Direct inlet negative ion chemical ionization tandem mass spectrometric analysis of triacylglycerol regioisomers in human milk and infant formulas

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Highlights

- A direct inlet MS/MS method was developed to analyze triacylglycerol regioisomers.
- Method was used to analyze human milk, bovine milk, and commercial infant formulas.
- The study revealed differences between human milk, bovine milk and infant formulas.
- Vegetable oil based formulas showed larger differences compared with human milk.

Abstract

A previously developed direct inlet tandem mass spectrometric method for analysis of triacylglycerol (TAG) regioisomers was updated and validated for operation with current instrumentation with improved sensitivity and throughput. TAG regioisomers in pooled Chinese and Finnish human milk samples, two bovine milk samples and 11 infant formulas were identified and quantified. A total of 241 TAG regioisomers were identified and quantified, consisting of over 60 mol% of all TAGs in the human milk samples. The infant formulas deviated largely from human milk in regioisomeric composition of TAGs. In the Finnish and Chinese human milks, the most abundant ones were 1,3-dioleoyl-2-palmitoylglycerol (OPO; 7.4 and 8.8 mol% of all TAGs) and 1(3)-linoleoyl-2-palmitoyl-3(1)-oleoylglycerol (LPO; 4.7 and 8.3 mol% of all TAGs). In the infant formulas 1,2(2,3)-dioleoyl-3(1)-palmitoylglycerol (OOP) and 1(3)-linoleoyl-2-oleoyl-3(1)-palmitoylglycerol/1(3)-palmitoyl-2-linoleoyl-3(1)-oleoylglycerol (LOP/PLO) were more abundant than OPO and LPO. The differences between human milk and infant formula prompt for further development of current formulas.

Keywords

Human milk, infant formula, triacylglycerol, regioisomer, mass spectrometry, direct inlet tandem mass spectrometry, negative ion chemical ionization

1 Introduction

Human milk provides the optimal nutrition for infants, and World Health Organization recommends mothers to exclusively breastfeed infants up to six months of age (World Health Organization, 2011). Fat in breast milk supplies the infant with essential fatty acids (FA) and roughly half of the dietary calories. Approximately 98 % of the lipids in human milk are triacylglycerols (TAG) consisting of three FAs esterified in the middle/secondary (*sn*-2) and outer/primary *(sn*-1 and *sn*-3) positions of TAGs (Koletzko, 2016). Mother's diet (Much et al., 2013; Tian et al., 2019), age (Kim et al., 2017) and BMI (Mäkelä, Linderborg, Niinikoski, Yang, & Lagström, 2013) have been shown to affect the FA composition of milk. However, the regioisomeric distribution of FAs between *sn*-2 and *sn*-1/3 positions in human milk has not been affected by mother's diet or BMI (Linderborg et al., 2014). In human milk, typically oleic and palmitic acids combined cover more than half of all FAs (Bravi et al., 2016). The majority of palmitic acid (typically over 70 mol%) is located in *sn*-2 position, whereas the majority of oleic acid is located in *sn*-1/3 positions (Kallio, Nylund, Boström, & Yang, 2017; Linderborg et al., 2014; Sun, Wei, Su, Zou, & Wang, 2018).

Mixtures of vegetable oils, such as sunflower, palm, coconut and rapeseed oils, are commonly used as the main sources of fat in infant formulas. Plants typically locate palmitic acid to the *sn*-1/3 positions and unsaturated FAs such as oleic and linoleic acids in *sn*-2 position (Innis, 2011). Bovine milk is also often used as an ingredient in infant formulas. Location of palmitic acid in the TAGs of bovine milk fat is closer to that in human milk compared to vegetable oils, and thus it offers some advantage, but the proportion of palmitic acid in *sn*-2 position is still typically less than 50 mol% (Innis, 2011). Infant formulas are generally good at imitating the FA composition of human milk (Sun et al., 2016), but they often fail in simulating human milk in the positional distribution of FAs in TAGs. For example, the majority of palmitic acid remains in the *sn*-1/3 positions despite the addition of commercial OPO-containing ingredients. (Kurvinen, Sjövall, & Kallio, 2002; Sun et al., 2018).

Furthermore, the composition of individual molecular species of TAGs in human milk is far more complex than current infant formulas.

The *sn*-position of palmitic acid in TAGs affects the stool composition of infants (Kennedy et al., 1999; Lasekan, Hustead, Masor, & Murray, 2017; López-López et al., 2001), and it has been shown that calcium soaps of saturated FAs are a major factor in determining stool hardness (Quinlan, Lockton, Irwin, & Lucas, 1995). Gastric lipase hydrolyzes FAs from *sn*-3 position, resulting in *sn*-1,2-diacylglycerols, which are further hydrolyzed by pancreatic lipase, releasing free FAs and *sn*-2-monoacylglycerols. Monoacylglycerols are readily absorbed in the intestine, but the absorption of unesterified FAs is highly influenced by their melting points, which are above human body temperature for saturated FAs such as palmitic (63 °C) and stearic acid (70 °C). Compared to unsaturated FAs with lower melting points, the long chain saturated FAs released from the *sn*-1/3 positions have an increased tendency to form insoluble FA soaps, which are lost in the stool (Innis, 2011). Thus, FAs and calcium are less bioavailable from formula containing palmitic acid in the *sn*-1/3 positions compared with the *sn*-2 position. Stool of breast-fed infants has contained lesser amounts of FA soaps compared to those of infants fed with formulas (Kennedy et al., 1999). The bioavailability of calcium has been reflected in the whole body bone mineral content, which has been detected to be lower in infants fed with low *sn*-2 palmitate formula compared to infants fed with high *sn*-2 palmitate formula or breast milk (Kennedy et al., 1999). The positioning of palmitic acid also affects gut microbiota, sleep and crying behavior of infants, thereby having a significant role in infant health and wellbeing (Bar-Yoseph, Lifshitz, & Cohen, 2013).

Analysis of TAG regioisomers is not a straightforward task. Complex natural sample matrixes such as human milk contain hundreds of different TAG species, making the quantification of each isomer challenging. Analysis methods based on *sn*-specific enzymes have been used for decades (Brockerhoff, Hoyle, & Wolmark, 1966; Deng et al., 2018; Sun et al., 2018). However, one of the drawbacks of the enzymatic reactions is the possibility of acyl migration which potentially leads to

inaccurate results (Pacheco, Crapiste, & Carrín, 2015). Additionally, while enzymatic methods provide information on the portion of specific FAs in *sn*-1/3 and *sn*-2 positions, they do not give any structural information on individual TAG regioisomers.

A number of liquid chromatographic-tandem mass spectrometric (LC-MS/MS) methods utilizing normal phase liquid chromatography (Kalo, Kemppinen, Ollilainen, & Kuksis, 2003), silver-ion liquid chromatography (Holčapek et al., 2010), reversed-phase liquid chromatography (Cubero Herrera, Ramaley, Potvin, & Melanson, 2013) and ultra-high performance liquid chromatography (Kallio et al., 2017; Leskinen, Suomela, & Kallio, 2010) have been developed for TAG regioisomer analysis. Additionally, supercritical carbon dioxide chromatography has gained more interest likely due to low cost and environmentally friendly analytical conditions (Lee et al., 2014). Recently, it has also been used for separation and identification of TAGs in human milk (Zhang et al., 2019). While the chromatographic methods can be different, all current mass spectrometric methods essentially rely on the energetically favored neutral loss of FA from *sn*-1/3 positions in order to identify the TAG regioisomers.

Direct exposure probe coupled with ammonia negative ion chemical ionization tandem mass spectrometry (Kallio & Currie, 1993; Kallio, Yli-Jokipii, Kurvinen, Sjövall, & Tahvonen, 2001; Linderborg et al., 2014) has been applied to the analysis of individual TAG regioisomers. In this approach, the quantification of TAG regioisomers is based on the relative abundances of the unique ketone enolate $[M–H–RCOO–100]^-$ product ions. Direct inlet methods require no chromatographic separation and have the advantage of short analysis times, which can be practical for quick confirmation analyses of certain specific TAGs. However, due to low sensitivity, the existing studies utilizing this direct inlet method only show results for the most abundant TAGs.

The first aim of this study was to further develop and refine the existing direct inlet ammonia negative ion chemical ionization MS/MS method (Kallio & Currie, 1993) to operate with the modern instrumentation. With increased sensitivity of the new instrument it was possible to analyze lower

abundance TAGs with as low as 1 mol% abundance. Additionally, the increased sensitivity also allowed us to simultaneously track multiple product ion spectra during a single analysis, a feature which was not possible with the older method, resulting in a significant reduction in the workload required for the analyses. For method validation we had an exceptionally broad range of 41 different regiopure TAG standards. The developed method was then used to study the regioisomeric composition of TAGs in two pooled human milk samples, one of Finnish origin, and the other of Chinese origin, and to compare them with commercial infant formulas available on the Finnish retail market. According to the packaging information, all of the studied infant formulas contained vegetable oils, some contained also bovine milk fat, and one was enriched with *sn*-2 palmitate. Our hypothesis was that the regioisomeric composition of TAGs in infant formulas is noticeably different compared to human milk, especially in those containing fat mainly from vegetable sources.

2 Materials and methods

2.1 Abbreviations and nomenclature

Regioisomers of triacylglycerols are denoted as AAB, ABA or ABC, where A, B and C are different FAs esterified to *sn*-1, *sn*-2 and *sn*-3 positions on the glycerol backbone. No distinction is made between *sn*-1 and *sn*-3 positions. Abbreviations for individual FAs are denoted as Ln = 18:3 (mainly linolenic), L = 18:2, (linoleic); O = 18:1 (mainly oleic), S = 18:0 (stearic), Po = 16:1 (palmitoleic), P = 16:0 (palmitic), M = 14:0 (myristic), La = 12:0 (lauric) and C = 10:0 (capric acid).

2.2 Materials

2.2.1 Human milk and infant formula samples

Two pooled human milk samples were analyzed. The milk sample of Finnish origin was pooled from milk samples of volunteer mothers (n=7) living in the Turku area, and the milk of Chinese origin was pooled from milk samples of mothers (n=10) living in Beijing area. Approval for collecting and studying the Finnish and Chinese human milk samples was obtained from the Ethics Committee of Hospital District of Southwestern Finland and Medical Research Board of Peking University, respectively. All mothers gave written informed consent. Only healthy mothers, who had given birth to a normally grown full-term infant younger than 6 months of age, and were exclusively breastfeeding, were accepted.

Infant formulas (7 liquid and 4 powdered samples) were purchased from local grocery stores in Turku, Finland. The selected formulas were all intended for infants younger than 6 months, and the samples represented a majority of infant formulas available on the Finnish retail market. In addition, two bovine milk samples provided by Valio Ltd (Helsinki, Finland) were included in this study. A full list of the samples studied is presented in **Table 1**.

2.2.2 Reference compounds and reagents

All 49 TAG reference standards were purchased from Larodan (Malmo, Sweden) and were of at least 98 % purity or higher. 41 regiospecific standards (AAB/ABA and ABC/ACB/BAC type TAGs, **Supplementary Table 1**) were used as such and as mixtures at different ratios. Additionally, 8 AAA type TAG standards were used **(Supplementary Figure 1**). Standards of FA methyl esters (FAMEs), 37 component FAME mixture (Sigma-Aldrich, St. Louis, MO, USA), 68D and GLC-490 (both from Nu-Chek-Prep, Elysian, MN, USA), were used as external standards, and triheptadecanoin (Larodan, Malmö, Sweden) was used as internal standards in the FA composition analysis.

Potassium chloride, HPLC grade chloroform and LC-MS grade hexane and methanol were purchased from VWR international (Radnor, PA, USA), sodium-dried diethyl ether from Merck (Darmstadt, Germany), and methyl acetate and glacial acetic acid from Sigma-Aldrich (St. Louis, MO, USA). Sodium methoxide was prepared from sodium (Sigma-Aldrich, St. Louis, MO, USA) and methanol. Sep-Pak Vac silica 6cc (500 mg) solid phase extraction (SPE) columns were purchased from Waters (Milford, MA, USA).

Table 1 List of samples and the source of fat for each sample.

