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Tandem mass spectrometric analysis of human milk triacylglycerols from normal weight and overweight mothers on different diets

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ABSTRACT

The composition and structures of TAGs in the human milk from mothers with different food choices and prepregnancy body mass index were determined with two tandem mass spectrometric methods (negative APCI–MS/MS and positive UHPLC/ESI–MS/MS) at the infant's age of three months. The normal weight mothers with recommended food choices had more 18:3n-3 and less 18:0 in their milk than normal weight mothers with non-recommended food choices. A significant difference between the normal weight mothers on the non-recommended food choices and the other groups was seen in acyl carbon number: number of double bond (ACN:DB)-groups 54:6, 54:5, 54:3 and 54:2. In ACN:DB 52:7 and 52:6 the two recommended food choices–groups differed significantly from the two non-recommended food choices–groups. The regioisomerism of TAGs varied little despite differences in mother's weight and diet with *sn*-18:1-16:0-18:1 as the most prevalent regioisomer in the milk (13.8 ± 2.7%). The results of this study highlight the importance of structure specific human milk substitutes and the careful selection of the MS/MS methods for analysis of mixtures of several isobaric TAGs.

1. Introduction

Human breast milk provides the sole source of nutrition for the breast-fed infant during the first few months after birth. It provides about 50% of total energy from fat (Jensen, 1995). The fatty acids (FA) used by the mammary gland for synthesis of triacylglycerols (TAG) to be secreted in milk are obtained by either the uptake of fatty acids from plasma or by *de novo* synthesis in the mammary gland (Neville & Picciano, 1997).

It is known that maternal diet influences the FA composition of the milk. The levels of polyunsaturated fatty acids, mainly eicosapentaenoic (20:5n-3), docosahexaenoic (22:6n-3), and *trans* fatty acids in human milk have varied depending on the dietary fat composition (Brenna et al., 2007). The synthesis and secretion of medium chain fatty acids into milk has increased in lactating women consuming high carbohydrate diets (Silber, Hachey, Schanler, & Garza, 1988). In contrast, the level of palmitic acid (16:0) in milk has been found to be relatively constant at 20–25% of the milk fatty acids despite differences in diets (Jensen, 1995). Factors outside the diet may also affect the milk FA composition. Recently, we found that the milk of overweight mothers contained higher amounts of saturated FAs and lower amount of n-3 FAs than the milk of normal weight mothers even after adjusting for maternal diet (Mäkelä, Linderborg, Niinikoski, Yang, & Lagström, 2013).

Despite the FA composition, the location of the FA in the glycerol backbone plays a role in the infant nutrition as recently reviewed (Bar-Yoseph, Lifshitz, & Cohen, 2013; Innis, 2011). Human milk is known to contain palmitic acid (16:0) predominantly in the *sn* (stereospecific numbering) -2 position and unsaturated fatty acids predominantly in the *sn*-1/3 positions of the TAG molecules (Breckenridge, Marai, & Kuksis, 1969; Tomarelli, Meyer, Weaber, & Bernhart, 1968). In fact, over 50% of the fatty acids in the *sn*-2 position of human milk TAG are 16:0 (Jensen, 1995; López-López, López-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargalló, 2002). In contrast to milk fats, most plant and animal TAG, including human adipose tissue but excluding lard, have an unsaturated fatty acid in the *sn*-2 position and saturated fatty acids such as 16:0 in the *sn*-1/3 positions (Kagawa, Matsubara, Kimura, Shiono, & Fukui, 1996).

Although the structure of TAGs is known to affect the infant's lipid metabolism, the composition of TAGs in the infant formulas that are based on vegetable oils is different from the corresponding

Abbreviations: ACN, acyl carbon number; APCI, atmospheric pressure chemical ionisation; DB, number of double bond; ESI, electrospray ionisation; FA, fatty acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TAG, triacylglycerol; UHPLC, ultra high performance liquid chromatography.

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composition in human milk. Little is known on how much the composition of individual *sn*-2 specific TAGs depends on mother's weight or diet, mainly due to lack of suitable methods of analysis. Traditionally used enzymatic methods combined with chromatographic techniques are susceptible to acyl micration during the analysis, and they do not give information of individual TAGs present in the lipid mixture.

This study aimed at determining the TAG composition and structure of the milk samples from mothers with different prepregnancy BMI (body mass index) and dietary food choices. Additionally, the material was used to investigate the suitability of two different tandem mass spectrometric (MS/MS) methods in the analysis of TAG regioisomers in complex natural mixtures.

2. Materials and methods

2.1. Subjects

The milk samples were obtained from 40 mothers who participated in the STEPS (Steps to healthy development) study in South-West Finland (Lagström et al., 2012). The mothers were selected from a cohort of 90 overweight (prepregnancy BMI $\geqslant$ 25 kg/m^2) women and 73 normal weight (prepregnancy BMI < 25 kg/m^2) women based on differences in the food frequency questionnaires and self-reported prepregnancy weight and height. The milk samples were divided into four groups of ten. These were 1. Milk from normal weight mothers with recommended food choices, 2. Milk from overweight mothers with recommended food choices, 3. Milk from normal weight mothers with non-recommended food choices, and 4. Milk from overweight mothers with non-recommended food choices.

