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Review

Recent advances in the analysis of human milk oligosaccharides by liquid phase separation methods

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ABSTRACT

Human milk is a complex, dynamically changing biological fluid, which contains a large amount of non-conjugated carbohydrates, referred to as human milk oligosaccharides (HMOs). These HMOs are very important for the infants as they play important roles in the formation of the gut microbiome, the immune system and support brain development. HMOs show highly complex structural diversity due to numerous linkage possibilities of the building monosaccharides. In order to elucidate their structure–function relationship and to develop more effective infant formulas, cutting-edge analytical technologies are in great demand. In this paper, we review the current strategies for HMO analysis based on liquid phase separation methods. High performance liquid chromatography, capillary electrophoresis and their hyphenation with mass spectrometry are critically reviewed, emphasizing their advantages and disadvantages from practical point of views. Recent advances of the methods are categorized according to their application fields.

1. Introduction

Human milk is the main source of nutrition for newborns, but also contains a wide variety of bioactive components, among them a large number of unique oligosaccharides [1]. These human milk oligosaccharides (HMOs) are the third most abundant ingredient in breast milk after lactose and lipids. Due to their indigestibility, HMOs are non-nutritive for infants, but contribute to multiple important biological effects. These unconjugated oligosaccharides reach the colon intact, where they act as prebiotics, stimulate the growth of beneficial bacteria; especially *Bifidobacteria* and thereby influence the composition of healthy intestinal microflora [2,3]. It is well known that the gut microbiota plays an important role in the development of the neonate immune system [4]. In addition, HMOs may modulate the immune function by altering host epithelial and immune cell responses and assist in the maintenance of balanced inflammatory responses [5,6]. Several studies revealed that HMOs provide protection against pathogens, such as *Vibrio cholerae*, *Streptococcus pneumoniae, enteropathogenic Escherichia coli, Campylobacter jejuni* or even HIV [7–9]. Furthermore, HMOs act as decoys, i.e., binding to pathogens to inhibit bacterial and viral adhesion to cell surface receptors and apparently protect breastfed infants against infections [10]. Besides their prebiotic, immunomodulatory and anti-adhesive effects, HMOs may also influence the cognitive functions of newborns. Recent studies have shown that HMOs appear in the bloodstream of the infant [11] and sialic acid residues play an important role in brain development and cell–cell interaction modulation [12] (see Table 1).

HMOs are synthesized by glucosyltransferases in the mammary gland and built up from the following five monosaccharide monomers: D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (in human, only *N*-acetylneuraminic acid (Neu5Ac)) (Fig. 1A). All HMOs carry lactose at their reducing end, which can be elongated by the addition of β1–3 or β1–6-linked lacto-*N*-biose (LNB) (Galβ1–3GlcNAc-, type 1 chain) or *N*-acetyllactosamine

Abbreviations: 2-AB, 2-aminobenzamidine; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; CGE, capillary gel electrophoresis; ESI, electrospray ionization; FLD, fluorescence detection; HILIC, hydrophilic interaction liquid chromatography; HMO, human milk oligosaccharide; HPAEC-PAD, High-performance anion-exchange chromatography with pulsed amperometric detection; LC, liquid chromatography; LIF, laser-induced fluorescence; MALDI, matrix-assisted laser desorption/ionization; PGC, porous graphitized carbon; RI, refractive index; RPLC, reversed phase liquid chromatography; SPE, solid phase extraction; TMT, target mass tag.

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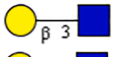
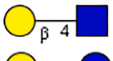
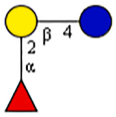
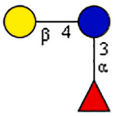
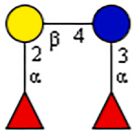
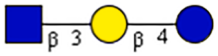
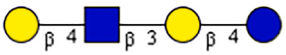
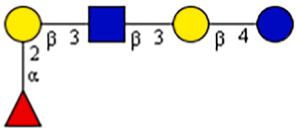
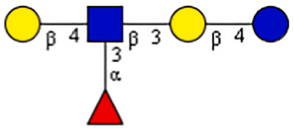
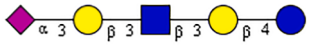
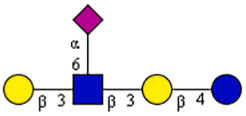
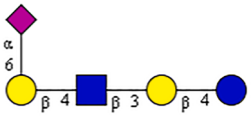
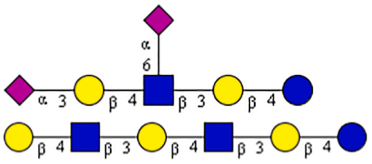
Table 1
Names, abbreviated names, monoisotopic masses and molecular structures of several important HMOs.

