (maintained at 40-60 mBar for analyte separation), a microwell-plate autosampler (maintained at 4°C by a thermostat), a 6200 Nano-HPLC chip cube as the interface, and an Agilent 6210 TOF-MS instrument.

A microfluidic chip (Agilent Technologies) made of biocompatible polyimide and having an equivalent functionality to a conventional nano-spray LC-MS system was used as the column. It consisted of an enrichment column of 40 nL volume and an analytical column of 43 mm × 0.075 mm i.d. Both columns were packed with graphitized carbon material of 5 μm pore size. The pumps used a binary solvent composed of solution A1 [(3.0% ACN/water (v/v) with 0.1% formic acid)] and solution B1 [(90% ACN/water (v/v) with 0.1% formic acid)]. Solution A1 at a flow rate of 3 μL/min was used for sample loading, with a 1 μL sample injection volume. A 60-min gradient optimized for glycan separation and delivered by a nanoflow pump with a flow rate of 0.3 μL/min was used for separation: 2.5-20.0 min, 0-16% B1; 20.0-30.0 min, 16-44% B1; 30.0-35.0 min, 44-99% B1; 35.0-45.0 min, 99% B1; and a 20 min equilibration time at 100% B1. A reference HMO pool was also injected every 12 samples to check the reproducibility of the MS data.

Electrospray was used as the ionization source, and data were collected in the positive mode. The instrument was calibrated using a dual nebulizer electrospray source with internal calibrant ions with a wide mass range: m/z 118.086, 322.048, 622.029, 922.010, 1221.991, 1521.972, 1821.952, 2121.933, 2421.914, 2721.895. A single calibrant ion (m/z 1,221.991) was used as reference. The optimized mass spectrometric parameters for human breastmilk samples reported by Wu et al. (2010) were used in our oligosaccharide analysis.

Data Processing. The chromatogram and MS data from Nano-HPLC chip/TOF-MS were collected using Agilent Mass Hunter Workstation Acquisition software and analyzed using Agilent Mass Hunter Workstation Qualitative Analysis B.07.00, Profinder B.08.00 and Microsoft Excel 2016. These programs were used to filter and examine the chromatographic and mass spectral results obtained. HMOs were rapidly identified by matching their retention times and accurate masses (within 20 ppm mass error) with those of HMOs in the in-house annotated library of the Lebrilla research group. The experimental masses, retention times, peak areas, volumes, and heights generated using Mass Hunter Qualitative software were exported to Microsoft Excel for data analysis. A library of HMOs (without duplicates) with their respective formula, retention times, and experimental masses was generated. The HMO library was further analyzed using Agilent Profinder B.08.00 to find the specific signal in each chromatogram that matches with a specific HMO. Chromatographic spectral alignment across multiple data files was done to minimize both false positive and false negative results. The targeted feature extraction mode of the software enabled rapid HMO identification based on chromatographic peaks, providing more accurate peak assignment. The output of the software was a list of HMOs detected in all the samples with their peak areas, experimental masses, and retention times.

An in-house script program developed by the Lebrilla research group using the Python algorithm was used to categorize HMOs according to sugar composition: total fucosylated, total sialylated, and non-fucosylated neutral HMOs. Both the relative and absolute abundances of each glycan type were generated and used to analyze the oligosaccharide distribution in all the samples. For each set, means and relative standard deviations (%RSDs) of the absolute and relative abundances of each glycan type were calculated.

Evaluation of Instrument Stability and Reproducibility. A reference HMO pool randomly collected within the sample sets was used to check the stability and reproducibility of the instrument over a long period of analysis. The pooled milk sample was repeatedly injected every 12 samples for three sets of analysis. The mean and % RSD relative abundance of different HMO types from the reference HMO pool were calculated, providing the basis of reproducibility and stability as well as the retention shifts of some common abundant oligosaccharides.

