Published in final edited form as:

Lipids. 2014 August ; 49(8): 839–851. doi:10.1007/s11745-014-3918-3.

Fast Transmethylation of Total Lipids in Dried Blood by Microwave Irradiation and its Application to a Population Study

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Abstract

A methodology combining finger-pricked blood sampling, microwave accelerated fatty acid assay, fast gas chromatography data acquisition, and automated data processing was developed, evaluated and applied to a population study. Finger-pricked blood was collected on filter paper previously impregnated with 0.05 mg of the antioxidant butylated hydroxytoluene and air-dried at room temperature. Transmethylation was accelerated by microwave irradiation in an explosion-proof multimode microwave reaction system. The chemical procedure was based on a one-step direct transmethylation procedure catalyzed by acetyl chloride. The short-term stability of PUFA in blood dried on filter paper and stored overnight at room temperature was examined using venous blood. The recoveries ranged from 97–101 % for the categorized fatty acids as well as the ratios of n-6 to n-3 PUFA and the n-3% highly unsaturated fatty acid. Specifically, recoveries were 99, 98, 97, and 97 % for linoleic acid (18:2n-6), arachidonic acid (ARA), α -linolenic acid (ALA), and docosahexaenoic acid (DHA), respectively. The mol% (mean $\pm$ SD, 95% confidence interval) of fatty acid composition in subjects from the population study was determined as 36.2 $\pm$ 3.8 (35.8, 36.7), 23.2 $\pm$ 3.0 (22.8, 23.5), 36.8 $\pm$ 3.5 (36.4, 37.2) and 3.79 $\pm$ 1.0 (3.68, 3.91) for the saturated, monounsaturated, n-6 and n-3 PUFA, respectively. Individually, the mean mol% (95% CI) was 22.6 (22.3, 22.9) for 18:2n-6, 9.5 (9.3, 9.7) for ARA, 0.51 (0.49, 0.53) for ALA, 0.42 (0.38, 0.47) for eicosapentaenoic acid (EPA), and 1.67 (1.61, 1.73) for DHA. This methodology provides an accelerated yet high-efficiency, chemically safe, and temperature-controlled transmethylation, with diverse laboratory applications including population studies.

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DISCLOSURE

The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army and/or the U.S. Department of Defense.

Keywords

Finger pricked blood; Filter paper; Dried blood spot; Microwave reaction system; PUFA; omega-3; omega-6

INTRODUCTION

Fatty acids have been measured in a variety of venous blood samples (e.g., plasma, serum, and erythrocytes) to represent fatty acid compositions in both circulating and visceral organs [1, 2]. Whole blood was successfully applied as an alternative to erythrocytes in a study by Rizzo et al. [3]. During the last decade, researchers have incorporated a dried blood sampling technique [4] into fatty acid research; collecting the capillary blood on filter paper by puncturing finger tips [5–8] or ear lobes [9]. Neither Marangoni et al [5] or Min et al [10] reported significant differences between the fatty acid profiles in venous blood and the capillary blood from the fingertips. An established body of research indicates that finger prick blood sampling on filter paper is both practical and useful for population studies. It is especially important when venous blood is impractical or not possible due to special circumstances, such as for new-born infants [11], remote geographical areas [9], quick feedback following dietary supplement use [12], during military deployments [13], and when health professionals are not available to perform sample collection.

The literature contains many techniques for fatty acid measurement, including both manual and automated approaches. Several examples include: conventional lipid extraction by chloroform/methanol [14] followed by boron trifluoride catalyzed transesterification [15], one step direct transesterification employing methanol with acetyl chloride [16], the high throughput method of Glaser using HCl [17, 18], and automated high throughput fatty acid analysis [19]. All of these procedures utilize conductive heating for transmethylation of the fatty acids in blood-derived samples, between temperatures of 70 and 100 °C for 1–3 h. However, these commonly used procedures are both labor intensive and/or time-consuming, from sample collection and preparation, to data acquisition, processing, and reporting.

Microwave processing holds promise to improve efficiency and safety. Armstrong et al. [7, 12] pioneered microwave irradiation assisted transesterification of lipids in combination with finger-pricked blood sampling by using a domestic microwave oven. This microwave irradiation greatly facilitates the chemical procedures, for as short as 45 s per reaction, enabling faster estimation of the fatty acid composition. Nevertheless, due to the low efficiency of transesterification (a recovery of 78 % for the total fatty acids), as well as the safety issues when using a domestic microwave oven for this procedure, this technique has been difficult to apply to quantitative analysis of fatty acids. Previously, we developed a high-efficiency and chemically safe microwave irradiation accelerated fatty acid assay (microwave assay) for lipids in serum that was based on one-step direct transesterification with methanol and catalyzed by acetyl chloride [20]. An explosion-proof and temperature-controlled multimode microwave reaction system was employed for this method. In the present study, we further developed the microwave fatty acid assay for fingertip pricked blood dried on filter paper. This technique was then applied to soldiers deploying to a

combat theater as a pilot to evaluate if this accelerated fatty acid measurement system could be applied to determine the fatty acid compositions in population studies.

MATERIALS and METHODS

Chemicals and Materials

Methanol was purchased from Burdick & Jackson (Muskegon, MI); hexane was from EMD Chemicals Inc (Gibbstown, NJ); acetyl chloride was from Sigma-Aldrich (St. Louis, MO); butylated hydroxytoluene (BHT) was from Acros (Geel, Belgium); sodium carbonate (anhydrous powder) was from Mallinckrodt Baker Inc (Paris, KT). Standard docosatrienoic ethyl ester (22:3n-3 EE) and GC reference standards GLC-462 containing 28 fatty acid methyl esters (FAME) were purchased from Nu-Chek Prep (Elysian, MN). All chemicals were of analytical grade and used without further purification.

Filter paper was prepared according to Ichihara [21] from Whatman 3MM chromatography paper (Whatman Inc., Florham Park, NJ; 185 g/m²) which was made entirely from the highest quality of cotton linters. Pieces of 1.5 × 1.5 cm² filter paper were soaked in a solution of acetone and the antioxidant BHT (0.5 mg/mL; 0.05 %). This procedure was repeated once with a fresh solution of acetone and BHT unless indicated in the text. Then, the dried BHT-impregnated filter paper was stored in a Ziploc® at room temperature, and utilized within four weeks.

Method Development

Several studies were conducted in order to develop and validate microwave fatty acid assay in dried blood, as presented in table 1. All blood samples employed in studies 1–3 were prepared from venous blood from two healthy research donors (Donors A and B) and four healthy volunteers with omnivore diets. After treatments, the samples were aliquoted, frozen on dry ice, and stored at –80 °C until analysis. In summary, the effect of filter paper on fatty acid quantities was examined in liquid serum and liquid whole blood to isolate the effect of the filter paper. Both the microwave assay and the reference method were applied to analyze these liquid samples and subsequently the blood dried on filter paper. The recoveries, precision of the microwave assay of blood dried on filter paper, and the stability of PUFA were evaluated. Finally, this microwave assay was applied to a population study (study 4) to assess the fatty acids in total lipids of finger prick blood that was dried on filter paper.

