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# Mass spectrometric detection of multiple extended series of neutral highly fucosylated *N*-acetyllactosamine oligosaccharides in human milk

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ABSTRACT

Complex mixtures of high-molecular weight fractions of pooled neutral human milk oligosaccharides (obtained via gel permeation chromatography) have been investigated. The subfractions were each permethylated and analyzed by high-resolution mass spectrometry, using matrix-assisted laser desorption/ionization (MALDI)–Fourier transform ion cyclotron resonance (FTICR) mass spectrometry, in order to investigate their oligosaccharide compositions. The obtained spectra reveal that human milk contains more complex neutral oligosaccharides than have been described previously; the data show that these oligosaccharides can be highly fucosylated, and that their poly-*N*-acetyllactosamine cores are substituted with up to 10 fucose residues on an oligosaccharide that has 7-*N*-acetyllactosamine units. This is the first report of the existence in human milk of this large range of highly fucosylated oligosaccharides which possess novel, potentially immunologically active structures.



## 1. Introduction

The oligosaccharide fraction of human milk comprises the quantitatively third most prominent constituent after lactose and lipids. The concentration of oligosaccharides varies between 6 and 12 g/L, depending on the status and course of lactation as well as the secretor and Lewis blood group [1–9].

The biological functions of oligosaccharides are not yet fully understood [10–12] and the oligosaccharides of human milk, in particular, are still the focus of much research interest [6,13–26]. One of the physiological effects of ingested human milk oligosaccharides could be the inhibition of adhesion of pathogenic bacteria, viruses, toxins or protozoa to the surface structures of epithelia of the digestive, respiratory or urinary tract of the breast-fed infant [23,27,28]. Glycoconjugates with distinct epitopes, such as sialyl-Lewis$^X$, may act as ligands for E- and P-selectins that play key roles in inflammation and tissue injury [13,15,21,22,29–32]. The roles of milk oligosaccharides in the intestine and other metabolic aspects of these glycans have been explored by Kunz et al. [33,34]. The human milk oligosaccharides have been reported to promote growth of *bifidobacteria* dominated indigenous microbiota and thereby suppressing growth of undesirable bacteria [35,36]. There is increasing evidence that the indigestible human milk oligosaccharides (HMOS) are involved in immune modulation either via supporting the beneficial complex indigenous microbiota or by directly interfering with immune competent cells of the host, as recently reviewed by Vos et al. [37].

Adhesion processes are, in general, dependent on the strength of binding of complementary structures and therefore on the polyvalence of the reactive structures [38]. For example, it could be shown that the binding affinity of Gal-terminated conjugates for the hepatic asialoglycoprotein receptor increases from 0.5 mM for monovalent to 100 nM for trivalent versions [39]. The polyvalence of human milk oligosaccharides may be particularly relevant in

*Abbreviations:* CMBT, 5-chloro-2-mercaptobenzothiazol; DHB, 2,5-dihydroxybenzoic acid mixture with NaCl (5 mM in water); DP, degree of polymerization; FTICR, Fourier transform ion cylotron resonance; Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetyl-glucosamine; HMOS, human milk oligosaccharides, L$x/y-z$ composition of human milk oligosaccharides (with L = lactose, $x$ = # of *N*-acetyllactosamine subunits, $y$ = # of fucose residues, and $z$ = # of sialic acid residues); LacNAc, *N*-acetyllactosamine; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NeuAc, sialic acid (*N*-acetyl neuraminic acid); NMR, nuclear magnetic resonance spectroscopy; OS, oligosaccharides; TOF, time-of-flight.

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vivo for the high-molecular weight oligosaccharides analyzed in this report. The occurrence of multiple Lewis-type epitopes in the structures could be relevant for all recognition processes that involve these fucosylated structures. Furthermore, the adherence of *Streptococcus pneumoniae* to buccal cells was found to be inhibited by the Gal(β1 → 4)GlcNAc(β1 → 3)Gal sequence [40]. Since this sequence could occur repetitively in the compounds found here, we propose that the high-molecular weight oligosaccharides could provide both more efficient inhibitors for the adhesion of pathogens to epithelia and higher affinity ligands for some carbohydrate-recognition receptors than are presented by the currently investigated low-molecular weight structures.

