Scientific Papers of the University of Pardubice, Series A; Faculty of Chemical Technology
26 (2020) 157–168.

UNIVERSITY OF PARDUBICE
FACULTY OF CHEMICAL TECHNOLOGY

Determination and profiling of fatty acids in dried breast milk spot

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Received: May 28, 2020; Accepted: June 8, 2020

A method was developed for the determination of fatty acids in dried breast milk spot and plasma using gas chromatography with mass spectrometry and flame ionization detection. The breast milk was sampled onto a collection paper treated with butylated hydroxytoluene. Fatty acids were derivatized with acetyl chloride in methanol to form fatty acids methyl esters. Our results indicate that the concentrations of fatty acids found in human milk can be used for assessing the intake and needs of infants.

Keywords: Fatty acid; Dried milk spot; Human breast milk; Gas chromatography

Introduction

There is some evidence implying the importance of human breast milk for the short-term health of infants, their development and decreasing risk of disease [1]. Breast milk fatty acids (FA) have a great impact, because they have several structural, energetic and bioactive functions [2]. Arachidonic acid (ARA) and eicosapentaenoic acid (EPA) are used for eicosanoid synthesis [3]. Docosahexaenoic acid (DHA) is an important structural part of cortical neuronal membranes [4]. The composition of FA in breast milk affects the brain and retinal development of infants [5,6] and reduces the risk of allergic diseases [7]. Analysis of fatty acids in breast milk can be used to determine nutritional conditions.

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However, conventional methods for the profiling of FA require a strict temperature control during the transport and storage of milk samples. Another problem is the oxidation of polyunsaturated FA during the sample storage and processing [8,9].

Some of these issues can be overcome by using a dried matrix spot sampling method. It is a relatively non-invasive method for the sample collection of biological fluids onto a special filter paper. The most widespread and commonly used technique is the sampling of whole blood for new-born screening of inborn errors of metabolism [10]. The dried matrix spot technique has a number of advantages, such as a low sample volume, simple transport conditions to laboratories, easy storage, and the increased stability of analytes [11]. Dried milk spot (DMS) technique is — in comparison with dried blood spot (DBS) — fully non-invasive method with a high level of biosafety. The aim of this study was to develop a method for the determination of FA in human breast milk using DMS technique and gas chromatography.

Materials and methods

Reagents and chemicals

Fatty acid methyl esters (FAME 37 = a mixture of 37 fatty acid methyl esters), internal standard (heneicosanoic acid), butylated hydroxytoluene (BHT), potassium carbonate, and acetyl chloride were from Sigma Chemical Company (St. Louis, MO, USA). Methanol (HPLC gradient grade), ethanol, dichloromethane, *n*-hexane, *tert*-butyl methyl ether, toluene, and isopropanol were purchased from Merck (Darmstadt, Germany).

Instrumentation

Chromatographic analyses were performed with a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 Ultra) and gas chromatograph (GC-2010 Plus) with a flame ionization detector (Shimadzu, Kyoto, Japan). The data were collected and processed by a software (GC Solution; Shimadzu, Kyoto, Japan).

Subjects

The study had included a grand total of 17 women, from which 8 colostrum samples were collected from mothers breastfeeding up to 14 days from childbirth, 9 mature breast milk samples taken from mothers breastfeeding over 14 days from childbirth plus blood samples collected from 2 persons; all the subjects being

anonymous healthy volunteers. They received a complete description of the study and gave written informed consent prior to providing the breast milk and blood samples. The ethical principles for medical research of the components of human beings have not been violated and all experiments with human samples were done in accordance with the WMA Declaration of Helsinki, June 1964.

Breast milk and blood collection

The first one was collected after breastfeeding. Before the spotting of milk, Specimen Collection Paper #903 (Whatman, Dassel, Germany) was treated with BHT solution (1 g L^{-1}) in ethanol. About 30 μL of BHT solution was pipetted onto the collection paper and allowed it to dry for at least 1 hour at room temperature. The milk was collected as a drop (approximately 20 μL) onto the collection paper containing BHT and, again, left to dry for at least 3 hours at room temperature. These samples were either stored at −20 °f C^{-1} or processed immediately. Blood was collected from anonymous donors and transferred into plastic tubes coated with spray-dried K_2EDTA.

