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DETERMINATION OF SELECTED FATTY ACIDS IN DRIED BLOOD SPOT BY GAS CHROMATOGRAPHY-FLAME IONIZATION DETECTION

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With the aim to minimize the amount of biological material needed for analysis, a new type of sample preparation has been used for the determination of the percentage content of fatty acids in human blood. The blood samples from the volunteers (n = 30) were processed in two ways, as a plasma and as dried blood spot (DBS) samples. The fatty acids were firstly derivatized into the respective esters and, subsequently, separated and detected using gas chromatography (GC) with a flame ionization detector (FID). The results obtained from the DBS sample analysis were compared with those obtained by the established method for the determination of fatty acids in plasma.

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Introduction

Fatty acids (FAs) are significant components of lipids. They play many important roles in human organisms; e.g., as energy sources, parts of cell membranes, and precursors for lipid mediators, when they can influence gene transcription or signal transduction pathways [1]. Also, FAs act in many diseases, such as *diabetes mellitus* [2], metabolic syndrome [3], Refsum disease or Zellweger syndrome [4] that cause changes in their concentration. Humans can synthesize only the saturated FAs and unsaturated FAs with a double bond before the atom "C9"; all the other unsaturated FAs are essential and must be consumed in the diet. The lack of essential FAs is associated with the growth retardation, increased skin permeability, infertility, kidney failure, neuropathies, etc. [5]. The positive effect of FAs can be seen, for example, in the prevention of atherosclerosis, ischemic heart disease, or hypertension [6].

FAs can be determined by two different approaches. Lipids can first be separated into the individual fractions using hydrophilic interaction liquid chromatography (HILIC) [7] or thin-layer chromatography (TLC) [8] in order that a particular fraction of the lipid can be analysed. Direct analysis of FAs in the sample, without their previous separation, can also be performed and these methods are less time-consuming. Analysis of FAs sample can be perfomed either by high-performance liquid chromatography (HPLC) [9-11] or, more often, by gas chromatography (GC) [8,12-14]. The stability of polyunsaturated FAs during storage may be problematic, but feasiable by adding an antioxidant – usually, butylated hydroxytoluene (BHT) [15,16].

In the search for new types of logistically less challenging samples, one's attention is focused on dried blood spots (DBS) that could be also used for direct analysis of FAs. This sampling method is standardly used in newborn screening, but, due to a lot of advantages, it has also expanded into other analytical fields [17-19]. The advantages of the DBS sampling method are a less-invasive sample collection, only a small volume of blood is needed, the procedure is bio-safe, and there is good stability of the analytes at room temperature and thus no need for freezing, which also means easy storage and transportation.

Experimental

Reagents and Chemicals

Standards of fatty acids (myristic acid, C14:0; 13-methylmyristic acid, 13-Me-C14:0; 12-methylmyristic acid, 12-Me-C14:0; palmitic acid, C16:0; sapienic acid, *cis*-C16:1 *n*-10; palmitoleic acid, *cis*-C16:1 *n*-7; 14-methylpalmitic acid, 14-Me-C16:0; 16-methylmargaric acid, 16-Me-C17:0; stearic acid, C18:0; oleic acid, *cis*-

C18:1 *n*-9; *cis*-vaccenic acid, *cis*-C18:1 *n*-7; *trans*-vaccenic acid, *trans*-C18:1 *n*-7; linoleic acid, all-*cis*-C18:2 *n*-6; linolenic acid, all-*cis*-C18:3 *n*-6; linolenic acid, all-*cis*-C18:3 *n*-3; dihomo-linolenic acid, all-*cis*-C20:3 *n*-6; arachidonic acid, all-*cis*-C20:4 *n*-6; timnodonic acid, all-*cis*-C20:5 *n*-3; adrenic acid, all-*cis*-C22:4 *n*-6; cervonic acid, all-*cis*-C22:6 *n*-3; nervonic acid, *cis*-C24:1 *n*-9; osbond acid, all-*cis*-C22:5 *n*-6; clupanodonic acid, all-*cis*-C22:5 *n*-3), butylated hydroxytoluene (BHT), potassium bicarbonate, toluene, acetyl chloride, and BF_3 – ethanol were obtained from Sigma Aldrich (St. Louis, USA). Furthermore, internal standard (all-*cis*-13,16,19-docosatrienoic acid) was purchased from Larodan (Malmö, Sweden), HPLC gradient-grade methanol, ethanol, 2-propanol, diethyl ether, dichloromethane, *n*-pentane, and *n*-hexane from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade.

