SUPPLEMENT
The Glycobiology of Human Milk Oligosaccharides

Advances in Analysis of Human Milk Oligosaccharides[1–3]

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ABSTRACT

Oligosaccharides in human milk strongly influence the composition of the gut microflora of neonates. Because it is now clear that the microflora play important roles in the development of the infant immune system, human milk oligosaccharides (HMO) are studied frequently. Milk samples contain complex mixtures of HMO, usually comprising several isomeric structures that can be either linear or branched. Traditionally, HMO profiling was performed using HPLC with fluorescence or UV detection. By using porous graphitic carbon liquid chromatography MS, it is now possible to separate and identify most of the isomers, facilitating linkage-specific analysis. Matrix-assisted laser desorption ionization time-of-flight analysis allows fast profiling, but does not allow isomer separation. Novel MS fragmentation techniques have facilitated structural characterization of HMO that are present at lower concentrations. These techniques now facilitate more accurate studies of HMO consumption as well as Lewis blood group determinations. *Adv. Nutr. 3: 406S–414S, 2012.*

Introduction

Human milk is the sole source of nutrition for neonates during their first period of life. Over centuries, human milk is evolutionary shaped to nourish the newborn (1,2) and is regarded as the nutritional gold standard for term infants. Indeed, several benefits of breastfeeding have been described for term infants (3). Human milk is composed of lactose, lipids, free oligosaccharides, and proteins, of which the free oligosaccharides are important constituents at a concentration ranging from 5 to 23 g/L (4–6).

Free oligosaccharides in human milk are linear and branched structures of 3 to 14 monosaccharides (7,8). More than 200 free oligosaccharide structures have so far been identified from human milk samples (7–10). Nearly all of them originate from a lactose [Gal(β1–4)Glc] core that is extended with *N*-acetyllactosamine repeats. These repeats can either be linked Gal(β1–3)GlcNAc, type I or Gal (β1–4)GlcNAc, type II. The linear or branched structures thus formed can be decorated with fucose and/or *N*-acetylneuraminic acid, where the *N*-acetylneuraminic acid residues may be attached either with an (α2–3) or an (α2–6) linkage.

A close relationship exists between the Lewis blood group system and the structures of human milk oligosaccharides (HMO)[4] found in an individuals' milk (11,12); fucose residues are attached to HMO according to the expression of both the secretor gene (*fucosyltransferase 2*) and the Lewis gene (*fucosyltransferase 3*) (13). Fucose residues may be α1–2 linked to galactose residues when *fucosyltransferase 2*

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[4] Abbreviations used: CE, capillary electrophoresis; FTICR, Fourier transform ion cyclotron resonance; HMO, human milk oligosaccharides; LC, liquid chromatography; LIF, laser-induced fluorescence; MALDI, matrix-assisted laser desorption ionization; PGC, porous graphitic carbon; RP, reverse phase; SRM, selective reaction monitoring; TOF, time of flight.

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is active, whereas fucose residues may be α1–4 linked when *fucosyltransferase 3* is active (14,15). Activity of the fucosyltransferases is regulated by genetic variation and is thus inherited.

HMO are the third abundant compound in human milk, and yet they have no direct nutritional values because they are not digestible by the human gastrointestinal tract. However, evolution has shaped the structure of HMO (1,2), so they must have important functions. Indeed, several studies showed that HMO are important during the development of the newborn (1,16–18).). Milk oligosaccharides stimulate the growth of probiotic bacteria by interfering with the bacteria-host interactions through several mechanisms (1,16), thus constituting a prebiotic function. HMO have been shown to have antiadhesive properties (16). It is proposed that the oligosaccharides mimic the natural ligands of the bacteria, thus occupying their natural binding sites and inhibiting their adhesion. Such antiadhesive properties have been described for several bacteria and viruses, including *Streptococcus pneumonia*, *Listeria monocytogenes*, *Vibrio cholera*, *Salmonella fyris*, HIV, enteropathogenic *Escherichia coli*, and *Campylobacter jejuni* (19–21). *C. jejuni* is one of the major causes of diarrhea and was shown to adhere to 2′-fucosyllactosamine (21). Later, it was observed that the incidence of diarrhea in breast-fed infants was directly related to the levels of 2′-fucosyllactosamine in their mother's breast milk (22).