Sample preparation

Heneicosanoic acid was used as an internal standard (IS). This fatty acid does not normally occur in human breast milk or plasma and it has similar chromatographic properties to other fatty acids measured.

Fatty acids in DMS were derivatized to FAME by the following procedure. A disc, 6 mm in diameter, was punched from DMS and transferred into a well-capped glass tube. Then, 200 μL of IS solution (10 μg mL^{-1}) in methanol and 300 μL of derivatization reagent (methanol/acetyl chloride, 19:1, v/v) were added. The tube was closed and the mixture incubated at 100 °C for 1 hour. After cooling down to room temperature, 1.25 mL of 3% potassium carbonate was added and the mixture vigorously vortexed for 5 min. Thereafter, 1 ml of *n*-hexane was added, the mixture vortexed for 5 min and centrifuged (1970 × g, 5 min). The upper organic layer was transferred into a glass tube, the hexane extraction was repeated and the combined extracts evaporated to dryness under a stream of nitrogen. The dried residue was re-suspended in 100 μL of dichloromethane, vortexed for a few seconds and then transferred into a vial with glass insert.

Plasma was separated from the cells by centrifugation (1970 × g, 10 min). 100 μL of plasma was mixed with 100 μL of internal standard (12 μg mL^{-1}) in ethanol and the mixture was incubated for 5 minutes in a freezer (−20 °C) to enhance deproteination. To mixture, 500 μL of *n*-hexane was added, the mixture was vortexed (5 min) and centrifuged (1970 × g, 5 min). The hexane extraction was repeated, the combined extracts transferred into a well-capped glass tube and evaporated to

dryness under a stream of nitrogen. Derivatization reagent (1 mL of methanol/acetyl chloride, 19:1, v/v) was added to the dried residue and the mixture was incubated under continuous stirring at 100 °C for 1 hour. The following procedure was otherwise the same as that for the determination FA in DMS samples.

Chromatographic method

The GC separation of FAME was performed on an HP-88 capillary column (100 m length, 0.25 mm internal diameter, 0.2 μm film thickness) coated with 88% cyanopropyl and 12% arylpolysiloxane (Agilent® J&W; Folsom, CA, USA). The injector temperature was adjusted at 250°C and the inlet split ratio set to 10:1. A programmed temperature ramp was applied according to the following sequences: the initial temperature at 75 °C for 3 min., then increased to 200 °C at 10 °C min^{-1}, again increasing to 230 °C at 0.8 °C min^{-1} and held for 5 minutes. The total analysis time was 58 min. Helium was used as a carrier gas with a flow rate of 3 mL min^{-1}. The injection volume of the sample was 0.5 μL. The flame ionization detector temperature was set at 250°C and quadrupole mass spectrometer with electron ionization (70 eV) used for detection. FAME standards were identified by using mass spectra library (NIST´14 Mass Spectral Library) and identities of the sample FA peaks were determined by direct comparison of their relative retention times with those of the FAME standards. The results were expressed as a concentration in μmol L^{-1}.

Results and discussion

The conventional method for the determination of FA by gas chromatography in different matrices is their derivatization to FAME [12]. The purpose of this step is to increase the volatility of FA, which is an important attribute required for gas chromatography analysis. Different approaches exist for derivatization of FA into the corresponding FAME. Generally, FA derivatization methods can be divided into two groups: acidic methods (using hydrochloric acid, acetyl chloride or sulphuric acid as a catalyst) and basic methods (using sodium methoxide or KOH as a catalyst) [12]. Blood and plasma fatty acids are bound in phospholipids, acylglycerols, cholesterol esters or they could be free. After derivatization, all the original bonds are broken and the origin of FA is lost. In human breast milk, FA are bonded especially in triacylglycerols (98 %) [13]. We chose derivatization using acetyl chloride as a catalyst due to the high efficiency [12]. A mixture of acetyl chloride with methanol in the required ratio was prepared freshly every day.

During the optimization of the method, different times and temperatures during derivatization procedure were tested. The next step was to try different extraction solvents for isolation of lipids before derivatization and a comparison with direct derivatization of DMS. Another optimization step was testing of different extraction solvents of FAME. During the derivatization step, different amounts of methanol and acetyl chloride were tested. Each measurement was done in triplicate. For the optimization process, 9 items were chosen and this group is listed in Table 1 as major FA.

