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ORIGINAL ARTICLE

EPA and DHA Levels in Whole Blood Decrease More Rapidly when Stored at −20 °C as Compared with Room Temperature, 4 and −75 °C

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Abstract High-throughput n-3 fatty acid profiling is enabled by collection techniques such as venous whole blood and fingertip prick (FTP) sampling, but the resulting increased sample numbers increases storage demand. Highly unsaturated fatty acids (HUFA) in erythrocytes are susceptible to oxidation, but this tendency is poorly characterized in venous and FTP whole blood. Presently, whole blood samples with low and high n-3 content collected with ethylenediaminetetraacetic acid were stored on chromatography paper with and without BHT pre-treatment for up to 180 days at different temperatures (room, 4, −20, −75 °C). Whole blood prepared with heparin and BHT and stored in cryovials was also examined. Eicosapentaenoic acid (EPA, 20:5n-3) + docosahexaenoic acid (DHA, 22:6n-3) is relatively stable when stored at −75 °C under various conditions but rapidly decreases in whole blood when stored at −20 °C. At −20 °C, BHT + heparin prepared whole blood can prevent decreases in cryovials up to 180 days but BHT only slows the decreases on chromatography paper. Surprisingly, whole blood stored at 4 °C and room temperature was less susceptible to decreases in EPA + DHA as compared with −20 °C storage. Assessments of n-3 blood biomarkers indicate the % n-3 HUFA in total HUFA was more stable as compared with the sum of the relative % of EPA + DHA. In conclusion, FTP and venous whole blood for fatty acid analysis should be stored at −75 °C whenever possible. In the absence of −75 °C storage conditions, BHT should be added and 4 °C or room temperature appear to be better alternatives to −20 °C.

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Abbreviations

ARA	Arachidonic acid
BHT	Butylated hydroxytoluene
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FTP	Fingertip prick
HUFA	Highly unsaturated fatty acid
PUFA	Polyunsaturated fatty acid

Introduction

Fingertip prick (FTP) blood and venous whole blood fatty acid analyses are fast and economical collection methods [1]. In FTP, blood is typically collected on an absorbent material such as chromatography paper and does not require a trained phlebotomist for administration. Simpler blood collection methods enable larger clinical and field studies to be carried out; however, this can generate larger sample sets that can challenge storage capacities. Stabilities of fatty acids in whole blood stored on chromatography paper and in cryovials have not been adequately assessed previously. Currently, only four studies have assessed the stability of fatty acids in FTP blood stored on paper [2–5] with one other assessing the stability of whole blood in vials [6]. Briefly, polyunsaturated fatty acid (PUFA) stability was determined to be between <2 and 8 weeks at room temperature, between 21 and 90 days at 4 °C and for

14 days at −20 °C. Whole blood PUFA appears to remain stable for less than 24 h at 24 °C, 24 h at 4 °C and longer than 30 days at −40 °C.

Prior studies assessing erythrocyte and plasma fatty acid stability are numerous with the existence of approximately ten storage studies for each blood fraction. This is reflective of the frequent use of these blood fractions for fatty acid profiling. More investigation is required to determine whole blood fatty acid storage stability on paper and in vials, and the present study aims to reconcile this void in the literature. Understanding the fatty acid stability of whole blood is crucial for application of high-throughput collection techniques. Comprehensive storage assessments are needed to guide medical, clinical and academic research professionals in practical aspects of sample collection and storage prior to analysis.

FTP fatty acid analysis has been used previously in infants [7, 8] and the elderly [9] among others [2, 10–12], and the accuracy of FTP fatty acid analysis is comparable to other venous blood fractions [2, 9, 12, 13]. Similarly, venous whole blood sampling is a simpler blood fraction for fatty acid analysis compared to erythrocyte and plasma analysis as it does not require centrifugation to obtain, but can still be used for more extensive lipid analyses. Although erythrocyte and plasma fatty acid profiling remain the most popular blood fractions analyzed, they are tedious and can dissuade the inclusion of fatty acid assessments during large population cohort studies. Increased eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) in whole blood is associated with decreased risk of sudden cardiac death [14] and coronary heart disease [15], and limitations of standard fatty acid analytical protocols can be minimized with the collection and storage of whole blood samples on paper or in vials for fatty acid analyses.

Therefore, the goal of the present study is to provide a comprehensive analysis of n-3 highly unsaturated fatty acids (HUFA), particularly EPA + DHA, and n-3 biomarker stability in venous whole blood stored on paper and cryovials during long-term storage. Blood from one individual was used to allow a mechanistic focus on numerous storage effects. Storage variables examined included temperature [room temperature (RT), 4, −20 and −75 °C], time (1–180 days), anticoagulant [heparin or ethylenediaminetetraacetic acid (EDTA)], and presence of antioxidant [butylated hydroxytoluene (BHT)]. Blood n-3 status (low and high) on oxidation rates was also examined through a fish oil intervention in the single participant. Briefly, n-3 biomarkers and concentrations of EPA + DHA are most stable during −75 °C storage and least stable at −20 °C in low and high n-3 blood. Degradation can be prevented or reduced with BHT treatment and storage at 4 °C or room temperature may be better alternatives when −75 °C storage is not available. In addition, blood high in n-3 HUFA may be slightly less susceptible to loss of EPA + DHA over the short term.

Materials and Methods

Participant and Study Design

All procedures and protocols in this study received clearance from the University of Waterloo Human Ethics Committee. Blood used in the study was provided by one 29 year old male participant. The participant provided fasting blood on two occasions for storage studies that corresponded to before and after a fish oil supplement intervention. The participant consumed fish oil supplements that provided approximately 3 g per day EPA (2 g) + DHA (1 g) as ethyl esters (Ocean Nutrition Canada, Dartmouth, NS) for 60 days. The fish oil intervention was considered successful as the n-3 status of the participant more than doubled as determined by the % n-3 HUFA in total HUFA n-3 biomarker. For each blood storage condition, fresh blood samples were used as control and are indicated as day 0 of storage.

Whole Blood Storage on Chromatography Paper

Venous whole blood samples before (low n-3) and after fish oil supplementation (high n-3) were collected from the antecubital vein into an EDTA-lined vacutainer by a trained technician. Fresh whole blood from both low and high n-3 conditions (25 μL) was pipetted to an area of ~ 1 cm^2 onto pre-washed (2:1 chloroform:methanol), 1 × 4 cm chromatography paper strips (Grade 3MM Chr, Whatman Ltd., Sanford, ME). The application area had been pre-treated with either 0 or 50 μg of BHT in methanol (2 μg/μL) per sample, and methanol was allowed to dry for 10 min prior to application of blood. Paper strips were prepared fresh prior to each blood collection. Blood was stored after being saturated fully into the paper and prior to drying. This approach controlled for the volume of blood applied to the chromatography strips thereby allowing quantitative fatty acid analyses, but still using the storage and analytical protocol for FTP blood. We have compared the use of venous whole blood on paper in long-term storage relative to a standard capillary FTP sampling method, and no differences in the fatty acid profiles between the two methods were detected (unpublished data). Subsequently, samples were analyzed for fatty acid composition immediately after application (time 0) or after storage for 1, 3, 7, 14, 21, 30, 60, 90, 120, 150 and 180 days at either room temperature, 4, −20 or −75 °C. Fatty acid analysis for all time points and conditions was performed in triplicate.

