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New strategies for profiling and characterization of human milk oligosaccharides

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Abstract

Human breast milk is an incredibly rich and complex biofluid composed of proteins, lipids and complex carbohydrates, including a diverse repertoire of free human milk oligosaccharides (HMOs). Strikingly, HMOs are not digested by the infant but function as prebiotics for bacterial strains associated with numerous benefits. Considering the broad variety of beneficial effects of HMOs, and the vast number of factors that affect breast milk composition, the analysis of HMO diversity and complexity is of utmost relevance. Using human milk samples from a cohort of Bangladeshi mothers participating in a study on malnutrition and stunting in children, we have characterized breast milk oligosaccharide composition by means of permethylation followed by liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-MS/MS) analysis. This approach identified over 100 different glycoforms and showed a wide diversity of milk composition, with a predominance of fucosylated and sialylated HMOs over nonmodified HMOs. We observed that these samples contain on average 80 HMOs, with the highest permethylated masses detected being >5000 mass units. Here we report an easily implemented method developed for the separation, characterization and relative quantitation of large arrays of HMOs, including higher molecular weight sialylated HMOs. Our ultimate goal is to create a simple, high-throughput method, which can be used for full characterization of sialylated and/or fucosylated HMOs. These results demonstrate how current analytical techniques can be applied to characterize human milk composition, providing new tools to help the scientific community shed new light on the impact of HMOs during infant development.

Key words: CID, human milk oligosaccharides (HMOs), LC-NSI-MS/MS, MALDI-TOF-MS, structural analysis

Introduction

Breast milk is a vital biological fluid, providing 100% of mammalian infant nutrition at the beginning of life. While lipids, proteins and lactose act as major caloric nutrients (Tao et al. 2011; Oursel et al. 2017), breast milk of many species also contains considerable amounts (1–2%, w/v) of indigestible milk oligosaccharides (Smilowitz et al. 2014) that reach the neonate’s large intestine. Human milk oligosaccharides (HMOs) are a diverse group of free soluble oligosaccharides displaying a wide array of biological functions, acting as prebiotics...
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(Chaturvedi et al. 1997, 2001; Sumiyoshi et al. 2003; Morrow et al. 2001; Charbonneau et al. 2016). Not only can HMOs be structurally complex but multiple positional and linkage isomers can also be found for the same monosaccharide composition (and thus mass), which further complicates their analysis. So far, over 200 individual HMO molecular species and more than 100 structures of HMOs are complex glycans formed by glucose (Glc), galactose (Gal), N-acetylgalactosamine (GalNAc), fucose (Fuc) and N-acetylenuraminic acid (Neu5Ac). With very few exceptions (Kunz et al. 2000), all HMOs are formed by a reducing lactose core that can be extended enzymatically by lacto-N-acetyllactosamine (Galα1,3-GlcNAc, type 1 LacNAc) or N-acetyllactosamine (Galβ1,4-GlcNAc, type 2 LacNAc) motifs. These structures can be further decorated by the addition of Fuc residues in α1,2-, α1,3- and α1,4-linkages and/or Neu5Ac residues in α2,3- and α2,6-linkages, providing an array of Lewis structures and blood group antigens (Figure 1). The relative abundance of each group of HMOs has been described (Totten et al. 2001; De Leoz et al. 2013; Wu et al. 2017), providing high-throughput retention time, mass and fragmentation information (Wu et al. 2010; Wu et al. 2011), allows for separation of >200 structures in a porous graphitized carbon (PGC) column and has been widely used in primates (Tao et al. 2011) and human samples (Totten et al. 2012; De Leoz et al. 2013; Wu et al. 2017), providing high-throughput and reproducible analysis (Totten et al. 2014). However, this method is fully dependent on in-house software and instrument stability which prevents its widespread use. Furthermore, the MS/MS analysis of native samples lacks structural information which is retained by permethylation. Permethylation is a widely used derivatization method, particularly in carbohydrate chemistry, which consists in the replacement of all hydrogens attached to oxygen and nitrogen atoms with methyl groups, providing higher sensitivity, better ionization and protection of labile groups (e.g. Neu5Ac), among other advantages (Costello et al. 2007). Currently, aside from electronic excitation dissociation (EED) and fixed-charge derivatization (Tang et al. 2018), only permethylation can enable MS² to provide some sequence, branching and linkage information for glycans (Ashline et al. 2017). Emerging work from a NIST group (Remorozza et al. 2018), who developed a searchable, reference MS library of annotated oligosaccharides, was proposed as a method to identify unknown reduced (or nonreduced) HMOs, as well as, potentially, permethylated HMOs based on spectral matching. Impressively, this library currently contains 469 positive and negative ion spectra resulting from by HILIC-ESI-MS/MS analysis and will be an exciting option for future work. However, the database is still built upon existing standards, and high molecular weight (11–12 sugar units) oligosaccharides had been only partially identified at the time of publication.

