Structural Analysis of Underivatized Neutral Human Milk Oligosaccharides in the Negative Ion Mode by Nano-Electrospray MS^n (Part 2: Application to Isomeric Mixtures)

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A complex mixture of isomeric neutral oligosaccharides from pooled human milk was analyzed by nano-electrospray ionization (ESI) in a quadrupole ion trap mass spectrometer (QIT-MS) in the negative ion mode. Since deprotonated molecules of neutral oligosaccharides follow distinct fragmentation rules, which have been elucidated by using model compounds (see [1]), spectra obtained from consecutive CID experiments allowed the differentiation of isomers out of this highly complex mixture. With this method new human milk oligosaccharides of previously unknown isomeric structures have been identified, e.g., the occurence of three isomeric fucosylated lacto-N-hexaoses could be determined precisely, which have not been described before: (1) Fuc ($\alpha 1 \rightarrow 2$) Gal ($\beta 1 \rightarrow 3$) GlcNac ($\beta 1 \rightarrow 3$) Gal ($\beta 1 \rightarrow 4$) GlcNac ($\beta 1 \rightarrow 3$) Gal ($\beta 1 \rightarrow 4$) Glc, (2) Gal ($\beta 1 \rightarrow 4$) GlcNAc [($\alpha 1 \rightarrow 3$) Fuc] ($\beta 1 \rightarrow 3$) Gal ($\beta 1 \rightarrow 4$) GlcNac ($\beta 1 \rightarrow 3$) Gal ($\beta 1 \rightarrow 4$) Glc, (3) Gal ($\beta 1 \rightarrow 4$) GlcNAc ($\beta 1 \rightarrow 3$) Gal ($\beta 1 \rightarrow 4$) GlcNac [($\alpha 1 \rightarrow 3$) Fuc] ($\beta 1 \rightarrow 3$) Gal ($\beta 1 \rightarrow 4$) Glc. (J Am Soc Mass Spectrom 2002, 13, 1341–1348) © 2002 American Society for Mass Spectrometry

Over the past decades, glycosylation of specific molecular structures or cell surfaces have been recognized to play an important role in physiology [2]. Also, free oligosaccharides, which are present either extracellular or in cell matrices, are believed to have other than nutritional function. As the (bio)chemical basis of many life processes is known, both recognition of diseases and the development of medical treatment becomes possible. Also preventional health treatment gains importance; especially nutrition is known to be an essential factor. This leads to the development of nutritional additives with healing and precautional character (functional food).

As revealed by MALDI/MS human milk contains neutral as well as acidic oligosaccharides of high molecular weight [3, 4], of which the biological function is yet not known, but there is some evidence that these compounds have essential biological functions [5, 6]. It is hypothesized that due to their structural similarity to the sugars in N-and O-glycans and their resistance against digestion by glycosidases specific for N- and O-glycans, human milk oligosaccharides are able to reach the colon. There they may be potential, water-soluble receptor analogues which prevent interactions of pathogenic bacteria, viruses, fungi, or effects of toxins. They also may have benificial effects for the development of a specific intestinal flora of breast-fead infants. As they are potential nonimmunological factors, human milk oligosaccharides are under discussion as supplements for infant formula. Therefore, knowledge of their specific biological activity and the crucial concentration is necessary.

This demands analytical tools that provide structural elucidation of bioactive compounds and their active concentration. Today, there is no universal method for the characterization of oligosaccharides. Traditional methods for structural analysis of oligosaccharides are time and cost consuming. Even though NMR spectroscopy as well as X-ray crystallography have been successfully applied [7, 8], both methods need high amounts of sample in high purity. Mass spectrometry is a useful and sensitive method to investigate samples of low absolute amount and concentration or in complex mixtures. The useful combination of classical enzymatic approach and mass spectrometry is not only time consuming and costly, it is also limited to samples, that

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Table 1. Summary of observed fragment ions for test compounds

	LNT	LNnT	LN*para*	LNFP I	LNFP II	LNFP III	LNFP III	LNDFH I	LNDFH II	LNH	LNnH	TFLNH
MS^2	★*706*	★*706*	★*1071*	★*852*	★*852*	★*852*	★*852*	★*998*	★*998*	★*1071*	★*1071*	★*1509*
	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
	646	646	1011	792	792	792	688	938	834	1011	1011	1449
	628	628	993	774	774	774	544	920	690	993	993	1431
	544	544	909	690	690	690	382	836	528	909	909	1347
	382	382	747	528	528	528		674	348	526	526	672
	202	281	646	325	348	364		348		508	508	600
		263	628					325		454	454	
			544							382	382	
			382									
MS^3	★*706*	★*706*	★*1071*							★*1071*	★*1071*	★*1509*
	★*382*	★*382*	★*382*							★*909*	★*909*	★*1347*
	↓	↓	↓							↓	↓	↓
	202	281	202							526	526	672
	179	263	179							508	508	600
										382	382	528
										★*1071*	★*1071*	★*1509*
										★*526*	★*526*	★*672*
										↓	↓	↓
										508	508	654
										382	382	600
										281	281	528
										263	263	
										179	179	
MS^4										★*1071*	★*1071*	
										★526	★*526*	
										★382	★*382*	
										↓	↓	
										281	281	
										263	263	
										179	179	

contain pure compounds, i.e., structural analysis of complex mixtures is not possible. Some mass spectrometers provide the possibility to perform structure elucidation studies such as triple-quadrupole [9], quadrople ion trap [10, 11], or FTICR mass spectrometry [12], based on collision-induced fragmentation (CID) or MALDI-TOF-MS (post-source-decay analysis) [13, 14]. Therefore, single compounds from even complex mixtures can be selected and applied to MS/MS experiments.

