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Short communication

Effect of storage temperature in a Cambodian field setting on the fatty acid composition in whole blood ☆

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ABSTRACT

Fatty acid analysis requires standardized collection and storage of samples, which can be a challenge under field conditions. This study describes the effect of storage temperature on fatty acid composition in two sets of whole blood samples collected from 66 children in a rural area in Cambodia. The samples were stored with butylated hydroxytoluene at −20 °C and −80 °C and the latter required extra transfers due to storage facility limitation. Fatty acid composition was analyzed by high-throughput gas-chromatography and evaluated by paired *t*-tests and Bland–Altman plots. Total amounts of fat in −20 °C and −80 °C samples did not differ, but there was relatively more highly unsaturated fatty acids (15.8 ± 2.7 vs. 14.4 ± 2.5%, $p < 0.001$) and a lower n-6/n-3 ratio (6.4 ± 1.4 vs. 6.9 ± 1.4, $p < 0.001$) in the −20 °C samples. Our results indicate that the importance of storage temperature should be evaluated in the context of storage facility availability and risk of temperature fluctuations during transport.

1. Introduction

The health consequences of specific types of fatty acids have been examined in developed countries for decades. Dietary studies in developing countries have focused mainly on total energy, animal protein and various micronutrients. The importance of essential fatty acid status and the quality of dietary fatty acids in these populations is not clearly defined [1,2]. High throughput methods for fatty acid analysis to examine fatty acid intakes and metabolism are commonly used and scientifically acceptable [3]. However, high throughput analysis still requires standardized collection and storage of blood samples, which is a challenge under field conditions in developing countries.

The use of whole blood samples for fatty acid analyses rather than erythrocytes or plasma can reduce sample processing time and can also enable field studies as centrifugation equipment is not required. However, decreases in polyunsaturated fatty acids (PUFA) levels in whole blood have been observed in samples stored above −75 °C [4]. Similar results have been observed in erythrocytes and are likely due to iron mediated peroxidation [5]. These experiments were, however, performed under controlled laboratory conditions, whereas the stability of samples collected and stored under field conditions in developing countries needs to be examined. This is necessary, in order to give guidelines for surveying fatty acid status in food insecure populations, as well as assessing the impacts of interventions aimed to improve health and nutritional status. The present study describes effects of storage temperatures on fatty acid composition in a field setting. The paper focus on the PUFA due to the potential importance in relation to infant brain development [6] and beneficial effects on cardiovascular risk in adults [7].

2. Materials and methods

All procedures and protocols in the study received clearance from the National Ethics Committee for Health Research Cambodia. Written and oral consent was gained from the caretaker who accompanied the infant to the sampling site.

Abbreviations: BHT, butylated hydroxytoluene; FA%, percent of all fatty acids; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids (≥ 20C and ≥ 3 double bonds); EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DFPTQ, Department of Fisheries Post-harvest Technologies and Quality Control Ministry of Agriculture Forestry and Fisheries Cambodia; IPH, Institute of Public Health Cambodia

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2.1. Participant and study design

The blood samples for this storage study were provided by children, aged 13–17 months-old from seven communes in the Prey Veng province, Kingdom of Cambodia at the Pea Reang health center between December 7th, 2011 and February 23rd, 2012. The children were a sub-group of participants enrolled in the 'Win-Food' intervention study [8], which aimed to assess the impact of daily provision of nutritionally improved complementary foods on growth and nutritional status in infants. The blood samples for the present study were aliquoted from blood samples collected at the endline visit of the intervention, nine months after enrollement. Excess blood for this storage study was available from 66 of the 397 children reporting for the endline visit.

2.2. Sample collection and storage

When respondents arrived at the Pea Reang health center, approximately 1 h prior to blood sampling, local anesthetic cream (Emla®) was applied to the left and right arms of the child. Blood samples were collected by trained local nurses. A few drops of blood were immediately used for hemoglobin analysis. The remaining blood was subsequently injected into vacutainers containing heparin, and shaken at least eight times to avoid coagulation. Aliquots of blood (500 μL), were pipetted into 1.5 mL-microtubes containing 50 μL of 0.1% Butylated hydroxytoluene (BHT) in ethanol for fatty acid analysis. The microtubes were quickly covered and stored in a −20 °C freezer. The procedure was then repeated to get a parallel set of samples from the same blood in heparin vacutainers. Although there were interuptions in the electrical supply a few times, the samples always remained frozen.

After the completion of a sampling round in the field (3–5 days), frozen samples were transported 66 km to the Department of Fisheries Post-harvest Technologies and Quality Control (DFPTQ) at the Ministry of Agriculture, Forestry and Fisheries in Phnom Penh. During the transport, which usually took 1–2 h, samples were kept in an insulated cooler box with ice and the internal temperature never rose to more than 5 °C. Upon arrival at DFPTQ, the first set of samples were placed in a −20 °C freezer and stored there until shipment to the laboratory in Canada for analysis.

