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High-performance liquid chromatography/electrospray ionization ion-trap mass spectrometry for analysis of oligosaccharides derivatized by reductive amination and N,N-dimethylation

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Abstract—Milk oligosaccharides derivatized by reductive amination with benzylamine followed by N,N-dimethylation (DMBA-oligosaccharides), were analyzed by high-performance liquid chromatography/electrospray ionization ion-trap mass spectrometry (HPLC/ESI-ITMS). Separation of DMBA-oligosaccharides was achieved on a graphitized carbon column eluted with aqueous acetonitrile and the DMBA-oligosaccharides were detected by positive-ion mode ESI-ITMS allowing sample amounts down to ~30 fmol of single DMBA-oligosaccharides injected on the HPLC column. MS/MS operation of the mass spectrometer resulted in the detection of diagnostic fragments, mainly belonging to the Y-series, allowing differentiation between isomeric milk oligosaccharides. HPLC/ESI-ITMS/MS/MS experiments indicated the migration of fucose residues of the DMBA milk oligosaccharides to the modified reducing end glucose residue during analysis, a migration previously only observed for proton adduct ions.

Keywords: Graphitized carbon column; Oligosaccharides; Reductive amination; Electrospray ionization; Ion-trap mass spectrometry; Fucose migration; Milk oligosaccharides

1. Introduction

Mass spectrometry (MS) has over the last decades developed into one of the most important techniques for analyzing organic molecules. MS offers a number of different modes of analysis, which may yield, for example, positive sample identification using fragmentation patterns from electron-impact MS, elemental composition using high-resolution MS, or sequence information using MS/MS experiments. The development of soft ionization techniques such as matrix-assisted laser-desorption/ionization (MALDI) and electrospray ionization (ESI) has made also large biomolecules amenable to analysis by MS.

Oligosaccharides have been analyzed by MS using a large variety of instrument types, with the carbohydrate samples in their native states or as different derivatives. A number of derivatization procedures for carbohydrates have been developed for improving the MS analysis with respect to (i) sensitivity, (ii) fragmentation pattern, and (iii) simplicity of data interpretation. MALDI time-of-flight (TOF) MS has been successfully used for the analysis of oligosaccharides, and the sensitivity has been increased down to the low femto-mole level by, in particular, reductive amination of the samples, using, for example, 2-(*N*,*N*-diethylamino)ethyl 4-aminobenzoate (ABDEAE)[1] and 2-aminopyridine.[2] ESIMS analysis of carbohydrate derivatives prepared by reductive amination, has also resulted in very high sensitivities, as exemplified by the study of a *N*,*N*,*N*-trimethyl-*N*-(4-aminophenyl)ammonium derivative of maltopentaose[3] and by analysis of ABDEAE-maltohexaose.[4] In

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addition to increasing the sensitivity, the introduction of a labeling group may also influence the pattern of daughter ions obtained during MS/MS operation.[5] Furthermore, the labeling group at the original reducing end of the oligosaccharide simplifies the interpretation of MS/MS data, since the identification of the fragments retaining the reducing end (e.g., Y-type[6]) is greatly facilitated.

Due to its on-line mode of sample introduction ESIMS has successfully been hyphenated with HPLC, and today HPLC/ESIMS is a standard technique in many laboratories. For HPLC analysis of oligosaccharides a few different techniques are available, with high-performance anion-exchange chromatography (HPAEC) as the method of choice for many applications.[7] From a mass spectrometric point of view a drawback of HPAEC is the mobile phase, which typically consists of a gradient of aqueous sodium acetate in aqueous sodium hydroxide, a mobile phase not directly compatible with hyphenation with a mass spectrometer. This problem may, however, be solved by on-line desalting of the eluent.[8] An alternative type of HPLC column suitable for carbohydrate analysis is the graphitized carbon column,[9] which for carbohydrate analysis typically is eluted with a mobile phase consisting of aqueous acetonitrile, making it well-suited for hyphenation with ESIMS. During the last years, graphitized carbon columns have been used in many HPLC/ESIMS studies of oligosaccharides released from glycoproteins or from other sources.[10,11] By reducing the column diameter (e.g., 75 μm), or by using a column on a microchip, the detection limits for HPLC/MS analysis of native oligosaccharides have been reduced to the low femto-mole level, using both graphitized carbon columns and normal-phase amide columns.[12–14]