A strategy for structural analysis of underivatized, neutral human milk oligosaccharides has been presented based on CID-MS/MS experiments in an quadrupole ion trap in the negative ion mode using a nano-electrospray source (see [1]). Under these conditions, neutral oligosaccharides can be investigated as deprotonated molecules with high sensitivity [15, 16]; their fragmentation behavior has been elucidated based on certain isomeric glycoforms yielding both complete sequence and linkage information as well as fucose position and branching sites. A summary of the observed fragment ions for several test compounds is given in Table 1.

These fragmentation rules are applied to oligosaccharides, the complex total mixtures stemming from GPC fractions of human milk oligosaccharides in order to differentiate the isomers out of complex isomeric mixture.

Experimental

Sample Preparation

The separation of oligosaccharides from pooled human milk was performed as described in [4, 17–19]; in this study, GPC subfractions of neutral oligosaccharides were analyzed. For mass analysis the lyophilized GPC-subfractions were dissolved in deionized water to a total concentration of 1 g/L; ammonium acetate (10^{-3} M) was used as additive (3:1,vol:vol).

Instrumentation

For mass analysis and structure elucidation a quadrupole ion trap mass spectrometer was used (LCQ, Finnigan MAT, San Jose, CA) equipped with a nano-ESI source (Protana, Odense, Denmark). The spray needles used were laboratory-pulled, gold-coated glass capillaries with an orifice diameter in the range of 1–2 μm. Typically 1–3 μl of the sample solution were loaded. The spray voltage was between 600–1200 V, the transfer capillary was held at temperature of 200 °C. The LCQ

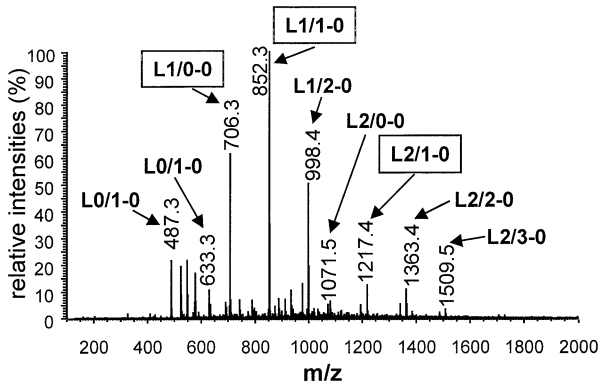

Figure 1. Nano-electrospray mass spectrum of the total fraction of neutral oligosaccharides from pooled human milk (negative ion mode). The ion signals are labeled with the corresponding composition Lx/y-z; the signals investigated in MS^n experiments are represented by L1/0-0, L1/1-0 and L2/1-0.

was used under standard settings [20]. All mass spectra shown were acquired over a period of approximately 30 s. For fragmentation by collision induced dissociation (CID) the relative collision energy in the QIT was set to 40–65% (corresponding to the LCQ software settings) depending on the charge and/or the chemical nature of the precursor ions. The precursor ion selection was carried out with an isolation width of 3 Da.

Results and Discussion

Figure 1 shows the nano-ESI mass spectrum of the total, neutral fraction of oligosaccharides from human milk in the negative ion mode; the observed deprotonated molecules show the major constituents present. In order to describe their composition out of the well-known building blocks, a simple nomenclature is introduced. The composition of human milk oligosaccharides is described as Lx/y-z, with the core structures: L = lactose (reducing end); Lac; x = number of N-Acetyllactosamine subunits, LacNAc (type I and/or II); and the residues: y = number of fucoses, Fuc; z = number of sialic acids, Sia.

For instance, L1/0-0 comprises one lactose as reducing end and one N-acetyllactosamine unit, and no fucose or sialic acid residues.

In a preceeding paper [1] it has been demonstrated, that deprotonated neutral oligosaccharides show consecutive C-type fragmentation yielding sequence information as well as cross-ring fragments of the "new" reducing ends which yield information about the linkage type of internal monosaccharide subunits. CID mass spectra can be interpreted by reading mass increments in the mass spectrum "from right to left".

The molecular ion at *m/z* 706, representing the deprotonated species of composition L1/0-0 (please refer to Figure 2) , was subjected to CID-fragmention in order to address the question whether it is possible to distinguish the two possible isomers, LNT ([Gal $\beta1\rightarrow3$ GlcNAc $\beta1\rightarrow3$ Gal $\beta1\rightarrow4$ Glc]) and LNnT ([Gal

