



# Label-free targeted LC-ESI-MS<sup>2</sup> analysis of human milk oligosaccharides (HMOS) and related human milk groups with enhanced structural selectivity

Marko Mank<sup>1</sup> · Philipp Welsch<sup>1</sup> · Albert J. R. Heck<sup>2</sup> · Bernd Stahl<sup>1</sup>

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## Abstract

Human milk (HM) supports the healthy development of neonates and exerts many of its beneficial effects via contained free human milk oligosaccharides (HMOS). These HMOS exhibit a complexity and structural diversity that pose a significant analytical challenge. A detailed characterization of HMOS is essential as every individual structure may have a different function/activity. Certain HMOS isomers may even fundamentally differ in their biological function, and especially their characterization by LC or LC-MS is often impaired by co-elution phenomena. Thus, more efficient analytical methodologies with enhanced structural selectivity are required. Therefore, we developed a negative ion mode LC-ESI-MS<sup>2</sup> approach featuring straightforward sample preparation, environmentally friendly EtOH gradient elution, and enhanced, semiquantitative characterization of distinct native HMOS by multiple reaction monitoring (MRM). Our MRM-LC-MS setup takes advantage of highly selective, glycan configuration-dependent collision-induced dissociation (CID) fragments to identify individual neutral and acidic HMOS. Notably, many human milk oligosaccharide isomers could be distinguished in a retention time-independent manner. This contrasts with other contemporary MRM approaches relying on rather unspecific MRM transitions. Our method was used to determine the most abundant human milk tri-, tetra-, penta-, and hexaoses semiquantitatively in a single LC-MS assay. Detected HMO structures included fucosyllactoses (e.g., 2'-FL), lacto-*N*-difucotetraose (LDFT), lacto-*N*-tetraoses (LNTs), lacto-*N*-fucopentaoses (e.g., LNFP I, LNFP II and III), lacto-*N*-difucohexaoses (LNDFHs) as well as sialyllactoses (SLs) and tentatively assigned blood group A and B tetrasaccharides from which correct human milk type assignment could be also demonstrated. Correctness of milk typing was validated for milk groups I–IV by high pressure anion exchange chromatography (HPAEC) coupled to pulsed amperometric detection (HPAEC-PAD).

**Keywords** Human milk oligosaccharides (HMOS) · Structural identification · Milk typing · Targeted LC-MS<sup>2</sup> · MRM · Label-free relative quantitation

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✉ Marko Mank  
Marko.Mank@danone.com

<sup>1</sup> Danone Nutricia Research, Uppsalalaan 12, 3584 CT Utrecht, the Netherlands

<sup>2</sup> Biomolecular Mass Spectrometry and Proteomics Division, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CH Utrecht, the Netherlands

## Introduction

With concentrations between 3 and 18 g/l [1–6] and next to lactose and lipids [7], human milk oligosaccharides (HMOS) represent the third most abundant class of biomolecules in human milk (HM). Distinct structure–function relationships have been attributed to various HMOS which may be a crucial factor in the healthy development of infants. The beneficial early life functions of HMOS which are non-digestible in the upper gastrointestinal tract [8] range from direct anti-infective [9] and immunomodulatory effects to modulation of the developing infant's microbiome [10–12], e.g., via prebiotic activity. Many of these beneficial effects are also highly dependent on the primary fine structures of these glycans.

Furthermore, the genetic predisposition of the mother with regard to polymorphisms of Se/Le genes [13], in addition to other early life factors, considerably influences individual HMO glycoprofiles. Se and Le genes code for fucosyltransferase 2 (FUT2, active Se gene) and fucosyltransferase 3 (active Le gene). FUT2 is described to primarily catalyze fucosylation of galactose (Gal)-terminating glycans like for instance lactose or other HMO core structures (e.g., lacto-*N*-tetraose (LNT)) via alpha 1–2 glycosidic linkages [14]. The resulting fucosylated HMOS are, e.g., 2'-fucosyllactose (2'-FL), difucosyllactoses (DFL), or lacto-*N*-fucopentaose I (LNFP I). In contrast, FUT3 activity forms alpha 1–3 or alpha 1–4 linkages between fucose and type I or type II oligosaccharide backbone structures. In this case, for example, lacto-*N*-fucopentaose II (LNFP II) or lacto-*N*-difucohexaose II (LNDFH II) may result. As a result of the donor-specific, individual FUT2 and FUT3 expression patterns and the related HMO profiles detectable in human milk, to date four different human milk groups or types have been defined: milk groups I, II, III, and IV [15]. Independent from milk group-related glycans, a plethora of other compositional and structural HMO variants (> 1000) may be inferred from matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) data of high molecular weight human milk oligosaccharide fractions obtained by gel permeation chromatography (GPC) [2].

Research in the field of early life nutrition continuously strives to better understand biological functions of different human milk types, particular HMOS and other dietary glycans, by deciphering the active molecular structures. Here, one prerequisite is the analytical ability to resolve, identify, and quantify as many individual HMO structures as possible including configurational isomers. In the optimal case, multiple target molecules should be reliably detectable and in one quick analytical run. This would facilitate the analysis of larger sample numbers from cohorts or clinical studies and thereby help to describe biological benefits of HMOS with improved statistical quality. (Semi)quantitative assays may be preferred over mere qualitative ones as some biological HMOS effects may be concentration dependent in nature [16].

To address these analytical needs, many sensitive and accurate methods have already been developed to characterize native and derivatized human milk oligosaccharides, dairy prebiotics, and other glycans. These methods include preparative and analytical size exclusion chromatography (SEC) combined with refractive index (RI) detection [17, 18] or offline MALDI-MS detection [19–23], high pressure anion exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD) [15, 24–27], and high pressure liquid chromatography (HPLC) offering high sensitivity [28, 29]. Furthermore, electrophoretic attempts like polyacrylamide gel electrophoresis [30] and capillary electrophoresis (CE) [31, 32] have been employed. More hyphenated

techniques such as fast atom bombardment mass spectrometry (FAB-MS) [33, 34], CE with laser-induced fluorescence and MS detection (CE-LIF-MS) [35–37], and capillary gel electrophoresis (CGE)-LIF [38, 39] were applied, too. Furthermore, nuclear magnetic resonance spectroscopy (NMR) [40–44], MALDI-MS [19, 45–47], untargeted or targeted liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) [48], multiple fragmentation stage ESI-MS (ESI-MS<sup>N</sup>) [49, 50], or isotope ratio mass spectrometry (IRMS) [51] proved to be suited for compositional or structural identification and quantitation of known or even unknown glycans.

Most of these carefully designed methods perform quite well, but especially LC- or CE-based analytical attempts often depend on the availability of pure oligosaccharide (OS) standards to calibrate and assign correct retention times (RTs) to specific OS structures. Moreover, the identification of compounds on the basis of specific RTs also requires very stable chromatographic systems. Even if provided, co-elution of difficult to resolve isomers can still impair detection and quantitation of target molecules. In addition, standards of, e.g., rare HMOS like LNFP V or more complex glycans may be not commercially available or very expensive.

To mitigate those analytical challenges, novel automated, targeted, and sensitive nano- or micro-LC-MS applications like capillary-HPAEC-ESI-MS [52] have been developed. Introducing the option to determine molecular weights of eluting compounds via MS allowed individual HMO structures to be analyzed with more specificity and selectivity compared to traditional HPLC/HPAEC methods with photometric, fluorescence, or amperometric detection. Thereby, identification of target glycans is achieved by combining structure-specific retention time information with intact molecular mass and MS<sup>2</sup> fragment ion monitoring. In consequence, additional information about the monosaccharide composition, linkage, and sequence can be obtained.

Further improvement of HMOS quantitation was published by Bao et al. in 2013 [53]. Here, a simple, efficient, and robust quantitative LC-ESI-MS method was described. It operates in negative ion mode and can resolve 11 neutral HMOS (tri- to Hexaoses, including LNFP I, II, III, V, and VI) after reduction into alditols. Identification is based on retention time and precursor mass information. Wu et al. presented highly sensitive chipLC-nESI-TOF-MS approaches for analysis of 45 neutral [54] and 30 acidic [55] HMO structures after reduction with sodium borohydride. A combination of structure-specific retention times together with selected precursor and fragment ions served to identify glycan structures and allow construction of an HMOS database. Another approach by Tao in 2011 employed chipLC-nESI-TOF-MS in positive ion mode for identification and relative quantification of milk OS from seven different primate species including human milk. On the basis of retention time and accurate mass detection,

approximately 50–100 different milk OS per species were claimed to be found [56]. Furthermore, Fong et al. demonstrated the use of hydrophilic interaction HPLC-HRMS with selected reaction monitoring (SRM) for measuring six different oligosaccharides in bovine milk, bovine colostrum, and infant milk formulae [57]. This method is able to detect, e.g., native 3'-SL, 6'-SL, and disialyllactose (DSL) in negative ion mode by using distinct pairs of precursor and selected fragment ions. Further targeted, label-free MRM UHPLC-MS methods have been introduced: they can monitor native monosaccharides (fucose, sialic acid, glucose), lactose, 2'-FL, 3'-SL, 6'-SL, and LNneoT in rat serum or detect HMOS alditols after reduction with NaBH<sub>4</sub> in negative [58] or in positive ion mode [6]. These approaches utilize specific retention time information combined with either specific or in the case of HMO alditols rather unspecific MRM transitions for absolute quantitation of individual compounds.

Until today, no efficient analytical strategy for HMOS has been developed to overcome both the dependence on availability of pure standards for compound identification and the frequent impairment of compound quantitation by co-elution effects. A key element to progress from here would be to unveil and to exploit structure-specific diagnostic MS<sup>2</sup> or MS<sup>N</sup> fragments for direct mass spectrometric identification and quantification of important HMOS. This basic idea has already been suggested a decade ago by, e.g., Pfenninger et al. [49, 50] who characterized several isolated HMO structures in static nESI-MS<sup>N</sup> experiments with high sensitivity on a classical quadrupole ion trap (QIT) instrument. Thereby, fundamental fragmentation rules could be established which can aid in characterization of composition, sequence but also partly in determination of glycosidic linkages in known or novel HMOS. As a result of the nature of static nESI, which requires manual change of spraying needles and reassembling of the nESI ion source for every new experiment, the sample throughput was quite limited.