Previously, we introduced a novel type of derivatization procedure for oligosaccharides based on reductive amination with benzylamine followed by N,N-dimethylation using iodomethane, resulting in derivatives (DMBA-oligosaccharides) with a fixed positive charge at the former reducing terminus.[15] Analysis by MALDI-TOFMS and MALDI-PSDMS resulted in a high sensitivity, as well as informative and simple daughter-ion mass spectra.[15] The daughter-ion mass spectra were dominated by fragments of Y-type, due to the presence of the fixed positive charge. DMBA-oligosaccharides have subsequently been analyzed by MALDI Fourier transform (FT) MS, which resulted in similar results.[16]

In the present study, the analysis of DMBA-oligosaccharides (LNF-I, LNF-II, LND-I, and MFLNH-III) is extended to HPLC on a graphitized carbon column hyphenated with ESI ion-trap MS (HPLC/ESI-ITMS). The chromatographic and mass spectrometric performance of the DMBA-derivatives is evaluated, as well as the sensitivity of the analysis.

2. Results and discussion

DMBA-oligosaccharides were analyzed by HPLC/ESI-ITMS at a very high sensitivity. The approximate detection limit for DMBA-oligosaccharides was 30 fmol injected on the HPLC column (10 μL of a 3 nM sample), as observed for a DMBA-derivative of maltoheptaose (DMBA-G7, Fig. 1). When analyzing native G7 a similar signal-to-noise ratio was obtained for a 500 fmol sample injected on the column (Fig. 1), indicating a 15- to 20-fold improvement of the sensitivity for DMBA-G7 over native G7. Previously, when these derivatives were studied by MALDI-TOFMS, the detec-

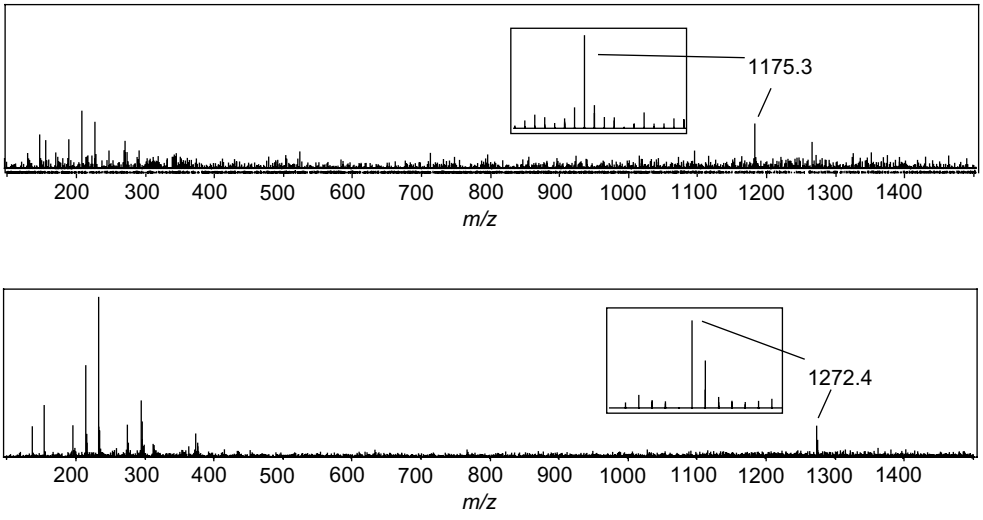

Figure 1. Mass spectra from HPLC/ESI-ITMS analysis of native maltoheptaose (G7) and G7 derivatized by reductive amination with benzylamine, followed by N,N-dimethylation (DMBA-G7). Top: 500 fmol G7 injected on the graphitized carbon column. Below: 30 fmol DMBA-G7 injected on the column.

tion limit for DMBA-G7 was determined to be ~50 fmol applied to the target plate corresponding to a 10-fold increase in sensitivity over that of native G7.[15] As comparison, ESIMS analysis (direct infusion) of a 10 nM ABDEAE-maltohexaose sample[4] resulted in a signal-to-noise ratio similar to the 3 nM sample of DMBA-G7 in Figure 1.

The DMBA-derivatives of LNF-I, LNF-II, LND-I, and MFLNH-III were found to produce informative and simple MS/MS spectra when analyzed by HPLC/ESI-ITMS/MS (Figs. 2 and 3). The amount of fragments formed was highly dependent on the fragmentation energies used (Fig. 2), and there was a clear difference between the studied DMBA-oligosaccharides with respect to which fragmentation energy that was required to form significant amounts of daughter ions. The DMBA-G7 required a fragmentation amplitude of 1.5 V to yield daughter ions of intensities exceeding the precursor ion, whereas DMBA-LNF-I and DMBA-LNF-II only required 0.9 V. DMBA-LND-I and DMBA-MFLNH-III were both intermediate in this respect (1.1 and 1.2 V, respectively). The milk oligosaccharides all contain at least one terminal Fuc residue, which is easily cleaved off to produce the corresponding Y-type fragment, as are most other deoxy-hexose residues. On the other hand, the glycosidic linkages between the Glc units of G7 are less prone to fragmentation, thus leading to a demand for higher fragmentation energies