Recent studies focused on the consumption of oligosaccharides by gut bacteria. Several strains of bifidobacteria can grow well on HMO; however, it was observed that some strains prefer fucosylated oligosaccharides, whereas others prefer nonfucosylated structures (23). Similarly, different galacto-oligosaccharide polymers were consumed differently by bifidobacteria strains (24). Marcobal et al. (25) reported recently that milk oligosaccharide consumption is not specific for bifidobacteria, but can also be observed for bacteroides species.

Overall, it may be concluded that HMO have a strong influence on the composition of the gut microflora. It is proposed that a well-balanced intestinal microflora is important for the development of the infant's immune system (26), indicating that HMO play an important role in the infants well-being.

To allow further evaluation of the role of HMO in the development of infants, methods to characterize these structures both in-depth and on a larger scale are necessary. Traditionally, milk oligosaccharide analysis has been performed using NMR, high-pH anion exchange chromatography with pulsed amperometric detection, or lectin affinity. With the introduction of MS for the analysis of oligosaccharides, large numbers of additional analytical techniques have been applied (27,28), such as hydrophilic interaction chromatography (10) and porous graphitic carbon (PGC) (7,8,29) separations with or without coupling to MS or stand-alone matrix-assisted laser desorption ionization (MALDI) MS (12). This review aims to give an overview of the current state-of-the-art analytical techniques used in milk oligosaccharide analysis and its biological and clinical implications.

Analysis of HMO

Because HMO are complex structures, of which the monosaccharide building blocks may be linked at different locations and with different linkages, resulting in several isomers, complex strategies are necessary for their detailed analysis. Over the years, several analytical strategies have been applied, both for profiling and in-depth characterization.

HMO profiling

A nonexhaustive overview of methods used for HMO fingerprinting can be found in **Table 1**. Traditionally, anion exchange chromatography, particularly high-pH anion exchange chromatography with pulsed amperometric detection, has been used for the analysis of HMO (11,30–38). Using anion exchange columns, HMO can be separated, resulting in separation of several isomers. However, previous separation of the neutral and acidic oligosaccharides may be required, resulting in doubled analysis times.

A second mode of separation often used for HMO analysis is reverse phase (RP) HPLC. Native oligosaccharides are not retained on RP material because of their hydrophilic properties, and therefore derivatization is required. Retention and separation of the HMO on RP liquid chromatography (LC) depend thus mainly on the method of derivatization; some isomer separation was obtained so far, but no method has emerged that provides comprehensive isomer separation. Labeling with chromophoric active tags such as 1-phenyl-3-methyl-5-pyrazolone, 2-aminopyridine, and 2-aminobenzoic acid as well as perbenzoylation have been applied for the analysis of HMO. These labels served 2 major purposes. They provided a chromophore for detection with HPLC and a hydrophobic label to allow chromatographic separation in stationary phases such as C_{18}, which do not normally retain or separate native oligosaccharides. Additional labels have been used in the analysis of other oligosaccharides (27,39) and may also be applicable for the analysis of HMO.

More recently, HMO have been separated using hydrophilic interaction chromatography HPLC (10), a method that has already been applied extensively for the analysis of *N*- and *O*-glycans (40–42). The oligosaccharides are labeled with 2- aminobenzamide using reductive amination to allow fluorescence detection, but retention is mostly based on the oliogosaccharide portion, and the elution order is mainly influenced by the number of monosaccharide residues. Several sialylated isomers can be separated.

Electromigration-based separation techniques have also been applied in the analysis of HMO (43,44). Using micellar electrokinetic chromatography, native sialylated milk oligosaccharides were separated, showing good isomer separation. For electromigrative separation techniques, charged analytes are necessary. Therefore, native neutral human milk oligosaccharides cannot be analyzed using such techniques.