In study 1, experiment 1, serum was prepared from Donor A's blood which was held at room temperature for about one hour and centrifuged at 1,700g for 15 min at 4 °C. Portions of 45 µL human serum that were not dried were tested with or without filter paper. A calibrated pipette was used to transfer the serum into reaction glassware immediately before chemical analysis. Whole blood samples from Donor B were prepared with the anti-coagulant heparin. In study 1, experiment 2 was a repeat of experiment 1 except that 45 µL of whole blood was used instead of serum. In study 2, 45 µL of blood was added to BHT impregnated filter paper which was washed once in a BHT acetone solution (0.5 mg/mL), air-dried for 3 h at room temperature, individually wrapped in aluminum foil, and then kept in Ziploc® bags at –80 °C. In study 3, the pooled blood of 4 volunteers was pipetted on

BHT impregnated filter paper and air-dried for 3 h at room temperature, then stored in a closed container at room temperature for another 14 h before being frozen and stored at -80°C . This study 3 was conducted to examine the short-term stability of PUFA on dried blood over 17 h and mimic the drying conditions of samples in the population study (study 4).

Population Study (Study 4)

Subjects—The study proposal was approved by, and was conducted in accordance with, the ethical standards of the Kansas State University, Institutional Review Board. Study subjects were recruited and consisted of U.S. Army Active Duty soldiers (Fort Riley, Kansas) and National Guard soldiers (Fort Bliss, Texas). The sample collection took place from October 2009 through March 2011. All of the recruited soldiers were given a complete description of the study and provided written informed consent prior to participation. There were 287 samples available for fatty acid assay, consisting of 210 samples from soldiers prior to their deployment and 77 from soldiers who had returned from deployment of which 30 were the same subjects. Age (yr) ranged from 18 to 54 with a mean age of 29.4. Mean BMI was 27.4 kg/m^2 ($\text{SD} = 3.9$). Additional demographic and health related characteristics are summarized in supplemental table 1.

Sample collection—Samples of non-fasting capillary blood were collected on BHT impregnated filter paper through the fingertip prick method. This used a high flow 18 G safety lancet (Assure Haemolance Plus) which was made by Arkay Inc. (Edina, MN). Samples were air-dried for 3 h at room temperature and stored in a closed sample container overnight during transportation. They were shipped from the two military bases to the laboratory on Kansas University campus at room temperature. The handling of samples was adjusted to allow samples from each location to be at room temperature for the same period of time (17 h), prior to storage at -80°C . Samples were then shipped to NIH on dry ice and stored at -80°C until analysis. The chemical analysis of fatty acids was completed within 12 months.

Instrumentation

Analog Drybath Incubator—An analog heatblock from VWR International, LLC (West Chester, PA) was the conductive heating source utilized in the reference method for fatty acid determination.

Multimode Microwave Accelerated Reaction System—A safety-proofed, temperature-controlled multimode microwave accelerated reaction system (MARS) from CEM Corporation (Matthews, NC) was employed in microwave fatty acid analysis as reported previously [20]. It could process multiple samples and up to 36 Pyrex glass vessels (20 mL) which were explosion-proof and pressurized. A reaction temperature was pre set, monitored, and controlled through a fiber optic probe in one vessel for every batch. The probe was inserted into a thermowell and attached to a special vent plug, which was in direct contact with the sample in the reagent mixture. An illustration of this system with accessories was supplied as supplemental figure 1.

Gas Chromatography—We employed an Agilent 7890A fast gas chromatograph coupled with flame ionization detector and a 7683B Series Injector (Agilent Technologies, Inc., Santa Clara, CA) to acquire the chromatograms of FAME. GC parameters were slightly modified from our previous report [22], as described below. A fused-silica, narrow-bore, high-efficiency DB-FFAP capillary column (15 m length $\times$ 0.1 mm ID $\times$ 0.1 μ m film thickness) was used for chromatographic separation of FAME with hydrogen as the carrier gas at a constant pressure of 51.5 psi (355 kPa). A total of 28 FAME in GLC-462 were eluted in about 8 min with an extended 25 min hold to bake off the column. GC ChemStation Rev. B.04.03 (SP52) was employed for data acquisition and peak integration. A sample chromatogram of FAME from a finger-pricked blood sample that was dried on filter paper is presented in Figure 1.

Fatty Acid Analysis

The one-step direct transesterification Lepage & Roy fatty acid assay [16, 19] was applied as the reference method. Forty-five μ L of blood or serum were added either with filter paper or directly to a 16 $\times$ 100 mm disposable borosilicate glass test tube placed in ice. Then, 100 μ L of normal saline (only added to the dried blood samples) and the internal standard (ISTD) 22:3n-3 EE (27.6 nmol or 10 μ g per sample) were added. This was followed by the addition of a mixture of 1.6 mL of methanol containing 50 μ g of BHT, 0.4 mL of hexane, and 0.2 mL of acetyl chloride. The test tubes were then capped and placed in an analog heating block at 100 $^{\circ}$ C for 60 min. Afterwards, the samples were chilled and neutralized by the addition of 5 mL of 6 % Na_2CO_3 solution followed by centrifugation at 1,700g for 4 min. The upper phase hexane containing FAME was collected and the volume was reduced to $\sim$ 30 μ L under a stream of nitrogen. In general, 2 μ L of aliquot was injected into the GC inlet for data acquisition.

The microwave assay was previously developed [20] as the method “M5–125 $\times$ 5”, which was the optimal transesterification at 125 $^{\circ}$ C for 5 min in the multimode microwave accelerated reaction system. The reaction temperature was programmed with a constant air pressure of 200 psi (1,379 pKa) to prevent the reaction vessels from exploding. Initially the temperature was around 15 $^{\circ}$ C and reached 125 $^{\circ}$ C in 3.5 min. When there were 8 or more samples, the initial power was 1,600 W, but was 400 W when there were $<$ 8 samples. A holding reaction temperature of 125 ± 1.3 $^{\circ}$ C ($n = 11$) for 5 min utilized 40–60 % of the initial power. At the end of the reaction, the vessels were transferred to ice before further chemical processing. The remaining procedures were the same as the reference fatty acid assay.

Calculation and Statistics

Data were expressed as mean $\pm$ SD in concentrations, μ mol/L serum or blood, and/or mol% for each fatty acid of the total identified fatty acids. Automated data processing was performed for the calculation as reported in a previous study [19]. Recovery of each fatty acid was defined as the percentage of fatty acids determined by microwave assay compared to those by the reference method; or, the percentage of fatty acids in dried blood to those in liquid blood frozen shortly after collection, as indicated in the text. The percentage

difference (%Diff) of the two experimental values was calculated by dividing the absolute difference of the two values by the reference values and then multiplying by 100.