To further enhance throughput and parallel characterization of relevant isomeric HMO structures in human milk up to pentaoses, we developed a novel targeted LC-ESI-MS<sup>2</sup> method operating in negative ion MRM mode. This approach builds on the aforementioned diagnostic ion strategy for identification and relative quantitation of particular HMOS. The novelty of this approach is to exploit highly specific, HMOS configuration-dependent, diagnostic MS<sup>2</sup>-CID fragments for identification and relative quantification of distinct oligosaccharide structures rather than using the more unspecific MRM transitions published so far. Thereby our method circumvents challenges in HMO quantitation posed by co-elution phenomena, the need to use rare HMO standards for compound identification, and also tedious derivatization of HMOS prior to analysis.

## Materials and methods

### Chemicals and reagents

1,5- $\alpha$ -L-Arabinopentaose (> 95%) was purchased from Megazyme International, Ireland. Ammonium acetate (p.a.), ACETONITRILE (LC-MS GRADE), and ethanol (gradient grade for HPLC) were from Merck, Darmstadt, Germany. LC-MS grade H<sub>2</sub>O (HiPerSolv Chromanorm) came from Prolabo, VWR International, Darmstadt, Germany. Alternatively, Ultrapure lab water (18 k $\Omega$ , < 5 ppm TOC) was produced using a MilliQ Advantage Ultrapure Water System from Merck Millipore, Darmstadt, Germany.

### Isolation and characterization of defined molecular weight HMO fractions by gel permeation chromatography (GPC) and sub-fractionation into isomerically pure lacto-*N*-tetraoses (LNTs), lacto-*N*-fucopentaoses (LNFPs), and lacto-*N*-difucohexaoses (LNDFHs) by high performance liquid chromatography (HPLC)

Availability of defined molecular weight HMO fractions and isomerically pure LNT, LNnT, LNFP I, LNFP II, LNFP III, LNFP V, LNDFH I, and LNDFH II was mandatory for subsequent method development, i.e., assignment of isomer-specific, diagnostic CID MS<sup>2</sup> fragments, further definition of specific MRM transitions, and future quantitation purposes, e.g., construction of calibration curves. Defined molecular weight HMO fractions (ranging from human milk-derived trioses to hexaoses) and individual isomers were isolated from pooled human milk as described in more detail by Stahl et al. [19]. Briefly, the protocol included milk skimming by centrifugation (3000 rpm, 4 °C, 30 min), protein precipitation (2 volumes of cold EtOH added to 1 volume of skimmed milk), and chromatographic fractionation of the resulting carbohydrate (CH) mineral fraction by preparative GPC into defined molecular weight HMO fractions (trioses, tetraoses, pentaoses, and hexaoses). Defined GPC fractions were sub-fractionated using C18rp-HPLC [19] to yield pure HMO isomers. In modification of the original procedure, no anion exchange chromatographic (AEC) separation of neutral and acidic HMOS was employed. Instead, the CH mineral fraction was lactose depleted prior to GPC and C18rp-HPLC fractionation by simulated moving bed chromatography as outlined by Geisser et al. [59]. Further technical details about GPC and c18rp-HPLC fractionation including pretreatment and storage of obtained HMO fractions can be found in the Electronic Supplementary Material (ESM) of this article. Purity of HMO fractions was assessed by MALDI-MS [19], HPAEC [24], and CGE-LIF [38]. In case of pure LNT and LNFP isomers, only purity grades exceeding 95% were accepted to

be used for preparation of calibration solutions and determination of HMO structure-specific tandem MS fragments.

### Oligosaccharide solutions for determination of HMO structure-specific diagnostic tandem MS fragments and $\alpha$ -arabinopentaose ( $\alpha$ -AP) internal standard solution

HMO GPC fractions or pure OS isomers also served for tandem MS experiments via flow injection analyses (FIA) or LC-MS<sup>2</sup> (see below). Solutions of defined molecular weight HMO GPC fractions and isomerically pure HMO fractions (see above) were prepared by using ultrapure lab water (18 k $\Omega$ , < 5 ppm TOC). The final concentrations after 0.2-micron filtration through a PES syringe filter (Minisart, Sartorius, Göttingen, Germany) were approximately 0.01  $\mu$ g/ $\mu$ l.  $\alpha$ -Arabinopentaose internal standard (ITS) was introduced to continuously monitor instrumental performance. Furthermore, we intended to (at least partly) compensate for possible deviations in ionization efficiency due to expected biological intersample variations of the human milk matrix. Hence, 5 mg  $\alpha$ -arabinopentaose ( $\alpha$ -AP, Megazyme International, Ireland) was dissolved in 1 ml of ultrapure water to yield a 5 mg/ml stock solution. Subsequently, 33.9  $\mu$ l of this stock solution was pipetted into a glass vial and filled up to 5.0 ml with water (equal to 0.05 mmol/l or 0.0339 mg/ml). This solution was stored at – 20 °C until further use.

### Eluent solutions for MRM LC-ESI-MS

LC-MS eluent solutions A and B were prepared as follows:

Preparation of 5 mM ammonium acetate solution (eluent A)

Ammonium acetate (385.4 mg) was dissolved in 1 L of water (LC-MS grade) and the solution was filtered through a 0.22- $\mu$ m filter device (bottle top filter, Sartolab, 0.22  $\mu$ m PES, 500 ml, Sartorius, Göttingen, Germany) directly into a 1-L Duran glass bottle (Schott, Mainz, Germany) by applying appropriate vacuum. The final pH of the eluent solution was approximately 6.0. The bottle was connected to the 1200 series binary HPLC system.

Preparation of 5 mM ammonium acetate solution in 80 % EtOH (eluent B)

Ammonium acetate (385.4 mg) was dissolved in 200 ml ultrapure lab water and 800 ml gradient-grade EtOH (Merck, Darmstadt, Germany) was added. The solution was filtered through a 0.22- $\mu$ m filter device into a 1-L Duran glass bottle (Schott, Mainz, Germany) as described for eluent A and connected to the 1200 series binary HPLC system.

### Four human milk samples of known milk group type

In order to verify if our MRM LC-ESI-MS approach can distinguish between the four commonly known human milk groups by deciphering the sample-specific LNFP patterns, a subset of four donor milks with known milk group type from previous studies conducted by Thurl et al. [15, 60] were used. These human milks had been provided by healthy 20- to 35-year-old Caucasian volunteers from the regions of Frankfurt/Main or Dresden in Germany who had given birth to healthy infants. Already in this study, each human milk sample had been assigned to one of the four different milk groups via HPAEC by virtue of its specific HMO pattern. Also, donor Lewis blood group status had been confirmed by hemagglutination blood testing in the same study. Samples were anonymized before re-analyses by HPAEC and LC-MS.

### Fast sample cleanup by ultrafiltration and addition of internal standard $\alpha$ -arabinopentaose prior to MRM LC-ESI-MS

The purity of the target compounds (oligosaccharides) is crucial to protect the analytical LC-MS system against contamination, clogging, and to enhance the sensitivity for lead molecules by depleting competing matrix compounds. Ultrafiltration (UF) is a fast means to facilitate sample cleanup by separating smaller molecules like HMOS from most proteins and other compounds of the sample matrix exceeding the UF cutoff. In this study, cleanup of all samples (i.e., human milks and isolated HMO standards) was performed by ultrafiltration with a 3-kDa membrane centrifugal device at room temperature before LC-MS as follows:

500  $\mu$ l Amicon Ultra centrifugal filter devices (Millipore, Billerica, MA, USA) with 3-kDa nominal cutoff were first cleaned by adding 450  $\mu$ l of H<sub>2</sub>O (LC-MS grade) into each device and centrifuging at 14,000 $\times$ g for 20 min at room temperature. This step removed possible residual surfactants from the UF membranes. Filtrate was discarded afterwards. Then, human milk samples were thawed at room temperature and vortexed well until an optically homogeneous milk solution was achieved. Afterwards, 135  $\mu$ l of milk was mixed with 15  $\mu$ l of  $\alpha$ -arabinopentaose internal standard (ITS) solution (0.05 mmol/l; for preparation, see above). This combined sample-ITS solution was further diluted 1:11 (v/v) in 2-ml Eppendorf tubes by adding 1350  $\mu$ l H<sub>2</sub>O (LC-MS grade). Then, 450  $\mu$ l of the diluted mixture was transferred into the previously washed filter device. After the mixture was centrifuged at 14,000 $\times$ g for 1 h at room temperature, the complete filtrate containing HMOS was vortexed again for 5 s and pipetted into a clean 300- $\mu$ l glass LC-MS vial with screw top (Thermo Fisher Scientific, Waltham, MA, USA). Samples were either directly subjected to MRM LC-ESI-MS or stored at – 20 °C until further use.

## HPAEC analysis of four human milk samples with known donor Le/Se gene status

Classification of four human milk samples with known donor Le/Se gen status (see above) into one of four human milk groups was reconfirmed by HPAEC following the approach published by Coppa et al. [61]. In deviation from the method of Coppa et al., we applied a CarboPac PA-200 column (Dionex, Idstein Germany) instead of a PA-1 column. Unlike in the method formerly described by Thurl et al. [15], lactose was not removed prior to HPAEC in our approach. Nevertheless, the resulting milk group assignments were identical with both approaches: the four selected samples comprised milk groups I, II, III, and IV. Aliquots of these four human milk samples had been prepared for HPAEC and MRM LC-ESI-MS analysis by 3-kDa ultrafiltration as described before.

## Qualitative and semiquantitative analysis of human milk oligosaccharides by negative ion MRM LC-ESI-MS

General MRM LC-ESI-MS setup: the employed LC-ESI-MS configuration consisted of a 3200 Qtrap triple quadrupole linear ion trap mass spectrometer (MS) equipped with a Turbo V-ion source (all ABSciex, Framingham, MA, USA) connected to a 1200 series HPLC stack (Agilent, Santa Clara, CA, USA). All MS experiments were carried out in negative ion mode. The modules of the HPLC stack included a binary pump, a degasser, a temperature-controlled autosampler kept at 22 °C, a column compartment kept at 45 °C, and a DAD monitoring possible peptide or protein contaminations at 215 and 280 nm wavelength. A Hypercarb 2.1 × 30-mm porous graphitized carbon (PGC) column with 5-μm particle diameter and a 2.1 × 10-mm Hypercarb precolumn (Thermo Fisher Scientific, Waltham, MA USA) were used as stationary LC phase. An approximately 18-min gradient elution at 400 μl/min flow rate was used to separate HMOS by LC before online infusion into the mass spectrometer. The gradient was formed by varying the ratio of eluents A (5 mM aqueous NH<sub>4</sub>CH<sub>3</sub>COO) and B (80 % EtOH (v/v) with 5 mM aqueous NH<sub>4</sub>CH<sub>3</sub>COO) over time as described in Table S1 (ESM). The sample injection volume per analytical run was 5 μl. After each injection, the autosampler needle was flushed externally for 5 s with 20% aqueous isopropanol to minimize sample carryover. The in-built Valco diverter valve of the 3200 Qtrap was directing the PGC column effluent either to the ion source or to waste. It was switched towards the MS ion source 0.2 min after sample injection and switched back to waste after 17 min. Effluent switching was introduced to protect the ion source from excess effluent between runs or during automated column cleaning procedures. In addition to the MS detection, UV profiles of each sample were also recorded at 215 and 280 nm wavelength and 360 nm reference wavelength. An

Agilent 1200 series diode array detector and an analytical flow cell were used sampling UV signals with a frequency greater than 20 Hz. At the start of each run the UV signal was set to zero automatically. Analyst 1.4 software (ABSciex, Framingham, USA) was used to control the complete LC-MS configuration and for further evaluation of LC-MS data.