Table 1. Overview of separation methods used in milk oligosaccharide analysis [1]

Separation	Column	Detection	Sample	Derivatization	Isomer separation	Notes	Ref.
Strong cation exchange	Aminex HPX 87 C	Refractive index	Oligo- and monosaccharides from human milk	—	Separation of glucose and galactose	Oligosaccharides not separated except fucosyllactose	5
HPAEC	CarboPac PA-100	Pulsed amperometric detection	HMO, neutral and acidic separated	—	Separation of several isomers (2′- and 3′- FL, 3′- and 6′—SL, LNFPs,	Use of salts for elution does not allow immediate coupling to MS	11,34, 37,38
HPAEC	CarboPac PA-1	Pulsed amperometric detection	HMO and FOS/GOS mixtures	—	Separation of several isomers (2′- and 3- FL, 3′- and 6′ SL, LNFP's)		30–33, 35,36
Hydrophilic interaction chromatography	TSK-gel amide-80	Fluorescence	BMO, neutral and acidic separated	Reductive amination using 2-aminobenzamide	Separation of several isomers	Retention times can be compared using GU index. Structural assignment confirmed using exoglycosidases and ESI-Q-TOF	10
Carbon LC	Porous Graphitic carbon-chip	nano-ESI-TOF	HMO	Reduction	Separation of most isomers	Library based on mass and retention time, containing 74 structures. Structural assignment confirmed using exoglycosidases and MALDI-FTICR	7,8,46,53
RP LC	Rainin C-8	UV at 229 nm	Neutral HMO	Reduction and perbenzoylation	Limited isomer separation	Elution mostly based on increasing molecular weight; linkage has smaller effect	22,72,73
RP LC	Inertsil ODS-3V and ODS-100Z	UV at 245 nm and 310 nm, for PMP and PA, respectively	Neutral HMO	PMP derivatization and reductive amination using PA	Partial isomer separation	Separations were different for PMP compared with PA derivatization	65
RP LC	Inertsil ODS-3V	UV at 310 nm	Neutral HMO	Reductive amination using PA	Partial isomer separation		74
RP LC	TSKgel ODS-100Z	Fluorescence	HMO, neutral and acidic separated	Reductive amination using 2-aminobenzoic acid	Separation of several isomers (2′- and 3′- FL, 3′- and 6′—SL, LNDFHs)		75,76
Micellar electrokinetic chromatography	—	UV at 205 nm	Sialylated HMO	—	Separation of several isomers	Only sialylated HMO can be separated using this method because the separation is charge based	43,44
Capillary electrophoresis	—	Fluorescence	HMO	Reductive amination using 8-aminopyrene-1,3,6-trisulfonic acid	Separation of most isomers	Very fast separation (10 min). Structural assignment confirmed using ESI MS MS	47–49

[1] BMO, bovine milk oligosaccharides; ESI-Q-TOF, electrospray ionization-quadupole-time-of-flight; FL, fucosyllactose; FOS, fructo-oligosaccharides; FTICR, Fourier transform ion cyclotron resonance; GOS, galacto-oligosaccharides; GU, glucose units; HMO, human milk oligosaccharides; HPAEC, high-pH anion exchange chromatography; LNDFHs, lacto-n-difucohexaose; LNFPs, lacto-n-fucopertaose; MALDI, matrix-assisted laser desorption ionization; PA, 2-aminopyridine; PMP, 1-phenyl-3-methyl-5-pyrazolone; RP LC, reverse phase liquid chromatography; SL, sialyl lactose.

Methods for separation of permethylated oligosaccharides have also been developed (45). Permethylation is often used to stabilize oligosaccharides during ionization and to increase sensitivity. However, because it does requires additional steps and the analysis can be complicated by incomplete derivatization. Standard RP columns such as C_{18} can provide some isomeric separation, but it too lacks comprehensive separation of isomeric species.

All the analytical techniques described so far are based on separation alone; however, structural confirmation can in such cases only be obtained based on standards. These standards are expensive and not available for all HMO. Moreover, co-elution/migration cannot be excluded. Because elution or migration is not perfectly identical in all runs and HMO samples from different donors may have very different patterns, identification of the signals in each of the samples may be ambiguous. For better identification, coupling of the separation with mass spectrometry has proven to be effective.

Our laboratory recently introduced nano-LC PGC chip time-of-flight (TOF) MS in the positive mode for the analysis of HMO (7,8,46). In this method, good isomer separation is combined with unambiguous identification using MS, as illustrated in **Figure 1**. Both neutral and sialylated compounds may be separated in 1 run, and, using a library containing retention time, mass, and fragmentation information, immediate identification is possible (7,8). Using this method, >200 HMO structures can be separated. Reduction of the reducing end of the oligosaccharides is necessary because the α- and β-anomers are separated on the PGC stationary phase.