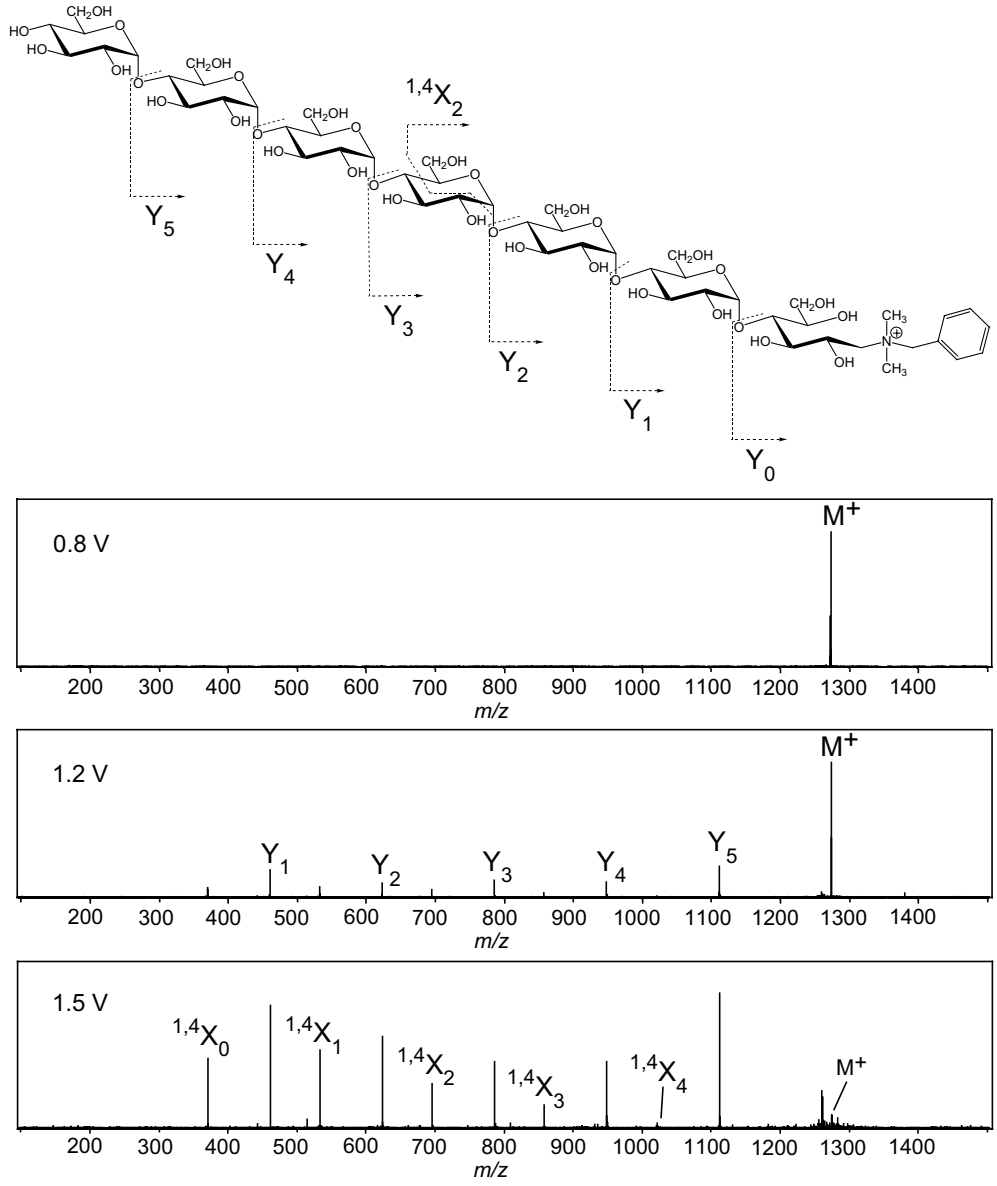

Figure 2. Mass spectra from HPLC/ESI-ITMS/MS analysis of maltoheptaose (G7) derivatized by reductive amination with benzylamine followed by N,N-dimethylation (DMBA-G7). The structure of DMBA-G7 is shown on top. MS/MS spectra obtained by isolation of DMBA-G7 (M^+, m/z 1272) and fragmentation at different fragmentation amplitudes are shown below.