## Optimization of ion source and compound-specific MS parameters for HMOS

To achieve maximum sensitivity for EPI-ESI-MS and the final MRM LC-ESI-MS assay, ion source and target compound-related parameters of the 3200 Qtrap were optimized. Flow injection analysis (FIA) MS of a GPC-derived lacto-*N*-fucopentaose mixture (for purification, see above) was employed to infer the optimized settings as shown by Table S2 in the ESM.

## Determination of HMO structure-specific diagnostic tandem MS fragments for construction of MRM transitions

In order to determine/confirm isomer-specific diagnostic fragment masses, MS<sup>2</sup> experiments were performed by direct syringe injection or HPLC infused flow injection MS/MS analysis or LC-MS analysis in negative ion mode. HMO GPC fractions or pure OS isomers were analyzed using the general linear ion trap triple quadrupole LC-MS setup and optimized ion source parameters already described above in enhanced product ion (EPI) mode. A quadrupole 3D ion trap (QIT) instrument (LCQ, Thermo Fisher) equipped with a syringe pump was used alternatively. All experiments were performed at room temperature with or without PGC column. HPLC or syringe flow rates were 400 μl/min using an isocratic eluent consisting of 25 % EtOH (v/v) and 5 mM aqueous NH<sub>4</sub>CH<sub>3</sub>COO. Both MS instruments were operated with standard μ-spray ion sources in negative ion mode using default ion source and MS<sup>2</sup> conditions if not further specified below. Isolation widths for precursor ions of interest were set to ±3 *m/z* in case of QIT-MS and to unit resolution in case of the linear ion trap MS instrument. Collision energies were adjusted to yield precursor ion intensities of approximately 10–30% relative to the most intense fragment ion in the resulting tandem MS spectra. Fragment ions were considered to be diagnostic if they were uniquely detected in MS/MS spectra of individual HMO structures but not in others and exceeded at least 1% of the signal intensity of the most prominent peak in the MS<sup>2</sup> spectrum. The identified diagnostic molecular fragment masses were further employed in MRM transitions of the final MRM LC-ESI-MS method after compound-specific ESI-MRM optimization on the 3200 Qtrap MS instrument.

## Application of final negative ion MRM LC-ESI-MS method for analysis of HMOS in human milk samples

The optimized final MRM LC-ESI-MS setup was used to analyze the human milk samples in a randomized manner. Each sample was followed by two blanks to minimize possible carryover. The first blank was 80 % isopropyl alcohol and the second blank was ultrapure water.

## Software settings for identification of HMOS, peak integration, and relative quantitation after negative ion mode MRM LC-ESI-MS analysis

Identification of most individual HMOS primarily relied on the specific MRM transitions (see “Results”). As the employed Analyst software also stipulates to assign specific LC retention times for compound recognition, a window of 6 min retention time (RT) tolerance was allowed to ensure correct peak assignment to HMO structures for most runs. Larger, rather erratic RT shifts were occasionally observed, especially for Hex<sub>2</sub>, 3-FL, 2'-FL, and DFL in some exceptional cases. Then the RT tolerance window was manually extended to up to 15 min. To also compensate for different LC peak shapes, bunching factors were adapted accordingly between 5 and 30 to achieve complete integration of peak areas. The complete software settings to ensure proper glycan peak assignment and quantitation are summarized in Table S3 (ESM).

## Manual assignment of human milk groups by individual inspection of LC-MS and HPAEC data

For further processing, generated LC-MS data sets were exported from Analyst 1.4 software to Microsoft Excel version 14.0.7145.5000 (32 bit) or ABSciex Markerview version 1.2.1.

To facilitate relative quantitation, dimensionless responses were calculated for each individual glycan in Excel or Analyst software according to Eq. 1:

$$R = \frac{A_s}{A_{its}} \quad (1)$$

where  $R$  is the response,  $A_s$  is the peak area for a specific OS, and  $A_{its}$  is the peak area of  $\alpha$ -arabinopentaose (internal standard, its). If needed, dimensionless responses obtained for individual HMO structures after analyses of different HM samples were compared to each other and used to calculate, e.g., changes in abundance between samples in percent.

Assignment of human milk samples to one of the four known milk groups was based on the absence, presence, or concomitance of LNFP I and LNFP II [15] as determined by MRM LC-ESI-MS. Peaks were

considered for evaluation if the detected peak responses  $R$  exceeded the value of 0.2. In case of the four human milk samples with known donor Le/Se gen status, additional HPAEC analysis (see above) was performed prior to MRM LC-ESI-MS to cross-validate milk group assignment. Determination of milk groups by HPAEC was facilitated by detection of milk group-specific marker HMOS like LNFP I, LNFP II, 2'-FL, DFL, LNDH I, and LNDH II as proposed in the literature [15]. HMO peaks were inspected manually in each LC-MS or HPAEC plot.

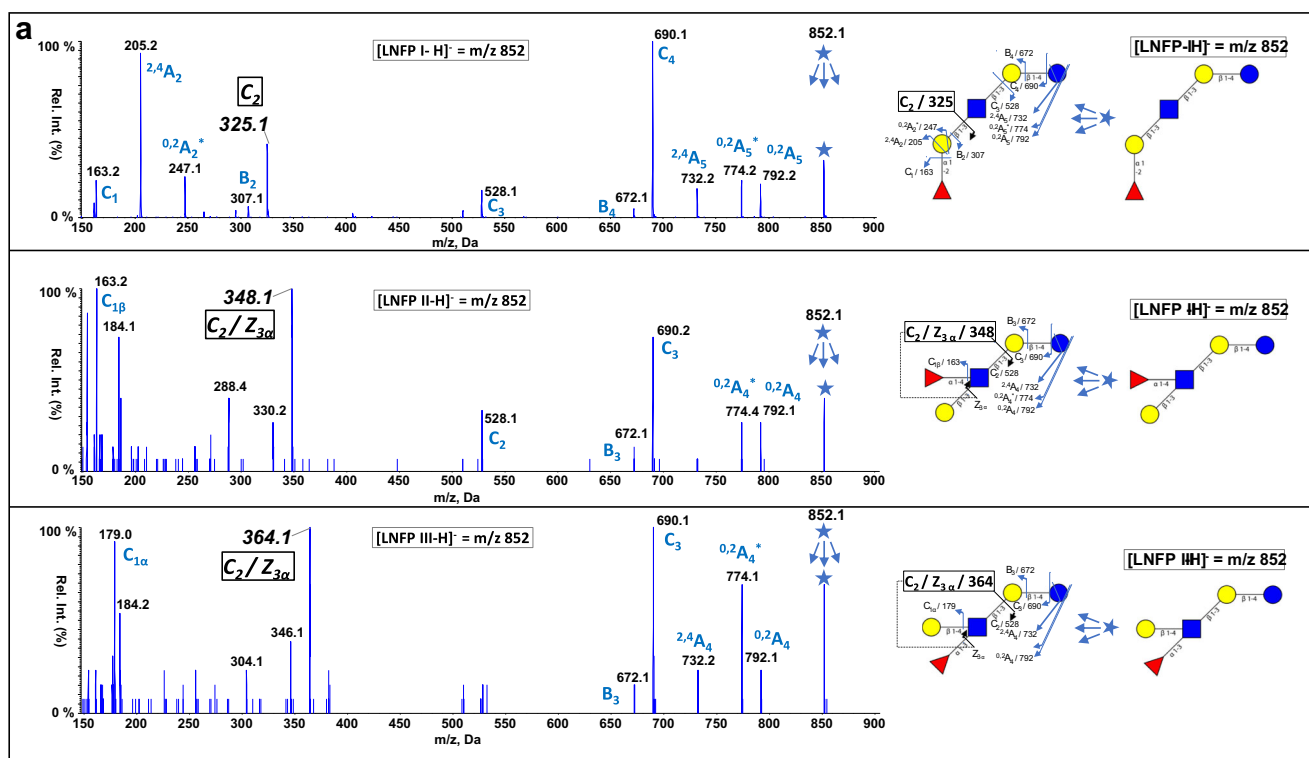
## Estimation of HMO quantitative inter-run variations in MRM LC-ESI-MS

To estimate the inter-run variation of detected HMOS quantities, a human milk sample was injected 10 times after sample pretreatment (see above). Between every injection an 80% isopropanol blank and an ultrapure water blank were run. Responses  $R$  (Eq. 1) for all detected HMOS were averaged over the 10 runs and relative standard deviations were calculated in percent.