Statistical Analysis. The mean and standard deviation of oligosaccharide intensities were reported in both relative and absolute abundances. Student's t-test was used to compare the mean abundance of oligosaccharide expression in breastmilk. One-way analysis of variance (ANOVA) was used to test variations in absolute and relative abundances of oligosaccharides at different stages of lactation, while using the Tukey-Kramer test to compare pair means. All statistical analyses were done using JMP program version 14.0.

RESULTS AND DISCUSSION

HMO Sample Preparation and Optimization. Previous studies used methods of HMO analysis that are optimized for a single sample (individual analysis). This study used the rapid-throughput method developed by Wu et al. (2010), with some modifications, to analyze Filipino breastmilk samples. These modifications include using Nano-HPLC Chip/TOF-MS for HMO analysis. The use of MALDI FTCIR was omitted for HMO structure identification. The use of exoglycosidase enzymes for digestion was also not performed since a library of HMOs was already developed at the time of our analysis. The sample preparation started with HMO extraction from the breastmilk sample in a 96-well plate with a multichannel pipettor. The initial step involved 1:1 dilution of breastmilk with deionized water to increase the volume for extraction. The optimized volume of breastmilk sample was determined to be within the dynamic range of 10-50 μL. Beyond 50 μL, the linearity of the instrument response became inconsistent. Plate centrifugation for 30 min immediately followed after dilution to remove lipids. Previous studies have also used the Folch method (2:1 methanol:chloroform) to remove lipids from breastmilk samples (Niñonuevo et al., 2006; Strum

et al., 2012; De Leoz et al., 2013; Nicholas et al., 2015). However, Totten et al. (2014) found that the elimination of Folch lipid extraction is not crucial in the development of the 96-well-plate-based extraction protocol. Although they observed a decrease in the absolute abundance of HMO types, the losses were equal for all HMO species and uniform across all sample sets. Less than 2% RSD was reported when HMO extractions with and without the use of the Folch method were compared. The exclusion of the Folch method significantly reduced the extraction time for hundreds of breastmilk samples without resulting in specific oligosaccharide losses.

The aqueous solution was then subjected to protein precipitation using 2 volumes of absolute ethanol to eliminate proteins, glycoproteins, or glycopeptides. Free oligosaccharides were the target of this precipitation technique so as not to oversaturate the porous graphitized carbon (PGC) column during purification. The PGC column also has the ability to separate α- and β-anomers of carbohydrate species (the reason why reduction using sodium borohydride is necessary). It is considered a versatile and effective column in the separation of neutral or acidic sugars, alditols, N- and O-linked glycans, and glycopeptides (Hua et al., 2011; Alley et al., 2009; Aldredge et al., 2012). Some glycoproteins, if not removed, can bind to the PGC column during purification and lead to oligosaccharide losses during flow-through. Furthermore, unseparated proteins can potentially lead to oligosaccharide signal suppression accompanied by difficulty in maintaining a stable current in the electrospraying during MS (Totten et al., 2014).

Free oligosaccharides were then subjected to sugar reduction using 2.0 M $NaBH_4$ and incubated at 65°C for 1.5 h. In the reduction process, the reducing end of the oligosaccharides is converted from its aldose form into its alditol form, as shown in Figure 2.

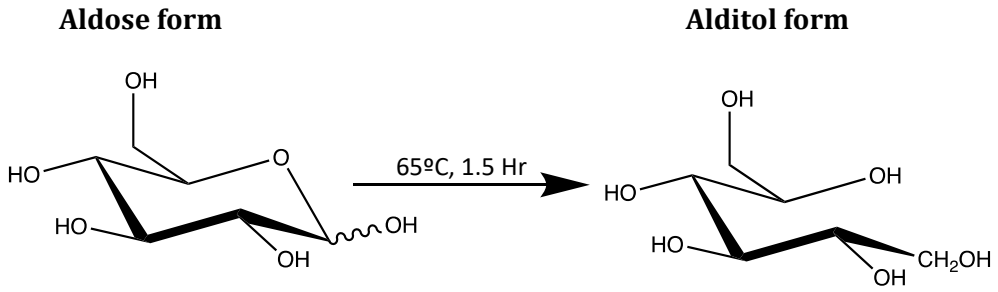


Figure 2. Reduction of aldose to alditol using $NaBH_4$ to eliminate anomers at the reducing end of the sugar.