Instrumentation

Chromatographic analyses were performed with a gas chromatograph Agilent (model 7890A; Agilent Technologies, Santa Clara, USA). Data were collected digitally with a Chem Station software (version 04.03; Agilent Technologies, Santa Clara, USA).

Subjects

The samples were obtained from 30 blood donors (19 women aged 20-28, with mean age 24 years and 11 men aged 21-28, mean age 24 years). All participants were treated according to standard protocols and gave written consent to participate in this research study, which was approved by the Hospital Committee on Human Research (Regional Hospital of Pardubice, Czech Republic).

Sample Collection

Blood samples were collected from cubital vein between 8 and 9 a.m. after minimal 10 hours of fasting into plastic EDTA tubes (Vacuette Detection Tube, No. 454246; Greiner Labortechnik, Kremsmünster, Austria). Immediately after blood collecting, 30 µl of whole blood was pipetted onto pre-treated filter papers and allowed to dry for at least 3 h. Prior to analysis, DBS samples were stored at –20 °C (maximally 2 months). Plasma was separated from the clot by centrifugation (1 700×g, 10 min, 8 °C) and immediately after pipetting 600 µl into well-capped 1.5 ml polypropylene tubes (Thermo Fisher Scientific, Pardubice, Czech Republic) pre-treated with BHT, stored at –20 °C prior to analysis

(maximum of 2 months).

Before blood collection, Specimen Collection Paper (#903; Whatman, Dassel, Germany) and 1.5 ml polypropylene tubes (Thermo Fisher Scientific, Pardubice, the Czech Republic) were treated with an antioxidant solution of BHT (10 g l^{-1}) in 100% ethanol. About 30 µl of BHT solution was pipetted onto the filter paper and left to dry at room temperature for at least 1 hour and then stored at 4 °C (maximally 1 week). Into polypropylene tubes, a volume of 200 µl BHT solution was pipetted and, under nitrogen (Linde Gas, Prague, the Czech Republic), evaporated to dryness at room temperature. Thus, treated polypropylene tubes were stored at 4 °C (maximum of one week).

Information about height and weight from which the body mass index (BMI) was calculated as a body mass (kg) divided by height (m) squared, and other specification (about sport activities, chronic diseases, usage of drugs, and relation to alcohol and cigarettes) were obtained *via* questionnary.

Sample Preparation

In this study, an internal standard was used only for verification of the derivatization process and stability of retention times and it was not involved in quantification of the individual FAs. Since all-*cis*-13,16,19-docosatrienoic acid is absent in human plasma and has quite similar chromatographic properties, it was chosen for this purpose.

For analysis of the composition of FAs in plasma, a volume of 500 µl plasma was pipetted into a glass tube and 2.5 ml mixture for protein precipitation added (2-propanol/heptane/2M H_3PO_4, 40:20:1, v/v/v). After vortexing, the mixture was left for 10 min to be conditioned and then vortexed again. Next, 1 ml of internal standard solution (with concentration of 10 µg ml^{-1}) and 1.5 ml distilled water was added. The organic layer was separated from aqueous by centrifugation (1 700×g, 10 min, 4 °C) transferred into clean glass tubes, and evaporated to dryness in the nitrogen atmosphere. The dried residue underwent re-suspension in 2 ml methanol/toluene solution (4:1, v/v), properly vortexed, and then 200 µl acetyl chloride was added. The glass tube was closed tightly and the mixture incubated under continuous stirring at 100 °C for 1 hour. After cooling down to room temperature, 5 ml circa 6% K_2CO_3 was added and the mixture vortexed for min. 2 minutes. Afterwards, the resultant mixture was centrifuged (1 700×g, 10 min, 4 °C) and the upper organic layer removed and subsequently dried under the stream of nitrogen. The dried residue was re-suspended in 100 µl methanol/toluene solution (4:1, v/v) and transferred into 0.1 ml vial insert.

In order to determine FAs in the DBS samples, a disc with diameter of 6 mm was punched out from DBS and transferred to well-capped glass tube.

Then, 20 µl the internal standard solution (10 µg ml^{-1}) and 200 µl

derivatization solution (approximately 10% solution of BF_3 in ethanol) were added. The mixture was incubated under continuous stirring at 60 °C for 3 hours. After cooling to room temperature, 400 µl deionised water and 400 µl saturated solution of NaCl were added and vortexed for 5 min.

For extraction of FAs esters 1.5 ml *n*-hexane was added and the mixture vortexed for another 10 min, then centrifuged (1 700×g, 10 min, 4 °C) and 1.3 ml of the upper organic layer removed to clean glass tube. The content was evaporated to dryness under nitrogen and the dried residue re-suspended in 100 µl 2-propanol, and transferred into insert of 0.1 ml crimped vial. All the stock solutions of FAs were prepared in ethanol and stored at –20 °C (maximally for 3 months). Working solutions of the individual FAs and the internal standard solution were prepared fresh daily.