For logistical reasons, the parallel set of sample aliquots were kept in the −20 °C freezer at DFPTQ for about a week. Subsequently, they were moved to a −80 °C freezer at the Institute of Public Health (IPH), 6 km from the DFPTQ office in Phnom Pehn. The samples were brought to −80 °C storage facility in three bathces, following the sampling schedule in the field. Overall, samples were kept at −80 °C storage for 10–17 weeks, depending on sampling date. The samples from the −80 °C storage in IPH were then transported back to −20 °C storage in DFPTQ a few days before shipment to Canada. During transport to and from IPH, samples were kept in an insulated ice box with ice bags and the journey typically took 30 min.

Both sets of samples were shipped by courier service (TNT Express Worldwide) from Phnom Penh to the University of Waterloo, Waterloo, Canada on June 11th 2012. Samples were packed in Styrofoam boxes with dry ice and the courier service checked and added dry ice during transport. The samples arrived in Waterloo on June 15th 2012, where they were stored at −75 °C until immediately before analysis. Storage time in the field prior to arrival in Canada were 4–6 months and total storage period before analysis varied from 9 to 11 months.

2.3. Fatty acid analysis

Fatty acids from 50 μL of thawed blood were directly transesterified by addition of 1 mL 14% boron-trifluoride in methanol, 300 μL hexane containing 50 μg/mL BHT and 33.33 μg/mL 22:3n-3 ethyl ester (internal standard, Nu-CheckPrep, Elysian, MN, USA), and heating at 95 °C on a heating block for 1 h [9,10]. The organic and aqueous layers were separated after the addition of 1 mL hexane and 1 mL water, and centrifugation at 1734 *rcf* for 5 min. The upper organic hexane layer containing the fatty acid methyl esters was collected, fully dried under a stream of nitrogen gas, reconstituted into 65 μL of hexane and stored in vials until analysis by gas chromatography.

A Varian 3900 gas chromatograph equipped with a DB-FFAP 15 m × 0.10 mm i.d. × 0.10 μm film thickness, nitroterephthalic acid modified, polyethylene glycol, capillary column (J & W Scientific, Agilent Technologies, Mississauga, ON) with hydrogen as the carrier gas [11], was used to analyze the fatty acid methyl esters. Fatty acid methyl esters in hexane (1 μL) were introduced by a Varian CP-8400 autosampler into the injector heated to 250 °C with a split ratio of 100:1. The initial oven temperature was 150 °C with a 0.25 min hold, followed by a 35 °C/min ramp to 200 °C, an 8 °C/min ramp to 225 °C with a 3.2 min hold and then an 80 °C/min ramp up to 245 °C with a 15 min hold at the end [12]. The flame ionization detector temperature was 300 °C with air and nitrogen make-up gas flow rates of 300 and 25 mL/min, respectively, and a sampling frequency of 50 Hz. An external mixed standard sample (GLC-462, Nu Chek Prep Inc., Elysian, MN, USA) was used to identify peaks based on retention time. Fatty acid composition data was expressed both quantitatively (concentrations) and qualitatively (relative weight percentages, percent of fatty acid, FA%).

2.4. Statistical analysis

All statistical analyses were performed by SPSS (Version 20.0.0, IBM Corp., Kgs. Lyngby, Denmark). Normality of the data was checked by manual inspection of histograms. Quantitative values for individual fatty acids and fatty acid groups are expressed as mean ± SD.

Differences were calculated for value in samples stored at −20 °C over value in −80 °C samples and relative difference compared to the mean of the two samples (%). Student paired *t*-test was used to compare the individual and grouped fatty acids in samples stored at −20 °C and −80 °C. Variation was visualized by principal component analysis as well as by Scatter plots and the Bland–Altman plots. Correlation coefficients were calculated by Pearsons. Significance was inferred at $p < 0.001$.

3. Results

The total fatty acid content of the samples (μg/dL) was not affected by storage temperature (Table 1). Correlation analysis showed significant relationships between the measured content of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and PUFA in the samples at the two storage conditions (both in absolute values and FA%) (data not shown). The amount of SFA was not significantly different between the two storage temperatures, but the relative amount (FA%) of MUFA was significantly lower in samples stored at −20 °C compared to samples stored at −80 °C (Table 1). Conversely, the concentration of total PUFA, both n-6 and n-3 PUFA and specifically the more highly unsaturated PUFA (HUFA) were significantly higher in samples stored at −20 °C (both in relative and absolute amount) and there was also a significant shift in the n-6/n-3 PUFA ratio.

The whole blood content of eicosapentaenoic acid (EPA, 20:5n-3)+docosahexaenoic acid (DHA, 22:6n-3) in the samples stored at −20 °C and −80 °C were correlated, but there was a consistent difference in the total concentration of DHA+EPA (Fig. 1(A)). Bland–Altman plots showed quite large variation in the concentrations of DHA+EPA, and the absolute differences increased with the mean