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Research Article

# CE-LIF-MS$^n$ profiling of oligosaccharides in human milk and feces of breast-fed babies

Mixtures of the complex human milk oligosaccharides (HMOs) are difficult to analyze and gastrointestinal bioconversion products of HMOs may complicate analysis even more. Their analysis, therefore, requires the combination of a sensitive and high-resolution separation technique with a mass identification tool. This study introduces for the first time the hyphenation of CE with an electrospray mass spectrometer, capable to perform multiple MS analysis (ESI-MS$^n$) for the separation and characterization of HMOs in breast milk and feces of breast-fed babies. LIF was used for on- and off-line detections. From the overall 47 peaks detected in off-line CE-LIF electropherograms, 21 peaks could be unambiguously and 11 peaks could be tentatively assigned. The detailed structural characterization of a novel lacto-*N*-neo-tetraose isomer and a novel lacto-*N*-fucopentaose isomer was established in baby feces and pointed to gastrointestinal hydrolysis of higher-Mw HMOs. CE-LIF-ESI-MS$^n$ presents, therefore, a useful tool which contributes to an advanced understanding on the fate of individual HMOs during their gastrointestinal passage.

**Keywords:**
Baby feces / Gastrointestinal tract / Human milk oligosaccharides / On-/off-line CE-LIF-MS
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## 1 Introduction

Still today, in times of rapid innovations in food technology with respect to nutritional needs, human breast milk is unambiguously the generally recommended form of nutrition for neonate infants [1–3]. Human milk contains a broad range of bioactive components [4] among them a complex mixture of oligosaccharides (3–19 g/L) [5, 6]. Research on human milk oligosaccharides (HMOs) is challenging due to their enormous structural complexity, which is substantiated by the presence of a large number of isomers. HMOs contain a lactose (L) unit at their reducing end, elongated by *N*-acetylglucosamine (GlcNAc) (either β1,3- or β1,4- linked to the galactose (Gal) residue) as well as by consecutive linear or branched extension consisting of alternating Gals and GlcNAcs. The sugar units can be fucosylated (either α1,2- α1,3- or α1,4-linked) or sialylated (either α2,3 or α2,6-linked) [7]. So far, more than 100 structures have been unraveled in human milk [6, 8, 9]. The structural composition of HMOs is closely related to their biological function (*e.g.* bifidobacterial stimulation, anti-inflammatory properties [6, 7, 10–13]. However, the exact relationship is not yet known. Referring to this, the gastrointestinal tract plays a key role as the location of possible bioconversion and brush border absorption of HMOs. Feces of breast-fed babies are rich in oligosaccharides [14, 15] and their analysis can give essential information on the fate HMOs undergo throughout the gastrointestinal tract passage. So far, only few publications have reported on the analysis of HMOs extracted from the feces of breast-fed babies [14–17].

In the past, normal- and reversed-phase HPLC, high-performance anion exchange chromatography and MALDI-TOF MS analysis were the methods most frequently used for HMO analysis [18–21]. Nowadays, the hyphenation of effective separation techniques with an MS device plays an important role in the structural analysis of complex analyte mixtures [22]. HPLC-Chip/TOF-MS has been recently introduced as a potential analytical tool for the analysis of HMOs in breast milk [8, 23].

CE and CE-MS coupling are other state-of-the-art methods, which recently show a tremendous development. They were introduced to a broad range of research fields (*e.g.* biomedical analysis) and are outstanding in view of separation efficiency and selectivity [22]. Regarding the

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**Abbreviations: HMO,** human milk oligosaccharides; **APTS,** 9-aminopyrene-1,4,6-trisulfonate; **DF-L,** difucosyllactose; **DS-LNT,** disialyllacto-*N*-tetraose; **F,** fucose; **FL,** fucosyllactose; **F-LNH,** fucosyllacto-*N*-hexaose; **Gal,** galactose; **Glc,** glucose; **GlcNAc,** N-acetylglucosamine; **Hex,** hexose; **L,** lactose; **LNDFH,** lacto-*N*-difucosylhexaose; **LNFP,** lacto-*N*-fucopentaose; **LNT,** lacto-*N*-tetraose; **LnNT,** lacto-*N*-neotetraose; **SL,** sialyllactose; **S-LNT,** sialyllacto-*N*-tetraose

analysis of oligosaccharide mixtures, CE is well known for its high resolving power and sensitivity [24–27]. Some commercially available HMO isomeric standards have even been successfully separated by CE, *e.g.* 2′fucosyllactose (2′FL)/3′FL or lacto-*N*-fucopentaose I/II (LNFP I/II) [28–31]. LIF detection presents one of the most favorable and most frequently used ways of routine detection for carbohydrates separated by CE [32]. The nonchromophoric carbohydrates are commonly derivatized with a fluorophore, *e.g.* 9-aminopyrene-1,4,6-trisulfonate (APTS) or 8-aminonaphtalene-1,3,6-trisulfonate *via* reductive amination [33, 34] prior to CE-LIF analysis. This results in a low-picomole sensitivity, mol-based fluorescent detection and the provision of charged groups, which is a prerequisite for electrophoretic separation [35]. CE-LIF presents an attractive technique for the analysis of complex biological samples, but the identification of the migrating analytes depends on the analysis of reference standards, which are often expensive, difficult to obtain or even not available. Furthermore, co-migration cannot be excluded solely based on migration times of standards. CE-MS hyphenation presents an attractive way of overcoming these limitations. Some of the parameters applied for routine CE-LIF analysis have to be modified due to technical and compatibility limitations between CE and MS device, *e.g.* the extension of capillary length and the composition of the separation buffer [27, 32, 36–38]. In contrast to LC-(UV/DAD)-MS, CE-LIF analysis implies a proceeding separation in the CE capillary, even after the LIF-detector window, due to the voltage set between cathode and anode at the MS interface. A solution for interlinking off-line CE-LIF analysis (no MS detector included) and CE-MS is the inclusion of an on-line LIF detector in front of the MS inlet to assure comparison between off-line CE-LIF electropherograms with MS base peak chromatograms. The first studies on the use of on-line CE-LIF-MS for the analysis of *N*-linked glycans showed promising results [36].