Sample	Producer	Form	Source of fat according to the package information
Arla Little Baby Organic	Arla	Liquid	Vegetable oil (palm oil, rapeseed oil, soy oil, coconut oil), *mortierella alpina oil*
Nestle NAN Pro	Nestle	Liquid	Vegetable oil (palm oil, rapeseed oil, coconut oil, sunflower oil), fish oil, *mortierella alpina* oil
Nestle NAN Organic	Nestle	Liquid	Vegetable oil (sunflower oil, rapeseed oil), fish oil
Nestle NAN Pro (powder)	Nestle	Powder	Vegetable oil (sunflower oil, coconut oil, soy oil), fish oil, *mortierella alpina* oil
Nestle NAN H.A. (powder)	Nestle	Powder	Vegetable oil (sunflower oil, coconut oil, rapeseed oil), fish oil, *mortierella alpina oil*
Nestle NAN Sensilac (powder)	Nestle	Powder	Vegetable oil (palm oil, rapeseed oil, coconut oil sunflower oil), fish oil, *mortierella alpina oil*
Nutrilon Standard	Nutricia	Liquid	Vegetable oil (palm oil, rapeseed oil, coconut oil, sunflower oil), fish oil, *mortierella alpina oil*
Tutteli Plus	Nutricia	Liquid	Vegetable oil (palm oil, rapeseed oil, coconut oil, sunflower oil), fish oil, *mortierella alpina oil*
Nutrilon Omneo (powder)	Nutricia	Powder	Vegetable oil (modified vegetable oil, rapeseed oil, sunflower oil), fish oil, *mortierella alpina oil*
Semper Baby Semp	Semper	Liquid	Bovine milk, vegetable oil (sunflower oil, rapeseed oil, palm oil, coconut oil), fish oil, *mortierella alpina oil*

Valio Tuuti	Valio	Liquid	Bovine milk, vegetable oil (rapeseed oil, sunflower oil), *mortierella alpina* oil
Bovine milk	Valio	Liquid	Bovine milk
Organic bovine milk	Valio	Liquid	Bovine milk
Human milk, Chinese origin, pooled (n=10)		Liquid	Human milk
Human milk, Finnish origin, pooled (n=7)		Liquid	Human milk

2.3 Methods

2.3.1 Extraction of lipids, isolation of TAG and PL fractions, preparation of FA methyl esters and FA analysis

Before extraction the powdered infant formulas were prepared and diluted according to the instructions on the packaging. 0.5 mL $CHCl_3$ was added to 0.5 mL sample of infant formula, bovine milk or human milk, and the mixture was vortexed briefly. Internal standard triheptadecanoin (0.85 mg) in $CHCl_3$ was added to the sample. For lipid extraction, 1.5 mL MeOH, 2.5 mL $CHCl_3$, and 0.8 mL 0.88 % KCl were added and the sample was vortexed briefly after each addition. Thereafter, the samples were centrifuged at 1100 *g* for 5 min, and the lower chloroform phase was collected. Lipid was further extracted from the upper phase by adding 1.5 mL $CHCl_3$, vortexing briefly, centrifuging, and collecting the chloroform phase. The chloroform phase of the two extractions were combined and evaporated to dryness under gentle nitrogen flow at 50 °C, after which 1 mL dry diethyl ether was added to dissolve the lipids.

For isolation of TAGs from the total lipids, a Sep-Pak Vac silica 6cc (500 mg) solid phase extraction column was conditioned by elution with 5 mL diethyl ether. The extracted lipid sample dissolved in 1 mL diethyl ether was applied to the column. The sample vial was washed with 2x1 mL diethyl ether, which was transferred into the column. TAG fraction was collected by elution with 9 mL diethyl ether. TAG extract was evaporated to dryness under gentle nitrogen flow at 50 °C and dissolved in 1 mL hexane. 100 µL of TAG fraction was taken for methylation.

Solvent was evaporated from the extracted TAGs. The samples were dissolved in 1 mL sodium-dried diethyl ether. 25 µL methyl acetate and 25 µL 1 M sodium methoxide were added. After a brief vortexing the sample was left to incubate at room temperature for 5 min and mixing it occasionally during the incubation. The reaction was stopped by adding 6 µL of acetic acid with brief agitation with vortex. The mixture was centrifuged at 1100 *g* for 5 min. The supernatant was collected into an autosampler vial. Solvent was gently evaporated under nitrogen flow at room temperature. TAGs were dissolved in 1 mL hexane. Vials were thoroughly mixed before analysis.

Gas chromatographic analysis of fatty acid composition of TAG fraction was carried out using a Shimadzu GC-2010 with AOC-20i auto injector and flame ionization detector (Shimadzu Corporation, Kyoto, Japan). The equipment was controlled by GC Solution software. The column was a wall coated open tubular column DB-23 (60 m x 0.25 mm i.d., liquid film 0.25 µm, Agilent Technologies, J.W. Scientific, Santa Clara, CA, USA). Helium was used as the carrier gas. Injector temperature was set at 270 °C. Split/splitless injection mode was used and the split was opened after 1 min. The injection volume was 0.5 µL. The column temperature program was as follows: initial temperature 130 °C and hold for 1 min, increase at 4.5 °C/min to 170 °C, increase at 10 °C/min to 220 °C and hold for 14.5 min, increase at 60 °C/min to 230 °C and hold for 3 min. The detector temperature was set at 280 °C. Fatty acids were identified using external fatty acid methyl ester standards. The quantification was performed by comparing the peak area of each fatty acid with that of the internal standard. Correction factors were determined by analysis of standard mixtures and applied in the quantification to correct the difference in detector response between each fatty acid and the internal standard. The fatty acid composition was calculated as weight percentage of the total fatty acids.

2.3.2 MS analysis of the TAG molecular weight distribution

Mass spectrometric analyses of TAGs were carried out using a Thermo Scientific TSQ 8000 EVO mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a direct exposure

probe (DEP). The equipment was controlled by Xcalibur software. The system was used in negative chemical ionization mode with ammonia (purity 6.0) as the ionization gas. 1 µL of the TAG fraction of each sample containing approximately 20 µg of TAGs was applied onto the rhenium wire on the tip of the probe. The probe was placed inside the ion source via the vacuum interlock and after a short period of vacuum stabilization the probe tip was heated at a steadily increasing rate. There was no gas flow coming from the GC side of the instrument and the transfer line was blocked.

Instrument parameters were optimized with regiopure PPO, POP, LLO and LOL with the goal of having the highest obtainable intensity for $[M–H]^-$ ions. Low ion source temperature (100 °C) yielded the highest abundance of $[M–H]^-$ ions in the tested temperature range (80-220 °C). For tandem MS analysis of TAG regioisomers, the ion source temperature was increased to 340 °C to keep the ion source clean and to gain better sensitivity.

The instrument settings for molecular weight analysis were: ion source temperature 100 °C, ammonia gas flow rate 1.5 mL/min, electron energy 70 eV, emission current 300 mA and scan time 0.1 s. In addition, the effects of probe heating rate were investigated, but the heating rate did not have a significant effect on the results, so a fast heating rate of 100 mA/s (0–800 mA) was chosen. MS scans between *m/z* 400–1000 were acquired in quadruplicate. The number of acyl carbons and double bonds were calculated according to the *m/z* values of $[M–H]^-$ ions. Relative molar proportions of different molecular weight species were calculated using the abundances of $[M–H]^-$ ions. The amount of naturally occurring ^{13}C was taken into account when the proportions of TAGs were calculated.

2.3.3 MS/MS analysis of the TAG regioisomers

The MS scan data was used to create a product ion scan method for each selected pseudomolecular $[M–H]^-$ ion. Fragmentation of pseudomolecular TAG ions $[M–H]^-$ was performed using collision-induced dissociation (CID) with argon gas, which favors dissociation of FAs from *sn*-1/3 positions. Selection of precursor ions was performed within a range of *m/z* ±0.5 of the theoretical *m/*z value of the pseudomolecular TAG ion. For our quadrupole mass spectrometer, which is a low resolution

instrument, this range was broad enough to allow acceptable sensitivity, but narrow enough not to select adjacent TAG ions with different *m/z* ratio. Product ions were scanned between *m/z* 100–650 to determine the primary (*sn*-1/3) and secondary (*sn*-2) positions of FAs. Ion source temperature was set at 340 °C, ammonia gas flow rate at 1.5 mL/min, electron energy at 70 eV, emission current at 300 mA, collision energy at 20 eV and 0.1 s scan time was used. Calculations of the TAG regioisomer abundances were based on the relative proportions of $[M–H–FA–100]^-$ and $[RCOO]^-$ ions, and the results were calculated using MSPECTRA 1.4 software (Kurvinen, Rua, Sjövall, & Kallio, 2001).

A total of 21 different ACN:DB species (44:2, 44:1, 46:1, 46:0, 48:3, 48:2, 48:1, 50:4, 50:3, 50:2, 50:1, 52:5, 52:4, 52:3, 52:2, 52:1, 54:6, 54:5, 54:4, 54:3 and 54:2), each representing at least 1 mol% of the total ACN:DB species in the human milk samples, were selected for regioisomeric analysis. In infant formulas and bovine milks out of the selected 21 ACN:DB species, only the ones containing more than 1 mol% of the total TAGs were quantified. Seven different ACN:DB species could be tracked and analyzed during a single run. Each selected ACN:DB species was analyzed in quadruplicate, and the results were expressed as average ± standard deviation.

2.3.4 Validation

Response factors correcting the possible effects of different acyl chain lengths and number of double bonds for the molecular weight distribution calculations were determined with a mixture of eight AAA type reference TAG compounds (CCC, LaLaLa, MMM, PoPoPo, PPP, LLL, OOO and SSS) with equal molar concentrations (0.45 mM each in hexane). Statistical analysis on the results of the TAG standard MS analyses was performed with Tukey test and Levene's test ($p<0.05$) using Origin 2016 software (OriginLab, Northampton, MA, USA).

The discrimination factor is the measured probability of a FA to be dissociated from *sn*-2 position instead of *sn*-1/3 positions. The calculation software (MSPECTRA 1.4) used to calculate the ratios of regioisomers uses an universal discrimination factor for all TAGs disregarding the impact of the number of acyl carbons and double bonds of fatty acids (Kurvinen et al., 2001). Discrimination factors

were established for 10 different ABA TAG regioisomer standards (MOM, PMP, POP, PSP, SLS, SOS, SPS, OLaO, OLO and OSO). The average discrimination factor of all 10 TAG standards was used as the universal discrimination factor for the subsequent calculations of the regioisomer composition of all TAGs. Additionally, in order for MSPECTRA to be able to determine the proportions of FAs within each ACN:DB group, correction factors for FAs were calculated from the intensities of $[RCOO]^-$ ions of the 10 ABA type TAG standards.

The regioisomeric analysis method and calculations were validated with reference compounds of 16 pairs of ABA and AAB TAGs. For each pair, validation was done with five binary mixtures of ABA and AAB TAGs (0, 25, 50, 75 and 100 % ABA). Additionally, the method was validated with reference TAGs containing three different FAs (ABC), including three different triplets of regioisomers ABC, ACB and BAC type TAGs. For each triplet TAG, four different samples were prepared containing, respectively, 100 % ABC, 100 % ACB, 100 % BAC, and 33 % of each of ABC, ACB and BAC. All ABA/AAB and ABC/ACB/BAC standard mixtures had a total concentration of 0.1 mM.

Further, a data conversion tool was developed for transforming exported Xcalibur raw data to ASCII format, which is supported by the MSPECTRA software, resulting in significant reduction in the time needed for data handling after the MS and MS/MS analysis. Reliability of the conversion tool was tested by comparing the results gained with automatic data conversion with results gained with manual picking of the ions from the raw data.

3 Results and discussion

3.1 FA composition in TAGs

A total of 37 different FAs, including the internal standards, were identified from the samples (see **Supplementary Table 2**). A majority of the total FAs were in the TAG fraction (human milk samples 98.5–98.9 %, bovine milk samples 98.4–98.9 % and infant formulas 97.3–99.4 %), and most of the identified FAs (75.2–95.0 %) in all samples consisted of just four FAs (16:0, 18:2n-6, 18:1n-9 and 18:0, **Figure 1**).