Whole Blood Storage in Cryovials

Venous whole blood samples collected from the participant after fish oil supplementation were also examined using three separate anticoagulant conditions, including: (1) an ethylenediaminetetraacetic acid (EDTA)-lined vacutainer, (2) a heparin-lined vacutainer, (3) a heparin-lined vacutainer with additional BHT added prior to storage. The whole blood samples from EDTA and heparin vacutainers were separated into 500-μL aliquots and stored at room temperature, 4, −20 and −75 °C for up to 180 days in cryovials. In the heparin + BHT condition, 50 μg of BHT in methanol (2 μg/μL) was added to the aliquot just prior to storage [16–18]. This storage condition was designed to provide maximum protection against fatty acid peroxidation in which peroxidation protection is provided by both BHT and heparin [19, 20].

Fatty Acid Analysis of Blood Samples

Whole blood from vial and on chromatography paper samples (25 μL each) were directly transesterified with the addition of 1 mL of 14 % BF_3 in methanol, 300 μL of hexane and 3 μg of an internal standard (22:3n-3 ethyl ester; Nu-Check Prep, Elysian, MN, USA) [4, 21], and heating at 95 °C for 1 h in a heating block. Organic and aqueous phases were then separated and the organic upper hexane layer containing fatty acid methyl esters was collected, dried under a stream of nitrogen, reconstituted in hexane and stored in a GC vial until analysis.

Fatty acid methyl esters were analyzed on a Varian 3900 gas chromatograph (GC) 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 and W Scientific from Agilent Technologies, Mississauga, ON) with hydrogen as the carrier gas [22]. Samples (2 μL) were introduced by a Varian CP-8400 autosampler into the injector heated to 250 °C with a split ratio of 200:1. The initial 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 [23]. 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. Peaks were identified by retention times through comparison to an external mixed standard sample (GLC-462, Nu Chek Prep Inc., Elysian, MN, USA).

Multi-participant Whole Blood Storage on Chromatography Paper

An additional ten healthy university students, including five males (21.6 years ± 2.1, mean ± SD) and 5 females (23.6 years ± 3.6) were recruited. Fasting blood samples were collected by a trained technician into an EDTA-lined vacutainer. Whole blood samples (25 μL) were applied to washed chromatography strips without BHT as described above and stored at −20 or −75 °C. Briefly, a single fresh sample was analyzed as control, and samples were removed from storage after 3, 7, 14 and 30 days and fatty acids were analyzed by gas chromatography after direct transesterification.

Statistical Analyses

Concentrations (μM) of fatty acids in whole blood were determined using the internal standard. Relative weight percentages and various n-3 blood biomarkers were also determined to enable comparison to other studies in the literature examining fatty acid profiling. All statistical analyses assessing the effects of time, antioxidant/anticoagulant treatment and storage temperature were assessed individually by one-way ANOVA. Statistical analyses for the multi-subject storage condition were performed by repeated measures one-way ANOVA. Post hoc tests were performed using Tukey's Honestly Significant Difference test and statistical significance was inferred as $P < 0.05$ after a significant F-value as determined by the one-way ANOVA. SPSS for Windows statistical software (release 11.5.1; SPSS Inc., Chicago, IL, USA) was used. Data are presented as the means ± SD.

Results

Initial Fatty Acid Compositions

Fatty acid compositions were determined before and after fish oil supplementation for 2 months without storage (Table 1). The n-3 blood biomarkers, the % EPA + DHA and the % n-3 HUFA in total HUFA increased by 297 and 215 %, respectively, and the n-6/n-3 ratio decreased by 61 % following supplementation. The relative percentage of six of seven n-6 PUFA determined decreased significantly following supplementation, with linoleic acid (18:2n-6) decreasing by 11 % and arachidonic acid (20:4n-6) decreasing by 18 %. The concentrations of these two fatty acids, however, did not change as the concentration of total fatty acids in the blood spots increased with fish oil supplementation. The relative percentage of EPA, n-3 docosapentaenoic acid (DPAn-3, 22:5n-3), DHA and total n-3 PUFA increased by 673, 31, 92 and 117 %, and concentrations (μM) increased by 854, 62, 138 and 174 %, respectively. The relative percentage of arachidonic acid, EPA, DPAn-3, DHA and n-3 PUFA were significantly higher in whole blood stored in cryovials compared with blood stored on chromatography paper.

Table 1 Fatty acid compositions before and after 4 weeks of fish oil supplementation