Groups were compared using a one-way ANOVA followed by Post Hoc Tukey's HSD test using SPSS 17.0 (SPSS Inc.; Chicago, IL). Statistical significance was set at $P < 0.01$. The 95 % confidential interval (95 % CI) was calculated for the mean by Microsoft® Excel 2010 (Microsoft Corp.; Seattle, WA).

RESULTS

Study 1. Effect of filter paper on liquid samples

Fatty acid concentrations of human serum and whole blood, with or without the presence of filter paper, were presented in Table 2 and Table 3, respectively. Values were determined by both microwave assay and the reference method.

Filter paper and liquid serum (Experiment 1)—As presented in Table 2, it was noted that the %Diff between fatty acid concentrations in serum with filter paper compared to without filter paper were less than 5 % for all fatty acids except 20:0, 22:0, and 20:2n-6 which were 7–9 %. There was no significant difference observed comparing groups with and without filter paper determined by either reference or microwave assays. The results of serum without filter paper were consistent with those in our previous report [20], in which the same human serum was applied.

When compared to the reference Lepage & Roy assay, the recoveries of fatty acids in serum measured by microwave assay was at a range of 94–101 % without filter paper, and a range of 96–104 % at the presence of filter paper. This excluded the fatty acids: 20:0, 22:0, 24:0, and 24:1n-9, which ranged from 71–83 %. Overall, the serum fatty acid values that were determined by microwave assay were slightly less than the reference method when using filter paper. Particularly, docosahexaenoic acid (DHA) measured by MW assay showed a recovery of 98.4 % with filter paper and no significant difference was detected between the two methods. Without filter paper, DHA as measured by MW assay was significantly ($P < 0.01$) lower than by Lepage & Roy method, with a recovery of 94.8 %. No significant difference was observed comparing assays for 18:2n-6, 20:4n-6, and 18:3n-3, as well as the proportions of n-6 to n-3 PUFA, arachidonic acid (ARA) to eicosapentaenoic acid (EPA), and n-3 % HUFA, between the four groups. The assays had good precision for all fatty acid concentrations in the four groups, in which CV % was less than 5 % for all fatty acids, except for 24:0 (8 %) in the microwave assay without filter paper (group MW-FP).

Filter paper and liquid whole blood (Experiment 2)—The effect of filter paper on fatty acid values in liquid whole blood was also examined and the results were presented in Table 3. The %Diff comparing fatty acid concentrations of blood, with and without filter paper, was 10 % for all fatty acids except 14:0 (30) and 20:0 (19) for the microwave assay and 14:0 (20), 20:0 (17), 18:3n-6 (14), and 20:3n-9 (12) for the reference assay. When compared to the reference Lepage & Roy method, the recoveries of the majority of the fatty acids measured by microwave assay were 95–122 % without filter paper, and 95–115 % with filter paper. However, recoveries of several minor fatty acids were in the range of 79–

90 %, including 20:0, 22:0, 24:0, 24:1n-9, and 20:2n-6. A greater concentration of 18:3n-3 was observed when using microwave assay with and without filter paper than when the reference method was used. When using the microwave assay, the recoveries of total n-6 PUFA and total n-3 PUFA were in the range of 100–103 % in comparison to the reference assay. No significant differences were detected for the concentrations of 18:2n-6, 20:4n-6, 18:3n-3, and 22:6n-3 in liquid blood comparing the four groups, or the ratio of n-6 to n-3 PUFA, ARA to EPA, and n-3 % HUFA.

Study 2. Fatty acid measurements in dried whole blood

Fatty acids in whole blood that was dried on filter paper were analyzed by microwave assay and the Lepage & Roy method. The concentrations and compositions of fatty acids, as well as the within-run precisions and between-run precisions were presented in Table 4. The recoveries of fatty acid concentrations determined by microwave assay were 95–103 % for the categorized fatty acids, n-6/n-3 PUFA, and n-3 % HUFA. In particular, recoveries of 101, 98, 106, and 98 % were observed for 18:2n-6, 20:4n-6, 18:3n-3, and 22:6n-3, respectively. A wider range of the recoveries (91–114 %) was observed for the majority of the individual fatty acids. Consistent with study 1, 20:0, 22:0, 24:0, and 24:1n-9 exhibited lower recoveries with a range of 70–78 %. However, the inefficiencies of these minor fatty acids did not interfere with the sum of the saturated or monounsaturated.

Measurements of within-run precision of microwave assay for the dried blood ($n = 30$) indicated a CV less than 4 % for the concentrations of each of the categorized fatty acids and 9 % for individual fatty acids, except 14:0 (20 %), 20:0 (12 %), and 18:3n-6 (13 %). CV of the between-run precision ($n = 8$) was slightly lower for the mol% of most individual fatty acids except 14:0 (40 %), 18:1n-7 (11 %), 18:3n-6 (18 %), and 20:5n-3 (16 %). The CV value for n-3 % HUFA was 2 % for both precisions.

Study 3. Fatty acid short-term stability in dried blood

The compositions of the various fatty acids and their corresponding recoveries were presented in Table 5. When compared to liquid blood as 0 h, the mean recoveries of PUFA in dried blood that stored at room temperature for 17 h ranged 97–101 % for the categorized fatty acids, the ratios of n-6 to n-3 PUFA, and n-3 % HUFA. The recoveries of the individual PUFA were 95 % or over except 22:5n-6 which was 87 %. Specifically, recoveries were 99, 98, 97, and 97 % for 18:2n-6, 20:4n-6, 18:3n-3, and 22:6n-3, respectively. The application of 0.05 mg of BHT on filter paper protected PUFA in dried blood spots from degradation when samples were stored at room temperature in closed containers over 3 and 17 h. Data were presented in supplement table 2.

Study 4 Fatty acid profiles in an observational study

The fatty acid compositions of dried blood samples from deploying soldiers were determined by microwave assay and presented in Table 6. The mean $\pm$ SD (95 % CI) of the measured fatty acid compositions (mol%) in deploying soldiers were 36.2 ± 3.8 (35.8, 36.7), 23.2 ± 3.0 (22.8, 23.5), 36.8 ± 3.5 (36.4, 37.2) and 3.79 ± 1.0 (3.68, 3.91) for the saturated, monounsaturated, n-6 PUFA, and n-3 PUFA, respectively. For major PUFA, the mean

values of mol% (95 % CI) were 22.6 (22.3, 22.9) for 18:2n-6, 9.5 (9.3, 9.7) for ARA, 0.51 (0.49, 0.53) for ALA, 0.42 (0.38, 0.47) for EPA, and 1.67 (1.61, 1.73) for DHA.