Table 1 Major fatty acids used for optimization of method and measured in real samples

Fatty acid	Summary	RT [min]	IRF
Myristic	C14:0	19.943	1.00
Palmitic	C16:0	21.939	0.97
Palmitoleic	*cis*-C16:1 n-7	22.677	1.04
Stearic	C18:0	24.268	0.99
Oleic	*cis*-C18:1 n-9	25.114	0.97
Linoleic	all-*cis*-C18:2 n-6	26.441	1.06
Linolenic	all-*cis*-C18:3 n-3	28.195	1.03
Arachidonic	all-*cis*-C20:4 n-6	32.446	1.15
Docosahexaenoic	all-*cis*-C22:6 n-3	42.911	1.33

RT, retention time; IRF, internal response factor

Minor fatty acids (see Table 2) were evaluated and quantified in real samples; the results of intra-assay precision being presented in Table 3. FA concentrations were evaluated and compared with the published values.

Table 2 Minor fatty acids measured in real samples

Fatty acid	Summary	RT [min]	IRF
Myristoleic	*cis*-C14:1 n-5	20.696	1.05
Pentadecanoic	C15:0	20.898	1.04
Heptadecanoic	C17:0	23.016	1.54
Arachidic	C20:0	27.222	0.98
Eicosenoic	*cis*-C20:1 n-9	28.284	1.08
Eicosadienoic	all-*cis*-C20:2 n-6	30.045	1.07
Eicosatrienoic	all-*cis*-C20:3 n-6	31.452	1.21
Eicosapentaenoic	all-*cis*-C20:5 n-3	35.242	1.32

RT, retention time; IRF, internal response factor

Table 3 Intra-assay precision of the determination of selected fatty acids in dried milk spot

Fatty acid	Mean [μmol L^{-1}]	SD	CV [%]
C14:0	7134	692	9.7
cis-C14:1 n-5	301	31	10.4
C15:0	564	41	7.3
C16:0	35019	2511	7.2
cis-C16:1 n-7	2969	233	7.8
C17:0	703	47	6.7
C18:0	9787	796	8.1
cis-C18:1 n-9	73405	5338	7.3
all-*cis*-C18:2 n-6	16979	1891	11.1
C20:0	339	23	6.8
all-*cis*-C18:3 n-3	2134	372	17.4
cis-C20:1 n-9	771	54	7.0
all-*cis*-C20:2 n-6	227	22	9.7
all-*cis*-C20:3 n-6	355	42	12.0
all-*cis*-C20:4 n-6	476	100	21.0
all-*cis*-C20:5 n-3	79	13	16.2
all-*cis*-C22:6	252	79	31.4

SD, standard deviation; CV, coefficient of variance; IRF, internal response factor

We evaluated the amount of breast milk in each spot. Different volumes of about 7, 8, 9, 10, 11, 12, and 13 μL were pipetted onto the collection card and the diameter of the spot precisely measured. A regular puncher makes the disc about 6 mm in diameter and we found that such a diameter corresponds to 10 μL of breast milk.

Derivatization time and temperature were optimized. We tested temperatures 60, 80, and 100 °C at 30, 60, 120, and 180 min.; the best results being obtained at a temperature 100 °C for 1 hour (Fig. 1).

Several approaches to extraction of FA from DMS were tested and compared with direct derivatization. Tested extraction solvents were methanol, ethanol, *n*-hexane, and mixture chloroform, and methanol (2:1, v/v) commonly used for lipid extraction. Double extraction was done in 500 μl of each solvent. Direct derivatization was carried out by the addition of derivatization reagent to the DMS punched disc. The highest yield of FA extraction was when *n*-hexane had been used as an extraction solvent, the yield of direct derivatization was satisfactory, too (Fig. 2). Direct derivatization is preferred, because no extra solvent is used and one step can be eliminated, so it is less time-consuming. During the comparison of different punches from the collection card, we have

found that a punch directly from the middle of the target is the only right option. Punches from the edge of the target have a lower yield of FAME (Fig. 2), so we assume that lipids hold in the middle of the spot and their concentration decreases towards the edge.

