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Direct quantification of fatty acids in human milk by gas chromatography

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ABSTRACT

Human milk provides the key nutrients necessary for the infants' growth and development. The fatty acid composition of human milk has been extensively studied over the last 20 years and the results obtained by analyzing the fatty acid profile followed by lipid extraction and expressing data as g per 100 g of fatty acids. The main drawback is that normalizing data set does not give any information on the amount of fatty acid mother's milk and therefore the level of intake by the infant. The objective of the present study was to develop and validate a direct method to analyze the fatty acid content in liquid human milk samples. Hydrochloric acid in a solution of methanol was selected as the catalyst and methyl undecanoate (11:0) as the internal standard together with tritridecanoin (13:0 TAG) to monitor transesterification performance. The separation of fatty acid methyl esters (FAME) was performed using a 100 m highly polar capillary column and a certified calibration mixture used to calculate experimental response factors. The method is suitable to quantify fatty acids in human milk from a 250 μL sample and allow expression of the data in mg of fatty acids per deciliter of human milk as well as weight % of fatty acids. The method has been validated and show a good repeatability [CV(r) < 15% and CV(r) < 20% for the concentrations close to the LOQ] and a good intermediate reproducibility [CV(iR) < 15% and CV(iR) < 20% for the concentrations close to the LOQ]. The method was applied to analyze human milk samples obtained from 50 mothers 4 weeks post partum and the data are provided in absolute and relative quantity. These results show that the inter-individual variability of the fatty acid content in human milk is of prime importance and such information cannot be captured with normalized data sets.

1. Introduction

Human milk is a source of key nutrients necessary for the infant's growth and development [2,3]. Some nutrients in human milk may evolve with the stages of lactation and may even change with nutritional status of the mother [4]. It has been shown that the lipid content changes within a feed, as well as diurnally [5]. The fatty acid composition of human milk has been extensively studied over last 20 years and the main conclusion is that the fatty acid composition of human milk reflects the fatty acid composition of the maternal diet [6]. Indeed, it has been shown that the long-chain polyunsaturated fatty acids (LC-PUFA) content in human milk can substantially vary according to the level of fish consumption [7]. Another example is the content and profile of *trans* fatty acids (TFA) and conjugated linoleic acid (CLA) which reflect, in human milk, the dietary preferences of the mother [*i.e.* consumption of full cream dairy products or processed foods containing high-level of partially hydrogenated vegetable oils [6].

In almost all of these studies, results were obtained by analyzing the fatty acid profile after lipid extraction [8–17] with data generally normalized as g of fatty acid per 100 g of fatty acids [8–17]. Molto-Puigmarti and co-workers [18], reported human milk fatty acid concentrations in μg/mL by using a 40 m long capillary column, although no response factor or transesterification performance is reported and validation method is based on relative determinations. Some authors [19,20] are using direct methods enabling fatty acid methyl ester (FAME) preparation from the original method published by Lepage and Roy [1]. In these studies, an aliquot of liquid milk is mixed with a solution of internal standard such as triheptadeconoin and submitted to either acid catalyzed transesterification [19] or based-catalyzed transesterification [20]. Even though such methodology allows for the expression of results as quantity of fatty acids per volume of liquid human milk (*i.e.* mg of fatty acid per dl of human milk) as originally done by Lepage and Roy [1], authors using such an approach are reporting fatty acid data as normalized profile [19,20]. There are many advantages to normalizing fatty acid data as it corrects the bias related to

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incomplete lipid extraction or reaction. However, the main drawback is that expressing data as g per 100 g of fatty acids does not give information on the absolute concentration of fatty acid in mother's milk. This drawback can be eventually overcome if lipid content is determined as well and use to estimate the absolute concentration of fatty acids.

This lack of basic nutritional information is consequential to the way lipid experts perform and express fatty acids, particularly while methods such as the one proposed by Lepage and Roy are available [1]. The objective of the present study was to develop and validate a direct method to analyze the fatty acid content in liquid human milk samples. The basic approach selected was a direct methylation procedure conducted using hydrochloric acid in methanol in presence of internal standards to monitor transesterification performance. In short, the purpose of the developed methodology is to improve the knowledge on individual fatty acid secreted by mothers' mammary glands for intake by the infants over the different stages of lactation.

2. Materials and methods

2.1. Internal standard solutions

Internal standard solution containing methyl undecanoate (11:0; Nu-Check-Prep, Elysian, MN) solution was prepared as follows. Into a 20 mL volumetric flask, 62 mg of methyl undecanoate was weighed to the nearest 0.1 mg. The mixture dissolved and volume adjusted to 20 mL with *n*-hexane. Solution of tritridecanoin (13:0 TAG; Nu-Check-Prep, Elysian, MN) was also prepared in *n*-hexane to assess the transesterification performance as follows: into a 20 mL volumetric flask, 62 mg of 13:0 TAG was weighed to the nearest 0.1 mg. The mixture was dissolved and volume adjusted to 20 mL with *n*-hexane.

