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A method for long term stabilisation of long chain polyunsaturated fatty acids in dried blood spots and its clinical application

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Abstract

Conventional assays of omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) status in humans involve venous blood collection and expensive, multi-step processes that limit their usefulness as screening tools. This study aimed to develop a capillary dried blood spot (DBS) system capable of protecting n-3 LCPUFA from oxidation for up to 2 months at room temperature (20-25°C). We demonstrated that a DBS system comprising both an antioxidant and chelating agent on silica-gel coated paper prevented any significant change in the n-3 LCPUFA profile after 2 months. Our DBS assay was then tested in fifty subjects, and this demonstrated the presence of strong and significant correlations between the results obtained from the DBS system and those obtained from conventional measures for all fatty acids, in particular the n-3 LCPUFA EPA and DHA ($r>0.96$, $P<0.0001$). This study therefore validates our DBS system as a reliable method for the assessment of n-3 LCPUFA status in humans.

Keywords:

LCPUFA, dried blood spot, oxidation, fatty acids, chelating agent, silica gel coated paper

Introduction

Increased consumption of omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) and the concomitant increase in n-3 LCPUFA concentrations in the circulation has been associated with a number of health benefits in humans, including prevention of preterm birth [1], primary and secondary protection against cardiovascular disease [2], and strengthened immune defences [3].

As a result of these health benefits, there is a growing interest in assessing the relationship between dietary n-3 fatty acid intake and LCPUFA status in human populations. There is particular interest in the bioactive n-3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the sum of which, termed the omega-3 index, has been proposed as a measure of risk for death from coronary heart disease [4, 5]. However, conventional approaches to assaying fatty acids in blood involve venous blood collection and an expensive, time consuming multi-step process that limit their usefulness as a screening tool.

Attempts have been made to adopt the dried blood spot (DBS) technique as a quick, inexpensive and minimally invasive tool for measuring fatty acid status in humans [6-9]. Nishio *et al.* described a rapid fatty acid assay using a DBS absorbed on filter paper to diagnose adrenoleukodystrophy, an X-linked metabolic disorder [6]. However, their study only focused on the levels of specific saturated fatty acid (SFA) biomarkers of adrenoleukodystrophy, including C22:0, C24:0 and C26:0 which are inherently stable in air [6]. Ichihara *et al.* described a method which utilised filter paper impregnated with the antioxidant, butylated hydroxytoluene (BHT), to collect blood or milk spots for the analysis of C_{20}-C_{22} LCPUFA status [7]. However, the alkaline methylation conditions used in their study lacked the ability to transesterify free fatty acids, steryl esters and sphingolipids, limiting the usefulness of the method [7]. A widely quoted study by Marangoni *et al.* described an assay for rapid profiling of the fatty acids in whole blood using a filter paper based DBS [8], and the authors claimed that this method was sufficiently sensitive to detect changes in blood fatty acid levels due to various lifestyle and dietary factors [9]. However, the DHA values obtained using their DBS method differed from those obtained using a conventional whole blood fatty acid assay [8]. In addition, the potential fatty acid contaminants released from the collection papers [10], and evidence of substantial oxidative losses

of LCPUFA in DBS samples collected in this manner when stored in air [11] has led to concerns about the reliability of the method. Due to the high sensitivity of LCPUFA to oxidation, previous studies have taken the approach of processing the DBS samples immediately after blood collection [12, 13], treating them with high levels of BHT [14] or storing them at -20°C to stabilise the LCPUFA [15]. However, none of these approaches fulfils the criteria for them to be considered as a stable and convenient clinical assay for large population studies, clinical trials or for pathology services.

The objective of this study was to develop a DBS method that would allow samples to be stored at room temperature for at least 2 months without significant change in the LCPUFA profile and to validate the clinical application of our DBS method through a direct comparison with established methods.

Materials and Methods

Subjects

Ethical approval for the collection of blood and body fluids for this study was obtained from The University of Adelaide Human Research Ethics Committee. All volunteers were fully informed of the nature of the study and provided written informed consent prior to blood collection. We undertook the initial development of our method using the blood of the donor who had consumed 15ml of MaxEPA fish oil daily for several years and had a very high blood content of EPA and DHA (each ~7% of the total fatty acids), since it was reasoned that blood from such a donor would provide the greatest opportunity of detecting even small losses in n-3 LCPUFA if they occurred. The final DBS method was evaluated in 50 subjects to establish the relationship between the fatty acid status obtained from our DBS method and traditional methods used to measure fatty acids in different blood fractions. In this study, blood samples were collected at the same clinic appointment both using the DBS system and through the antecubital vein from each of the 50 subjects (26 females and 24 males) aged between 22 and 71 years. The participants included 23 people who reported consuming relatively high amounts of fish oil supplements (≥3 gram per day), 14 people who reported seldom consuming any fish oil supplements (<1 gram per week) but had a regular consumption of fish (≥1 serving per week), and 13 people

who reported seldom consuming any fish (<1 serving per week) or fish oil supplements (<1 gram per week) (Table 1). The DBS method was further applied to plasma and breast milk samples donated by one healthy volunteer aged 33.

Development of DBS method

Blood collection papers

Four types of papers were used for blood collection: Fluka blood collection paper (Sigma-Aldrich, Buchs, Switzerland), Whatman 903 specimen collection paper, Whatman 3MM chromatography paper, and Whatman ion exchange paper (46x57cm, Whatman, Buckingham, UK). The Fluka blood collection paper is currently part of a kit marketed by Sigma-Aldrich for the evaluation of n-3 and n-6 PUFA status in blood, thus, it was used as an industry standard to compare with the other papers.

Protectants

Two phenolic antioxidants, BHT, and tertiary butylhydroquinone (TBHQ) (Sigma-Aldrich, St Louis, MO) at three concentrations (0.5mg/ml, 2mg/ml and 4mg/ml), and three chelating agents, L-ascorbic acid, citric acid, and ethylenediaminetetraacetic acid (EDTA) (Chem-supply, Gillman, Australia) at three concentrations (1mg/ml, 5mg/ml and 20mg/ml) were tested separately or in combination in order to determine the optimal protectant concentration to limit lipid oxidation in DBS. 50µl of protectant solution was spread evenly onto the collection area (~1.5x1.5cm) of strips (~1.5x3cm) of each collection paper and air dried prior to collection of blood onto the paper.