Chromatographic Analysis

The chromatographic separation of prepared methyl/ethyl esters of FAs was carried out with a HP88 capillary column (100 m × 0.25 mm id; Agilent Technologies, Santa Clara, USA) coated with 88 % cyanopropyl and 12 % arylpolysiloxane (with a film thickness of 0.25 µm) as a stationary phase. Helium was used as the carrier gas with a constant flow rate of 3 ml min^{-1}. The same chromatographic conditions were used for both methyl- and ethyl esters of FAs. The injection volume of sample was 1 µl at the temperature of 250 °C and with inlet split ratio 10:1. The separation on the column was performed under the following temperature programme: 130 °C held for 1 min, then increased to 176 °C at a rate of 2 °C min^{-1}, held at this temperature for 2 min, then increased to 186 °C at a rate of 1 °C min^{-1}, held at this temperature for 1 min, then increased to 190 °C at a rate of 0.2 °C min^{-1}, held at this temperature for 1 min, then increased to 220 °C at a rate of 1 °C min^{-1}, and finally held at this temperature for 4 min.

The total analysis time was 92 min; the detector temperature set to 280 °C. The qualification of FAs peaks in plasma or DBS sample was done by direct comparing their retention times to those of the known standards. The results of quantitative analysis were expressed as a percentage of total FAs measured in the sample.

Statistical Analysis

The data obtained in this study were statistically analysed using Sigmastat (version 3.5; Systat Software, Point Richmond, USA) and presented as the median and the interquartile range (IRQ); alternatively, as mean and the standard deviation (*SD*).

Differences between the percentage content of FAs in plasma and DBS samples were analysed using the Student *t*-test, when the values at $p < 0.050$ and $(1–\beta) > 0.800$ were considered as significant. For detecting the differences between men and women, the Rank Sum Test according to Mann & Whitney was used. Statistical dependence of the percentage of FAs upon the sex, age, BMI, and sport activities was also analysed using a 2D ANOVA.

Results and Discussion

Sample Collection and Preparation

Blood samples were collected in tubes with EDTA, which is a well-known chelating agent. It entraps many transition metal ions and, thus, prevents the oxidation or so-called lipid peroxidation of FAs. Further, due to the expected lower stability of polyunsaturated FAs, BHT was added to the plasma and into the DBS samples as an antioxidant, hindering their oxidation during storage and sample-processing [15,16].

An internal standard was added for monitoring the extraction and the derivatization process. In clinical practice, it is often difficult to add a stabilizing solvent or an internal standard solvent immediately after sample collecting. And, since it is desirable to introduce the internal standard to the real sample as soon as possible, in most cases, the respective substance is added together with the first extraction step. This, however, does not fully reflect the behaviour of FAs present in the sample. The application of both solutions on the filter paper before the blood-sampling was tested. When the filter paper soaked with BHT was treated with internal standard solution of hexane, chromatographic separation and the corresponding effects caused displacement of the BHT. Therefore, the internal standard had to be diluted in the BHT solution beforehand and applied at the filter paper together. This method of sample preparation was successful, but, due to the sample collection that had already begun, it was not the case of our study; nevertheless, this information was considered in next studies.

Optimization of Sample Processing

Since the method for determination of FAs in plasma has been developed and applied in several studies [8,20,21], only a variant for the DBS sample preparation was optimised. From the variety of derivatization temperatures (from 60 to 90 °C) and time periods (from 10 to 180 min, and incubation overnight) that had been tested to ensure maximal formation of fatty acid esters, the best results were reached when derivatizing for 3 h at 60 °C. A longer derivatization or a higher