Human milk contains a highly complex mixture of neutral and acidic oligosaccharides; up to now, more than 100 different structures have been elucidated. Mass spectrometry (MS) has shown great utility for the structure elucidation of large glycans, with progress being accelerated by the development of fast atom bombardment (FAB), laser desorption (LD), matrix-assisted laser desorption/ionization (MALDI) and/or electrospray ionization (ESI); $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectroscopy (NMR) are also important structural methods [1,15,16,21,22,41–43].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) provided early evidence for the presence of extended series of neutral high-molecular weight components in HMOS [44]. These results were confirmed in a later study [45] in which pooled human milk oligosaccharides were fractionated by anion-exchange chromatography and by an improved gel filtration procedure that allowed the separation of large oligosaccharides. The results demonstrated that there are significantly more free oligosaccharides in human milk than had previously been reported. It was shown that the gel filtration methods applied for the fractionation of higher molecular weight neutral (as well as higher molecular weight acidic) oligosaccharides, as exemplified by the human milk oligosaccharides, are a prerequisite for subsequent high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) and MALDI-MS analyses [46].

Acidic oligosaccharides containing up to 20 monomers were detected in the molecular mass range extending to 3700 Da, but their detailed structures, including extensive fucosylation, have not yet been fully elucidated. In addition, neutral structures containing up to 35 monosaccharides were observed, thereby indicating the suitability of the combination of chromatography and MALDI mass spectrometry for determinations of oligosaccharides in this high-molecular-mass range. With MALDI-TOF MS, we have obtained evidence for the existence of HMOS with more than 50 monosaccharide residues [47]. Overall, the monosaccharide composition of the oligosaccharide pool detected here was found to be the same as those previously identified in oligosaccharides of lower mass: the monosaccharide subunits in HMOS are limited to the following five: D-glucose (Glc), D-galactose (Gal), D-*N*-acetyl-glucosamine (GlcNAc), L-fucose (Fuc) and the sialic acid D-*N*-acetyl neuraminic acid (NeuAc). The structures and weights of the native and permethylated monosaccharide building blocks are listed in Table 1. The reducing end is a lactose unit, followed by *N*-acetyllactosamine (LacNAc) subunits (either linear or branched) of the lacto- (Gal(β1 → 3)GlcNAc) or lacto-neo-series (Gal(β1 → 4)GlcNAc). These core structures bear fucose and/or sialic acid residues.

The individual isomeric structures cannot be elucidated based on the mass alone, but the overall monosaccharide composition can be deduced from the observed mass; for convenience in Section 3 the composition of these human milk oligosaccharides is abbreviated by the following nomenclature: L$x$/$y$ – $z$, with L = lactose, $x$ = # of *N*-acetyllactosamine subunits, $y$ = # of fucose residues, and $z$ = # of sialic acid residues.

Even though the monosaccharide subunits are well known, the results of an earlier study in this series [45] showed that compositional analysis of the underivatized high-molecular weight oligosaccharides could not be achieved on the basis of the masses observed for the molecular species. This was due to the following difficulties: (1) five fucose residues have nearly the same mass as two *N*-acetyllactosamine subunits ($\Delta m$ = 0.042 Da or 0.025 u monoisotopic mass), (2) two fucose residues have a mass nearly identical to one sialic acid ($\Delta m$ = 1.029 Da or 1.0204 u monoisotopic mass). Therefore, for a given nominal mass value, several compositions fall within the experimental limits of low to medium resolution mass spectrometers, in general:

neutral oligosaccharides : $\mathrm{L}x/(y+5)-0$ or $\mathrm{L}(x+2)/y-0$ with $x \geq 3$

acidic oligosaccharides : $\mathrm{L}x/(y+2)-z$ or $\mathrm{L}x/y-(z+1)$ with $x \geq 2$

An enormous structural heterogeneity was observed by high-pH anion-exchange chromatography with HPAEC–PAD [45], but, due to the difficulties described above, most of the ion signals in the obtained MALDI-TOF mass spectra could not be assigned to unique compositions; the degree of fucosylation or sialylation was not fully revealed by the analysis of underivatized oligosaccharides with the MALDI-TOF mass spectrometer employed for these studies.

The research reported in this paper focused on the investigation of complex mixtures of neutral human milk oligosaccharides which have been obtained via gel permeation chromatography, as described by Finke et al. [45]. The analyzed subfractions are all mixtures that were found to contain high-molecular weight oligosaccharides that had not permitted discrimination between the possible "high-fucose compositions" and the "low-fucose compositions", e.g., L5/5 – 0 and L7/0 – 0. In order to answer the question whether only one or both compositions (high and low fucose) are present, these subfractions were permethylated and the derivatives were analyzed by MALDI–FTICR MS.