While some reports have previously described LC-MS separation of permethylated HMOs (Dong et al. 2016; Oursel et al. 2017), these methods focus on the most abundant, low molecular weight, species and only describe a fairly limited number of HMOs. Here we present a much improved nanoliquid chromatography nanospray tandem mass spectrometry (nLC-NSI-MS/MS) method for HMOs analysis, using reversed-phase chromatography of permethylated samples and collision-induced dissociation (CID) MS/MS fragmentation. This approach also includes oligosaccharide profiling by matrix-assisted
Fig. 1. Structural diversity of human milk oligosaccharides. With very few exceptions, all HMOs are formed by a lactose core (Gal-β1→4-Glc, center), which can be extended enzymatically in repeats of lacto-N-biose (Gal-β1→3-GlcNAc) or N-acetyllactosamine (Gal-β1→4-GlcNAc) (upper right). Lactose can also be fucosylated or sialylated in different linkages (upper left). Linkage and position of fucose residues generate isomers, which can result in structures that are phenotypically related to secretor status and Lewis blood group (lower left). Isomerization can also occur as a result of sialylation of elongated core structures (lower center). HMOs can also be simultaneously fucosylated and sialylated, resulting in highly complex structures (lower right). The monosaccharide key is shown at the bottom. Abbreviations: 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LNFP I, II, III, V, lacto-N-fucopentaose I, II, III, V, LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNnH, lacto-N-hexaose; LNnH, lacto-N-neohexaose; pLNH, para-lacto-N-hexaose; LST a, b, c, sialyl-lacto-N-tetraoses a-c; S-LNF II or F-LST a, sialylfucosyllacto-N-tetraose; DS-LNF II or FDS-LNT I, fucosyldisialyllacto-N-tetraose; TetraF-LND III, tetrafucosyl-lacto-N-decaose III. Monosaccharide symbols follow the Symbol Nomenclature for Graphical Representation of Glycans (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). This figure is available in black and white in print and in colour at Glycobiology online.

laser-induced time-of-flight mass spectrometry (MALDI-TOF-MS). We show how this method can be applied to milk glycomic profiling and how fragmentation of permethylated HMOs can provide structural information and isomer distinction.

Results and Discussion

HMO profiling by MALDI-TOF-MS analysis

Permethylated samples were profiled initially by MALDI-TOF-MS to characterize major HMOs present in human milk in terms of mass and monosaccharide composition (Figure 2). MALDI-TOF-MS analysis revealed the expected complexity of breast milk. Using this approach, up to 32 of the highest abundance permethylated masses were identified in human milk. While the number of HMO structures described in the literature is much higher than this (Chen 2015), since structural isomers do not differ in terms of mass, the total number of glycoforms present in this spectrum is in reality much higher. This is displayed in Figure 2 by the different possible structures for the same mass (shown in brackets). In addition, many of the high molecular weight species are present in human milk at very low abundances (<1%, Tao et al. 2011), which makes their detection by MALDI-TOF-MS more difficult. In agreement with previous reports (Totten et al. 2014; Chen 2015; Charbonneau et al. 2016), the samples contained a wide variety of fucosylated and/or sialylated HMOs, with increasing degrees of complexity. It should be noted here that while free lactose is the main oligosaccharide of these samples (see ahead), it is not considered a true HMO because, unlike HMOs, it is readily digested by the infant gut, thus being a primary source of nutrition (Newburg 2013). Different approaches have been used to remove free lactose from human milk, from size-exclusion chromatography using Sephadex G-25 (Marino et al. 2011) and small scale PGC cartridges (Xu et al. 2017), to large scale simulated moving bed chromatography (Mank et al. 2019). While lactose removal can help generate cleaner and more purified HMO samples, it also increases the chances of...
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Fig. 2. Representative MALDI-TOF-MS spectrum of permethylated human milk oligosaccharides. All masses correspond to fully permethylated, free reducing end, sodium adducts of HMOs. Possible structures for each mass are shown. Free lactose was excluded from the spectrum. HMOs were identified using Glycomod (web.expasy.org/glycomod) as a search engine. This figure is available in black and white in print and in colour at Glycobiology online.

HMO loss and bias, which is especially relevant for most of the low abundance species. This is detrimental to breast milk studies because although the 10 most abundant HMOs make up ~75% of the total HMO mass (Grabarics et al. 2017), the greatest structural (and likely functional) diversity of HMOs resides in the 25% of high molecular weight glycoforms. For this reason, we chose not to remove lactose and take advantage of a selected mass window in the case of MALDI-TOF-MS and chromatographic separation in the case of LC-MS (see ahead).