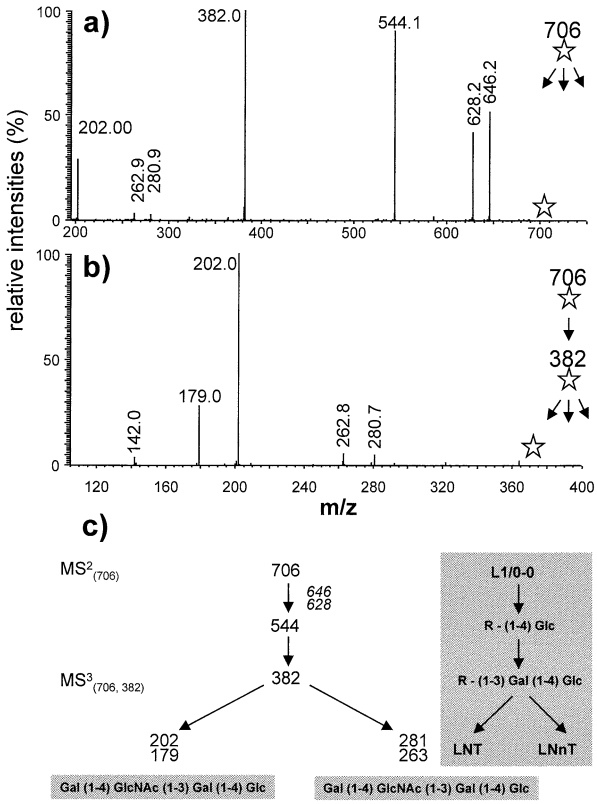

Figure 2. (**a**) MS^2 spectrum of *m/z* 706 (composition L1/0-0). (**b**) MS^3 spectrum of the fragment ion at *m/z* 382 as precursor ion (★ *m/z* 706 ★ *m/z* 382). (**c**) Fragmentation pathways for the two possible isomers with the composition L1/0-0, LNT and LNnT, present in the mixture. The grey region illustrates the meaning of the observed fragment ions [R_1 = (LacNAc + Gal) and R_2 = (LacNAc) with LacNAc of the lacto- or lacto-neo-series].

$\beta1\rightarrow4$ GlcNAc $\beta1\rightarrow3$ Gal $\beta1\rightarrow4$ Glc]), which differ in the linkage in their terminal LacNAc; whereas the LacNAc in LNT belongs to the lacto-series (type I, Gal $\beta1\rightarrow3$ GlcNAc), LNnT comprises a terminal LacNAc of the lacto-neo-series (type II, Gal $\beta1\rightarrow4$ GlcNAc). It is known that the mixture of neutral oligosaccharides contains both isomers, however, pooled human milk contains 0,5– 1,5 g/l LNT whereas the corresponding isomer LNnT is found in lower amounts (~0.1 g/L) [21].

The MS^2 spectrum of the precursor ion at *m/z* 706 is shown in Figure 2a; the occurence of *m/z* 281, 263, and 202 in the MS^2 spectrum indicates that both isomers are present in the mixture. This is confirmed by a MS^3 experiment of the fragment ion *m/z* 382 (Figure 2b). While LNT produces the characteristic fragment ions of *m/z* 202 and 179, the $1\rightarrow4$-linkage of the GlcNAc in LNnT is reflected by the fragment ions at *m/z* 281 and 263. As expected, the fragment ions of LNT are observed with stronger signal intensities because the concentration of this isomer is higher than that of LNnT. Figure 2c summarizes the fragmentation pathway of both isomers. For comparison, the observed masses of fragment ions in MS^n experiments of the individual

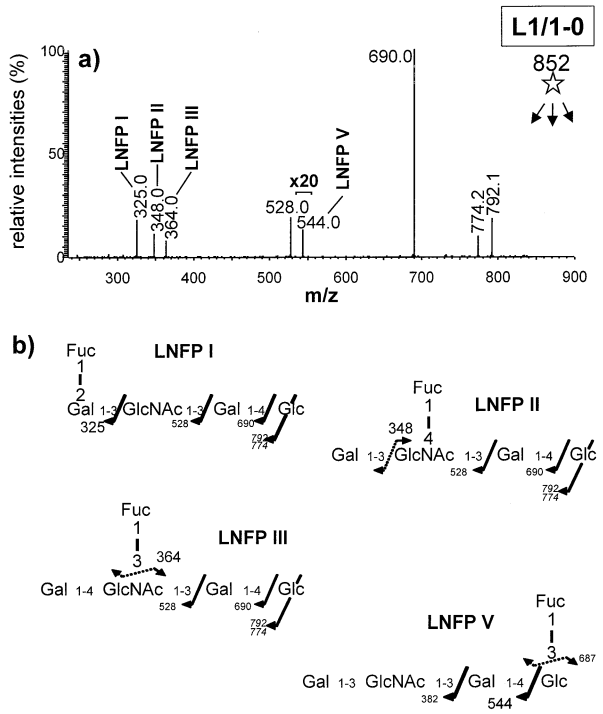

Figure 3. MS^2 experiment of *m/z* 852 as precursor ion (corresponding to the monofucosylated composition L1/1-0).

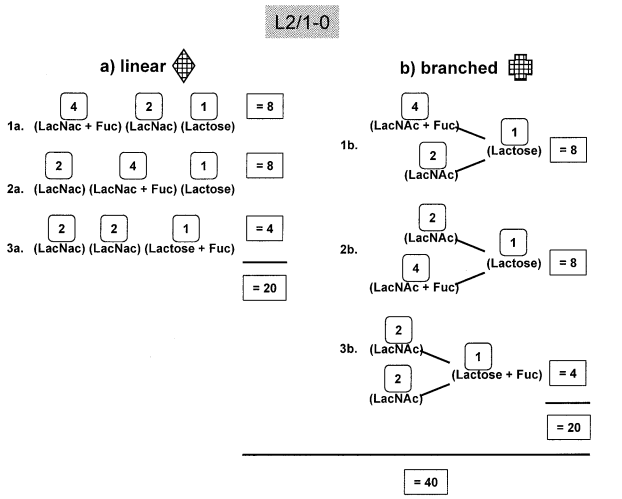

Figure 4. Possible basic structures of isomers with L2/1-0 as composition [(**a**) linear and (**b**) branched structures]. The number of possible substructures is indicated above each unit; for further explanations see text.

isomeric structures are summerized in Table 1 (for detailed discussion and complete structures see [1]).