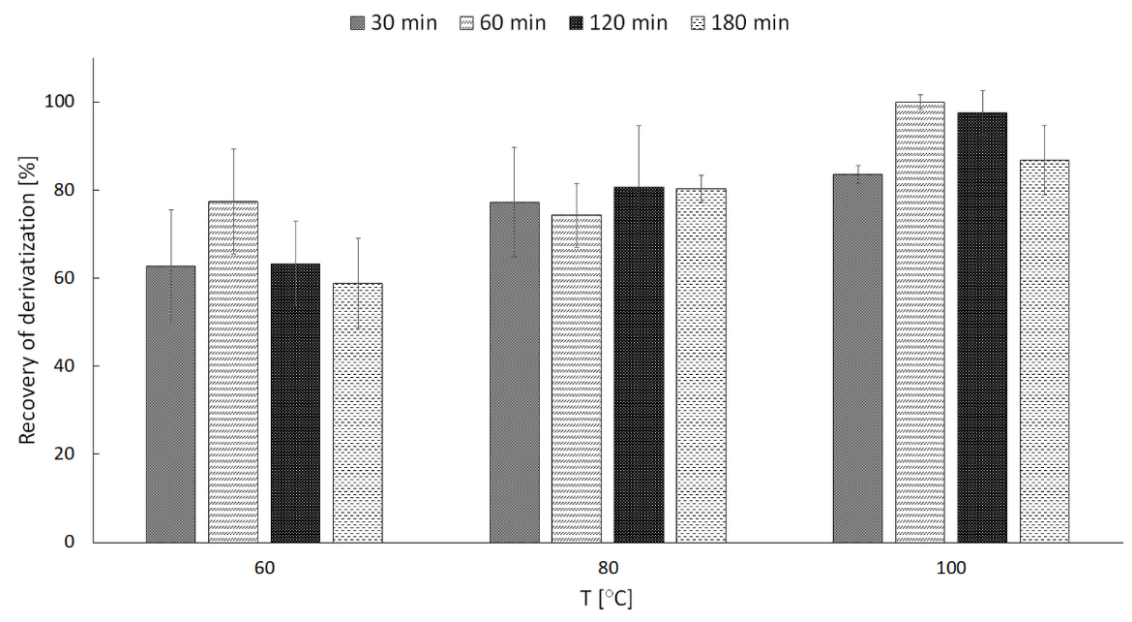

Fig. 1 Derivatization time and temperature optimization

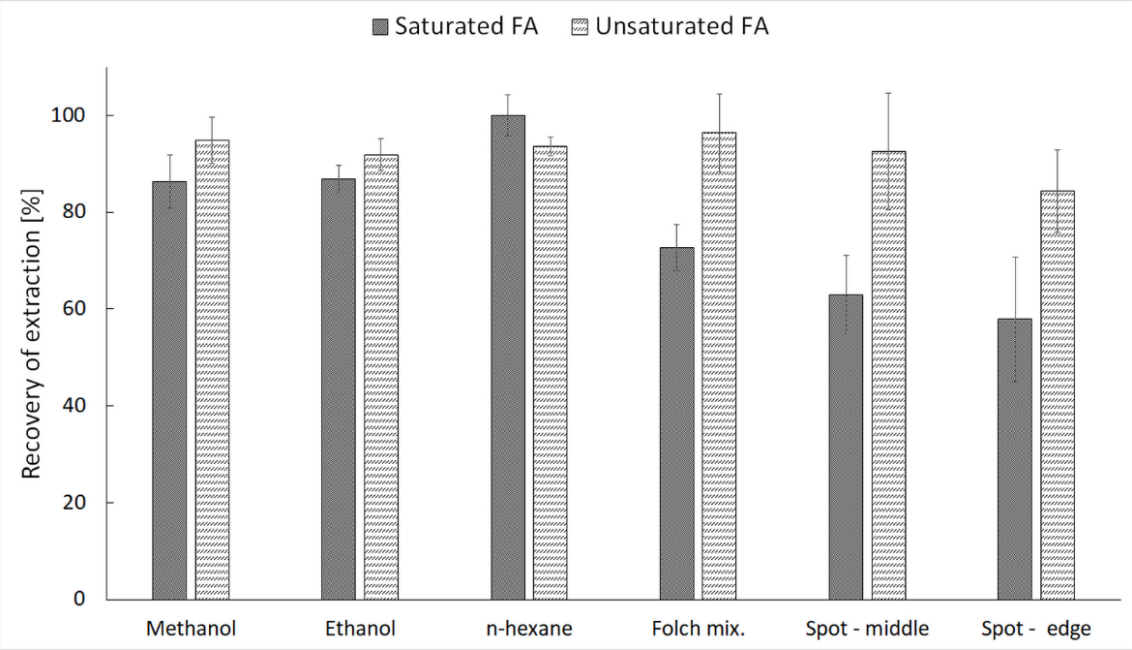

Fig. 2 Liquid-liquid extraction techniques of lipids with different solvents and direct derivatization

Folch mix. – mixture of chloroform and methanol (2:1, v/v);
Spot – middle, direct derivatization of a disc punched from the centre of the spot;
Spot – edge, direct derivatization of a disc punched from the edge of the spot

Different organic solvents and mixtures of solvents were tested for the best extraction of FAME. We tested *n*-hexane, *tert*-butyl methyl ether (TBME), toluene, *n*-hexane with isopropanol (3:2, v/v), TBME with methanol (3:1, v/v), and toluene with methanol (3:1, v/v). We obtained the best results with *n*-hexane and the mixture of TBME with methanol. Saturated FAME was better extracted with *n*-hexane, while unsaturated FAME with TBME. The best extraction yield of all FAME was obtained with *n*-hexane as an extraction solvent (Fig. 3).

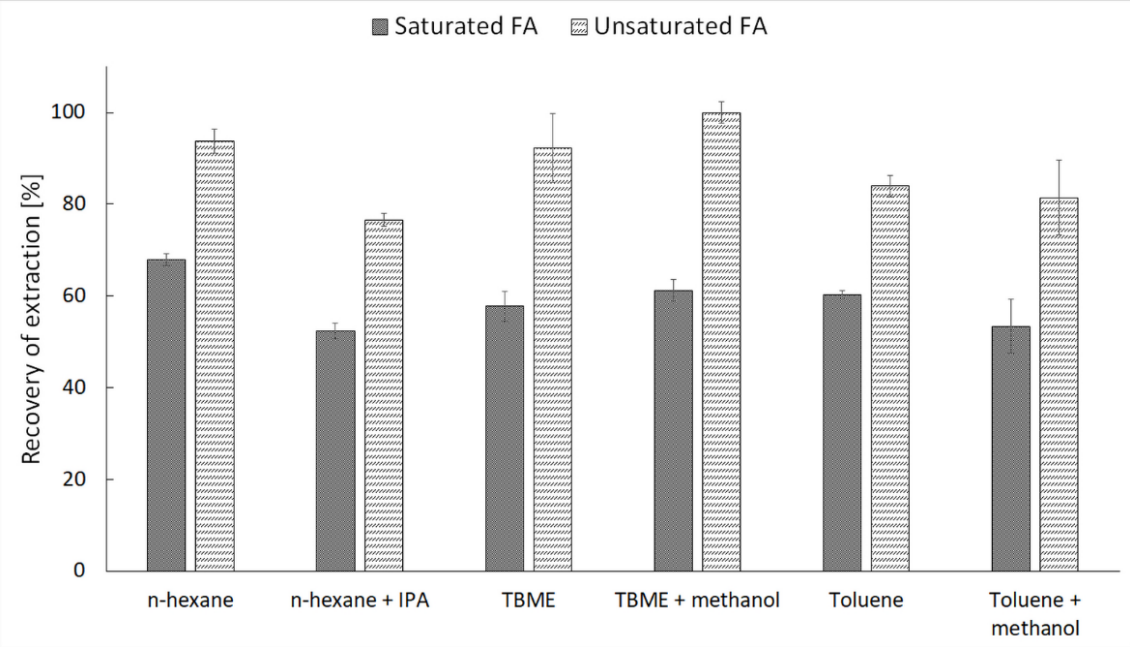

Fig. 3 Liquid-liquid extraction techniques of FAME with different solvents and solvent mixtures

IPA, isopropyl alcohol; TBME, *tert*-butyl methyl ether

Different ratios and volumes of the derivatization reagent were tested. Tested ratios of methanol and acetyl chloride were 4:1, 9:1, 19:1, and 49:1 (v/v). The efficiency tested ratios did not differ much and, therefore, a value of 19:1 (v/v) was the ratio of choice for analysis. Different volumes (250, 500, 750, 1000, and 1500 µL) of a mixture of methanol with acetyl chloride (19:1, v/v) was added to the 6 mm disc of DMS. Again, the recovery did not differ with the increasing amount of added mixture, so we chose 500 µL as optimal.