This study introduces for the first time the hyphenation of ESI-MS with CE for the characterization of HMOs in breast milk and feces of breast-fed babies, simultaneously making use of an on-line LIF detector. Special attention was paid to the elucidation of a lacto-*N*-neo-tetraose (LnNT) and LNFP isomer not present in breast milk, but present in the feces of breast-fed babies.

## 2 Materials and methods

### 2.1 Sample material, HMO reference material and chemicals

Breast milk samples of five human individuals and fecal slurries (1:10 w/v in phosphate-buffered saline) of three individual breast-fed preterm babies were provided by FrieslandCampina (FrieslandCampina, Deventer, The Netherlands). Preterm birth took place at 32 wk of pregnancy. Feces were collected 22–24 days after birth.

3′FL, lacto-*N*-tetraose (LNT), LNFP I-III, lacto-*N*-difucosylhexaose (LNDFH), monofucosyl-lacto-*N*-hexaose III and a mixture composed of 3′sialyllactose, 6′sialyllactose and 6′sialylGlcNac were purchased from Dextra Laboratories (Reading, UK). LNFP V was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

### 2.2 HMO extraction from breast milk and fecal extracts

The extraction of carbohydrates from breast milk was accomplished as performed by Stahl *et al.* [19] and included a mild temperature treatment (30 min, 70°C), defatting at 4°C as well as a protein precipitation by adding the double amount v/v of cold ethanol. Diluted fecal slurries were kept over night at 4°C and filtered through a 0.22 μm membrane according to Moro *et al.* [39], after a short enzyme inactivation (5 min, 100°C).

The carbohydrates extracted from breast milk and fecal extracts were purified by SPE on graphitized carbon column cartridges (150 mg bed weight, 4 mL tube size; Alltech, Deerfield, IL, USA) [8]. The cartridges were washed with 1.5 mL of 80/20 v/v ACN/water containing 0.1% v/v TFA followed by 1.5 mL of millipore water. One milliliter of the sample extract was loaded onto a cartridge, which was subsequently eluted with 1.5 mL millipore water to remove salts. Next, monomers and L were largely removed by eluting with 1.5 mL of an aqueous 2% v/v ACN solution. This fraction was not further considered for analysis. The remaining carbohydrates (HMOs) on the cartridge were eluted with 1.5 mL of 40/60 v/v ACN/water containing 0.05% v/v TFA. The solution was dried under a stream of air and the dried sample was then rehydrated with 500 μL of millipore water.

### 2.3 Off-line CE-LIF

For CE analysis, SPE-extracted carbohydrates present were derivatized with the fluorescent APTS overnight at room temperature as reported elsewhere [26]. Briefly, 1 nmol xylose was added to each sample volume (100–200 μL) as internal standard and mobility marker. The solutions containing the fluorescent derivatives were diluted 20 times before CE-LIF analysis. Off-line CE-LIF was performed on a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA, USA), equipped with a LIF detector (excitation: 488 nm, emission 520 nm) (Beckman Coulter) and a polyvinyl alcohol (N–CHO) coated capillary (50 μm id × 50.2 cm (Beckman Coulter), detector after 40 cm), kept at 25°C. The samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL sample solution) on the capillary. Separation was performed in the reversed polarity mode (30 kV, 20 min) in the 25 mM sodium acetate buffer containing 0.4% polyethylene oxide provided in the derivatization kit. Due to the low $pK_a$ of the sialic acid residues ($pK_a = 2.6$) and in order

 

to provide the same pH as for CE-MS, the separation buffer was adjusted to pH 2.4 by adding 1.2% v/v formic acid [38]. Peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA, USA).

### 2.4 On-line CE-LIF-ESI-MS$^n$

On-line CE-LIF-ESI-MS$^n$ experiments were performed on a P/ACE$^{TM}$ System MDQ (Beckman Coulter). For the fluorescent detection, a Picometrics ZetaLIF discovery system was used (Picometrics, Toulouse, France) (excitation: 488 nm, emission 520 nm). The LIF detection cell was placed approx. 25 cm from the ESI-MS source by an adjustable arm. Separation was performed on a fused silica capillary (50 μm id × 85 cm (Beckman Coulter), capillary window fitted with an ellipsoid for LIF detection after 60 cm) in reversed polarity mode. LIF signals were sent to Beckmann 32Karat software *via* an Agilent SSXL4002 converter (Agilent Technologies, Santa Clara, CA, USA). Best separation results were obtained at 20 and 15 kV in 0.3% v/v formic acid (pH 2.4) and a capillary temperature of 15°C, after sample injection at 10 psi for 2 s. The sample solutions containing the fluorescent derivatives were diluted four times or ten times before CE-LIF-MS analysis. The ESI-MS (LTQ ion trap, Thermo Fisher Scientific, Waltham, MA, USA) was operated in the negative mode using a spray voltage of 1.9 kV and an MS-capillary temperature of 190°C. The end of the CE capillary was installed in front of the ESI source by leading it through a T-part designed in our laboratory [37] and provided the coaxial addition of a sheath liquid (50/50 isopropanol/water) at 2 μL/min. Mass spectra were acquired from $m/z$ 300 to 2000. MS$^n$ was performed in the data-dependent mode using a window of $m/z$ 1 and collision energy of 35%. For increasing the $S/N$, ions of $m/z$ 311, 314 and 329 were excluded from detection in MS$^n$ experiments.

MS$^n$ data were interpreted using Xcalibur software 2.0.7 (Thermo). Base peak chromatograms are shown in their smoothed forms.