Name	Abbreviation	Monoisotopic mass [Da]	Molecular structure
Lactose	Lac	477.2	
Lacto-*N*-biose	LNB	518.3	
N-acetyllactosamine	LacNAc	518.3	
2-fucosyllactose	2′FL	651.3	
3-fucosyllactose	3FL	651.3	
Difucosyllactose	DFL	825.4	
Lacto-*N*-triose	LNT2	722.4	
3-sialyllactose	3′SL	838.4	
6-sialyllactose	6′SL	838.4	
Lacto-*N*-tetraose	LNT	926.5	
Lacto-*N*-neotetraose	LNnT	926.5	
Lacto-*N*-fucopentaose I	LNFPI	1100.5	
Lacto-*N*-fucopentaose III	LNFPIII	1100.5	
Sialyllacto-*N*-tetraose a	LSTa	1287.6	
Sialyllacto-*N*-tetraose b	LSTb	1287.6	
Sialyllacto-*N*-tetraose c	LSTc	1287.6	
Disialyllacto-*N*-tetraose	DSLNT	1648.8	
Para-lacto-*N*-neohexaose	pLNnH	1375.7	

(Galβ1–4GlcNAc-, type 2 chain) (Fig. 1B). Lactose or the elongated oligosaccharide chains can be fucosylated or sialylated via different linkages. As a matter of fact, HMOs have unusually high structural diversity because of the numerous linkage/anomericity options of the building monosaccharide units. The total concentration of these oligosaccharides in human milk ranges from approximately 20 mg/mL in colostrum to 5–15 mg/mL in mature milk [13]. Studies found that the level of HMOs and their composition vary among women and over the stage of lactation [13,14]. This diversity is influenced by many factors including geographical location and the genetic diversity within a population [6]. The greatest interpersonal variations have been observed in the fucosylation pattern, which depends on secretor and Lewis blood group statuses [15]. Reasons for individual variability include the frequency of breastfeeding, the lifestyle of the mother along

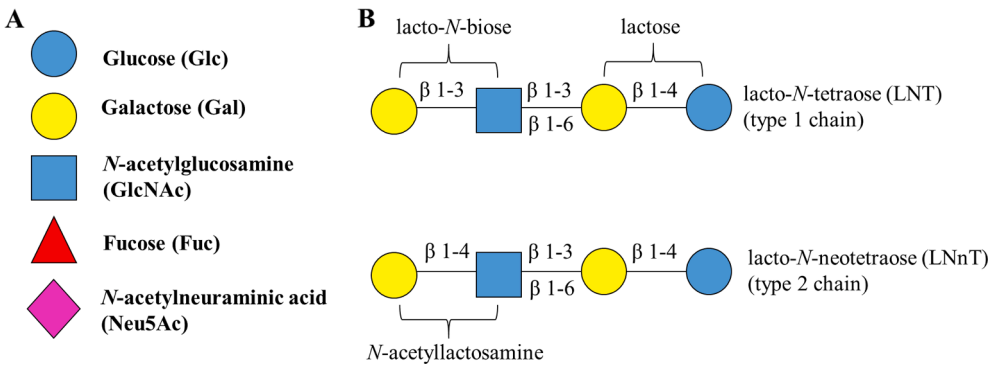


Fig. 1. (A) Monomer building blocks of the HMOs; (B) Elongation examples of with LNB or LacNAc with β1-3 or β1-6 linkages.

with her weight and dietary habits, especially the level of protein intake. Thanks for the currently available high resolution analytical techniques, as of today more than 200 unique oligosaccharides [16] have been identified in human milk differing in size, charge and abundance.

The structures of biomolecules determine their function, so various isomeric oligosaccharides can have to different biological effects, therefore, adequate identification of HMOs is essential. However, identifying and quantifying HMOs represent a major challenge due to the complexity of their structures. The most commonly used liquid phase separation methods for HMO analysis are liquid chromatography (LC) and capillary electrophoresis (CE). LC utilizes various stationary phases supporting reversed phase, anion exchange, porous graphitized carbon, and hydrophilic interaction modes. Capillary electrophoresis (CE) is another powerful analytical tool for HMO analysis in capillary zone (CZE) and capillary gel (CGE) electrophoresis modes. Both, LC or CE can be coupled to a mass spectrometer (MS) to obtain structural information. The purpose of this review is to summarize and describe the state-of-the-art liquid phase separation methods in the field of HMO analysis over the last five years and critically discuss their advantages and limitations.

2. Advanced analytical methods for HMO analysis

Investigation of human breastmilk started at the early 1900s [17] and since then various approaches have been developed for the HMO analysis. Elucidation of these oligosaccharide structures is highly important to reveal their biological function and thereby produce more suitable infant formulas. Although the analysis of glycan structures represents a separate field of science, i.e., part of glycomics, comprehensive analytical methods for the separation of oligosaccharides from human milk samples is still in progress. One reason for this is the great heterogeneity and complexity of their monosaccharide compositions and linkages. The low concentration of HMOs in the presence of the large lactose content further complicates their examination. In addition, oligosaccharides lack chromophores or fluorophores making their optical detection difficult.