Fatty acids transesterification

A baseline measurement for fatty acid status in whole blood was carried out using a modified direct transesterification method (16). Briefly, 50µl of fresh blood was added to 2ml of 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex, UK) in anhydrous methanol (Merck, Darmstadt, Germany) in a 5ml sealed vial (Wheaton, Millville, USA), and heated at 70°C for 3 hrs. The resultant fatty acid methyl esters (FAME) were extracted into heptanes (Merck, Darmstadt, Germany). All the samples were processed in triplicate.

Blood spot samples were obtained by absorbing a drop of fresh blood (~50 µl) onto the collection area (~1.5x1.5cm) of blood collection paper strips in the absence or presence of protectants, and all blood spots were dried in air at room temperature for 5 hrs prior to processing. The whole dried blood spot was directly transmethylated following the same procedure as for the fresh blood either immediately after air drying, or after 1 week, 2 weeks, 3 weeks, 4 weeks or 9 weeks of storage at room temperature. In all experiments, the dried blood/plasma/breast milk spot samples were stored in cellophane bags in the dark and in the presence of desiccants. All samples were processed in triplicate.

Clinical validation of the DBS method

Blood and body fluids collection

For the capillary samples, a drop of blood (~30µl) was collected from the thumb of each subject using an automatic lancing device (Unistick 2, Owen Mumford, UK). The blood was spotted onto the collection area of our blood collection paper, and air dried for 5 hrs at room temperature prior to fatty acid analysis.

For the venous blood samples, ~6 ml of blood were drawn from an antecubital vein of the forearm into Vacutainer tubes containing heparin as an anticoagulant (Vacutte, Greiner Bio One, Austria). 500µl of venous whole blood was retained for total lipid extraction. The remainder of the venous whole blood sample (~5 ml) was centrifuged at 3200 rpm for 15 mins at 4°C to separate plasma and erythrocytes. The erythrocytes were then washed three times with 0.9% sodium chloride, and the buffy coat removed. Whole blood, plasma, and erythrocytes were stored at 4°C, and processed for fatty acid composition within 24 hrs of blood collection.

Fatty acid analysis of DBS samples

The transesterification of lipids in DBS samples was carried out using the method described in the DBS method development section above.

Conventional fatty acid analysis of blood fractions

Total lipids from whole blood, plasma, and erythrocytes were extracted using a modified Folch method [16]. Briefly, total lipids from whole blood, plasma, and

erythrocytes were extracted by chloroform/methanol (2:1, v/v). After addition of the chloroform/methanol, the sample was shaken vigorously and allowed to stand at room temperature for 5 mins. The sample was then centrifuged at 3000 rpm for 10 mins to separate the aqueous and organic phase. The organic layer which contained the total lipids extract was transferred into a scintillation vial (Wheaton, USA) and evaporated to dryness under a steady nitrogen stream.

The phospholipid fraction of plasma and erythrocytes was separated from total lipids by one dimensional thin layer chromatography [17] using Silica gel 60H plates (Merck, Darmstadt, Germany). A petroleum spirit/acetone (3:1, v/v) solution was used as the mobile phase for phospholipid separation. The phospholipid band was visualized under ultraviolet light and scraped into scintillation vials for fatty acid transesterification. FAME were prepared from whole blood total lipids, plasma total lipids, plasma phospholipids, and erythrocyte phospholipids using the same transesterification method as described above for DBS samples. The resultant FAME were extracted into heptane, and injected into a Gas chromatography for analysis.

Gas Chromatography Analysis

FAME were separated and quantified using a Gas Chromatograph (Hewlett-Packard 6890; Palo Alto, CA, USA) equipped with a BPX70 capillary column 50m x 0.32mm, film thickness 0.25µm (SGC Pty Ltd., Victoria, Australia), programmed temperature vaporization injector and a flame ionisation detector (FID). The injector temperature was set at 250°C and the FID temperature at 300°C, a programmed temperature ramp (140-240°C) was used. Helium gas was utilised as a carrier at a flow rate of 35 cm per second in the column and the inlet split ratio was set at 20:1. The identification and quantification of FAME was achieved by comparing the retention times and peak area values of unknown samples to those of commercial lipid standards (Nu-Chek Prep Inc., Elysian, MN, USA) using the Hewlett-Packard Chemstation data system [17].

Statistical Analyses

All statistical analyses were conducted using PASW Statistic 18. Values for individual fatty acids are expressed as a percentage of total blood fatty acids, and presented as mean ± standard deviation (SD). The main effects and interactions of paper type, protectant formulation, and storage periods in relation to the changes in

fatty acid composition were determined using three-way ANOVA. Where significant interactions between protectant formulations and paper type over time were determined by multi-factorial ANOVA, significant differences in fatty acids percentage between protectant formulations within each paper type was determined at each storage period by one-way ANOVA with comparison of individual means by Tukey's post-hoc test. Due to the number of statistical comparisons made, $p<0.01$ was chosen as the level of statistical significance. Spearman's rank correlation coefficient was calculated to determine the strength of the association between capillary DBS fatty acid values and the corresponding fatty acid values in various lipid fractions from venous blood.

Results

Stability of DBS absorbed on different types of collection papers

Regardless of the collection paper used, significant decreases in percentages of arachidonic acid (AA), EPA and DHA in DBS samples were observed over 4 weeks of storage at room temperature, despite the fact that the papers had been impregnated with BHT (Figure 1). There was no significant difference in the fatty acid composition of the DBS between samples collected on Fluka blood collection paper, Whatman 3MM paper, and Whatman 903 paper at any time point over the storage period. However, the DBS samples collected on Whatman ion exchange paper exhibited a lower decline in the levels of all LCPUFA when compared to those DBS collected on other types of papers following 4 weeks of storage at room temperature (Figure 1).

