Mass spectrometric methods for analysis of oligosaccharides in human milk

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Establishing the analytical platforms for characterizing human milk oligosaccharides is important to fully assess their specific functionalities. The characterization of these biomolecules, however, is still considered challenging, owing to their overall complexity and diversity. Addressed here are the technical difficulties with an emphasis on the application of mass spectrometry to rapidly profile and quantify human milk oligosaccharides. Fundamental concepts and improvements in instrumentation and an overview of the biological functions and structures of these compounds are also discussed. Results reveal that small-chain oligosaccharides, evident in abundance in the early stage of lactation, are selectively consumed by specific stains of Bifidobacterium longum biovar, infantis.

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INTRODUCTION

Human milk is the only primary, natural source of food for infants during the early months of life. While human milk was previously presumed to have neither nutritional value nor added benefits to infants, and was perceived to be of no significant value, it was eventually shown that individuals who were breastfed as infants generally have higher IQs, longer life expectancy, and reduced susceptibility to infectious diseases.¹⁻⁵ A study to determine the relationship between infant feeding type and disease incidence was performed several decades ago with 20,000 mother-infant dyads. Breastfed infants were found to have lower risk of acquiring diseases like diarrhea and otitis media.⁶ In a more recent study conducted during a serious outbreak of diarrhea in Botswana in 2006, 93% of infants admitted to hospitals were not breastfed and were at greater risk of dying.⁷ Moreover, recent comprehensive studies on the role of breastfeeding have shown improvements in the health and growth of infants, as well as a correlation with the risk of acquiring asthma, chronic respiratory discomforts, atopy, and other diseases.⁸⁻¹⁴ Because of the nutritional and developmental advantages of breastfeeding, the need to monitor and/or manipulate the dietary intakes of infants in the vulnerable period of their growth and development is crucial. Feeding changes can potentially provide benefit or harm with short-term (early childhood) or long-term (adulthood) implications. Randomized trials of infants’ diets showed that brief and early manipulation in preterm infants has major effects on later cognitive function.⁵,¹⁵ Thus, the rapid growth and development of infants could be highly dependent on nutrient intakes during the early stages of life. Any benefit that breastfeeding could impart is possibly due to the unique and diverse nutritional contents of human milk, the remarkable abundance of which provides physiological and protective functions over and above simple nourishment.¹⁶

Although the myriad attributes of human milk are widely recognized, there are still conflicting notions on the optimal duration of breastfeeding. The World Health Organization (WHO) made a global recommendation that mothers should breastfeed exclusively for at least 6 months.¹⁷ This recommendation was based upon an
expert consultation in March 2001. However, the scientific evidence for the optimal duration of breastfeeding is still controversial and poses new and important research questions that need to be addressed. For instance, the detailed functional mechanisms of the bioactive components in human milk and their modes of action are poorly understood, so a demand exists for further studies of these important components in order to advance the limited knowledge of their functions. To advance the functional understanding of human milk, it is necessary to improve the current analytical capabilities for deconstructing its components. Having a broad understanding will lay the foundation for taking appropriate actions and measures to advance the health and well-being of infants.

HUMAN MILK OLIGOSACCHARIDES

Human milk is a complex biological fluid composed mainly of lipids, proteins, and lactose. Other components found in milk have potential immune effects in the form of antibodies and other defense agents (e.g., immunoglobulin A, leukocytes, bifidus factor, lysozyme, lactoferrin, nucleotides, cytokines, and interferon-γ). Not only do human milk components provide nutrition for infants, they also influence their growth, immune system, and mental and cognitive development, they help establish a healthy microflora, and they help protect the infant from pathogenic diseases. Oligosaccharides are the third most abundant components in human milk, after lactose and lipids, and they are currently receiving much attention. HMOs are present in large amounts, ranging between 5 and 23 g/L. While the biological functions of HMOs are not fully defined and understood, two possible roles have been identified: 1) HMOs serve as prebiotics, defined as non-digestible carbohydrates that promote the growth of beneficial gut bacteria; and 2) HMOs prevent adhesion of pathogens to the epithelial cells, which is the initial event of infection.