2.1. Sample preparation techniques

Before analysis, HMOs need to be isolated from the other components present in breast milk, such as lipids, proteins and lactose [18–21]. The currently reported HMO sample preparation methods have similar steps, generally starting with the removal of fats and proteins. The defatting step is often performed simply by centrifugation or by solvent extraction. Protein precipitation is usually completed with organic solvents, such as ethanol, chloroform/methanol, acetone or acetonitrile. In several studies membrane separation has been applied to eliminate milk proteins [21–24], because of the organic solvents, such as ethanol and acetonitrile result in lactose crystallization and oligosaccharides co-crystallization with lactose, which impairs HMO analysis [21]. Some potential interfering agents such as lactose, salts, derivatization and reduction agents may make the separation procedure more difficult. Several purification approaches like ion exchange chromatography, gel permeation chromatography, solid phase extraction (SPE) with porous graphitized carbon (PGC) or hydrophilic interaction liquid chromatography (HILIC) sorbents have also been used to eliminate these agents, which made the process more complicated and time-consuming. Another issue is the possible loss of HMOs during the purification steps. In 2017, Wu et al. have developed a high-throughput 96-well plate based sample preparation protocol [25] allowing HMO profiling in large sample sets. However, separation of closely related structures and their quantification still calls for higher resolution separation techniques.

2.2. High performance liquid chromatography for HMO analysis

For quantitative characterization of oligosaccharides and their downstream structural elucidation, e.g., with by MS, it is crucial to choose the appropriate separation technique. The method must be suitable to separate many polar and branched isomeric structures. During the past years, a wide variety of HPLC methods have been developed and become frequently used for HMO analysis. HPLC can be used with label-free detection modes, such as refractive index, evaporative light-scattering, charged aerosol, pulsed amperometric and MS detection. Unfortunately, without derivatization, HMOs cannot be detected with the most widely used optical detection methods due to their poor UV absorption and lack of fluorescent characteristics. However, a number of fluorescent or UV active labels are available, which increase detection sensitivity. The most frequently applied ones are 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 2-aminoacridone (2-AMAC), per-benzoylation and 1-phenyl-3-methyl-5-pyranozolone (PMP) [26].

There are a great number of stationary phases available for HMO separation by liquid chromatography. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is one of the well-established techniques for the analysis of oligosaccharides not requiring derivatization. This method is suitable for the separation of neutral or acidic HMOs, however, before the separation, pre-fractionation of the neutral and acidic oligosaccharides is required increasing the analysis time. It is important to note that the detection sensitivity of this approach is orders of magnitude less that of fluorescence or MS detection [27], thus in recent years, HPAEC-PAD became a less widely applied glycoanalytical tool.

HILIC is another frequently used analytical tool in the field of HMO analysis. HILIC was originally developed to analyze polar and ionic species. In case of sugar analysis, 2-AB is the most frequently used labeling reagent. The method is well suited for the separation of longer positional and linkage isomers, including sialic acid isoforms [28]. On the other hand, HILIC works poorly for very similar small carbohydrates, such as trisaccharides [18].

The use of PGC stationary phases represent another LC option to separate oligosaccharides from human milk. PGC provides unique