Stability of n-3 LCPUFA in DBS treated with a single antioxidant or a single chelating agent

Blood spots collected on Fluka blood collection paper or Whatman ion exchange paper in the absence or presence of a single antioxidant or a single chelating agent were analysed for fatty acid status either immediately after air drying (5 hrs), or after 2 weeks or 4 weeks storage at room temperature in cellophane bags as previously described.

The results are expressed as the sum of EPA+DHA level in total blood fatty acids, as these n-3 LCPUFA showed the greatest sensitivity to oxidation, and the sum of EPA+DHA, termed the omega-3 index, has been proposed as a measure of risk for death from coronary heart disease [4]. DBS collected on Whatman ion exchange papers consistently exhibited higher residual levels of n-3 LCPUFA when compared with the DBS samples collected on Fluka papers irrespective of the protectant formulations used (Figures 2-3).

Equivalent concentrations of BHT and TBHQ showed similar abilities to retard the decline of n-3 LCPUFA levels in DBS collected on Fluka paper over the 4 weeks of storage (Figure 2). However, the DBS collected on BHT impregnated Whatman ion exchange paper retained a higher level of n-3 LCPUFA than those collected on TBHQ impregnated Whatman ion exchange paper at all three antioxidant concentrations (Figure 2). BHT and TBHQ at a concentration of 2mg/ml exhibited a significantly improved capacity for protection of n-3 LCPUFA levels over the 4 weeks of storage than those samples collected on papers impregnated with 0.5mg/ml of the same antioxidant (Figure 2). Further increasing the BHT or TBHQ concentration to 4mg/ml did not result in any further improvements in the level of n-3 LCPUFA retained in DBS after storage (Figure 2).

In order to protect against potential oxidative effects of free iron from red blood cells we tested a range of chelating agents. Regardless of the type of chelating agent used, the blood samples collected on papers impregnated with either of the chelating agents at a concentration of 5mg/ml showed significantly higher residual n-3 LCPUFA content than the samples collected on papers impregnated with 1mg/ml of the chelating agent after 4 weeks of storage (Figure 3). Further increasing the chelating agent concentration to 20mg/ml did not result in any further improvements in the level of n-3 LCPUFA retained in DBS after storage (Figure 3).

As a result, BHT at a concentration of 2mg/ml and chelating agent at a concentration of 5mg/ml were chosen as the optimal protectant concentrations for the subsequent experiments.

Stability of DBS treated with a mixture of an antioxidant and a chelating agent over time

Blood spots collected on Fluka blood collection paper and Whatman ion exchange paper in the absence or presence of BHT, or a mixture of BHT and a chelating agent (Table 2) were analysed for fatty acid status either immediately after air drying (5 hrs), or after 2 weeks, 4 weeks or 9 weeks storage at room temperature in cellophane bags as previously described.

Even after 5 hrs of air drying at room temperature, there was a significant reduction in the levels of all n-3 LCPUFA in the DBS collected on both types of collection papers which had not been treated with any protectant compared to those measured when whole blood was directly transmethylated before drying (Table 2). Significant declines in the levels of EPA and DHA were also detected in DBS samples which were collected on either of the collection papers treated with BHT alone when compared with those measured from fresh blood by direct transesterification (Table 2). However, the fatty acid levels measured in DBS collected on papers impregnated with a mixture of BHT and either of the chelating agents (L-ascorbic acid, citric acid or EDTA) were not significantly different from the results obtained from fresh blood, irrespective of the type of collection paper used (Table 2).

During storage, there was a significant interaction between paper type and protectant formulation in relation to fatty acid composition, such that protectant formulations on different types of paper showed different stabilising ability. DBS collected on Whatman ion exchange paper consistently exhibited a fatty acid composition that was more similar to that in fresh blood when compared with DBS collected on Fluka paper, irrespective of the protectant formulation used or the storage period (Figure 4).

After 9 weeks of storage at room temperature, irrespective of the protectant formulation used, the DBS samples collected on Fluka paper showed significant losses in the level of all LCPUFA (Table 3). The levels of n-3 LCPUFA in DBS collected on Fluka paper were significantly lower than the corresponding baseline values obtained from fresh blood as early as 2 weeks after collection, and continued to decline over the 9 week storage period (Figure 4a).

Different protectant formulations showed different stabilising ability in regard to DBS collected on Whatman ion exchange paper. The blood spot samples treated with 2mg/ml BHT alone exhibited significant declines in the level of EPA+DHA after only 2 weeks of storage when compared with the baseline values obtained from fresh blood (Figure 4b). However, when 5mg/ml of either of the chelating agents (L-ascorbic acid, citric acid or EDTA) was added to the BHT impregnated Whatman ion exchange paper, the levels of all LCPUFA in the DBS samples did not differ significantly from the baseline values even after 9 weeks of storage at room temperature (Table 3).

Clinical Validation of our DBS method

There were no significant differences in the mean levels of any of the major fatty acids of interest between the capillary DBS and venous whole blood total lipids (Table 4) confirming that capillary DBS was representative of venous blood in relation to fatty acid composition. As expected, there were differences in the levels of most fatty acids between the whole blood sample (capillary DBS and venous whole blood total lipids) and those in the blood fractions (plasma total lipids, plasma phospholipids, and erythrocyte phospholipids). The only exception was EPA, which did not exhibit any significant differences between different sample types (Table 4).

The fatty acid results from capillary DBS were strongly and significantly correlated with those obtained using conventional measurements (Table 5). Of all the fatty acids evaluated, the n-3 LCPUFA (EPA, DPA and DHA) in capillary DBS samples exhibited the strongest correlation with the corresponding fatty acids from each of the respective blood fractions measured by conventional assays (Table 5). There was a particularly tight relationship between EPA (r = 0.998) and DHA (r = 0.996) levels measured using the capillary DBS assay and conventional assay of venous whole blood (Table 5, Figure 5).