HUMAN MILK OLIGOSACCHARIDES AS ANTI-ADHESIVE AGENTS

HMOs protect infants from enteric pathogens by binding with pathogenic bacteria before they can adhere to intestinal epithelial cell surfaces. The attachment is a crucial factor in pathogenesis, resulting in the potential prevention of infection when HMOs compete with cell surfaces against bacterial adhesion. The intervention with attachment by carbohydrates could then be an effective way of impeding the course of infection. Glycoconjugates from human milk inhibit binding of specific pathogens such as Escherichia coli, Streptococcus pneumoniae, Campylobacter jejuni, Helicobacter pylori, and Vibrio cholerae. In an in vitro study, oligosaccharides found in human milk were tested as antiadhesive drugs in rabbits and rats. Intratracheal and intranasal administration of either free oligosaccharides or neoglycoconjugates significantly decreased pneumonia caused by Streptococcus pneumoniae. Another study demonstrated that specific oligosaccharide (3’-sialylactose sodium salt), either alone or in combination with antiulcer drugs (bismuth subsalicylate, omeprazole), was observed to treat two of six rhesus monkeys infected with Helicobacter pylori permanently. Furthermore, human milk from secretor donors appears to bind competitively to host cell receptors with viruses that are major causes of diarrhea, thus imped ing infection of the host cell. The inhibition is attributed to the virus-binding ability of specific fucosylated moieties found in secretor donors.

HUMAN MILK OLIGOSACCHARIDES AS PREBIOTICS

HMOs are resistant to enzyme degradation in the upper gastrointestinal tract. They pass through the pancreas and brush border membranes and remain intact as they reach the colon. The colon is a primary region in which a variety of activities including nutritive, metabolic, immunologic, and protective functions could take place. Bifidobacterium, Eubacterium, Fusobacterium, Clostridium, and bacteroides are predominantly found in the colon. By arriving at the colon intact, HMOs can potentially serve as nutrients (“prebiotics”) to gut bacteria present in the large intestine. Prebiotics are defined initially as non-digestible carbohydrates that promote the growth of beneficial gut bacteria. The idea of prebiotics was first introduced more than a decade ago, but the concept has been recently revisited. There are three criteria for a carbohydrate to be classified as a prebiotic: 1) it has to be resistant to absorption and hydrolysis by gastric enzymes, 2) it should be fermentable by gut microflora, and 3) it can selectively stimulate the growth and/or activity of beneficial intestinal bacteria. The third criterion is the most demanding to fulfill because of the difficulty in obtaining evidence of selectivity.

The impact of prebiotics on human health has recently gained much attention. A healthy gut microflora exerts a major effect on the development of the immune system, and in the prevention of infectious diseases. Therefore, early dietary intervention becomes a critical facet in modulating the intestinal microflora. Companies that manufacture infant formula are attempting to mimic the health benefits that HMOs provide to infants by supplementing infant formula with the available prebiotics. To date, infant formula is supplemented with either fructooligosaccharides (FOS) or galactooli-
gosaccharides (GOS) showing somewhat similar health benefits as that of breast-fed infants. Supplementation with a small quantity of FOS leads to rapid growth of bifidobacteria in bottle-fed preterm infants while decreasing the population of pathogenic bacteria. 38 FOS and inulins are commonly isolated from plants. Whether or not these compounds can provide health benefits similar to those of breastfeeding and whether or not they pose potential hazards to infants later in life will require further long-term studies, especially since these compounds are not structurally similar to the oligosaccharides found in human milk. Further studies and well-designed randomized clinical trials are necessary to confirm the health benefits of prebiotics and to demonstrate potential side-effects. 16 Oligosaccharides found in human milk are still regarded as the most ideal for supplementation. Currently, inexpensive means to replicate and synthesize huge amount of HMOs are unavailable due to the structural complexity and diversity of these oligosaccharides.