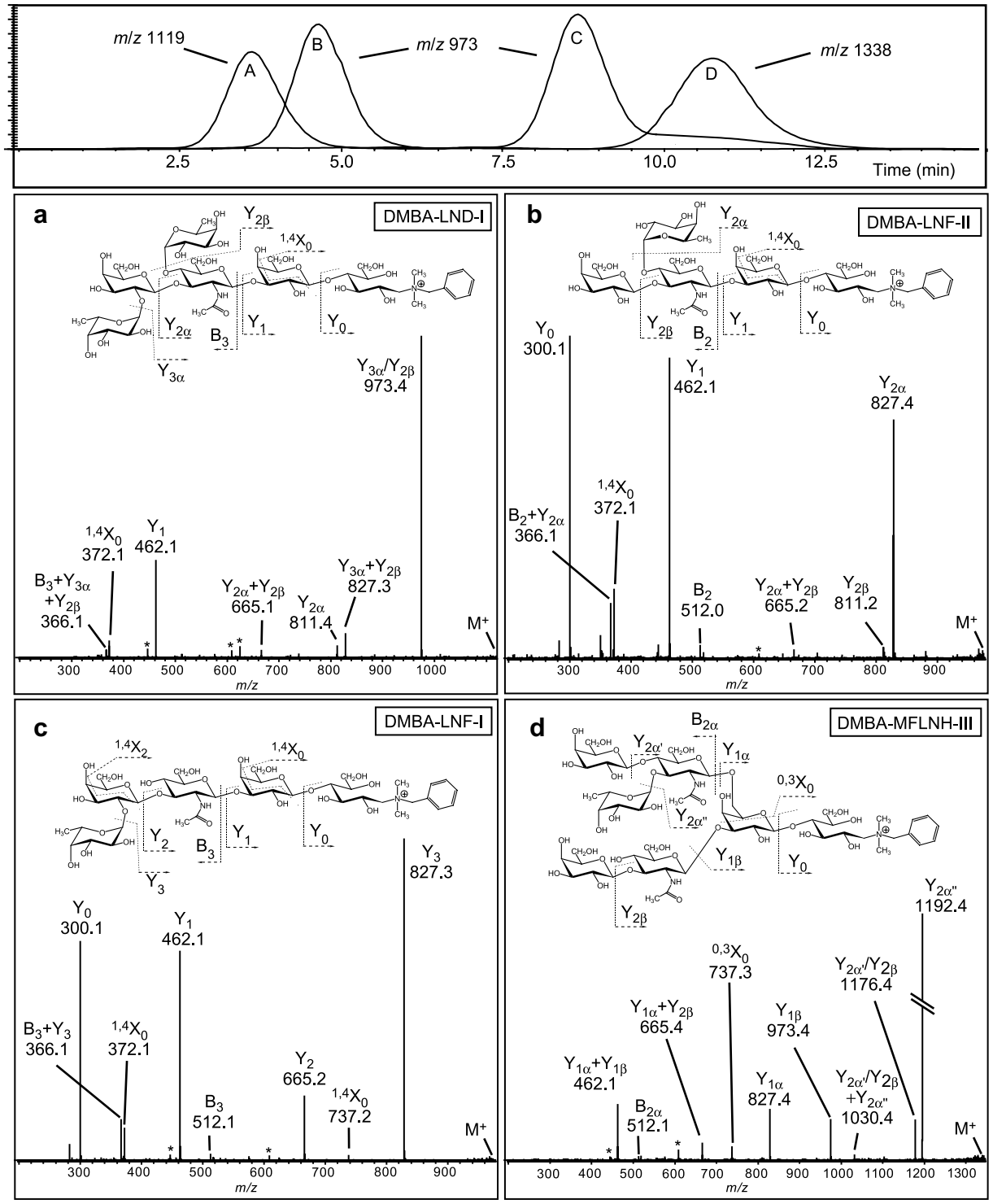

Figure 3. HPLC/ESI-ITMS/MS analysis of DMBA milk oligosaccharides [2.1 × 100 mm graphitized carbon column (5 μm) eluted with 13% aq MeCN at 0.2 mL/min, 10 pmol injected of each oligosaccharide]. Displayed on top are the extracted ion chromatograms, with the MS/MS spectra for peaks a–d displayed below. Tentative assignments of daughter ions are displayed. (X/Y: Two possible daughter ions. X + Y: Ion formation by combination of fragmentation leading to X ion and Y ion. *: Ion formation by glycosyl migration).