Correlations of n-3 biomarkers, including the omega-3 Index (sum of EPA+DHA% in erythrocyte phospholipids), AA/EPA ratio, and n-6/n-3 ratio were also compared between capillary DBS and whole blood total lipids, plasma total lipids, plasma phospholipids, and erythrocyte phospholipids. In general, values for n-3 biomarkers obtained from capillary DBS were strongly correlated with those from conventional

measurements (r >0.9) (Table 5). Furthermore, the EPA+DHA content in the capillary DBS was significantly correlated (r = 0.972) with the omega-3 index which is an accepted biomarker for the risk of coronary heart disease [4] (Figure 5).

Stability of fatty acids in other bio-fluids collected as dried spots

50µl of plasma or breast milk was collected using the Fluka collection kit (Fluka paper + 0.5mg/ml BHT) and our newly developed system (Whatman ion exchange paper + 2mg/ml BHT+ 5mg/ml EDTA). Plasma spots and breast milk spots were analysed for fatty acid status following the same procedure as for the DBS either immediately after air drying (5 hrs), or after 1 week, 2 weeks or 4 weeks storage at room temperature in cellophane bags as previously described.

The plasma spots collected on Fluka blood collection kit showed significant losses of EPA and DHA after 2 weeks of storage at room temperature (Figure 6a), and after 4 weeks of storage there was an ~20% reduction of all n-3 LCPUFA levels in the plasma collected on commercial collection paper when compared with direct transesterification of 50 µl fresh plasma (Table 3). However, there was no significant change in the fatty acid composition of plasma spots collected using our protection system over 4 weeks of storage at room temperature (Table 3).

In relation to the breast milk spots, irrespective of the protection system used, there was no significant difference in fatty acid composition in breast milk spots over 4 weeks of storage at room temperature (Figure 6b, Table 4).

Discussion and conclusions

DBS technology is increasingly being used in a variety of applications including newborn screening, epidemiological studies, and therapeutic drug monitoring [18-20]. The potential usefulness of DBS lies in the ability to undertake analyses using a small volume of capillary blood taken by auto-lancets rather than syringes, and ease of storage and transport. The system is reliant on the fact that the compounds to be measured are either completely stable on the collection paper or break down to a stable metabolite that can be readily measured. Previously reported DBS assays for the evaluation of LCPUFA status of individuals [7, 8] have been found to lack efficiency in stabilising LCPUFA [11, 15]. This is almost certainly due to the fact that

spotting blood on filter paper naturally exposes the LCPUFA in blood to the air, resulting in oxidation of the LCPUFA, and this process can be enhanced by the liberation of iron contained in blood haemoglobin during air drying [21, 22].

Many of the reports claiming stable DBS methodology in profiling fatty acids are flawed by the use of inappropriate controls or using bloods with low levels of n-3 LCPUFA so that small changes would not have been detected [7, 8, 11, 14, 15]. Some studies [7, 11, 15] have taken as their zero time point a sample of blood that has been recently spotted onto filter paper and dried in air, a process we have shown here to result in significant losses in n-3 LCPUFA even in the presence of an antioxidant (Table 1). Two studies [8, 14] claimed that their baseline blood spot samples were processed immediately at time zero, however, the actual air drying times were not indicated or controlled for. It is important to emphasise that our study used the fatty acid values from direct transesterification of 50µl of whole blood (that is, blood added directly to a tube containing transesterification reagent) immediately after phlebotomy as a baseline control for all of our studies, a process that ensures an accurate measure of original blood levels of n-3 LCPUFA [23]. Furthermore, we chose to conduct many of our experiments on blood from an individual who was a long time consumer of high dose fish oil to deliberately subject our methodology to the magnification of having high blood levels (>15%) of n-3 LCPUFA. This is contrast to other reports [11, 14] where the blood n-3 LCPUFA levels are only ~5% of the total fatty acids, such that small decreases in n-3 LCPUFA would be hard to detect.

Our study revealed that a combination of three components are necessary to ensure the stability of LCPUFA in blood spot samples during air drying and long term storage at room temperature: an antioxidant (BHT), a chelating agent (EDTA) and silica gel coated paper (Whatman ion exchange paper). This is consistent with previous studies which have shown that protection systems consisting of both antioxidant and chelating agents performed much better than systems which use antioxidant alone for protecting PUFA in avocado [24] and fish oil [25], and also vitamin A palmitate in ultra rice [26] from oxidation during storage. The mechanisms involved by which all three components achieve optimal stability of LCPUFA can be explained by the respective actions of these three components.

Antioxidants are frequently used in inhibiting the oxidation of lipids because they compete with lipids for oxidation, and stop the propagation of free radicals by undergoing oxidation to produce stable and relatively unreactive antioxidant radicals [27]. Fatty acids bind strongly to silica gel particles aiding their stability in chromatographic situations. Studies have shown that lipid oxidation rates are reduced with decreasing water activity on the surface of lipids [28, 29]. Thus, silica gel coated paper may diminish the lipid oxidation rate by reducing the water activity close to the water monolayer on the surface of dried blood spot [30].

The use of a chelating agent in our study was not accidental, but based on the hypothesis that by binding the iron ion in blood with chelators, it would be possible to greatly retard iron-mediated LCPUFA losses from DBS samples. This hypothesis is based on the known role of iron ions as oxidisers, and the fact that the liberation of iron from haemoglobin during air drying [31] enables the rapid catalysis of degradation of unsaturated lipids either by direct initiation and formation of lipid peroxyl and alkyl radicals, or by inducing the production of hydroxyl radicals through a Fenton reaction [32, 33]. The key property shared by all chelating agents is that they are able to bind iron ions very tightly, thereby rendering them chemically inert [33]. A study of lipid peroxidation in heart cells showed that iron loading led to significant reductions in the levels of LCPUFA in liposomal vesicles [34]. These changes were identical to those reported in previous studies of the fatty acid composition of RBC membrane lipids in beta-thalassemia, a disease in which iron overload is frequently observed in the blood [35].