## 2. Materials and methods

### 2.1. Chromatography

The high-molecular weight HMOS were separated out of the total carbohydrate fraction from pooled human milk that was

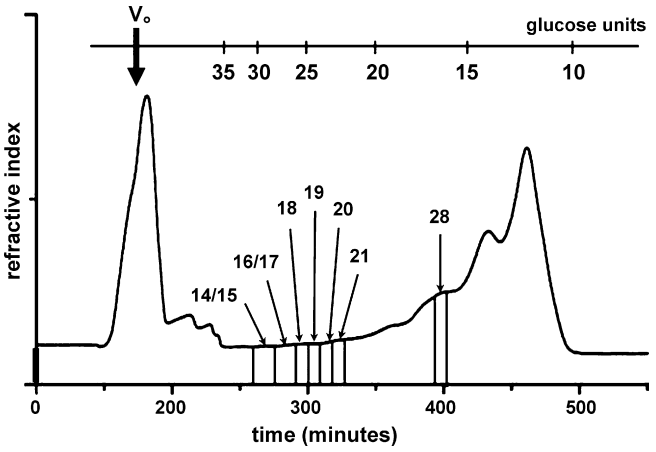
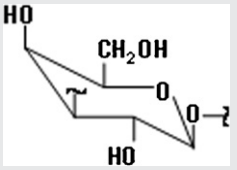
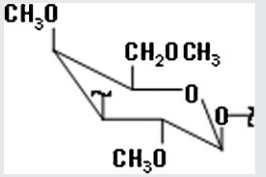
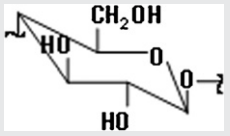
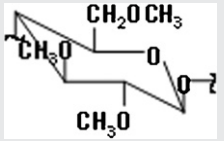
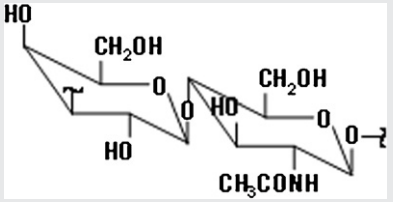
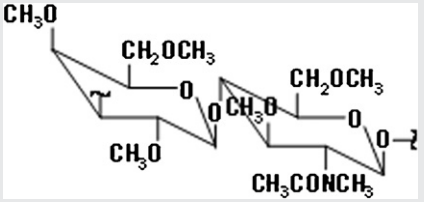
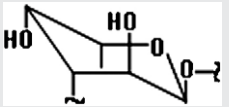
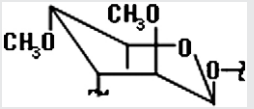
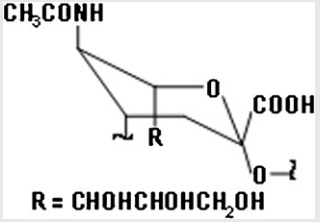
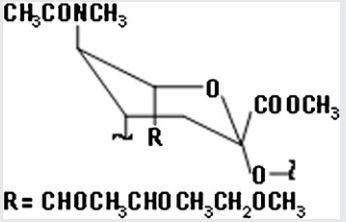



**Fig. 1.** Gel filtration elution profile of the neutral carbohydrate fraction. Elution was monitored by refractive index detection. The labeled subfractions have been analyzed by MALDI–FTICR MS; the major components of the subfractions are summarized in Table 3.

**Table 1**
Monoisotopic and average masses of the building blocks of HMOS in atomic mass units.

| Building block | Underiatized | Permethylated |
|---|---|---|
| β-D-galactose (shown as 3-linked residue) $C_8H_{10}O_6 \rightarrow C_8H_{18}O_6$<br>(mono) 162.05282→204.09977 (avg) 162.1424→204.2230 |  |  |
| β-D-glucose (shown as 4-linked residue) $C_8H_{10}O_6 \rightarrow C_8H_{18}O_6$<br>(mono) 162.05282→204.09977 (avg) 162.1424→204.2230 |  |  |
| *N*-Acetyl-lactose (shown as 3-linked unit) $C_{14}H_{23}NO_{10} \rightarrow C_{20}H_{36}NO_{10}$<br>(mono) 365.13219→449.22609 (avg) 365.3374→449.4986 |  |  |
| α-L-Fucose (shown as 3-linked residue) $C_8H_{13}NO_6 \rightarrow C_{11}H_{18}NO_6$<br>(mono) 146.05791→174.08921 (avg) 146.1430→174.1968 |  |  |
| *N*-Acetylneuraminic acid (shown as 3-linked residue) $C_{11}H_{17}NO_8 \rightarrow C_{18}H_{27}NO_6$<br>(mono) 291.09542→361.17367 (avg) 291.2579→361.3923 |  |  |