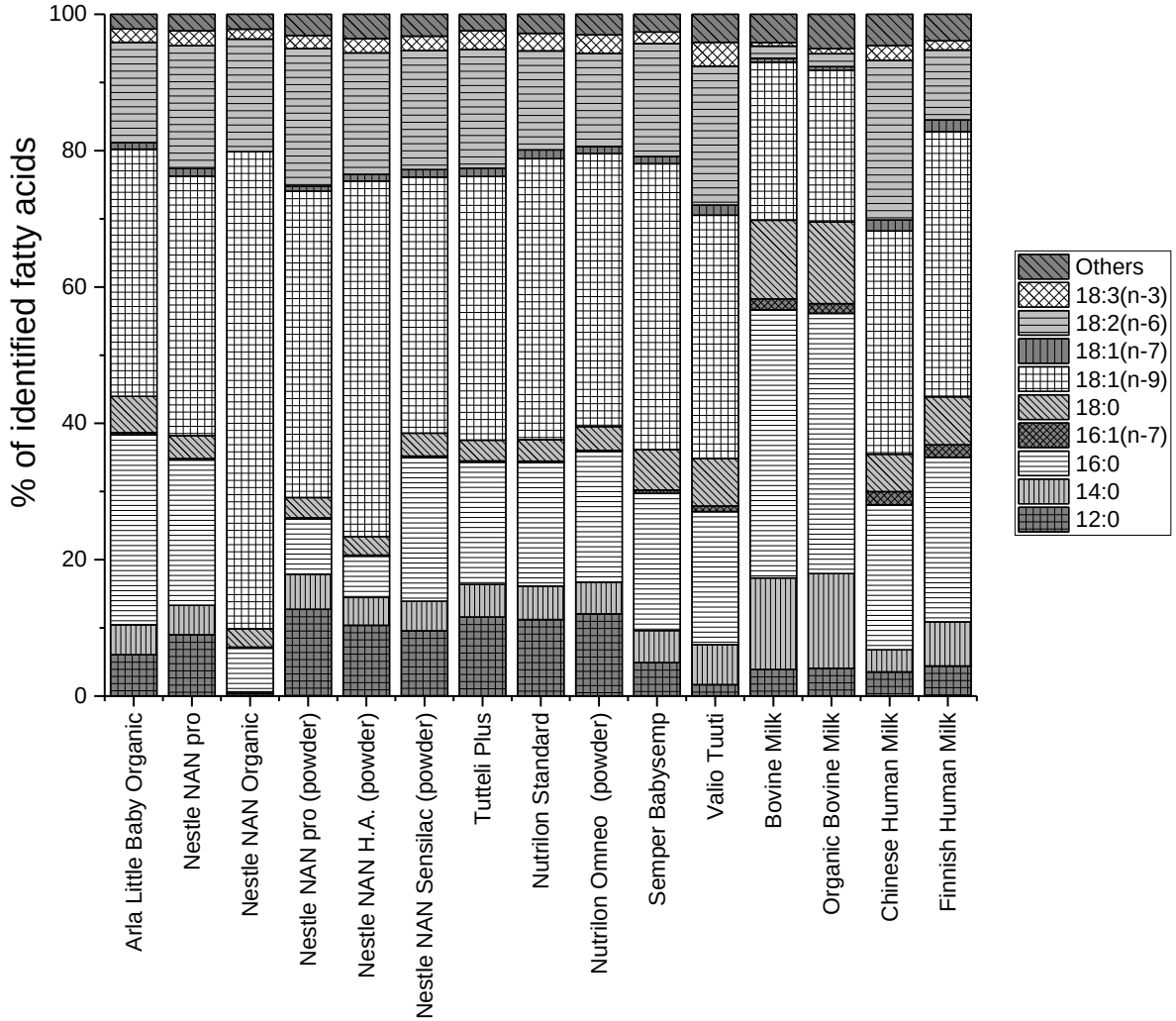

Figure 1 Identified FAs in the TAG fraction displayed as a percentage of all identified FAs

There were some differences between Chinese and Finnish human milk. Most notably, the percentage of 18:2n-6 in the Chinese human milk (23.5 %) was more than double compared to the Finnish human milk (10.3 %). The proportion of 14:0 in the Chinese human milk was half (3.2%) of the level in the Finnish human milk (6.4 %). Additionally, the proportion of 18:1n-9, 18:0, 16:0 and 12:0 in the Chinese human milk (32.8, 5.4, 21.2 and 3.5 %, respectively) was slightly lower compared to the corresponding values in the Finnish human milk (38.9, 7.0, 24.1 and 4.4 %, respectively).

Proportions of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FAs in Chinese (34.6 % SFA, 37.0 % MUFA and 28.4 % PUFA) and Finnish (42.8 % SFA, 43.5 % MUFA and 13.7 % PUFA) human milk samples are well in agreement with the findings of a previous study (Chinese 35.4 % SFA, 35.9 % MUFA and 27.2 % PUFA; Finnish 47.4 % SFA, 37.5 % MUFA and 12.6 % PUFA) (Kumar et al., 2016).

Compared to the human milk samples the bovine milks contained more 14:0 (13.3–13.8 %), 16:0 (38.0–39.1 %) and 18:0 (11.3–11.9 %). The bovine milk samples also contained less 18:1n-9 (22.9–23.5 %) and significantly less 18:2n-6 (2.3–2.5 %) than the human milk samples. The differences between the regular and organic bovine milk were very small. FAs with ACN ≤10, abundant in bovine milk, could not be quantified with this GC-method. Lower temperature at the beginning of the program would have allowed 8:0 and 10:0 to be identified but it also would have broadened the peaks of some longer chain FAs resulting in overlapping peaks. A longer column would likely have helped with better separation, but in this research the shorter chain FAs are of less significance. According to previous studies the amount of short chain FAs with ACN ≤10 in human milk is less than 3 % of the total FAs, whereas in bovine milk it can be closer to 20 % (Cruz-Hernandez, Goeuriot, Giuffrida, Thakkar, & Destaillats, 2013; Zou et al., 2013).

NAN organic, NAN Pro (powder) and NAN H.A. (powder) contained the lowest proportions of 16:0 (6.1–8.2 %), while the highest levels were found in bovine milk (38.0–39.1 %). The content of 16:0 in other infant formulas ranged from 17.9 % to 27.8 % of total FAs. The amount of 18:2n-6 in most

infant formulas was somewhat consistent, where the lowest amount was found in Nutrilon Omneo (13.6 %) and the highest amount in Valio Tuuti (20.2 %). The 18:1n-9 content in infant formulas ranged from 35.7 % to 52.0 %, excluding NAN Organic (69.4 %), which was exceptionally abundant in 18:1n-9.

The results of FA composition (see **Figure 1**) in human milk are in agreement with previous studies (Cruz-Hernandez et al., 2013; Zou, Jin, Guo, Xu, & Wang, 2016). The differences in FA composition between the Chinese and the Finnish human milk are likely a result of different dietary habits between the two regions. Higher abundance of 14:0 in the Finnish human milk could be a result of higher consumption of dairy products, as bovine milk contains a notable amount of 14:0 (13.3–13.8 %) and dairy products are a very common part of Finnish diet. This was also observed in a study with Swedish mothers (Xiang, Harbige, & Zetterström, 2005), where the main sources of dietary fat were cheese and meat. Higher amounts of 18:2n-6 in the Chinese human milk has also been documented earlier (Peng et al., 2009; Xiang et al., 2005), which may be explained by higher intake of vegetable oils rich in 18:2, such as soybean oil and peanut oil.

Most studied infant formulas were fairly good at imitating the human milk FA composition, but there were also some notable differences. The three Nestle infant formulas (NAN organic, NAN Pro (powder) and NAN H.A. (powder) contained significantly less 16:0 (6.1–8.2 %) than human milk (21.2–24.1 %). It is also worth noting that NAN Pro and NAN Pro (powder), despite having the same brand name, had different FA compositions. For example, NAN Pro contained more 16:0 (21.3 %) than NAN Pro (powder) (8.2 %). The difference can be explained by information provided by the package label, where the three major sources of fat listed for NAN pro are palm oil, rapeseed oil and coconut oil, whereas for NAN pro (powder) the main sources of fat are sunflower oil, coconut oil and soybean oil.

3.2 Molecular weight distribution

Molecular weight distribution expressed as ACN:DB ratios of TAGs in human milks, bovine milks and selected infant formulas is presented in **Table 2**. While it has been shown that diet has an effect on the FA composition of human milk, there are few studies that have reported the effects of diet on the molecular weight distribution of TAGs. It is logical to speculate that if the building blocks of TAGs are altered, also the TAGs themselves are likely altered. In our study we found some minor differences in the molecular weight distribution of TAGs between the Chinese and Finnish human milks, which could potentially be explained by the dietary differences.

The molecular weight distribution of the human milks fell mostly within the range of even-numbered ACN 48–54 (61.1 mol% in Finnish vs. 65.5 mol% in Chinese), the most abundant molecular species being 52:2 (12.2 vs. 10.4 mol%) and 52:3 (6.7 vs. 11.0 mol%). TAGs with even-numbered ACN of 28–46 represented only 23.5–24.5 mol% of the total molecular species. Other minor molecular species, including odd-numbered ACN 29–57 added up to a total of 9.7–13.1 mol% in the human milk samples. There were only small differences between Finnish and Chinese human milks, mostly in the relative abundance of 52:3 (6.7 mol% in Finnish vs. 11.0 mol% in Chinese), 52:4 (2.3 vs. 5.5 mol%) and 54:5 (1.6 vs 4.2 mol%). Previous studies (Kallio et al., 2017; Kurvinen et al., 2002; Linderborg et al., 2014) with Finnish human milk have also shown that the most abundant ACN:DB species in human milk are 52:2 and 52:3, but there are notable differences on the abundance of 52:2, which ranged from 10.8 to 21.6 mol% of total abundance of ACN:DB species depending on the study. The amount of OPO, which is the most abundant 52:2 TAG in human milk, varied also among different studies investigating Chinese human milk, ranging between 8.9–16.6 mol% of total amount of TAGs (Kallio et al., 2017; Yuan et al., 2019; Zhang et al., 2019). A majority of these differences is likely explained due to variation among the individual samples studied (Zhang et al., 2019), but the different types of methodologies used could also have contributed to the variation.

Molecular weight distributions of regular bovine milk and organic bovine milk were very similar with only small differences. Contrary to human milk, the molecular weight distribution of bovine milk was concentrated between even-numbered ACN 28–46 (71.6–71.7 mol% of total) and only 14.7–16.4 mol% of total molecular species were in the range of ACN 48–54. Other minor molecular species, including odd-numbered ACN 29–57, added up to a total of 12.0–12.6 mol% in the bovine milk samples. The most abundant ACN:DB species in bovine milk samples were 36:0 (9.7–9.9 mol%), 38:0 (6.3–6.6 mol%), 34:0 (6.5 mol%) and 38:1 (5.8–6.2 mol%).

Several infant formulas (NAN Pro, NAN pro (powder), NAN HA (powder), NAN Sensilac (powder), Little Baby Organic, Nutrilon Standard, Tutteli Plus and Nutrilon Omneo (powder) have noticeably higher amounts of molecular weight (ACN:DB) species 32:0, 34:0, 36:0, 38:0, and 40:0 adding up to a total of 16.7–26.3 mol% compared to human milk with only 2.0–2.3 mol% represented by these molecular species. Bovine milk also had significant amounts of these molecular species adding up to 29.2–29.9 mol% of total ACN:DB species of TAGs. However, the amount of these molecular species in these infant formulas cannot be explained by milk fat, because they only contained fat free milk ingredients. All of these formulas contained coconut oil, which is likely the reason for this kind of molecular weight distribution as close to 50 % of all FAs in coconut oil is 12:0 (Orsavova, Misurcova, Ambrozova, Vicha, & Mlcek, 2015). Infant formulas containing milk fat (Baby Semp, Valio Tuuti) also had higher amounts of these molecular species (13.7–14.2 mol%) compared to human milk.

All studied infant formulas had higher amounts of ACN:DB species 54:2, 54:3, 54:4, 54:5 and 54:6 (together representing 21.7–64.8 mol% of total ACN:DB) compared to human milk (11.5–15.2 mol%) and bovine milk (1.3–1.5 mol%). This is likely due to the use of various vegetable oils as a source of fat, most notably sunflower oil, rapeseed oil and palm oil, which all contain notable quantities of 18:1 (28.0–63.3 mol%) and 18:2 (9.0–62.4 mol%) (Orsavova et al., 2015). At least one of these three oils is a major source of fat in every studied infant formula, and in most of the studied infant formulas at least two of these oils are used.

382 **Table 2** Distribution of ACN:DB species of TAGs in infant formula, bovine milk and human milk samples presented as molar percentage of all TAGs.