Fatty acid	Low n-3 blood on paper Weight percentage	High n-3 blood on paper	High n-3 blood in cryovial	Low n-3 blood on paper Concentration, μM	High n-3 blood on paper	High n-3 blood in cryovial
C 14:0	0.43 ± 0.04[a]	0.76 ± 0.01[b]	0.55 ± 0.04[c]	59.6 ± 7.7[a]	131 ± 5[b]	75.4 ± 4.2[c]
C 16:0	17.9 ± 0.3[a]	20.4 ± 0.2[b]	20.4 ± 0.4[b]	2,212 ± 206[a]	3,156 ± 155[b]	2,491 ± 31[a]
C 17:0	0.26 ± 0.02[a]	0.35 ± 0.01[b]	0.32 ± 0.01[b]	31.5 ± 4.3[a]	51.8 ± 1.3[b]	38.1 ± 0.2[c]
C 18:0	11.4 ± 0.2	12.1 ± 0.7	11.3 ± 0.4	158 ± 17[a]	209 ± 9[b]	155 ± 1[a]
C 20:0	0.43 ± 0.12	0.36 ± 0.02	0.25 ± 0.02	43.2 ± 11.7[ab]	45.6 ± 3.3[a]	25.4 ± 1.7[b]
C 22:0	1.06 ± 0.06[a]	1.00 ± 0.05[a]	0.70 ± 0.06[b]	98.6 ± 4.1[a]	117 ± 11[b]	64.6 ± 3.9[c]
C 23:0	0.26 ± 0.05	0.28 ± 0.03	0.20 ± 0.02	23.3 ± 2.2[a]	30.7 ± 2.1[b]	17.8 ± 1.5[c]
C 24:0	2.19 ± 0.12[a]	1.99 ± 0.06[a]	1.54 ± 0.17[b]	188 ± 12[a]	214 ± 15[a]	131 ± 11[b]
SFA	37.0 ± 0.3	38.6 ± 0.7	38.2 ± 1.2	3,177 ± 274[a]	4,155 ± 176[b]	3,337 ± 10[a]
C 16:1	0.57 ± 0.02[a]	0.71 ± 0.01[b]	0.55 ± 0.04[a]	70.6 ± 8.3[a]	111 ± 6[b]	67.5 ± 7.4[a]
C 18:1n-7	1.70 ± 0.06[a]	1.51 ± 0.01[b]	1.59 ± 0.02[b]	191 ± 20[ab]	212 ± 7[a]	176 ± 5[b]
C 18:1n-9	15.2 ± 0.3[a]	15.0 ± 0.1[a]	13.9 ± 0.4[b]	1,705 ± 155[a]	2,099 ± 93[b]	1,542 ± 87[a]
C 20:1n-9	0.22 ± 0.01[a]	0.19 ± 0.01[b]	0.21 ± 0.01[ab]	22.9 ± 1.3	24.2 ± 2.2	21.0 ± 0.9
C 22:1n-9	0.21 ± 0.02[a]	0.43 ± 0.02[b]	0.17 ± 0.05[a]	19.6 ± 1.7[a]	49.8 ± 0.3[b]	15.8 ± 4.3[a]
C 24:1n-9	2.25 ± 0.09[a]	2.11 ± 0.09[a]	1.58 ± 0.18[b]	194 ± 11[a]	228 ± 18[a]	135 ± 11[b]
MUFA	20.7 ± 0.4[a]	20.1 ± 0.2[a]	18.5 ± 0.2[b]	2,267 ± 195[a]	2,755 ± 125[b]	2,012 ± 86[a]
C 18:2n-6	20.5 ± 0.4[a]	18.2 ± 0.3[b]	18.2 ± 1.2[b]	2,314 ± 154[ab]	2,568 ± 131[a]	2,037 ± 196[b]
C 18:3n-6	0.17 ± 0.10	0.22 ± 0.01	0.15 ± 0.02	18.6 ± 10.8	31.7 ± 1.6	16.7 ± 2.9
C 20:2n-6	0.26 ± 0.02[a]	0.19 ± 0.01[b]	0.21 ± 0.01[b]	26.3 ± 2[a]	24.3 ± 1.4[ab]	21.5 ± 0.4[b]
C 20:3n-6	1.57 ± 0.05[a]	1.17 ± 0.02[b]	1.30 ± 0.03[c]	162 ± 17[a]	151 ± 5[ab]	133 ± 4[b]
C 20:4n-6	11.0 ± 0.3[a]	8.99 ± 0.17[b]	10.6 ± 0.1[a]	1,146 ± 103	1,170 ± 72	1,094 ± 27
C 22:4n-6	1.70 ± 0.10[a]	1.01 ± 0.02[b]	1.28 ± 0.04[c]	162 ± 17[a]	120 ± 6[b]	120 ± 1[b]
C 22:5n-6	0.37 ± 0.03[a]	0.23 ± 0.02[b]	0.26 ± 0.01[b]	34.9 ± 0.1[a]	27.0 ± 3.0[b]	24.7 ± 1.0[b]
n-6 PUFA	35.6 ± 0.9[a]	30.1 ± 0.4[b]	32.1 ± 1.0[b]	3,872 ± 279[ab]	4,102 ± 216[a]	3,454 ± 227[b]
C 18:3n-3	0.37 ± 0.02[ab]	0.41 ± 0.01[a]	0.34 ± 0.02[b]	42.6 ± 1.9[a]	57.6 ± 3.6[b]	38.5 ± 3.2[a]
C 20:3n-3	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	4.5 ± 1.3	6.8 ± 2.0	4.7 ± 0.4
C 20:5n-3	0.37 ± 0.02[a]	2.86 ± 0.06[b]	3.07 ± 0.07[c]	39.2 ± 3.5[a]	374 ± 21[b]	319 ± 17[c]
C 22:5n-3	1.37 ± 0.05[a]	1.79 ± 0.02[b]	2.13 ± 0.05[c]	132 ± 8[a]	214 ± 12[b]	202 ± 4[b]
C 22:6n-3	1.64 ± 0.11[a]	3.15 ± 0.03[b]	3.92 ± 0.09[c]	159 ± 20[a]	379 ± 19[b]	374 ± 3[b]
n-3 PUFA	3.81 ± 0.17[a]	8.25 ± 0.13[b]	9.51 ± 0.05[c]	377 ± 34[a]	1,032 ± 56[b]	938 ± 27[b]
PUFA	39.4 ± 1.0[ab]	38.3 ± 0.5[a]	41.6 ± 1.0[b]	4,249 ± 312[a]	5,134 ± 272[b]	4,392 ± 253[a]
HUFA	18.1 ± 0.7[a]	19.2 ± 0.3[b]	22.6 ± 0.2[c]	1,840 ± 168[a]	2,442 ± 135[b]	2,271 ± 55[b]
EPA + DHA	2.02 ± 0.13[a]	6.00 ± 0.09[b]	6.99 ± 0.02[c]	198 ± 23[a]	753 ± 40[b]	693 ± 20[b]
n-6/n-3	9.35 ± 0.25[a]	3.65 ± 0.01[b]	3.38 ± 0.12[b]	10.3 ± 0.3[a]	4.0 ± 0.1[b]	3.7 ± 0.1[b]
% n-3 HUFA	19.0 ± 0.2[a]	40.8 ± 0.2[b]	40.5 ± 0.2[b]	18.2 ± 0.2[a]	39.9 ± 0.2[b]	39.6 ± 0.2[b]
Total	97.0 ± 1.5	97.1 ± 0.1	98.2 ± 0.1	9,693 ± 779[a]	12,043 ± 570[b]	9,740 ± 346[a]

Different letters within fatty acid values indicates significant differences between blood fractions as determined by Tukey's HSD post hoc test following a significant *F*-value by One way ANOVA

Data presented as means ± SD

SFA saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid, *HUFA* highly unsaturated fatty acid, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid

Changes in EPA + DHA During Storage of Whole Blood

Storage of Low n-3 and High n-3 Blood on Chromatography Paper

The largest decline of EPA + DHA in whole blood stored on chromatography paper was observed at the −20 °C temperature without BHT in low and high n-3 status as compared with all other conditions (Fig. 1). EPA + DHA in low n-3 blood on paper decreased from 198 ± 23 μM at day 0 to 61 ± 6 μM after 1 day and 21 ± 2 μM after 3 days and in high n-3 blood on paper EPA + DHA decreased from 753 ± 40 μM at day 0 to 649 ± 30 μM after 3 days, 114 ± 26 μM after 14 days and 86 ± 23 μM after 180 days of storage. Pre-treatment of the paper with BHT delayed EPA + DHA decreases in low n-3 blood to 157 ± 11 μM after 60 days and to 145 ± 11 μM after 180 days of storage and in high n-3 blood 588 ± 43 μM after 14 days of storage with additional decreases to 433 ± 22 μM after 180 days.

In low n-3 blood on chromatography paper without BHT, EPA + DHA decreases significantly after 120 days at room temperature and 14 days at 4 °C. Pre-treatment with BHT does not delay EPA + DHA decline at room temperature; however, BHT prevents a significant decrease from occurring at 4 °C until 60 days of storage (161 ± 13 μM). The EPA + DHA in high n-3 blood on paper stored without BHT decreased significantly to 619 ± 9 at room temperature and 619 ± 24 μM at 4 °C after 60 days. BHT pre-treatment had no effect on EPA + DHA in high n-3 blood at room temperature, but did prevent significant losses in EPA + DHA in high n-3 blood at 4 °C until 150 days of storage (576 ± 71 μM). At −75 °C, lower EPA + DHA was observed at day 7 (665 ± 18 μM) and day 180 (641 ± 30 μM) of storage in the high n-3 blood on paper without BHT only.