## 3 Results and discussion

### 3.1 Introducing on-line CE-LIF-MS for the analysis of breast milk and fecal samples

Assuring the compatibility of CE separation and ESI-MS analysis requires the adaption of several parameters commonly applied for off-line CE-LIF assays. In our system, it included the change of capillary material from neutral surface to fused silica [38] and the introduction of a volatile separation buffer (formic acid 0.3% v/v, pH 2.4) [32, 37]. Of main importance is the capillary length, which had to be extended considerably to a length of 85 cm in order to reach the MS inlet, leading to an on-going separation and longer migration times. In off-line CE-LIF analysis, capillaries with a detection window after 40 cm are generally used [26, 36–38]. Placing an on-line LIF detector just in front of the MS inlet presents, therefore, an attractive means for transferring mass information to the electropherograms recorded by the off-line CE-LIF detector [36]. In our system, this was accomplished by a Picometrics LIF detection cell located on an adjustable arm, which was placed after 60 cm capillary length. An overview of the off-line and on-line CE-LIF electropherograms as well as the respective MS base peak chromatograms is shown in Figs. 1A–E for a randomly chosen breast milk extract and a fecal extract of a breast-fed preterm baby. Off-line CE-LIF resulted in the most detailed separation profile of the samples and could be well compared with the MS base-peak chromatograms. On-line LIF electropherograms showed some peak overlap, which was not the case for the MS base peak chromatograms, due to ongoing electrophoretic separation between LIF and MS detector. The time difference between on-line LIF peak detection and MS detection was 5 min, which is small compared with ≥10 min reported in earlier studies [27, 38]. A sheath-flow interface was used for transferring liquid from the CE capillary outlet to the MS inlet. This results, however, in a dilution of the analyte and a reduced detection sensitivity [32]. Appropriate MS detection requires thus a sample injection of increased pressure and length (2 psi/10 s in place of 0.5 psi/7 s) and less dilution of the solutions containing the APTS-derivatized carbohydrates compared with off-line CE-LIF (4 times instead of 20 times). The higher injection volume resulted in overloaded on-line LIF electropherograms. Lowering the separation voltage from 20 to 15 kV and injecting a ten times instead of a four times diluted sample resulted in the most optimal on-line LIF separation profile, as shown for the electropherogram of breast milk (Fig. 2). The overall oligosaccharide migration window was extended from 7.4 min (20 kV) to 10.6 min (15 kV) ($\Delta t = 3.2$ min). As a consequence, baseline separation was improved (*e.g.* peaks 9/10 or 11/12 (Fig. 2)). Because of peak widening, which accompanies the separation at lowered voltage, now only the most abundant HMOs (Fig. 2, peaks 1–3, 5, 7, 8, 10/11) resulted in peaks in the base peak chromatograms (data not shown) and thus implies less mass information.

For breast milk, 19 peaks were detected in the on-line CE-LIF electropherograms, whereas 37 peaks were detected with off-line CE-LIF analysis. Off-line CE-LIF detection showed superior sensitivity, however, no mass information is obtained during off-line CE-LIF measurement. On-line LIF detection was, therefore, considered a valuable tool for properly interlinking the simultaneously obtained information on peak mass to the corresponding off-line CE-LIF electropherogram.

### 3.2 Characterization of breast milk and fecal extracts by CE-LIF-MS

#### 3.2.1 General overview

The off-line CE-LIF electropherograms of a breast milk extract and of a fecal sample of a breast-fed baby are shown

 