	28:2	28:1	28:0	30:2	30:1	30:0	32:2	32:1	32:0	34:3	34:2	34:1	34:0	36:3	36:2	36:1	36:0	38:3	38:2	38:1	38:0	40:3	40:2	40:1	40:0	42:3	42:2	42:1	42:0	44:3	44:2	44:1	44:0	46:3	46:2	46:1	46:0
Arla Little Baby Organic	0.0	0.6	0.4	0.0	0.3	0.7	0.7	0.2	2.5	0.5	0.3	0.6	3.5	0.3	0.5	0.9	4.9	0.0	0.5	1.6	4.1	0.0	0.3	1.0	1.8	0.0	0.4	0.7	1.3	0.0	0.3	0.7	0.9	0.0	0.3	0.7	0.6
Nestle NAN Pro	0.0	0.4	0.4	0.0	0.0	0.9	0.5	0.3	3.3	0.7	0.5	0.3	4.9	0.0	0.3	0.4	5.7	0.0	0.4	0.6	4.9	0.0	0.0	0.5	3.3	0.0	0.0	0.7	2.1	0.0	0.3	0.4	0.5	0.0	0.0	0.5	0.3
Nestle NAN Organic	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.2	2.0	0.3	0.2	0.1	0.2	0.2	0.2	0.3	0.0	0.4	0.2	0.3	0.0	0.2	0.2	0.2	0.0	0.0	0.1	0.2	0.0	0.2	0.4	0.3	0.0	0.0	0.0	0.0
Nestle NAN Pro (powder)	0.0	0.0	0.5	0.0	0.0	0.9	0.2	0.2	4.3	0.8	0.2	0.3	5.7	0.0	0.1	0.5	7.1	0.0	0.1	0.7	5.4	0.0	0.2	0.5	3.7	0.0	0.2	0.7	1.9	0.0	0.3	0.6	0.6	0.0	0.2	0.4	0.2
Nestle NAN HA (powder)	0.0	0.1	0.4	0.0	0.0	1.1	0.2	0.3	3.7	0.0	0.1	0.3	5.6	0.0	0.2	0.3	5.1	0.2	0.3	0.6	4.9	0.0	0.2	0.4	3.1	0.0	0.0	0.5	1.2	0.0	0.2	0.5	0.4	0.0	0.1	0.3	0.2
Nestle NAN Sensilac (powder)	0.0	0.0	0.4	0.0	0.2	0.9	0.4	0.2	3.4	0.4	0.2	0.2	4.2	0.0	0.1	0.2	4.1	0.0	0.2	0.7	4.6	0.0	0.1	0.4	2.7	0.0	0.2	0.5	1.2	0.0	0.2	0.4	0.5	0.0	0.2	0.4	0.2
Nutrilon Standard	0.0	0.4	0.3	0.0	0.2	1.3	0.5	0.2	4.1	0.5	0.3	0.5	4.9	0.0	0.2	0.4	5.4	0.0	0.3	0.7	4.0	0.0	0.1	0.7	3.2	0.0	0.0	0.7	1.2	0.0	0.2	0.5	0.5	0.0	0.2	0.4	0.3
Tutteli Plus	0.0	0.4	0.4	0.0	0.3	1.1	0.3	0.3	4.7	0.0	0.4	0.4	5.5	0.3	0.3	0.4	6.0	0.0	0.6	0.7	5.2	0.0	0.0	0.7	3.2	0.0	0.0	0.6	1.7	0.0	0.2	0.6	0.6	0.0	0.2	0.5	0.4
Nutrilon Omneo (powder)	0.0	0.0	0.2	0.0	0.1	0.5	0.4	0.3	2.3	0.5	0.1	0.2	2.7	0.0	0.0	0.4	5.9	0.0	0.1	0.6	4.0	0.0	0.0	0.5	3.6	0.0	0.2	1.2	1.3	0.0	0.3	1.0	0.4	0.0	0.3	0.8	0.3
Semper Baby Semp	0.0	0.2	0.4	0.0	0.2	0.7	0.3	0.3	1.7	0.0	0.3	0.5	2.8	0.0	0.4	0.7	3.5	0.0	0.7	1.5	3.6	0.2	0.7	1.4	2.1	0.2	0.4	1.3	1.3	0.0	0.4	0.7	0.8	0.0	0.4	0.7	0.7
Valio Tuuti	0.0	0.5	0.7	0.0	0.4	0.6	0.4	0.5	1.3	0.6	0.4	1.0	2.4	0.0	0.5	2.1	5.0	0.4	1.1	3.9	3.6	0.3	1.1	2.3	1.9	0.2	0.7	1.5	1.9	0.2	0.6	1.5	1.3	0.0	0.5	1.2	1.1
Bovine Milk	0.2	0.5	1.5	0.3	1.0	1.9	0.5	1.2	3.2	0.3	0.4	1.7	6.5	0.2	0.5	3.4	9.7	0.4	1.2	6.2	6.3	0.4	1.8	3.8	3.6	0.3	0.9	2.1	2.5	0.0	0.7	2.0	1.9	0.3	0.8	1.7	1.7
Organic Bovine Milk	0.3	0.8	2.3	0.3	1.1	2.3	0.6	1.2	3.3	0.2	0.4	1.6	6.5	0.2	0.6	3.5	9.9	0.3	1.1	5.8	6.6	0.5	1.7	3.9	3.5	0.3	0.8	2.0	2.5	0.2	0.7	1.9	2.0	0.3	0.6	1.7	1.6
Chinese Human Milk	0.3	0.5	0.2	0.4	0.4	0.2	1.2	0.8	0.2	0.6	0.3	0.2	0.3	0.0	0.1	0.2	0.3	0.0	0.3	0.4	0.5	0.0	0.4	0.7	0.7	0.2	0.8	1.3	0.9	0.5	1.2	2.0	0.8	0.9	2.1	2.5	1.0
Finnish Human Milk	0.2	0.2	0.0	0.3	0.2	0.0	0.9	0.2	0.1	0.5	0.2	0.2	0.2	0.0	0.0	0.3	0.5	0.0	0.2	0.7	0.7	0.2	0.3	1.0	0.7	0.2	0.5	1.9	1.1	0.3	1.1	3.0	1.4	0.7	1.9	3.4	1.3

	48:4	48:3	48:2	48:1	48:0	50:6	50:5	50:4	50:3	50:2	50:1	50:0	52:6	52:5	52:4	52:3	52:2	52:1	52:0	54:6	54:5	54:4	54:3	54:2	54:1	54:0	56:5	56:4	56:3	56:2	56:1	56:0	58:2	58:1	58:0	[a]Others
Arla Little Baby Organic	0.0	0.0	0.7	1.3	1.6	0.0	0.0	0.0	0.5	3.9	7.7	1.2	0.4	0.5	2.9	5.6	10.0	2.9	0.5	2.6	3.4	6.8	5.3	3.6	0.6	0.2	0.1	0.3	0.4	0.3	0.4	0.0	0.0	0.0	0.0	4.0
Nestle NAN Pro	0.0	0.2	0.6	0.6	0.4	0.0	0.0	0.2	0.4	3.0	10.6	0.6	0.0	0.6	2.3	4.6	8.4	2.2	0.3	3.1	6.4	4.7	8.9	1.8	0.6	0.1	0.0	0.4	0.5	0.3	0.2	0.1	0.0	0.0	0.0	4.8
Nestle NAN Organic	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.1	0.2	0.6	2.0	0.0	0.2	0.4	2.2	2.6	8.4	1.5	0.0	3.6	7.3	10.4	40.3	3.3	1.7	0.0	0.3	0.3	0.7	0.6	0.3	0.1	0.6	0.2	0.1	5.3
Nestle NAN Pro (powder)	0.0	0.2	0.4	0.3	0.2	0.1	0.2	0.2	0.3	0.6	0.8	0.1	0.0	0.7	2.7	2.4	3.9	0.5	0.1	4.5	8.3	7.8	21.2	2.4	1.0	0.2	0.2	0.4	0.4	0.3	0.1	0.0	0.3	0.1	0.0	2.5
Nestle NAN HA (powder)	0.0	0.0	0.2	0.2	0.1	0.0	0.1	0.1	0.2	0.3	0.5	0.1	0.0	0.3	1.4	2.2	5.5	0.6	0.2	5.1	6.7	9.3	23.4	4.6	0.5	0.1	0.4	0.6	0.5	0.4	0.2	0.1	0.4	0.1	0.0	5.3
Nestle NAN Sensilac (powder)	0.1	0.2	0.4	0.6	0.3	0.0	0.0	0.0	0.4	3.6	6.6	2.0	0.0	0.5	1.9	3.7	7.2	4.1	0.0	3.8	7.7	7.8	10.4	2.9	0.5	0.3	0.0	0.7	0.4	0.3	0.2	0.1	0.0	0.0	0.0	5.7
Nutrilon Standard	0.0	0.0	0.3	0.6	0.5	0.0	0.0	0.0	0.4	4.0	6.0	0.4	0.3	0.3	1.5	2.9	7.3	3.3	0.1	2.7	7.0	10.4	13.6	1.0	0.5	0.2	0.2	0.4	0.4	0.4	0.2	0.0	0.0	0.0	0.0	2.6
Tutteli Plus	0.0	0.0	0.4	0.6	1.3	0.0	0.0	0.3	0.5	3.3	6.2	0.8	0.0	0.0	2.4	3.2	8.4	2.1	0.4	3.9	5.0	6.8	14.0	0.8	0.8	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5
Nutrilon Omneo (powder)	0.0	0.0	0.6	0.8	1.5	0.0	0.2	0.1	0.4	1.7	7.6	1.5	0.0	1.0	2.5	4.8	10.8	3.7	0.4	2.8	5.8	7.5	7.4	3.6	0.3	0.4	0.3	0.5	0.4	0.4	0.2	0.1	0.0	0.0	0.0	4.7
Semper Baby Semp	0.0	0.0	0.6	0.9	0.8	0.0	0.0	0.0	0.5	2.4	6.2	0.7	0.2	0.3	1.8	4.7	6.3	1.5	0.7	4.1	5.5	5.3	14.2	3.4	1.0	0.1	0.2	0.4	0.5	0.4	0.3	0.0	0.2	0.1	0.0	7.4
Valio Tuuti	0.0	0.2	0.8	1.6	1.2	0.2	0.2	0.1	0.4	1.2	2.1	0.7	0.3	0.4	1.7	1.5	2.8	1.3	0.2	4.9	7.0	5.8	6.4	1.4	0.2	0.2	0.2	0.3	0.5	0.3	0.2	0.0	0.0	0.0	0.0	14.1
Bovine Milk	0.0	0.0	0.9	2.5	1.3	0.0	0.0	0.0	0.4	1.2	2.7	0.9	0.0	0.0	0.0	0.7	1.8	1.4	0.5	0.0	0.0	0.2	0.6	0.7	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.0
Organic Bovine Milk	0.0	0.3	0.8	2.3	1.2	0.1	0.1	0.2	0.4	1.1	2.1	0.9	0.0	0.0	0.2	0.5	1.5	1.1	0.4	0.0	0.0	0.2	0.5	0.6	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.6
Chinese Human Milk	0.9	1.8	2.6	1.5	0.6	0.0	0.2	1.1	2.2	3.7	3.1	0.5	0.0	1.3	5.5	11.0	10.4	2.9	0.5	1.8	4.2	4.3	3.5	1.3	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.0	0.0	0.0	9.7
Finnish Human Milk	0.4	1.5	3.4	3.4	1.0	0.3	0.3	0.7	2.2	4.7	4.3	0.8	0.0	0.6	2.3	6.7	12.2	3.8	0.5	0.8	1.6	3.3	4.1	1.7	0.4	0.2	0.3	0.3	0.2	0.2	0.1	0.2	0.0	0.0	0.0	13.1

383 [a] "Others" primarily includes odd-numbered ACN:DB species between ACN 29–57 and other minor species.

3.3 Regioisomerism of TAGs of selected molecular weight species

In most infant formulas the 21 ACN:DB species selected for regioisomer analysis constituted 55.2–68.9 mol% of all TAGs. Two exceptions were NAN organic (84.1 mol%) and Valio Tuuti (44.1 mol%), where the percentage was higher and lower than the range, respectively. In human milk these ACN:DB species added up to 64.5–64.7 mol% of the total TAGs, and in the bovine milk samples only 17.6–19.4 mol%. Regioisomeric compositions of the most abundant TAGs of the analyzed ACN:DB species are presented in **Table 3** and all the identified regioisomers of the selected 21 ACN:DB species are presented in **Supplementary Table 3**. ACN:DB species containing less than 1 mol% of the total amount of TAGs could not be quantified reliably, meaning that not all of the selected 21 ACN:DB species were quantified in all the samples. ACN:DB species 50:1, 52:3 and 52:2 are often viewed as important in human milk because of their high relative abundance and the interest in 16:0.

In all the samples the most abundant 50:1 isomer pair was PPO/POP. PPO was the most abundant regioisomer of 50:1 TAG in human milk (65.9–80.2 mol%), bovine milk (61.7–64.6 mol%), Nutrilon Omneo (75.5 mol%) and Valio Tuuti (57.0 mol%). In all other infant formulas POP was the most abundant molecular species of 50:1 TAG (69.3–95.2 mol%). Additionally, 50:1 TAGs in Finnish human milk, both bovine milk samples, as well as Valio Tuuti contained a notable amount of OMS (13.6–20.1 mol%) and smaller amounts of MOS (3.4–6.6 mol%) and MSO (0.1–3.8 mol%).

All the samples analyzed had PLO/LPO/POL as a majority of 52:3 TAGs. LPO was the most abundant 52:3 TAG in human milk (70.7–75.5 mol%), whereas the only infant formula with LPO as the most abundant of 52:3 TAGs was Nutrilon Omneo (39.0 mol%). A majority of 52:3 TAGs in the other infant formulas consisted mainly of PLO (21.6–53.5 mol%) and POL (32.4–51.2 mol%), while the amount of LPO was generally less than 10 mol%.

OOP/OPO was the most abundant regioisomer pair of the ACN:DB 52:2 group in all samples, but only human milk contained more OPO (70.7–72.3 mol%) than OOP (9.6–17.0 mol%). In the bovine milks and Nutrilon Omneo, OPO (38.8–46.3 mol%) and OOP (46.1–48.3 mol%) were more equally distributed, whereas Valio Tuuti contained more OOP (62.6 mol%) than OPO (21.9 mol%). In all other infant formulas OOP (87.4–94.0 mol%) was clearly dominating in 52:2 TAGs with OPO being almost non-existent (0.0–2.7 mol%). Ingredients enriched with OPO are sometimes used in infant formulas to increase the total amount of 16:0 in *sn*-2 position. In this study the only such infant formula was Nutrilon Omneo.

Average distribution of FAs in *sn*-positions in the selected 21 ACN:DB species is presented in **Table 4**. Majority of 16:0 and 14:0 in human milk samples was located in *sn*-2 position (74.0–77.8 mol% and 64.8–65.4 mol%, respectively), whereas the unsaturated FAs 18:1, 18:2, 18:3 and 16:1 were primarily located in *sn*-1/3 positions (83.2–83.5 mol%, 88.9–89.4 mol%, 82.1–88.2 mol% and 61.6–63.9 mol%, respectively). In contrast to 16:0, a majority of 18:0 (77.6–86.3 mol%) in human milks was in *sn*-1/3 positions.