## Results

### Determination of characteristic, isomer-specific HMO CID fragments and derived MRM transitions

A crucial step in MRM method optimization was to define suitable negative ion mode MRM transitions that were specific enough to distinguish between (partly isomeric) HMOS of interest such as 2'-FL, 3-FL, 3'-SL, 6'-SL, DFL, LNT, LNnT, LNFP I, LNFP II, and LNFP III. The necessary  $[M-H]^-$  precursor masses for definition of MRM precursor fragment ion pairs could be readily deduced from chemical sum formulae of the respective HMO compounds. Then, the corresponding MRM fragment ion masses were derived from the literature [49, 50, 62, 63] as described for negative ion mode CID-induced fragmentation pathways of linear or branched HMOS (ranging from tetraoses up to hexaoses). Afterwards the literature information was confirmed and completed by our own negative ion mode ESI-MS<sup>2</sup> CID experiments with purified HMO standards. A selection of resulting spectra for defined HMO structures by negative ion mode enhanced product ion ESI-MS<sup>2</sup> (EPI-ESI-MS<sup>2</sup>) are exemplified in Figs. 1, 2, and 3. The depicted EPI-ESI-MS<sup>2</sup> spectra were obtained for pure LNFP I, II, and III (Fig. 1a), a mixture of LNFP I, II, III and V (Fig. S1a in ESM), pure LNT and LNnT (Fig. 1b), a mixture of LNT and LNnT (Fig. S1b in



**Fig. 1** a Negative ion mode EPI-ESI-MS<sup>2</sup> spectra of pure LNFP I, II, III. [M–H]<sup>−</sup> precursor ions selected for MS<sup>2</sup> in EPI mode were at 852 *m/z*. Isomer-specific diagnostic MS<sup>2</sup> fragments are highlighted by boxes and in bold italic letters for LNFP I (325 *m/z*), LNFP II (348 *m/z*), LNFP III (364 *m/z*). b Negative ion mode EPI-ESI-MS<sup>2</sup> spectra of pure LNT and LNnT. [M–H]<sup>−</sup> precursor ions selected for MS<sup>2</sup> in EPI mode were at 706 *m/z*. Isomer-specific diagnostic MS<sup>2</sup> fragments are highlighted by boxes and in bold italic letters for LNT (202 *m/z*) and LNnT (263 *m/z*). Detected LNFP or LNT isomer-specific diagnostic CID fragments comply with published findings described for direct static nESI-QIT- and qTOF-MS/MS experiments [49, 50, 62]. All MS<sup>2</sup> spectra were acquired at −29 eV collision energy

ESM), as well as isomerically pure 2'-FL (Fig. 2a), 3-FL (Fig. 2b), 3'-SL (Fig. 3a), and 6'-SL (Fig. 3b).

Diagnostic C, C/Z, or A fragment ions of LNFP I, II, III, and V at *m/z* 325, 348, 364, and 544 and those of LNT and LNnT at *m/z* 202 and 263 have already been described by Pfenninger et al. [49, 50]. These authors used classical quadrupole 3D ion trap instruments and nESI in negative ion mode for structural MS<sup>N</sup> elucidation. Low energy resonant collision-induced dissociation (CID) was applied.

Interestingly, the same HMO isomer-specific diagnostic fragments could also be detected under non-resonant low energy CID conditions by us with triple quadrupole linear ion trap MS (Fig. 1), but also by Chai et al. [62] using quadrupole-quadrupole TOF-MS. This might be surprising as different ion dissociation processes take place during resonant and non-resonant CID. Encouraged by these findings, we integrated other structure-specific C, C/Z, or A fragments into our MRM setup which had been formerly described, e.g., for LNFP V, LNDFH I, or LNDFH II by Chai et al. Moreover, additional diagnostic fragment ions specific for 6'-SL at *m/z* 470, 3'-SL at *m/z* 408, 2'-FL at *m/z* 325, and 3-FL at *m/z* 179 (Figs. 2, 3) could be revealed

or reconfirmed by our EPI-ESI-MS<sup>2</sup> experiments on the triple quadrupole linear ion trap MS platform.

It is noteworthy that we did not find any indication for gas phase rearrangement of fucosylated human milk oligosaccharides and prompt fucose migration as described by Wührer et al. [64] and Aldredge et al. [65].

These authors conducted positive ion mode MS/MS analysis of, e.g., protonated OS precursor ions. Gas phase rearrangement can lead to misinterpretation of MS/MS data if structural elucidation of oligosaccharides is attempted. Obviously, this drawback can not only be circumvented by analyzing permethylated milk oligosaccharides as outlined by Dong et al. [66] and Oursel et al. [67] but also by utilizing negative ion mode CID of underivatized HMOS as demonstrated by us and others.

According to the literature, Kelly et al. [68] already successfully attempted to distinguish and to quantify native 3'-SL and 6'-SL in bovine milk by negative ion mode 3D-ion-trap-FIA-MS. Unlike in our results, the 470 *m/z* fragment was not exclusively found after CID fragmentation of 6'-SL, but also appeared in low abundance in 3'-SL standards. It is not clear if this divergence in results might stem from 3'-SL traces contained

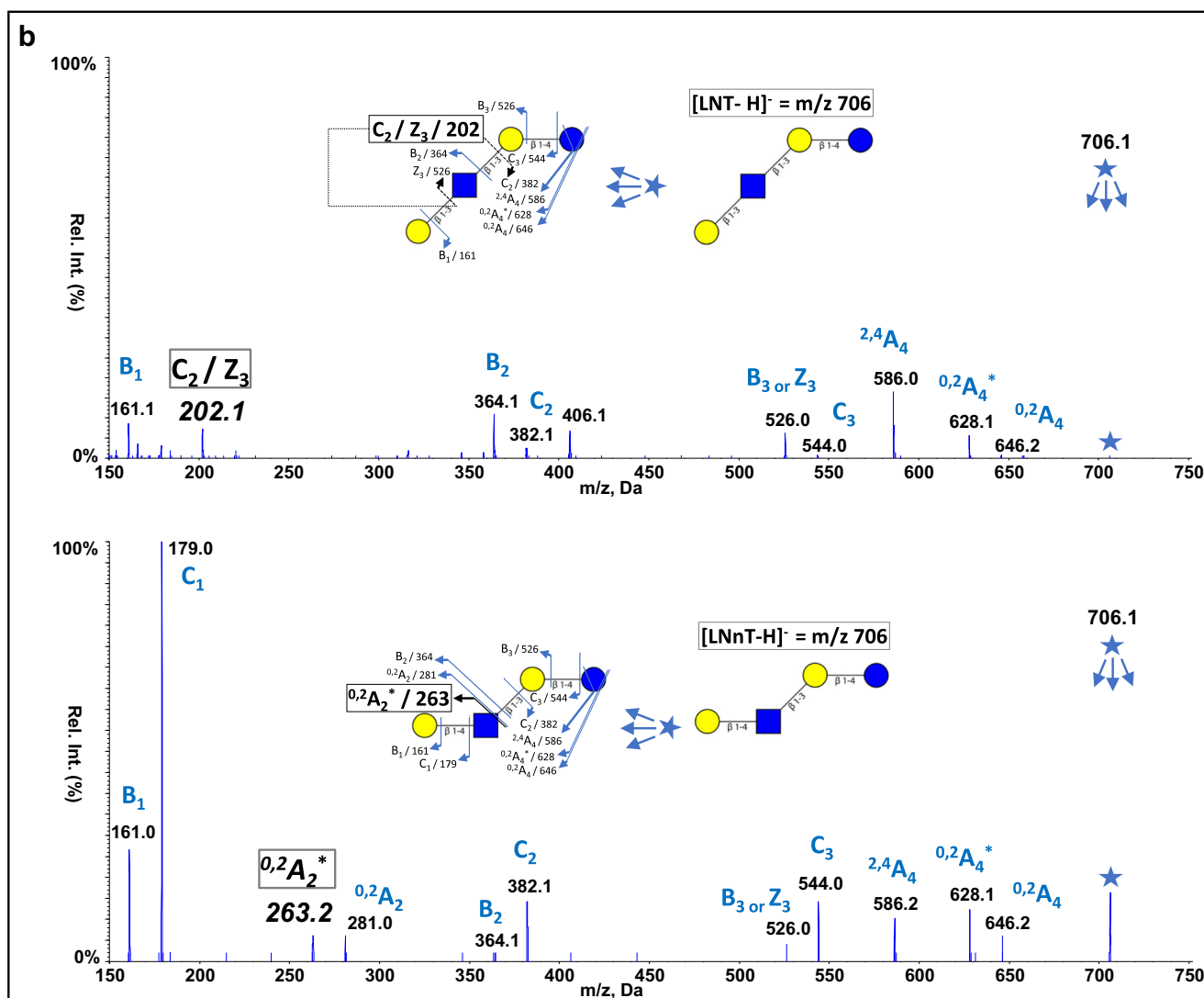
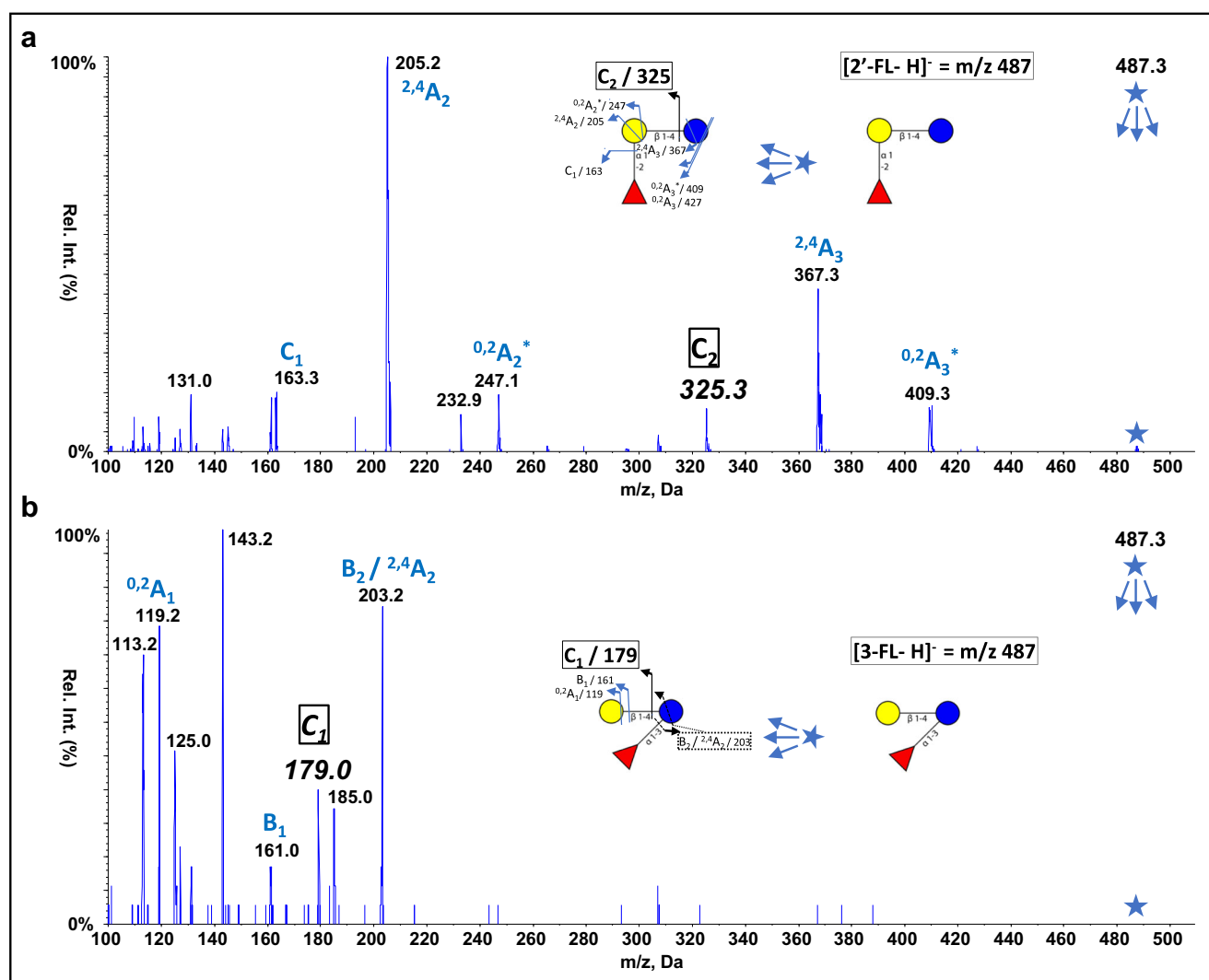