DISCUSSION

The microwave irradiation assay in combination with dried blood spot (DBS) sampling is not a novel technology for fatty acid measurement. It has been previously reported in other studies that used a domestic microwave oven for human blood [7] or long chain fatty acid esters [23]. However, by employing the novel multimode microwave reaction system, it became possible to perform a safe and well-controlled transesterification that was accelerated by microwave irradiation. Despite the accelerated processing, both the reaction efficiency and the reaction temperature were comparable to an accepted reference method.

Method development

Microwave fatty acid assay—This study reproduced our previously established microwave fatty acid assay for human serum [20] and extended it to dried blood on filter paper. In comparison to the reference Lepage & Roy method, the majority of the individual fatty acid concentrations determined by microwave assay were consistent in liquid human serum (Table 2), liquid whole blood (Table 3), and dried whole blood (Table 4). The recoveries for the majority of the fatty acids and the categorized fatty acids were in the range of 95–105 % for liquid serum and liquid whole blood, and were 94 % in dried blood. Both the within-run and between-run precisions were 9 % for the majority and <2 % for n-3 % HUFA. This microwave assay can be applied as an alternative to the reference method with a much shorter reaction time with consistently accurate results. With this accelerated technique, it took less than one hour to process an individual sample, from finger pricked sample collection (without the blood drying) to the generation of a fatty acid report.

Unfortunately, incomplete transmethyations of some minor fatty acid: 20:0, 22:0, 24:0, and 24:1n-9, were observed (recoveries range from 70–90 %) in all samples analyzed by microwave assay. Addition of 100 μ L of normal saline improved their recoveries by improving the energy absorption of samples. Neither increasing the transesterification duration (from 5 min to 7.5 min) nor reducing the number of reactions per batch improved reaction efficiencies. One possible explanation is that some lipids, such as sphingo- and glyco-lipids, that are linked via very stable amide bonds instead of esters, are more difficult to transesterify relative to other lipids [15], regardless of the experimental microwave irradiation power, duration, and temperature.

Filter paper and capillary blood sampling—The possible contaminations in filter paper were significantly reduced after the filter paper was washed twice with an acetone BHT solution [21]. However, mean values ($n = 10$) of 0.13, 0.49, and 0.24 μ g of background noises were observed at the same retention times as 14:0, 16:0, and 18:0 in one piece of blank filter paper in the current study. These accounted for 30, 4.0, and 3.9 % of the total amount of the respective fatty acids in the control dried blood samples. These values were subtracted from the fatty acids results of dried blood from the subjects in the population study (Table 6). When compared to the endogenous level, the interference peak at the

retention time for 14:0 in filter paper exhibited a low signal-to-noise ratio during data acquisition which contributed to the variance of the 14:0 values in blood.

This technique requires further improvement mainly for the estimation for the volume of dried blood on filter paper. A previously standardized DBS sampling on a specific filter paper disc with normalized blood collection technique has been routinely employed in clinical laboratories for neonatal screening programs [24]. In order to establish absolute concentrations, accurate estimations of the volume of the blood samples are needed. Presentation of fatty acid data in units of absolute concentration are optimal for a description of fatty acid status [25] and key physiological processes like essential fatty acid metabolism and production of bioactives like resolvins [26] or endocannabinoids [27] are driven by concentrations and not generally by fatty acid percentages. The finger prick sampling without filter paper [28] as well as a very low volume (15 μ L) of sample that has absorbed on filter paper [29] could also be applied for fatty acid measurement by gas chromatography coupled with mass spectrometry.

PUFA stabilities in dried blood—We found the incorporation of 0.05 mg of BHT on filter paper prior to blood collection could effectively maintain the PUFA compositions during short-term storage and transportation at room temperature while the exposure of air and the humidity was limited for the dried blood (Table 5). This was consistent with two previous reports [5, 21], in which filter paper was pre-treated with a 0.5 mg/mL BHT solution. Minimal changes in PUFA wt% were observed when samples were at room temperature for 5 days [21] or after two weeks [5]. The instabilities of fatty acid compositions in dried blood increased during long-term storage of up to 6 months, as reported in several studies [8, 10, 30]. A recent study on the long-term stabilities of fatty acids reported that pre-conditioning the filter paper with a higher concentration of BHT solution (5 mg/mL) could effectively maintain the n-3 % HUFA of dried blood, when stored at room temperature for 28 days [30].

However, even with the application of BHT [31], a slight degradation of PUFA (5 %) in dried blood spots occurred as indicated in Table 5. Air exposure and humidity are critical variables contributing to instability of PUFA in dried blood samples. Part of the degradation was likely due to ozone, which results in PUFA oxidation when PUFA is exposed to air [32]. By limiting air exposure, even without BHT, the fatty acid profiles in dried blood could be maintained for two weeks at 4 °C in a cellophane envelope within an airtight closure [5]. Any trace of moisture may result in the formation of mold, thus favoring the degradation of PUFA. This can be minimized through the use of a desiccator and desiccant in a sealed sample container. The presence of filter paper results in additional degradation, perhaps because of increased surface area, as suggested by Pottala et al [33]. This issue could be addressed by minimizing the blood volume which reduces the surface area exposed to air. In addition to these factors, the metal-catalyzed peroxidation, induced directly by copper and iron, might play a significant role in PUFA degradation as suggested by Knight et al [34]. A variety of metal chelators could suppress the metal-induced oxidation [35]. The degradation of PUFA in dried blood could also be reduced by another antioxidant system [13] or processing and storing the samples at an optimal low temperature. Under

circumstances of substantial degradation of PUFA, the correction method reported by Pottala et al [33] should be applied.

Gas Chromatography—Fast oven temperature ramps and a high-efficient, short capillary column in this GC technology greatly shortened the time for fatty acids to elute, from 45 min in conventional 30 m column to 8 min for all fatty acids with the last identified elution of 24:1n-9 [22]. Theoretical plates of 10,000 for the short column, compared to 4,300 in a conventional column, provided for good resolutions for the majority of the fatty acid peaks. Generally, a bakeout time of 9 min is applied for human serum and plasma. However, in this study, the lipids in whole blood were neither extracted nor pre-purified, but total lipids were transmethyated, leaving sterols and other neutral lipids present. Some of these lipids eluted at a retention time around 27 min and at a temperature around 245 °C. This makes it necessary to purge the column for an extended period of time after each run to avoid interference with subsequent data acquisitions. This problem could be minimized by the newly developed technology of capillary column backflush [36] that significantly reduces the bakeout time during our pilot experiments.

Limitations

The major constraint to a higher throughput of this microwave fatty acid assay was the manual pipetting for the additions of all reaction reagents, a very time-consuming step. Manual pipetting made it very difficult to keep up with the theoretical capability of the microwave system to perform 36 reactions in 5 min for multiple runs within one work day. The cleaning step of the recycle glass reaction vessels after sample analysis was also time-consuming. The cost of replacing the delicate reaction vessels added to the limitation of this method. But even with the limitation of manual pipetting, a throughput of chemical processing of 36 samples per chemist every three hours was still practical. However, when serum or plasma from venous blood were available in large scale studies, the automated high-throughput methodology [19], referred to as the robot, is preferred for determining the fatty acids profiles. The extended bakeout time could be a limitation to overall sample throughput in a single instrument-equipped laboratory.