The fatty acid levels measured using our novel DBS method in 50 subjects consuming a range of omega 3 fats were closely related not only to levels measured in whole blood, but also to levels in all blood fractions that were assessed. This is not unexpected given previous reports of strong correlations of individual fatty acid levels between blood fractions [22, 24], which is thought to be due to the exchange of fatty acids in phospholipids between plasma and other cell types, in particular the erythrocytes [25]. We found that these relationships were particularly strong for EPA and DHA ($r > 0.95$), which are considered to be the most reliable indicators of n-3 LCPUFA status in relation to disease risks [36]. In addition, established biomarkers of disease risk, including the omega-3 index and the AA/EPA ratio, obtained using our

DBS system were also closely correlated with those obtained using conventional measures. Whilst previous DBS studies [9, 10, 17] have reported strong correlations between the fatty acid levels obtained from the capillary DBS and conventional blood fractions, these studies were all conducted in populations with a relatively narrow range of whole blood n-3 LCPUFA content (EPA+DHA%, from 2.0 to 6.0). The present study demonstrates that these relationships extend to a much wider range of whole blood n-3 LCPUFA content (EPA+DHA%, from 2.0 to 20.0). The close relationships identified between our DBS measures of the n-3 LCPUFA and the conventional measures therefore validates the suitability of our capillary DBS assay for the measurement of fatty acid status in subjects with a wide range of dietary n-3 fatty acid intakes. Furthermore, these relationships make it possible to develop mathematical equations for converting the n-3 results obtained from our DBS system to equivalent results for conventional blood fractions in the same subject. This will enable researchers to directly compare the n-3 fatty acid status obtained from capillary DBS with those of fractions of blood separated by conventional means, and thus to relate the DBS results to n-3 fatty acid levels reported in previous studies.

We also compared the stability of LCPUFA in plasma and in breast milk collected using our protection system with those collected using the commercially available Fluka collection kit. The plasma spots collected using the commercial test kit showed a ~20% loss of n-3 LCPUFA after 4 weeks of storage at room temperature, whereas the plasma spots collected using our system were stable over the same period. On the other hand breast milk was stable in both collection systems. This could be explained by the fact that plasma (1~2mg/L) [37] has a higher iron content than breast milk (~0.35mg/L) [38]. However, it is important to note that the LCPUFA in both plasma spots and breast milk spots collected using our protection system remained stable over 4 weeks of storage at room temperature (Tables 3, 4). This indicates that our protection system may be suitable for protecting the LCPUFA in variety of bio-fluids, including plasma, breast milk, and possibly saliva.

In conclusion, we have developed a three component protection system comprising an antioxidant, a chelating agent, and silica gel coated paper which can prevent LCPUFA in blood spots from significant oxidative loss for at least 2 months when stored at room temperature. This system has the potential to allow accurate evaluation of fatty

acid status in DBS samples after long term storage and thus enable comparisons between samples which have been stored for different periods of time. The DBS test offers significant advantages over conventional methods for assessing fatty acid status in large clinical trials and population screening programs. It is because the capillary DBS assay does not necessitate the involvement of professional operators in blood sampling, and considerably reduces the analytical time and cost when compared with conventional blood fatty acid assays. Furthermore, since the LCPUFA in DBS are stable at room temperature, the use of this system elimates the cost and logistical problems involved in transporting samples that need refrigeration or freezing between clinical sites and diagnostic laboratories. The clinical data presented in this study further validate our DBS methodology by demonstrating that the results obtained using this approach can be directly related to those obtained by conventional methods. Our DBS method thus has significant potential for the application in large-scale clinical or epidemiological studies focused on the role of n-3 fatty acids consumption in human health.

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Conflict of interest statement

The authors have no conflicts of interest to this study.

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Figure legends

Figure 1. Stability of LCPUFA in blood spots on 4 types of collection papers impregnated with 0.5mg/ml BHT over 4 weeks storage at room temperature. Values are presented as mean ± SD (n=3 per time point), different superscripts indicate significant differences between treatments, $p<0.01$. Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time zero.

Figure 2. EPA+DHA% of DBS on Fluka paper or Whatman ion exchange paper in the presence or absence of single antioxidant determined after 4 weeks storage at room temperature. Values are presented as mean ± SD (n=3 per time point), different superscripts indicate significant differences between treatments, $p<0.01$. Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time zero.

Figure 3. EPA+DHA% of DBS on Fluka paper or Whatman ion exchange paper in the presence or absence of single chelating agent determined after 4 weeks storage at room temperature. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $p<0.01$. Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time zero.

Figure 4. EPA+DHA% in DBS on Fluka paper (a) or Whatman ion exchange paper (b) determined over 9 weeks storage at room temperature. Values are presented as mean ± SD (n=3). Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time zero. Data obtained from table 1 and 2.

Figure 5. Correlations for EPA+DHA content between capillary DBS and whole blood total lipids (A), plasma total lipids (B), plasma phospholipids (C), erythrocyte phospholipids (D). TLE, total lipid extract; PL, phospholipids

Figure 6. EPA+DHA% in plasma spots (a) or breast milk spots (b) collected using Fluka collection kit or new protection system determined over 4 weeks storage at

room temperatures. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $p<0.01$. Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl of plasma or breast milk at time zero.

Tables

	Number	Age (mean and range)	Gender (M/F)
High n-3 intake (≥3 grams of fish oil/week)	23	56 (24-75)	14/9
Moderate n-3 intake (Seldom fish oil consumption, but ≥1 serving of fish/week)	14	42 (23-59)	5/9
Low n-3 intake (Seldom fish oil consumption, and<1 serving of fish/week)	13	35 (23-54)	5/8

Table 1. Summary of participant characteristics

Table 2. Fatty acid composition of blood spots on two types of collection paper impregnated with different protectants after 5 hr air drying at room temperature