Fig. 1 (continued)

in the commercial 6'-SL standards they used or has other causes. No statement about the purity of the used standards was made in that article. Intriguingly, the 3'-SL-specific fragment which we detected at  $m/z$  408 was not at all described in the 3D-ion-trap 3'-SL spectra by Kelly and colleagues. In consequence, they used ratios of the fragment ions  $m/z$  572 and  $m/z$  290 rather than diagnostic fragment ions to determine proportions of 3'-SL and 6'-SL present in milk samples. Regarding ease of use in method development or application, our and other FIA or LC-MS approaches might be considered as an improvement in structural HMO analysis compared to static nESI tandem MS experiments, e.g., conducted by Pfenninger et al. [49, 50]. This is because these static nESI approaches were much more tedious to conduct because of the need for manual loading and reassembly of the whole ion source to introduce a new nano

spray needle containing the next sample. On the other hand, carryover is theoretically much less probable in such static nESI applications, and MS<sup>N</sup> for elucidation of monosaccharide sequences and glycosidic linkages can be conveniently performed with sub-microliter sample volumes. By analyzing further HMO standards, we succeeded in defining unambiguous MRM transitions for 11 HM isomers employing one unique diagnostic fragment ion per structure. These structures represent different configurational isomers of the following HMOS: fucosyllactoses (FLs), difucosyllactose (DFL), lacto-*N*-tetraoses (LNTs), lacto-*N*-fucopentaoses (LNFPs), and silalylactoses (SLs). It is noteworthy that the diagnostic fragment found for LNFP I at  $m/z$  325 presents an exception, as it would not be specific enough to distinguish between LNFP I and its type II core isomer LNFP IV [64]. This LNFP I  $C_2$  fragment

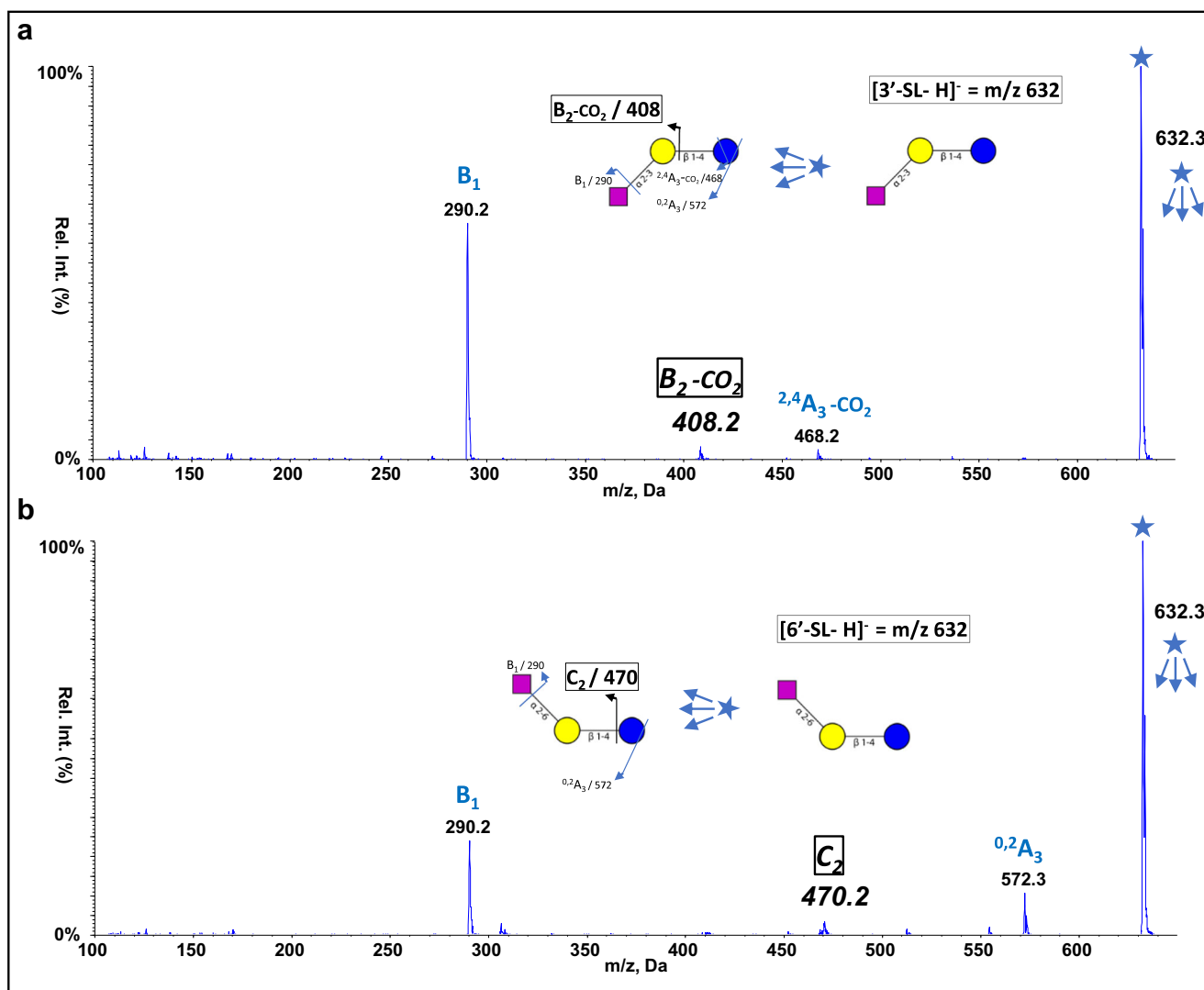


**Fig. 2** Negative ion mode EPI-ESI-MS<sup>2</sup> spectra of **a** pure 2'-FL and **b** pure 3-FL. Isomer-specific diagnostic MS<sup>2</sup> fragments are highlighted by boxes and bold italic letters and are found for 2'-FL at 325 *m/z* and for 3-FL at 179 *m/z*. The [M-H]<sup>-</sup> precursor ion selected for MS<sup>2</sup> of both isomers was at 487 *m/z*. MS<sup>2</sup> spectra were acquired at -29 eV collision energy. Monosaccharide symbols and structural representations of

oligosaccharides were drawn with Glycoworkbench [71] according to Consortium for Functional Glycomics (CFG) proposals [72]: glucose (Glc), blue circles; galactose (Gal), yellow circles; fucose (Fuc), red triangles. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

(Table 1) indicates the position of fucose at the glycan's non-reducing end which does not differ between LNFP I and IV. Fortunately, the existence of LNFP IV has not been reported for human milk but for seal milk so far [69]. Therefore, it still appears to be reasonable to employ the C<sub>2</sub> fragment at *m/z* 325 as a marker for LNFP I in human milk analysis. Moreover, if LNFP IV were present in significant abundance in HM, the appearance of an additional LC-MS peak or at least peak broadening might be expected. This was not the case in the subsequent MRM LC-ESI-MS analyses we conducted with the four HM samples. Also for the MRM transition which we established for LNFP V, in theory another structure called LNFP VI could be picked up. LNFP

V is known to have a fucose residue at the reducing end of a type I core LNT backbone. Its type II core counterpart LNFP VI could theoretically produce the same C<sub>3</sub> fragment at *m/z* 544. LNFP VI was recently detected by Bao et al. [53] in four different human milks collected between 3 and 29 days post-partum (pp). Here, contained HMOS were converted into alditols prior to LC-MS. With and without reduction of HMOS into alditols, LNFP V and LNFP VI were discernable by gradient elution on a 100 × 2.1-mm PGC column via different retention times. In contrast to Bao et al., we used a 2.1-mm-ID PGC stationary phase but with a shorter column length of 30 mm. However, in our HM sample set we could not detect a second peak in



**Fig. 3** Negative ion mode EPI-ESI-MS<sup>2</sup> spectra of **a** pure 3'-sialylactose and **b** pure 6'-sialylactose standards. Isomer-specific diagnostic MS<sup>2</sup> fragments are found for 3'-SL at 408 *m/z* and for 6'-SL at 470 *m/z* and are highlighted by boxes and bold italic letters. [M-H]<sup>−</sup> precursor ion selected for MS<sup>2</sup> in EPI mode was at 632 *m/z* for both isomers. All MS<sup>2</sup> spectra were acquired at −29 eV collision energy. Monosaccharide symbols and

structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]; glucose (Glc), blue circles; galactose (Gal), yellow circles; *N*-acetylneuraminic acid (Neu5Ac), pink diamonds. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

addition to LNFP V for the transition *m/z* 882 to 544, which might have been an indication for presence of LNFP VI. With our setup, only one peak with two shoulders was detected, most probably reflecting the two anomeric isomers of LNFP V. Assignment to LNFP V was made as this compound is a well-known and confirmed part of the HMO composition in HM [70]. It will be interesting to investigate if this outcome will persist if more milks, e.g., from an HM cohort, are analyzed in the future. Biological differences between the characterized sample sets regarding gestational ages of donors as well as a better sensitivity of Bao's method for LNFP VI and co-elution of LNFP V

and VI in our case may account for the diverging observations, too.