nLC-NSI-MS/MS analysis of permethylated milk oligosaccharides

Considering the limitations of MALDI-TOF-MS, and to allow for HMO isolation from lactose, the samples were further separated and analyzed by nLC-NSI-MS/MS (Figure 3). Given the overwhelming abundance of free lactose in relation to HMOs, the first 5 min of the separation result almost exclusively in the ionization of lactose (m/z 477.23) and lactose dimers that are formed during ionization (m/z 931.47) as a consequence of high concentration (Figure 3A). After 20 min, and even though lactose is still the main ion present, HMOs start to elute (Figure 3A and B), up until 35 min. The benefits of LC separation are illustrated in Figure 3B, where we can see the extracted ion chromatograms (EICs) of some of the most abundant HMOs, and although they are not fully resolved, it is clear that reasonable separation can be achieved. When using electro/nanospray ionization, several multiply charged ions can be observed for the same mass, making data analysis much more complex. An example of this is the high number of doubly and triply charged ions visible in Figure 3A. However, LC-NSI-MS methods are also associated with higher sensitivity, allowing for the detection of a considerably higher number of glycoforms than MALDI-TOF-MS. In fact, using this method, a total of 102 HMOs permethylated masses were detected (Table I), including new HMOs that have not been described in the literature. Specifically, we were able to detect HMOs of higher molecular weights than previously reported using commercially available instrumentation and columns that are common to most academic laboratories, a considerable improvement over previous methods (Dong et al. 2016). It should be noted here that this list corresponds to oligosaccharide masses, and as mentioned before, many HMOs have structural isomers, implying that the number of structures found in these samples is much higher than this. Table I shows the full list of HMOs detected in the human samples analyzed in this study. The list of HMOs detected in each sample can be found in the Supplementary Table SI. Full separation of HMO peaks is not possible under these conditions, which is to be expected given sample complexity. It is true that the separation of native HMOs on PGC columns is better than that of permethylated HMOs in C18 columns (Oursel et al. 2017), and while PGC columns have even been used for the separation of permethylated N-glycans (Costello et al. 2007; Zhou et al. 2017), they are better suited to native oligosaccharide separations, and are not as widely available as reversed phase columns. However, while separation is definitely important, especially in terms of isomer distinction, permethylation is crucial in increasing sensitivity by
facilitating ionization, as well as in providing relevant structural information.

Importance of permethylation for HMO structure assignment

Among many other advantages (Shajahan et al. 2017), permethylation provides diagnostic fragment ions (Figure 4), increasing the yield of useful structural information obtained from MS/MS experiments (Oursel et al. 2017). Permethylation aids in the distinction of internal fragments from terminal fragments (including reducing end vs. nonreducing end) and branched structures from linear structures, as well as in the determination of fucose position and linkage isomers. As interest in HMOs progresses from simple glycoforms, for which standards are available, to the more complex but biologically interesting forms, methodologies that do not automatically rely on standards will be needed. A particularly important advantage of permethylation is the fact that it precludes the occurrence of “internal residue loss” (Kovácik et al. 1995; Harvey et al. 2002), a phenomenon characterized by rearrangement reactions involving migration of fucose, and sometimes other monosaccharide residues (Kovácik et al. 1995), typically found in CID spectra of native glycans or even glycans derivatized at their reducing end (e.g. 2-AB, 3-AQ). This migration is known to happen in N- and O-glycans, as well as HMOs (Harvey et al. 2002; Zhou et al. 2017), and is affected by the type of adduct ions (Ernst et al. 1997; Brüll et al. 1998), aglycon (Ma et al. 2000), and derivatization (Brüll et al. 1997; Franz and Lebrilla 2002; Wührer et al. 2006; Nwosu et al. 2015).

Experiments by Wührer et al. (2006) found that fragmentation of fucosylated N-glycans resulted in additional fragmentation ions that had acquired fucose residues from other parts of the oligosaccharide. This transfer was found on native reducing or 2-AB labeled N-glycans with protonated charge carriers. It was also observed by both ion-trap-based CID as well as laser-induced dissociation via MALDI-TOF/TOF, even though both fragmentation methods occur under drastically different ionic lifetimes, suggesting that fucose transfer is an active part of the decomposition. This included the observed transfer of an effective fucose onto an existing Lewis X antenna which would improperly suggest a Lewis Y epitope. A similar outcome had previously been reported by Ernst et al. (1997), who described the formation of “false” sugar sequence ions from branched sialyl-Lewis-type tetrasaccharides as a result of fucose migration towards sialic acid residues in both [M + H]+ and [M + NH4]+ adduct ions.