The reduction step was important in resolving ambiguity among anomers present at the reducing end of the oligosaccharides. Anomers present at equilibrium yield split peaks in the chromatogram, thereby introducing structural ambiguity (Karlsson et al., 2004). This ambiguity can be resolved by converting the sugar at the reducing end into its alditol form. The alditol form of oligosaccharides is two mass units greater than the unreduced oligosaccharides. Furthermore, it produces a 500-fold response on mass spectrometer compared with the combined α/β anomeric aldose form of oligosaccharides (Bao et al., 2013).

The sugar reduction performed in the earlier step introduced salts (through $NaBH_4$) into the oligosaccharide mixture. Thus, a purification step was done using an SPE 96-well plate with a PGC column inside to remove the salts and other impurities. Prior to purification, the PGC column was conditioned and activated using 100% nanopure water and 80% acetonitrile (ACN) with 0.1% triflouroacetic acid (TFA) in nanopure water (TFA activates the porous carbon and maximizes the efficiency of oligosaccharide molecules to adsorb onto such material). This step was important for effective cleanup and the elution of oligosaccharides. The reduced oligosaccharide samples were introduced into the PGC column and eluted with two volumes each of 20% ACN and 40% ACN with 0.1% TFA, respectively, the former for eluting both acidic and neutral oligosaccharides,

while the latter for eluting neutral oligosaccharides. The eluted oligosaccharides were dried using a centrifugal evaporator on low to medium heat for 6–8 h and stored in a -80°C freezer prior to MS. The SPE process was considered a limiting step in oligosaccharide analysis owing to PGC limitation since the saturation point of the PGC column can affect the efficacy and efficiency of purification. Some HMOs such as 3'FL and other low-abundance oligosaccharides can be lost during PGC cleanup; however, this can be overcome using an optimized volume that will not saturate the column. The PGC column is also effective in removing monosaccharides and proteins including enzymes that have not been removed in the previous extraction process (Wu et al., 2010)

The rapid-throughput extraction method significantly reduced the time of analysis compared with the previous method of using individual PGC cartridges. Therefore, it is evident that it has advantages in terms of speed but at the same time of uniformity of sample preparation. Generally, the manual protocol requires a period of over a week to perform. Our rapid-throughput extraction method significantly reduced this period to 2 days. Moreover, the exclusion of Folch lipid extraction from the previous method in the defatting step did not markedly alter the oligosaccharide profile.