### Implementation of HMO isomer-specific MRM transitions in the new negative ion mode MRM LC-ESI-MS method for identification and relative quantitation of individual HMO structures

On the basis of translation of all diagnostic HMO fragments described in the paragraphs above, we attempted to create a highly HMO structure-selective negative ion mode MRM LC-ESI-MS approach.

An overview of the characteristic isomer-specific MRM transitions consisting of respective pseudomolecular precursor ions and diagnostic fragment ions is given in Table 1 for 10 HMO structures. The sketches used to visualize intact HMO structures and hypothetical fragment ion structures as shown in Tables 1 and 2 comply with the nomenclature first introduced by Domon and Costello [73] and continued by Pfenninger et al. [49], Chai et al. [62], and Harvey et al. [74, 75]. Although considering published suggestions [49, 62, 75], the shown structural representations of these fragment ions are only likely variants which may actually still differ from the real fragment ion configuration. Analyzing the exact nature of such fragment ions is beyond the scope of this study. An extended set of additional 13 MRM transitions conveying less configurational but at least compositional specificity was included in the final MRM method, too (Table 2).

Among other OS, this set contains MRM transitions for LNFP V, LNDFH I, LNDFH II or their respective type II core isomers, tentative histo blood group antigen A [76] and B-like tetrasaccharides, and  $\alpha$ -arabinopentaose which served as internal standard.

After implementation of the entire set of compound-specific MRM transitions into the new MRM LC-ESI-MS method and subsequent optimization of source and detector settings, analyses of GPC- and C18rp-HPLC-purified HMO structures were performed to assign individual retention times to the respective HMOS. Then, by analyzing HMOS in a complete human milk sample, we tested the specificity of the selected MRM transitions together with the resolving power of the employed multistep H<sub>2</sub>O/EtOH gradient on porous graphitized carbon (PGC) columns designed to resolve in particular the different LNFP isomers.

Both a good chromatographic resolution and the clear distinction between LNFP isomers I and II by the chosen MRM transitions would be valuable advantages for the desired determination of different human milk groups. As published by Thurl et al. [15] and others, human milk groups can be determined by monitoring the absence, presence, or concomitance of certain Secretor and Lewis gene-dependent oligosaccharides like LNFP I and LNFP II.

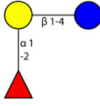
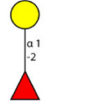


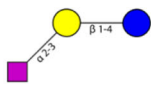

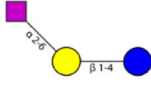

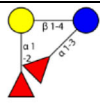
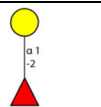
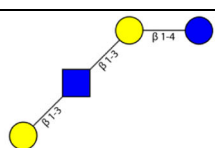

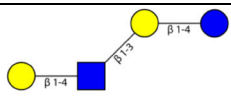
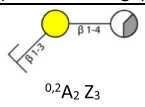
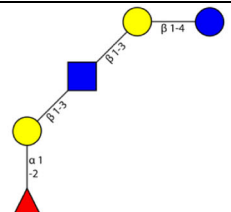
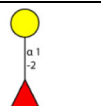
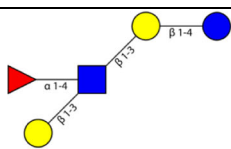
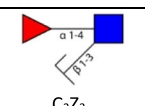
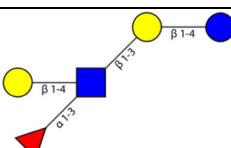
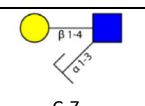
Figure 4 shows the resulting UV trace at 215 nm wavelength, the total ion chromatogram (TIC), and the MRM traces of multiple HMO structures. By tracking the UV absorbance of the LC effluent at 215 and 280 nm wavelength, we intended to monitor the presence of possible additional non-HMO compounds not targeted by MRM. For example, peptides or other small molecules might also pass through the 3-kDa ultrafiltration devices used for sample cleanup. Fortunately, no indication for abundant elution of such compounds

was found among samples subjected to analysis. The 3-kDa ultrafiltration obviously retained the majority of other compounds like, e.g., HM proteins or peptides. The peaks visible in the blue shaded ranges of the UV trace in Fig. 4a only represent absorbance variations triggered by changes in the used LC gradient rather than additional HM compounds or small molecules co-eluting with HMOS. Those eluent-dependent peaks reproducibly occurred throughout all sample runs but also in the blank runs.

If UV and TIC traces are compared to the MRM profiles (Fig. 4a–d), the advantage of increased specificity for particular HMO structures, which is inherent in the applied MRM methodology, becomes evident. The UV or TIC signals alone do not provide any structural or compositional information to confirm peak identity without prior calibration by appropriate and often rarely available standards. In contrast, the applied MRM LC-MS technology does: by using specific MRM transitions, multiple and partly isomeric HMO structures could be identified in parallel within only one run. Even HMO isomers with the same molecular weight as LNFP I, II, III, or V could be clearly distinguished. Moreover, the applied H<sub>2</sub>O/EtOH gradient also facilitated full separation of LNFP I from the other human milk LNFP isomers. LNFP II, III, and V were at least partly resolved as well. Compared with recently published LC-MS approaches of Wu et al. [54], the LC separation of LNFP I, LNFP III, and DFL was clearly improved with our adapted EtOH gradient. Those authors used PGC as the stationary phase in the form of an HPLC chip and converted HMOS into their respective alditols to avoid appearance of anomeric peaks. Nevertheless, LNFP III and DFL did still elute at identical retention times, whereas these HMOS were well separated with our gradient although we injected native HMOS. Also the LNFP I and LNFP III retention differed only by 0.14 min in the 45-min gradient applied in the method of Wu et al. In contrast, the difference in retention time was approximately 7 min in our approach which enabled secure distinction within a total LC run time of only 17 min.

However, despite optimization, some major HMOS like the lacto-*N*-tetraose isomers LNT and LNnT were still not sufficiently resolved and co-eluted (Fig. 4c). The same finding applied to (Hex)<sub>2</sub>/lactose, 3-FL, some low abundant HM glycans (like the tentative blood group B-tetrasaccharide/(Hex)<sub>3</sub>(Fuc)<sub>1</sub>), but also to one of the different detected (Hex)<sub>3</sub> peaks. Usage of the applied compound-specific MRM transitions clearly removed the dependence on (baseline) resolution for both identification and (relative) quantitation. Although at the current state of development, our approach could

**Table 1** Characteristic, isomer-specific MRM transitions (monoisotopic  $m/z$  values) for HMOS including pseudomolecular precursor ions  $[M-H]^-$  and diagnostic fragment ions as employed in final negative ion mode MRM LC-ESI-MS method

Nr.	Oligosaccharide	Structure	MRM-Transitions		Possible Fragment ion types [73,49,62,75]
			Precursor [ $m/z$ ]	Fragment [ $m/z$ ]	
1	2'-FL		487.1668	325	 C <sub>2</sub>
2	3-FL		487.1668	179	 C <sub>1</sub>
3	3'-SL		632.2044	408	 B <sub>2</sub> <sup>1,3</sup> X <sub>3</sub> (double cleavage)
4	6'-SL		632.2044	470	 C <sub>2</sub>
5	DFL		633.2248	325	 C <sub>2</sub>
6	LNT		706.2411	202	 C <sub>2</sub> Z <sub>3</sub> (double cleavage)
7	LNnT		706.2411	263	 <sup>0,2</sup> A <sub>2</sub> Z <sub>3</sub> (double cleavage)
8	LNFP I		852.2990	325	 C <sub>2</sub>
9	LNFP II		852.2990	348	 C <sub>2</sub> Z <sub>3</sub> <sub>α</sub> (double cleavage)
10	LNFP III		852.2990	364	 C <sub>2</sub> Z <sub>3</sub> <sub>β</sub> (double cleavage)

Monosaccharide symbols and structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]: glucose (Glc), blue circles; galactose (Gal), yellow circles; *N*-acetylglucosamine (GlcNAc), blue squares; fucose (Fuc), red triangles; *N*-acetylneuraminic acid (Neu5Ac), pink diamonds. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

**Table 2** Characteristic, composition-specific MRM transitions (nominal  $m/z$  values) for oligosaccharides, tentative blood group-related haptens, and internal standard  $\alpha$ -arabinopentaose including pseudomolecularprecursor ions  $[M-H]^-$  and fragment ions as used in final negative ion mode MRM LC-ESI-MS method

Nr.	Oligosaccharide	Oligosaccharide Structure	MRM-Transitions		Possible Fragment ion types
			Precursor $[m/z]$	Fragment $[m/z]$	
11	(Hex) <sub>2</sub> / Lactose		341.1089	179	
12	(Hex) <sub>3</sub> / Galactosyllactose		503.1618	341	
13	SL / (Hex) <sub>2</sub> (NeuAc) <sub>1</sub>		632.2044	290	
14	(Hex) <sub>4</sub>		665.2146	503	
15	$\alpha$ -Arabinopentaose (internal standard)		677.2148	587	
16	LST / (Hex) <sub>3</sub> (HexNAc) <sub>1</sub> (Neu5Ac) <sub>1</sub>		997.3365	290	
17	LNH / (Hex) <sub>4</sub> (HexNAc) <sub>2</sub>		1071.3733	909	
18	Tentative Blood Group B-Tetrasaccharide / (Hex) <sub>3</sub> (Fuc) <sub>1</sub>		649.2197	179	
19	Tentative Blood Group A-Tetrasaccharide / (Hex) <sub>2</sub> (HexNAc) <sub>1</sub> (Fuc) <sub>1</sub>		690.2462	220	
20	LNFP V or LNFP VI		852.2990	544	
21	LNDFH I or LNDFH I		998.3569	325	
22	LNDFH I or LNDFH I		998.3569	836	
23	LNDFH II or LNDFH II		998.3569	690	

Structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]: hexose (Hex), white circles; *N*-acetylhexosamine (HexNAc), white squares;  $\alpha$ -arabinose (Ara), white pentagons; glucose (Glc), blue circles; galactose (Gal), yellow circles; *N*-acetylglucosamine (GlcNAc), blue squares; fucose (Fuc), red triangles; *N*-acetylneuraminic acid (Neu5Ac), pink diamonds. Annotation of fragments follows the nomenclature introduced by Domon and Costello [73]

monitor less HMO compositions compared to the impressive MRM or chip TOF LC-MS methods published, e.g., by Hong [6] et al. or De Leoz et al. [77], we benefited from higher throughput owing to shorter analysis times. Furthermore and in contrast to the MRM approaches published before [6], higher HMO compound specificity is achieved here by replacement of rather unspecific oxonium ions in MRM transitions by more diagnostic fragment ions (Tables 1, 2). Usage of such diagnostic fragment ions allows one to discern the following major structural features of HMOS: position of fucose (Fuc) residues and neuraminic acid (Neu5Ac) residues relative to the HMO backbone and also type 1 or 2 backbone structures. Thus, glycan inherent structural chemical features can now directly be monitored through targeted MS/MS provided by MRM. In theory, this should be possible even without prior LC separation.