obtained under approved conditions. The subfractions of neutral oligosaccharides under investigation (14/15 → 28) were separated via gel permeation chromatography using a procedure that has been described in detail elsewhere [4]. The subfractions (see chromatogram in Fig. 1) were characterized by the determination of the refractive index. The subfractions eluting with higher volumes exhibited decreasing molecular mass. The lower-mass fractions have been analyzed by ESI-MS$^n$ [48,49].

### 2.2. Permethylation

Permethylation was carried out twice in order to minimize by-products and the degree of under-methylation that may occur when following the microscale procedure, as described elsewhere [50–52]. The permethylated subfractions were diluted in water/methanol (1:1, v/v) to a total concentration of 1 μg/μL.

### 2.3. Mass analysis

A HiResMALDI–FTICR (IonSpec Corp., Irvine, CA, USA) with a 7-T actively shielded magnet, equipped with an $N_2$-Laser (337 nm), a rf-quadrupole ion guide, and an open cylindrical cell, served as mass analyzer. The instrument was operated in the broadband mode. The observed resolution (which is inversely proportional to $m/z$) was ca. 8–10,000 at $m/z$ 5000. Data were acquired over the range $m/z$ 2800–6500 and processed using the IonSpec software. Each spectrum represents the sum of 50–100 laser shots. The mass scale was calibrated with a mixture of permethylated malto-oligosaccharides and insulin. For MALDI-MS analysis two different matrices were used: 2,5-dihydroxybenzoic acid [2,5-DHB, 10 g/L in water/methanol (1:1, v/v)] in a 1:1 (v/v) mixture with NaCl (5 mM in water) and 5-chloro-2-mercaptobenzothiazole [CMBT, 5 g/L in THF/water/ethanol (1:1:1, v/v/v)]. For each sample spot, a co-crystallization of an analyte/2,5-DHB matrix mixture (0.5 μL each) was produced and then the CMBT matrix solution (0.5 μL) was added on top of this co-crystallization.

## 3. Results and discussion

During any investigation of the biological activities of human milk oligosaccharides, knowledge of the number of compounds and their specific individual structures is a prerequisite for exploration of the structure–function relationships that are expected to underlie their proposed protective roles. In this paper, we demonstrate that the combination of gel permeation chromatography and MALDI-MS is a powerful tool for the analysis of oligosaccharides from complex mixtures. Utilization of the FT-ICR mass spectrometer and investigation of the oligosaccharides as permethylated derivatives enabled precise determination of the degree of fucosylation. The extent of fucosylation was found to be high, including 7-LacNAc-oligosaccharide species that are substituted with up to 10 fucose residues.

Several different approaches could have been used, alone or in combination, to obtain more information about the composition of neutral, underivatized HMOS for which ambiguity arises because of the nearly identical mass of five fucose and two *N*-acetyllactosamine units (Table 2). These include: (1) elucidation of the fucose number through fragmentation studies, (2) mass analysis with high mass accuracy, (3) mass analysis with high mass resolution and (4) derivatization (e.g., permethylation) of the oligosaccharides. In the study reported here, we employed strategies (2–4).

Elucidation of the fucose number is, in principle, possible with MS$^n$ experiments, e.g., in a nano-ESI-quadrupole ion trap [48,49,53] or ESI-QIT-TOF MS [54], but this approach is not very feasible for the mixtures of compounds with ambiguous composition. This is also true for mass analysis with high mass accuracy but limited resolution [55], because the presence of two compounds with nearly identical mass broadens the ion signal and accurate mass determination of partially resolved peaks becomes extremely difficult. High mass resolution is needed to determine the existence of more than one compound with nearly identical mass within a mixture. This high mass resolution is, in theory, provided by an FT-ICR mass analyzer. However, measurement of high-resolution mass spectra of MALDI-derived, singly sodiated ions of underivatized high-molecular weight HMOS requires a long transient (duration of mass analysis), which could not be achieved with the usual matrices or their combinations, due to the relative instability of the ions from the high-molecular-mass HMOS. For the mass analysis reported here, the selection of the matrix and the matrix preparation were very carefully optimized. These results show that matrix-assisted laser desorption/ionization of intact permethylated high mass HMOS is possible, but that it requires a new matrix-sample preparation protocol (compared to those previously established for MALDI-TOF MS [43,56–59]). The results obtained during the present study indicate that applying a CMBT matrix solution on top of a co-crystallization of an analyte/2,5-DHB mixture facilitates high-resolution FTICR mass analysis of the high mass HMOS, without the occurrence of an unacceptable degree of fragmentation. Although increase in the local pressure during desorption [60–62] or analysis at atmospheric pressure [63] has proven to relieve the vibrational excitation introduced during the ionization/desorption of native oligosaccharides or glycoconjugates, such approaches did not appear to be necessary for this study, since selection of an optimum matrix preparation provided stabilization sufficient for profiling the permethylated oligosaccharides investigated here. Nevertheless, desorption/ionization at increased pressure could be useful when examination of even higher molecular weight fractions is undertaken. However, analysis of these fractions would present a further difficulty, since the singly charged $[M+Na]^+$ ions generated from these larger species would exceed the operational mass range of the instrument.