More importantly, while these rearrangements have long been associated with activation during CID fragmentation in tandem MS experiments, recent work by Mucha et al. (2018) using cold-ion spectroscopy has shown that fucose migration is not limited to fragments obtained in the gas phase during tandem MS, but it can also occur in intact glycan ions. The exact mechanism by which this process occurs is still not fully understood, although it is known that the reaction happens in the presence of a proton and is inhibited in its absence (thus, it is typically not observed with sodium adducts (Brüll et al. 1998; Kovacik et al. 1999; Franz and Lebrilla 2002)), and recent data have demonstrated that the mobility of the proton, not CID fragmentation, is a necessary prerequisite for migration reactions (Lettow et al. 2019).
Table I. Full list of HMOs (not including isomers) detected in human milk samples by nLC-NSI-MS/MS analysis

| Permethylated theoretical mass [M + Na]^+ | Native reduced mass [M] | Most abundant charge state | Monosaccharide composition |
|------------------------------------------|------------------------|----------------------------|----------------------------|
| 651.3200                                 | 490.1897               | 1                         | Hex2 Fuc1                  |
| 722.3572                                 | 547.2112               | 1                         | Hex3 HexNAc1               |
| 825.4092                                 | 636.2476               | 1                         | Hex2 Fuc1                  |
| 838.4045                                 | 635.2272               | 1                         | Hex2 NeuAc1                |
| 896.4464                                 | 693.2691               | 1                         | Hex2 HexNAc1 Fuc1          |
| 926.4570                                 | 709.2640               | 1                         | Hex3 HexNAc1               |
| 1100.5462                                | 855.3219               | 1                         | Hex3 HexNAc1 Fuc1          |
| 1130.5568                                | 871.3168               | 1                         | Hex3 HexNAc1               |
| 1274.6354                                | 1001.3798              | 1                         | Hex3 HexNAc1 Fuc2          |
| 1287.6307                                | 1000.3594              | 1                         | Hex3 HexNAc1 NeuAc1        |
| 1304.6460                                | 1017.3747              | 1                         | Hex4 HexNAc1 Fuc1          |
| 1345.6726                                | 1038.4013              | 1                         | Hex4 HexNAc2 Fuc1          |
| 1375.6832                                | 1074.3962              | 1                         | Hex4 HexNAc1               |
| 1461.7199                                | 1146.4173              | 1                         | Hex4 HexNAc1 Fuc1 NeuAc1   |
| 1478.7352                                | 1163.4326              | 1                         | Hex4 HexNAc1 Fuc2          |
| 1491.7305                                | 1162.4122              | 1                         | Hex5 HexNAc1 NeuAc1        |
| 1519.7618                                | 1204.4592              | 1                         | Hex5 HexNAc2 Fuc2          |
| 1549.7724                                | 1220.4541              | 2                         | Hex5 HexNAc2 Fuc1          |
| 1579.7830                                | 1236.4490              | 2                         | Hex5 HexNAc2 Fuc3          |
| 1648.8045                                | 1291.4548              | 2                         | Hex5 HexNAc2 Fuc1 NeuAc1   |
| 1706.8464                                | 1349.4967              | 2                         | Hex5 HexNAc2 Fuc1 NeuAc1   |
| 1723.8617                                | 1366.5120              | 2                         | Hex5 HexNAc2 Fuc2          |
| 1736.8569                                | 1365.4916              | 2                         | Hex5 HexNAc2 NeuAc1        |
| 1753.8722                                | 1382.5069              | 2                         | Hex5 HexNAc2 Fuc1          |
| 1822.8937                                | 1437.5127              | 2                         | Hex5 HexNAc2 Fuc1 NeuAc2   |
| 1824.9094                                | 1439.5284              | 2                         | Hex5 HexNAc3               |
| 1897.9509                                | 1512.5699              | 2                         | Hex5 HexNAc2 Fuc3          |
| 1910.9462                                | 1511.5495              | 2                         | Hex5 HexNAc2 Fuc1 NeuAc1   |
| 1927.9615                                | 1528.5648              | 2                         | Hex5 HexNAc2 Fuc2          |
| 1940.9567                                | 1527.5444              | 2                         | Hex5 HexNAc2 Fuc3          |
| 1998.9986                                | 1585.5863              | 2                         | Hex5 HexNAc3 Fuc1          |
| 2085.0354                                | 1657.6074              | 2                         | Hex5 HexNAc2 Fuc2 NeuAc1   |
| 2098.0307                                | 1656.5870              | 2                         | Hex5 HexNAc2 NeuAc2        |
| 2102.0507                                | 1674.6227              | 2                         | Hex5 HexNAc2 Fuc3          |
| 2115.0460                                | 1673.6023              | 2                         | Hex5 HexNAc2 Fuc1 NeuAc1   |
| 2173.0879                                | 1731.6442              | 2                         | Hex5 HexNAc3 Fuc2          |
| 2203.0984                                | 1747.6391              | 2                         | Hex5 HexNAc3 Fuc3          |
| 2272.1199                                | 1802.6449              | 2                         | Hex5 HexNAc2 Fuc1 NeuAc2   |
| 2347.1771                                | 1877.7021              | 2                         | Hex5 HexNAc3 Fuc3          |
| 2360.1724                                | 1876.6817              | 2                         | Hex5 HexNAc3 Fuc1 NeuAc1   |
| 2377.1877                                | 1893.6970              | 2                         | Hex5 HexNAc3 Fuc2          |
| 2448.2249                                | 1950.7185              | 2                         | Hex5 HexNAc4 Fuc1          |
| 2459.2044                                | 1947.6824              | 2                         | Hex5 HexNAc2 NeuAc3        |
| 2521.2664                                | 2023.7600              | 2                         | Hex5 HexNAc3 Fuc4          |
| 2534.2616                                | 2022.7396              | 2                         | Hex5 HexNAc3 Fuc2 NeuAc1   |
| 2547.2569                                | 2021.7192              | 2                         | Hex5 HexNAc3 NeuAc2        |
| 2622.3141                                | 2096.7764              | 2                         | Hex5 HexNAc4 Fuc2          |
| 2635.3094                                | 2095.7560              | 2                         | Hex5 HexNAc4 Fuc3          |
| 2695.3556                                | 2169.8179              | 2                         | Hex5 HexNAc3 Fuc5          |
| 2708.3509                                | 2168.7975              | 2                         | Hex5 HexNAc3 Fuc1 NeuAc1   |
| 2721.3461                                | 2167.7771              | 2                         | Hex5 HexNAc3 Fuc1 NeuAc2   |
| 2796.4033                                | 2242.8343              | 2                         | Hex5 HexNAc4 Fuc3          |
| 2809.3986                                | 2241.8139              | 2                         | Hex5 HexNAc4 Fuc1 NeuAc1   |
| 2895.4354                                | 2313.8350              | 2                         | Hex5 HexNAc3 Fuc3 NeuAc2   |
| 2970.4926                                | 2388.8922              | 2                         | Hex5 HexNAc4 Fuc4          |
| 2983.4878                                | 2387.8718              | 2                         | Hex5 HexNAc4 Fuc5 NeuAc1   |
| 2996.4831                                | 2386.8514              | 2                         | Hex6 HexNAc4 NeuAc2        |