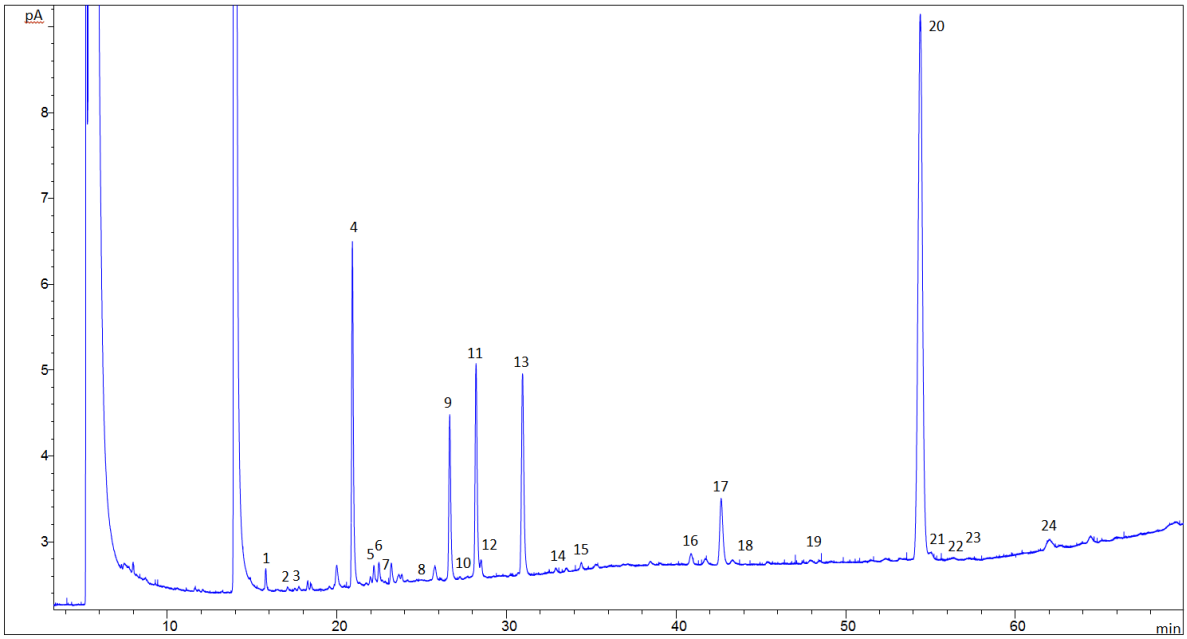

Fig. 1 Chromatogram of a dried blood spot sample analysed by GC-FID. Peaks: 1 – C14:0, 2 – 13-Me-C14:0; 3 – 12-Me-C14:0; 4 – C16:0, 5 – *cis*-C16:1 *n*-10; 6 – *cis*-C16:1 *n*-7; 7 – 14-Me-C16:0; 8 – 16-Me-C17:0, 9 – C18:0; 10 – *trans*-C18:1 *n*-7; 11 – *cis*-C18:1 *n*-9; 12 – *cis*-C18:1 *n*-7; 13 – all-*cis*-C18:2 *n*-6; 14 – all-*cis*-C18:3 *n*-6; 15 – all-*cis*-C18:3 *n*-3; 16 – all-*cis*-C20:3 *n*-6; 17 – all-*cis*-C20:4 n-*6*; 18 – all-*cis*-C22:4 n-*6*; 19 – all-*cis*-C20:5 *n*-3; 20 – all-*cis*-C22:3 *n*-3 (internal standard); 21 – *cis*-C24:1 *n*-9; 22 – all-*cis*-C22:5 *n*-6; 23 – all-*cis*- C22:5 n-3; 24 – all-*cis*-C22:6 n-3. Experimental conditions for GC analysis: temperature ramp 130 °C for 1 min, 2 °C min^{-1} to 176 °C, 176 °C for 2 min, 1 °C min^{-1} to 186 °C, 186 °C for 1 min, 0.2 °C min^{-1} to 190 °C, 190 °C for 1 min, 1 °C min to 220 °C, 220 °C for 4 min. Injection volume 1 µl (with split mode, 10:1) at 250 °C. Detector temperature: 280 °C. Flow rate of helium 3 ml min^{-1}, stationary phase: HP88 capillary column (100 m × 0.25 mm id) coated with 88 % cyanopropyl and 12 % arylpolysiloxane (with film thickness of 0.25 µm)

temperature had no additional effect on the peak intensity. For the extraction of FAs ethyl esters from the aqueous to the organic-phase diethyl ether, 2-propanol, *n*-pentane and *n*-hexane were tested; the latter having shown the best results.

Liquid/liquid extraction should always be performed with the excess of the organic solvent used; therefore, various volumes (from 500 µl to 1.5 ml) of *n*-hexane were tested. For the proper analyses, the volume of 1.5 ml was chosen. The last step before injecting the sample into the GC system was to redissolve the dried residue in the organic solvent. Several organic solvents were tested: ethanol, *n*-pentane, *n*-hexane, diethyl ether, 2-propanol, and dichloromethane. The lowest peak intensities were observed when using the *n*-hexane or *n*-pentane solvents. At the end, due to a high volatility of diethyl ether and dichloromethane at laboratory temperature (even although the other results were satisfactory), the solvent with the most similar result was chosen: 2-propanol. A typical chromatogram of the ethyl esters of FAs in the DBS sample analysed by GC-FID is shown in Fig 1.