The *trans* fatty acids, dimethylacetal, and extra-long or very short chain fatty acids were not identified using the current method. This caused the slight over-estimation of the individual fatty acid compositions in blood samples but would not affect absolute concentration measurements.

Population Study

The PUFA compositions observed in this study (Table 6) were close to those found in liquid blood of male undergraduates (n=10) in a Canadian university [12]. However, a variety of differences were observed comparing previous studies on fatty acids in dried blood. A similar study on U.S. service members [13] reported lower wt% of 18:2n-6 (18.5) and ALA (0.32), but greater wt% of ARA (12.9) and DHA (2.61). Among our subjects, the compositions of 18:2n-6 and ARA were greater by 26 and 31 %, but lower for ALA, EPA, and DHA by 22, 35, and 13 %, respectively, compared to healthy Italians [37]. The mean age for the Italian study subjects was 34 years with a range of 20 to 70 years. Another large-

scale study [38] on Norwegian and Swedish subjects indicated values comparable to our subjects in the wt% of 18:2n-6 (21.7) and ARA (9.2), but was much greater (1–2 fold) for EPA (1.4) and DHA (3.6) among the subjects that did not use n-3 supplements (n = 1,555 out of a total of 3,476, average age of 46 years). These differences most likely arose from the high dietary intake of fish in Norway and Sweden, relative to fish intake levels in the U.S [39, 40]. However, the difference of ages among these studies could also contribute to the variance in fatty acid compositions, as reported by Harris et al [41] from the observations of 160,000 patients in which the older patients had greater n-3 PUFA compositions in erythrocytes. The age may, however, be associated with other lifestyle habits, such as dietary habits and physical activities that may have influenced the results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to acknowledge the service members who took part in the study as well as Dr. Norman Salem Jr. and Dr. Charlie N. Serhan for the valuable advice and discussion on the method development. The authors thank Mr. Brian Brown, NIH Library Writing Center, for manuscript editing assistance. This project was partially funded by the Intramural Research Program of the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health.

Abbreviations

ARA	Arachidonic acid, 20:4n-6
BHT	Butylated hydroxytoluene
DBS	Dried blood spot
DHA	Docosahexaenoic acid, 22:6n-3
%Diff	Percentage difference
EPA	Eicosapentaenoic acid, 20:5n-3
FAME	Fatty acid methyl ester
GC	Gas chromatography with flame ionization detector
HUFA	Highly unsaturated fatty acid(s)
ISTD	Internal standard
ALA	α -Linolenic acid, 18:3n-3
PUFA	Polyunsaturated fatty acid(s)

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Summary

Despite a few limitations, the accelerated transmethylation of the temperature-controlled, explosion-safe, high-efficiency microwave technique and the minimally invasive finger-pricked sampling are practical, efficient, and promising. This microwave assay, coupled with finger prick blood collection, fast GC technology, and automated data processing, advances the goal of providing an accelerated clinical study methodology for fatty acid determination in human subjects, with diverse laboratory applications.

Table 1

Study design and result presentation

Exp	Study	Sample	Source	Collection	Phase	Filter paper	Reference assay	MW assay	Result presentation	Study purpose
1	Study 1	serum	Donor A	vein	liquid	±	+	+	Table 2	apply MW assay to serum; effect of filter paper on serum
2	Study 1	blood	Donor B	vein	liquid	±	+	+	Table 3	apply MW assay to blood; effect of filter paper on blood
3	Study 2	blood	Donor B	vein	dry	+	+	+	Table 4	apply MW assay to blood that was dried on filter paper
4	Study 3	blood	Pooled	vein	dry & liquid	+	+	-	Table 5	examine PUFA degradation in dried blood on filter paper
5	Study 4	blood	study subjects	capillary	dry	+	-	+	Table 6	application of MW assay in finger-pricked dried blood