Chromatography Paper Versus Cryovial Storage

Changes in EPA + DHA in whole blood stored on chromatography paper versus stored in a cryovial at different temperatures were examined with the high n-3 blood (Fig. 2). During storage at −20 °C, EPA + DHA of whole blood in cryovials was higher compared with on chromatography paper across all time points between 7 and 180 days. The largest difference was 367 μM after 30 days and the smallest was 46 μM after 180 days. At room temperature EPA + DHA in whole blood stored on paper was 173 μM higher compared with storage in cryovials after 14 days and 168 μM after 60 days of storage, while at 4 °C, EPA + DHA on paper was higher for all storage days assessed between 7 (67 μM) and 60 (88 μM) days. These differences are reversed or lost completely after 180 days of storage. At −75 °C, EPA + DHA was higher on chromatography paper at 60 days by 159 μM and lower by 64 μM at 180 days of storage.

Anticoagulant and Antioxidant Effects on Storage in Cryovials

Storing whole blood in cryovials with heparin anticoagulant plus BHT maintains EPA + DHA concentrations as compared with EDTA anticoagulant at all temperatures assessed (Fig. 3). The protective effect of heparin plus BHT was particularly obvious with storage at −20 °C. In addition, there were no significant differences in EPA + DHA between baseline and 180 days of all storage temperatures with heparin + BHT, although there were some significant differences at day 7 and day 60 at room temperature and 4 °C storage conditions. EPA + DHA responses with EDTA and heparin alone treatments were largely similar; expect heparin alone prevented less loss of EPA + DHA over 180 days at room temperature.

Changes in n-3 Blood Biomarkers During the Storage of Whole Blood

Given the increasing use of n-3 blood biomarkers in clinical studies, the effects of all storage conditions on the sum of the relative weight percentage of EPA and DHA or "n-3 index" [24] and the percentage of n-3 HUFA in total HUFA [25, 26] were examined in the high n-3 blood. Storage of whole blood on chromatography paper with and without BHT for 180 days at −75 °C did not change the % EPA + DHA (Table 2). The % EPA + DHA in whole blood on paper at both room temperature and 4 °C began to decrease significantly (−13 %) at day 60 of storage. Pre-treatment of paper with BHT delayed decreases in % EPA + DHA at −20 °C until day 14 (−7 %) as compared with day 7 without BHT (−20 %). The total decline after 180 days of storage at −20 °C was also higher without BHT (−85 %) as compared with BHT treated paper (−22 %). EPA + DHA in whole blood stored in cryovials was decreased significantly at the first measured time point for all storage temperatures when collected with EDTA, and at room temperature and 4 °C when collected with heparin and heparin + BHT. At −20 °C, EPA + DHA in cryovial blood at day 180 decreased 65 % when prepared with EDTA and 48 % when prepared with heparin, but only 7 % when prepared with heparin + BHT.

Similar to the relative weight % EPA + DHA, the % n-3 HUFA in total HUFA decreased significantly after 60 days of storage at room temperature (−4.4 and −4.4 %) and 4 °C (−2.9 and −5.1 %) with and without BHT, respectively (Table 3). The % n-3 HUFA in total HUFA in

blood on chromatography paper was not affected by storage at −75 °C. The % n-3 HUFA in total HUFA was lower when stored on paper without BHT at −20 °C between day 14 and day 90, but returned to baseline at day 180. Treatment of paper with BHT delayed this decrease with −20 °C storage until day 60 (−7.8 %) with values remaining significantly lower after 180 days.

In whole blood in cryovials with EDTA, the decreases in the % n-3 HUFA in total HUFA were significant at day 14 at room temperature, day 60 at 4 °C (−4.4 %) and day 30 at −20 °C (−4.2 %). In whole blood stored with heparin, the % n-3 HUFA in total HUFA was lower at day 60 (−4.4 %) and day 180 (−4.9 %) at room temperature, and day 60 at 4 °C (−3.9 %). The % n-3 HUFA in total HUFA in the heparin plus BHT condition was largely stable across all temperatures although day 60 at room temperature (−2.5 %) and day 180 at −75 °C, were slightly, but significantly lower.

Changes in EPA + DHA During Storage of Whole Blood from Multiple Participants

The rapid loss of EPA and DHA in blood stored on chromatography paper at −20 °C in comparison with −75 °C was confirmed in an additional ten participants. The n-3 status of the participants ranged from 19.8 to 36.4 % n-3 HUFA in total HUFA (27.1 ± 5.0, mean ± SD) or 2.5–5.0 % of EPA + DHA in total fatty acids (3.5 ± 0.8) (data not shown). There was a consistent pattern of decreases in the concentrations of EPA + DHA for all participants, with concentrations at day 14 and day 30 with −20 °C storage being significantly lower (Fig. 4). Conversely, no significant changes in EPA + DHA occurred during storage at −75 °C. The pattern of decreases in EPA + DHA was reflected in concentrations of EPA and DHA when plotted individually, with similar statistical results.

Discussion

Results of this study indicate that whole blood samples stored on chromatography paper or in cryovials are best preserved at −75 °C for the purpose of fatty acid profiling, regardless of the n-3 status of the blood samples. However, these storage conditions are not always available to the academic or clinical researcher, and as such, whole blood fatty acid stability with other storage methods need to be considered and adequately assessed across various conditions. Freezing at −20 °C is often considered an alternative if −75 °C storage is not available, but our findings indicate that the storage of blood samples at −20 °C is the most detrimental to HUFA stability even in comparison to refrigeration at 4 °C and storage at room temperature. The degradation of n-3 HUFA at −20 °C has been reported previously in erythrocyte samples [16–18] and in FTP samples stored in the presence of BHT for 28 days [5]. Although the focus of present study was restricted to one subject, we confirmed excessive degradation of EPA + DHA in whole blood stored on chromatography paper at −20 °C in an additional ten participants. Degradation of unsaturated fatty acids has also been observed in erythrocytes stored at −20 °C in a large multi-subject pool from the Women's Health Initiative study [27]. Interestingly, the present findings indicate that the different n-3 biomarkers may be more or less susceptible to the degradation of unsaturated fatty acids. Specifically, the sum of the relative weight percentages of EPA and DHA is susceptible to decreases during all storage conditions, while the percentage of n-3 HUFA in total HUFA appears relatively robust. This phenomenon is largely due to the relative similar degradation of individual HUFA, and slower degradation of less unsaturated fatty acids and the inclusion of only HUFA in the % n-3 HUFA biomarker [4]. The similar degradation of HUFA was confirmed in this study as the degradation patterns of EPA and DHA individually from the ten participants were very similar (Fig. 4).