Whereas the incorporation of prebiotics helps establish a healthy microflora, the addition of living bacteria (probiotics) that can be sustained and thrive in the colon also alters the gut microflora. 21 The beneficial effects of probiotics, particularly those isolated from breast milk, include promotion of immune system maturation, defense against infection, and anti-inflammatory properties. 39 The presence of bifidobacteria and lactobacili in large proportions in the intestinal microflora of breastfed infants, compared with those of bottle-fed infants further reaffirms the effect of breastfeeding on the composition of gut microflora. 40, 41

**STRUCTURES OF HUMAN MILK OLIGOSACCHARIDES**

Oligosaccharides in human milk are diverse and complex in nature. The monosaccharides used for the biosynthesis of human milk glycans are glucose, galactose, N-acetylglucosamine, fucose, and sialic acid. The biosynthesis of HMOs in the mammary glands starts with a housekeeping enzyme, β-galactotransferase connecting galactose (Gal) to glucose (Glc) to form lactose in the presence of α-lactalbumin. Repeating units of N-acetylglucosamine (GlcNAc) linked to galactose by β(1-3) or β(1-4) linkages are attached with β(1-3) or β(1-6) linkages to the lactose core. The disaccharide (N-acetyllactosamine) can be as long as 25 units. The core structure is further elongated by fucose (Fuc) connected either with α(1-2), α(1-3) or α(1-4) linkages and/or sialic acid (NeuAc) residues attached by α(2-3) or α(2-6) linkages in the terminal positions. 22 With few exceptions, all HMO structures consist of the lactose core. The addition of fucose is also genetically determined and affected by the action of three specific fucosyltransferases. Approximately 77% of Caucasians are classified as secretors expressing α(1-2) fucosyltransferase. 26 As a consequence, female secretors are expected to produce HMOs with these motifs that lead to greater structural variations (individual variability). The possible combinations of monosaccharide compositions, including plausible linkages, contribute to the overall diversity and complexity of HMO structures. There are currently >150 HMOs identified and >80 structures elucidated. 42, 43

**CHARACTERIZATION OF HUMAN MILK OLIGOSACCHARIDES**

Systematic characterization of HMOs started in the 1950s, yet HMO analysis remains a challenging task due to the number of unique structures and their overall complexity. More than 200 neutral and anionic oligosaccharides have been identified, including some with high molecular weights of up to mass 6000 Da, using methods such as high-performance liquid chromatography (HPLC), high pH anion-exchange chromatography, capillary electrophoresis, nuclear magnetic resonance, and mass spectrometry (MS). 25, 44–70 HMO analysis was previously performed using gas chromatography by employing derivatization techniques to yield methylated and/or acetylated glycans. However, this method suffered from low sensitivity as well as the slow and tedious nature of the analysis. More recently, HPLC has emerged as the preferred method for the characterization of HMOs. However, HPLC analysis alone provides little structural information, while still requiring large numbers of samples. Coupling HPLC with mass spectrometry has solved the problem associated with structural information. HPLC has also evolved from anion exchange (which separated oligomers but was not highly effective for isomers compared to graphitized carbon chromatography), to a method that can separate isomers of both neutral and anionic oligosaccharides.

Nanoflow HPLC or nanoLC is the latest evolution of the technique providing high sensitivity on minute amounts of material. When coupled with MS, the method is exceptionally capable of profiling complicated glycan mixtures. Miniaturization is also revolutionizing analytical biochemistry by offering a number of advantages such as reduced analyte quantity, lower solvent consumption, and better reproducibility. 71 Devices employing microfluidic technologies are now commercially available. Laser ablation is used to create laminated polyimide HPLC chip devices. For HMOs, stationary phases for the enrichment and analytical column of the microfluidic device are packed with porous graphitized carbon, which has been proven to effectively separate isomeric glycans, even those with closely related structures, from different biological sources. 72–75 These devices all integrate nanoLC.
components, enrichment and separation columns, as well as ESI emitter to produce devices that can handle small amounts of sample with high sensitivity and reproducibility. Cross-contamination and sample loss are further minimized. When coupled with high-performance mass spectrometry, such as high-accuracy TOF mass analyzer, nanoLC provides an invaluable tool for analyzing biomolecules such as HMOs.