BHT, butylated hydroxytoluene

Capillary, blood collected through finger tip

Dry, whole blood dried on filter paper

Exp, experiment

MW assay, microwave accelerated fatty acid assay

Liquid, whole blood that was not dried

Pooled, mixed venous blood from 4 volunteers

Reference assay, Lepage & Roy assay

Vein, blood collected through antecubital vein

“+”, applied

“-”, not applied

Table 2

Effects of filter paper on fatty acid quantities in liquid human serum

Fatty acids	LR-FP			LR+FP			MW-FP			MW+FP			%Diff (+/-FP)			Recy (MW/LR)	
	mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		LR	MW		-FP	+FP
<i>Fatty acid concentrations (μmol/L)</i>																	
14:0	91.5 ± 3.4	4		89.6 ± 0.6	1		89.2 ± 1.1	1		89.8 ± 0.8	1		2.1	0.7		97.5	100
16:0	1763 ± 49	3		1759 ± 19	1		1740 ± 24	1		1769 ± 23	1		0.2	1.7		98.7	101
18:0	551 ± 17	3		559 ± 9.2	2		540 ± 7.4	1		567 ± 2.5	0		1.4	5.0		98.0	101
20:0*	23.4 ± 0.6	3 ^a		24.2 ± 0.7	3 ^a		18.8 ± 0.6	3 ^b		20.1 ± 0.7	3 ^b		3.6	7.2		80.5	83.3
22:0*	44.8 ± 1.0	2 ^a		46.3 ± 1.0	2 ^a		33.5 ± 1.1	3 ^b		36.7 ± 0.7	2 ^b		3.4	9.3		74.8	79.1
24:0*	39.2 ± 0.6	2 ^a		39.3 ± 0.8	2 ^a		29.7 ± 2.3	8 ^b		30.8 ± 0.7	2 ^b		0.2	3.6		75.8	78.4
16:1n-7	127 ± 3	3		123 ± 0	0		123 ± 1	1		123 ± 1	0		3.1	0.1		96.8	100
18:1n-7	142 ± 4	3		139 ± 1	1		137 ± 1	1		139 ± 3	2		1.7	0.9		97.1	99.7
18:1n-9	1776 ± 57	3		1736 ± 13	1		1721 ± 9	1		1727 ± 5	0		2.3	0.3		96.9	99.5
20:1n-9	10.5 ± 0.2	2		10.6 ± 0.2	2		10.5 ± 0.3	3		11.0 ± 0.5	4		1.1	4.2		101	104
24:1n-9*	104 ± 1	1 ^a		101 ± 1	1 ^a		74.0 ± 3.8	5 ^b		73.2 ± 0.5	1 ^b		3.1	1.1		71.3	72.8
18:2n-6	1695 ± 56	3		1654 ± 19	1		1623 ± 7	0		1625 ± 3	0		2.4	0.2		95.7	98.3
18:3n-6	50.1 ± 1.4	3		49.9 ± 0.6	1		48.2 ± 0.9	2		48.1 ± 0.4	1		0.3	0.0		96.2	96.4
20:2n-6	10.8 ± 0.6	5		10.1 ± 0.3	3		10.2 ± 0.4	4		10.0 ± 0.2	2		6.7	1.9		94.4	99.3
20:3n-6	112 ± 4	3		109 ± 1	1		108 ± 0.3	0		109 ± 0.5	0		2.8	0.5		96.6	99.8
20:4n-6	760 ± 24	3		741 ± 9	1		723 ± 3	0		725 ± 0.6	0		2.5	0.3		95.2	97.9
22:4n-6*	19.9 ± 0.5	2 ^{abc}		20.7 ± 0.1	1 ^b		19.3 ± 0.6	3 ^c		20.1 ± 0.3	2 ^{abc}		4.2	4.2		96.9	96.8
22:5n-6	9.0 ± 0.3	3		8.8 ± 0.3	3		8.8 ± 0.2	2		9.0 ± 0.4	5		2.4	2.0		97.3	102
18:3n-3	22.6 ± 0.8	4		21.9 ± 0.4	2		22.2 ± 0.9	4		22.5 ± 0.7	3		3.1	1.5		98.3	103
20:5n-3	77.4 ± 1.9	2		75.1 ± 1.2	2		74.8 ± 1.0	1		76.5 ± 0.2	0		3.0	2.3		96.6	102
22:5n-3	48.1 ± 1.8	4		46.9 ± 0.9	2		45.7 ± 0.3	1		45.8 ± 0.3	1		2.6	0.2		94.9	97.6
22:6n-3*	205 ± 6	3 ^a		199 ± 2	1 ^{ab}		194 ± 2	1 ^b		196 ± 0	0 ^{ab}		2.9	0.8		94.8	98.4
20:3n-9	21.6 ± 0.8	3		20.6 ± 0.5	2		20.3 ± 0.2	1		20.2 ± 0.1	0		4.5	0.9		94.2	97.8
Total FA	7752 ± 236	3		7630 ± 79	1		7456 ± 44	1		7538 ± 24	0		1.6	1.1		96.2	98.8
Summary	<i>mol% of Total fatty acids</i>															<i>Percentage</i>	

Fatty acids	LR-FP			LR+FP			MW-FP			MW+FP			%Diff (+/-FP)			Recy (MW/LR)		
	mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		LR	MW		-FP	MW	+FP
Fatty acid concentrations (μmol/L)																		
Percentage																		
Saturates	30.9 ± 0.1	0		31.4 ± 0.1	0		31.2 ± 0.3	1		31.4 ± 0.3	1		1.4	0.7		101	101	100
Mono*	26.6 ± 0.0	0 ^a		26.3 ± 0.1	0 ^b		26.3 ± 0.1	0 ^b		25.9 ± 0.0	0 ^c		1.1	1.5		99.0	99.0	98.5
n-6 PUFA*	32.7 ± 0.0	0 ^a		32.3 ± 0.1	0 ^b		32.3 ± 0.1	0 ^b		31.8 ± 0.1	0 ^c		1.2	1.6		98.9	98.9	98.5
n-3 PUFA	4.34 ± 0.0	0		4.27 ± 0.0	0		4.28 ± 0.0	1		4.25 ± 0.0	0		1.7	0.8		98.6	98.6	99.6
Proportion																		
n-6/n-3 PUFA	7.5 ± 0.0	0		7.6 ± 0.0	0		7.5 ± 0.1	1		7.5 ± 0.0	0		0.5	0.9		100	100	98.9
ARA/EPA	9.8 ± 0.1	1		9.9 ± 0.1	1		9.7 ± 0.1	1		9.5 ± 0.0	0		0.5	2.0		98.6	98.6	96.1
n-3% HUFA	26.4 ± 0.1	0		26.3 ± 0.0	0		26.3 ± 0.1	0		26.5 ± 0.0	0		0.3	0.5		99.9	99.9	101

Data were presented as means ± SD, n = 3 or 4 measurements. “0.0” indicates data < 0.05 and “0” indicates data < 0.5. Fatty acids with * and different alphabetical superscripts were statistically significant when using a One-way ANOVA followed by Tukey’s HSD at *P* < 0.01; %Diff (+/-FP) indicates the difference between fatty acids in samples with and without filter paper, within each of the LR and MW methods. Recy (MW/LR) indicates the recoveries of fatty acids determined by the microwave assay to those by the reference method; LR Lepage & Roy method; MW microwave assay; “+/-” FP with/without filter paper; Mono monounsaturates; HUFA highly unsaturated fatty acids [42]