The stability of fatty acids during storage is important to understand. Previously, only one study has assessed whole blood PUFA stability [6], and determined the stability of unesterified or free PUFA to be less than 24 h at 24 °C, 24 h at 4 °C and at least 1 month at −40 °C. The composition of free fatty acids in whole blood is not assessed frequently for fatty acid profiling, making stability determinations from this study difficult to extrapolate. PUFA stability in FTP has been assessed previously [2–5], and in each case BHT was used as an antioxidant for the prevention of fatty acid peroxidation. BHT-treated FTP samples remain stable for <2 months at RT, and for between 3 weeks and 3 months at 4 °C. These determinations are in relatively good agreement with our findings that EPA + DHA (μM) in whole blood stored on chromatography paper is stable between 30 and 90 days at room temperature with or without BHT, and 7–30 days at 4 °C without BHT, and 30–120 days at 4 °C with BHT (Fig. 1). These ranges in stability appear to be dependent on the n-3 content of the blood samples being stored. Interestingly, the pre-treatment of chromatography paper with BHT does not extend the stability of EPA + DHA under room temperature conditions, and may indicate a non-peroxidation related mechanism of HUFA loss such as thermal degradation. The prevalence of thermal fatty acid degradation during storage at room temperature has not been proposed previously. Fatty acid losses at room temperature have previously been reported to occur in <24 h in whole blood

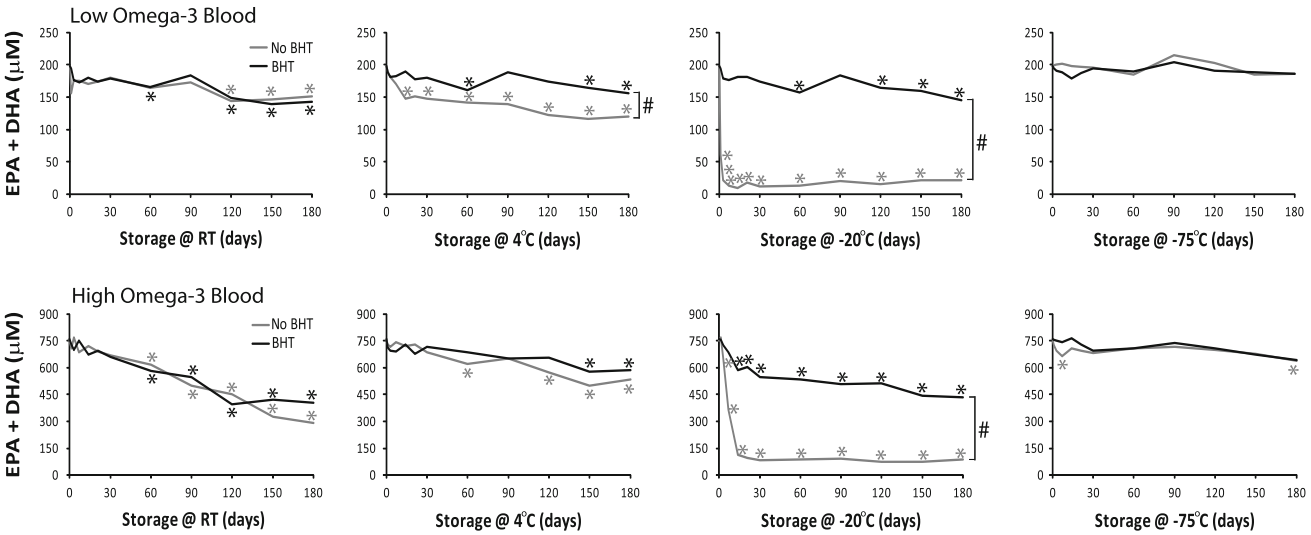

Fig. 1 Concentrations of EPA + DHA in whole blood when stored on chromatography paper with and without butylated hydroxytoluene (*BHT*) at different temperatures for 180 Days. *Indicates fatty acid values significantly lower than baseline (day 0) as determined by Tukey's HSD post hoc test following a significant *F*-value by one-way ANOVA. Data presented as means. *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *FTP* fingertip prick, *RT* room temperature

[6] and plasma [28] and in under 2 months in FTP whole blood in the presence of BHT [3].

To our knowledge, this is the first study to assess the stability of whole blood samples stored on chromatography paper at −75 °C, and only the second at −20 °C. As expected, storage at −75 °C results in the least amount of change in EPA + DHA. Conversely, storage at −20 °C results in rapid declines in EPA + DHA from baseline values that were greater than declines at 4 °C and room temperature. The mechanism for the accelerated degradation of EPA and DHA at −20 °C is unknown, but may involve disruption of the erythrocyte structure with freezing and/or −20 °C not being cold enough to suppress oxidative processes as BHT seems to partially prevent the effect. In low n-3 blood, the EPA + DHA stability at −20 °C was <1-day. Initially, EPA + DHA in high n-3 blood decreased less as compared with low n-3 blood stored on chromatography paper in both absolute (−104 vs. −177 μM) and relative (−14 vs. −89 %) amounts from baseline to day 3. This suggests a slower rate of HUFA degradation in the high n-3 blood despite a higher unsaturation level following fish oil supplementation. Usually, an increase in fatty acid unsaturation of whole blood samples is expected to increase susceptibility to lipid peroxidation. However, osmotic fragility, a marker of hemolytic susceptibility, has been positively associated with erythrocyte n-6 PUFA content [29], and is frequently used as a measure of erythrocyte integrity [30, 31]. In addition, diets with high n-3 PUFA content decrease the osmotic fragility of erythrocytes from humans [32] and rats [33]. These changes suggest a more stable erythrocyte membrane that may reduce fatty acid susceptibility to peroxidation during storage as a result of fish oil supplementation. Alternatively, osmotic fragility is also affected by erythrocyte vitamin E [34] and vitamin C [35] blood content. Vitamin E supplementation in pigs is shown to improve oxidative stability of fatty acids in pork meat during storage [36]. High levels of vitamin A, D and E in fish oils [37] provides an alternative explanation for the reduction in degradation of EPA + DHA in high n-3 whole blood samples.

EPA + DHA in blood stored in cryovials demonstrate a similar decline when stored at −20 °C as compared with blood stored on chromatography paper (Fig. 2). The cryovial response, however, reflects a more linear decrease as compared with the exponential decrease observed in chromatography paper that plateaued after ~30 days. The slower decrease rate in EPA + DHA and n-6 PUFA (data not shown) in cryovials as compared with chromatography paper may be related to the sample volume and surface area. Presently, blood in cryovials was stored in 500-μL aliquots, compared with 25-μL aliquots on chromatography paper. A larger volume-to-surface ratio has previously been shown to slow the rate of fatty acid peroxidation in erythrocytes [27], and may be indicative of reduced oxygen exposure in higher volume blood samples. Briefly, the aforementioned study determined weekly decreases in erythrocyte HUFA content of 3.5 % for 250-μL and 5.9 % for 80-μL aliquots during the first two weeks of storage. This is compared to weekly decreases in the EPA + DHA content of whole blood of 6.0 % for 500-μL cryovial aliquots and 21.5 % paper aliquots presently. Although differences between the two studies in collection and handling protocols exist, a comparison of the two studies suggests