2.2. Human milk sample

The protocol and collection of human milk was reviewed and approved by the local ethical committee of Singapore. The study took place at National University of Singapore. Volunteer mothers of term infants, who were apparently healthy and non smokers ($n = 50$; 31.1 ± 3.1-year old) provided breast milk samples (approximately 30 mL; 4 weeks post partum). Samples were collected after full expression from one breast using milk pump and while the baby was fed on the other breast. We made all efforts to collect complete feed that included fore-milk, mid-milk and hind-milk as a representation of one feed and to avoid within feed variation of lipid content. Approximately 30 mL aliquot was separated in a conical polypropylene tube for this study and the rest was fed to the infant. Samples collected for research were stored at −80 °C until analyses.

2.3. Direct method procedure to prepare fatty acid methyl esters (FAME) from human milk

FAME were prepared using HCl/Methanol (3N) as a catalyst [21]. The methylation procedure was as follows: In a 15 mL test tube equipped with Teflon-lined screw caps, 250 μL of human milk was added followed by 300 μL of internal standard FAME 11:0 and 300 μL of internal standard TAG 13:0, 2 mL of methanol, 2 mL of methanol/HCL (3N) and 1 mL of *n*-hexane. Test tubes were firmly capped, shaken vigorously and heated at 100 °C for 60 min, with occasional additional shaking. Care was taken to fit the cap tightly with cap liner to avoid leaks when tubes are heated at 100 °C. After cooling down to room temperature, 2 mL of water is added and shaken vigorously for centrifugation at 1200 g for 5 min followed by the transfer of the upper phase (*n*-hexane) into GC vials.

2.4. Gas chromatography (GC) analysis of fatty acid methyl esters (FAME)

For optimal GC separation, the use of a long (100 m), highly polar capillary column is recommended [21,22,24,25]. These columns allow to obtain an accurate separation of FAME, including the *cis* and *trans* isomers. GC analyses have been performed according to previously described conditions [21] as followed. A 7890A gas-chromatograph with a 7693 autosampler with preparative station module (Agilent Technologies, Palo Alto, CA) equipped with a fused-silica CP-Sil 88 capillary column (100% cyanopropylpolysiloxane; 100 m, 0.25 mm id, 0.25 μm film thickness; Agilent, Palo Alto, CA) was used with a split injector (1:25 ratio, 1 μl injected) heated at 250 °C and a flame-ionization detector operated at 300 °C. Oven temperature programming used was 60 °C isothermal for 5 min, increased to 165 °C at 15 °C/min, isothermal for 1 min at this temperature, and then increased to 195 °C at 2 °C/min and held isothermal for 14 min and then increased to 215 °C at 5 °C/min and held isothermal for 8 min at 215 °C. Hydrogen was used as carrier gas under constant flow mode at 1.5 mL/min.

2.5. Identification, quantification of fatty acids and monitoring of the chromatographic performances

Identification and quantification was performed by injecting authentic FAME standard solutions as described elsewhere [21]. Relative response factors were calculated for each FAME using commercially available certified FAME standard mixtures obtained from Nu-Check-Prep (Elysian, MN, USA) under the code name Nestlé 36. This mixture contains the following methyl esters: butyric acid (4:0), caproic acid (6:0), caprylic acid (8:0), capric acid (10:0), undecanoic acid (11:0), lauric acid (12:0), tridecanoic acid (13:0), myristic acid (14:0), myristoleic acid (14:1 n-5), pentadecanoic acid (15:0), pentadecenoic acid (15:1 n-5), palmitic acid (16:0), palmitoleic acid (16:1 n-7), heptadecanoic acid (17:0), heptadecenoic acid (17:1 n-7), stearic acid (18:0), elaidic acid (*trans*-9 18:1), oleic acid (18:1 n-9), linolelaidic acid (all *trans*-18:2 n-6), linoleic acid (18:2 n-6), arachidic acid (20:0), γ-linoleic acid (18:3 n-6), eicosenoic acid (20:1 n-9), α-linolenic acid (18:3 n-3), heneicosanoic acid (21:0), eicosadienoic acid (20:2 n-6), behenic acid (22:0), eicosatrienoic acid (20:3 n-6), erucic acid (22:1 n-9), eicosatrienoic acid (20:3 n-3), arachidonic acid (20:4 n-6), docosadienoic acid (22:2 n-6), lignoceric acid (24:0), eicosapentanoic acid (20:5 n-3), nervonic acid (24:1 n-9) and docosahexaenoic acid (22:6 n-3). Calibration samples were prepared in hexane at around 10 mg/mL.

The response factor *Ri* (mean of three injections of the calibration standard solution) for each FAMEi present in the calibration standard solution is calculated relative to the 11:0 internal standard as follows:

$$Ri = \frac{m'i * A'_o}{m'_o * A'i}$$

where $m'i$: % mass of FAMEi in the calibration standard solution; A'_o: peak area of 11:0 in the calibration standard solution chromatogram; m'_o: % mass of 11:0 in the calibration standard solution; $A'i$: peak area of FAMEi in the calibration standard solution chromatogram