**MASS SPECTROMETRY AS A TOOL FOR ANALYZING OLIGOSACCHARIDES IN HUMAN MILK**

Recent advances in MS techniques enable us to obtain meaningful information on the molecular details of oligosaccharides. The inherent attributes of MS in terms of sensitivity, specificity, and robustness are ideal for profiling and quantifying oligosaccharides in human milk. There are basic components common to all MS methods (Figure 1) – the sample inlet, ionization source, a mass analyzer (with associated data system), and an ion detector. Both the mass analyzer and the ion detector are maintained under high vacuum. The sample can be introduced into the mass spectrometer as a gas, a solid, or a liquid. Gaseous analytes are introduced directly into the ion source and are ionized by electron impact or chemical ionization. Solids are placed on a sample probe and irradiated by laser in matrix-assisted laser desorption/ionization. Liquids are introduced directly into the ionization source as in electrospray ionization (ESI). ESI is typically used to couple liquid chromatography to mass spectrometry. Sample introduction into the instrument is performed while maintaining the vacuum inside the system, typically below 10⁻⁶ torr. The sample molecules are converted to ions in the ionization source before being extracted electrostatically into the mass analyzer and separated according to their mass-to-charge ratio (m/z). Ions are then detected as electrical signals and transmitted to a data system.

Mass analyzers have improved significantly over the years and have always provided capabilities for high sensitivity and high resolution. However, it was the development of better ionization sources, specifically matrix-assisted laser desorption ionization (MALDI) and ESI, which led to significant developments and the use of mass spectrometry to analyze biological samples. MALDI MS was initially developed by Karas and Hillenkamp in 1988 at the University of Frankfurt in Germany and first reported by Tanaka of Shimadzu Corp. While ESI MS was first conceptualized by Dole of Northwestern University in the 1960s, it was only in the 1980s that Fenn of Yale University first applied the use of ESI MS in the analysis of biomolecules. The research efforts of Fenn and Tanaka to develop these two valuable ionization techniques resulted in their sharing the 2002 Nobel Prize in Chemistry.

**MATRIX-ASSISTED LASER DESORPTION IONIZATION**

In MALDI, ions are generated by pulsed irradiation of the sample, leading to the desorption of ions from a solid surface. The sample is cocrystallized with a matrix that can absorb the wavelength of light emitted by the laser. Typically, UV lasers such as nitrogen (337 nm) and Nd:YAG (266 nm and 355 nm) are used, but less commonly, IR lasers (CO₂) are employed at 10.6 mm. Sample preparation consists of mixing the sample and matrix on a target probe that is inserted into the ion source maintained under vacuum. The desorbed molecules are then extracted into the mass analyzer.

HMOs are particularly amenable to MALDI MS analysis. The ions produced by MALDI are typically alkali metal-coordinated species, due to the strong chelating properties of oligosaccharides. Often, samples are purposely doped with sodium chloride to specifically obtain the sodium-coordinated species.