Table I Intra-assay precision of the method for determination of the percentage content of FAs in DBS samples

FAs formula	FAs percentage content		*CV*, %			
	AVG	*SD*	< 5	5-10	10-20	> 20
C14:0	0.65	0.10				*
13-Me-C14:0	0.21	0.05				*
12-Me-C14:0	0.21	0.05				*
C16:0	20.21	0.69	*			
cis-C16:1 *n*-10	0.31	0.05			*	
cis-C16:1 *n*-7	1.00	0.05		*		
14-Me-C16:0	6.32	0.41		*		
16-Me-C17:0	0.16	0.01		*		
C18:0	10.38	0.48	*			
cis-C18:1 *n*-9	15.52	0.48	*			
cis-C18:1 *n*-7	1.64	0.23			*	
trans-C18:1 *n*-7	0.57	0.01	*			
all-*cis*-C18:2 *n*-6	18.23	0.59	*			
all-*cis*-C18:3 *n*-6	0.35	0.02		*		
all-*cis*-C18:3 *n*-3	0.80	0.16			*	
all-*cis*-C20:3 *n*-6	1.47	0.07	*			
all-*cis*-C20:4 *n*-6	10.73	0.11	*			
all-*cis*-C20:5 *n*-3	0.98	0.11			*	
all-*cis*-C22:4 *n*-6	1.44	0.35				*
all-*cis*-C22:6 *n*-3	2.17	0.33			*	
cis-C24:1 *n*-9	0.53	0.12				*
all-*cis*-C22:5 *n*-6	0.50	0.19			*	
all-*cis*-C22:5 *n*-3	1.28	0.20			*	

* indicates to which category a particular FA belongs

Determination of Selected Fatty Acids in Plasma and Dried-Blood Spot Samples

In this pilot study, the content of FAs in plasma and DBS samples was evaluated as the percentage of the individual fatty acids in relation to the total area of these substances measured in plasma or DBS. This approach is very popular, but does not allow one the comparison of the results among particular laboratories if different FAs are to be determined. Thus, in the future, a concentration could be used instead in order to simplify not only the comparison among the involved work-sites, but also the comparison of the samples themselves; for instance, those with a high variety of FAs. To determine the same-day precision, DBS sample was analysed ten times (ten filter spots) in the same day under identical conditions (see Table I). Coefficients of the variation for a low abundance FAs in the DBS were over almost ten percent. An improvement can be achieved by using a splitless injection or with the aid of a more sensitive detector; for example, in the mass spectrometry (MS or MS/MS).

Statistical Analysis

The percentage content of FAs in DBS and plasma from blood donors was compared. The percentage content of FAs in DBS and plasma differed (Table II) and there were no indications for any associations, as not many linear relations were found (Table III). These results suggest us that the FAs composition of these two sample types are different, which could be caused by several factors; whereas the FAs content in plasma is rather variable and significantly influenced by FAs consumed in the diet [22,23], the FAs content in the erythrocyte membranes is relatively stable — their life cycle is usually 120 days — and reflect a long-term dietary intake [24]. A DBS sample was prepared from the whole blood (i.e. from a mix of plasma and erythrocytes) and, thus, it may exhibit a different content of FAs than that of plasma.

The percentage content of DBS and plasma FAs in a group of blood donors is given in Table IV. Significant differences in the results for some FAs in both, DBS and plasma, were observed between men and women (data not shown). An interesting fact is that women had significantly increased percentage content of C16:0, *cis*-C16:1 *n*-7, and 16M-C17:0 ; both in plasma and DBS samples. Other significant differences in the percentage content of FAs between men and women were found only in plasma samples (13-Me-C14:0; 12-Me-C14:0; all-*cis*-C20:3, *n*-6; all-*cis*-C20:5, *n*-3; all-*cis*-C22:5, *n*-3; all being increased in samples from women). Otherwise, no other significant difference was found regarding our statistical analysis.

The pilot study described above has included the samples from 30 blood donors only, so that the results should be interpreted with certain caution. In the

future, it is planned to make a larger scale study, where not only DBS and plasma samples but also whole-blood samples will be analysed and the results compared. (It is expected that the results could be more similar in the composition of FAs with the DBS samples than those in plasma).