Fatty acids[1]	Baseline[2]	Whatman ion exchange paper					Fluka blood collection paper				
		No Protectant	BHT*	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA
16:0	23.6±0.3^{a}	24.4±0.3^{ab}	24.3±0.2^{ab}	23.7±0.2^{a}	23.7±0.2^{a}	23.6±0.2^{a}	25.0±0.3^{b}	24.2±0.3^{ab}	23.8±0.2^{a}	23.8±0.3^{a}	23.8±0.2^{a}
18:0	13.4±0.1^{a}	14.0±0.2^{b}	13.9±0.2^{ab}	13.4±0.1^{a}	13.4±0.1^{a}	13.4±0.2^{a}	14.4±0.1^{b}	13.8±0.2^{ab}	13.5±0.1^{a}	13.4±0.1^{a}	13.5±0.1^{a}
22:0	0.4±0.01^{b}	0.4±0.02^{b}	0.4±0.02^{b}	0.4±0.01^{b}	0.4±0.01^{b}	0.4±0.01^{b}	0.5±0.02^{a}	0.4±0.01^{b}	0.4±0.02^{b}	0.4±0.01^{b}	0.4±0.01^{b}
24:0	0.6±0.02^{b}	0.7±0.02^{a}	0.6±0.01^{b}	0.6±0.02^{b}	0.6±0.02^{b}	0.6±0.01^{b}	0.6±0.02^{b}	0.7±0.02^{a}	0.6±0.02^{b}	0.6±0.01^{b}	0.6±0.02^{b}
16:1 n-7	1.4±0.02	1.4±0.02	1.4±0.02	1.4±0.02	1.4±0.02	1.4±0.02	1.5±0.02	1.4±0.02	1.4±0.02	1.4±0.02	1.4±0.02
18:1 n-7	1.5±0.03	1.5±0.02	1.5±0.02	1.5±0.02	1.5±0.02	1.5±0.02	1.6±0.02	1.5±0.02	1.5±0.02	1.5±0.02	1.5±0.02
18:1 n-9	18.6±0.2^{a}	19.1±0.1^{ab}	19.0±0.2^{ab}	18.5±0.1^{a}	18.5±0.1^{a}	18.5±0.1^{a}	19.4±0.2^{b}	19.0±0.1^{ab}	18.5±0.1^{a}	18.5±0.1^{a}	18.6±0.2^{a}
24:1 n-9	0.8±0.02	0.8±0.01	0.8±0.01	0.8±0.01	0.8±0.01	0.8±0.01	0.8±0.02	0.8±0.02	0.8±0.01	0.8±0.01	0.8±0.02
18:2 n-6	19.8±0.2^{a}	19.7±0.2	19.8±0.1	19.9±0.2	19.7±0.2	19.9±0.1	19.5±0.1	19.8±0.1	19.8±0.2	19.8±0.2	19.8±0.2
20:3 n-6	1.2±0.02^{a}	1.2±0.01^{a}	1.2±0.01^{a}	1.2±0.01^{a}	1.2±0.01^{a}	1.2±0.02^{a}	1.1±0.01^{b}	1.2±0.01^{a}	1.2±0.01^{a}	1.2±0.01^{a}	1.2±0.01^{a}
20:4 n-6	7.4±0.1^{a}	7.1±0.2^{ab}	7.2±0.1^{ab}	7.3±0.1^{a}	7.4±0.1^{a}	7.3±0.1^{a}	6.8±0.1^{b}	7.1±0.1^{ab}	7.3±0.1^{a}	7.4±0.2^{a}	7.3±0.1^{a}
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:5 n-3	6.3±0.1^{a}	5.6±0.1^{b}	5.7±0.1^{b}	6.3±0.1^{a}	6.3±0.1^{a}	6.3±0.1^{a}	5.3±0.1^{b}	5.6±0.1^{b}	6.2±0.1^{a}	6.2±0.1^{a}	6.2±0.1^{a}
22:5 n-3	3.6±0.06^{a}	3.3±0.05^{b}	3.5±0.08^{ab}	3.6±0.05^{a}	3.6±0.05^{a}	3.6±0.05^{a}	3.2±0.05^{b}	3.4±0.03^{ab}	3.6±0.03^{a}	3.5±0.04^{a}	3.5±0.11^{ab}
22:6 n-3	6.8±0.1^{a}	6.1±0.1^{b}	6.2±0.1^{b}	6.9±0.2^{a}	6.9±0.1^{a}	6.8±0.1^{a}	5.9±0.2^{b}	6.1±0.1^{b}	6.8±0.1^{a}	6.8±0.1^{a}	6.7±0.1^{a}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $p<0.01$.

[2]Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time zero.

*****BHT was used at a concentration of 2mg/ml, citric acid, L-ascorbic acid, and EDTA were used at a concentration of 5mg/ml.

Table 3. Fatty acid composition of blood spots on two types of collection paper impregnated with different protectants after 9 weeks of storage at room temperature