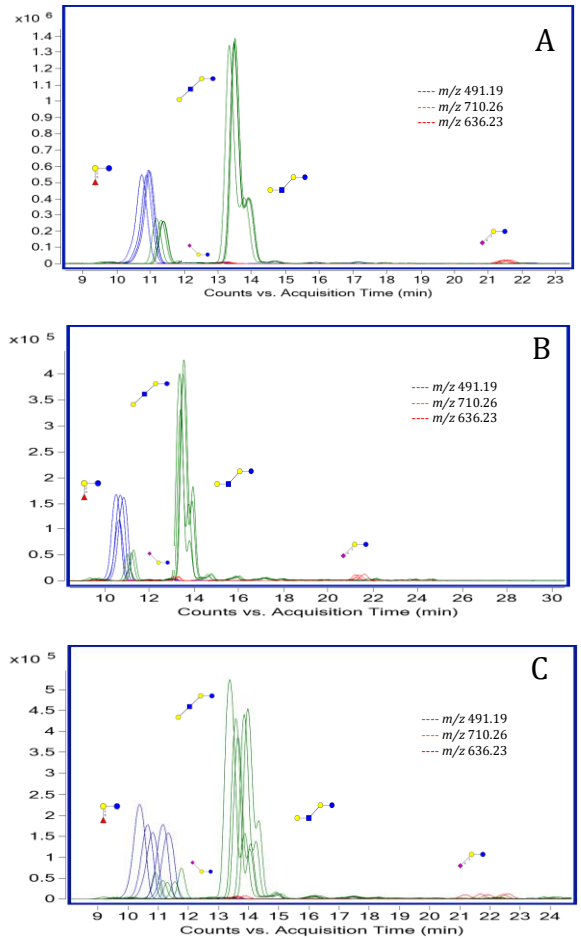


Figure 3. Extracted ion chromatogram (EICs) of m/z 491.19, 710.26, and 636.23. (A) Set 1:72 h; (B) Set 2: 67 h; and (C) Set 3: 112 h. The three chromatograms labelled A, B and C respectively show the extracted ion chromatograms of m/z 491.19 (fucosylated species), m/z 636.23 (sialylated species), and m/z 710.26 (non-fucosylated neutral species) HMO compounds. These species were chosen to represent each oligosaccharide type because of their high absolute abundance in the samples. The y-axis represents the ion counts and the x-axis represents the retention time in minutes.

Reproducibility of Nano-HPLC Chip/TOF-MS. The purified oligosaccharide samples dried earlier were reconstituted with nanopore water prior to MS. The oligosaccharides were diluted 50-fold and analyzed in three batches. The method of comprehensive HMO analysis using Nano-HPLC Chip/TOF-MS was optimized to be 1 h per run per sample. In addition, a reference pool oligosaccharide sample was also analyzed every 10 sample runs to check the reproducibility and stability of nano-HPLC Chip/TOF-MS. From the evaluation of the reproducibility and stability of the instrument, we obtained extracted ion chromatograms (EICs) from three instrument runs of the reference pool oligosaccharide sample.

In Figure 3, Runs A and B showed highly reproducible results with less than 5% RSD for fucosylated and non-fucosylated neutral HMOs. Run C showed less reproducible results owing to the exceeded lifetime of the microfluidic-chip-based PGC column used during HMO separation. A high RSD in the abundance of sialylated HMOs was observed in three runs. Sialylated HMOs are often suppressed by the signal of non-sialylated HMOs in the MS positive mode owing to the higher ionization efficiencies of the latter (Totten et al., 2012).

In terms of effective PGC isomer separation, the EIC for *m/z* 1221.45 (MFLNH series) was evaluated for effective separation (Figure 4). Five individual isomers were detected and baseline-separated, proving the effective use of the microfluidic-chip-based PGC column.

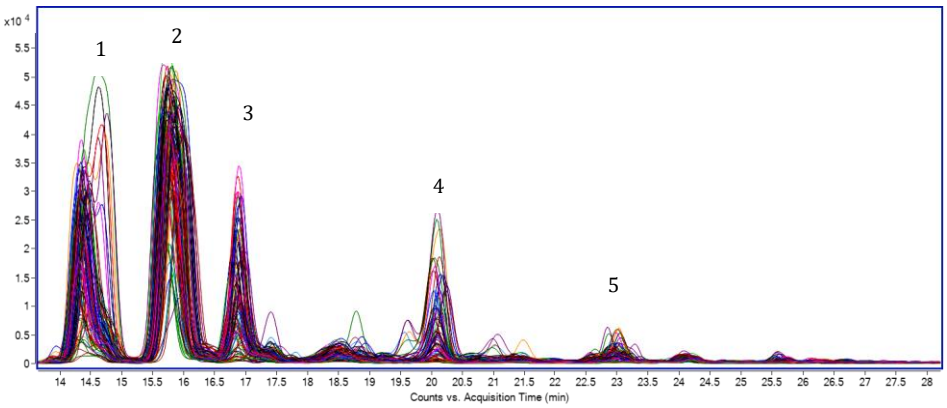


Figure 4. Overlaid extracted ion chromatogram (EIC) of m/z 1221.45 demonstrating effective isomer separation by PGC (N=155). The y-axis represents the ion counts and the x-axis represents the retention time in minutes. Numbers denote isomers.