More recently, a method consisting of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) coupled to MS of 8-aminopyrene-1,3,6-trisulfonic acid–labeled milk oligosaccharides was developed (47–49). Labeling of HMO with 8-aminopyrene-1,3,6-trisulfonic acid introduces a fluorophore for the LIF detection, simultaneously adding the negative charge needed for the separation. Although good separation can be achieved using offline CE with LIF using very fast runs ($\sim$9 min), both resolution and separation times must be compromised when coupling CE to MS (50). The separations obtained using both offline and online CE with LIF are depicted in **Figure 2**. The authors decided to use the offline CE with LIF method for their clinical applications (47–49).

Compositional profiling of HMO

Although separation allows identification of isomers, it also is a time-consuming and, in high-throughput studies, rate-limiting step. Offline MS profiling of HMO using MALDI TOF MS was first described by Stahl et al. (51), who were able to observe neutral oligosaccharides in a positive mode as monosodium adducts as well as acidic oligosaccharides in both the positive and negative modes. It was noticed that desialylated fragments could be observed in the acidic fraction.

More recently, our group developed a strategy using MALDI Fourier transform ion cyclotron resonance (FTICR)

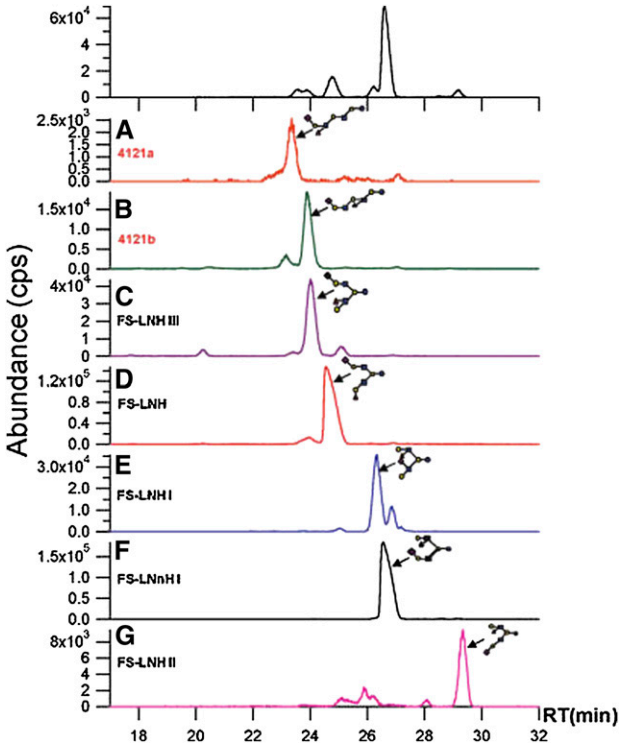

Figure 1 Separation of isomers of reduced FS-LNH using nanoliquid chromatography porous graphitic carbon chip time-of-flight MS. RT, retention time; FS-LNH, fucosyl-lacto-N-hexaose. Reprinted from Reference (7) with permission.

MS to monitor bacterial consumption of HMO (52). Using 2,5-dihydroxybenzoic acid as the ionizing matrix, neutral oligosaccharides were observed as sodiated adducts. This is illustrated in **Figure 3**. The high resolution of the FTICR MS allowed application of deuterium-labeled internal standards, which was shown to be beneficial for relative quantitation (23,52,53).

A similar approach was applied recently for the determination of Lewis blood group by HMO fingerprinting. After automated oligosaccharide purification, HMO were analyzed using MALDI TOF with 6-aza-2-thiothymine as the matrix (12). Neutral oligosaccharides and sialyllactose could be observed as sodium and potassium adducts in the positive mode, whereas other sialylated HMO were detected as deprotonated molecular ions in the negative mode. Using this method, 93.8% of the samples could be assigned the correct blood group.

Overall, direct MS strategies may be a fast alternative for HMO analysis because no separation is needed; however, it is not possible to distinguish isomeric structures, which may be necessary, for example, to determine whether only specific types of linkages are affected.