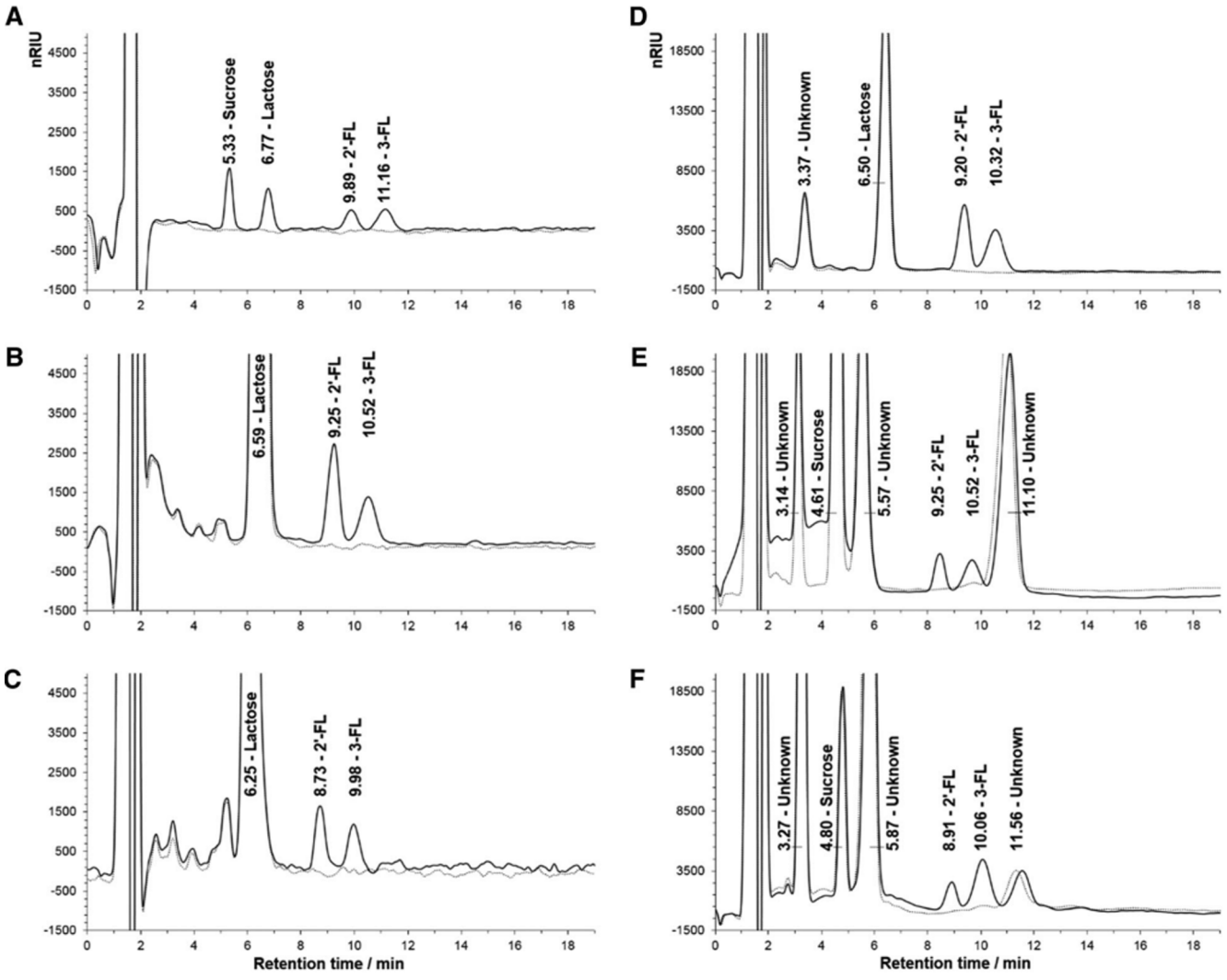


Fig. 2. HILIC-HPLC analysis of 2′FL and 3FL spiked sucrose and lactose standard in water (A), whole milk (B), UHT milk (C), yoghurt (D), infant formula (E) and cereal bars (F). Dotted lines are blank chromatograms for the corresponding matrices. nRIU: nano refractive index units. With permission from [32].

isomer separation, not only linkage isomers but also α/β anomeric forms. In cases when both anomers are present, it doubles the number of peaks and therefore complicates the evaluation of the resulting chromatograms. Borohydride reduction is a widely used method to alleviate this issue [18,25,29,30].

In recent years, infant formulas have been supplemented with 2′-fucosyllactose (2′FL) and lacto-*N*-neotetraose (LNnT) [31], thus qualitative and quantitative control of these ingredients are of high importance. Christensen et al. have been published a robust, easily applicable HPLC method with refractive index (RI) detection for the quantification of 2′FL and 3FL in different food samples, such as in infant formulas, cereal bars and milk-based products [32]. The separation was performed with HILIC column using isocratic elution with acetonitrile - water mobile phase containing triethylamine. The method was validated, highly robust and reproducible for the absolute quantification of 2′FL and 3FL in different food supplements by HPLC-RI as shown in Fig. 2.

Similarly, Austin et al. developed two approaches for the analysis 2′FL and LNnT oligosaccharides in infant formulas [33]. One method was based on HPAEC-PAD and the other on HILIC with fluorescence detection (FLD) after 2-AB labeling. Both methods worked well when used with spiked infant formulas. However, when the species were tested from production, the HPAEC-PAD method showed lower than expected abundance of 2′FL and LNnT. The authors hypothesized that the analytes and matrix components interacted during the infant formula manufacturing process, apparently depending on the production conditions.

In another study, ultrafiltration and centrifugation pretreatment methods were compared for 15 neutral and acidic HMOs for quantification using UHPLC with FLD analysis of the products [24]. This simple sample preparation method only required dilution, centrifugation or ultrafiltration and the derivatization steps ensured high sensitivity. While in the ultrafiltration pretreatment step the recovery rate of disialyllacto-*N*-tetraose (DSLNT) was less than 50%, the centrifugation method provided good yield.

The major drawback of LC based methods is the requirement of the use of oligosaccharide standards to determine retention time based structural elucidation with corresponding publicly available glycan libraries. However, the standards are frequently not available commercially and if so they are very expensive or their purity is less adequate [33]. Therefore, in the future it will be necessary to produce good quality oligosaccharide standards due to the increasing need of using HMOs in infant formulas. Furthermore, the long chromatographic run times of typically 30–60 min is considered as a limitation.