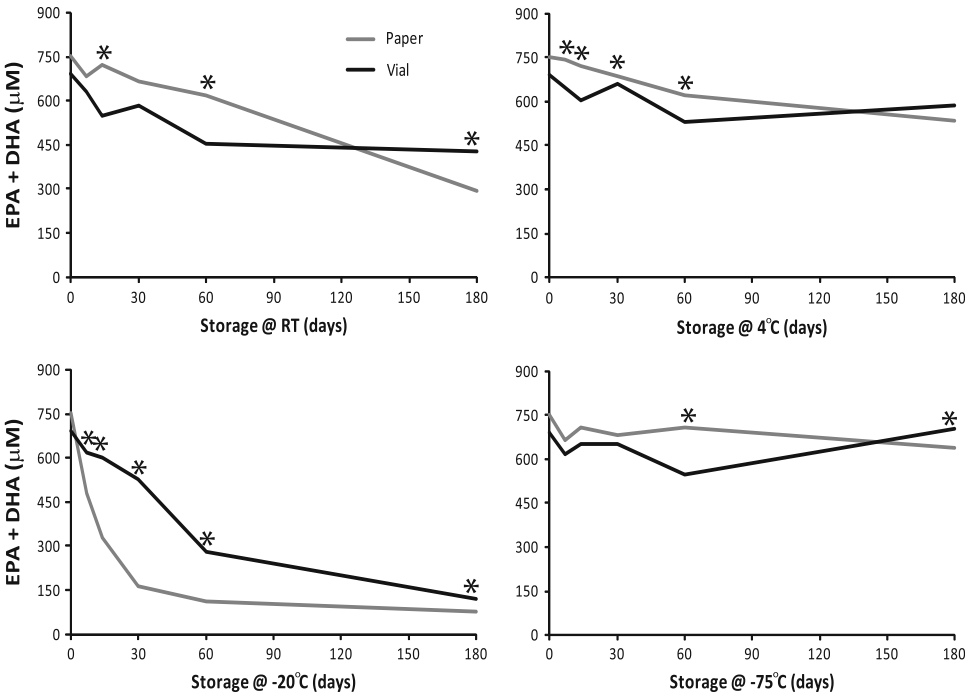

Fig. 2 Differences between EPA + DHA concentrations in whole blood when stored on chromatography paper and in cryovials at different temperatures for 180 Days. *Indicates fatty acid values significantly different within time points between paper and cryovial storage conditions as determined by Tukey's HSD post hoc test following a significant *F*-value by one-way ANOVA. Data presented as means. *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *RT* room temperature

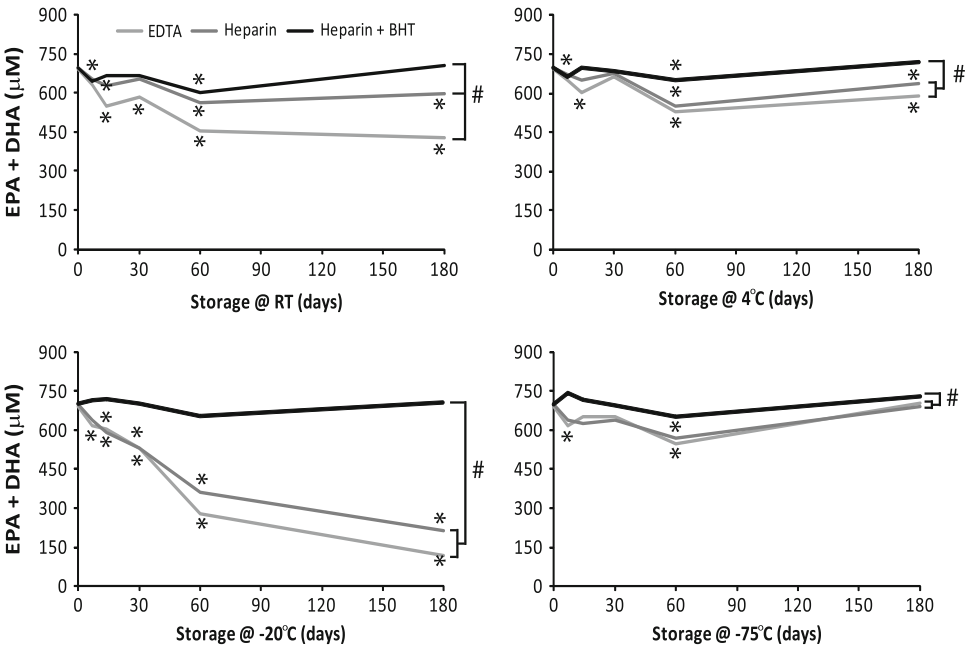

Fig. 3 The effects of anticoagulant and/or antioxidant on concentrations of EPA + DHA in whole blood stored in cryovials at different temperatures for 180 Days. #Indicates a significant main effect of anticoagulant and/or antioxidant treatment by one-way ANOVA. *Indicates EPA + DHA concentration significantly lower than baseline (day 0) as determined by Tukey's HSD post hoc test following a significant *F*-value by one-way ANOVA. Data presented as means. *BHT* butylated hydroxytoluene, *DHA* docosahexaenoic acid, *EDTA* ethylenediaminetetraacetic acid, *EPA* eicosapentaenoic acid, *RT* room temperature

that whole blood may be more susceptible to degradation than erythrocytes and storage on paper greatly increase the degradation process.

The relative weight % of EPA + DHA (Table 2) and the % n-3 HUFA in total HUFA blood biomarkers (Table 3) demonstrate an interesting responses to storage. Specifically, the % n-3 HUFA in total HUFA decreases after 14 days in blood stored on paper and after 30 days in EDTA-treated blood in cryovials. In each of these storage conditions, the % n-3 HUFA in total HUFA biomarker trends back towards baseline values at 180 days of storage, with storage on paper becoming statistically equal to baseline. This has been demonstrated previously in blood stored on paper and exposed to air at room temperature [4]. This response is indicative of an earlier and faster degradation of the more peroxidizable n-3 fatty acids. The higher degree of unsaturation of n-3 fatty acids relative to n-6 fatty acids generally results in greater peroxidation [38]. Decreases in the relative weight % of EPA + DHA in EDTA-treated whole blood are demonstrated during the