Table 3

Effects of filter paper on fatty acid quantities in liquid human whole blood

Fatty acids	LR-FP			LR-FP			MW-FP			MW-FP			%Diff (+/-FP)			Recy (MW/LR)		
	mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		LR	MW		-FP	+FP	
<i>Fatty acid concentrations (μmol/L)</i>																		
<i>Percentage</i>																		
14:0	34.2 ± 2.0	6	27.5 ± 7.2	>10	33.9 ± 2.9	9	23.8 ± 20	>10	20	30	99.1	86.7	20	30	99.1	86.7		
16:0	1475 ± 88	6	1536 ± 50	3	1419 ± 31	2	1515 ± 37	2	4.1	6.8	96.2	98.6	4.1	6.8	96.2	98.6		
18:0	735 ± 46	6	758 ± 25	3	726 ± 20	3	768 ± 17	2	3.1	5.7	98.8	101	3.1	5.7	98.8	101		
20:0*	25.3 ± 1.7	7 ^a	29.6 ± 0.9	3 ^b	21.7 ± 0.1	0 ^c	25.8 ± 1.1	4 ^a	17	19	85.6	87.2	17	19	85.6	87.2		
22:0*	92.7 ± 5.6	6 ^a	94.4 ± 4.9	5 ^a	75.8 ± 0.8	1 ^b	79.1 ± 4.3	5 ^b	1.8	4.4	81.7	83.8	1.8	4.4	81.7	83.8		
24:0*	177 ± 11	6 ^{ac}	188 ± 9	5 ^a	142 ± 1	1 ^b	156 ± 8.0	5 ^{bc}	6.0	10	80.0	83.2	6.0	10	80.0	83.2		
16:1n-7	44.2 ± 1.9	4	46.9 ± 1.8	4	42.0 ± 1.7	4	44.4 ± 2.1	5	6.1	5.9	94.9	94.8	6.1	5.9	94.9	94.8		
18:1n-7	103 ± 6	6	106 ± 4	4	100 ± 1	1	110 ± 7.4	7	3.2	9.4	97.7	104	3.2	9.4	97.7	104		
18:1n-9	829 ± 51	6	842 ± 25	3	803 ± 17	2	862 ± 52	6	1.7	7.3	96.9	102	1.7	7.3	96.9	102		
20:1n-9	10.1 ± 0.6	6	10.2 ± 0.4	4	10.0 ± 0.2	2	10.7 ± 0.3	3	0.7	6.8	99.1	105	0.7	6.8	99.1	105		
24:1n-9*	191 ± 11	6 ^a	195 ± 9	5 ^a	151 ± 1	1 ^b	161 ± 8.3	5 ^b	2.4	6.8	79.0	82.4	2.4	6.8	79.0	82.4		
18:2n-6	1653 ± 99	6	1716 ± 50	3	1590 ± 27	2	1698 ± 36	2	3.8	6.8	96.2	99.0	3.8	6.8	96.2	99.0		
18:3n-6*	16.2 ± 0.7	4 ^a	18.5 ± 0.9	5 ^{ab}	18.8 ± 0.9	5 ^{ab}	19.7 ± 1.6	8 ^b	14	5.0	116	106	14	5.0	116	106		
20:2n-6*	19.9 ± 0.8	4 ^{ac}	20.7 ± 0.7	3 ^a	17.1 ± 1.1	7 ^b	17.7 ± 0.5	3 ^{bc}	4.1	3.8	85.9	85.7	4.1	3.8	85.9	85.7		
20:3n-6	130 ± 8	6	132 ± 4	3	126 ± 2	2	135 ± 0.4	0	1.6	7.1	97.1	102	1.6	7.1	97.1	102		
20:4n-6	1041 ± 63	6	1056 ± 24	2	996 ± 12	1	1049 ± 21	2	1.4	5.3	95.7	99.4	1.4	5.3	95.7	99.4		
22:4n-6	123 ± 9	7	122 ± 3	2	117 ± 0	0	124 ± 1.8	1	0.9	5.7	95.7	102	0.9	5.7	95.7	102		
22:5n-6	26.7 ± 2.1	8	27.0 ± 0.8	3	25.5 ± 0.4	2	26.6 ± 1.2	5	1.2	4.4	95.4	98.4	1.2	4.4	95.4	98.4		
18:3n-3	23.6 ± 2.5	10	25.1 ± 2.2	9	28.8 ± 2.2	8	28.9 ± 1.9	7	6.4	0.4	122	115	6.4	0.4	122	115		
20:5n-3	34.2 ± 1.8	5	34.8 ± 1.0	3	34.1 ± 0.6	2	37.1 ± 1.8	5	1.6	8.8	99.6	107	1.6	8.8	99.6	107		
22:5n-3	73.5 ± 4.3	6	72.5 ± 1.4	2	70.0 ± 0.8	1	73.2 ± 1.8	2	1.4	4.6	95.1	101	1.4	4.6	95.1	101		
22:6n-3	192 ± 11	6	191 ± 2	1	182 ± 1	1	190 ± 4.2	2	0.3	4.4	95.3	99.8	0.3	4.4	95.3	99.8		
20:3n-9	3.3 ± 0.2	5	3.7 ± 0.3	8	3.5 ± 0.1	3	3.4 ± 0.3	10	12	3.7	105	90.4	12	3.7	105	90.4		
Total FA	7070 ± 424	6	7250 ± 161	2	6758 ± 116	2	7170 ± 209	3	2.5	6.1	95.6	98.9	2.5	6.1	95.6	98.9		
Summary	<i>mol% of Total fatty acids</i>															<i>Percentage</i>		

Fatty acids	LR-FP			LR+FP			MW-FP			MW+FP			%Diff (+/-FP)			Recy (MW/LR)		
	mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		mean ± SD	CV		LR	MW		-FP	+FP	
Fatty acid concentrations (µmol/L)																		
Percentage																		
Saturates	35.9 ± 0.2	0		36.1 ± 0.4	1		35.8 ± 0.2	1		35.8 ± 0.3	1		0.6	0.1		99.6	99.2	
Mono	16.8 ± 0.1	0		16.6 ± 0.5	3		16.6 ± 0.0	0		16.5 ± 0.5	3		1.1	0.2		98.7	99.7	
n-6 PUFA	42.7 ± 0.1	0		42.7 ± 0.2	0		42.9 ± 0.1	0		43.0 ± 0.6	1		0.1	0.2		100	101	
n-3 PUFA	4.58 ± 0.1	1		4.49 ± 0.1	2		4.71 ± 0.1	2		4.63 ± 0.1	2		1.8	1.6		103	103	
Proportion																		
n-6/n-3 PUFA	9.3 ± 0.1	1		9.5 ± 0.2	2		9.1 ± 0.2	2		9.3 ± 0.1	1		2.0	1.8		98	98	
ARA/EPA	30.4 ± 0.4	1		30.4 ± 0.4	1		29.2 ± 0.4	1		28.3 ± 0.9	3		0.2	3.1		96	93	
n-3% HUFA	18.4 ± 0.1	0		18.2 ± 0.2	1		18.5 ± 0.2	1		18.4 ± 0.1	0		0.8	0.7		100	101	

Footnote is the same as Table 2 except n = 4 or 5 measurements.

Table 4
Fatty acid quantities in dried blood as determined by microwave assays in comparison to Lepage & Roy assays

Fatty acids	LR+FP (n=11)		MW+FP (n=30)		MW+FP (n=8)	
	Within-run precision		Recy (%)		Between-run	
	Concentration (μmol/L)		mol% Total FA			
14:0	40.4 ± 13.4	33	46.2 ± 9.3	20	114	0.30 ± 0.1
16:0	1415 ± 104	7	1550 ± 86	6	110	23.6 ± 0.8
18:0	698 ± 36	5	704 ± 45	6	101	10.7 ± 0.4
20:0	24.9 ± 1.2	5	19.4 ± 2.3	12	78	0.45 ± 0.01
22:0	91.5 ± 2.5	3	69.5 ± 3.9	6	76	1.48 ± 0.07
24:0	159 ± 6	4	116 ± 7	6	73	2.50 ± 0.13
16:1n-7	47.1 ± 3.5	7	51.6 ± 2.9	6	109	0.80 ± 0.02
18:1n-7	103 ± 5	5	118 ± 11	9	114	13.4 ± 1.4
18:1n-9	805 ± 62	8	775 ± 59	8	96	1.63 ± 0.0
20:1n-9	8.18 ± 0.8	9	8.92 ± 0.7	8	109	0.18 ± 0.01
24:1n-9	175 ± 5	3	123 ± 7	6	70	2.68 ± 0.12
18:2n-6	1402 ± 78	6	1414 ± 82	6	101	22.4 ± 0.6
18:3n-6	9.01 ± 0.9	10	8.68 ± 1.2	13	96	0.10 ± 0.02
20:2n-6	20.3 ± 1.7	8	23.9 ± 1.9	8	118	0.32 ± 0.01
20:3n-6	106 ± 4	4	106 ± 7	6	99	1.73 ± 0.06
20:4n-6	810 ± 40	5	796 ± 49	6	98	12.4 ± 0.6
22:4n-6	96.6 ± 4.9	5	94.2 ± 4.7	5	97	1.50 ± 0.09
22:5n-6	21.3 ± 0.9	4	19.7 ± 1.3	6	93	0.27 ± 0.02
18:3n-3	16.0 ± 0.8	5	17.1 ± 1.6	9	106	0.26 ± 0.02
20:5n-3	24.7 ± 1.2	5	22.5 ± 1.7	7	91	0.32 ± 0.05
22:5n-3	53.6 ± 3.1	6	50.5 ± 2.9	6	94	0.83 ± 0.06
22:6n-3	134 ± 7	6	131 ± 8	6	98	2.10 ± 0.14
Total FA	6285 ± 307	5	6278 ± 307	5	100	6349 ± 513
Summary	mol% Total FA					
Saturates	38.9 ± 0.7	2	40.1 ± 1.1	3	103	39.1 ± 1.2
Mono	18.1 ± 0.8	4	17.1 ± 0.6	3.6	95	18.7 ± 1.3