**Table 2**
Monoisotopic masses and mass differences between five fucose and two *N*-acetyllactosamine residues (underivatized and permethylated) in atomic mass units [u].

| | Underivatized | Permethylated |
|---|---|---|
| 5× fucose | 730.290 | 870.446 |
| 2× *N*-acetyllactosamine | 730.264 | 898.452 |
| $\Delta m$ | 0.025 | 28.006 |

In addition to stabilizing the desorbed ions and providing for making the response of glycans that may contain acidic and/or labile groups similar to that of neutral glycans, permethylation of the human milk oligosaccharides provides an increased mass difference, relative to the native sugars, as illustrated in Table 2. Upon permethylation, the mass difference between the high and low-fucose composition of neutral oligosaccharides rises from 25 mDa to 28 Da, a mass difference which can be distinguished even in lower resolution instruments.

While the permethylation step provided useful mass shifts (Table 2), and increased sensitivity and volatility, it served a further purpose in that it also eliminated concern for the possibility of oligosaccharide rearrangement upon ionization [64] and minimized the possibility of fucose elimination (see below).

Gel permeation chromatography provides a low-resolution separation that is roughly based on molecular size, but does not distinguish well among isomers. As a consequence, the same mass may appear in two or more adjacent fractions. Fig. 2 shows the