for DMBA-G7. For all DMBA-oligosaccharides, the MS/MS spectra were dominated by Y-type fragments (Figs. 2 and 3), whereas DMBA-G7 also produced significant amounts of X-type fragments ($^{1,4}X$ or $^{0,3}X$) at higher fragmentation energies, that is, fragments all retaining the modified reducing terminus. This was also found when DMBA-oligosaccharides were analyzed by MALDI-PSDMS[15] as well as by MALDI-FTMS.[16] This

preference is due to the fixed positive charge at the modified reducing terminus, formed by the derivatization procedure. Similar results have also been obtained when oligosaccharides derivatized with benzylamine have been analyzed by MS/MS as the corresponding proton adduct ions.[17,18] Benzylamine derivatives are preferentially protonated at the labeling group due to the high proton affinity of the secondary amine formed in the reductive amination. Consequently, the positive charge thus developed affects the fragmentation pattern obtained in MS/MS analysis, just as the positive charge in DMBA-oligosaccharides. In addition to the high abundance Y-type and low intensity X-type fragments, the ESI-ITMS/MS spectra of the DMBA-derivatives of LNF-I, LNF-II, LND-I, and MFLNH-III contain low-abundance B-series fragment ions resulting from cleavage of the GlcNAc glycosidic linkages and/or internal fragment ions resulting from cleavages of B-type in combination with Y-type (e.g., *m*/*z* 512 and 366, Fig. 3). These B-type fragments are of the same relative intensities as the corresponding fragments formed during MALDI-PSDMS analysis.[15]

When a mixture of DMBA-LNF-I, DMBA-LNF-II, DMBA-LND-I, and DMBA-MFLNH-III (~10 pmol injected of each) was analyzed by HPLC/ESI-ITMS/MS, on a graphitized carbon column eluted with 12% aq MeCN, the DMBA-oligosaccharides were baseline separated (data not shown). The time of elution of these oligosaccharide derivatives proved to be highly sensitive to small changes in MeCN concentration. When changing the mobile phase from 12% to 14% MeCN, the elution time for DMBA-LNF-I decreased from 16.0 min to 5.2 min. To reduce the time of analysis as well as to avoid excessive peak broadening, an intermediate MeCN concentration (13%) was selected for the subsequent HPLC/ESI-ITMS/MS experiments (Fig. 3).

The HPLC/ESI-ITMS/MS data allowed the discrimination between the different DMBA milk oligosaccharides (Fig. 3). The substance eluting at 3.6 min (peak A) generated a molecular ion at *m*/*z* 1119 with fragments all in accord with the structure of DMBA-LND-I as illustrated in Figure 3. DMBA-LND-I has only terminal Fuc residues as manifested by the presence of an ion at *m*/*z* 973 and the absence of an ion at *m*/*z* 957, which would correspond to the loss of a hexose residue. The terminal disaccharide Fuc-Gal is illustrated by the ion at *m*/*z* 811, whereas the ion at *m*/*z* 665, formed by two Y-type cleavages, indicates that the central GlcNAc residue is substituted by the Fuc-Gal disaccharide as well as by another Fuc residue. The peak at 4.7 min (peak B) yielded an ion at *m*/*z* 973, that is, corresponding to DMBA-LNF-I or DMBA-LNF-II, with diagnostic daughter ions at *m*/*z* 827 (loss of a Fuc residue) and *m*/*z* 811 (loss of a Gal residue, Fig. 3). This points to the presence of a terminal Fuc as well as a terminal Gal residue, which indicates this peak to contain DMBA-LNF-II. Furthermore, the low intensity of the ion at *m*/*z* 665 indicates this ion to be formed by the breaking of two bonds, also in accord with the structure of DMBA-LNF-II. The compound eluting at 8.6 min (peak C) generated an ion at *m*/*z* 973, and the MS/MS data pointed to the presence of a terminal Fuc residue only (*m*/*z* 827: loss of a Fuc residue), indicating this peak to consist of DMBA-LNF-I (Fig. 3). Moreover, the MS/MS data also showed the presence of a daughter ion at *m*/*z* 665 of moderate abundance, which is in accord with the Y_2 fragment of DMBA-LNF-I, formed by the cleavage of one single bond. The ESIMS/MS data obtained on DMBA-LNF-I and DMBA-LNF-II is very similar to ESIMS/MS data previously published for LNF-I and LNF-II derivatized with benzylamine and analyzed as the corresponding proton adduct ions.[18] The compound eluting at 10.6 min (peak D) generated MS/MS data in accordance with DMBA-MFLNH-III (M^+ at *m*/*z* 1338, Fig. 3). The daughter ions at *m*/*z* 1192 and 1176 are in agreement with the loss of the terminal Fuc and Gal residues, respectively. The fragment at *m*/*z* 973 is consistent with the loss of a Gal residue together with a GlcNAc residue, that is, loss of the terminal disaccharide Gal-GlcNAc. The daughter ion at *m*/*z* 827 corresponds to the loss of a terminal trisaccharide containing Fuc, Gal, and GlcNAc, which is supported by the presence of a low-abundance B-type fragment ion at *m*/*z* 512 ($B_{2\alpha}$) formed by cleavage of the glycosidic linkage of the GlcNAc residue. Moreover, if this trisaccharide residue was linear, the cleavage of one single bond could result in the loss of a disaccharide containing Fuc and Gal residues, which would be manifested as a Y-type daughter ion at *m*/*z* 1030 of significant abundance. In the MS/MS spectrum the corresponding ion is of low-abundance indicating that two bonds were cleaved for its formation, and thus that the trisaccharide containing Fuc, Gal, and GlcNAc is a branched residue. The MS/MS data thus enable the identification of all three terminal residues of DMBA-MFLNH-III. A daughter ion at *m*/*z* 737 is formed by a cross-ring cleavage, possibly $^{0,3}X_0$ (Fig. 3), resulting in the loss of the branched trisaccharide and thus yielding information concerning the linkages of the oligosaccharide.