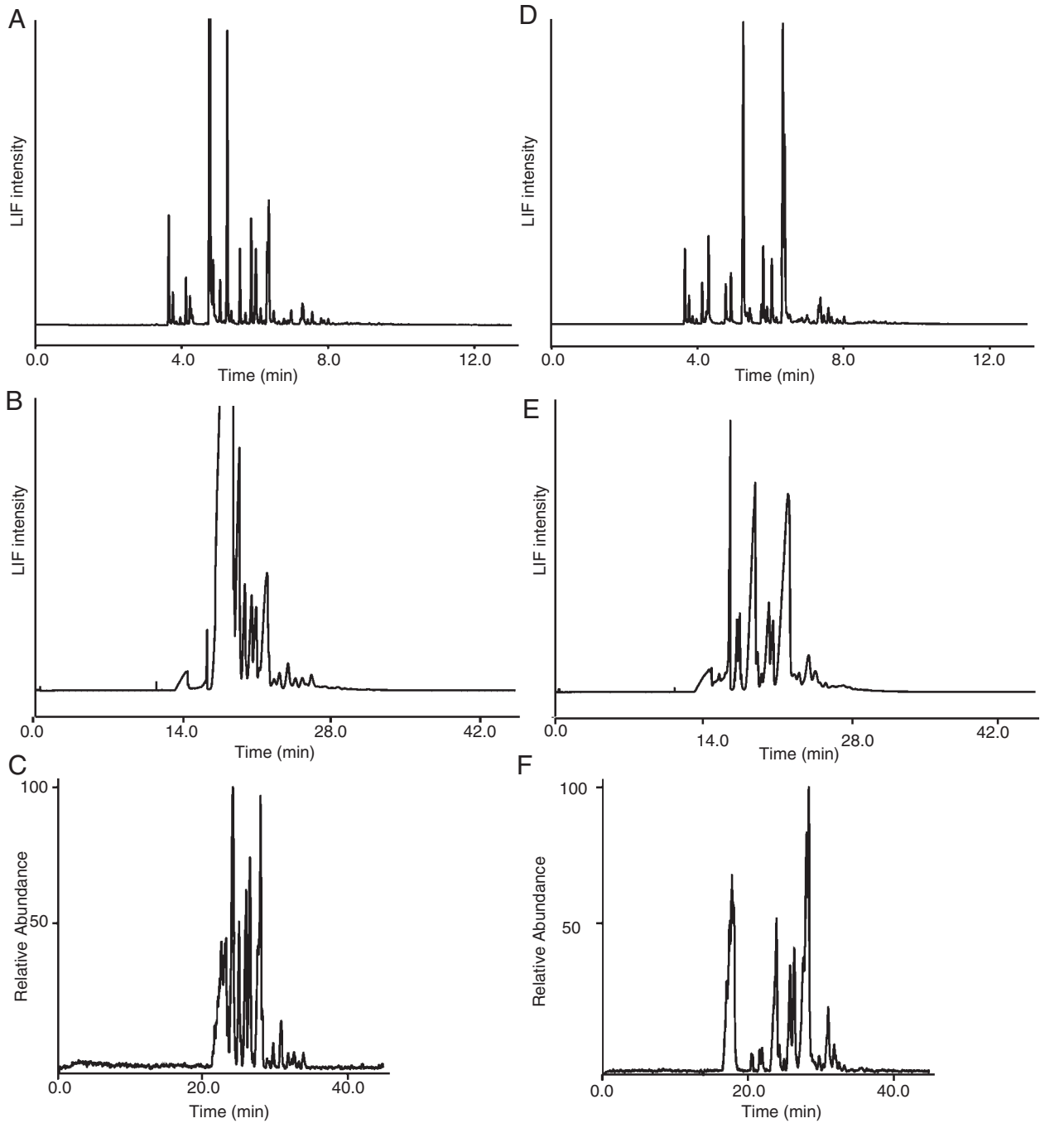


**Figure 1.** APTS-derivatized HMOs extracted from breast milk (A–C) and feces of a breast-fed baby (D–F). (A, D) Off-line CE-LIF electropherograms. (B, E) On-line CE-LIF electropherograms. (C, F) MS base peak chromatograms.

in Fig. 3. The samples chosen represent the oligosaccharide patterns of individuals and the breast milk depicted does not correspond to the breast milk the preterm baby was fed with. However, the oligosaccharide pattern is representative for the patterns commonly observed in all samples extracted, taking into account individual variation. For both extracts, a detailed profile of major and minor peaks was obtained within a separation window of 4.5 min. Peaks observed between 3.5 and 4 min were defined as degradation products of the fluorescent APTS, as defined in an earlier study [37]. The peak marked with an asterisk (*) represents the internal standard and mobility marker xylose, which was added during sample preparation. For a better overview, peaks were classified as ''low-Mw HMOs'' ($t_{migr}$ = 4.8–6.4 min), ''intermediate-Mw HMOs'' ($t_{migr}$ = 6.5–8 min) and ''high-Mw HMOs'' ($t_{migr}$ > 8 min) in this study. The number of peaks detected were 37 and 36 for breast milk and fecal sample, respectively. Summing up,

 

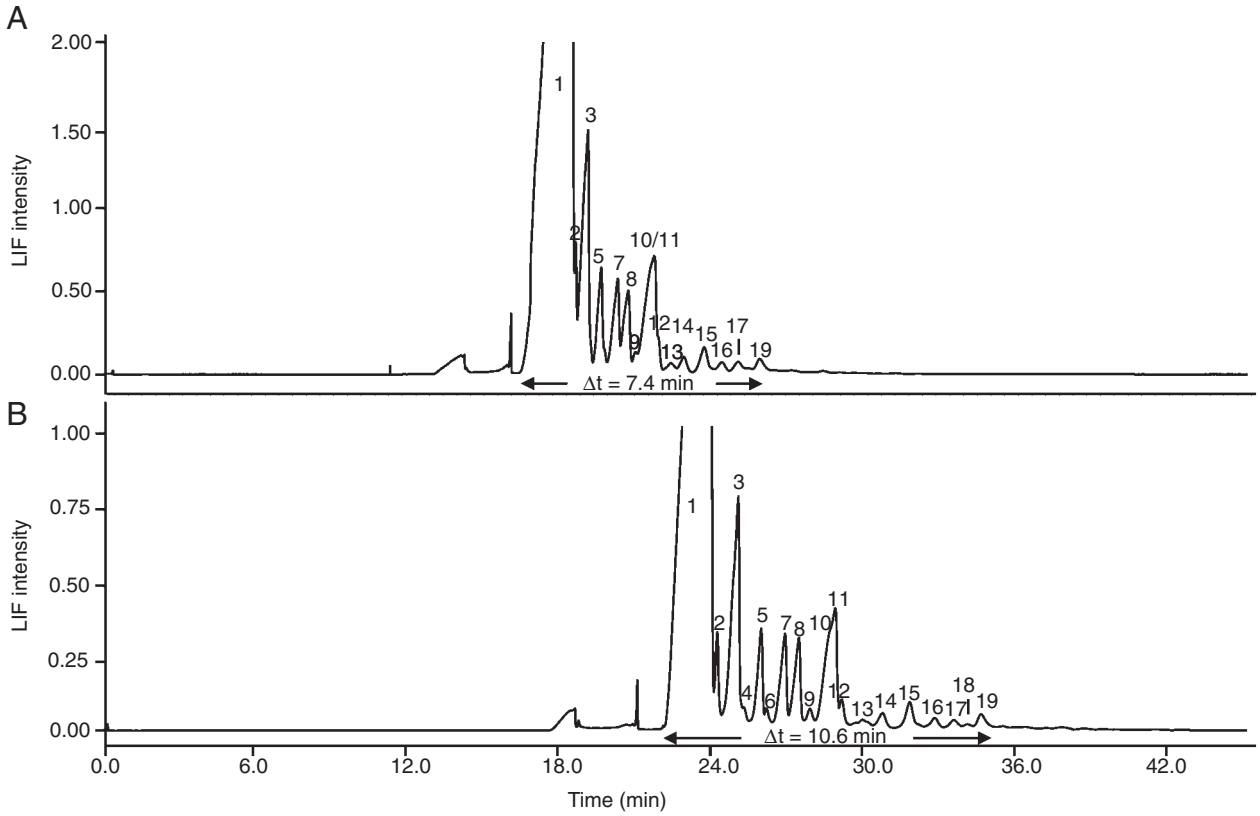


**Figure 2.** On-line CE-LIF electropherograms of APTS-derivatized HMOs extracted from breast milk. Separation conditions: FA buffer (0.3%, pH 2.4); separation-T: 15°C; injection: 2 s, 10 psi; (A) separation voltage: 20 kV, sample dilution: 4 ×. (B) separation voltage: 15 kV, sample dilution: 10 ×. *1–19*: peaks detected; partly identified by assigning with MS data (Table 1). $\Delta t$ indicates the total sample migration range.