16:0 and 14:0 were more evenly distributed in both bovine milk samples between the outer and central positions, but slightly more concentrated in *sn*-1/3 positions (59.0–59.2 mol% and 51.0–54.4 mol%, respectively), whereas 18:0, 18:1, 18:2 and 16:1 were mainly in *sn*-1/3 positions (78.8–85.6 mol%, 78.9–83.2 mol%, 88.9–89.4 mol% and 61.6–63.9 mol%, respectively). 18:3 was not detected at all in the selected 21 ACN:DB species of the two bovine milk samples.

No infant formula had the majority of 16:0 in *sn*-2 position, highest levels being in Nutrilon Omneo (43.4 mol%), followed by Valio Tuuti (29.9 mol%). All other infant formulas had less than 10 mol% of 16:0 in *sn*-2 position. FAs with ACN 18 were more concentrated in *sn*-1/3 positions in all infant formulas, the highest levels being of 18:0 (72.8–83.1 mol%) and 18:3 (67.6–85.7 mol%), whereas 18:1 (53.9–67.4 mol%) and 18:2 (57.8–71.7 mol%) had a slightly smaller majority in *sn*-1/3 positions.

The amount of 21 regiospecifically quantified ACN:DB species represented 66.0 mol% and 69.1 mol% of all TAGs in the Finnish and Chinese human milk samples, respectively. This means that approximately one third of the TAGs in the human milk, mostly in the molecular weight range below ACN 44, were not quantified. These TAGs likely also contain smaller amounts of 16:0, and according to (Kallio et al., 2017), in human milk TAGs consisting of 16:0 and shorter chain saturated (14:0, 12:0, 10:0 or 8:0) FAs, 16:0 no longer has a strong preference to *sn*-2 position.

The 21 selected ACN:DB species added up to two thirds of all TAGs in human milk, but in bovine milk they formed only a small minority, highlighting the differences in molecular weight distribution and FA composition between bovine milk and human milk. Only 13.4–15.2 mol% of bovine milk TAG regioisomers were quantified, mainly because the TAGs were so heavily distributed between ACN 32–40, which were outside of the range, which could be reliably quantified with the current method. Even though the proportion of identified TAGs is small, the regioisomeric distribution of FAs in the quantified bovine milk TAGs is generally closer to human milk than the distribution of FAs in vegetable oils.

Out of the selected 21 ACN:DB species (**Table 4**), the amount of non-quantifiable TAGs was generally less than 10 mol% of the total amount of FAs in the selected 21 ACN:DB species. However, in the bovine milk samples, which already had a low total amount TAGs within the selected 21 ACN:DB species (17.6–19.4 mol% of total TAGs), the proportion of unidentified TAGs is also higher (22.7 and 24.9 mol% of the selected 21 ACN:DB species). As a result, regioisomers of roughly only 15 mol% of all TAGs in the bovine milk samples were quantified.

When comparing the regioisomeric distribution of FAs in the Finnish and Chinese human milk samples to the findings of our previous study based on UPLC-MS/MS analysis of TAGs as lithium adducts (Kallio et al., 2017), the results are fairly well in agreement. In our current study, 74.0 and 77.8 mol% of analyzed 16:0 was in *sn*-2 position, 83.5 and 83.2 mol% of 18:1 in *sn*-1/3 positions, 88.9 and 89.4 mol% of 18:2 in *sn*-1/3 positions and 65.4 and 64.8 mol% of 14:0 in *sn*-2 position in

the Finnish and Chinese milk sample, respectively, whereas in our previous study the respective values for Finnish and Chinese human milk were 71.2 and 75.7 mol% of 16:0 in *sn*-2 position, 89.6 and 93.0 mol% of 18:1 in *sn*-1/3 positions, 84.8 and 83.7 mol% of 18:2 in *sn*-1/3 positions and 60.4 and 66.2 mol% of 14:0 in *sn*-2 position (see **Figure 2**).

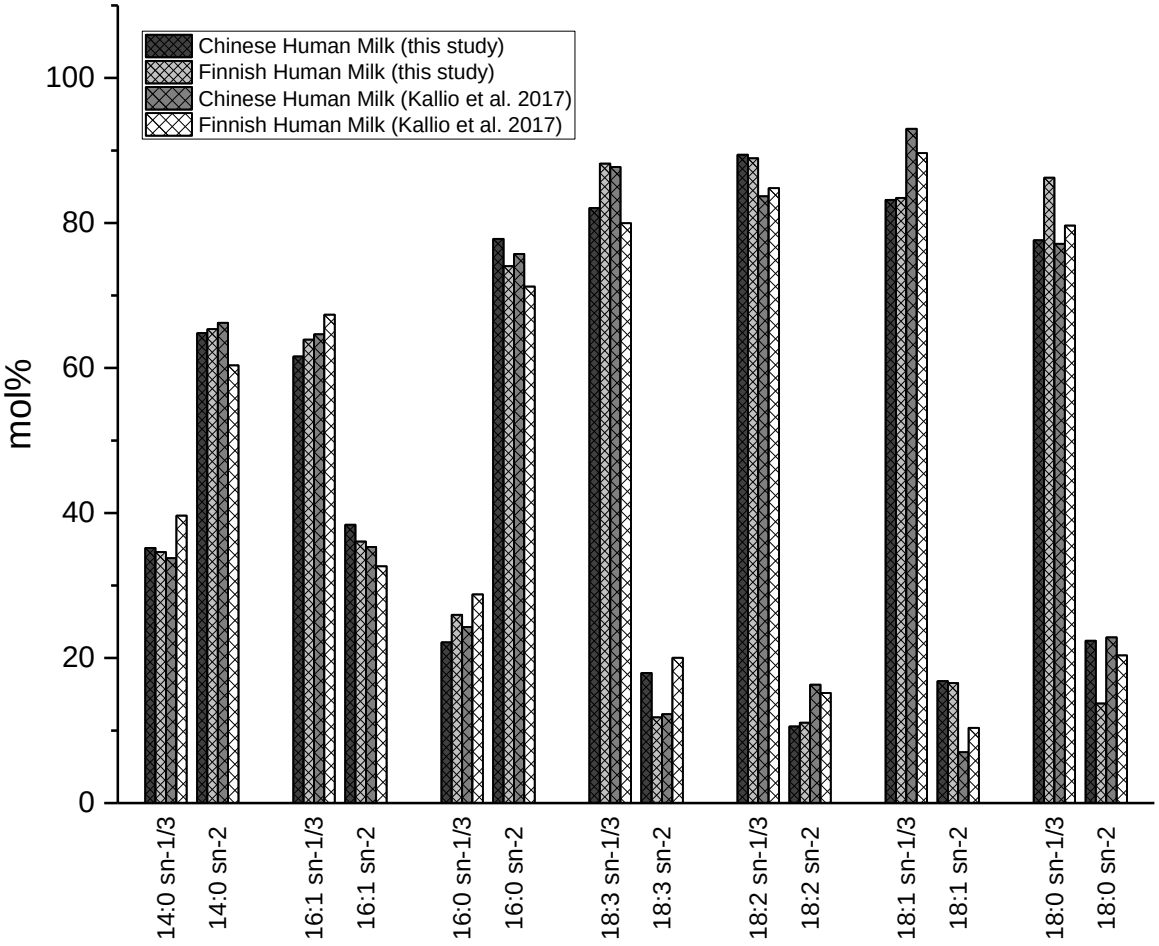

Figure 2 Positional distribution of selected FAs in Finnish and Chinese human milk compared to a previous study (Kallio et al., 2017)

A recent study where pancreatic lipase was used for regiospecific determination of FA positions in TAGs of human milk and infant formula concluded that in Chinese human milk out of all FAs occupying the *sn*-2 position 44.5 % (w/w) is 16:0, 12.3 % 18:1, 11.4 % 18:2, and 10.8 % 14:0 (Sun et al., 2018). Respective values for FAs in *sn*-2 position in the Chinese human milk in our study were 52.3 % (w/w) 16:0, 20.2 % 18:1, 6.8 % 18:2 and 7.3 % 14:0. In both studies 16:0 occupied nearly half of all *sn*-2 positions in Chinese human milk, and similar results have also been reported earlier

(Deng et al., 2018; Kallio et al., 2017; Zou et al., 2013). Further, the current findings on the *sn*-2 FA composition of infant formulas are similar as those of the previous study on 180 commercial infant formulas available on the Chinese market (Sun et al., 2018), although the referred study did not investigate the regioisomers of TAGs of individual ACN:DB species. In vegetable oil based formulas, 16:0 occupied 11.5 % of all *sn*-2 positions, and in bovine milk formulas the value was higher (20.2 %), but still significantly less than the level in human milk (44.5 %). Subsequently, the highest amount of 18:1 occupying *sn*-2 positions was found in vegetable oil based formulas (37.0 %), followed by bovine milk based formulas (29.2 %) and human milk with only 12.3 % (Sun et al., 2018).

Most infant formula manufacturers use vegetable oils to adjust the FA composition, which often results in the regioisomeric composition being skewed further away from human milk. This may potentially result in different dietary absorption of FAs and calcium between formula fed and human milk fed infants as well as other effects on infant wellbeing.

Table 3 Abundance and regioisomeric composition of the 21 analyzed ACN:DB species (mol% ± std). The most abundant TAG species within each ACN:DB species in human milk are shown. All identified TAG regioisomers, including the minor ones, are presented in **Supplementary Table 3**.