Fatty acids	LR+FP (n=11)		MW+FP (n=30)		Recy (%)	MW+FP (n=8)	
	Within-run precision		Within-run precision			Between-run	
	Concentration (μmol/L)		Concentration (μmol/L)			mol% Total FA	
n-6 PUFA	39.2 ± 0.9	2	39.2 ± 1.1	3	100	38.7 ± 1.2	3
n-3 PUFA	3.63 ± 0.2	5	3.52 ± 0.1	3.7	97	3.25 ± 0.2	7
n-6/n-3 PUFA	10.8 ± 0.4	4	11.2 ± 0.2	2	103	11.1 ± 0.5	5
n-3% HUFA	17.0 ± 0.4	2	16.7 ± 0.2	1	98	17.0 ± 0.4	2

Footnote is the same as Table 2; data were presented as mean ± SD and CV (%) ; sample sizes were presented in the column headings.

Table 5

Fatty acid compositions (mol%) of liquid blood and blood spots dried on BHT impregnated filter paper

Fatty acids	<u>Blood (0 h)</u>	<u>DBS (17 h)</u>	Recovery (%)
	Mean $\pm$ SD	Mean $\pm$ SD	
18:2n-6	23.2 $\pm$ 0.05	22.9 $\pm$ 0.11	98.5
18:3n-6	0.20 $\pm$ 0.00	0.20 $\pm$ 0.00	98.7
20:2n-6	0.17 $\pm$ 0.01	0.16 $\pm$ 0.00	94.6
20:3n-6	1.08 $\pm$ 0.01	1.06 $\pm$ 0.01	98.2
20:4n-6	9.35 $\pm$ 0.04	9.15 $\pm$ 0.03	97.8
22:4n-6	0.76 $\pm$ 0.01	0.76 $\pm$ 0.01	100
22:5n-6	0.44 $\pm$ 0.01	0.38 $\pm$ 0.01	87.2
18:3n-3	0.50 $\pm$ 0.00	0.48 $\pm$ 0.00	97.1
20:5n-3	0.96 $\pm$ 0.00	0.91 $\pm$ 0.01	94.5
22:5n-3	0.98 $\pm$ 0.01	0.97 $\pm$ 0.01	99.0
22:6n-3	3.57 $\pm$ 0.04	3.46 $\pm$ 0.02	97.0
Saturates	36.9 $\pm$ 0.2	37.4 $\pm$ 0.09	101
Monounsaturates	21.9 $\pm$ 0.1	22.2 $\pm$ 0.04	101
n-6 PUFA	35.2 $\pm$ 0.09	34.6 $\pm$ 0.07	98.2
n-3 PUFA	6.00 $\pm$ 0.04	5.82 $\pm$ 0.02	96.9
n-6/n-3 PUFA	5.86 $\pm$ 0.04	5.94 $\pm$ 0.03	101
n-3% HUFA	32.2 $\pm$ 0.12	32.0 $\pm$ 0.07	100

Data were presented as mean of n = 3 measurements of a pooled blood from 4 volunteers;

Blood (0 h) indicates blood that was frozen shortly after collection;

DBS (17 h) indicates dried blood spots on filter paper and kept at room temperature for 17 h;

Recovery (%) was defined as the percentage of fatty acids in DBS (17 h) to those in blood (0 h);

BHT, butylated hydroxytoluene

TABLE 6

Fatty acid profiles of dried blood from study subjects

Fatty acids	Fatty acid composition (mol%)	
	Mean SD	95% CI
14:0	0.62 ± 0.7	0.54, 0.71
16:0	19.6 ± 3.1	19.2, 19.9
18:0	10.9 ± 2.3	10.6, 11.2
20:0	0.66 ± 0.3	0.62, 0.70
22:0	1.77 ± 0.4	1.72, 1.82
24:0	2.68 ± 0.5	2.61, 2.74
16:1n-7	1.23 ± 0.7	1.15, 1.31
18:1n-9	18.0 ± 2.7	17.7, 18.3
18:1n-7	1.74 ± 0.3	1.70, 1.77
20:1n-9	0.19 ± 0.1	0.18, 0.20
24:1n-9	2.01 ± 0.5	1.95, 2.06
18:2n-6	22.6 ± 2.7	22.3, 22.9
18:3n-6	0.39 ± 0.3	0.35, 0.42
20:2n-6	0.33 ± 0.5	0.28, 0.39
20:3n-6	1.52 ± 0.4	1.47, 1.56
20:4n-6	9.49 ± 1.7	9.29, 9.69
22:2n-6	0.44 ± 0.4	0.39, 0.48
22:4n-6	1.50 ± 0.3	1.46, 1.54
22:5n-6	0.58 ± 0.5	0.52, 0.63
18:3n-3	0.51 ± 0.2	0.49, 0.53
20:5n-3	0.42 ± 0.4	0.38, 0.47
22:5n-3	1.18 ± 0.6	1.12, 1.25
22:6n-3	1.67 ± 0.5	1.61, 1.73
<i>Summary</i>		
Saturates	36.2 ± 3.8	35.8, 36.7
Mono	23.2 ± 3.0	22.8, 23.5
n-6 PUFA	36.8 ± 3.5	36.4, 37.2
n-3 PUFA	3.79 ± 1.0	3.68, 3.91
n-6/n-3 PUFA	10.2 ± 2.1	9.94, 10.4
n-3% HUFA	19.4 ± 4.3	18.9, 19.9

Data were presented as means ± SD (95 % Confidence Interval), n = 287 samples

Measured data were determined by microwave assay