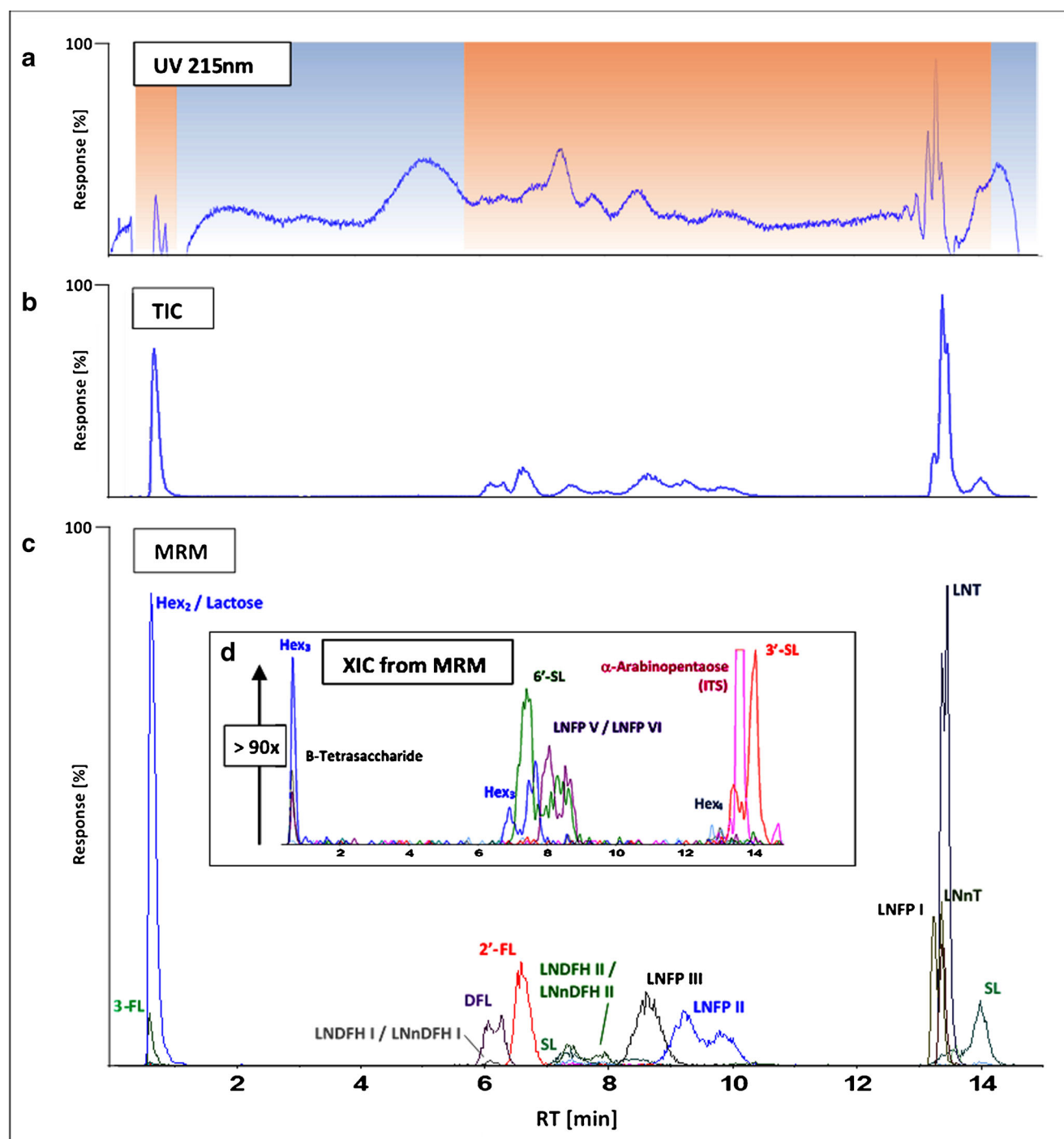
Quantitative variation in the detection of individual HMOS was estimated by 10 repetitive injections of the same HM sample and subsequent determination of relative standard deviations (RSDs) per HMO. The resulting RSDs ranged from less than 12% for LNFP I, LNFP II, LNFP III, LNT, and LNnT; over up to 20% for total SL, (Hex)<sub>3</sub>, 3'-SL, 6'-SL, (Hex)<sub>2</sub>/Lac; to at most 38% for all other HMOS detectable in this sample (Fig. S2 in ESM). One reason for HMO structure-related differences in precision of quantitation could be that the properties of the internal standard  $\alpha$ -arabinopentaose better resemble LNFP I, LNFP II, LNFP III, LNT, LNnT, and the sialyllactoses. Therefore, additional complementary internal standards like  $\alpha$ -arabinoheptaose or  $\alpha$ -arabinotriose might be used in future to also achieve better results for HMOS now detected with less precision.

In summary, with negative ion mode MRM LC-ESI-MS we achieved parallel identification and relative quantitation for more than 20 human milk glycan structures in one efficient approach (Tables 1, 2; Figs. 4, 5). Applicability of this method could be proven for purified, defined HMO fractions but also for whole human milk samples. Identified glycan structures included (Hex)<sub>2</sub> (= lactose by specification via retention time), neutral non-fucosylated or partly fucosylated HMOS such as trioses (Hex<sub>3</sub> (e.g., galactosyllactoses), 2'-FL or 3'-FL), tetraoses (DFL, LNT, LNnT), pentaoses (LNFP I–V), and hexaoses (e.g., LNDFH I–II or LNnDFH I–II). Also, acidic HMOS (3'-SL, 6'-SL) and tentative blood group A and B-like determinants in the form of free human milk tetraoses could be traced. Moreover, the introduction of an affordable internal standard ( $\alpha$ -arabinopentaose) facilitated normalization and relative quantitation of MS raw data. Given the fact

that ethanol, a less harmful replacement of more commonly used acetonitrile, was employed for gradient formation, this LC-MS method might be considered as an environmentally friendlier analytical alternative for HMO characterization.

### Suitability of negative ion mode MRM LC-ESI-MS for determination of human milk groups in comparison with HPAEC

Four human milk samples representing the four currently known human milk groups were then compared with the final MRM LC-ESI-MS method. The milk group status of each individual sample had been thoroughly characterized by HPAEC before. Thereby it was possible to benchmark the milk-typing performance of our label-free MRM LC-ESI-MS method against the well-established HPAEC methodology (Figs. 5, 6) for oligosaccharide analysis. Compared to HPAEC or to other commonly used analytical methods like untargeted LC-MS or CE, targeted MRM LC-ESI-MS offered the advantage of relatively short run times and better selectivity. For example, it took only about 17 min to perform one MRM LC-ESI-MS run. In contrast, 40–70 min is consumed on average to finish OS analyses by HPAEC [60, 78], by nanoESI-LC chipTOF-MS [79], or by CE [38, 80]. As an exception, Xu et al. described an impressive label-free 10-min MRM LC-ESI-MS approach similar to ours [81]. This ultrahigh-pressure LC-MS (UPLC-MS) approach relies on positive ion mode. Although being very fast and able to monitor at least 31 different HMOS monosaccharide compositions, the method lacks the ability to separate between isomers like type I and type II tetrasaccharides LNT and LNnT. Also 3'-SL, 6'-SL, or lacto-*N*-fucopentaose isomers different from LNFP I can not be distinguished. One of those LNFP isomers is LNPF II, which is another Le/Se gene-related compound which can be used for determination of human milk groups in conjunction with the Le/Se gene-dependent LNFP I. As outlined before, our negative ion MRM LC-ESI-MS setup on the other hand provided clear distinction between such (partly isomeric) HMOS as shown in Fig. 4. Thus, this methodological advantage helped to perform human milk group analysis based on detection of marker HMOS like LNFP I and LNFP II. The extracted ion chromatograms displayed in Fig. 5 show that group I milk contains, e.g., LNFP I and LNFP II, group II milk lacks LNFP I but contains LNFP II, group III milk only contains LNFP I and group IV milk finally does not show any of these lacto-*N*-fucopentaose markers. The same findings had been elicited by prior HPAEC analysis of these samples. Consequently, MRM LC-ESI-MS correctly confirmed the HPAEC results in line with the biological expectations dictated by the known Se/Le