**ELECTROSPRAY IONIZATION**

In ESI, ions are produced at atmospheric pressure by passing a solution-based sample through a small capillary that is at a potential difference (1–3 kV) relative to a counter electrode. Electrospraying of a sample solution initially generates an aerosol of charged droplets, customarily with the aid of a drying gas (e.g., nitrogen) to facilitate the nebulization process. Droplets undergo "Coulomb
explosion” as the droplets reduce in size. Eventually, ions become fully desolvated resulting from complete solvent evaporation as they travel towards the mass analyzer. The electrospray processes can be separated into three stages: droplet formation, droplet shrinkage, and gaseous ion formation. During droplet formation, a solution is delivered through the tip of a small capillary in the presence of a high electric field. Ions then accumulate at the surface forming a “Taylor cone.” When the surface tension exceeds the applied electrostatic force, the “budding” process begins followed by shrinkage of the droplet. In this process, the droplets traverse towards the mass analyzer, with changing pressure resulting in a reduction in the droplet’s diameter; this is followed by Coulombic explosion, which occurs when the droplet reaches the “Rayleigh limit.” At this time, the surface tension holding the droplet together surpasses the charge magnitude.

Currently, there is no consensus regarding the exact mechanism for the ESI process, but two models have been proposed that attempt to explain it. The first model is the single-ion droplet theory, in which a “naked” ion in a single droplet is generated after droplet fission and evaporation of solvent. The second model is the ion evaporation theory (ejection), which proposes that once the Rayleigh limit is reached, charge-charge repulsion occurs, resulting in ejection of the ions from the droplet’s surface. In both cases, gaseous ions are transferred and focused towards the mass analyzer through a series of focus lenses, skimmers, and multipoles (e.g., quadrupole, hexapole, octapole).

Oligosaccharide ions produced by electrospray are typically protonated, but they can also be found as ammonium (NH₄⁺) and alkali metal ion-coordinated species. Profiling mixtures with ESI is facilitated by the coupling of LC, allowing for the separation of isomeric species.

**NANO-ELECTROSPRAY IONIZATION**

ESI has evolved to be the method of choice for ionizing biomolecules, including proteins, peptides, glycans, and metabolites. However, the method has major limitations, particularly the degree of sample consumption during measurements. The introduction of nano-electrospray ionization (nanoESI) significantly expanded the utility of ESI for analyzing biomolecules by requiring significantly less sample material. Typical flow rates for nano-ESI are in the low nL/min, with a total of 0.2–2 mL injection volume resulting in high sensitivity, even down to atto- and femtomole levels. A stable spray of solvents with high surface tension, such as water, is more attainable, which is ideal since water is the universal solvent of nearly all biological molecules. A further limitation of ESI that is perhaps mitigated, but not fully solved, by nanoESI is the limited sample throughput, making high-throughput analysis difficult.

**MASS ANALYZERS**

The mass analyzer separates gas phase ions according to their m/z ratio. The ions are transferred from the ion source, where they are produced, to the mass analyzer and maintained under high vacuum to allow ions to travel unperturbed towards the detector. For the study of HMOs in our laboratory, high-accuracy mass analyzers such as Fourier transform ion cyclotron mass spectrometry (FTICR MS) and high performance time-of-flight mass spectrometry (TOF MS) were used.

**FOURIER TRANSFORM ION CYCLOTRON RESONANCE MASS SPECTROMETRY**

There are four basic components in all FTICR MS instruments – a high field magnet, an analyzer cell, an ultrahigh vacuum system, and a data system. The high-field-superconducting magnet is the most prominent part of the FTICR MS instrument, ranging from 3 to 15 Tesla. Most of the high-field FTICR MS magnets are shielded to prevent excessive fringe fields. The detector in the FTICR MS instrument consists of an analyzer cell positioned within the homogenous region of the magnetic field. In the analyzer cell, the ions are mass analyzed and detected. Ions are trapped by electric and magnetic forces in the cell, which consists of three major components – the excitation, detection, and trapping plates. In a cubic cell configuration, ions are injected through an opening in one of the trapping plates. Ions are kept inside the cell by applying a small voltage (typically 1 V) to the trapping plates. In the presence of a magnetic field that is perpendicular to the direction of the ion’s velocity, ions tend to move in small cyclotron orbits. However, the radius of gyration is not sufficient for detection. The ions are also out of phase and need to be excited to a larger radius by applying a dipolar radio frequency (RF) electric field that is in resonance with the ions’ cyclotron frequency. Excited ions orbit with larger radii and are cyclotroning coherently. The packet of ions induces an image current on the detector plates. The image current is further amplified, digitized, and Fourier transformed.