Continued
| Permethylated theoretical mass [M + Na]^+ | Native reduced mass [M] | Most abundant charge state | Monosaccharide composition |
|------------------------------------------|-------------------------|-----------------------------|-----------------------------|
| 3069.5246                                | 2459.8929               | 2                           | Hex5 HexNAc3 Fuc3 NeuAc2    |
| 3071.5403                                | 2461.9086               | 2                           | Hex7 HexNAc3 Fuc2           |
| 3082.5199                                | 2458.8725               | 3                           | Hex3 HexNAc3 Fuc3 NeuAc3    |
| 3099.5352                                | 2475.8878               | 3                           | Hex4 HexNAc3 Fuc2 NeuAc1    |
| 3144.5818                                | 2534.9501               | 3                           | Hex5 HexNAc4 Fuc5           |
| 3157.5771                                | 2533.9297               | 3                           | Hex6 HexNAc4 Fuc3 NeuAc3    |
| 3170.5724                                | 2532.9093               | 3                           | Hex6 HexNAc4 Fuc3 NeuAc2    |
| 3245.6296                                | 2607.9665               | 3                           | Hex7 HexNAc5 Fuc3           |
| 3269.6044                                | 2603.9100               | 3                           | Hex5 HexNAc3 NeuAc4         |
| 3273.6244                                | 2621.9457               | 3                           | Hex6 HexNAc3 Fuc3 NeuAc2    |
| 3275.6401                                | 2623.9614               | 3                           | Hex8 HexNAc5 Fuc2           |
| 3311.6663                                | 2679.9876               | 3                           | Hex6 HexNAc4 Fuc4 NeuAc3    |
| 3344.6616                                | 2678.9672               | 3                           | Hex6 HexNAc4 Fuc3 NeuAc2    |
| 3419.7188                                | 2754.0244               | 3                           | Hex7 HexNAc5 Fuc4           |
| 3432.7141                                | 2753.0040               | 3                           | Hex7 HexNAc5 Fuc3 NeuAc3    |
| 3443.6936                                | 2749.9679               | 3                           | Hex5 HexNAc3 Fuc3 NeuAc4    |
| 3449.7294                                | 2770.0193               | 3                           | Hex5 HexNAc3 Fuc3           |
| 3518.7508                                | 2825.0251               | 3                           | Hex6 HexNAc4 Fuc3 NeuAc2    |
| 3531.7461                                | 2824.0047               | 3                           | Hex7 HexNAc4 Fuc3 NeuAc3    |
| 3593.8080                                | 2900.0823               | 3                           | Hex7 HexNAc5 Fuc5           |
| 3595.7873                                | 2874.0302               | 3                           | Hex9 HexNAc4 Fuc2 NeuAc1    |
| 3606.8033                                | 2899.0619               | 3                           | Hex7 HexNAc5 Fuc3 NeuAc3    |
| 3617.7829                                | 2896.0258               | 3                           | Hex7 HexNAc5 Fuc3 NeuAc4    |
| 3619.7986                                | 2898.0415               | 3                           | Hex7 HexNAc5 Fuc3 NeuAc2    |
| 3692.8401                                | 2971.0830               | 3                           | Hex5 HexNAc4 Fuc3 NeuAc5    |
| 3694.8558                                | 2973.0987               | 3                           | Hex5 HexNAc4 Fuc3           |
| 3705.8354                                | 2970.0626               | 3                           | Hex6 HexNAc4 Fuc3 NeuAc3    |
| 3718.8306                                | 2969.0422               | 3                           | Hex8 HexNAc4 NeuAc4         |
| 3793.8878                                | 3044.0994               | 3                           | Hex7 HexNAc5 Fuc3 NeuAc5    |
| 3868.9450                                | 3119.1566               | 3                           | Hex8 HexNAc6 Fuc4           |
| 3881.9403                                | 3118.1362               | 3                           | Hex8 HexNAc6 Fuc3 NeuAc4    |
| 3892.9199                                | 3115.1001               | 3                           | Hex8 HexNAc6 Fuc4 NeuAc3    |
| 3967.9771                                | 3190.1573               | 3                           | Hex7 HexNAc5 Fuc3 NeuAc2    |
| 4043.0343                                | 3265.2145               | 3                           | Hex5 HexNAc6 Fuc3           |
| 4056.0295                                | 3264.1941               | 3                           | Hex6 HexNAc6 Fuc3 NeuAc1    |
| 4142.0663                                | 3336.2152               | 3                           | Hex7 HexNAc5 Fuc4 NeuAc2    |
| 4217.1235                                | 3411.2724               | 3                           | Hex8 HexNAc6 Fuc6           |
| 4230.1188                                | 3410.2520               | 3                           | Hex8 HexNAc6 Fuc3 NeuAc1    |
| 4243.1140                                | 3409.2316               | 3                           | Hex8 HexNAc6 Fuc3 NeuAc5    |
| 4417.2033                                | 3555.2895               | 3                           | Hex9 HexNAc7 Fuc2 NeuAc2    |
| 4692.3403                                | 3774.3638               | 4                           | Hex10 HexNAc3 Fuc4          |
| 4767.3975                                | 3849.4210               | 4                           | Hex11 HexNAc5 Fuc2          |
| 4868.4452                                | 3922.4374               | 4                           | Hex11 HexNAc9 Fuc2          |
| 5042.5344                                | 4068.4953               | 4                           | Hex11 HexNAc6 Fuc3          |
| 5317.6714                                | 4287.5696               | 4                           | Hex12 HexNAc10 Fuc2         |