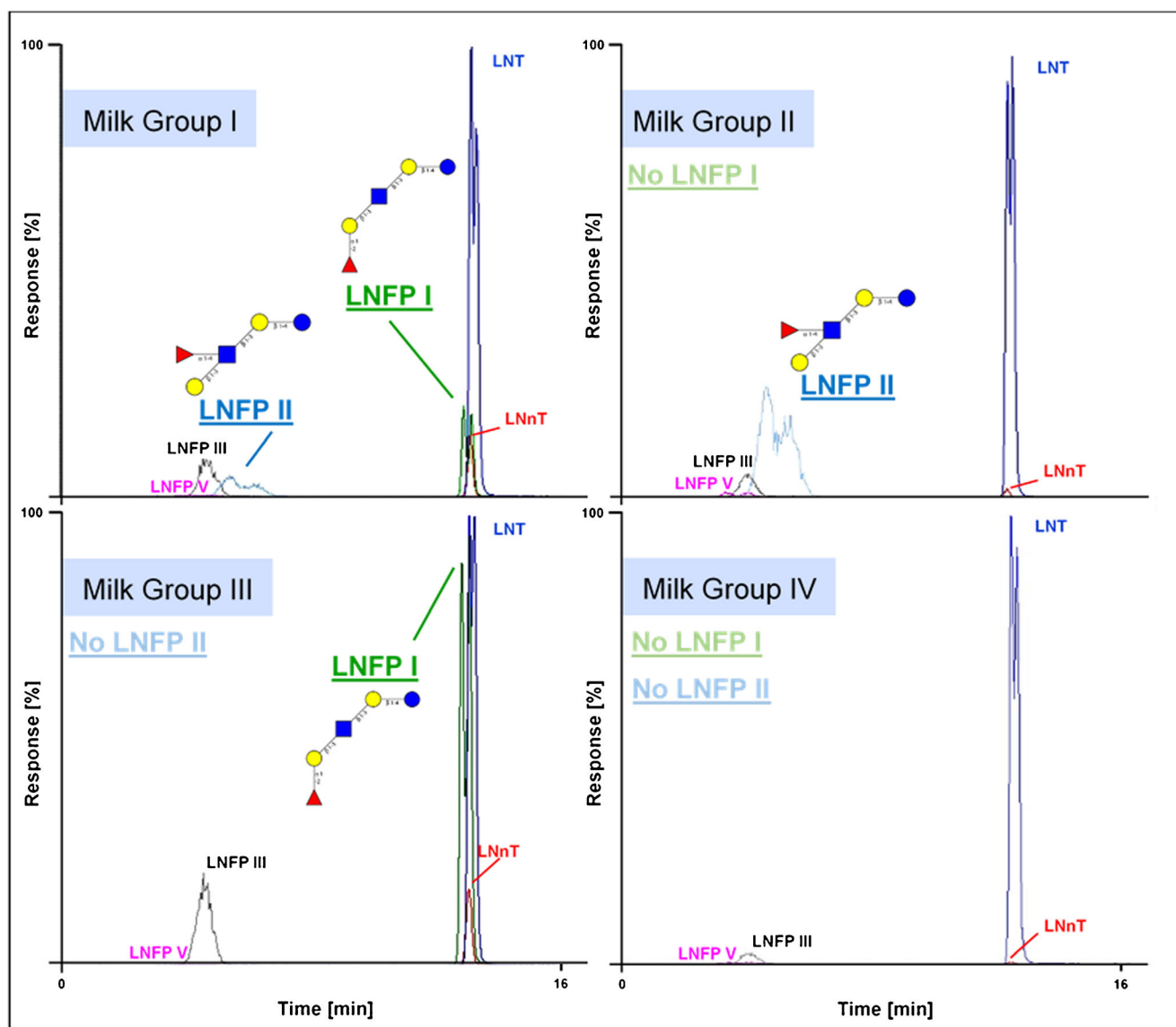


**Fig. 4** MRM LC-ESI-MS analysis of OS in human milk: **a** UV trace at 215 nm wavelength (HMO range shaded in red); **b** total ion chromatogram (TIC); **c** MRM traces of individual HMOS (for compound-specific MRM transitions and related HMO structures, see Tables 1 and 2); HMOS in order of elution: Hex<sub>2</sub>/Lactose (blue); 3-FL (green); LNDHF II/LNnDFH II (gray); DFL (magenta); 2'-FL (red); LNDHF I/LNnDFH I

(green); SL (blue-green); LNFP III (black); LNFP II (blue); LNFP I (dark yellow); LNnT (brown); LNT (dark blue). **d** Magnified section of extracted ion chromatograms (XIC) derived from **c** and normalized to 3'-SL, showing low abundance glycans: Hex<sub>3</sub> (blue), tentative blood group B-tetrasaccharide (gray); Hex<sub>3</sub> (blue); LNFP V (magenta); 6'-SL (green); Hex<sub>4</sub> (light blue); α-arabinopentaose (internal standard, pink), 3'-SL (red)

status of the tested HM specimens [82]. For comparison with LC-MS (Fig. 5), Fig. 6 exemplifies one HPAEC HMO profile obtained for the group I human milk sample. Furthermore, HPAEC and MRM LC-ESI-MS not only

corresponded in lacto-*N*-fucopentaose or lacto-*N*-tetraose profiles but also in all other HMO structures accessible by both methods (data not shown). This suggests comparable suitability of HPAEC and MRM LC-ESI-MS for



**Fig. 5** Extracted ion chromatograms (XIC) of specific LNT and LNFP isomers after MRM LC-ESI-MS analyses of human milk samples belonging to either human milk group I, II, III, or IV. Multi-compound detection of HMOS including LNFP and LNT isomers was achieved. The four different milk groups are discernible by absence or presence of LNFP I, LNFP II, or other fucosylated HMOS which are products of fucosyltransferases FUT2 and FUT3. FUT2 and FUT3 are controlled by Le and Se gene activity. Milk group I is characterized by, e.g., presence of LNFP I and LNFP II. Milk group II contains LNFP II but no LNFP I.

Milk group III has LNFP I but not LNFP II. Finally, milk group IV neither contains LNFP I nor LNFP II. All milk groups display FUT2 and FUT3 unrelated HMOS like LNT or LNnT. Unlike with HPAEC (Fig. 6), all present LNT and LNFP isomers including the low abundance LNFP V could be clearly distinguished from each other owing to selectivity of MRM LC-ESI-MS. Structural representations of oligosaccharides were drawn with Glycoworkbench [71] according to CFG proposals [72]: glucose (Glc), blue circles; galactose (Gal), yellow circles; *N*-acetylglucosamine (GlcNAc), blue circles; fucose (Fuc), red triangles

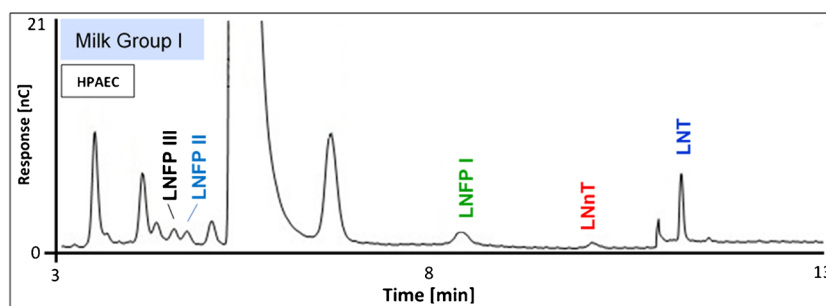
human milk typing and glycoprofiling with the advantage of shorter analysis times using MRM LC-ESI-MS.

## Discussion

In this study, several diagnostic, HMO configuration-dependent fragment ions could be described and translated into a novel MRM LC-ESI-MS method. Thereby, multiplexed and

targeted MRM LC-ESI-MS analysis of relevant HMOS up to DP 6 (neutral and acidic) became possible by utilizing more than 20 MRM transitions in parallel in negative ion mode. Identification as well as relative quantitation of such HMOS and tentative blood group A and B determinants could be accomplished with efficient LC-MS run times of about 17 min.

Compared to similar contemporary LC-MS approaches [6], a higher selectivity for particular HMO structures was obtained by exploiting specific MS/MS



**Fig. 6** Section of an HMO HPAEC-PAD profile obtained for a milk group I sample. For easier comparison with extracted ion chromatograms (XICs) shown in Fig. 5, only LNT and LNFP isomers have been labeled. With HPAEC and in contrast to MRM LC-ESI-MS (Fig. 5), LNFP V could not be unambiguously assigned as it was either below detection

signatures and their translation into MRM transitions. In particular, this allowed the determination of the position of fucose residues relative to the HMO backbone and, moreover, the HMO backbone type itself (type I or type II). The analytical LC-MS setup was further facilitated by an environmentally benign ethanol gradient. Overall our MRM LC-ESI-MS approach offered increased efficiency and ease of use also with respect to a more effortless sample pretreatment (only aqueous dilution of HM, addition of internal standard, and 3-kDa UF).

Application of this MRM LC-ESI-MS concept to real-world human milk study samples provided correct distinction of partly isomeric HMOS and assignment of individual HM samples to one of the four currently known human milk groups.

The high specificity of this MRM LC-ESI-MS concept for particular HMO structures bears potential for further methodological improvement like column-free MRM ESI-MS milk group typing. This might allow for even faster direct detection and identification of several prominent fucosylated or sialylated human milk oligosaccharides like 2'-FL, 3'-FL, 3'-SL, 6'-SL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III, and LNFP V (Table 1). Of course, possible adverse influences of LC-free MRM ESI-MS due to co-elution of major compounds like lactose and less abundant HMOS should be carefully investigated before. They could compromise ionization efficiency and sensitivity for HMOS present in low concentration.

Although our current approach is limited to HMOS up to DP 6, theoretically the identification and relative quantitation of higher molecular weight glycans with more than two fucose or one sialic acid residue might be possible. In this case, information derived from multiple specific or unspecific MRM transitions needs to be combined to achieve a reliable determination. The reason behind this is that we could not reveal any single diagnostic MS/MS fragment by low energy CID which

limit or co-eluted with other HMOS. HPAEC requires more extensive sample preparation and longer elution times to accommodate sufficient resolution and proper identification of HMOS compared to MRM LC-ESI-MS

enabled unambiguous identification of (multiply) fucosylated or sialylated HMOS exceeding DP 6.

Owing to the advantages of relatively short run times and ease of use, we hope that our new method will facilitate HMO analyses of larger sample sets and complete human milk cohorts [83–85]. The expected expanded and thus statistically more meaningful HMO data sets could be useful to decipher the influence of certain early life factors (e.g., maternal genetic background and nutrition) on HMOS patterns. This might in turn help to better understand the impact of human milk oligosaccharides on offspring's longitudinal health trajectories.

Finally, also absolute quantitation of recently introduced next-generation infant milk ingredients like 2'-FL, LNnT, or other HMOS could be imagined. Fortunately, necessary pure 2'-FL and LNnT standards needed for validation of absolute HMOS quantitation are becoming commercially available at lower costs now. Apart from mere scientific application of MRM LC-ESI-MS and, after slight adaptation, quality control (QC) of HMOS, mammalian milk OS or dietary prebiotic glycans like galacto- or fructo-oligosaccharides (GOS/FOS) may be an interesting additional option.

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## Compliance with ethical standards

The usage and analysis of human milk samples described in this study were performed in accordance with ethical standards and guidelines as laid down in the Declaration of Helsinki. Ethical approval and written consents of donors were given as stated in the publications by Thurl et al. [15, 60, 86]. The same samples were already subjected to HMOS analysis in these prior studies. They were now used for optimization of a new LC-MS method but for the same purpose of usage (HMO analysis) as stated in the former publications by Thurl et al. [60, 86] for which ethical approval was received.

**Conflict of interest** Marko Mank and Bernd Stahl are employees of Nutricia Research B.V. Philipp Welsch was enrolled as a PhD student at the Technical University of Dresden during this study and performed the practical part of his PhD thesis at Danone Nutricia Research in Utrecht. Albert J.R. Heck is a professor at Utrecht University. The present research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. None of the authors have further conflicts of interest with regard to the content of this manuscript.

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