Table II Comparison of the percentage content of Fas in plasma and DBS (n = 30)

FAs formula	Plasma Mean (*SD*)[a]	DBS Mean (*SD*)[a]	t	p[b]	Power (1–ß)
C14:0	1.003 (0.374)	1.184 (0.350)	–1.935	0.058	0.355
13-Me-C14:0	0.033 (0.028)	0.218 (0.107)	–9.154	**< 0.001**	**1.000**
12-Me-C14:0	0.017 (0.010)	0.141 (0.116)	–5.843	**< 0.001**	**1.000**
C16:0	21.952 (2.281)	24.505 (2.079)	–4.530	**< 0.001**	**0.996**
cis-C16:1 *n*-10	0.466 (0.068)	0.932 (0.515)	–4.902	**< 0.001**	**0.999**
cis-C16:1 *n*-7	2.034 (0.872)	1.235 (0.540)	4.268	**< 0.001**	**0.991**
14-Me-C16:0	0.110 (0.150)	0.148 (0.044)	–1.338	0.186	0.132
16-Me-C17:0	0.151 (0.038)	0.149 (0.038)	0.247	0.806	0.050
C18:0	7.017 (1.736)	13.112 (1.890)	–13.008	**< 0.001**	**1.000**
trans-C18:1 *n*-7	0.117 (0.047)	0.284 (0.095)	–8.643	**< 0.001**	**1.000**
cis-C18:1 *n*-9	21.413 (3.222)	18.568 (2.275)	3.951	**< 0.001**	**0.977**
cis-C18:1 *n*-7	1.941 (0.324)	1.961 (0.330)	–0.243	0.809	0.050
all-*cis*-C18:2 *n*-6	29.434 (4.469)	20.131 (3.448)	9.028	**< 0.001**	**1.000**
all-*cis*-C18:3 *n*-6	0.342 (0.117)	0.145 (0.096)	7.125	**< 0.001**	**1.000**
all-*cis*-C18:3 *n*-3	0.750 (0.280)	0.908 (0.424)	–1.704	0.094	0.258
all-*cis*-C20:3 *n*-6	1.567 (0.527)	1.443 (0.426)	1.003	0.320	0.050
all-*cis*-C20:4 *n*-6	6.783 (1.087)	8.268 (1.592)	–4.218	**< 0.001**	**0.989**
all-*cis*-C20:5 *n*-3	0.086 (0.039)	0.473 (0.145)	–14.100	**< 0.001**	**1.000**
all-*cis*-C22:4 *n*-6	0.236 (0.046)	0.916 (0.323)	–11.443	**< 0.001**	**1.000**
cis-C24:1 *n*-9	0.904 (0.247)	0.549 (0.281)	5.203	**< 0.001**	**1.000**
all-*cis*-C22:5 *n*-6	0.188 (0.072)	0.353 (0.104)	–7.148	**< 0.001**	**1.000**
all-*cis*- C22:5 *n*-3	0.460 (0.115)	2.438 (0.584)	–19.350	**< 0.001**	**1.000**
all-*cis*-C22:6 *n*-3	2.995 (0.887)	1.939 (0.512)	5.649	**< 0.001**	**1.000**

Legend to Table II

[a]*SD* – standard deviation; [b]Unpaired *t*-test; Content of fatty acids in DBS and plasma is stated in percentages; *t* – *t*-statistic. The *t*-test statistic is the ratio between difference between the means of the two groups and standard error of the difference between the means. A large *t* indicates that the differences between two groups are statistically significant; *p* – *p*-value. The *p* value is the probability of being wrong in concluding that there is a true difference in the two groups. There are significant differences if $p < 0.050$; The power, or sensitivity, of a *t*-test is the probability that the test will detect a difference between the groups if there really is a difference; The closer the power is to 1, the more sensitive the test. Traditionally, the power of the performed test should be > 0.8.

Table III Regression data from the method comparison for DBS and plasma samples by GC-FID