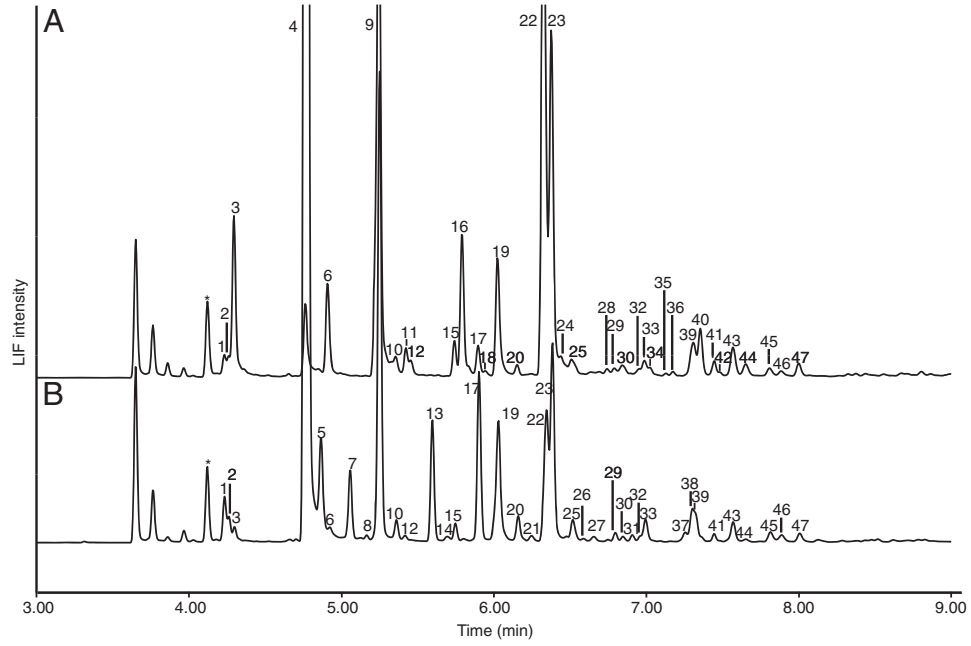


**Figure 3.** Off-line CE-LIF electropherograms of APTS-derivatized HMOs extracted from (A) feces of a breast-fed baby and (B) breast milk.. *1–47*: peaks detected; partly identified by assigning with MS data (Table 1). *internal standard xylose.

there were 47 different peaks present in the two electropherograms (peaks present in both electropherograms were counted once only). In total, 22 of these 47 peaks were unambiguously and 11 were tentatively assigned (Table 1), mainly by means of MS data obtained from on-line CE-LIF-MS analysis and supported by $MS^2$ fragmentation and the

 

**Table 1.** Structural assignment, composition and relative abundancy of peaks detected in representative off-/on-line CE-LIF electropherograms of APTS-derivatized HMOs extracted from breast milk and feces of a breast-fed baby. APTS-derivatized carbohydrates detected in threefold negatively charged state by ESI-MS analysis (APTS = three protoneable sulfonate groups)

| Peak off-line CE-LIF (Fig. 3) | Peak on-line CE-LIF (breast milk) (Fig. 2) | $t_{migr}$ (min) off-line CE-LIF (breast milk) (Fig. 3) | $t_{migr}$ (min) off-line CE-LIF(fecal extract) (Fig. 3) | *m/z* (APTS 3-) | Hex | GlcNAc | Fucose | Neu5Ac | Name (abbreviation) | Peak area rel. to ISTD (Fig. 3, breast milk) | Peak area rel. to ISTD (Fig. 3, fecal extract) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| * | – | 4.12 | 4.13 | 196 | – | – | – | – | Xylose ISTD | 1 | 1 |
| 1 | – | 4.23 | 4.24 | 206 | 1 | 0 | 0 | 0 | Glc | n.d. | n.d. |
| 2 | – | 4.26 | 4.27 | 206 | 1 | 0 | 0 | 0 | Gal | n.d. | n.d. |
| 3 | – | 4.30 | 4.30 | 201 | 0 | 0 | 1 | 0 | F | n.d. | n.d. |
| 4 | 1 | 4.75 | 4.77 | 260 | 2 | 0 | 0 | 0 | L | n.d. | n.d. |
| 6 | – | 4.92 | 4.92 | 255 | 1 | 0 | 1 | 0 | F-Hex | 0.2 | 1.5 |
| 7 | 2 | 5.06 | – | 357 | 2 | 0 | 0 | 1 | SL | 1.0 | – |
| 9 | 3 | 5.25 | 5.25 | 309 | 2 | 0 | 1 | 0 | FL | 6.9 | 9.6 |
| 11 | – | – | 5.43 | 382 | 3 | 1 | 0 | 0 | LnNT Y | – | 0.4 |
| 13 | 5 | 5.60 | – | 358 | 2 | 0 | 2 | 0 | DF-L | 1.8 | – |
| 15 | 6 | 5.75 | 5.75 | 382 | 3 | 1 | 0 | 0 | LnNT | 0.2 | 0.5 |
| 16 | – | – | 5.80 | 431 | 3 | 1 | 1 | 0 | LNFP Y | – | 2.2 |
| 17 | 7 | 5.90 | 5.91 | 382 | 3 | 1 | 0 | 0 | LNT | 2.5 | 0.5 |
| 19 | 8 | 6.03 | 6.03 | 431 | 3 | 1 | 1 | 0 | LNFP II | 2.4 | 2.0 |
| | | | | 576 | 3 | 1 | 0 | 2 | DS-LNT | | |
| 20 | 9 | 6.16 | 6.16 | 479 | 3 | 1 | 0 | 1 | S-LNT | 0.4 | 0.2 |
| 22 | 10 | 6.34 | 6.34 | 431 | 3 | 1 | 1 | 0 | LNFP I/III | 2.5 | 9.2 |
| 23 | 11 | 6.38 | 6.39 | 479 | 3 | 1 | 2 | 0 | LNDFH | 3.2 | 5.5 |
| 25 | 12 | 6.52 | 6.52 | 479 | 3 | 1 | 2 | 0 | LNDFH | 0.4 | 0.4 |
| | | | | 479 | 3 | 1 | 0 | 1 | S-LNT/ | | |
| 26–32 | 13 | 6.59–6.95 | | 479 | 3 | 1 | 2 | 0 | LNDFH | 0.3 | 0.1 |
| | | | | 504 | 4 | 2 | 0 | 0 | LNH | | |
| | | | | 528 | 3 | 1 | 1 | 1 | FS-LNT | | |
| 33 | 14 | 6.99 | 7.00 | 552 | 4 | 2 | 1 | 0 | F-LNH II | 0.4 | 0.3 |
| 38 | 15 | 7.30 | – | | | | | | | 0.6 | – |
| 39 | 15 | 7.32 | 7.32 | 601 | 4 | 2 | 2 | 0 | DF-LNH | 0.4 | 0.7 |
| 40 | 15 | – | 7.37 | | | | | | | – | 0.8 |
| 43 | 16 | 7.57 | 7.58 | 650 | 4 | 2 | 3 | 0 | TF-LNH | 0.4 | 0.5 |
| | | | | 601 | 4 | 2 | 2 | 0 | DF-LNH/ | | |
| | | | | 601 | 4 | 2 | 0 | 1 | S-LNH | | |
| 44–47 | 17–19 | 7.65–8.01 | | 649 | 4 | 2 | 1 | 1 | FS-LNH | 0.6 | 0.7 |
| | | | | 650 | 4 | 2 | 3 | 0 | TF-LNH | | |
| | | | | 674 | 5 | 3 | 1 | 0 | F-LNO | | |
| | | | | 723 | 5 | 3 | 2 | 0 | DF-LNO | | |
| | | | | 771 | 5 | 3 | 3 | 0 | TF-LNO | | |
| – | – | < 8.01 | | 820 | 5 | 3 | 4 | 0 | tetra-F-LNO<br>Sialylated HMOs | – | – |

