Measurement of the Omega-3 Index in Dried Blood Spots

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Abstract

Background: The roles that the omega-3 fatty acids play in health and disease remain unclear. The conduct of epidemiological studies to explore this relationship can be facilitated by the use of dried blood spots (DBS) for sample collection and shipment. The Omega-3 Index [i.e. erythrocyte levels of the two major marine-derived omega-3 fatty acids, eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA)], is an emerging risk factor for cardiovascular disease mortality. The purpose of this report is to describe how the Omega-3 Index can be derived from an analysis of DBS fatty acid levels.

Methods: A comparison of the Omega-3 Index and DBS EPA+DHA levels was conducted in 147 random samples received in the laboratory, and in 106 samples prospectively collected and processed through the US mail. The "in field" coefficient of variation of the DBS-derived Omega-3 Index was calculated from 128 duplicate samples collected in an epidemiological study in Mexico. The stability of DBS EPA+DHA levels when collected on antioxidant-treated paper was tested for 44 days at room temperature, and for up to 4 years in the freezer.

Findings: We found that the correlation between the DBS EPA+DHA level and the Omega-3 index was 0.98 (p<0.0001), hence the latter can be accurately estimated from the former. As a storage and transport system, DBS cards (pretreated with an antioxidant cocktail) provide protection from degradation for blood EPA+DHA levels (i.e. >15% loss) for at least 6 weeks at room temperature, 4 weeks in a refrigerator, 3 years at -20°C and 4 years at -80°C.

Conclusion: These data confirm the utility of this DBS system for estimating the Omega-3 index.

Keywords: Omega-3 fatty acids; Epidemiology; Dried blood spots; Red blood cells

Introduction

The roles that the omega-3 fatty acids play in health and disease remain unclear. Epidemiological studies based on dietary intake surveys suggest that higher intakes of fish (the primary source of omega-3 fatty acids) are associated with better health outcomes1, but such tools are blunt instruments, unable to clearly define omega-3 status in an individual subject. Measurement of circulating omega-3 fatty acid levels is a preferred approach and has shown promise as a biomarker of risk for cardiovascular disease [1]. Conducting large-scale epidemiological investigations into the relationship between omega-3 fatty acid bio status and disease is hampered by challenges in collecting, processing, storing and transporting blood in the developed, and especially the developing, world. Many of these logistical challenges can be overcome by the use of dried blood spots (DBS) for sample collection and shipment. Marangoni et al. were the first to propose such a method in 2004 [2], and variations of it have since been used in a variety of research projects by us [3-8]. and by others [9-12]. We have been particularly interested in deriving the "Omega-3 index [13]" from DBS samples since this metric-the erythrocyte content of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA)-is becoming widely accepted as both a biomarker of omega-3 biostatus [14] and a risk factor for cardiovascular [15] and possibly even neurocognitive health [16-18]. Since whole blood contains both serum and cellular components (all of which contain fatty acids in unique proportions specific to each component), some means must be found to derive the erythrocyte EPA+DHA level from the EPA+DHA level from whole blood (i.e. a DBS). The purpose of this report is to describe how this conversion is made and to document the validity of DBS-derived Omega-3 index values.

Methods

Laboratory methods

A drop of blood (~50 uL) is collected on Perkin Elmer 226 or Whatman 903 filter paper that had been pre-treated with a multi-component antioxidant cocktail (FAPS™, OmegaQuant Analytics, LLC) and allowed to dry at room temperature for 15 min. (Results for both papers are identical, data not shown.) One punch from the DBS card (about 10 uL of blood) is

transferred to a screw-cap glass vial followed by addition of mixture of methanol toluene and boron trifluoride (14%, Sigma-Aldrich, St. Louis, MO). The vial is briefly vortexed and heated in a hot bath at 100˚C for 45 minutes. After cooling, HPLC grade water and hexane (EMD Chemicals, USA) are added, the tubes are recapped, vortexed and centrifuged to separate layers. An aliquot of the upper (hexane) layer is transferred to a gas chromatography (GC) vial. GC is carried out using a GC2010 Gas Chromatograph (Shimadzu Corporation, Columbia, MD) equipped with a SP2560, 100-m fused silica capillary column (0.25 mm internal diameter, 0.2 um film thickness; Supelco, Bellefonte, PA). Hydrogen (carrier and flame) is generated by hydrolysis of water, and air (flame) is compressed and filtered using equipment from Peak Scientific (Billerica, MA). Fatty acids are identified by comparison with a standard mixture of fatty acids characteristic of erythrocytes (GLC OQ-A, NuCheck Prep, Elysian, MN; this mixture is also used to construct individual fatty acid calibration curves). In addition to EPA (20:5n-3) and DHA (22:6n-3), the following 22 fatty acids (by class) are identified: saturated (14:0, 16:0, 18:0, 20:0, 22:0 24:0); cis monounsaturated (16:1, 18:1, 20:1, 24:1); trans unsaturated (16:1, 18:1, 18:2); cis n-6 polyunsaturated (18:2, 18:3, 20:2, 20:3, 20:4, 22:4, 22:5); cis n-3 polyunsaturated (18:3, 22:5). The sum of these 24 fatty acids constituted the total fatty acid content of the blood, and each individual fatty acid was expressed as a percent of the total.