In many papers, relative amounts of FA are presented as a percentage [2,9,14]. We determined the absolute amounts when using of internal response factors. Generally, it is a single point internal standard calibration. An internal standard, as a substance that is not normally present in the sample, has to be added in the known concentration. The commercially available mixture of FAME with known concentrations of the internal standard and other determined fatty acids is measured in a batch of unknown samples.

The respective concentrations are calculated from the internal standard concentration using internal response factors (*IRF*); the *IRF* of each FA being defined from the integrated FAME 37 chromatogram as follows:

$$IRF = \frac{\text{area}_{IS} \times \text{amount}_{FA}}{\text{amount}_{IS} \times \text{area}_{FA}} \quad (1)$$

Then, the concentration of FA can be calculated from the equation:

$$\text{Amount of FA} = \frac{\text{amount}_{IS} \times \text{area}_{FA} \times IRF_{FA}}{\text{area}_{IS}} \quad (2)$$

IRF serve as the correction factors for errors caused by different responses of the flame ionization detector to each FA. The advantage of this approach is its simplicity and there is no need of possessing each FA standard. Unfortunately, there is no commercially available certified standard sample of breast milk for assessing the accuracy of the method. The concentrations of FA in 2 plasma samples and 2 breast milk samples were determined and compared with published results for plasma [15,16] and for breast milk FA [17]. Concentrations of FA in plasma were similar to those found in literature, whereas concentrations in breast milk were slightly higher compared to the published data; especially, the levels of oleic acid. As known, human breast milk fat composition is not the same during breastfeeding — the amount of fat increases —, which is the reason for distinguishing between the FA concentrations in foremilk and hindmilk. In terms of the fat content, the foremilk is less concentrated and more watery. The function of foremilk is to quench the thirst of a baby and hindmilk is the proper feed. We had analysed the hindmilk samples and compared the FA levels with those of Cruz-Hernandez et al. [17]; however, it should be added here that they determined FA levels in the samples obtained from the whole breastfeeding process.

The FA levels in 17 samples are presented in Table 4, GC chromatogram of the determination of FA in a DMS sample is shown in Fig. 4. As can be seen, the most abundant fatty acids in human breast milk are oleic and palmitic acid. These two FA, together with stearic, linoleic and myristic acid, form more than 87 % of all the FA.

Table 4 Concentrations [μmol L^{-1}] of the fatty acids measured in dried milk spots

Fatty acid	Colostrum (n = 8)				Mature milk (n = 9)			
	Mean	SD	Median	IQR	Mean	SD	Median	IQR
C14:0	8149	2873	8088	3951	15959	6267	15113	11925
cis-C14:1 n-5	334	122	386	228	609	285	479	321
C15:0	636	188	651	256	961	404	848	276
C16:0	37766	12385	38452	17116	60663	14985	59670	29933
cis-C16:1 n-7	3464	1528	4002	2587	6123	2064	5762	3864

Table 5 Concentrations [μmol L^{-1}] of the fatty acids measured in dried milk spots (continued)

Fatty acid	Colostrum (n = 8)				Mature milk (n = 9)			
	Mean	SD	Median	IQR	Mean	SD	Median	IQR
C17:0	742	240	756	305	1083	291	1015	371
C18:0	8129	2218	8198	2054	15872	4260	15931	5053
cis-C18:1 n-9	42073	11890	45360	10119	85830	15158	91161	16273
all-*cis*-C18:2 n-6	12465	4097	13718	3570	32518	7120	28952	9898
C20:0	251	57	238	65	377	90	347	125
all-*cis*-C18:3 n-3	1050	418	1095	329	2198	842	2096	1284
cis-C20:1 n-9	889	263	804	258	912	262	899	435
all-*cis*-C20:2 n-6	703	255	617	257	566	93	559	147
all-*cis*-C20:3 n-6	695	144	671	174	746	195	758	337
all-*cis*-C20:4 n-6	910	256	947	276	1028	257	1004	478
all-*cis*-C20:5 n-3	84	38	90	35	187	122	138	81
all-*cis*-C22:6	530	133	530	146	571	309	523	351