HMO Analysis Using Nano-HPLC Chip/TOF-MS. From the Nano-HPLC Chip/TOF-MS results, majority of the ionized oligosaccharides detected in the positive ion mode were singly protonated $[M+H]^+$ or doubly protonated $[M+2H]^{2+}$ molecular ions. The oligosaccharides were one unit higher (if singly protonated) in mass owing to an additional hydrogen ion from protonation.

Figure 5 shows a two-dimensional deconvoluted plot of mass vs retention time of HMOs from a reference pooled breastmilk sample. The plot shows the spread and distribution of masses as well as the number of oligosaccharides present. All HMOs eluted within 35 min of the 1-h instrument run per sample. 141 HMOs (Figure 5) were found in the reference pooled sample from 25 representative donors with less than 15 ppm RSD.

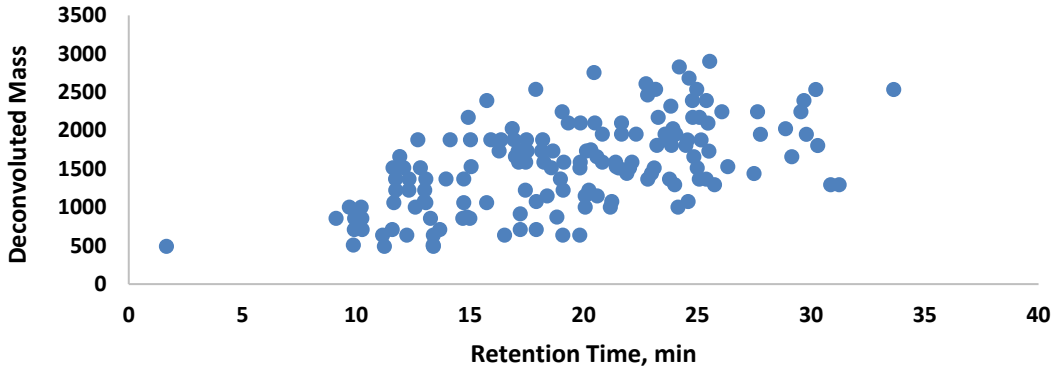


Figure 5. Deconvoluted plot of mass vs retention time of HMOs from a reference pooled breastmilk sample.

Figure 6 shows the extensive glycoprofile of the reference pooled milk sample. Each HMO, shown in different colors together with their number, structural composition and structural formula, was assigned a specific structure and structural composition except those not yet fully elucidated. Lacto-*N*-tetraose (LNT) was the most abundant HMO in the pooled milk sample and in all the samples analyzed (Figure 7). This neutral HMO is highly abundant in all populations and only varies depending on secretor status and lactation stage (Niñonuevo et al., 2007; Thurl et al., 2010; Totten et al., 2012).