FAs formula	*k*	*Q*	*R*	*F*	p^b	Power (1–ß)
C14:0	0.876	-0.033	0.819	57.047	**< 0.001**	**1.000**
13-Me-C14:0	–0.087	0.052	0.336	3.564	0.069	0.443
12-Me-C14:0	–0.025	0.020	0.294	2.644	0.115	0.349
C16:0	0.675	5.408	0.615	17.072	**< 0.001**	**0.962**
cis-C16:1 *n*-10	–0.035	0.499	0.268	2.167	0.152	0.297
cis-C16:1 *n*-7	1.539	0.132	0.954	282.742	**< 0.001**	**1.000**
14-Me-C16:0	0.505	0.035	0.149	0.632	0.433	0.119
16-Me-C17:0	0.457	0.083	0.468	7.838	0.009	0.750
C18:0	0.427	1.424	0.464	7.701	0.010	0.743
trans-C18:1 *n*-7	0.049	0.131	0.099	0.280	0.601	0.075
cis-C18:1 *n*-9	1.061	1.720	0.749	35.768	**< 0.001**	**0.999**
cis-C18:1 *n*-7	0.646	0.673	0.659	21.528	**< 0.001**	**0.984**
all-*cis*-C18:2 *n*-6	0.974	9.822	0.752	36.350	**< 0.001**	**0.999**
all-*cis*-C18:3 *n*-6	0.172	0.317	0.142	0.573	0.455	0.111
all-*cis*-C18:3 *n*-3	0.026	0.727	0.039	0.042	0.840	0.039
all-*cis*-C20:3 *n*-6	1.037	0.070	0.840	66.958	**< 0.001**	**1.000**
all-*cis*-C20:4 *n*-6	0.308	4.238	0.451	7.140	0.012	0.713
all-*cis*-C20:5 *n*-3	0.056	0.060	0.206	1.243	0.274	0.191
all-*cis*-C22:4 *n*-6	0.026	0.211	0.187	1.016	0.322	0.165
cis-C24:1 *n*-9	0.130	0.832	0.148	0.626	0.435	0.118
all-*cis*-C22:5 *n*-6	0.320	0.075	0.461	7.570	0.010	0.737

Table III – Continued

FAs formula	*k*	*Q*	*R*	*F*	*p*[b]	Power (1–ß)
all-*cis*- C22:5 *n*-3	0.086	0.250	0.409	5.638	0.025	0.618
all-*cis*-C22:6 *n*-3	0.874	1.301	0.504	9.552	**0.004**	**0.822**

x – DBS; *y* – plasma; *k* – intercept; *Q* – slope; *R* – the correlation coefficient. *R* value near 1 indicates that the straight line is a good description of the relation between the independent and dependent variable; *F* – *F*-statistic. The *F*-test statistic gauges the contribution of the independent variable in predicting the dependent variable. If *F* is a large number, it can be concluded that the independent variable contributes to the prediction of the dependent variable; *p* – *p*-value. The *p*-value is the probability of being wrong in concluding that there is an association between the dependent and independent variables. Traditionally, it can be concluded that the independent variable can be used to predict the dependent variable when $p < 0.050$. Power (1-β) is power of performed test. Traditionally, the power of the performed test should be > 0.8.

Table IV Comparison of the percentage content of FAs in DBS and plasma samples between men and women

FAs formula	Women (*n* = 19) Median (*IQR*)[a]	Men (*n* = 11) Median (*IQR*)[a]	*p*[b]
BMI	23.12 (6.56)	24.26 (3.18)	0.22
Age	24.00 (2.75)	24.00 (5.25)	0.64
P-C14:0	0.93 (0.49)	0.99 (0.34)	0.90
P-13-Me-C14:0	0.03 (0.02)	0.02 (0.01)	**0.05**
P-12-Me-C14:0	0.02 (0.01)	0.01 (0.01)	**0.03**
P-C16:0	22.01 (2.72)	20.54 (2.52)	**0.03**
P-*cis*-C16:1 *n*-10	0.47 (0.07)	0.49 (0.12)	0.58
P-*cis*-C16:1 *n*-7	2.05 (0.58)	1.50 (0.64)	**0.01**
P-14-Me-C16:0	0.09 (0.06)	0.07 (0.04)	0.07
P-16-Me-C17:0	0.17 (0.03)	0.14 (0.04)	**0.04**
P-C18:0	7.20 (2.22)	6.42 (1.17)	0.37
P-*trans*-C18:1 *n*-7	0.10 (0.06)	0.10 (0.05)	0.70
P-*cis*-C18:1 *n*-9	20.15 (4.57)	22.65 (4.93)	0.07
P-*cis*-C18:1 *n*-7	1.94 (0.49)	1.90 (0.31)	0.55
P-all-*cis*-C18:2 *n*-6	29.55 (6.29)	30.02 (6.25)	0.30
P-all-*cis*-C18:3 *n*-6	0.31 (0.09)	0.33 (0.13)	0.18