In the next step the mono-fucosylated compounds of composition L1/1-0 were inspected, i.e., the deprotonated molecules at *m/z* 852. It is known that four different isomers of this composition exist in pooled human milk; these are LNFP I, II, III, and V (the structures are given in Figure 3b, for detailed discussion see [1]). For comparison the fragment ions found in MS^2 experiments for the individual isomeric glycoforms are summerized in Figure 3b as well as in Table 1. Figure 3a shows the MS^2 spectrum of the precursor ion *m/z* 852; the spectrum shows characteristic signals of LNFP I, II, and III at *m/z* 325, 348 and 364, respectively. Magnification (20×) for *m/z* 544 reveals that the isomer LNFP V is present, too.

Whereas the presence of the isomers with the composition L1/0-0, LNT, and LNnT, as well the isomers with the composition L1/1-0, LNFP I, LNFP II, LNFP III, and LNFP V, is well known for the total fraction of neutral oligosaccharides from pooled human milk (e.g., [21]), only a few complete isomeric structures are known for larger neutral (and acidic) oligosaccharides.

For testing this method for its application to larger molecules, an isomeric mixture of molecules with the composition L2/1-0 ($[M - H]^-$ *m/z* 1217) has been investigated. Again, the total fraction of neutral oligosaccharides from pooled human milk was used as sample. Figure 4 shows the basic structures that may be

Table 2. Isomeric structures

Abbreviation	Structures	Reference	This study
F-LNH I	Gal (β1 → 4) GlcNAc (β1 → 6) Gal (β1 → 4) Glc Fuc (α1 → 2) Gal (β1 → 3) GlcNAc (β1 → 3)	[22]	Represented by G
F-LNH II	Gal (β1 → 4) GlcNAc [(α1 → 3) Fuc] (β1 → 6) Gal (β1 → 4) Glc Gal (β1 → 3) GlcNAc (β1 → 3)	[23] [27]	Represented by F
"F-LNneoH"	Fuc α1 → { Gal (β1→4) GlcNAc (β1→6) Gal (β1→4) Glc ; Gal (β1→4 GlcNAc (β1→3) }	[24]	Represented by F and G
F-LNparaH	Gal(β1 → 3)GlcNAc (β1 → 3)Gal (β1 → 3) Gal (β1 → 4) GlcNac[(α1 → 3)Fuc](β1 → 3) Gal (β1 → 4) Glc	[25]	
		[26] [27]	D
	Gal (β1 → 3)GlcNAc[(α1 → 4)Fuc] (β1 → 3) Gal (β1 → 4) GlcNac (β1 → 3) Gal (β1 → 4) Glc	[27]	B

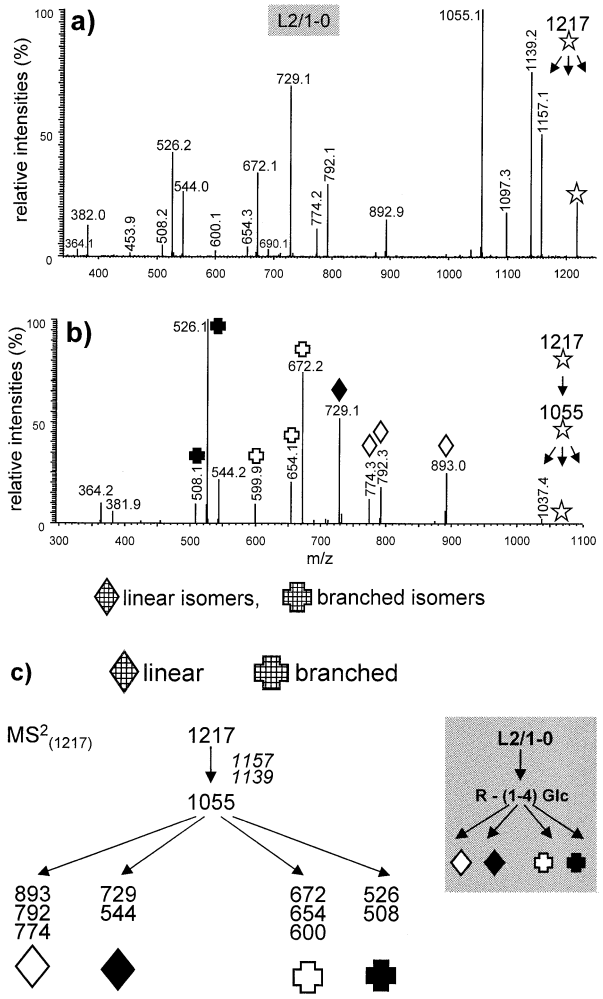

Figure 5. (**a**) MS^2 spectrum of *m/z* 1217 (composition L2/1-0). (**b**) MS^3 spectrum of *m/z* 1055 as precursor ion (★ *m/z* 1217 ★ 1055). The fragment ions corresponding to linear isomers are indicated with diamond symbols; those corresponding to branched isomers are marked with cross symbols (branched isomers). (**c**) Fragmentation pathway for the possible isomers with the composition L2/1-0 present in the mixture. Distinct fragmentation channels are observed for different subgroups of linear isomers (indicated with white and black diamond symbols) and branched isomeric structures (marked with white and black cross symbols). Again, the grey region illustrates the meaning of the observed fragment ions, with R = (LacNac + LacNAc + Fuc + Gal).

either linear (Figure 4a) or branched (Figure 4b) and may contain either LacNAc subunits of the lacto- or lacto-neo-series. Fucosylation may occur either at the C(3) of the reducing end glucose (Glc) or at each N-acetyl lactosamin subunit (attached to Gal or GlcNAc). The number of possible isomeric substructures is indicated above each subunit; therefore, for the composition L2/1-0 twenty different linear and twenty different branched isomeric structures are theoretically possible.