The Ministry of Social Affairs and Health, and the Ethics Committee of the Hospital District of Southwest Finland have approved the STEPS Study (2007-02-27). The parents gave a written informed consent. They have been informed of their right to withdraw from the study at any point. The description of the scientific data file is formulated according to the standards given by the Office of the Data Protection Ombudsman. The data are stored under lock and key in computers at the Turku Institute for Child and Youth Research (CYRI), University of Turku.

2.2. Dietary evaluation

Maternal diet was evaluated with a short food frequency questionnaire. The questionnaire focused on foods rich in different FAs or in foods that constitute one of the main sources of a certain FA. The questionnaire contained questions on fish, fish-oil supplements, vegetable oils, spreads, and foods rich in saturated fatty acids such as fast food, snacks, sausages, high-fat dairy products, and chocolate. The foods included in the questionnaire represented the typical sources of different FAs in Finland (Pietinen, Paturi, Reinivuo, Tapanainen, & Valsta, 2010). The food frequency questionnaire was answered on the day of the sampling in order to define the current intake of FAs which could partly explain the FA profile of the milk samples. Recommended food choices were fat free or low fat (less than 1%) milk, low fat (less than 17% fat) cheese, vegetable-based margarines, low fat cold cuts and no sausage as the main meal within the last week.

2.3. Milk collection

The samples were collected at infant's age of 3 months. The collection procedure was standardised by written instructions. The mothers collected the samples by manual expression in the morning, first milking a few drops to waste, and after that collecting the actual sample into a plastic container. The mothers brought the samples to the research centre or the samples were collected from their homes on the day of sampling. At home, and during transportation the samples were kept in +4–8 °C. At the research centre, the samples were frozen and stored at −70 °C until analysis.

2.4. Isolation of lipids

Triheptadecanoylglycerol (Larodan Fine Chemicals, Malmö, Sweden), was added to the thawed milk sample as an internal standard. 1.5 mL of methanol, 3 mL of chloroform and 0.8 mL of 0.88% KCl in water were added and the blend was thoroughly vortexed after each addition. The tubes were centrifuged 2000*g* for 3 min to separate the layers, and the chloroform-rich layer containing the lipids was removed and evaporated to dryness.

2.5. Fatty acid analysis

FA methyl esters were prepared from total lipids of the milk with boron trifluoride (Ågren, Julkunen, & Penttilä, 1992). The samples were analysed by gas chromatography (GC-2010 with Auto Injector/Auto Sampler and a flame ionisation detector, Shimadzu, Japan) with a DB-23 column (60 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies, DE). The peaks of FAs were identified by comparing their retention times with those of known reference mixtures FAME 37 (Supelco, Bellefonte, PA) and 68D (NuChek Prep, Elysian, MN) and quantified in relation to the internal standard. The FA composition of the total lipids of the milk was expressed as weight percentage of total FAs.

2.6. Triacylglycerol molecular weight distribution

A tandem mass spectrometer (Waters Quattro Premier, Waters Corp., Milford, MA) operated with Mass Lynx v4.1 (Waters Corp., Milford, MA) software was used for collection of the full scan mass spectra of the extracted lipids. TAG molecular weight distribution was analysed in triplicate with negative ion atmospheric pressure chemical ionisation mass spectrometry (negative APCI–MS) with ammonia (purity 5:0; Linde AG, Munich, Germany) as nebuliser gas. The gas flow was optimised and other instrument parameters were tuned for MS and MS/MS operations to generate maximal intensity and stability for negative $[M-H]^-$ ions of TAGs using *sn*-16:0-16:0-18:1 + *sn*-18:1-16:0-16:0 as a reference compound. The corona current was set at 30 μA and the cone voltage at 35 V. Ion source temperature was set at 120 °C and APCI probe temperature at 425 °C. The desolvation and cone gas flows were set at 180 L/h and 100 L/h, respectively. The samples were delivered manually to APCI probe through injection valve with a 5 μL sample loop while simultaneously introducing the solvent acetone/acetonitrile (50:50, v/v) at flow rate 0.4 mL/min. Liquid chromatograph Acquity UPLC™ (Waters Corp., Milford, MA) acted as a solution delivery device only. Full scan mass spectra of *m*/*z* 700–1000 were collected and analysed. An acquisition speed was 1500 amu/s. The MS analyses were corrected for the natural occurrence of the ^{13}C isotope.

Additionally, the single ion recording was used to quantify 18 selected abundant TAG species with atmospheric pressure chemical ionisation mass spectrometry (APCI–MS). The corona current was set at 6 μA and the cone voltage at 40 V. Ion source temperature was set at 150 °C and APCI probe temperature at 670 °C. The desolvation and cone gas flows were set at 199 L/h and 37 L/h, respectively. In this analysis, Kinetex™ C18 reversed phase column (100 × 2.1 mm, i.d. 1.7 μm, Phenomenex, Torrance, CA) was used as the UHPLC column and acetone (A) and acetonitrile (B) were as the mobile phases: the initial A/B (0:100, v/v); from 0 min to 31 min A/B (100:0) at 0.4 mL/min. Ammoniumadducts $[M+NH_4]^+$