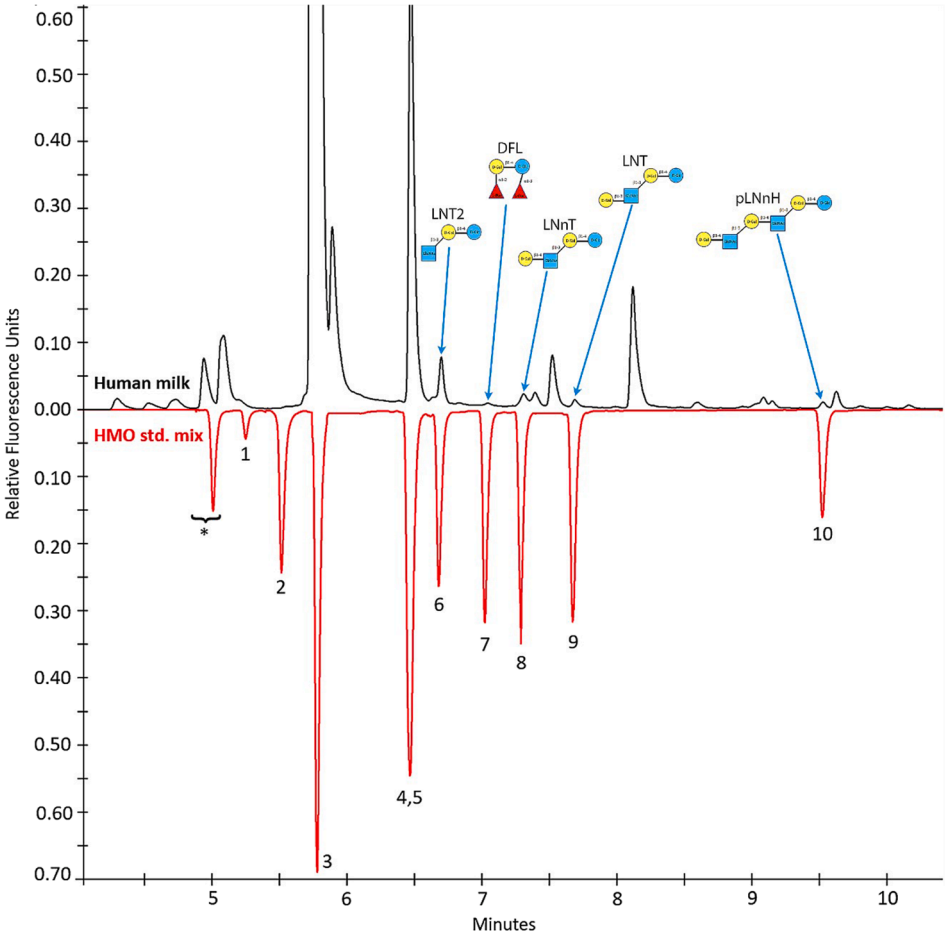


Fig. 3. Quantitative analysis of low concentration HMOs in a human milk sample. With permission from [34].

2.3. Capillary electrophoresis for HMO analysis

In addition to chromatographic based techniques, capillary electrophoresis is also broadly used as a powerful glycoanalytical tool, because of the low sample volume requirement, reduced buffer consumption, rapid and high resolution separations. CE offers several separation modes and the conditions can be easily altered during operation. For CE, derivatization of the mostly uncharged carbohydrates is necessary to ensure their proper electromigration. Applying a charged fluorescent label, such as 9-aminopyrene-1,3,6-trisulfonic acid (APTS), the sensitivity of the method reaches the 10^{-11}–10^{-12} M level [34]. In 2007, Bao et al. developed and validated a CE based method for the quantification of 12 major acidic HMOs using UV detection [35]. Later, this method was applied by others to test the concentration of 3-sialyllactose (3′SL), 6-sialyllactose (6′SL) and DSLNT from cow, goat and equine milk [36] in search for potential sources of these oligosaccharides as infant formula additives. It was found that milk from monogastric animals contained more oligosaccharides than milk from ruminants, and mare milk may be a good source of infant formula oligosaccharides.

Volpi and co-workers developed a CE method with simple UV detection at 254 nm to identify HMO standards and oligosaccharides from human milk samples [37]. They applied simple pretreatment steps and derivatization of oligosaccharides with 2-AMAC, an uncharged and hydrophobic fluorescent label. After derivatization, the labeled oligosaccharides were separated in borate buffer containing 20% methanol. This CE approach was capable to separate the main neutral and acidic oligosaccharides from breast milk in the presence of lactose and other high concentration impurities such as the excess fluorophores, proteins and salts. HMOs were tested from the four secretory groups Lewis-positive Secretors (Se +/Le +), Lewis-positive non-Secretors (Se −/Le +), Lewis-negative Secretors (Se+/Le−), and Lewis-negative non-Secretors (Se −/Le−). Comparing their results to HPAEC-PAD separations [38] and previous HPLC-ESI-MS characterization [27], this CE-UV approach provided high resolution and sensitivity for HMOs from these distinct genetic subgroups. It is important to note that the UV detection limit was significantly inferior to laser-induced fluorescence (LIF) and the long separation times limited the applicability of this method. Identification of unknown peaks in instances like this also require sequential exoglycosidase digestion [39] of MS based [40] analysis.