Out of the 40 possible isomers, five isomeric structures with the composition L2/1-0 have been identified before. These are F-LNH I [22], F-LNH II [23], "F-LNneoH" [24] (the fucose position has been determined

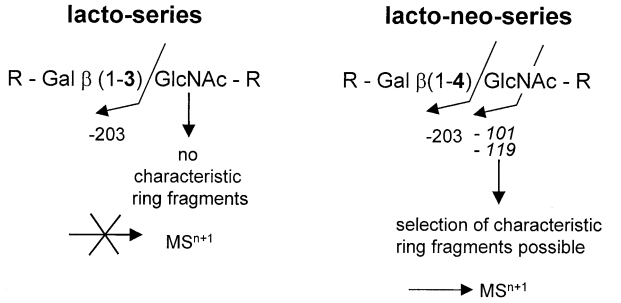

Figure 6. Illustration for the possibility to identify a subgroup of isomers containing LacNAcs of the lacto-neo-series (1 → 4-linkage) out of a complex mixture by mass spectrometry via the selection of characteristic fragment ions for further collision-induced dissociation. The identification of isomers containing LacNAcs of the lacto- series (1 → -linkage) is not possible as those do not produce characteristic cross-ring fragments.

to be at one of the branches), and F-para-LNH [25–27] and another linear structure described in [22]. Their structures are exhibited in Table 2a.

Figure 5a shows the MS^2 spectrum of the precursor ion at *m/z* 1217; a mass increment of 164 is not observed. This indicates that the mixture does not contain a measurable amount of isomers which contain a fucose at the reducing-end glucose. Therefore, the possible isomeric structures 3a and 3b as indicated in Figure 4 of either linear or branched structures are not present in this mixture, which leads to the exclusion of the presence of 8 out of 40 possible isomeric structures.

The mass increments of 60 and 78 Da reflect the 1 → 4-linked hexose (glucose) of the reducing-end lactose. A loss of this glucose results in *m/z* 1055; this fragment ion was chosen for a subsequent MS^3 experi-

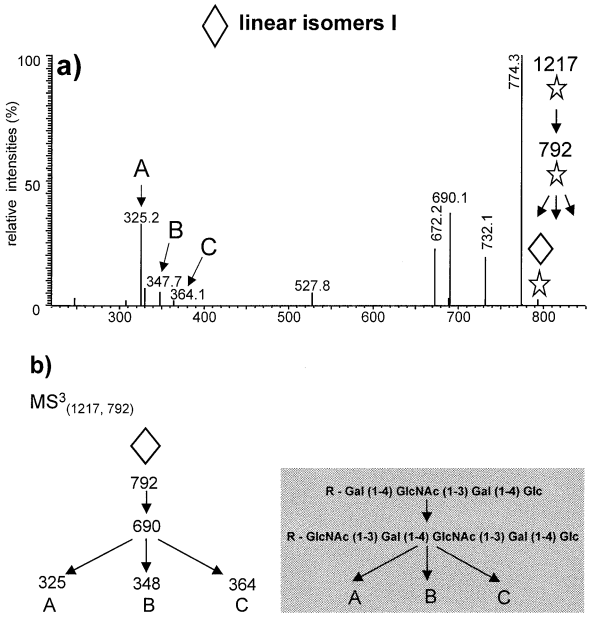

Figure 7. (**a**) MS^3 spectrum of the fragment ion at *m/z* 792 as precursor ion (★ *m/z* 1217 ★ 792). (**b**) Fragmentation pathway leading to the identification of the isomers A, B, and C (see also text and Figure 9).

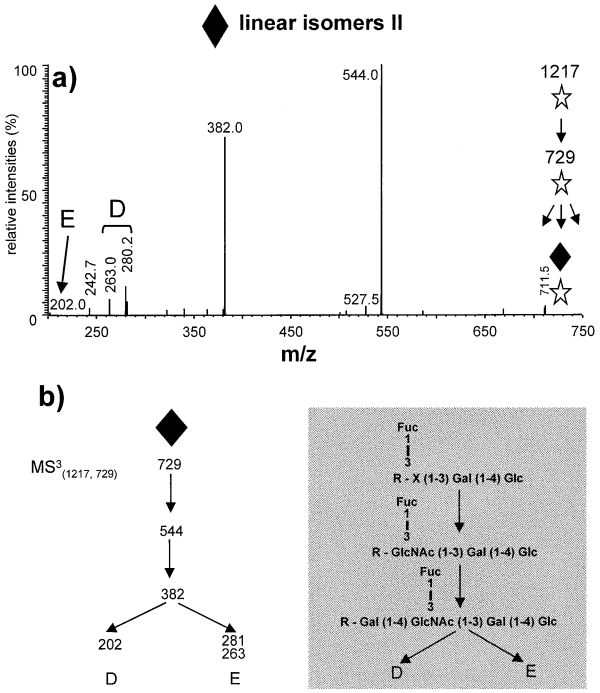

Figure 8. (**a**) MS^3 spectrum of the fragment ion at *m/z* 729 as precursor ion (★ *m/z* 1217 ★ *m/z* 729). (**b**) Fragmentation pathway leading to the identification of isomers D and E (see also text and Figure 9).