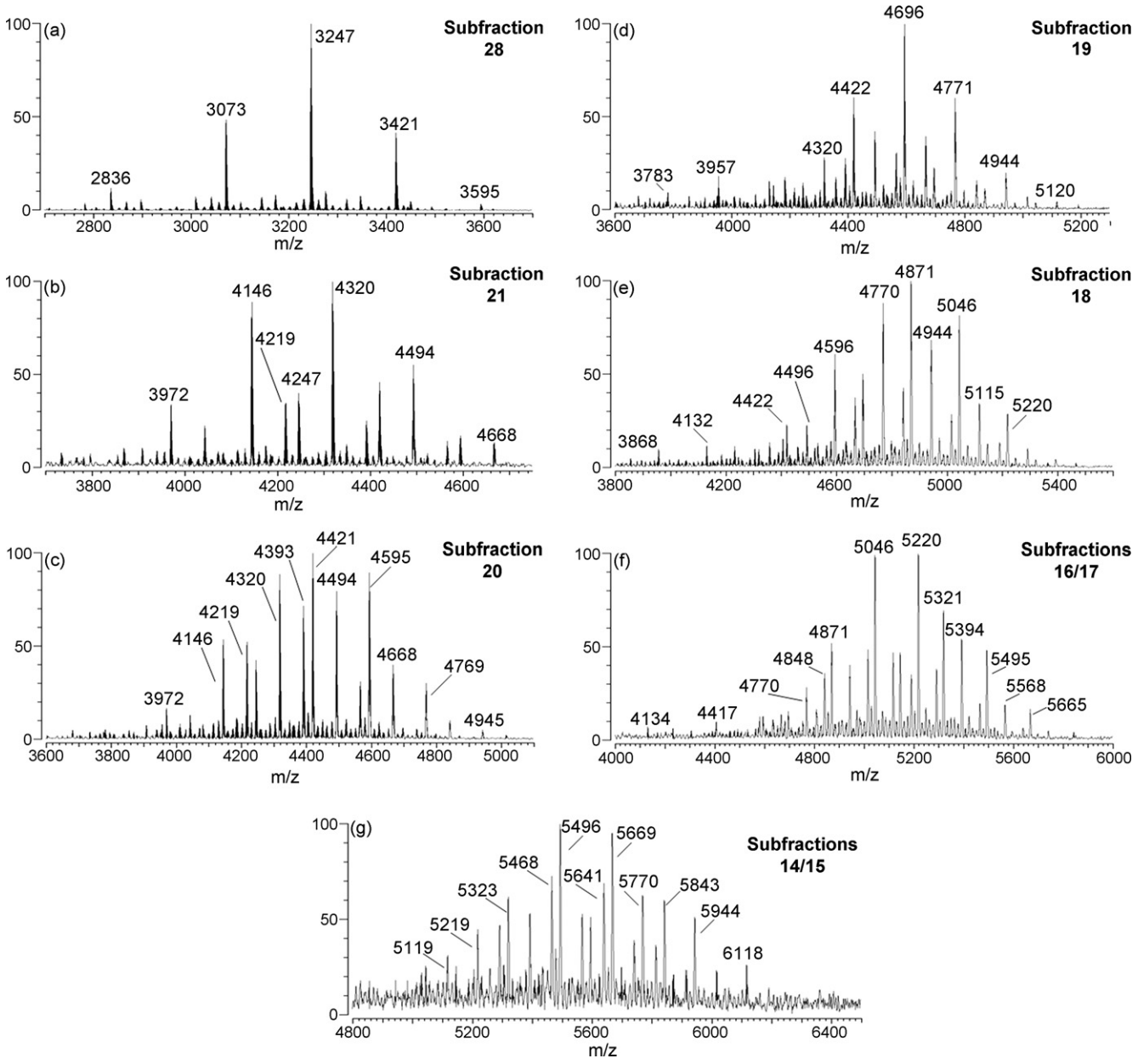



**Fig. 2.** MALDI–FTICR mass spectra of permethylated high-molecular weight subfractions of neutral milk oligosaccharides. Peak labels indicate centroid *m*/*z* values for the isotopic clusters. The assignments for the contents of these subfractions are summarized in Table 3.

MALDI–FTICR mass spectra of the oligosaccharide subfractions 28 → 14/15. The ion signals have been analyzed concerning the presence of oligosaccharides with compositions based on the list of monosaccharides described above; the results are summarized in Table 3.

Individual reports have indicated the presence in human milk of one or several fucosylated oligosaccharides [13,15,21,22,30]. The data that is summarized in Fig. 2 and Table 3 indicates that high-performance mass spectrometric analysis of the permethylated oligosaccharides enables the determination that milk contains multiple homologous series of high-molecular weight fucosylated oligosaccharides that cover a broad range of substitution, bearing only a few or many fucose residues, sometimes exceeding one fucose/*N*-acetyllactosamine unit. The degree of fucosylation corresponds to occupancy of up to two-thirds of the theoretical limit of the potential core fucosylation sites that have been previously established (e.g., for a L7/*y* – 0 core-structure, *y* = 15 is the maximum degree of fucosylation). For oligosaccharides containing more than seven LacNAc units, the molecular weights of the glycans with very high fucosylation levels would exceed the available mass range, so their full distributions of the fucosylated species were not determined. Comparison of these results with the results from TLC IR-MALDI-TOF MS analysis of milk oligosaccharides reported by Dreisewerd et al. suggests that analysis of the native compounds results in fucose loss, since these investigators reported observation of a maximum of four fucose substituents on L4 and also noted that some in-source loss of fucose did occur when pure standards of low-molecular-weight milk-derived glycans were analyzed [25]. Nevertheless, even though rearrangements are prevented and the response of oligosaccharides with different compositions is "normalized" by methylation so that profiles of fractions can provide a reliable survey of the content of each, molar response can be

**Table 3**
Summary of *N*-acetyllactosamine-based compositions found within the range *m*/*z* 2800–6000 in the MALDI-FTMS spectra of neutral, permethylated oligosaccharides from human milk (L = lactose, *x* = # of LacNAc, *y* = # of Fuc, *z* = # of NeuAc, DP = degree of polymerization).