Permethylated masses correspond to sodium adducts. Native reduced masses are given for easier comparison with other references. Bold rows indicate HMOs that have not been previously reported in the literature.

This type of transfer-related misassignment would be a particularly relevant problem for HMOs, where Lewis structures may be related to a number of biological functions including the determination of secretor status. Remarkably, permethylation was found to prevent this type of fucose migration reaction during MS/MS experiments (Aldredge et al. 2013; Zhou et al. 2017; Mank et al. 2019). Considering that many HMO isomers are related with fucose position, fucose migration during MS/MS fragmentation can severely hamper structure determination. Thus, MS/MS analysis of permethylated HMOs can assist in structure assignment without further enzymatic digestion of the glycan (Figure 5).

Structure assignment and isomer differentiation based on MS/MS fragmentation

Structural assignment of low molecular weight HMOs is fairly simple, considering the small number of fragment ions resulting from these glycans. For instance, in the case of m/z 651.32, we can detect...
two fucose position isomers of fucosyllactose, which can be distinguished by the fragments at \( m/z \) 415.19 corresponding to terminal fucosylation, and \( m/z \) 433.20 corresponding to core fucosylation (Figure 5A). In the case of sialylated HMOs, such as sialyllactose (\( m/z \) 838.40), it is more difficult to detect the presence of isomers because the glycosidic bonds linking sialic acid residues are especially labile, meaning that upon fragmentation, the neutral loss of Neu5Ac is the main fragment ion, as observed in Figure 5B. Hence, and although the EIC of this mass could suggest the presence of isomers (Figure 3), the distinction between 2,3- and 2,6-linked sialic acids (which would be a source of isomerization) requires further MS\(^n\) fragmentation (Anthony et al. 2008). In many cases, HMOs can show isomerization of both position and linkage of fucose. This is illustrated in Figure 5C, where two fucose positional isomers can be observed for \( m/z \) 1100.55 (inset). These isomers can be distinguished by the fragments at \( m/z \) 433.20 and 637.30, which indicate core fucosylation, as well as fragments at \( m/z \) 660.32 and 864.42, which indicate the presence of a Lewis epitope. Aside from these positional isomers, Figure 5C also shows the presence of linkage isomers of fucose, as demonstrated by fragments at \( m/z \) 894.43 and 912.44, which correspond to the loss of a 4-linked fucose and a 3-linked fucose residue, respectively (Terada et al. 2005). With the increase in molecular weight, there is a proportional increase in glycoform structure complexity, which is distinctly demonstrated in Figure 5D. The MS/MS spectrum of \( m/z \) 1549.77 reveals both position and linkage isomers of fucose, as well as linear and branched structures, as suggested by the presence of a fragment at \( m/z \) 449.20. Thus, we can say that there are four possible core structures (inset) for this mass, and that each of these can potentially contain further fucose linkage isomers (\( m/z \) 1343.66). These results reflect the importance of proper chromatographic separation for isomer distinction. As discussed previously, using a common reversed-phase C18 column, co-elution of isomers is unavoidable (Dong et al. 2016; Mank et al. 2019), although some separation can be accomplished, as shown in Figure 6. Physical separation of glycoforms in the column facilitates analysis of MS\(^n\)MS results, as peaks eluting at different times will have different MS\(^2\) spectra. Here we show the example of the HMO Hex\(_4\)HexNAc\(_2\)Fuc\(_2\)NeuAc\(_1\), with mass 2085.04. Three isomers were found for this mass, two of them co-eluting at 28.5 min and the third eluting separately at 35.7 min. These isomers are distinguishable by their characteristic MS\(^2\) fragments (highlighted).
Fig. 5. Structure assignment based on MS/MS analysis. CID MS² spectra of (A) m/z 651.32, (B) m/z 838.40, (C) m/z 1100.55 and (D) m/z 1549.77. Permethylated fragments aid in structure determination and evaluation of number of isomers for a given mass. Fragments marked with asterisk represent fucose linkage isomers. This figure is available in black and white in print and in colour at Glycobiology online.