An ultrahigh vacuum system (<10⁻⁷ torr) is necessary for FTICR MS instruments to obtain the required mean free path (>10⁻⁷ cm). The pressure must be within 10⁻⁹–10⁻¹⁰ torr or lower to prevent collisions inside the cell. The pressure requirement can be achieved using cryogenic pumps, turbo molecular pumps, or a combination of both. The data system requirements for FTICR MS instruments consist of a fast transient digitizer,
broadband preamplifier and amplifiers, a frequency synthesizer, and a delay generator. The system should be able to process large amounts of FTICR MS data sets, depending on the number of data points taken per acquisition.98

For the studies in our laboratory, a MALDI-FTICR MS (IonSpec ProMALDI FTMS, IonSpec Corp., Irvine, CA, USA) was used to analyze oligosaccharides in human milk. The system is equipped with a multi-well plate capable of analyzing multiple samples, a pulsed laser for target irradiation (Nd:YAG, 355 nm), an ultrahigh vacuum system (one turbo molecular pump and two cryopumps) to maintain a pressure of at least 10⁻¹⁰ torr, a quadrupole ion guide for efficiently transporting ions into the FTICR analyzer cell, and a 7.0 Tesla shielded-superconducting magnet. The MS system also includes hexapole accumulation rods to collect ions prior to transmission to the analyzer cell.

**TIME-OF-FLIGHT MASS SPECTROMETRY**

An orthogonal-axis time-of-flight mass spectrometer (oa-TOF MS) consisting of an ion source, ion transfer optics, beam shaping optics, ion pulser, flight tube, and a detector coupled with a microfluidic device was also employed in this study. The key feature of oa-TOF MS is the independence of TOF directions with the velocity of ions in the ion beam. The advantages of oa-TOF MS compared to standard TOF are increased resolution and sensitivity due to the following factors: 1) reduction of the average initial ion velocity and reduction in the spread of velocity components in the TOF direction, and 2) regulation of continuous ion beam energy and the ion drift energy, independently resulting in about the same amount of time required for the ion beam to fill the orthogonal accelerator.99

With oa-TOF MS, ions are generated from the source and are electrostatically guided while simultaneously introduced to drying gas (typically N₂ gas) as they traverse a heated capillary. A voltage is applied to fragment the ions as they pass through the capillary, a metal skimmer, and focusing by the first octapole ion guides. Ions exit the first octapole and are directly injected through the second octapole, which accelerates and transfers the ions to the TOF MS analyzer. A portion of the ion beam is then pulsed into the TOF region by applying a high-voltage orthogonal beam to the original ion beam. Ions travel through the 1-meter long flight tube and reflect back to the detector via a reflectron.

**DEVELOPMENT OF MS-BASED METHODS FOR HMO ANALYSIS**

A new approach towards characterizing oligosaccharides isolated from human milk was developed using MS techniques employing a combination of high-mass-accuracy and high-resolution MS analyzers (FTICR, TOF) and microfluidic devices.