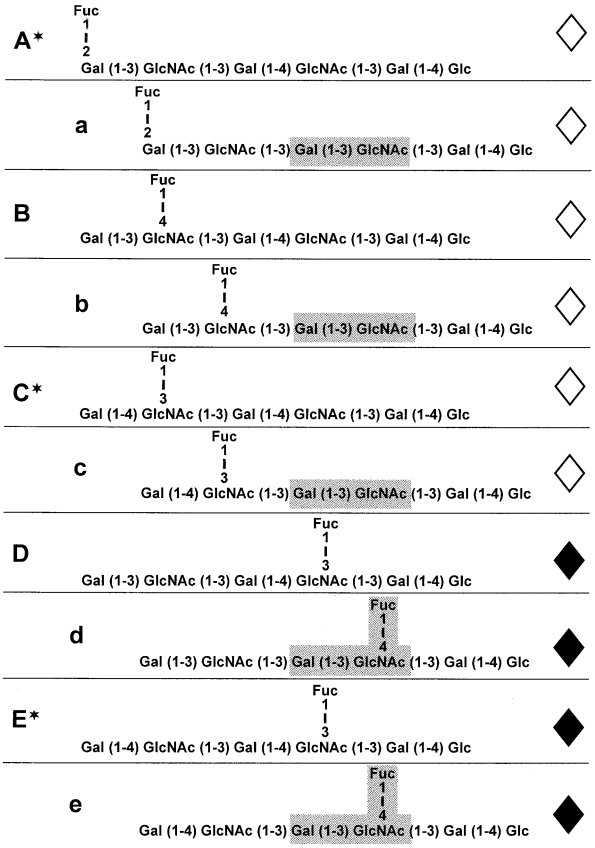

Figure 9. Five linear isomers with the composition L2/1-0 found in the mixture. The grey attributions indicate that there may be also isomers present in the mixture which exhibit other linkages within the internal LacNAc (see text).

ment (spectrum shown in Figure 5b), since the next fragmentation will be different for linear or branched structures. For example, the fragment ion at *m/z* 893 shows a further elimination of another hexose (galactose, Gal); this means that linear isomers are present in the mixture (marked with a diamond symbol). Thus, this fragment ion and the following cross-ring fragment signals at *m/z* 792 and 774 belong to the subgroup of linear isomers as well as the fragment ion at *m/z* 729.

The observed elimination of LacNAc (with or without additional fucose) moreover indicate the presence of branched structures in this isomeric mixture (marked with a cross symbol), those will be discussed later in the text. The corresponding fragmentation pathway is illustrated in Figure 5c.

First, the subgroup of linear isomers was subjected to further mass spectrometric investigations. The fragment ion at *m/z* 792 and 774 are cross-ring fragments of a 1 → 4-linked GlcNAc, indicating the presence of a subgroup of isomers present in the mixture (symbolized by white diamond symbol), whereas 729 (elimination of fucose) proves the presence of second series of components with a 1 → 3-fucosylation (preventing cross-ring fragment ions); in the following, the latter series is symbolized by a black diamond symbol.

As 1 → 4-linked N-acetyl-glucosamins (GlcNAc) produce characteristic cross-ring fragments, it can be determined unambiguously that the mixture contains isomers with a 1 → 4-linkage (lacto-neo-series) within this LacNAc (for further MS^n studies, selection of these cross-ring fragments is required). But 1 → 3-linked N-acetyl-glucosamines do not produce characteristic cross-ring fragment ions; therefore, mass spectrometric identification of a subgroup of linear isomers containing LacNAcs of the lacto-series (1 → 3-linkage) out of a complex mixture is not possible, as no characteristic fragment ion may be selected for further collision-induced dissociation (as illustrated in Figure 6).

In a first step, the precursor ion at *m/z* 792 was chosen for a further CID experiment; the MS^3 spectrum (Figure 7a) shows characteristic fragment ions at *m/z* 325, 348, and 364 indicating that the last (= non-reducing) LacNac-subunit exhibits the fucosylation pattern of LNFP I, II, or III, respectively (see above). Figure 7b illustrates the fragmentation pathway leading to the identification of the isomers A, B, and C (see also Figure 9).

In a next step, the selection of *m/z* 729 defined by the elimination of reducing lactose and a fucose (either in the MS^3 or MS^4) leads to a subgroup of isomers exhibiting the fucosylation at the internal LacNAc in a α 1 → 3-linkage to GlcNAc. CID experiments with this precursor ion show a consecutive loss of this GlcNAc (−185 Da to form *m/z* 544, see Figure 8a). Fragment ions at *m/z* 202, 281, and 263 reveal the presence of two