Fatty acids[1]	Baseline[2]	Whatman ion exchange paper					Fluka blood collection paper				
		No Protectant	BHT*	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA
16:0	23.6±0.3^{a}	28.0±0.3^{d}	26.0±0.3^{c}	24.4±0.3^{ab}	24.5±0.2^{b}	24.2±0.3^{ab}	29.7±0.3^{e}	27.7±0.3^{d}	27.1±0.3^{d}	27.3±0.3^{d}	27.3±0.2^{d}
18:0	13.4±0.1^{a}	16.1±0.1^{e}	14.7±0.2^{c}	14.0±0.1^{b}	14.1±0.1^{b}	14.0±0.2^{ab}	16.9±0.2^{c}	15.8±0.1^{de}	15.6±0.1^{de}	15.7±0.1^{de}	15.4±0.2^{cd}
22:0	0.4±0.01^{b}	0.5±0.02^{a}	0.4±0.01^{b}	0.4±0.01^{b}	0.4±0.01^{b}	0.4±0.01^{b}	0.5±0.02^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.02^{a}
24:0	0.6±0.01^{c}	0.7±0.01^{b}	0.7±0.01^{b}	0.6±0.01^{c}	0.6±0.02^{c}	0.6±0.01^{c}	0.8±0.02^{a}	0.7±0.02^{bc}	0.7±0.01^{b}	0.7±0.01^{b}	0.6±0.01^{c}
16:1 n-7	1.4±0.02^{c}	1.6±0.02^{ab}	1.5±0.02^{bc}	1.4±0.02^{c}	1.4±0.02^{c}	1.4.±0.03^{c}	1.7±0.02^{a}	1.6±0.04^{ab}	1.5±0.03^{bc}	1.5±0.03^{bc}	1.5±0.02^{bc}
18:1 n-7	1.5±0.03^{c}	1.7±0.02^{ab}	1.5±0.02^{c}	1.5±0.02^{c}	1.5±0.03^{c}	1.5±0.02^{c}	1.8±0.01^{a}	1.6±0.02^{bc}	1.6±0.02^{bc}	1.6±0.02^{bc}	1.6±0.02^{bc}
18:1 n-9	18.6±0.2^{a}	21.4±0.2^{b}	19.3±0.2^{a}	19.0±0.2^{a}	19.0±0.1^{a}	19.0±0.2^{a}	23.1±0.2^{c}	21.1±0.2^{b}	21.4±0.2^{b}	21.7±0.2^{b}	21.3±0.2^{b}
24:1 n-9	0.8±0.02	0.8±0.01	0.8±0.01	0.8±0.02	0.8±0.02	0.8±0.01	0.9±0.02	0.8±0.02	0.8±0.02	0.8±0.01	0.8±0.02
18:2 n-6	19.8±0.2^{a}	18.3±0.1^{b}	19.3±0.2^{a}	19.6±0.1^{a}	19.4±0.1^{a}	19.5±0.1^{a}	17.5±0.2^{c}	18.6±0.2^{b}	18.8±0.2^{b}	18.7±0.1^{b}	18. 8±0.2^{b}
20:3 n-6	1.2±0.02^{a}	0.8±0.01^{d}	1.0±0.01^{b}	1.1±0.02^{ab}	1.2±0.02^{a}	1.2±0.02^{a}	0.7±0.01^{e}	0.9±0.01^{c}	0.9±0.01^{c}	1.0±0.01^{b}	1.0±0.01^{b}
20:4 n-6	7.4±0.1^{a}	6.0±0.2^{c}	6.6±0.1^{b}	7.0±0.1^{ab}	7.0±0.1^{ab}	7.1±0.1^{a}	4.8±0.2^{e}	5.4±0.1^{d}	5.5±0.1^{cd}	5.4±0.1^{d}	5.6±0.2^{cd}
22:4 n-6	0.5±0.01^{a}	0.4±0.01^{b}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.4±0.01^{b}	0.5±0.02^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}
18:3 n-3	0.4±0.01	0.3±0.01^{b}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.3±0.01^{b}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.02^{a}
20:5 n-3	6.3±0.1^{a}	3.4±0.1^{e}	4.9±0.1^{c}	5.8±0.2^{ab}	5.7±0.2^{b}	5.9±0.2^{ab}	2.7±0.2^{f}	4.0±0.2^{d}	4.2±0.1^{d}	4.1±0.1^{d}	4.2±0.1^{d}
22:5 n-3	3.6±0.06^{a}	2.4±0.04^{c}	3.2±0.04^{b}	3.4±0.05^{ab}	3.5±0.05^{a}	3.4±0.05^{ab}	1.7±0.12^{d}	2.4±0.04^{c}	2.4±0.06^{c}	2.4±0.06^{c}	2.5±0.04^{c}
22:6 n-3	6.8±0.1^{a}	4.0±0.1^{c}	5.5±0.1^{b}	6.3±0.2^{a}	6.3±0.2^{a}	6.4±0.2^{a}	3.1±0.1^{d}	4.3±0.2^{c}	4.4±0.1^{c}	4.3±0.1^{c}	4.5±0.2^{c}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $p<0.01$.

[2]Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time zero.

*****BHT was used at a concentration of 2mg/ml, citric acid, L-ascorbic acid, and EDTA were used at a concentration of 5mg/ml.

Table 4. Fatty acid profiles of lipids in 50 subjects measured from capillary DBS, venous whole blood, plasma and erythrocytes

Fatty acids	Capillary DBS	Whole blood TLE	Plasma TLE	Plasma PL	Erythrocyte PL
16:0	*23.4±1.8^{a}	23.6±2.0^{a}	23.3±2.5^{a}	29.4±1.9^{c}	25.4±1.3^{b}
18:0	11.7±1.2^{b}	10.9±1.1^{b}	7.4±0.9^{a}	14.3±1.9^{d}	13.7±1.4^{d}
SFA	35.1± 1.9^{b}	34.5±1.9^{b}	30.7±2.5^{a}	43.7±2.4^{d}	39.0±1.4^{c}
16:1 n-7	0.6±0.04^{a}	0.6±0.05^{a}	0.7±0.11^{a}	0.4±0.02^{b}	0.3±0.03^{c}
18:1 n-9	19.0±2.8^{a}	19.0±3.0^{a}	20.8±3.9^{a}	10.5±2.5^{c}	15.7±1.7^{b}
MUFA	19.6±2.7^{a}	19.6±2.6^{a}	21.5±3.7^{a}	11.0±2.4^{c}	16.0±1.5^{b}
18:2 n-6(LA)	26.7±4.2^{b}	27.0±4.6^{b}	34.3±5.8^{c}	24.4±4.7^{b}	14.3±2.8^{a}
20:4 n-6(AA)	9.1±1.6^{b}	9.1±1.7^{b}	6.5±1.2^{a}	9.5±2.0^{b}	14.1±2.4^{c}
22:4 n-6	0.9±0.3^{b}	1.0±0.3^{b}	0.2±0.04^{a}	0.3±0.12^{a}	2.9±1.0^{c}
n-6 PUFA	36.7± 4.9^{b}	38.0±5.3^{b}	40.9±6.0^{c}	35.2±5.2^{b}	31.3±5.2^{a}
18:3 n-3(ALA)	0.5±0.1^{b}	0.5±0.1^{b}	0.7±0.2^{c}	0.2±0.1^{a}	0.2±0.1^{a}
20:5 n-3(EPA)	2.5± 2.4	2.8±2.5	2.8±2.4	3.2±2.5	2.7±2.3
22:5 n-3(DPA)	1.8±0.5^{b}	1.8±0.5^{b}	0.8±0.3^{a}	1.4±0.4^{b}	3.9±1.1^{c}
22:6 n-3(DHA)	4.4±1.3^{b}	4.5±1.5^{b}	3.3±1.4^{a}	5.7±1.9^{c}	7.3±1.9^{d}
EPA+DHA	6.9±3.3^{a}	7.3±3.5^{a}	6.1±3.7^{a}	8.9±4.0^{ab}	9.9±3.3^{b}
n-3 PUFA	9.2±3.3^{b}	9.5±3.6^{b}	7.6±3.7^{a}	10.6±4.0^{b}	14.0±3.8^{c}
Total PUFA	45.9±3.5^{a}	46.5±3.8^{ab}	48.5±5.2^{b}	45.8±2.8^{a}	45.3±2.1^{a}
AA/EPA	8.4 ± 4.9^{ab}	7.3±5.1^{ab}	5.3±3.9^{a}	6.4±4.9^{a}	10.7±6.9^{b}
n-6 /n-3	4.9±2.0^{b}	4.9±2.1^{b}	7.2±3.4^{c}	4.2±2.0^{b}	2.6±1.0^{a}