Sarkozy et al. compared the electromigration properties of 10 major HMOs by multicapillary electrophoresis (12-capillary cartridge) in two separation matrices, the industry standard carbohydrate separation gel and borate-buffered dextran gel [34]. 2′FL, 3FL, lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) isomeric structures were present in the HMO test mixture. 2′FL and 3FL isomers were co-migrating in the standard carbohydrate separation gel matrix, while the LNT and LNnT isomers were co-migrating in borate-buffered dextran gel. Importantly, 12 samples were simultaneously analyzed in less than 10 min and separation of all ten test mixture components were obtained by mixing the two separation gel buffer systems. They also quantified five low abundant HMOs from human milk as shown in Fig. 3, proving that this system provided a means for rapid, sensitive and high-throughput HMO analysis.

2.4. MS coupled to liquid phase HMO separation methods

Mass spectrometry is frequently applied in the field of glycan analysis and also successfully used for milk oligosaccharide analysis for

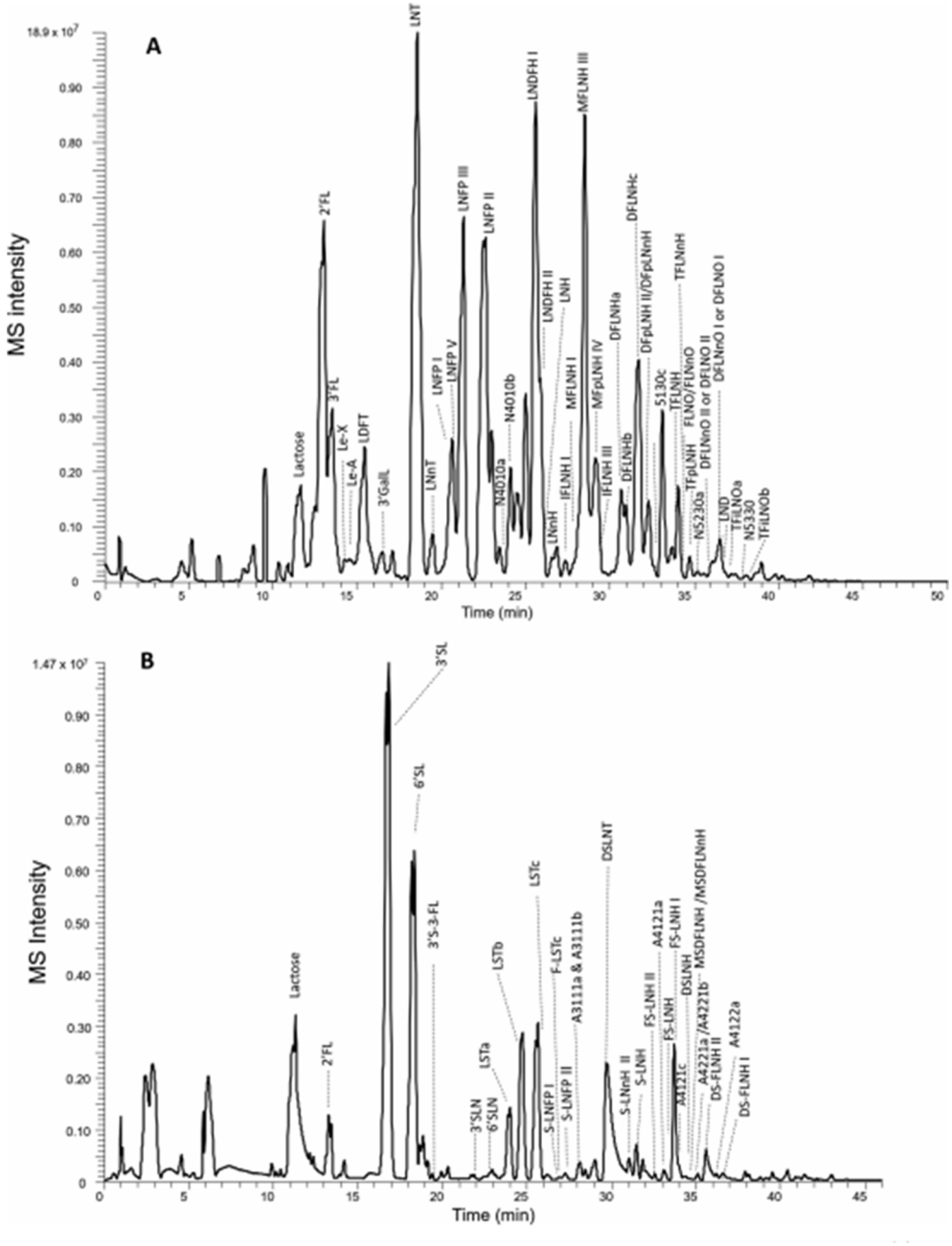


Fig. 4. Base peak HILIC-MS analysis of free oligosaccharides in human milk using positive detection mode. (A) Neutral oligosaccharides. (B) Acidic oligosaccharides. With permission from [47].