Structural characterization of HMO

Whereas HMO fingerprinting can provide an overview of the oligosaccharides present in a given sample, the structural assignments mostly rely on previous literature or databases, in which HMO structures have been thoroughly characterized.

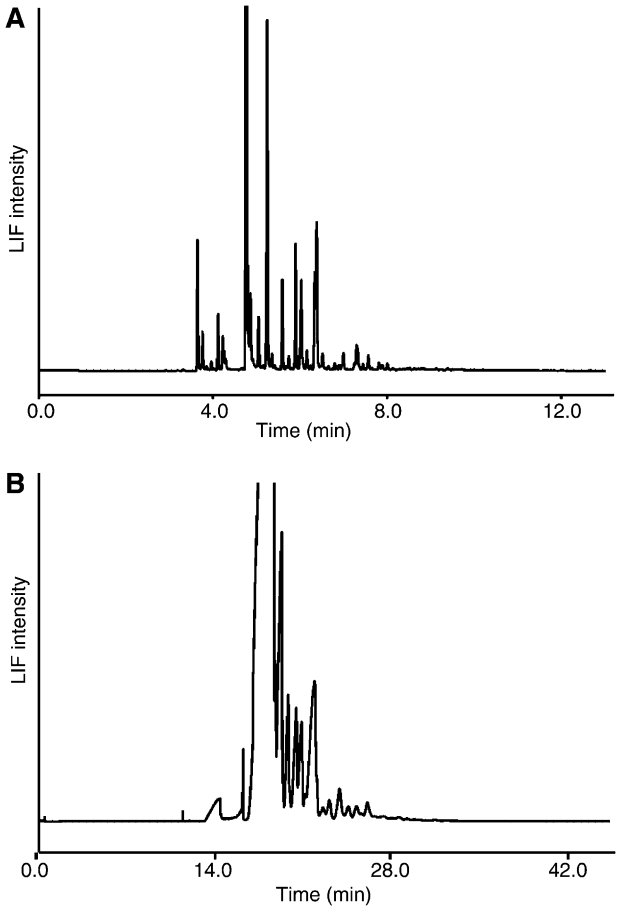

Figure 2 Separation of 8-aminopyrene-1,3,6-trisulfonic acid–labeled human milk oligosaccharides using capillary electrophoresis with laser-induced fluorescence (LIF) (*A*) and capillary electrophoresis with LIF MS (*B*). The adjustments needed for hyphenation with MS result in reduced resolving power and longer analysis times. MS, however, facilitates direct identification. Reprinted from Reference (47) with permission.

As pure oligosaccharides are needed for structural characterization, substantial purification is needed, which often results in small amounts (picomoles) of material. Offline MS and NMR (54,55) are most often used for HMO characterization. Although larger amounts of pure oligosaccharides are needed for NMR (typically micromoles of pure compounds), MS-based fragmentation techniques can be performed on picomole quantities in complicated mixtures with the proper LC couple.

Traditionally, fragmentation of HMO has been performed using collision-induced dissociation on a quadrupole ion trap MS instrument (56,57) and on FTICR MS instruments (58–60); an example of a fragmentation spectrum for 3 fucosylated HMO is depicted in **Figure 4**. Fragmentation behavior of milk oligosaccharides in both positive and negative modes has been reviewed extensively (61,62), and it was observed that cleavages of the glycosidic bond are most common. It must be noted that glycan rearrangements may occur in fragmentation studies performed by collision-induced dissociation (63). More recently, structural characterization is also obtained using quadrupole TOF MS instrumentation (7,8).

Recently, the use of electron capture methods such as electron transfer dissociation for characterization of reduced and permethylated milk oligosaccharides was reported (64). It was observed that electron transfer dissociation resulted mainly in cross-ring cleavages, allowing unambiguous linkage identification. So far, however, only simple, linear or minimally branched structures have been analyzed. Although the first results indicate electron transfer dissociation is a promising complementary fragmentation technique for milk oligosaccharides, further studies will need to be performed. These methods remain far from routine with oligosaccharides because they require multiply charged species, preferably triply positively charged, which are difficult to produce with nonbasic and even acidic milk oligosaccharides.