In all ESI-ITMS/MS spectra of the DMBA milk oligosaccharides, a low-abundance fragment ion at *m*/*z* 608 is present (labeled with * in Fig. 3). This ion indicates a DMBA-labeled Y-type fragment constituted by two hexose residues and one deoxy-hexose residue. From the studied oligosaccharides this fragment can only be formed by the migration of a Fuc residue to the common central Gal residue or to the original reducing end Glc residue during the analysis. The fragment at *m*/*z* 446 (also labeled with * in Fig. 3) may be formed from the fragment at *m*/*z* 608 by the loss of a Gal residue, indicating that the migrating Fuc residue is linked to the original reducing end Glc residue. To further

investigate this, all DMBA milk oligosaccharides were subjected to HPLC/ESI-ITMS/MS/MS analysis, by isolation and fragmentation of the respective molecular ion and subsequently of the first generation daughter ion at *m*/*z* 608 (Fig. 4). In the MS/MS/MS spectrum of each DMBA-oligosaccharide, Y-type daughter ions corresponding to the loss of a Fuc residue (*m*/*z* 462) as well as a Gal residue (*m*/*z* 446) were present, together with a Y-type ion consistent with the loss of both a Fuc and a Gal residue (*m*/*z* 300) (Fig. 4). This is consistent with the migration of the Fuc residue to the original reducing end Glc residue of the DMBA-derivative. Furthermore, an ion at *m*/*z* 356 is hypothetically formed by the cleavage of the C-3–C-4 bond of the original reducing end Glc residue (illustrated as X in Fig. 4), indicating that the Fuc residue is linked to C-2 or C-3 of the original reducing end Glc residue. In the ESI-ITMS/MS spectrum of DMBA-LND-I, one additional fragment ion (*m*/*z* 624) is present (Fig. 3), corresponding to three hexose residues together with the DMBA-label, possibly originating from the migration of a Gal residue to the central Gal residue or to the original reducing end Glc residue. The observation of the migration of Fuc residues in derivatives of LND-I, LNF-I, and LNF-II is in accordance with the previous studies by MALDI-FTMS on derivatives prepared by reductive amination with benzylamine or 9-aminofluorene,[19] and by ESI-quadrupole-TOFMS on derivatives prepared by reductive amination with 2-aminobenzamide.[20] Moreover, Fuc migration has also been observed for proton adduct ions of native *N*-glycans,[21] and glycan chain migration has been detected for proton adduct ions of native and per-O-methylated glycan chains of saponins and steroidal glycosides.[22] In all these studies, it was noted that the rearrangement only occurred when the proton adduct ion was subjected to MS/MS analysis and never when the corresponding sodium adduct ion was studied, indicating the protonation to be crucial to this migration. However, the Fuc migration observed on the DMBA-oligosaccharides in this study indicates that protonation is not a prerequisite for the migration. Migrating glycosyl residues may result in incorrect assignment of oligosaccharide structures, particularly if glycosyl migrations result in daughter ions of high relative intensity. The relative intensities of the fragment ions caused by Fuc migrations in the present study (<4% relative to the most intense fragment ion of all tested fragmentation amplitudes) appeared to be substantially lower than in the previously described studies, where fragment ions arising from migrating glycosyl residues in some cases were the most intense daughter ions.[22] It is possible that the same migration mechanism is active for the DMBA-oligosaccharides and for the proton adducts discussed above, but that the rate of the migration is enhanced by the protonation. Alternatively, another mechanism, resulting in a lower migration rate, is active in the DMBA-oligosaccharides. Regardless of the migration mechanism, a low rate of migration is desirable to avoid the risk of misinterpretation of the MS/MS data, which may make the DMBA label a better choice than benzylamine, 9-aminofluorene or 2-aminobenzamide for sequence analysis of oligosaccharides.