Table 2 Weight percent of EPA + DHA during storage of blood for 180 days

Storage vessel	Condition	Temperature	Storage day							
			0	3	7	14	30	60	90	180
Paper	No BHT	RT	6.00 ± 0.09	6.04 ± 0.05	6.12 ± 0.09	6.18 ± 0.15	5.90 ± 0.28	5.22 ± 0.18*	4.71 ± 0.26*	3.62 ± 0.53*
		4 °C		5.96 ± 0.08	6.24 ± 0.08	6.23 ± 0.06	6.07 ± 0.03	5.21 ± 0.09*	5.41 ± 0.20*	4.88 ± 0.45*
		−20 °C		5.59 ± 0.12	4.78 ± 0.19*	3.42 ± 0.18*	2.01 ± 0.31*	1.26 ± 0.18*	0.93 ± 0.09*	0.93 ± 0.07*
		−75 °C		–	6.21 ± 0.08	6.24 ± 0.05	6.09 ± 0.10	5.81 ± 0.18	5.95 ± 0.03	5.74 ± 0.50
	BHT	RT	5.96 ± 0.09	6.06 ± 0.04	6.01 ± 0.03	5.91 ± 0.10	5.89 ± 0.19	5.09 ± 0.20*	4.63 ± 0.12*	4.38 ± 0.15*
		4 °C		5.87 ± 0.06	6.05 ± 0.04	5.97 ± 0.07	6.05 ± 0.05	5.41 ± 0.13*	5.32 ± 0.08*	5.39 ± 0.12*
		−20 °C		5.92 ± 0.13	5.70 ± 0.05	5.52 ± 0.10*	5.39 ± 0.13*	4.63 ± 0.12*	4.59 ± 0.03*	4.64 ± 0.05*
		−75 °C		–	6.07 ± 0.21	6.20 ± 0.11	6.22 ± 0.14	5.56 ± 0.03*	5.64 ± 0.21	6.00 ± 0.08
Cryovial	EDTA	RT	6.99 ± 0.02	6.28 ± 0.23*	6.24 ± 0.19*	5.57 ± 0.17*	5.67 ± 0.04*	4.66 ± 0.22*	–	4.38 ± 0.01*
		4 °C		6.54 ± 0.15*	6.43 ± 0.19*	6.03 ± 0.11*	6.46 ± 0.11*	5.37 ± 0.05*	5.94 ± 0.05*	5.80 ± 0.09*
		−20 °C		–	6.18 ± 0.33*	6.15 ± 0.26*	5.39 ± 0.06*	3.58 ± 0.08*	2.84 ± 0.05*	2.44 ± 0.07*
		−75 °C		–	6.23 ± 0.33*	6.49 ± 0.06*	6.25 ± 0.22*	6.07 ± 0.22*	6.59 ± 0.12	6.95 ± 0.12
	Heparin	RT	6.98 ± 0.02	6.02 ± 0.09*	6.49 ± 0.05*	6.16 ± 0.31*	6.36 ± 0.03*	5.55 ± 0.13*	–	5.71 ± 0.09*
		4 °C		6.61 ± 0.18*	6.63 ± 0.02*	6.39 ± 0.06*	6.48 ± 0.13*	5.51 ± 0.13*	6.20 ± 0.11*	6.35 ± 0.08*
		−20 °C		–	6.35 ± 0.25	6.03 ± 0.17*	5.47 ± 0.51*	4.25 ± 0.23*	4.05 ± 0.06*	3.64 ± 0.14*
		−75 °C		–	6.32 ± 0.09	6.18 ± 0.53	6.19 ± 0.32	6.26 ± 0.26	6.55 ± 0.17	6.62 ± 0.28
	Heparin + BHT	RT	7.06 ± 0.06	6.42 ± 0.07*	6.36 ± 0.09*	6.46 ± 0.06*	6.45 ± 0.09*	6.02 ± 0.12*	–	6.63 ± 0.05*
		4 °C		6.70 ± 0.05*	6.42 ± 0.26*	6.54 ± 0.14*	6.38 ± 0.05*	6.18 ± 0.06*	6.15 ± 0.02*	6.73 ± 0.07*
		−20 °C		–	6.92 ± 0.14	6.70 ± 0.05*	6.88 ± 0.11	6.68 ± 0.04*	6.46 ± 0.13*	6.56 ± 0.06*
		−75 °C		–	7.11 ± 0.09	6.98 ± 0.15	6.74 ± 0.05	6.92 ± 0.14	6.52 ± 0.04*	6.32 ± 0.53*

Data presented as means ± SD

BHT butylated hydroxytoluene, *DHA* docosahexaenoic acid, *EDTA* ethylenediaminetetraacetic acid, *EPA* eicosapentaenoic acid

* Indicates values significantly lower than day 0 (baseline) as determined by Tukey's HSD post hoc test following a significant *F*-value by One way ANOVA

Table 3 Weight percent of omega-3 HUFA in total HUFA during storage of blood for 180 days

Storage vessel	Condition	Temperature	Storage day							
			0	3	7	14	30	60	90	180
Paper	No BHT	RT	40.8 ± 0.2	40.7 ± 0.1	41.1 ± 0.3	41.2 ± 0.1	40.5 ± 0.5	39.0 ± 0.6*	38.1 ± 0.7*	37.7 ± 0.5*
		4 °C		40.5 ± 0.2	41.4 ± 0.4	41.3 ± 0.4	40.6 ± 0.2	38.7 ± 0.2*	39.6 ± 0.4	39.5 ± 1.0
		−20 °C		40.0 ± 0.3	38.7 ± 0.3	36.6 ± 0.2*	35.8 ± 1.3*	35.2 ± 1.1*	34.5 ± 1.8*	40.4 ± 1.2
		−75 °C		–	41.1 ± 0.2	41.1 ± 0.2	41.3 ± 0.1	40.0 ± 0.4	40.5 ± 0.1	41.3 ± 0.2
	BHT	RT	40.9 ± 0.2	41.4 ± 0.2	41.4 ± 0.6	41.5 ± 0.5	41.0 ± 0.2	39.1 ± 0.5*	37.9 ± 0.1*	38.0 ± 0.1*
		4 °C		41.3 ± 0.2	41.4 ± 0.1	41.1 ± 0.3	41.2 ± 0.2	39.7 ± 0.1*	39.8 ± 0.2*	40.1 ± 0.1*
		−20 °C		40.9 ± 0.1	40.7 ± 0.2	40.7 ± 0.3	40.3 ± 0.3	37.7 ± 0.5*	37.7 ± 0.2*	38.2 ± 0.1*
		−75 °C		–	41.4 ± 0.4	41.6 ± 0.3	41.6 ± 0.2	40.1 ± 0.2	40.5 ± 0.2	40.9 ± 0.3
Cryovial	EDTA	RT	40.5 ± 0.2	40.3 ± 0.3	40.4 ± 0.4	38.9 ± 0.4*	39.4 ± 0.4*	37.5 ± 0.6*	–	37.7 ± 0.1*
		4 °C		40.7 ± 0.4	40.4 ± 0.7	39.7 ± 0.2	40.4 ± 0.3	38.5 ± 0.1*	39.0 ± 0.1*	39.3 ± 0.3*
		−20 °C		–	39.8 ± 0.1	39.7 ± 0.6	38.8 ± 0.1*	36.9 ± 0.6*	39.4 ± 0.3*	38.7 ± 0.1*
		−75 °C		–	39.9 ± 0.6	40.3 ± 0.2	40.3 ± 0.3	39.0 ± 0.4*	40.2 ± 0.1	40.8 ± 0.2
	Heparin	RT	40.7 ± 0.1	40.1 ± 0.3	40.7 ± 0.2	40.0 ± 0.4	40.4 ± 0.5	38.9 ± 0.2*	–	38.7 ± 0.1*
		4 °C		40.7 ± 0.7	40.9 ± 0.2	40.6 ± 0.1	40.6 ± 0.3	39.1 ± 0.3*	40.0 ± 0.2	40.5 ± 0.3
		−20 °C		–	40.0 ± 0.4	39.8 ± 0.6	39.1 ± 0.9	39.8 ± 0.7	39.7 ± 0.5	39.6 ± 0.4
		−75 °C		–	40.2 ± 0.5	39.9 ± 1.0	39.9 ± 0.4	39.4 ± 0.2	40.3 ± 0.3	40.9 ± 0.1
	Heparin + BHT	RT	40.7 ± 0.3	40.3 ± 0.1	40.5 ± 0.2	40.7 ± 0.1	40.6 ± 0.3	39.7 ± 0.2*	–	40.5 ± 0.1
		4 °C		41.2 ± 0.3	40.6 ± 0.8	40.8 ± 0.3	40.6 ± 0.2	40.2 ± 0.2	39.9 ± 0.1	40.7 ± 0.2
		−20 °C		–	41.0 ± 0.2	41.2 ± 0.2	41.0 ± 0.2	40.5 ± 0.2	40.5 ± 0.1	40.7 ± 0.4
		−75 °C		–	41.3 ± 0.2	41.2 ± 0.3	40.8 ± 0.3	40.3 ± 0.4	40.4 ± 0.1	39.6 ± 0.2*