| L*x*/*y* − *z* | *y* | [M+Na]$^+$ mono-isotopic *m*/*z* | Subfraction | | | | | | | DP |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 28 | 21 | 20 | 19 | 18 | 16/17 | 14/15 | |
| L4/*y* − 0 | 4 | 2970.493 | *x* | | | | | | | 14 |
| | 5 | 3144.582 | *x* | | | | | | | 15 |
| | 6 | 3318.671 | *x* | | | | | | | 16 |
| L5/*y* − 0 | 1 | 2897.451 | *x* | | | | | | | 13 |
| | 2 | 3071.540 | *x* | | | | | | | 14 |
| | 3 | 3245.629 | *x* | | | | | | | 15 |
| | 4 | 3419.719 | *x* | | | | | | | 16 |
| | 5 | 3593.808 | *x* | | | | | | | 17 |
| L6/*y* − 0 | 0 | 3172.588 | *x* | | | | | | | 14 |
| | 1 | 3346.677 | *x* | | | | | | | 15 |
| | 2 | 3520.766 | *x* | | | | | | | 16 |
| | 5 | 4043.034 | | *x* | *x* | | | | | 19 |
| | 6 | 4217.123 | | *x* | *x* | | | | | 20 |
| | 7 | 4391.212 | | *x* | *x* | *x* | | | | 21 |
| | 8 | 4565.302 | | *x* | *x* | *x* | | | | 22 |
| | 9 | 4739.391 | | | *x* | | | | | 23 |
| L7/*y* − 0 | 2 | 3969.992 | | *x* | *x* | | | | | 18 |
| | 3 | 4144.082 | | *x* | *x* | | | | | 19 |
| | 4 | 4318.171 | | *x* | *x* | *x* | *x* | | | 20 |
| | 5 | 4492.260 | | *x* | *x* | *x* | *x* | | | 21 |
| | 6 | 4666.349 | | *x* | *x* | *x* | *x* | *x* | | 22 |
| | 7 | 4840.438 | | *x* | *x* | *x* | *x* | *x* | | 23 |
| | 8 | 5014.528 | | | | *x* | *x* | *x* | | 24 |
| | 9 | 5188.617 | | | | | *x* | *x* | | 25 |
| | 10 | 5362.706 | | | | | *x* | *x* | | 26 |
| L8/*y* − 0 | 1 | 4245.129 | | *x* | *x* | *x* | | | | 19 |
| | 2 | 4419.219 | | *x* | *x* | *x* | | | | 20 |
| | 3 | 4593.308 | | *x* | *x* | *x* | *x* | *x* | | 21 |
| | 4 | 4767.397 | | | *x* | *x* | *x* | *x* | | 22 |
| | 5 | 4941.486 | | | *x* | *x* | *x* | *x* | | 23 |
| | 6 | 5115.575 | | | | *x* | *x* | *x* | *x* | 24 |
| | 7 | 5289.665 | | | | | *x* | *x* | *x* | 25 |
| | 8 | 5463.754 | | | | | *x* | *x* | *x* | 26 |
| | 9 | 5637.843 | | | | | | | *x* | 27 |
| | 10 | 5811.932 | | | | | | | *x* | 28 |
| L9/*y* − 0 | 0 | 4520.266 | | | | *x* | | | | 20 |
| | 1 | 4694.355 | | | | *x* | *x* | *x* | *x* | 21 |
| | 2 | 4868.445 | | | | *x* | *x* | *x* | *x* | 22 |
| | 3 | 5042.534 | | | | | *x* | *x* | *x* | 23 |
| | 4 | 5216.623 | | | | | *x* | *x* | *x* | 24 |
| | 5 | 5390.712 | | | | | *x* | *x* | *x* | 25 |
| | 6 | 5564.801 | | | | | | *x* | *x* | 26 |
| | 7 | 5738.891 | | | | | | *x* | *x* | 27 |
| | 8 | 5912.980 | | | | | | | *x* | 28 |
| L10/*y* − 0 | 0 | 4969.492 | | | | | *x* | *x* | | 22 |
| | 1 | 5143.582 | | | | | *x* | *x* | *x* | 23 |
| | 2 | 5317.671 | | | | | *x* | *x* | *x* | 24 |
| | 3 | 5491.760 | | | | | *x* | *x* | *x* | 25 |
| | 4 | 5665.849 | | | | | | *x* | *x* | 26 |
| | 5 | 5839.938 | | | | | | *x* | *x* | 27 |
| L11/*y* − 0 | 1 | 5592.808 | | | | | | | *x* | 25 |
| | 2 | 5766.897 | | | | | | | *x* | 26 |
| | 3 | 5940.986 | | | | | | | *x* | 27 |
| | 4 | 6115.075 | | | | | | | *x* | 28 |

expected to drop as mass increases, and thus highly accurate quantification over the wide mass range investigated here would require the use of internal standards.