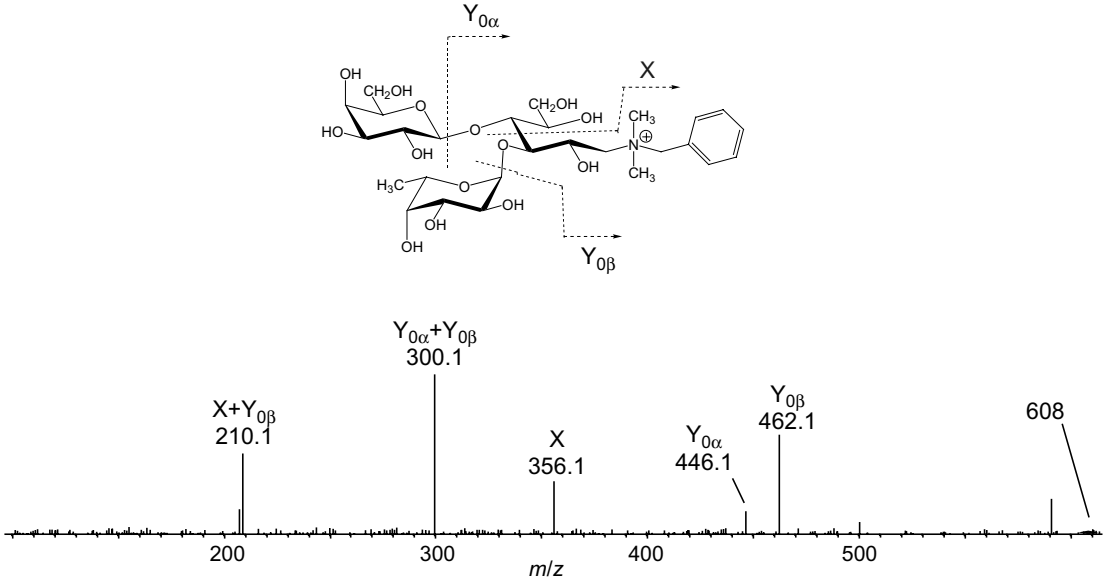

Figure 4. ESI-ITMS/MS/MS spectrum of DMBA-LNF-I, resulting from the isolation and fragmentation of the molecular ion of DMBA-LNF-I (*m*/*z* 973) and subsequently the first-generation daughter ion at *m*/*z* 608, which supposedly is formed by the migration of a Fuc residue. The Fuc residue is arbitrarily illustrated as linked to C-3 of the original reducing end Glc residue, but could equally well be linked to C-2. Tentative assignments of daughter ions are displayed. (X + Y: Ion formation by combination of fragmentation leading to X ion and Y ion.)

The existence of fragments containing a Fuc residue linked to the original reducing end Glc residue may also depend on the hypothetical presence of low amounts of contaminating oligosaccharides in the commercial samples. This, however, seems less likely due to the fact that the commercial samples all were at least 95% pure and that a HPLC separation step preceded the ESI-ITMS analysis.

3. Conclusions

When analyzed by HPLC/ESI-ITMS and HPLC/ESI-ITMS/MS, different DMBA milk oligosaccharides were efficiently separated on a graphitized carbon column and yielded predominantly Y-type daughter ions. Moreover, on-line detection allowed detection at the low femto-mole sample level. Low-intensity fragments, explained by the migration of Fuc residues from different terminal positions on DMBA-oligosaccharides to the original reducing end Glc residue, were observed by HPLC/ESI-ITMS/MS experiments. However, the Fuc migration appeared to be less pronounced than for oligosaccharides derivatized with benzylamine, 9-aminofluorene or 2-aminobenzamide,[19,20] as well as for native oligosaccharides.[21,22] These findings indicate that this methodology is suitable for analysis of, for example, oligosaccharides released from glycoproteins or oligosaccharides from other sources, that is, analytical problems imposing high demands with respect to separation efficiency, sensitivity, and simplicity of MS/MS data interpretation.