HMO quantitation

For comparison of milk and feces of mother-baby dyads, as well as in bacterial consumption studies, accurate (relative) quantitation of the individual HMO is necessary. Although fluorescent and UV detection is traditionally regarded to be more robust for quantitation (65), MS detection requires more effort for quantitation. Oligosaccharides in mixtures tend to suppress each other, resulting in different ionization efficiencies for specific components. For example, neutral oligosaccharides (those not containing sialic acids) will tend to suppress sialylated species in the positive mode, whereas the reverse happens in the negative mode. However, separating the mixture into individual components produces responses to ionization and detection that are generally similar. For most cases, therefore, detector response is sufficient in LC/MS.

For more accurate quantitation, isotopic labeling is performed (23,52,53). Reduction of the aldehyde with sodium borodeutoride adds a deuterium to the resulting alditol. Mixtures can be analyzed by adding a standard mixture with deuterium to the sample, which has been reduced with sodium borohydride. With accurate mass instruments such as FTICR and TOF, quantitation is obtained by comparing the monoisotopic peaks of the hydrogenated and deuterated compounds, subtracting for ^{13}C abundances. This method is typically used for quantitating oligosaccharide consumption profiles of bacteria (23) or for characterizing enzymatic properties of glycosidases from bacteria.

Discussion

Recent advances in analytical approaches, especially coupling of PGC separation to MS, allows rapid simultaneous profiling and quantitation of >100 HMO. Using this approach, it may now be studied whether HMO-consuming bacteria have specificities for specific linkage isomers. Moreover, in-depth studies of the relationship between (linkage-specific) HMO profile and Lewis blood group are now feasible. Recent studies also focused on the uptake and clearance of HMO by the neonate (35,48). So far, analytical approaches based on separation with UV or fluorescent

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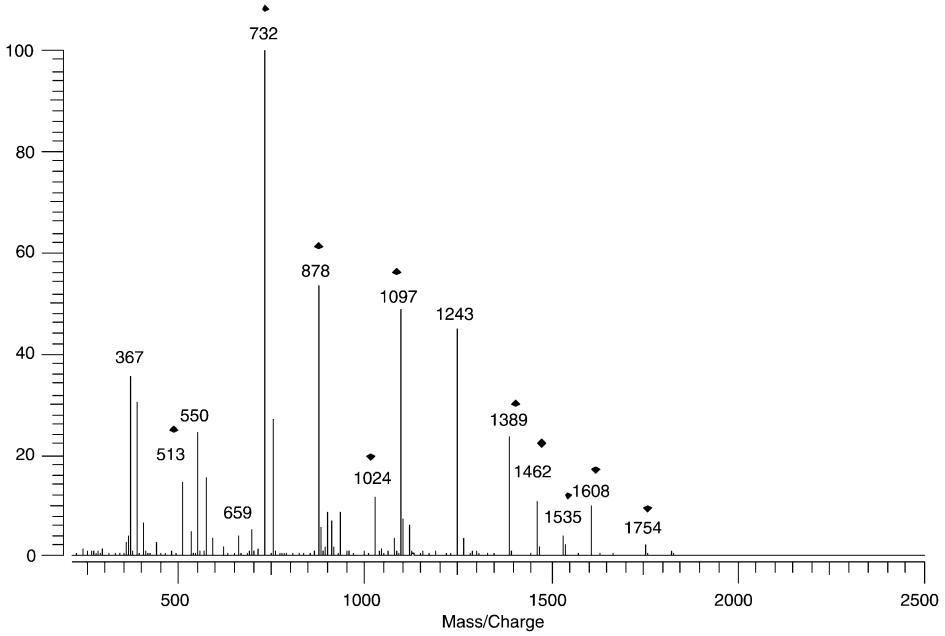

Figure 3 Matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance MS spectrum of reduced human milk oligosaccharides (HMO) using 2,5-dihydroxybenzoic acid matrix in the positive ionization mode. Signals originating from HMO are marked with a diamond. Reprinted from Reference (52) with permission.

detection have been used, which allow monitoring of a maximum of 40 structures. Using nano-LC PGC chip TOF MS, it is now feasible to monitor >100 structures, and combine data from milk, feces, and urine.