	Arla Little Baby Organic	Nestle NAN Pro	Nestle NAN Organic	Nestle NAN Pro (powder)	Nestle NAN HA (powder)	Nestle NAN Sensilac (powder)	Nutrilon Standard	Tutteli Plus	Nutrilon Omneo (powder)	Semper Baby Semp	Valio Tuuti	Bovine Milk	Organic Bovine Milk	Chinese Human Milk	Finnish Human Milk
54:2	**3.6**	**1.8**	**3.3**	**2.4**	**4.6**	**2.9**	**1.0**	**0.8**	**3.6**	**3.4**	**1.4**	**0.7**	**0.6**	**1.3**	**1.7**
18:1/18:1/18:0															
sn-18:1/18:1/18:0 + *sn*-18:0/18:1/18:1	77.1 ± 7.8	78.4 ± 8.9	79.3 ± 1.4	84.7 ± 9.1	79.9 ± 0.7	69.3 ± 5.9	75.9 ± 7.4	78.5 ± 5.9	72.1 ± 5.4	75.1 ± 4.8	76.6 ± 5.8			47.0 ± 4.0	69.8 ± 2.1
sn-18:1/18:0/18:1	15.1 ± 7.9	14.5 ± 7	18.6 ± 1.0	12.7 ± 8.9	16.4 ± 0.8	25.3 ± 5.6	18.9 ± 5.8	16.2 ± 5.0	22.9 ± 5.0	20.9 ± 4.1	18.0 ± 5.4			41.5 ± 4.2	21.7 ± 1.8
54:3	**5.3**	**8.9**	**40.3**	**21.2**	**23.4**	**10.4**	**13.6**	**14.0**	**7.4**	**14.2**	**6.4**	**0.6**	**0.5**	**3.5**	**4.1**
18:2/18:1/18:0															
sn-18:2/18:1/18:0 + *sn*-18:0/18:1/18:2	8.3 ± 6.2	6.5 ± 3.5	3.4 ± 1.3	3.5 ± 0.8	4.0 ± 1.0	6.7 ± 4.7	4.7 ± 3.4	7.2 ± 2.1	5.1 ± 3.4	5.0 ± 0.9	8.0 ± 3.5			15.9 ± 4.8	8.6 ± 3.8
sn-18:1/18:2/18:0 + *sn*-18:0/18:2/18:1	7.3 ± 7.3	7.5 ± 4.3	0.9 ± 1.4	1.2 ± 0.7	0.9 ± 0.7	6.6 ± 3.2	4.0 ± 1.7	3.7 ± 2.9	4.0 ± 2.4	3.1 ± 0.9	6.1 ± 1.5			15.2 ± 3.8	9.2 ± 2.5
sn-18:2/18:0/18:1 + *sn*-18:1/18:0/18:2	2.3 ± 2.7	0.8 ± 1.6	0.4 ± 0.4	0.7 ± 0.6	1.3 ± 0.9	1.7 ± 2.1	2.3 ± 1.9	1.3 ± 1.8	2.6 ± 2.5	1.7 ± 1.0	1.8 ± 2.6			6.9 ± 4.1	5.4 ± 3.9
18:1/18:1/18:1															
sn-18:1/18:1/18:1	81.7 ± 2.3	85.2 ± 2.1	95.2 ± 0.5	94.6 ± 0.1	93.8 ± 0.7	85.1 ± 0.7	88.8 ± 0.7	87.9 ± 0.4	88.3 ± 0.7	90.1 ± 0.6	83.5 ± 0.4			61.4 ± 0.2	76.2 ± 1.3
54:4	**6.8**	**4.7**	**10.4**	**7.8**	**9.3**	**7.8**	**10.4**	**6.8**	**7.5**	**5.3**	**5.8**	**0.2**	**0.2**	**4.3**	**3.3**
18:2/18:1/18:1															
sn-18:2/18:1/18:1 + *sn*-18:1/18:1/18:2	56.2 ± 4.8	54.0 ± 5.2	64.7 ± 3.0	78.9 ± 6.2	67.3 ± 8.2	53.9 ± 12.2	66.4 ± 8.4	58.8 ± 7.1	65.8 ± 2.8	62.2 ± 3.1	63.1 ± 7.8			57.9 ± 8.6	57.0 ± 6.1
sn-18:1/18:2/18:1	30.7 ± 4.5	37.1 ± 4.2	24.9 ± 3.7	7.3 ± 6.9	21.9 ± 6.3	34.9 ± 11.2	25.3 ± 8.3	31.7 ± 7.2	24.9 ± 2.8	26.9 ± 3.4	24.6 ± 7.4			27.9 ± 6.4	32.5 ± 4.6
54:5	**3.4**	**6.4**	**7.3**	**8.3**	**6.7**	**7.7**	**7.0**	**5.0**	**5.8**	**5.5**	**7.0**	**0.0**	**0.0**	**4.2**	**1.6**
18:2/18:2/18:1															
sn-18:2/18:2/18:1 + *sn*-18:1/18:2/18:2	22.4 ± 6.4	42.6 ± 1.2	46.2 ± 4.0	48.9 ± 6.0	42.0 ± 4.8	43.0 ± 5.3	42.2 ± 5.0	49.6 ± 3.4	34.3 ± 8.1	58.7 ± 4.2	48.9 ± 7.9			52.8 ± 8.6	48.1 ± 3.1
sn-18:2/18:1/18:2	46.8 ± 4.8	29.7 ± 2.2	32.8 ± 1.7	38.8 ± 6.1	31.5 ± 6.1	29.3 ± 2.0	20.8 ± 2.6	14.0 ± 5.5	30.0 ± 6.1	19.9 ± 4.4	25.4 ± 4.2			32.6 ± 8.1	29.0 ± 10.8
54:6	**2.6**	**3.1**	**3.6**	**4.5**	**5.1**	**3.8**	**2.7**	**3.9**	**2.8**	**4.1**	**4.9**	**0.0**	**0.0**	**1.8**	**0.8**
18:3/18:2/18:1															
sn-18:3/18:2/18:1 + *sn*-18:1/18:2/18:3	19.4 ± 14.9	13.1 ± 3.7	5.0 ± 2.8	5.7 ± 3.9	8.6 ± 5.2	15.5 ± 8.2	23.9 ± 10.5	12.3 ± 10.8	17.9 ± 5.5	3.1 ± 4.7	7.7 ± 5.0			11.0 ± 10.0	
sn-18:2/18:3/18:1 + *sn*-18:1/18:3/18:2	0.0 ± 0.0	7.7 ± 7.2	4.1 ± 5.6	1.2 ± 1.6	6.4 ± 5.3	4.0 ± 3.9	11.3 ± 11.4	15.9 ± 14.0	1.0 ± 1.7	12.7 ± 2.3	10.0 ± 2.7			20.9 ± 8.5	
sn-18:3/18:1/18:2 + *sn*-18:2/18:1/18:3	15.6 ± 13.7	15.8 ± 8.4	16.2 ± 1.9	16.5 ± 2.6	13.7 ± 6.2	13.3 ± 7.4	11.4 ± 14.2	22.9 ± 9.8	25.9 ± 8.6	12.7 ± 2.6	20.2 ± 6.8			14.0 ± 7.8	
18:2/18:2/18:2															
sn-18:2/18:2/18:2	65.0 ± 2.2	63.4 ± 3.4	74.6 ± 3.1	76.7 ± 1.0	71.3 ± 3.2	67.1 ± 0.8	53.4 ± 5.7	48.8 ± 1.7	55.3 ± 4.7	71.5 ± 1.4	62.1 ± 1.0			54.1 ± 0.9	
52:1	**2.9**	**2.2**	**1.5**	**0.5**	**0.6**	**4.1**	**3.3**	**2.1**	**3.7**	**1.5**	**1.3**	**0.7**	**1.1**	**2.9**	**3.8**
16:0/18:1/18:0															
sn-16:0/18:1/18:0 + *sn*-18:0/18:1/16:0	57.0 ± 5.5	66.7 ± 7.4	57.8 ± 11.7			65.9 ± 3.8	65.3 ± 6.3	65.7 ± 5.4	11.5 ± 8.9	49.3 ± 7.3	22.5 ± 3.4	17.5 ± 10.2	19.9 ± 12.5	1.5 ± 3.0	0.9 ± 1.8
sn-18:1/16:0/18:0 + *sn*-18:0/16:0/18:1	13.5 ± 7.8	14.4 ± 9.7	8.9 ± 4.0			0.0 ± 0.0	7.4 ± 9.7	11.2 ± 14.9	61.3 ± 6.3	22.4 ± 3.8	58.1 ± 3.3	49.5 ± 5.3	54.9 ± 9.2	89.8 ± 7.7	89.4 ± 2.1
sn-16:0/18:0/18:1 + *sn*-18:1/18:0/16:0	29.3 ± 8.9	17.7 ± 13.8	33.1 ± 8.6			33.3 ± 3.5	26.3 ± 3.9	22.1 ± 10.6	26.4 ± 6.8	26.5 ± 6.1	18.2 ± 6.3	30.5 ± 8.1	22.6 ± 4.2	7.2 ± 8.5	7.4 ± 1.2
52:2	**10.0**	**8.4**	**8.4**	**3.9**	**5.5**	**7.2**	**7.3**	**8.4**	**10.8**	**6.3**	**2.8**	**1.8**	**1.5**	**10.4**	**12.2**
16:0/18:1/18:1															
sn-16:0/18:1/18:1 + *sn*-18:1/18:1/16:0	87.6 ± 4.1	87.4 ± 4.0	94.0 ± 2.2	90.0 ± 1.3	92.7 ± 1.3	90.5 ± 1.1	90.8 ± 0.8	89.7 ± 1.3	48.3 ± 2.8	89.3 ± 1.9	62.6 ± 4.4	46.5 ± 8.6	46.1 ± 4.8	9.6 ± 5.8	17.0 ± 5.7
sn-18:1/16:0/18:1	2.7 ± 3.4	2.0 ± 4.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	46.3 ± 3.4	0.7 ± 1.4	21.9 ± 3.9	41.5 ± 8.7	38.8 ± 3.9	70.7 ± 5.2	72.3 ± 4.1
52:3	**5.6**	**4.6**	**2.6**	**2.4**	**2.2**	**3.7**	**2.9**	**3.2**	**4.8**	**4.7**	**1.5**	**0.7**	**0.5**	**11.0**	**6.7**
16:0/18:2/18:1															
sn-16:0/18:2/18:1 + *sn*-18:1/18:2/16:0	53.5 ± 3.7	44.6 ± 6.4	45.3 ± 10.1	35.5 ± 10.2	42.0 ± 6.1	44.4 ± 6.6	48.7 ± 3.3	39.9 ± 3.8	20.3 ± 7.9	41.5 ± 8.6	42.7 ± 3.6			0.0 ± 0.0	0.5 ± 0.9
sn-18:2/16:0/18:1 + *sn*-18:1/16:0/18:2	0.0 ± 0.0	3.7 ± 6.4	7.4 ± 11.9	0.0 ± 0.0	3.8 ± 5.0	3.2 ± 6.3	2.3 ± 3.7	6.5 ± 6.2	39.0 ± 7.1	1.3 ± 2.6	0.0 ± 0.0			75.5 ± 0.9	70.7 ± 4.1
sn-16:0/18:1/18:2 + *sn*-18:2/18:1/16:0	38.9 ± 4.3	43.8 ± 1.8	35.1 ± 6.5	51.2 ± 10.4	36.9 ± 1.9	45.1 ± 8.3	39.4 ± 5.3	44.5 ± 5.4	34.5 ± 2.9	46.6 ± 6.1	40.6 ± 3.2			8.7 ± 2.3	5.2 ± 3.5