4. Experimental

4.1. Materials

Maltoheptaose, $BnNH_2$, $NaCNBH_3$, trifluoroacetic acid, and HOAc were purchased from Sigma (St. Louis, MO, USA), whereas CH_3I, MeOH, and MeCN were from Merck–Schuchardt (Hohenbrunn, Germany). The human milk oligosaccharides α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc (LNF-I), β-D-Gal-(1→3)-[α-L-Fuc-(1→4)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc (LNF-II), α-L-Fuc-(1→2)-β-D-Gal-(1→3)-[α-L-Fuc-(1→4)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc (LND-I), and β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-β-D-GlcNAc-(1→6)-[β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)]-β-D-Gal-(1→4)-D-Glc (MFLNH-III) were purchased from IsoSep AB (Tullinge, Sweden).

4.2. Formation of DMBA-derivatives

Reductive amination with $BnNH_2$, followed by N,N-dimethylation, was performed as previously described.[15] A reagent mixture containing $NaCNBH_3$ (10 mg), MeOH (100 μL), HOAc (12 μL), and $BnNH_2$ (3 μL) was prepared. Each oligosaccharide sample (5–10 nmol in 10 μL H_2O) was mixed with 4 μL of the reagent mixture and 6 μL of MeOH and subsequently treated at 80 °C for 45 min in a sealed Reacti-Vial (Pierce, Rockford, IL, USA). The sample was cooled to room temperature and diluted with H_2O (2 mL) and loaded onto a Carbograph solid-phase extraction column (Alltech, Deerfield, IL, USA) pre-conditioned with 80% aq MeCN (5 mL) containing 0.1% trifluoroacetic acid, followed by H_2O (5 mL). After washing with H_2O (5 mL), the oligosaccharide derivative was eluted with 20% aq MeCN (1 mL), and dried in a vacuum centrifuge in a 1.5 mL plastic tube. The dried sample was dissolved in EtOH (50 μL) and anion-exchange resin (Dowex 1, Sigma, St. Louis, MO, USA) was added. Prior to use, the anion-exchange resin was equilibrated with 1 M aq $NaHCO_3$ and subsequently washed with H_2O and EtOH. CH_3I (5 μL) was added to the mixture, and then it was heated at 60 °C for 20 min. The solution was withdrawn, the ion-exchange resin was washed twice with EtOH (200 μL), and the combined supernatants were dried in a vacuum centrifuge. Prior to analysis, all carbohydrate samples were dissolved in and diluted to the desired concentrations with H_2O.

4.3. HPLC/ESI-ITMS analysis

HPLC/ESI-ITMS, MS/MS, and MS/MS/MS analyses were performed on an HP1100 system (Hewlett-Packard, Palo Alto, CA) using a Hypercarb graphitized carbon column (2.1 × 100 mm, 5 μm; ThermoQuest Runcorn, Cheshire, UK) connected to a Bruker Esquire ion-trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an ESI ion source. Samples (10 μL of DMBA-G7, DMBA-LNF-I, DMBA-LNF-II, DMBA-LND-I, and DMBA-MFLNH-III in H_2O, ~1 μM each for MS/MS operation and 100 μM for MS/MS/MS operation) were injected, and the column was eluted at 0.2 mL/min with aq MeCN (12%, 13% or 14% MeCN). For sensitivity determination, the eluent was 25% MeCN, and 10 μL samples (100 μM to 1 nM) of DMBA-G7 as well as native G7 were injected. Nitrogen gas was used in the nebulizer at a pressure of 40 psi, whereas the spray voltage was 4.5 kV and a flow of nitrogen gas of 9 L/min at 365 °C assisted in the drying process. The ion optics and ion-trap settings were adjusted by the 'smart' option of the software. For each analysis, compound stability was set to 80%, trap drive 120%, and target mass to the actual mass of the analyte (m/z 1100 when analyzing mixtures). Ions were scanned in the range m/z 100–1500 at a scan speed of 13,000 Da/s, and 15 scans were averaged for each spectrum. The maximum number of ions allowed in the ion-trap was set to 15,000 with a maxi-

mum acquisition time of 50 ms. For MS/MS operation the precursor ion isolation width was 2 mass units, and the fragmentation amplitudes (which controls the fragmentation energies) were varied between 0.6 and 2.0 V. For MS/MS/MS the precursor ion isolation width was 3 mass units and the first-generation daughter ion isolation width was 2 mass units, and the fragmentation amplitudes were 1.5 V in both fragmentation cycles.

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