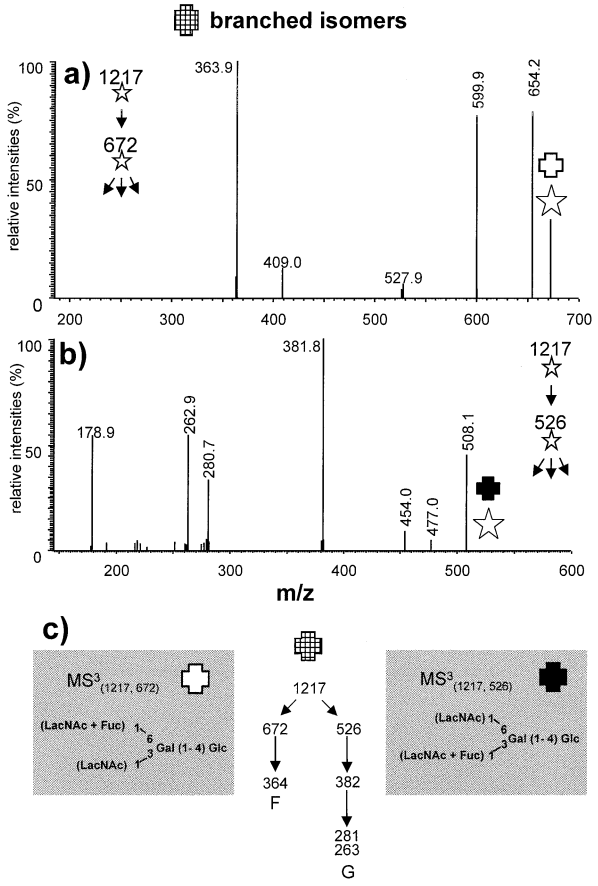

Figure 10. MS^3 experiments of branched isomers with the composition L2/1-0. (**a**) MS^3 spectrum of the fragment ion at *m/z* 672 as precursor ion. (**b**) MS^3 spectrum of the fragment ion at *m/z* 526 as precursor ion. (**c**) Fragmentation pathway of the branched isomers with the fucose linked to the 1 → 6-branches (F) or the 1 → 3-branches (G).

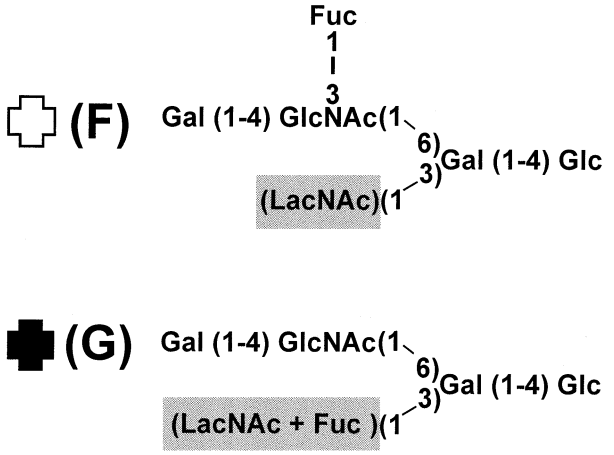

Figure 11. Branched isomers with the composition L2/1-0 found in the mixture. The grey fields indicate LacNac subunits which cannot be characterized in detail. For structure F there are two possible isomers, whereas structure G may represent four isomers (see text).

isomers, D and E, which contain a non-reducing LacNAc of both the lacto-series and the lacto-neo-series, as indicated in Figure 8b.

Whereas the presence of an internal LacNAc of the lacto-neo series with a 1 → 3-linked fucose is thus clearly shown, it cannot be determined unambiguously whether this internal LacNAc is also present in the lacto form with a 1 → 4-linked fucose, since this would result in the direct loss of both monosaccharide units to form *m/z* 544 (loss of 349 Da). Figure 9 summarizes the different linear isomers A–E determined to be present in the mixture. The grey attributions in the structures a–e indicate that there may be also isomers present in the mixture, which exhibit other linkages within the internal LacNAc, such as β 1 → 3 in the Structures A, B, and C (internal LacNAc corresponds to the lacto-series) and β 1 → 4 in Structures D and E (internal Lac-neo-Nac, therefore the fucose is α 1 → 4-linked to the GlcNAc).

As derived above, also branched structures are present in the mixture. In principle, two different basic structures are observed (Figure 11), as indicated by the fragment ions at *m/z* 672 and 526 representing the elimination of the 1 → 3-branch (for detailed discussion see [1]). The latter results from a elimination of a LacNAc subunit, which contains a fucose, whereas *m/z* 672 is produced by the loss of a LacNAc; in this case, the fucose is linked to the 1 → 6-branch. Both losses result in mass increments of 383 and 529 Da (from *m/z* 1055), respectively. As discussed in detail in [1], these losses occur because of the elimination of the 1 → 3-branch; a further structural analysis of this branch becomes thereby impossible.

In the next step the linkage in the remaining "tri- or tetrasaccharide" has to be determined. MS^3 experiments of fragment ion *m/z* 672 (Figure 10a) and *m/z* 526 (Figure 10b) prove the presence of structures F and G, as illustrated in Figure 10c. The presence of *m/z* 364 in the MS^3 spectrum of *m/z* 672 reveals that the fucose is linked to the GlcNAc of a Lac-neo-NAc subunit (like in LNFP III, compare to Table 1). Structure F (white cross) represents two possible isomers, where the LacNAc subunit belongs either to the lacto-series (1 → 3-linkage of galactose and N-actyl-lactosamin) or to the lacto-neo-series (1 → 4-linkage).

Figure 10b shows the MS^3 spectrum of the precursor ion *m/z* 526; the presence of fragment ions at *m/z* 281 and 263 demonstrates the 1 → 4-linkage of this LacNAc subunit (Lac-neo-NAc), whereas the absence of *m/z* 202 proves that no isomer with a LacNAc of the lacto-series in this region is present in the mixture. Therefore, structure G represents four different possible isomers: Either the fucose may be linked to the non-reducing galactose or it may also be linked to a GlcNAc in a 1 → 4-linkage (LacNAc) or in a 1 → 3-linkage (Lac-neo-NAc). Figure 10c summerizes the branched isomeric structures corresponding to the composition of L2/1-0 indicating the uncertainties remaining within the 1 → 3 branch (marked in black).

Conclusion

It could be demonstrated that MS^n experiments of deprotonated neutral oligosaccharides can be applied for structural analysis of oligosaccharides out of complex isomeric mixtures. Linear, fucosylated, and branched structures can be differentiated by making use of specific fragmentation channels which result in a separation into subgroups of the present isomeric mixture. For the first time, the presence of both linear and branched structures of neutral oligosaccharides, out of complex isomeric mixtures, has been revealed by mass spectrometry. Problems arise for internal LacNAcs that do not produce specific fragment ions for either lacto-series or for GlcNAc-fucosylated lacto-neo constituents. Moreover, structure elucidation of $1 \rightarrow 3$-branches of branched structures is not possible due to its elimination.