Although some studies have described isomer-specific CID fragments for the distinction of low molecular weight HMOs by MS² analysis (Mank et al. 2019), MS² fragmentation alone cannot provide enough unambiguous information to completely determine entire glycan structures, especially for HMOs of high molecular weight. Thus, we provide here examples of structural information that can be obtained from MS² experiments, but full determination would require further MSⁿ experiments. For example, milk group determination would require up to MS⁴ experiments to distinguish fragments coming from different Lewis epitopes. However, in studies with high numbers of samples, full structure characterization is impractical, and often the goal of these studies is to compare milk profiles from
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Fig. 6. Isomer distinction based on chromatographic separation and MS/MS fragmentation. CID MS² spectra of EIC peaks of [M + Na]⁺ 2085.04, eluting at 28.45 and 35.65 min. Characteristic fragments that allow for isomer(s) identification are highlighted. This figure is available in black and white in print and in colour at Glycobiology online.

different individuals, as samples from different donors can have very different HMO patterns (Ruhaak and Lebrilla 2012). In such cases, the approach presented here can be used as a profiling tool, providing information on the diversity and relative abundance of HMOs between subjects.

Conclusions
HMO characterization is an analytical challenge as a result of the heterogeneity of molecular species that are typically present in a sample. We present here a method for milk oligosaccharide analysis, based on high-resolution chromatography and mass spectrometry. This method, which does not aim to be quantitative, takes advantage of instrumentation that is increasingly more accessible and allows for sensitive, high-resolution analysis of carbohydrate structures. Using reversed-phase separation of permethylated HMOs and CID MS/MS fragmentation, we identified over 100 HMOs masses, many of them displaying structural isomers. We also detected new HMOs of higher molecular weight than previously reported (permethylated mass 5317.67). We recognize that permethylation is not a perfect method. Permethylation can result in partially methylated products, prohibits the use of enzymes for structural analysis and diminishes the ability to perform extensive isomer separation. However, we believe the benefits of permethylation outweigh the shortcomings, which is why this is an established procedure in carbohydrate chemistry. We now hope to improve this method by introducing an internal standard to allow for full quantification of glycoforms and also plan to improve chromatographic separation of isomeric species. Furthermore, emerging software tools such as SimGlycan (Apte and Meitei 2010) and, more recently, GRITS toolbox (Weatherly et al. 2019), designed for archiving, processing and interpreting analytical data, can assist in structural characterization of HMOs, facilitating the development of new high-throughput methods. This, in addition to sample reduction to prevent peak splitting, would be another valuable improvement of the current method. In summary, we have developed a method for HMO analysis that has the advantage of providing structural data. This method, which can be further optimized into high-throughput, has a potential to be applied in screening of breast milk from mothers from different countries and accessing heterogeneity on HMO structural motifs from different regions and socioeconomical backgrounds. This method can also be used to screen milk oligosaccharides from other mammals and commercial products.

Materials and methods
Chemicals and reagents
Reagent water used throughout this study was obtained from a Barnstead™ Nanopure™ water purification system (Thermo-Fisher, Waltham, MA, USA). Solvents, LC-MS grade, were obtained from Thermo-Fisher Scientific (Waltham, MA, USA) and Sigma Aldrich (St. Louis, MO, USA) and were of the highest available quality.
**Milk samples**

Human milk samples were from the PROVIDE birth cohort study carried out in Dhaka, Bangladesh by the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) (Kirkpatrick et al. 2015). Breast milk samples collected within 6 weeks post-partum from 130 mothers were used in this study. From this group of 130 samples, 6 were randomly selected and used for the current analysis.