SD, standard deviation;
IQR, interquartile range – the difference between the upper quartile and the lower quartile

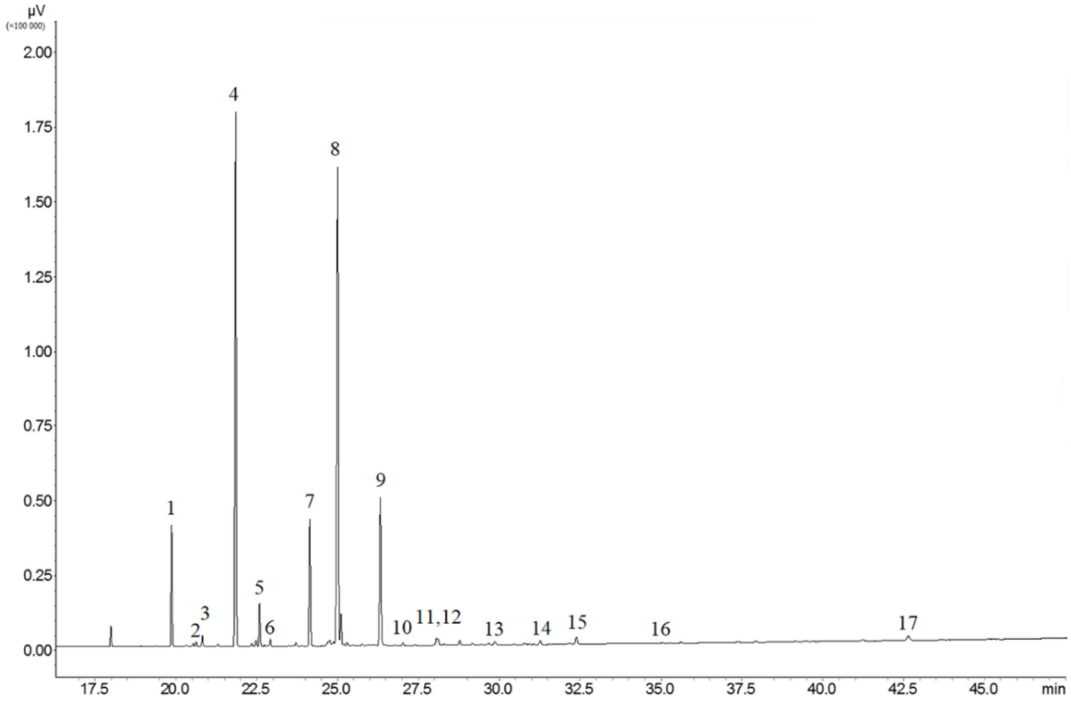

Fig. 4 Typical chromatogram (real record) of FA after derivatization to corresponding FAME in dried breast milk sample

Peaks: 1, C14:0; 2, *cis*-C14:1 n-5; 3, C15:0; 4, C16:0; 5, *cis*-C16:1 n-7; 6, C17:0; 7, C18:0; 8, *cis*-C18:1 n-9; 9, all-*cis*-C18:2 n-6; 10, C20:0; 11, all-*cis*-C18:3 n-3; 12, *cis*-C20:1 n-9; 13, all-*cis*-C20:2 n-6; 14, all-*cis*-C20:3 n-6; 15, all-*cis*-C20:4 n-6; 16, all-*cis*-C20:5 n-3; 17, all-*cis*-C22:6

Conclusions

In this article, a method developed for the determination of FA levels in the human breast milk dried spot has been reported. The presented method requires a small amount of sample with the advantage of simple and non-invasive collection, easy transport and storage of the sample. Information about the concentrations of FA in human milk can be used for assessing the intake and needs of infants, thus ensuring the proper development of the child.

In the future, there is a plan of comparing our method with the standard calibration curve method and, in prospect, the detailed characterisation via evaluation of the accuracy, linearity, and the limits of detection and quantification. Moreover, we intend to enlarge the population studied.

Acknowledgement

This work was financially supported by grant SGS_2020_005.

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