Table IV – Continued

FAs formula	Women (n = 19) Median (IQR)[a]	Men (n = 11) Median (IQR)[a]	p[b]
P-all-*cis*-C18:3 *n*-3	0.69 (0.34)	0.89 (0.50)	0.16
P-all-*cis*-C20:3 *n*-6	1.63 (0.51)	1.07 (0.75)	**0.01**
P-all-*cis*-C20:4 *n*-6	6.71 (0.83)	6.66 (1.44)	0.55
P-all-*cis*-C20:5 *n*-3	0.09 (0.02)	0.06 (0.05)	**0.03**
P-all-*cis*-C22:4 *n*-6	0.23 (0.07)	0.22 (0.06)	0.52
P-*cis*-C24:1 *n*-9	0.94 (0.30)	0.91 (0.36)	0.52
P-all-*cis*-C22:5 *n*-6	0.19 (0.07)	0.14 (0.08)	**0.01**
P-all-*cis*-C22:5 *n*-3	0.46 (0.17)	0.48 (0.13)	0.13
P-all-*cis*-C22:6 *n*-3	2.64 (1.65)	2.64 (1.42)	0.32
D-C14:0	1.17 (0.52)	1.19 (0.58)	0.83
D-13-Me-C14:0	0.17 (0.21)	0.22 (0.11)	0.30
D-12-Me-C14:0	0.07 (0.07)	0.18 (0.22)	0.08
D-C16:0	24.84 (2.30)	23.29 (1.08)	**0.01**
D-*cis*-C16:1 *n*-10	0.59 (0.82)	1.09 (0.61)	0.44
D-*cis*-C16:1 *n*-7	1.21 (0.39)	0.80 (0.33)	**0.00**
D-14-Me-C16:0	0.16 (0.04)	0.12 (0.08)	**0.01**
D-16-Me-C17:0	0.15 (0.05)	0.12 (0.03)	**0.03**
D-C18:0	13.13 (1.30)	13.48 (1.15)	0.44
D-*trans*-C18:1 *n*-7	0.27 (0.15)	0.29 (0.14)	1.00
D-*cis*-C18:1 *n*-9	18.39 (3.37)	18.69 (2.75)	0.55
D-*cis*-C18:1 *n*-7	1.95 (0.47)	1.85 (0.37)	0.61
D-all-*cis*-C18:2 *n*-6	19.94 (4.43)	20.01 (1.55)	0.86
D-all-*cis*-C18:3 *n*-6	0.11 (0.06)	0.12 (0.07)	0.41
D-all-*cis*-C18:3 *n*-3	0.68 (0.50)	0.98 (0.42)	0.06
D-all-*cis*-C20:3 *n*-6	1.54 (0.31)	1.11 (0.67)	0.07
D-all-*cis*-C20:4 *n*-6	8.33 (1.35)	8.88 (1.56)	0.64
D-all-*cis*-C20:5 *n*-3	0.44 (0.25)	0.46 (0.13)	1.00

Table IV – Continued

FAs formula	Women (n = 19) Median (IQR)[a]	Men (n = 11) Median (IQR)[a]	p[b]
D-all-*cis*-C22:4 *n*-6	0.80 (0.26)	0.98 (0.61)	0.20
D-*cis*-C24:1 *n*-9	0.38 (0.29)	0.61 (0.37)	0.28
D-all-*cis*-C22:5 *n*-6	0.36 (0.14)	0.34 (0.19)	0.70
D-all-*cis*- C22:5 *n*-3	2.46 (0.73)	2.81 (0.85)	0.18
D-all-*cis*-C22:6 *n*-3	1.98 (0.66)	1.91 (0.91)	0.61

[a]*IQR* – interquartile range is the difference between the upper quartile and the lower quartile; [b]Mann–Whitney rank-sum test; Body mass index (BMI) calculated as body mass (kg) divided by height (m) squared; P – plasma; D – DBS; Content of fatty acids in DBS and plasma is stated in percentages.

Conclusion

Currently, there is a great interest in simple and noninvasive methods of sample collection. The sampling of biological material on the special filter paper is one of possible choices. Many studies have focused on the determination of analytes from samples in the dried form [13,17,18,25-28]. The DBS sampling technique has extended from the NS programme thanks to many advantages, such as stability, easy storage, simple shipment to laboratories, and no biohazard. This sample collection is much less invasive and the reason of why a DBS sample could be used for preliminary detection of desired analytes in patients. Despite the small volume of samples tested, more than 20 FAs could be successfully determined.

The aim of this study was to optimise the method of DBS-sample processing and to compare the percentage content of FAs between the DBS and plasma samples (and, in this case, to find the respective correlations). The FAs composition of plasma seems to be different from that of the DBS sample. One possible explanation is the different characterisation of both samples: the DBS is prepared from whole blood and, hence, it contains not only plasma FAs, but also FAs from erythrocytes membranes. Since the number of processed samples in the entire study was limited, the results should be interpreted with caution. In this area, a further research is necessary with a larger group of samples, and also, with larger variety of sample types; e.g., whole blood, DBS and plasma.

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