**Sample preparation**

Initial processing of samples was carried out at icddr,b. Breast milk samples were centrifuged in 15 mL Falcon tubes at 4,000 rpm for 10 min. After centrifugation, breast milk appears as three layers. The middle layer (defatted) was taken into a 1.5 mL micro centrifuge tube, then preservative (10% sodium azide and 0.1 M PMSF) was added at a concentration of 20 μL/mL, mixed and stored at −70°C. Samples were shipped in dry ice to the University for analysis at the Complex Carbohydrate Research Center. Fifty microliters of defatted milk were diluted in half with water and subjected to ethanol precipitation (two volumes of ethanol, −80°C, 1 h) for protein removal. After 30 min of centrifugation at 14,000 rpm, 4°C, the upper liquid fraction was collected and lyophilized in preparation for permethylation. To the lyophilized HMOs (200 μg), dissolved in 200 μL of anhydrous dimethyl sulfoxide (DMSO), 300 μL of sodium hydroxide (NaOH) base in anhydrous DMSO (prepared separately by mixing NaOH and DMSO) was added and vortexed. A volume of 100 μL of CH3I was added to the sample, and the reaction mixture was vortexed vigorously for 10 min. The permethylation reaction was quenched by the addition of 2 mL of ddH2O, excess CH3I was bubbled off by a stream of nitrogen gas and glycans were purified by liquid-liquid extraction with dichloromethane. After solvent removal by a stream of nitrogen gas, the permethylated HMOs were dissolved in a final volume of 50 μL of methanol and filtered (0.2 μm).

**MALDI-TOF-MS analysis**

A 1 μL aliquot of the permethylated HMOs was mixed with 1 μL of 2,5-dihydroxy benzoic acid (DHB) matrix (20 mg/mL in 1:1 methanol/ddH2O) and spotted on a MALDI plate. The samples were analyzed on a MALDI-TOF-MS instrument (AB SCIEX TOF/TOF 5800, Applied Biosystem MDS Analytical Technologies) in reflector positive ion mode. Structural annotation of glycan MALDI-TOF-MS data was performed manually and the masses were searched against the search engine Glycomod (https://web.expasy.org/glycomod/).

**nLC-NSI-MS/MS analysis**

nLC-NSI-MS/MS analysis was performed using a Dionex UltiMate 3000 LC system coupled with Orbitrap Fusion Trubrid Mass Spectrometer (ThermoFisher) equipped with a nanospray ion source. Samples (2 μL) were injected onto an Acclaim PepMap™ 100 C18 column (75 μm × 15 cm, nanoViper) (ThermoFisher). Separation was performed using 2% acetonitrile, 0.1% formic acid, 1 mM sodium acetate as solvent A and 80% acetonitrile, 0.1% formic acid as solvent B. Oligosaccharides were eluted for 15 min with 20% B. In 1 min, solvent B was increased to 38%, and a 32 min linear gradient of solvent B was used to increase the percentage to 60%. Finally, a clean-up step was added at the end of the run and the conditions were returned to the original settings for 9 min. A constant flow rate of 0.3 μL/min was used. The mass spectrometer was operated in "top-down" data-dependent mode controlled by Thermo Xcalibur (version 3.0.63). This method selected the highest intensity ions within mass range for fragmentation based on the full mass spectra, collected as much data as possible within a 3 s window and excluded additional spectra after 2 within a 120 s window. Using a spray voltage of 1900V, a full FTMS spectrum was collected with the Orbitrap detector at a resolution of 120 000 at m/z from 400 to 2000. The ion transfer tube temperature was set to 275°C and MS analysis was performed in the positive ion mode. Product ion trigger analysis was performed using CID (collision energy (%): 35) and HCD (collision energy (%): 28). Structural annotation of glycan MS/MS data was performed manually and MS/MS fragments were assigned based on predicted theoretical fragments provided by GlycoWorkBench (Ceroni et al. 2008).

**Supplementary data**

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest statement**

None declared.

**Abbreviations**

CE, capillary electrophoresis; CID, collision-induced dissociation; EIC, extracted ion chromatogram; HILIC, hydrophilic interaction chromatography HPLC; HMOs, human milk oligosaccharides; HPAEC-PAD, high pH anion-exchange chromatography with pulsed amperometric detection; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MALDI-FTICR-MS, matrix-assisted laser-induced Fourier-transform ion cyclotron resonance mass spectrometry; MALDI-TOF-MS, matrix-assisted laser-induced time-of-flight mass spectrometry; nLC-NSI-MS/MS, nanoliquid chromatography nanospray tandem mass spectrometry; nano-LC chip-TOF MS, nanoliquid chromatography chip time-of-flight mass spectrometry; PGC, porous graphitized carbon; RP-HPLC, reversed-phase high-performance liquid chromatography; TIC, total ion current

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