Although current separations using PGC or CE provide good separation and allow identification of large numbers of milk oligosaccharides, these techniques require relatively long analysis times (10–60 min). Current separations occur on solid interphases, but ions can also be separated on a gas interphase by ion mobility, under the influence of a weak electric field (66). Recently, ion mobility coupled to MS was applied for the separation of *N*-glycans from patients with liver cancer and liver cirrhosis (67,68). Clear differences could be observed in the drift patterns of several *N*-glycans. So far, this technique has not been applied in milk oligosaccharide analysis; however, its use may well

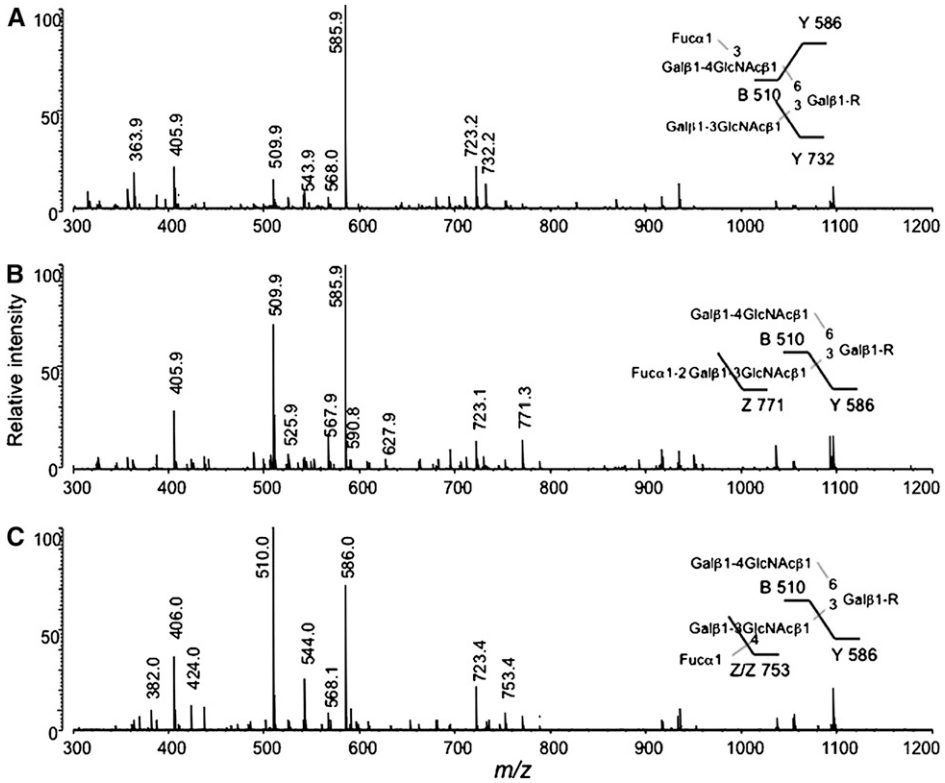

Figure 4 Differentiation of 3 fucosylated human milk oligosaccharides (HMO) using collision-induced dissociation fragmentation of deprotonated species in the negative ionization mode. MS^3 fragmentation spectra of *m/z* 1079 for the 3 fucosylated HMO (*A–C*) are depicted together with fragmentation patterns explaining the most important ions. Reprinted from Reference (56) with permission.

result in linkage-specific determinations with very short analysis times (<1 min).

Recently, the use of selective reaction monitoring (SRM) on triple quadrupole MS was developed as a selective method for quantitative proteomics. This method has advantages in both selectivity and sensitivity over traditional quantitative proteomics (69). Currently, applications of SRM for glycomics and glycoproteomics are being developed, and recent studies on quantitation of fucosylated glycopeptides (70) as well as bovine milk oligosaccharides (71) revealed good reproducibility data in terms of quantitation. Application of SRM strategies in bacterial consumption studies as well as comparisons of milk and fecal oligosaccharides will probably result in superior quantitative results.

Overall, application of the novel analytical approaches will greatly enhance our knowledge on the specificities of bacterial consumption of milk oligosaccharides and allow better understanding of the interaction between gut microflora (and thus the development of an infant's immune system) and HMO. Such knowledge should facilitate the development and clinical application of better infant formula as well as personalized formula.

Acknowledgments

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