52:4	**2.9**	**2.3**	**2.2**	**2.7**	**1.4**	**1.9**	**1.5**	**2.4**	**2.5**	**1.8**	**2.1**	**0.0**	**0.2**	**5.5**	**2.3**
16:0/18:3/18:1															
sn-16:0/18:3/18:1 + *sn*-18:1/18:3/16:0	0.0 ± 0.0	4.4 ± 2.4	3.1 ± 5.4	2.2 ± 2.7	7.4 ± 7.4	7.4 ± 7.4	1.9 ± 1.9	8.6 ± 11.4	7.9 ± 8.0	7.3 ± 4.8	11.5 ± 12.6			0.0 ± 0.0	0.0 ± 0.0
sn-18:3/16:0/18:1 + *sn*-18:1/16:0/18:3	2.2 ± 3.8	1.4 ± 1.2	0.5 ± 0.9	1.4 ± 2.8	3.6 ± 3.5	3.6 ± 3.5	3.0 ± 5.3	0.6 ± 1.1	1.9 ± 3.7	0.9 ± 1.9	0.0 ± 0.0			16.1 ± 2.6	31.6 ± 3.0
sn-16:0/18:1/18:3 + *sn*-18:3/18:1/16:0	17.1 ± 7.6	10.8 ± 3.6	9.7 ± 3.7	3.8 ± 3.0	5.2 ± 3.3	5.2 ± 3.3	15.9 ± 1.3	17.3 ± 5.1	17.0 ± 4.0	8.5 ± 5.0	14.6 ± 4.9			1.9 ± 1.6	3.8 ± 5.4
16:0/18:2/18:2															
sn-16:0/18:2/18:2 + *sn*-18:2/18:2/16:0	52.6 ± 2.4	74.4 ± 3.2	75.2 ± 3.7	75.9 ± 9.7	71.6 ± 6.4	71.6 ± 6.4	67.7 ± 0.7	49.8 ± 8.4	49.9 ± 12.8	63.0 ± 9.7	49.5 ± 6.2			2.2 ± 4.4	0.9 ± 1.6
sn-18:2/16:0/18:2	19.3 ± 4.3	2.7 ± 4.6	0.4 ± 0.6	10.7 ± 9.0	3.1 ± 2.9	3.1 ± 2.9	0.0 ± 0.0	10.8 ± 11.4	11.6 ± 13.5	11.8 ± 10.6	12.6 ± 1.9			51.9 ± 2.6	32.2 ± 6.5
52:5	**0.5**	**0.6**	**0.4**	**0.7**	**0.3**	**0.5**	**0.3**	**0.0**	**1.0**	**0.3**	**0.1**	**0.0**	**0.0**	**1.3**	**0.6**
16:1/18:2/18:2															
sn-16:1/18:2/18:2 + *sn*-18:2/18:2/16:1									4.0 ± 7.0					16.3 ± 17.4	10.1 ± 12.6
sn-18:2/16:1/18:2									0.0 ± 0.0					24.8 ± 11.0	16.2 ± 10.2
16:0/18:3/18:2															
sn-16:0/18:3/18:2 + *sn*-18:2/18:3/16:0									0.0 ± 0.0					0.0 ± 0.0	2.3 ± 2.0
sn-18:3/16:0/18:2 + *sn*-18:2/16:0/18:3									55.5 ± 17.6					39.2 ± 8.6	42.8 ± 9.5
sn-16:0/18:2/18:3 + *sn*-18:3/18:2/16:0									15.2 ± 14.4					1.6 ± 1.4	2.2 ± 3.8
50:1	**7.7**	**10.6**	**2.0**	**0.8**	**0.5**	**6.6**	**6.0**	**6.2**	**7.6**	**6.2**	**2.1**	**2.7**	**2.1**	**3.1**	**4.3**
14:0/18:1/18:0															
sn-14:0/18:1/18:0 + *sn*-18:0/18:1/14:0	0.3 ± 0.6	0.4 ± 0.4	0.0 ± 0.0			0.3 ± 0.1	0.4 ± 0.3	0.1 ± 0.2	0.7 ± 0.6	1.7 ± 1.4	6.6 ± 7.6	4.4 ± 4.6	5.0 ± 3.7	4.6 ± 2.2	3.4 ± 1.9
sn-18:1/14:0/18:0 + *sn*-18:0/14:0/18:1	0.9 ± 0.6	0.0 ± 0.0	0.0 ± 0.0			0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 0.8	13.6 ± 6.4	16.3 ± 5.2	17.8 ± 2.9	3.8 ± 3.6	20.1 ± 2.2
sn-14:0/18:0/18:1 + *sn*-18:1/18:0/14:0	0.5 ± 0.6	0.2 ± 0.3	0.5 ± 0.3			0.1 ± 0.1	0.0 ± 0.1	0.6 ± 0.5	0.5 ± 0.7	1.5 ± 2.0	2.9 ± 4.0	1.7 ± 2.2	3.8 ± 4.7	2.4 ± 2.1	0.1 ± 0.1
16:0/16:0/18:1															
sn-16:0/16:0/18:1 + *sn*-18:1/16:0/16:0	20.2 ± 3.2	13.0 ± 7.3	2.0 ± 1.7			6.4 ± 5.1	13.2 ± 3.4	9.7 ± 4.0	75.5 ± 2.9	23.2 ± 3.6	57.0 ± 4.2	61.7 ± 4.4	64.6 ± 3.6	80.2 ± 2.2	65.9 ± 3.8
sn-16:0/18:1/16:0	76.6 ± 3.2	85.2 ± 7.2	95.2 ± 2			92.2 ± 5.2	85.2 ± 3.6	88.6 ± 3.9	22.2 ± 3.5	69.3 ± 3.2	14.4 ± 8.1	10.2 ± 7.0	3.1 ± 3.9	0.7 ± 1.3	4.4 ± 3.7
50:2	**3.9**	**3.0**	**0.6**	**0.6**	**0.3**	**3.6**	**4.0**	**3.3**	**1.7**	**2.4**	**1.2**	**1.2**	**1.1**	**3.7**	**4.7**
14:0/18:1/18:1															
sn-14:0/18:1/18:1 + *sn*-18:1/18:1/14:0	9.3 ± 0.2	7.1 ± 0.8				7.6 ± 1.4	9.7 ± 2.0	10.4 ± 1.0	19.2 ± 7.4	17.8 ± 2.7	16.4 ± 6.0	17.3 ± 9.0	17.5 ± 5.3	4.4 ± 5.9	10.3 ± 4.1
sn-18:1/14:0/18:1	0.3 ± 0.6	1.5 ± 2.1				0.5 ± 1.1	1.7 ± 1.9	0.4 ± 0.6	8.9 ± 4.1	5.1 ± 3.3	39.0 ± 4.0	42.6 ± 11.4	40.7 ± 5.8	29.1 ± 10.1	45.3 ± 5.3
16:0/16:0/18:2															
sn-16:0/16:0/18:2 + *sn*-18:2/16:0/16:0	13.7 ± 5.7	9.0 ± 3.2				17.7 ± 2.0	15.1 ± 7.3	15.8 ± 6.0	59.7 ± 2.0	17.7 ± 3.2	19 ± 5.7	19.7 ± 5.0	21.5 ± 6.5	38.8 ± 3.0	15.7 ± 3.5
sn-16:0/18:2/16:0	62.2 ± 5.8	71.5 ± 4.2				61.4 ± 3.1	64.3 ± 5.5	61.6 ± 8.5	0.5 ± 1.0	41.4 ± 1.4	11.6 ± 8.5	2.3 ± 2.6	2.6 ± 3.8	0.0 ± 0.0	0.0 ± 0.0
50:3	**0.5**	**0.4**	**0.2**	**0.3**	**0.2**	**0.4**	**0.4**	**0.5**	**0.4**	**0.5**	**0.4**	**0.4**	**0.4**	**2.2**	**2.2**
14:0/18:2/18:1															
sn-14:0/18:2/18:1 + *sn*-18:1/18:2/14:0														0.9 ± 1.7	0.0 ± 0.0
sn-18:2/14:0/18:1 + *sn*-18:1/14:0/18:2														56.3 ± 8.9	67.7 ± 1.3
sn-14:0/18:1/18:2 + *sn*-18:2/18:1/14:0														5.2 ± 6.3	5.0 ± 4.4
50:4	**0.0**	**0.2**	**0.1**	**0.2**	**0.1**	**0.0**	**0.0**	**0.3**	**0.1**	**0.0**	**0.1**	**0.0**	**0.2**	**1.1**	**0.7**
14:0/18:3/18:1															
sn-14:0/18:3/18:1 + *sn*-18:1/18:3/14:0														6.6 ± 8.8	1.1 ± 1.9
sn-18:3/14:0/18:1 + *sn*-18:1/14:0/18:3														9.8 ± 9.5	38.6 ± 13.5
sn-14:0/18:1/18:3 + *sn*-18:3/18:1/14:0														0.0 ± 0.0	0.0 ± 0.0
14:0/18:2/18:2															
sn-14:0/18:2/18:2 + *sn*-18:2/18:2/14:0														0.9 ± 1.5	0.0 ± 0.0
sn-18:2/14:0/18:2														63.0 ± 2.6	36 ± 13.7
48:1	**1.3**	**0.6**	**0.6**	**0.3**	**0.2**	**0.6**	**0.6**	**0.6**	**0.8**	**0.9**	**1.6**	**2.5**	**2.3**	**1.5**	**3.4**
12:0/18:1/18:0															
sn-12:0/18:1/18:0 + *sn*-18:0/18:1/12:0	0.1 ± 0.1										1.6 ± 2.8	4.0 ± 1.6	4.6 ± 3.5	5.9 ± 3.7	4.1 ± 2.1
sn-18:1/12:0/18:0 + *sn*-18:0/12:0/18:1	5.4 ± 7.9										5.3 ± 2.1	5.2 ± 3	5.7 ± 0.2	13.7 ± 6.6	12.8 ± 4
sn-12:0/18:0/18:1 + *sn*-18:1/18:0/12:0	1.7 ± 1.9										6.1 ± 6.3	0.0 ± 0.0	1.1 ± 1.9	5.7 ± 5.9	1.9 ± 1.8
14:0/16:0/18:1															
sn-14:0/16:0/18:1 + *sn*-18:1/16:0/14:0	6.7 ± 11.5										27.1 ± 6.5	22.7 ± 7.6	30.1 ± 1.5	48.8 ± 5.0	49.8 ± 4.1
sn-16:0/14:0/18:1 + *sn*-18:1/14:0/16:0	26.0 ± 13.5										46.4 ± 8.7	39.8 ± 7.7	42.7 ± 4.5	13.9 ± 10.1	18.6 ± 6.4

sn-14:0/18:1/16:0 + *sn*-16:0/18:1/14:0	47.5 ± 11.6										0.0 ± 0.0	14.4 ± 8.6	4.5 ± 4.1	0.5 ± 0.9	5.7 ± 7.0
48:2	**0.7**	**0.6**	**0.0**	**0.4**	**0.2**	**0.4**	**0.3**	**0.4**	**0.6**	**0.6**	**0.8**	**0.9**	**0.8**	**2.6**	**3.4**
12:0/18:1/18:1															
sn-12:0/18:1/18:1 + *sn*-18:1/18:1/12:0														15.6 ± 4.1	23.0 ± 2.4
sn-18:1/12:0/18:1														38.7 ± 8.9	36.0 ± 5.5
14:0/16:0/18:2															
sn-14:0/16:0/18:2 + *sn*-18:2/16:0/14:0														12.7 ± 4.2	8.5 ± 6.3
sn-16:0/14:0/18:2 + *sn*-18:2/14:0/16:0														9.5 ± 5.5	13.3 ± 6.4
sn-14:0/18:2/16:0 + *sn*-16:0/18:2/14:0														3.0 ± 5.9	0.0 ± 0.0
48:3	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.3**	**1.8**	**1.5**
12:0/18:2/18:1															
sn-12:0/18:2/18:1 + *sn*-18:1/18:2/12:0														2.0 ± 3.5	11.1 ± 9.4
sn-18:2/12:0/18:1 + *sn*-18:1/12:0/18:2														56.3 ± 11.7	54.5 ± 10.6
sn-12:0/18:1/18:2 + *sn*-18:2/18:1/12:0														27 ± 7.9	13.1 ± 3.9
46:0	**0.6**	**0.3**	**0.0**	**0.2**	**0.2**	**0.2**	**0.3**	**0.4**	**0.8**	**0.7**	**1.1**	**1.7**	**1.6**	**1.0**	**1.3**
12:0/16:0/18:0															
sn-12:0/16:0/18:0 + *sn*-18:0/16:0/12:0											3.8 ± 6.6	11.5 ± 6.1	7.2 ± 7.5	55.8 ± 8.0	50.0 ± 5.2
sn-16:0/12:0/18:0 + *sn*-18:0/12:0/16:0											6.3 ± 6.0	5.1 ± 4.4	6.5 ± 3.9	0.0 ± 0.0	0.0 ± 0.0
sn-12:0/18:0/16:0 + *sn*-16:0/18:0/12:0											8.0 ± 7.6	2.3 ± 3.9	5.9 ± 6.8	0.4 ± 0.7	0.0 ± 0.0
14:0/16:0/16:0															
sn-14:0/16:0/16:0 + *sn*-16:0/16:0/14:0											46.5 ± 21.1	58.1 ± 2.7	55.4 ± 9.4	27.2 ± 3.3	33.7 ± 6.9
sn-16:0/14:0/16:0											14.0 ± 14.2	11.9 ± 3.1	10.6 ± 6.5	10.8 ± 5	7.7 ± 8.9
46:1	**0.7**	**0.5**	**0.0**	**0.4**	**0.3**	**0.4**	**0.4**	**0.5**	**0.8**	**0.7**	**1.2**	**1.7**	**1.7**	**2.5**	**3.4**
12:0/16:0/18:1															
sn-12:0/16:0/18:1 + *sn*-18:1/16:0/12:0											14.5 ± 4.9	22.0 ± 1.6	9.6 ± 10.8	66.4 ± 10.9	64.2 ± 5.6
sn-16:0/12:0/18:1 + *sn*-18:1/12:0/16:0											32.1 ± 12.5	18 ± 5.4	17.7 ± 3.6	0.9 ± 1.7	1.2 ± 2.3
sn-12:0/18:1/16:0 + *sn*-16:0/18:1/12:0											0.0 ± 0.0	4.6 ± 3.2	13.7 ± 8	9.6 ± 11.7	3.6 ± 4.2
44:1	**0.7**	**0.4**	**0.4**	**0.6**	**0.5**	**0.4**	**0.5**	**0.6**	**1.0**	**0.7**	**1.5**	**2.0**	**1.9**	**2.0**	**3.0**
10:0/16:0/18:1															
sn-10:0/16:0/18:1 + *sn*-18:1/16:0/10:0									1.3 ± 2.6		18.9 ± 7.4	18.3 ± 6.2	11.2 ± 8.5	46.6 ± 12.2	36.8 ± 3.4
sn-16:0/10:0/18:1 + *sn*-18:1/10:0/16:0									2.7 ± 5.5		41.5 ± 2.9	26.6 ± 5.4	20.6 ± 15.1	3.4 ± 3.2	0.5 ± 0.9
sn-10:0/18:1/16:0 + *sn*-16:0/18:1/10:0									5.4 ± 2.5		0.0 ± 0.0	15.5 ± 4.7	27.6 ± 10.3	6.5 ± 9.2	9.2 ± 2.0
12:0/18:0/14:1															
sn-12:0/18:0/14:1 + *sn*-14:1/18:0/12:0									0.0 ± 0.0		0.0 ± 0.0	0.7 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
sn-12:0/14:0/18:1 + *sn*-18:1/14:0/12:0									15.8 ± 17.7		7.6 ± 13.1	10.8 ± 4.8	12.6 ± 3.7	28.7 ± 8.3	36.8 ± 5.5
sn-14:0/12:0/18:1 + *sn*-18:1/12:0/14:0									19.6 ± 22.0		6.8 ± 10.1	8.6 ± 6.6	10.4 ± 8.9	5.5 ± 6.4	6.3 ± 2.2
44:2	**0.3**	**0.3**	**0.2**	**0.3**	**0.2**	**0.2**	**0.2**	**0.2**	**0.3**	**0.4**	**0.6**	**0.7**	**0.7**	**1.2**	**1.1**
10:0/16:0/18:2															
sn-10:0/16:0/18:2 + *sn*-18:2/16:0/10:0														48.5 ± 5.4	34.3 ± 11.7
sn-16:0/10:0/18:2 + *sn*-18:2/10:0/16:0														4.8 ± 5.8	0.8 ± 1.3
sn-10:0/18:2/16:0 + *sn*-16:0/18:2/10:0														0.0 ± 0.0	0.0 ± 0.0
12:0/14:0/18:2															
sn-12:0/14:0/18:2 + *sn*-18:2/14:0/12:0														25.9 ± 1.8	39.2 ± 9.9
sn-14:0/12:0/18:2 + *sn*-18:2/12:0/14:0														8.3 ± 7.3	0.6 ± 1.0
sn-12:0/18:2/14:0 + *sn*-14:0/18:2/12:0														2.0 ± 3.4	2.5 ± 4.4

Blank spots indicate a molar abundance of less than 1 mol% of all TAGs, which could not be quantified reliably.

Table 4 Positioning of FAs in the selected 21 ACN:DB species (mol%).