Derivation of the Omega-3 index from DBS samples

In order to obtain the Omega-3 index from a DBS sample, a comparison of the former with the EPA+DHA level of the latter was made in 147 de-identified, randomly selected blood samples sent to the laboratory for analysis. Prior to centrifuging the blood tubes to isolate the erythrocyte fraction, each tube was inverted several times, opened and one drop removed and placed on a DBS card. The card was then stored for 7 days in a Ziplock bag in a drawer at room temperature. The tube was then centrifuged, an aliquot of the erythrocyte pellet was removed and analyzed for the Omega-3 index. Similarly, the DBS samples were analyzed for EPA+DHA levels. Regression analysis was conducted comparing the erythrocyte-based Omega-3 index and the DBS-derived EPA+DHA value.

In a second experiment that was designed to replicate handling of DBS samples in the "real world," we collected blood by venipuncture from 106 random subjects into EDTA tubes, and at the same time, collected one drop of blood by finger stick and placed it on a DBS card. The blood tubes were immediately processed for erythrocytes which were frozen at -80°C; the DBS samples were placed individually into return mail envelopes, the envelopes were sealed and then express shipped in batches of about 20 to colleagues in five cities around the USA. Upon receipt, the express mail envelop was opened and the individual sample envelopes, each containing a DBS sample, were sent by regular US mail back to OmegaQuant for routine analysis. When all samples had

arrived back in the laboratory, the DBS and frozen erythrocyte samples were analyzed in the same batch, and regression analysis was carried out as above.

In a third experiment, 98 de-identified, left-over blood samples collected from a local hospital laboratory were separated by fasting status: 50 non-fasting and 48 fasting (since DBS samples in practice are collected in both fasting and fed states). Comparisons between the EPA+DHA content of DBS and erythrocytes were performed as described above. Thereafter, these 98 samples were randomly separated into a 49-sample derivation and a 49-sample validation set. In the derivation set, an equation to convert DBS EPA+DHA into the erythrocyte Omega-3 index was generated, and in the validation set, that equation was used to predict the latter metric from the former. The intra-class correlation between the predicted and observed Omega-3 index values was computed using MedCalc Statistical Software version 16.8.4 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2016).

Reproducibility of the Omega-3 Index from DBS samples

Each batch of DBS samples tested for the Omega-3 index is run with DBS controls spotted with a high (omega-3) and low (omega-3) blood sample. Within-run imprecision is defined as the coefficients of variation (CVs) for each of these control samples. Data from 400 runs across 7 GCs over 11 months are reported here. The reproducibility of the method has also been tested under field conditions in an epidemiologic study of 1306 Mexican women from Chiapas and the Yucatan [6]. Samples were collected in local clinics and express mailed at ambient temperature to OmegaQuant Analytics (Sioux Falls, SD) where they were stored at -80°C. As a part of this study, 128 randomly collected blind duplicate pairs of DBS samples were blindly analyzed, and reproducibility was calculated as both the average CV and the average percent difference (APD). The time between collection and freezing at -80°C was <14 days.

Minimum amount of blood needed

For an analyte expressed as a percent of total fatty acids and not as a concentration, defining the 'lower limit of detection' is challenging. This is especially true when analyzing erythrocytes (or whole blood) where one cannot purchase samples of defined composition but must utilize samples sent to the laboratory. Accordingly, the more practical question is, "How much blood must be on the DBS card to get an accurate analysis of the Omega-3 index?" To address this question, we did triplicate analyses of blood spots ranging from 2 to 50 μL with two different blood specimens: one with a high Omega-3 index (about 9%) and one with a low index (about 3%). Our routine "volume" is about 10 μL which is the approximate amount of blood contained in 1 punch from a fully saturated DBS card.