n.d., not defined; Neu5Ac, sialic acid; FS-LNT, fucosyl-sialyllactotetraose; LNH, lacto-*N*-hexaose; DF-LNH, difucosyllacto-*N*-hexaose; TF-LNH, trifucosyllacto-*N*-hexaose; F-LNO, fucosyllacto-*N*-octaose; DF-LNO, difucosyllacto-*N*-octaose; TF-LNO, trifucosyllacto-*N*-octaose.
*, internal standard xylose.

analysis of standard substrates. The mol-based labeling with APTS allowed to calculate the relative amounts of HMOs present *per* sample, based on the area of internal standard added (xylose, 1 nmol) (Table 1). Monomers and L were not considered as the sample pretreatment on graphitized carbon material and the ethanolic extraction of breast milk is connected with a loss of monomers and L [40, 41].

Components migrating $t > 8$ min could not be identified due to their low abundance. As the electrophoretic mobility is generally decreasing with the Mw of the oligosaccharides [26, 37], these are most probably representing high-Mw HMOs. MS data gave first indications hereof (Table 1).

### 3.2.2 Identification of oligosaccharides in breast milk and fecal extracts

Obviously, HMOs are present in breast milk in adequate proportions for surviving the gastrointestinal tract passage and the overall CE pattern can be found back in the feces of breast-fed babies (Fig. 3). Though, considering individual peaks of the electropherograms, marked differences were observed concerning their presence and relative abundance in breast milk and feces. The fecal samples originated from preterm babies. Preterm birth might imply differing gastrointestinal bioconversion compared with term babies. However, this was not studied within the scope of this research.

#### 3.2.2.1 Low-Mw, neutral HMOs

L was clearly the most abundant peak found in breast milk samples (Fig. 3B, peak 4). Although *in-vivo* studies reported immature lactase activity with preterm babies [42], the low L peak present in fecal extracts (Fig. 3A, peak 4) indicated increased lactase activity at 22–24 days after birth or a different gastrointestinal utilization, *e.g.* membrane transport by intestinal bacteria. It was not possible to define peak 5 (Fig. 3B) present in breast milk, but not in the fecal extract, in spite of its abundance. MS base peak chromatograms of breast milk did not give any information here over. In fecal samples, a peak composed of a hexose and fucose (F) unit (F-Hex) migrated at $t = 4.92$ min (Fig. 3A, peak 6), which was present in much smaller quantities in breast milk. F-Hex was, therefore, considered as a fragment resulting from gastrointestinal degradation of HMOs. Within both, fecal extracts and breast milk, FL was the most abundant HMO (Fig. 3, peak 9). Unfortunately, it was not possible in this study to distinguish between 3′FL (Gal(β1,4)-[F(α1,3)]-Glc) and 2′FL (F(α1,2)-Gal(β1,4)-Glc), which is the more prominent isomer present in breast milk [7, 30]. Having a closer look on the HMOs up to an $m/z$ 500 in breast milk, LNDFH (Fig. 3B, peak 23), LNT (Fig. 3B, peak 17), LNFP I/III (Fig. 3B, peak 22), LNFP II (Fig. 3B, peak 19) and difucosyllactose (DF-L) (Fig. 3B, peak 13) were found to be prevailing (ratio 1:0.8:0.8:0.7:0.5). Within the fecal extract, these peaks were found to be present in a completely different ratio (ratio 1:0.1:1.7:0.4:0), indicating an active gastrointestinal bioconversion. The absence of DF-L within the fecal extract indicated gastrointestinal fucosidase activity (Fig. 3A) [13]. On the contary, the remaining high abundance of FL and LNFP isomers in fecal extracts (Fig. 3A, peaks 9 and 16, 19, 22), which probably results from the enzymatic degradation of higher-Mw HMOs and DF-L, indicates a certain limitation of gastrointestinal fucosidase activity toward HMO structures. LNT, which is a nondecorated tetramer, seems to be a highly susceptible substrate for enzymatic attack, as it was present in only low amounts in the fecal extract (Fig. 3A, peak 17). The peak ratios found were highly individual dependent, but always showed the same trend.