structural characterization. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the commonly used soft ionization modes for MS-based analysis of sugars. In MALDI ionization the sample is mixed or coated with an energy-absorbent matrix and ionized with a laser beam. This method generates single protonated ions from the analytes [41]. ESI, on the other hand, produces multiple-charged ions by utilizing electric field to convert solution-phase analytes into gas phase ions [42]. Permethylation is a commonly used technique to derivatize glycans for MS detection as it enhances and improves ionization and stabilizes the labile sialic acid moieties. Permethylation also help to prevent fucose rearrangement during MS analysis [40,43,44] and provides diagnostic fragment ions. MS coupled to chromatographic or electromigration separation methods makes structural identification of HMOs easier. Oursel et al. have described an LC-MS analysis of permethylated HMOs [45] comparing the analytical strategies with RPLC and PGC. Native HMOs were separated on a PGC column and permethylated species on an RPLC column, both were detected by an iontrap MS instrument. They concluded that the use of PGC column worked better for high-throughput detection of species, due to the faster sample preparation, while analysis of permethylated HMOs by RPLC-MS/MS was more suitable for structural characterization.

Utilization of biosynthetic routes to generate HMOs are not yet completely solved, but comprehensive bioanalytical methods can bring us closer to accomplish this endeavor. HMOs have a core structure carrying lactose at the reducing end, which can be connected to lacto-*N*-biose (LNB) in type 1 chain structure. In contrast, type 2 chain structure can be elongated by the addition of *N*-acetyllactosamine (LacNAc) moieties as shown in Fig. 1/B. LNB causes chain termination, while additional LNB or LacNAc moieties may be attached to LacNAc. Humans have higher amount of type 1 oligosaccharides than that of type 2. The

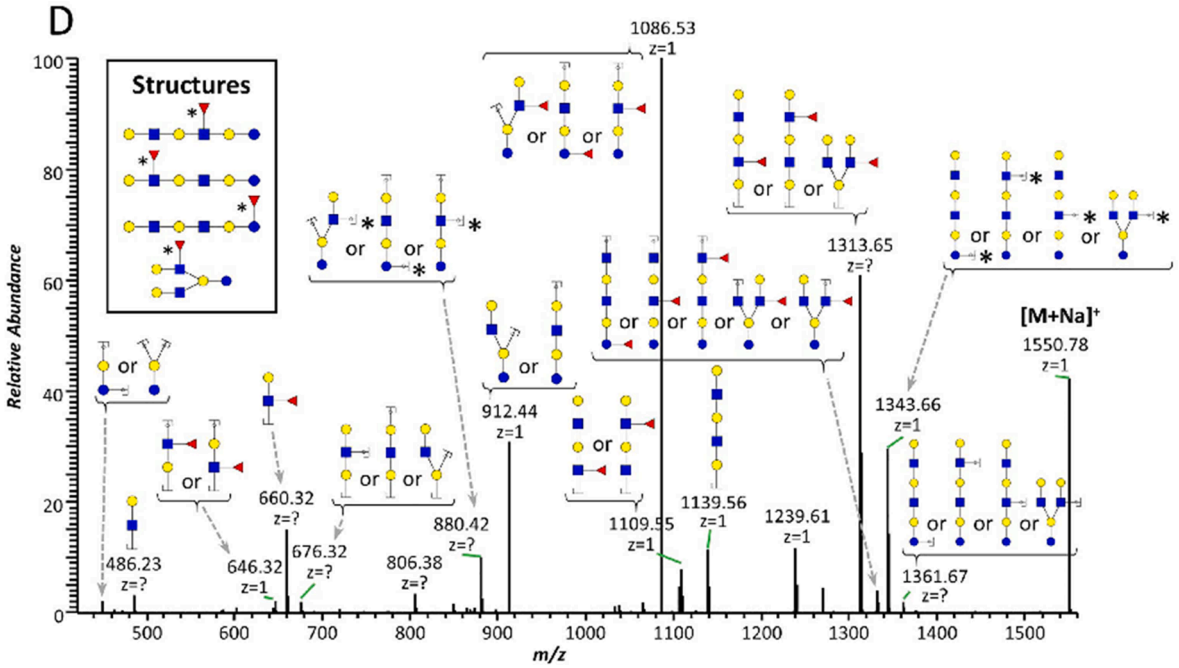


Fig. 5. Increasing molecular weight proportionally increases the structural complexity of glycoforms. With permission form [19].

predominance of type 1 oligosaccharides can be considered specific to humans, milk from other mammals contains mainly type 2 oligosaccharides [46]. Surprisingly, Balogh et al. concluded that non-conjugated LacNAc was present in a much higher concentration than non-conjugated LNB in human milk [18], just the opposite of the human type 1 and type 2 chain ratio. A PGC stationary phase based LC-MS/MS method was developed to identify and quantify these two important free disaccharides LNB and LacNAc from human milk. The method was used to track the concentration changes of these two HMO building block disaccharides throughout the first week of lactation and found them continuously decreasing during this period. Understanding the ratio of free LNB and free LacNAc in human milk contributed to the exploration of HMO biosynthesis.