Data presented as means ± SD

BHT butylated hydroxytoluene, *DHA* docosahexaenoic acid, *EDTA* ethylenediaminetetraacetic acid, *EPA* eicosapentaenoic acid, *HUFA* highly unsaturated fatty acid

* Indicates values significantly lower than baseline (day 0) as determined by Tukey's HSD post hoc test following a significant *F*-value by One way ANOVA

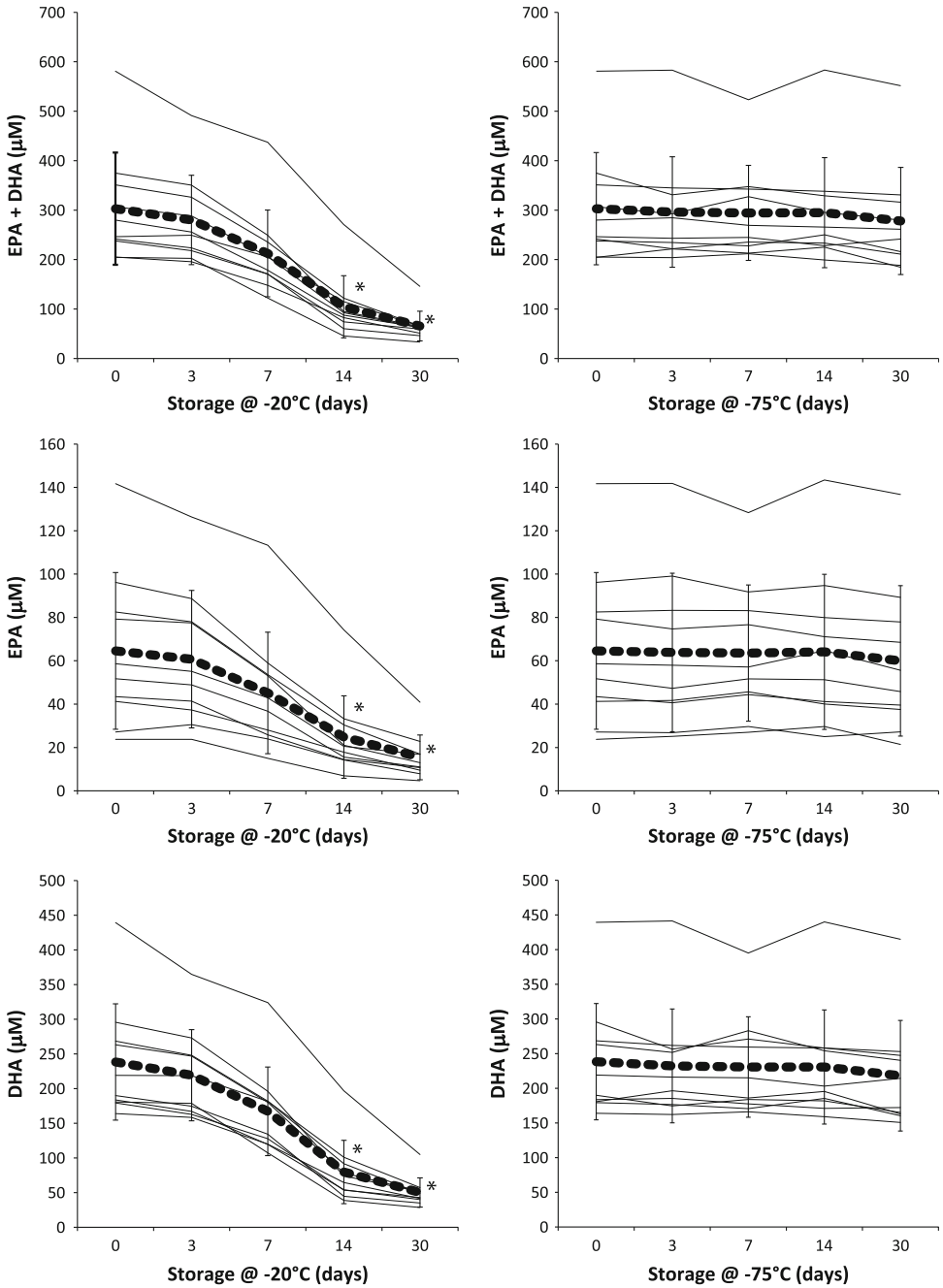

Fig. 4 Effect of storage at −20 and −75 °C on EPA + DHA, EPA and DHA concentrations in whole blood on chromatography paper. Heavy dashed line with *error bars* indicates mean ± SD. Light *solid lines* represent individual responses ($n = 10$). *Significantly lower than baseline (day 0) as determined by Tukey's HSD post hoc test following a significant *F*-value by repeated measures one-way ANOVA. *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid

first measured post-baseline time point for all storage temperatures (Table 2). A similar response was observed in heparin-treated blood, although no decline at −75 °C occurred. This result may suggest a limitation in blood handling techniques prior to storage of whole blood samples. For instance, rapid anticoagulation can cause an increase in erythrocyte lysis [39]. This hemolysis may then expose PUFA more readily to peroxidation initiators such as oxygen radicals and free iron.

The present study has limitations. In order to collect a large homogeneous blood pool to study numerous storage conditions, venous blood samples were collected in the presence of EDTA and then aliquoted on chromatography paper. Blood spot collection on chromatography paper typically involves the collection of capillary blood from a fingertip prick and EDTA is normally absent. Fatty acids in capillary erythrocytes may [18] be more susceptible to oxidation, and EDTA has the potential to function as an antioxidant through metal chelation. Therefore, it is possible that potential for degradation of EPA + DHA in fingertip prick blood samples on chromatography paper is greater than degradation in venous whole blood; however, we have not observed these differences (unpublished data). In addition, blood from a single participant was examined

for the storage condition experiments, thereby limiting extrapolation to large population studies. However, we did confirm the degradation pattern of EPA + DHA in blood stored at −20 °C and not at −75 °C over 1 month in a small sub-study ($n = 10$). Limiting the main study to triplicate analyses of blood from one participant enabled a more comprehensive assessment of stability across numerous storage protocols. The use of a single participant also restricted the inter-individual variation within the degradation process. An examination of whole blood on chromatography paper before and after fish oil intervention was assessed to broaden the findings. Unfortunately an examination of whole blood cryovial storage was not completed with low n-3 blood, as we originally hypothesized that the high n-3 blood would be more susceptible to oxidation during storage. The results from the present study will allow for targeted time point and storage condition assessments of fatty acid stability in future studies using larger cohorts that may account for other factors that influence pro- and anti-oxidant properties of blood.

In conclusion, blood samples for fatty acid analyses should be stored at −75 °C whenever possible, and conversely, −20 °C storage should be avoided particularly in the absence of an antioxidant such as BHT. Storage of whole blood samples on paper at 4 °C with BHT may be the next viable option if −75 °C is not available, although room temperature may be adequate if n-3 PUFA levels are low in the study population. Whole blood in vials should be stored in the presence of heparin + BHT to minimize degradation. Further investigation is required to elucidate the mechanism of degradation at −20 °C, as a better understanding of PUFA degradation in blood may lead to improved handling and storage protocols to extend fatty acid stability during long-term storage.

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