Out of 40 possible isomers with the composition L2/1-0, 24 isomers could be excluded to be present in the mixture. These are those eight with the fucose linked to the reducing glucose (Figure 4, 3a and 3b), four linear isomers that could contain the fucose at the galactose of the internal LacNac (Figure 4, 1a and 2a), eight branched structures that have the fucose linked to the terminal galactose and four branched isomers that have a LacNAc-subunit of the type II ($\beta 1 \rightarrow 3$-linkage) in their $1 \rightarrow 3$-branch (Figure 4, 1b and 2b).

In total, five isomers (A–E) could be determined completely based on their specific fragment ions; three of those have never been described before (A, C, and E, which are marked with an asterix in Figure 9). Isomeric linear structures that exhibit the fucose at the terminal galactose (e.g., A) and such based on paraLNneoH [22] (e.g., C and E) have not been reported before. Five more isomers (a–e) could not be exluded to be present in the mixture. Structure F and G may represent two and four isomeric structures, respectively, of which two have been described before (see Table 2).

All these experiments were carried out with pooled neutral human milk oligosaccharides which represent a very complex mixture. Therefore, the tools developed lend themselves to be applied to individual human milk oligosaccharides in future investigations since based on the individual enzyme equipment an informative differentiation and selective expression of oligosaccharide isomers is to be expected.

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References

1. Pfenninger, A.; Karas, M.; Finke, B.; Stahl, B.; Sawatzki, G. *J. Am. Soc. Mass Spectrom.* in press.
2. Dwek, R. A. *Chem. Rev.* **1996,** *96,* 683.
3. Stahl, B.; Thurl, S.; Zeng, J.; Karas, M.; Hillenkamp, F.; Steup, M.; Sawatzki, G. *Anal. Biochem.* **1994,** *223,* 218.
4. Finke, B.; Stahl, B.; Pfenninger, A.; Karas, M.; Daniel, H.; Sawatzki, G. *Anal. Chem.* **1999,** *71,* 3755.
5. Newburg, D. S.; Street, J. M. *Nutrit. Today* **1987,** *32,* 191.
6. Varki, A. *Glycobiol.* **1993,** *3,* 97.
7. Kennedy, J. F. Carbohydrate Chemistry; Clarendon Press: Oxford, 1988; 269.
8. Fievre, S.; Wieruszeski, J.; Michalski, J.; Lemoine, J.; Montreuil, J.; Strecker, G. *Biochem. Biophys. Res. Commun.* **1991,** *177,* 720.
9. Paul, W. *Angew. Chem.* **1990,** *102,* 780.
10. March, R. E. *J. Mass Spectrom.* **1997,** *32,* 351.
11. Cooks, R. G.; Glish, G. L.; McLuckey, S. A.; Kaiser, R. E. *Chem. Eng. News* **1991,** *69,* 26.
12. Amster, I. J. *J. Mass Spectrom.* **1996,** *31,* 1325.
13. Spengler, B. *J. Mass Spectrom.* **1997,** *32,* 1019.
14. Spengler, B.; Kirsch, D.; Kaufmann, R.; Lemoine, J. *J. Org. Mass Spectrom.* **1994,** *29,* 782.
15. Bahr, U.; Karas, M.; Schubert-Zsilavecz, M. *Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics*; Orlando, FL, May/June, 1998.
16. Bahr, U.; Pfenninger, A.; Karas, M. *Anal. Chem.* **1997,** *69,* 4530.
17. Stahl, B.; Thurl, S.; Zeng, J.; Karas, M.; Hillenkamp, F.; Steup, M.; Sawatzki, G. *Anal. Biochem.* **1994,** *223,* 218.
18. Thurl, S.; Offermanns, J.; Müller-Werner, B.; Sawatzki, G. *J. Chromatogr.* **1991,** *568,* 291.
19. Thurl, S.; Müller-Werner, B.; Sawatzki, G. *Anal. Biochem.* **1996,** *235,* 202.
20. Bier, M. E.; Schwartz, J. C. In Electrospray Ionization Mass Spectrometry. Cole, R. B., Ed.; John Wiley and Sons: New York, 1997; p 235.
21. Kunz, C.; Rudolf, S. *Acta Paediatr.* **1993,** *82,* 903.
22. Yamashita, K.; Tachibana, Y.; Kobata, A. *J. Biol. Chem.* **1977,** *252,* 5408.
23. Dua, V. K.; Goso, K.; Dube, V. E.; Bush, C. A. *J. Chromatogr.* **1985,** *328,* 259.
24. Kobata, A.; Ginsburg, V. *Arch. Biochem. Biophys.* **1972,** *150,* 273.
25. Sabharwal, H.; Nilsson, B.; Grönberg, G.; Chester, M. A.; Dakour, J.; Sjöblad, S.; Lundblad, A. *Arch. Biochem. Biophys.* **1988,** *265,* 390.
26. Sabharwal, H.; Nilsson, B.; Chester, M. A.; Lindh, F.; Grönberg, G.; Sjöblad, S.; Lundblad, A. *Carbohydr. Res.* **1988,** *178,* 145.
27. Bruntz, R.; Dabrowski, U.; Dabrowski, J; Ebersold, A.; Peter-Katalinic, J.; Egge, H. *Biol. Chem. Hoppe-Seyler* **1997,** *369,* 257.