Structural assignment of isomeric oligosaccharides in HMO analysis is one of the most challenging tasks. PGC stationary phase has been proven as a robust technique for the analysis of isomeric HMOs with LC-MS. In a Brazilian study, Tonon et al. quantified the 16 most abundant HMOs by LC-MS/MS method using a PGC column [21]. With a quick and simple sample preparation method, the neutral and acidic HMOs were simultaneously examined in a single run with the isomeric pair of LNT and LNnT well separated. The drawback of this study was the long chromatographic run time.

A UPLC-MS/MS method was developed with multi reaction monitoring (MRM) mode to separate 4 pairs of isomeric HMO structures (2′FL and 3FL; 3′SL and 6′SL; LNFPI and LNFPIII; LSTa as well as LSTb and LSTc) from a 12 oligosaccharide containing standard mixture [20]. Significant correlations were found between some HMO species from 61 mothers, suggesting that these HMOs may had common synthesis precursors. However, no separation of the abundant and important HMOs in human milk, LNT and LNnT core isomer pair was obtained in this study.

The above-mentioned studies mainly focused on the most frequent, low molecular weight oligosaccharides and only reported a limited number of HMOs. In a very recent and emerging work Stein and co-workers created a searchable reference MS library of annotated HMOs [47]. They characterized reference standards by HILIC-ESI-MS/MS and the library currently contains 469 positive and negative ion spectra, an example is shown in Fig. 4. The reference standard mixture contained human milk from 100 breastfeeding mothers and the library accommodated identification of unknown reduced or non-reduced oligosaccharides from human milk. When standards are not available, this information represents a promising option for future work. However, in this study high molecular weight oligosaccharides (>10 sugar units) have not been completely characterized so far.

In another interesting study, 100 different permethylated high molecular weight HMOs were identified by nano LC-MS/MS using RPLC by the Azadi group [19]. Previous studies usually focused on identification of HMOs in the lower molecular weight region, because higher molecular weight increased the complexity of structures, thus, complicated the analysis as shown in Fig. 5, but permethylation helped isomer analysis, due to partially methylated products. This approach may provide a high-throughput option in the future, may also be suitable for screening breast milk samples or commercial products.

CE-ESI-MS is another emerging tool in glycomics studies [39,48,49]. For negatively charged carbohydrates or sugars labeled with APTS, the CE system has to be coupled with negative ESI detection. In this mode the detection highly selective but less sensitive and more prone to corona discharge effects than in positive ESI mode. To alleviate the issue, a CE-ESI-CID-MS/MS method was developed utilizing multiple carbonyl-reactive target mass tag (TMT) labeled HMO standards [50]. Aminoxy TMT-label gave glycans are positively charged in acidic buffers, thus, allowed the use of positive ESI mode with enhanced sensitivity. In this study the authors separated aminoxy TMT-labeled isomeric HMO standards with increased resolution of the isomeric structures using traveling wave ion mobility as a second separation dimension.

3. Conclusion and future prospective

Continuously growing evidence shows the beneficial effects of HMOs on breast-fed infants by shaping the intestinal flora, modulating their immune responses and protecting them from infections and other diseases. These favorable effects are dependent on the structures of the HMOs, making their comprehensive characterization important for better understanding of the phenomena. However, analysis of HMOs is highly challenging because of their heterogeneity and diverse isomeric/anomeric structures. The lack of easily ionisable or chromophore/fluorophore groups on oligosaccharides further complicates the analysis.

Over the past decades, multiple analytical approaches have been developed for HMO analysis, most of them were based on liquid phase separation methods. In most instances, potential interfering agents had to be removed from the milk prior to analysis, making sample preparation an important first step. Proper sample preparation also improves the resolution of the resulting separation, but makes the process more time-consuming with the risk of losing low concentration sugars.

Oligosaccharide additives to infant formulas have to be controlled by well-established, reliable and rapid analytical techniques. While in early days mostly HILIC methods were developed to measure ingredients in milk formulas, CE has been rapidly catching up in the field of identification of milk oligosaccharides. One of the other major challenges of liquid phase separation-based methods is the need for standard reference libraries, to be utilized for retention/migration time based structural elucidation. The availability of such standards is very limited and often contain impurities. MS coupled to LC or CE proved to be an efficient approach to further elucidate HMO structures, but require proper glycoinformatics toolsets. While the recently introduced analytical strategies discussed in this review may shed light on the biomedical impact of oligosaccharides and contribute to the improvement of food supplements, further development in the field is necessary to obtain the higher selectivity and sensitivity to gain deeper understanding of the biological relevance of HMOs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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