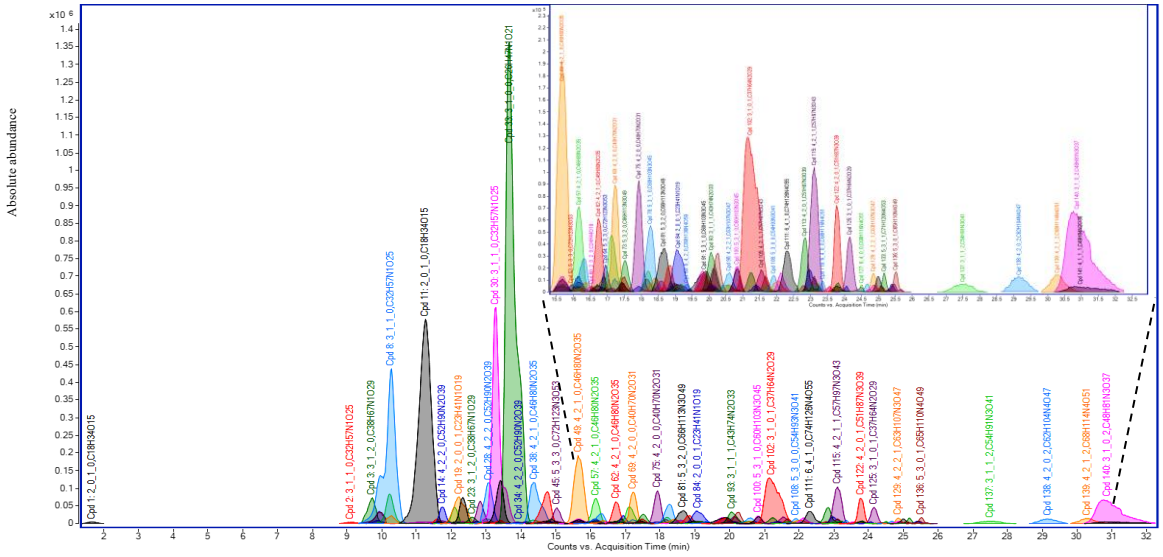


Figure 6. Overlaid extracted compound chromatogram (ECC) of reference pooled breastmilk sample obtained by HPLC Chip/TOF-MS. Each compound represents an HMO with a corresponding formula (Hex_HexNAc_Fuc_Sia). The inset chromatogram represents the extensive glycoprofile from HMO 49 to 141.

3'Fucosyllactose (3'FL, HMO 1: 2_0_1_0) eluted very early at 1.6 min, while the other HMOs eluted at 9-35 min. The presence of 3'FL in the reference pool showed the effectivity of PGC separation in the sample preparation. 3'FL HMO is often lost in the sample cleanup owing to its relatively low abundance (Xu et al., 2017).

Although overlapping was observed in the chromatogram, baseline-separated isomers, except LNT and lacto-*N*-neotetraose (LNnT), were still obtained. In this analysis, isomers were

chromatographically separated by differences in linkage position. These results clearly demonstrate the ability of PGC to separate oligosaccharide isomers.

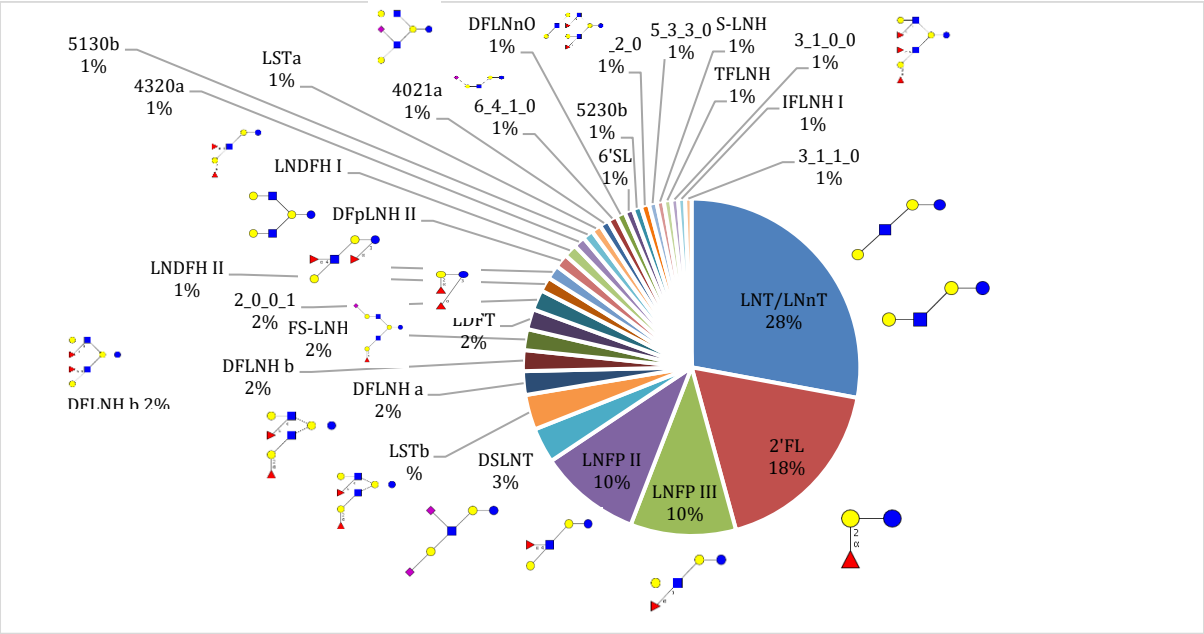


Figure 7. Distributions of abundances of different HMOs in reference pooled sample.