**HUMAN MILK OLGOSACCHARIDE PROFILING**

The general scheme of the analytical strategy to profile HMOs is shown in Figure 2A. Automated nano-ESI LC-MS coupled with an integrated microfluidic chip that is capable of separating complex glycan mixtures was performed with high chromatographic repeatability. The microfluidic chip consists of an enrichment column for sample concentration and an analytical column for separation, both packed with porous graphitized carbon. Nearly 200 potential HMOs from pooled human milk sample were identified based on accurate mass measurements using an orthogonal TOF mass analyzer.94 Fucosylated HMOs were found to be the most prominent component (~77%), while sialylated HMO accounted for about 16% of the total abundance of HMOs. In a separate study, the chromatographic reproducibility, prior to sample analysis of the nanoLC profiling method, was first examined by calculating the shift in retention times and deviations of the peak areas and peak heights of 10 selected peaks from nine consecutive injections of the same HMO sample. The repeatability of the retention times was high (0.1% RSD) while the peak areas and peak heights varied from 3% to 29% and from 3% to 15% RSDs, respectively. Using this profiling method, oligosaccharides in human milk from different individual donors and samples taken at different stages of lactation were analyzed to monitor any variations in oligosaccharides.100 Differences in the oligosaccharide profile among individuals were apparent, which is consistent with previous reports.96,101 However, little variation was observed in the total HMO content during the first few days of lactation. Another important feature of this method was its ability to separate isomers. Several isomers (fucosylated and non-fucosylated) were inspected over the course of the lactation period of a single donor. Additionally, abundant amounts of specific oligosaccharides preferentially consumed by *Bifidobacterium longum* bv. *infantis* were detected.27 These oligosaccharides were consistently found in abundance during the course of the monitored lactation period.

**HUMAN MILK OLGOSACCHARIDE QUANTIFICATION**

The relative quantification of HMOs after bacterial fermentation was accomplished using deuterated compounds as internal standards analyzed by FTICR MS equipped with MALDI and nanoESI ionization sources.
In this experiment, several bacterial samples were grown in media containing purified HMOs that serve as the only carbon source. Isotopically labeled compounds were added as internal standards and used to quantify bacterial HMO consumption. After bacterial fermentation, oligosaccharides were recovered, purified, and analyzed by mass spectrometry. Based upon the ratio of unlabeled sample to the deuterated internal standards, relative amounts of bacterial HMO consumption were obtained. The quantification method
was initially applied to compare a few bacterial strains (E. coli, B. longum bv. longum, B. longum bv. infantis, B. longum bv. breve) among which differential HMO consumption was clearly evident. B. longum bv. infantis, a predominant bifidobacteria species in the breastfed infant colon, manifested to preferentially consumed HMOs with degree of polymerization ≤7. This particular quantification method was further developed and refined to increase the throughput of the analyses, and it was later used to screen larger bacterial collections for probiotic properties.

In summary, the MS capabilities and modern microchip LC technology make it possible to separate complicated glycan mixtures and identify specific components with high accuracy and reproducibility. The ability to rapidly identify and quantify individual components makes it possible, in turn, to further elucidate the role of oligosaccharides in human milk. The results of this study clearly illustrate how advancements in analytical technology can accelerate the course of research and, specifically, provide a better understanding of the biological functions of carbohydrates.

**CONCLUSION**

Analytical methods using mass spectrometry to profile and quantify oligosaccharides in human milk have been presented. The methods are relatively rapid, allowing specific components to be separated with high sensitivity and in a reproducible manner. The methods employ the use of microfluidic chips for separation, coupled with high-performance mass analyzers (TOF MS and FTICR MS) for high resolution and high mass accuracy. Use of these advanced techniques in combination provides invaluable information for assessing a number of potential biological functions of HMOs in the infants’ health and development.

The high resolving power of FTICR MS and the TOF mass analyzers, along with the use of deuterium-labeled internal standards, allow precise measurements of HMO consumption to be made. A screening study is currently being conducted to examine different bacterial strains for their consumption of specific oligosaccharides. Further development is also underway to produce bioinformatics software to streamline the analysis. A software program is being developed to annotate mass spectral peaks and determine consumption automatically. A high-throughput method for performing consumption studies, as well as for sample clean-up and purification, is being developed to handle the large number of experiments and the data analysis.

Translational research is also underway. Clinical studies are planned to determine the relationship between the mother’s oligosaccharides and the bacterial strains present in their offspring’s gut. Investigating the path of oligosaccharides from the mother to the infant and the infant’s waste, specifically in feces and urine, will determine with better specificity the eventual fate of oligosaccharides. The new analytical tools described here will significantly enhance our understanding of the role of oligosaccharides and other glycoconjugates in the future.

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