*Values in the same row with different superscripts indicate values which are significantly different between groups, $P<0.01$.

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.; TLE, total lipid extract; PL, phospholipid

Table 5. Correlation for fatty acid composition between capillary DBS and conventional assays

Capillary DBS fatty acids	Corresponding fatty acids in venous blood fractions[a]			
	Whole blood TLE	Plasma TLE	Plasma PL	Erythrocyte PL
16:0	0.961**	0.962**	0.771**	0.626**
18:0	0.938**	0.843**	0.551**	0.7**
16:1 n-7	0.946**	0.878**	0.623**	0.511*
18:1 n-9	0.982**	0.971**	0.463*	0.674**
18:2 n-6 (LA)	0.991**	0.854**	0.896**	0.824**
18:3 n-3 (ALA)	0.951**	0.927**	0.578**	0.441*
20:4 n-6 (AA)	0.993**	0.85**	0.693**	0.754**
20:5 n-3(EPA)	0.998**	0.995**	0.988**	0.973**
22:4 n-6	0.980**	0.587**	0.436*	0.889**
22:5 n-3 (DPA)	0.915**	0.878**	0.838**	0.847**
22:6 n-3(DHA)	0.996**	0.976**	0.964**	0.971**
EPA+DHA	0.996**	0.982**	0.962**	0.972**
AA/EPA	0.993**	0.970**	0.974**	0.955**
n-6 FA/n-3 FA	0.993**	0.936**	0.927**	0.925**

TLE, total lipid extract; PL, phospholipids

[a] Spearman's rank correlation coefficient

* P <0.001, ** P <0.0001

Table 6. Fatty acid composition of plasma spots collected using Fluka collection kit or new protection system stored at room temperatures over 4 weeks

Fatty acids[1]	Baseline[2]	New protection system		Fluka collection kit	
		2 weeks	4 weeks	2 weeks	4 weeks
16:0	21.3±0.4^{a}	21.2±0.4^{a}	21.4±0.3^{a}	22.1±0.2^{ab}	22.4±0.2^{b}
18:0	5.8±0.2^{a}	5.9±0.1^{a}	6.0±0.3^{ab}	6.3±0.1^{ab}	6.5±0.1^{b}
18:1 n-9	27.7±0.2^{a}	27.9±0.4^{a}	27.9±0.3^{a}	28.3±0.2^{ab}	28.6±0.2^{b}
18:2 n-6	32.4±0.3^{a}	32.2±0.3^{a}	31.8±0.3^{ab}	31.7±0.2^{ab}	31.4±0.2^{b}
18:3 n-3	0.3±0.02	0.3±0.02	0.3±0.02	0.3±0.01	0.3±0.01
20:4 n-6	6.9±0.1^{a}	6.7±0.1^{a}	6.6±0.1^{a}	6.5±0.1^{ab}	6.2±0.1^{b}
20:5 n-3	1.5±0.02^{a}	1.5±0.05^{a}	1.4±0.04^{a}	1.3±0.02^{b}	1.2±0.03^{b}
22:4 n-6	0.6±0.02	0.6±0.02	0.6±0.02	0.5±0.02	0.5±0.02
22:5 n-3	0.8±0.03	0.8±0.02	0.8±0.02	0.8±0.03	0.7±0.02
22:6 n-3	3.0±0.1^{a}	3.0±0.1^{a}	2.9±0.1^{a}	2.6±0.1^{b}	2.3±0.1^{b}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $p<0.01$.

[2]Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh plasma at time zero.

Table 7. Fatty acid composition of breast milk spots collected using Fluka collection kit or new protection system stored at room temperatures over 4 weeks

Fatty acids[1]	Baseline[2]	New protection system		Fluka collection kit	
		2 weeks	4 weeks	2 weeks	4 weeks
16:0	30.2±0.2	30.3±0.1	30.3±0.1	30.5±0.2	30.5±0.2
18:0	8.1±0.1	8.2±0.1	8.1±0.1	8.3±0.1	8.3±0.1
18:1 n-9	43.1±0.3	43.4±0.2	43.4±0.1	43.4±0.1	43.3±0.1
18:2 n-6	15.1±0.1	14.7±0.1	14.8±0.1	14.7±0.1	14.6±0.1
18:3 n-3	2.1±0.1	2.0±0.04	2.1±0.01	2.0±0.03	1.9±0.04
20:4 n-6	0.09±0.00	0.09±0.00	0.08±0.00	0.08±0.00	0.08±0.00
20:5 n-3	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.01	0.5±0.01
22:4 n-6	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01
22:5 n-3	0.1±0.00	0.1±0.00	0.1±0.01	0.1±0.00	0.1±0.00
22:6 n-3	0.2±0.01	0.2±0.01	0.2±0.01	0.2±0.01	0.2±0.01

[1]Values are presented as mean ± SD (n=3), no significant differences were found between treatments for any of the fatty acids, $p<0.01$.

[2]Baseline refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh breast milk at time zero.