#### 3.2.2.2 Acidic HMOs and intermediate-Mw HMOs

Besides neutral HMOs, acidic HMOs decorated with one or more sialic acid groups form an important part of HMOs, although present in much lower amounts [6]. Sialyllactose (SL), disialyllacto-*N*-tetraose (DS-LNT) and sialyllacto-*N*-tetraose (S-LNT) (Fig. 3B, peaks 7, 19, 20) were identified in breast milk. Fecal extracts did not contain any SL or DS-LNT and showed a reduced abundance of S-LNT (Fig. 3A, peak 20). These observations support results of *in-vivo* studies on the gastrointestinal sialidase activity [13, 15]. The assignment of intermediate-Mw sialylated HMOs is complicated by the fact that their $m/z$ values partly coincide with the $m/z$ values of neutral HMOs. Therefore, peaks 26–32 and 44–47 present in breast milk and fecal extracts (Fig. 3) were assigned tentatively only. More detailed $MS^n$ analysis of these intermediate-Mw HMOs is necessary. In general, intermediate-Mw HMOs (Fig. 3, peaks 24–47) showed a large complexity. A definite assignment was not possible so far due to frequent co-migration caused by the increasing number of possible isomers for these structures.

#### 3.2.2.3 Unknown HMOs

The individual variation of HMOs in breast milk and their recovery in feces has been reported previously [7, 16, 23, 30]. However, in most cases the identification of HMOs was limited to the purified HMOs commercially available or connected with tedious preparative fractionation. CE-LIF-MS presents an efficient way for a sensitive and mol-based separation of the complex HMOs combined with a simultaneous availability of mass information. This is of special importance for the identification of peaks in the fecal extract, which were not present in breast milk. MS base peak chromatograms indicated the presence of a new LNFP structure (Fig. 3A, peak 16) and a new LNT structure (Fig. 3A, peak 11). Their characterization, based on MS fragmentations of commercially available HMO isomers, was of special interest in order to understand the gastrointestinal breakdown of HMO structures. It is, therefore, addressed separately.

 

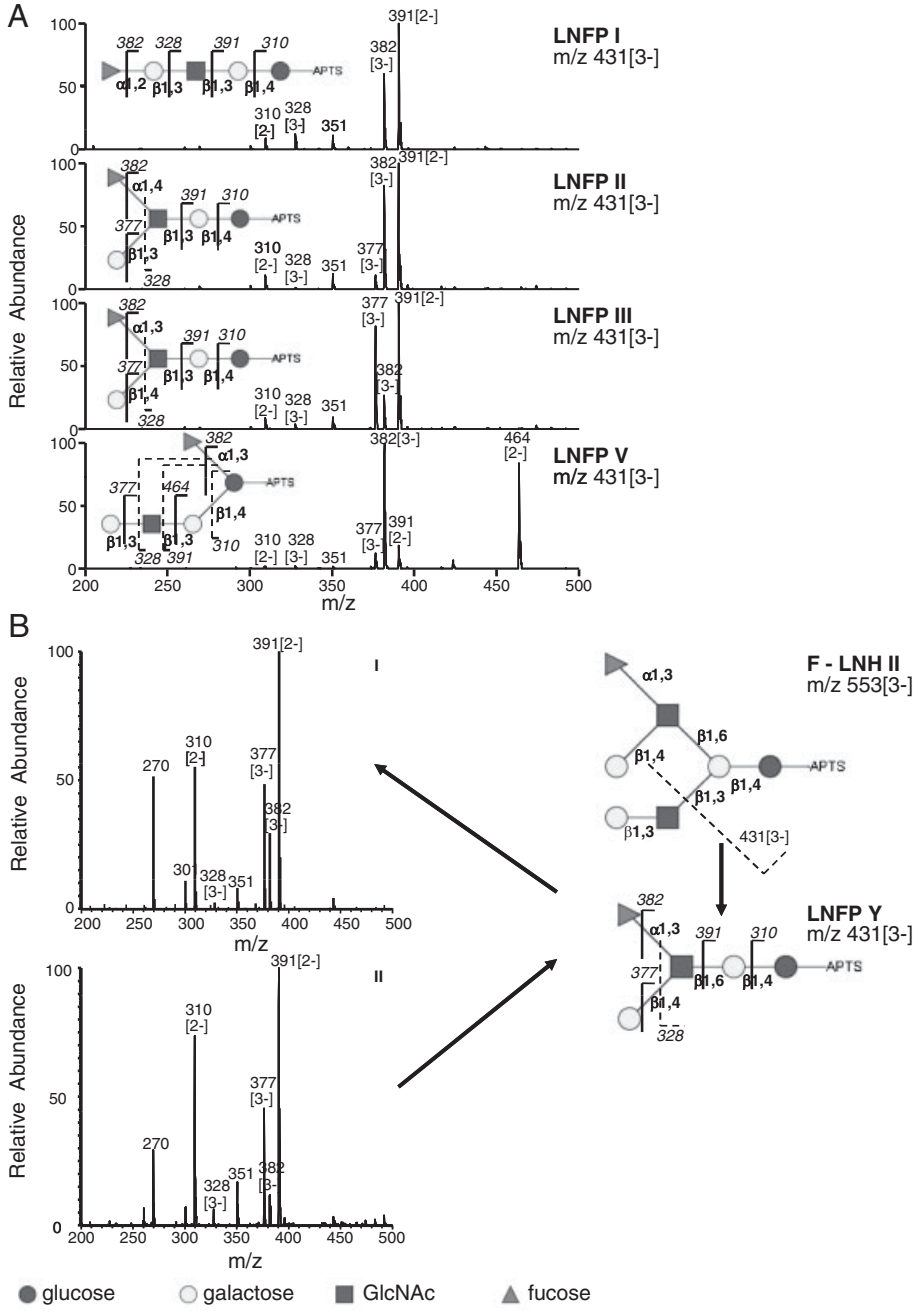


**Figure 4.** (A) $MS^2$ fragmentation patterns of APTS-derivatized standards LNFP I, II, III, V (*m/z* 431). (B) Identification of LNFP Y in feces of breast-fed babies. (B I) $MS^2$ fragmentation pattern of *m/z* 431 (precursor ion: APTS-derivatized standard F-LNH II, *m/z* 553). (B II) $MS^2$ fragmentation pattern of LNFP Y. [2-] [3-]: charge state of APTS-molecule.