However, not all isomeric oligosaccharides were baseline-separated, particularly LNT and LNnT. These isomers are receptor analogs against *Streptococcus pneumoniae* (Kuntz et al., 2008; Kunz et al., 2000). LNT was found to be the most abundant HMO in all the samples, which was consistent with previous results showing high LNT abundance in women. LNT and LNnT co-eluted during separation and are therefore not fully baseline-separated (Figure 8). This is probably due to the saturation of the PGC column by LNT owing to the high LNT concentration in breastmilk, at 25-60% of the total HMOs in all the breastmilk samples. 25 µL of breastmilk sample was used in this study so that the PGC column will not be saturated by LNT and will detect even low-abundance oligosaccharides. Unfortunately, even at this volume, the two isomers were not baseline-separated. Hence, the optimization of protocol and parameters for separating LNT and LNnT will be addressed in future studies.

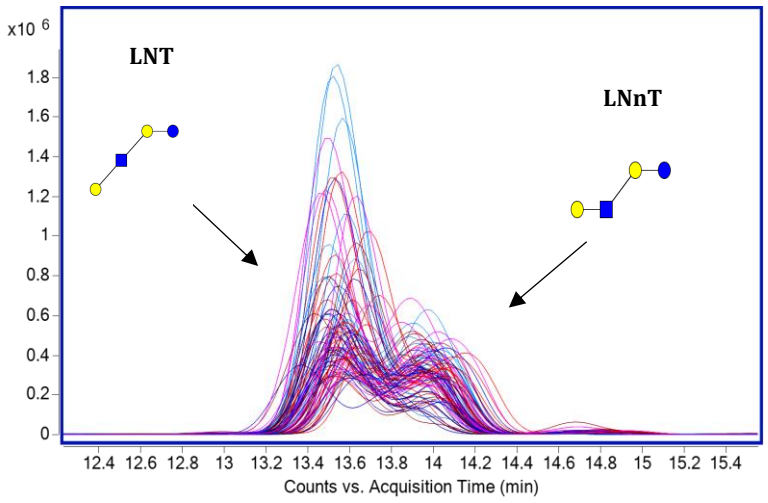


Figure 8. EIC of m/z 710.26 corresponding to isomers LNT and LNnT. These isomers are not fully resolved or baseline-separated by the PGC column.

Nano-HPLC Chip/TOF-MS has been proved to be effective in separating oligosaccharides in breastmilk samples. Approximately 100-200 oligosaccharides with masses ranging from 400 to 3700 Da were extracted and identified for each sample. The specific oligosaccharide structures were determined by comparing the accurate masses and retention times based on the annotated HMO library developed by the Lebrilla research group. Both neutral and acidic HMOs were analyzed and tabulated in this study. The HMO peaks were rapidly identified using automated software by filtering the LC-MS output against a library of exact masses, retention times, and monosaccharide compositions. Complex HMOs that are not yet fully elucidated were named according to their sugar composition.

CONCLUSIONS

In conclusion, a rapid-throughput extraction method using a 96-well plate format for unconjugated oligosaccharides coupled with Nano-HPLC Chip/TOF-MS for isomer-specific milk oligosaccharide analysis was proved to be effective and efficient in determining the HMO profile of Filipino breastmilk samples. Hundreds of HMOs were accurately detected and identified using an in-house annotated library sans the use of expensive commercial HMO standards and a labelling step, thereby cutting considerable amount of time in sample preparation (four 96-well plates can be analyzed simultaneously) and allowing for hundreds of samples to be processed simultaneously. Thus, the rapid-throughput method is highly efficient and effective for the profiling and structural determination of Filipino breastmilk HMOs in large sample population sets.

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