The spectrum in Fig. 3 illustrates that, when both high- and the low-fucose compositions (L6/6 − 0, L6/7 − 0 and L8/1 − 0) are present, the mass difference between a high-fucose composition, e.g., L6/6 − 0, monoisotopic peak at *m*/*z* 4217.1, and the nearest cluster at the high-mass side is 28.0 Da. This observed difference corresponds to the presence of a low-fucose composition L(*x* + 2)/(*y* − 5) − 0; here the low-fucose species is L8/1 − 0 (monoisotopic mass at *m*/*z* 4245.1). Corresponding pairs of peaks can be observed throughout the series, as indicated by the values listed in Table 3.

The permethylation process has advantages but can itself introduce complexity because it can generate some products that exhibit incomplete methylation and small amounts of permethylated by-products whose structures we have investigated in detail [51], whose contributions can be minimized by careful attention to the conditions used for derivatization [51]. To address confusion that could result from the presence of by-products, one must rely on

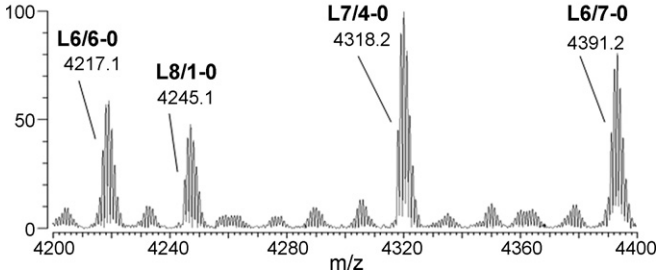



**Fig. 3.** MALDI–FTICR mass spectrum of the permethylated subfraction 20 of the neutral milk oligosaccharides in the range *m*/*z* 4200–4400. The spectrum indicates the presence of compounds having both high- and low-fucose composition, e.g., L6/6 – 0 and L6/7 – 0, as well as L8/1 – 0.

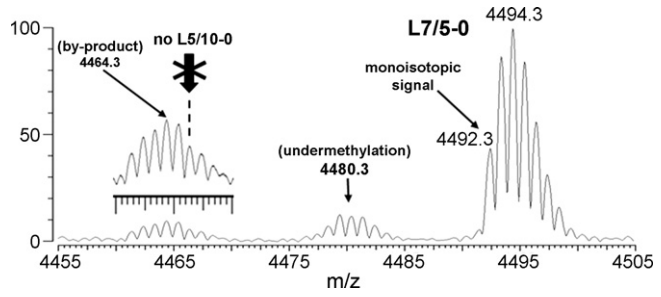



**Fig. 4.** MALDI–FTICR mass spectrum of the permethylated subfraction 20 of the neutral milk oligosaccharides in the range *m*/*z* 4455–4505. As the isotopic pattern of the by-product of L7/5 – 0 is not overlapping with the isotopic pattern of the high-fucose composition, the presence of L9/10 – 0 can be excluded. Inset expands the scale of the region *m*/*z* 4450–4460.

accurate analysis of the isotopic patterns, as well as accurate mass measurements. For example, the isotopic cluster corresponding to L7/5 – 0, monoisotopic $[M+Na]^+$ *m*/*z* 4492.3, can easily be compared to the cluster having its most abundant isotopic ion signal at *m*/*z* 4464.3, in the expanded spectral region shown in Fig. 4. The mass difference between the centers of the isotopic clusters is 30.0 Da (corresponding to the presence of the by-product) and not 28.0 Da (corresponding to the potential high-fucose composition L5/10 – 0 (calc. monoisotopic $[M+Na]^+$ *m*/*z* 4464.3, with most abundant peak predicted at *m*/*z* 4466.3). As the observed isotopic pattern of the [M+Na-30 Da] by-product of L7/5 – 0 does not show a distortion that would correspond to the overlap of signals from the [M+Na-30 Da] species with the theoretical isotopic pattern of the high-fucose composition, one can exclude the presence of L5/(10 – 0).

## 4. Conclusions

The results shown here affirm that the neutral human milk oligosaccharide fraction is a highly complex mixture. These data allow a deeper insight into the distribution of the diversity within the neutral fraction including the high and low fucose-containing compounds of human milk oligosaccharides. The high-fucose content provides the potential for numerous secretor- and Lewis-epitopes on each oligosaccharide molecule. Such structures create the potential for substantial immunomodulating activity within the neutral high-molecular weight HMOS subfractions.

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