### 3.3 Structural characterization of LNFP Y and LnNT *Y* in fecal extracts

#### 3.3.1 LNFP I, II, III and V

Compared with breast milk, additional peaks were detected in the feces of breast-fed babies. In order to be able to characterize the unknown LNFP structure in fecal extracts of breast-fed babies (LNFP Y, *m/z* 431, Fig. 3A, peak 16), CE-LIF-$MS^2$ experiments on commercially available LNFP isomers were performed. Four isomeric LNFP structures are known to be present in breast milk [44, 45], namely LNFP I, II, III and V (Fig. 4A). The migration time of the unknown LNFP isomer did not coincide with any of the LNFP standards.

$MS^2$ fragmentation of underivatized LNFP isomers has been studied earlier [28, 46]. Compared hereto, deviating $MS^2$ fragmentation patterns were obtained in this study due to the APTS derivatization of the isomers LNFP I, II, III and V (Fig. 4A). The fragments of interest were exclusively Y fragments [43]. Due to the derivatization at the reducing end, no cross-ring cleavages were expected [28, 45].

LNFP I has a linear structure and *m/z* 328 (corresponding to the loss of the terminal F and Gal unit) is

 

characteristic for this linear structure [28]. For the branched LNFP II, III and V, *m/z* 328 demands the cleavage of 2 glycosidic linkages and explains the lower abundance of this fragment. Another main characteristic feature is the ratio of the fragments *m/z* 377 (loss of the terminal Gal; not possible for LNFP I) and 382 (loss of the F residue). LNFP II and V, which both possess a (β3,1)-linked terminal Gal, show an abundance of *m/z* 377 < *m/z* 382, whereas for LNFP III, which possesses a (β4,1)-linked terminal Gal, the abundancy is *m/z* 377 > *m/z* 382.

LNFP V showed a predominating fragmentation ion of *m/z* 464 [Gal-[F]-Glc-APTS]. It is characteristic for an F residue being attached to the reducing glucose (Glc) moiety and, therefore, such fragmentation is not possible for any of the other LNFP isomers.

### 3.3.2 LNFP Y

The fragmentation pattern of LNFP Y (Fig. 3A, peak 16) did not coincide with any of the known LNFP isomers. Based on the assumption that LNFP Y results from gastrointestinal degradation, the structural characteristics of higher-Mw HMOs were studied. The unit -Gal(β1,6)- frequently occurs at the branching points of these higher-Mw HMOs (exemplified for F-LNH II (F-LNH, fucosyllacto-*N*-hexaose), *m/z* 553, Fig. 4B). F-LNH II (*m/z* 553) was chosen for a MS-fragmentation study. The $MS^2$ spectrum of F-LNH II contained the fragment *m/z* 431 (LNFP), which corresponds to the loss of a Gal and a GlcNAc unit at the branching point of F-LNH II. This *m/z* 431 fragment was of special interest as it contained the -Gal(β1,6)- unit and was, therefore, subjected to a subsequent MS fragmentation. The fragmentation pattern showed *m/z* 377 > *m/z* 382, which is representative for its (β1,4)-linked terminal Gal (see LNFP III), and an increased signal for *m/z* 310 (Hex-APTS), deduced from its -Gal(β1,6)- unit. The *m/z*–value 310 was not prominently present in any other LNFP isomer fragmentation pattern (Fig. 4B). LNFP Y in fecal extracts showed the same MS-fragmentation pattern and was, therefore, characterized as a breakdown product of higher-Mw HMOs, possessing a -Gal(β1,6)- unit and a (β4,1)-linked terminal Gal (Fig. 4B).

LNFP isomers are present in considerable quantities in fecal extracts. The hydrolysis of the -GlcNAc(β1,3)-Gal- linkage at branching points of higher-Mw HMOs (Fig. 4B) results in the formation of LNFP structures or larger structures further hydrolysable to LNFP. The -GlcNAc(β1,3)-Gal- linkage was therefore assumed to be a preferred site of enzymatic attack during the gastrointestinal passage. Compared hereto, de-fucosylation was considered to be of minor importance.

### 3.3.3 LNT, LnNT and LnNT Y

Further characterization was as well needed for the unknown LnNT structure found in fecal extracts of breast-fed babies (LnNT Y, *m/z* 382, Fig. 3A, peak 11). Two LNT isomers were detected in breast milk (Fig. 3B, peaks 15 and 17). For breast milk, the presence of two LNT isomers is known, namely LNT (Gal(β1,3)-GlcNAc(β1,3)-Gal(β1,4)-Glc) and LnNT (Gal(β1,4)-GlcNAc(β1,3)-Gal(β1,4)-Glc), representing nonfucosylated LNFP I, II, V and LNFP III, respectively [7, 8, 47]. The migration time and $MS^2$ pattern of the LNT standard was the same as peak 17 (Fig. 3). Peak 15 (Fig. 3) was, therefore, assigned as LnNT and showed a very similar $MS^2$ pattern to LNT, as expected. In the fecal extract, three isomeric LNT structures were detected (Fig. 3A, peak 11 [LnNT Y], 15 [LnNT] and 17 [LNT]). The $MS^2$ pattern of LnNT Y differed from the ones of LnNT and LNT, but showed the same pattern as LNFP Y, including an abundantly present fragment *m/z* 310, typical for a Gal(β1,6)- linkage. LnNT Y was therefore characterized as a de-fucosylated LNFP Y structure (Gal(β1,4)-GlcNAc(β1,6)-Gal(β1,4)-Glc) and was present in lower abundance compared with LNFP Y (1:0.2, Table 1).

## 4 Concluding remarks

The hyphenation of on-line CE-LIF with ESI-MS showed to be a useful tool for the annotation of off-line CE-LIF separation profiles of APTS-derivatized HMOs from breast milk. Analyzing fecal extracts of breast-fed babies gave a valuable insight in the fate of HMOs during the gastrointestinal passage. The identification of LnNT Y and LNFP Y, two new isomers not initially present in breast milk allowed to draw conclusions on the gastrointestinal degradation of higher-Mw HMOs.

A more detailed study on the oligosaccharide pattern in feces of breast-fed as well as formula-fed babies, taking into account time of birth and age of child, will be the focus of our further investigations.

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