Fatty acid	Position	Arla Little Baby Organic	Nestle NAN Pro	Nestle NAN Organic	Nestle NAN Pro (powder)	Nestle NAN HA (powder)	Nestle NAN Sensilac (powder)	Nutrilon Standard	Tutteli Plus	Nutrilon Omneo (powder)	Semper Baby Semp	Valio Tuuti	Bovine Milk	Organic Bovine Milk	Chinese Human Milk	Finnish Human Milk
10:0	*sn*-1/3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	1.8	1.9	0.9	1.0
	sn-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.1	1.1	0.2	0.1
12:0	*sn*-1/3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.5	1.9	1.9	2.4	3.1
	sn-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.5	1.5	1.6	1.4	1.5
14:1	*sn*-1/3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.6	0.6	0.1	0.2
	sn-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.1
14:0	*sn*-1/3	0.6	0.2	0.0	0.0	0.0	0.2	0.2	0.2	0.6	0.4	1.7	5.7	6.1	1.5	2.5
	sn-2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1	1.6	5.4	5.1	2.8	4.7
16:1	*sn*-1/3	0.6	0.5	0.2	0.2	0.2	0.3	0.4	0.5	0.5	0.5	0.5	1.6	1.1	2.4	2.3
	sn-2	0.4	0.1	0.1	0.1	0.2	0.3	0.1	0.2	0.2	0.2	0.6	0.7	0.8	1.5	1.3
16:0	*sn*-1/3	21.9	22.4	6.9	4.6	4.5	18.0	16.5	17.5	11.5	14.2	8.5	15.7	14.5	5.0	5.6
	sn-2	2.2	1.4	0.2	0.2	0.1	0.8	1.0	1.1	8.8	1.5	3.6	10.9	10.0	17.6	16.0
18:3	*sn*-1/3	1.4	1.3	0.8	1.2	1.6	1.7	1.7	1.9	2.2	1.1	2.6	0.0	0.0	1.5	1.0
	sn-2	0.3	0.6	0.3	0.2	0.5	0.4	0.8	0.8	0.6	0.5	0.8	0.0	0.0	0.3	0.1
18:2	*sn*-1/3	10.4	10.8	10.7	17.4	14.0	12.6	11.2	10.8	12.0	12.1	16.7	0.6	0.7	17.8	9.0
	sn-2	7.5	7.9	5.1	7.2	6.4	8.4	7.3	7.2	4.7	7.2	8.5	0.1	0.1	2.1	1.1
18:1	*sn*-1/3	22.8	23.7	43.3	34.5	39.2	26.3	30.4	29.1	31.7	29.6	28.0	18.6	17.9	30.5	35.6
	sn-2	19.2	20.3	25.9	22.0	23.4	20.2	21.4	21.4	15.3	20.0	14.4	5.1	4.8	6.2	7.1
18:0	*sn*-1/3	4.5	3.2	2.7	2.5	3.3	4.0	3.1	2.8	3.8	3.6	4.3	5.0	5.4	4.6	5.5
	sn-2	1.3	0.8	0.7	0.5	0.8	1.5	1.0	0.8	1.4	1.1	1.0	0.8	1.4	1.3	0.9
	Total identified FAs	93.3	93.2	97.0	90.6	94.2	94.8	95.2	94.1	94.1	92.0	95.0	77.3	75.1	100.0	98.8
	Unidentified FAs [a]	6.7	6.8	3.0	9.4	5.8	5.2	4.8	5.9	5.9	8.0	5.0	22.7	24.9	0.0	1.2

[a] Unidentified FAs consist of ACN:DB species within the 21 selected ACN:DB species with less than 1 mol% abundance of the total amount of TAGs.

3.4 Validation

Eight reference TAG compounds ranging from ACN 30 to ACN 54 with equal molar concentrations in a mixture (see **Supplementary Figure 1**) produced similar MS response with 100 ºC ion source temperature and the measured average molar percentage (12.5 ± 1.5 mol%) of all compounds was within the margin of error. The results indicated that no additional correction factors were needed to take into account in the variation of the MS response due to different acyl carbon lengths of the TAGs. The number of double bonds was 0, 3, or 6 in the TAGs tested. The tested number of double bonds did not have a major effect on the MS response either. Tukey test and Levene's test ($p<0.05$) showed no significant differences in the means or the variances of the results of different TAG standards. No competitive ionization or ion suppression effects were observed between the eight analyzed TAG reference compounds as they all produced statistically similar MS response. However, after we had concluded our MS analyses for molecular weight distribution it was noticed that using very high ion source temperature (340 ºC) the overall intensities were significantly higher. Whereas with low 100 ºC ion source temperature the MS response was statistically similar for each analyzed TAG, increasing temperature affected the MS response, which would have required extra correction factors to be calculated. This means that changing temperature for molecular weight distribution analysis has a trade-off between sensitivity and need for correction factors. Using low 100 ºC ion source temperature the sensitivity was sufficient for detection and quantification of the molecular ions, excluding the need for additional correction factors.

The discrimination factor was determined with 10 different ABA TAG regioisomer standards resulting in an average discrimination factor of 0.14 ± 0.02. The effect of using the same discrimination factor for all TAGs is displayed in **Supplementary Table 1**, which shows the differences between actual and measured ratios of regioisomers in various reference standard mixtures. FA chain length did not seem to have an obvious effect on the results of different TAGs. However, TAGs containing polyunsaturated FAs 18:2 or 18:3, especially LLLn/LLnL, OOLn/OLnO,

LLP/LPL and LLO/LOL had the most skewed results but the total number of double bonds in the TAG did not seem to have a clear effect. The results for TAGs containing only saturated or monounsaturated FAs were more in line with the actual regioisomeric ratios of the reference standards. This type of calculation using only one discrimination factor is not optimal if the aim is to accurately quantify individual regioisomers, but it can be applied for the purpose of this study, where the main interest is comparison among different samples of human milk, bovine milk, and infant formulas.

Correction factor 1.00 was given to 18:0. Correction factors using the 10 ABA type standards were calculated for 18:1 (1.33), 18:2 (1.56), 16:0 (1.44), 14:0 (2.38), 12:0 (2.78) and 8:0 (4.16). Shorter chain length and higher degree of unsaturation increased the correction factor. Based on this information the correction factors for 18:3 (1.80), 16:1 (1.70) and 10:0 (3.50) were extrapolated.

Functionality of the automated data conversion tool was tested with 4 samples and 4 different ACN:DB species. There were only minor differences between the handpicking of product ions and automated data conversion. Therefore, the conversion tool is suitable for the calculation process. An example comparison of automated and manual data conversion is presented in **Supplementary Figure 2**. The automatic processing of the MS/MS spectral data significantly reduces the time required for the calculations.

Increasing the ion source temperature from 100 ºC to 340 ºC increased sensitivity by more than an order of magnitude, making it possible to acquire multiple product ion spectra during a single analysis, whereas previously it was possible to track only one. The reason for such a dramatic increase in sensitivity may be the fact that at lower temperatures the ion source would get contaminated relatively quickly, requiring cleaning after 40–50 samples, but using a higher temperature the contamination was barely noticeable even after several hundred samples. The initial tests demonstrated that it was possible to track 7 product ion spectra during a single analysis from ACN:DB species that contained at least 1 mol% of the total TAGs in the sample. This is a major improvement

compared to the previous studies using similar DEP instrumentation (Kallio et al., 2005; Leskinen et al., 2010; Linderborg et al., 2014). Consequently, the total analysis time to study the majority of ACN:DB species in a complex sample such as human milk is as low as 10 min. Compared to LC-MS methods with often significantly longer analysis times, it is also easier to pick certain ACN:DB species for reanalysis if required without having to spend time on the analysis of entire sample again.

With a reasonably experienced worker one sample cycle takes roughly three minutes, making it possible to analyze 20 samples for a total of 140 ACN:DB species per hour. Compared with many LC methods this direct inlet application is fast as it requires no time-consuming pre-separation with chromatography. Additionally, the automated data conversion and MSPECTRA software makes data processing convenient and reduces the chance of user error. In most cases MSPECTRA calculation algorithm is not disturbed by overlapping masses, but with lower mass (mainly ACN <40) TAGs some $[M–H–FA–100]^-$ and $[RCOO]^-$ fragment masses may overlap, making them impossible to quantify with this algorithm. No overlapping fragment masses were found in the 21 selected ACN:DB species of this study. The overlapping issue with lower mass TAGs need to be resolved in future studies in order to enable more comprehensive elucidation of regioisomeric profile of TAGs consisting of short chain fatty acids such as bovine milk TAGs.

A major challenge in accurate quantification of TAG regioisomers has typically been the amount of reference standards required to produce calibration curves. The reference standards are very expensive and in natural samples there may be hundreds of different TAGs, making it extremely impractical and costly to generate calibration curves for all of them. While the correlation is not entirely obvious, there is an indication in our results that the number of double bonds in the *sn*-2 FA could affect fragmentation of the TAGs and thus result in different calibration curves. This can be demonstrated by constructing calibration lines (linear fit) for all ABA/AAB type standards (See **Supplementary Table 4**), where both the slopes of the calibration lines and the y-intercept values correlate with the number of double bonds in the “B” FA. The effect of the number of double bonds

564 in *sn*-2 FA on the dissociation of *sn*-2 FAs from TAGs in tandem MS analysis was also observed in
565 a recently published study in our laboratory using a UPLC-MS/MS method (Tarvainen, Kallio, &
566 Yang, 2019). This observation would need to be further tested with additional reference standards
567 before making any firm conclusions. If a clear pattern of impact of the number of double bonds in the
568 *sn*-2 FA on the calibration curve is found, it could be possible to update MSPECTRA by taking into
569 consideration the impact. This could lead to a major advancement in accurate quantification of TAG
570 regioisomers in complex samples since to our knowledge this effect has not been reported in any
571 other study published so far. Therefore, it is definitely worth investigating in the future with more
572 reference TAG standards.

573 Compared to traditional injection methods, the direct exposure probe seems to be more sensitive to a
574 multitude of different factors that may affect analysis reproducibility. For example, the probe might
575 not always lock in the exact same position between each analysis as there is some room for small
576 movements. Additionally, the metal tip of the probe is extremely fragile and bends easily while
577 sample is being applied, potentially causing differences in the sample vaporization and ionization.
578 However, both issues could be minimized by careful operation of an experienced operator.
579 Reproducibility was a major challenge of the method when the previous generation TSQ 700
580 instrument was used. In the current updated method using the TSQ 8000, significant improvement
581 has been achieved in both sensitivity and reproducibility, although reproducibility remains a
582 challenge for minor TAG species of low abundance. Despite the high deviation for ACN:DB species
583 TAGs of low abundance, the results provide information on the regioisomers of TAGs with
584 dominating fatty acid combinations. If necessary, reproducibility can be improved significantly by
585 analyzing the product ion spectra of only a single molecular weight species during each analysis at
586 the cost of time and sample throughput.

587 The method was also tested with TAG reference standards containing long chain polyunsaturated
588 20:5n-3 (EPA) and 22:6n-3 (DHA), but the quantification of TAGs with these FAs requires further

589 method development. With the current method many of the appropriate product ions were either not
590 detected at all or the intensities were very inconsistent (data not shown).

4 Conclusions

Regiospecific analysis of TAGs in natural fats and oils is highly challenging task, for which there are no perfect methods. Most of MS based analysis methods require pre-separation of TAGs using ultra-high performance liquid chromatography, which makes them both time consuming and expensive. A fast regiospecific analysis method based on direct inlet tandem mass spectrometry and automatic calculation software MSPECTRA has been previously developed in our laboratory. Applications have proved the method to be fast and efficient without need of chromatographic separation of TAGs. In this study, the method was updated for modern instrumentation. Increased sensitivity resulted in major improvement in throughput and reproducibility. Automatic processing of the MS/MS spectral data reduced the time required for the calculations significantly. The updated method was validated with a broad range of regiopure TAG reference compounds. The method proved suitable for fast analysis of complex samples. However, equal *m/z* ratio of formed fragments hindered the calculation of some TAGs with medium or short chain FAs. A possible association of the calibration curves with the number of double bonds in *sn*-2 FA was observed, which should be further investigated and could potentially lead to further improvements of this analysis method.

The updated method was applied for analysis of the regioisomeric composition of TAGs of human milk, infant formulas and bovine milk. The results of human milk TAG regioisomers were well in agreement with previous studies. The regioisomeric composition of infant formulas largely deviated from human milk. OPO and LPO, which are important regioisomers for the infant due to the palmitic acid in *sn*-2 position, were the most abundant regioisomers in the human milk samples. In contrast, OOP and LOP/PLO with palmitic acid in the primary positions were more abundant in every studied infant formula. In the studied 21 ACN:DB species of human milk, over 70 mol% of palmitic acid was located in *sn*-2 position, whereas in most infant formulas palmitic acid was heavily concentrated in *sn*-1/3 positions, the only exception being the OPO-enriched formula, in which roughly 40 % of

615 palmitic acid was in the *sn*-2 position in the identified TAGs. For bovine milk, regioisomers of only
616 roughly 15 mol% of all TAG were identified, mainly due to the dominance of TAGs of ACN < 40.
617 The proportion of *sn*-2 palmitic acid in the identified TAGs in bovine milk was close to that in the
618 OPO-enriched formula. However, further method development is still required to properly elucidate
619 the regioisomeric composition of TAGs of bovine milk.

5 Conflict of interest

Authors have declared no conflicts of interest.

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