Stability during storage of the Omega-3 index from DBS samples

The stability of the Omega-3 index derived from DBS samples has been tested in two experiments. In the first, 3 blood samples (with Omega-3 indexes of 3.7%, 6.2% and 12.0%) were tested while stored at room temperature over 44 days with and without prior treatment of the cards with a proprietary antioxidant cocktail (FAPS™). At each time point, the three values were averaged and compared to baseline levels. Acceptable stability was defined as being within 15% of baseline values per reproducibility guidelines from the Food and Drug Administration [19]. Statistical differences between the values from the 3 anti-oxidant protected and the 3 unprotected cards at any given time point were not formally evaluated.

In the second experiment, DBS cards were pretreated with the antioxidant cocktail and then spotted with blood from 5 pools ranging in Omega-3 index from 2.2% to 10.6%. DBS cards were then stored in a refrigerator, a -20°C freezer and in a -80° freezer, and analyzed periodically over 4 weeks (refrigerator) and 4 years (freezers).

The blood samples used in the laboratory experiments were deidentified clinical samples. For the "mail-back" experiment described above, the study was approved by the Sanford Research Institutional Review Board (IRB). Samples from the study conducted in Mexico[6] were collected under a protocol approved by the IRB at National Institute of Public Health. All participants provided informed consent.

Results

Validity of the Omega-3 Index from DBS samples: comparison with erythrocyte EPA +DHA

The first experiment compared the DBS-derived EPA+DHA value with the erythrocyte-based Omega-3 index in 147 samples. The correlation between the two was 0.98 (P<0.0001). For the second comparison using "real world" mailed-in samples, the correlation between the EPA+DHA content of the two was r=0.96 (p<0.0001), and the 95% confidence interval for the DBS-estimated Omega-3 index value was ±1%. In the third comparison using 98 fasted and fed samples to develop and validate a conversion metric, the observed Omega-3 index was reproduced with high accuracy by the predicted values **(Figure 1)**. The mean (SD) observed Omega-3 index was 4.56% (2.08%) compared to the predicted of 4.52% (1.90%) (p=0.64). The mean intra-class correlation coefficient (95% CI) was 0.96 (0.93-0.98).
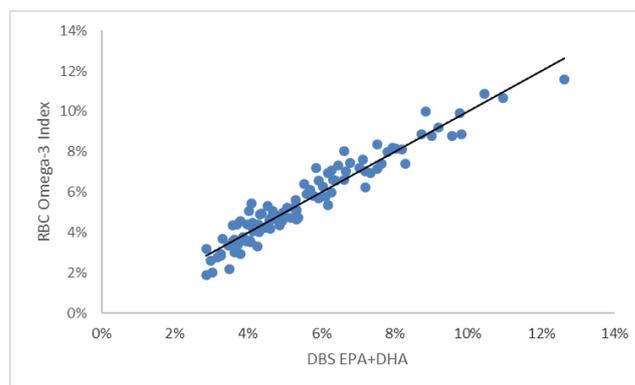

Figure 1 Comparison of the predicted vs observed erythrocyte EPA+DHA (Omega-3 index) in 98 samples. Omega-3 index= 1.0 × DBS EPA+DHA -0.000049, r=0.96.

Reproducibility of the Omega-3 index from DBS samples: Within lab and in field conditions

The in-laboratory, inter-assay coefficient of variability (CV) for a control sample with a mean Omega-3 index of 7.9% is 3.6%, and for a sample with an index of 4.6%, the CV is 4.9%. In the Mexican field study, the CV for the Omega-3 index was <5%, the APD was <4%. The CVs (and APDs) for all the fatty acids measured in DBS samples are shown in **Figure 2** as a function of their levels. For 7 of the 8 FAs present at 2% abundance or greater, the CVs and APDs were <6%.

Minimum amount of blood needed

As is evident from **Figure 3**, the Omega-3 index was unaffected by the volume of blood on the DBS card, down to 2 μL.

Stability of the Omega-3 index from DBS samples: Time and temperature

At room temperature, the Omega-3 index measured from DBS cards pretreated with the antioxidant cocktail was stable for up to 44 days, whereas storage on cards without prior antioxidant treatment resulted in >15% loss of the Omega-3 index by day 7 **(Figure 4)**. In the long-term storage experiments, the Omega-3 index was stable for 4 weeks in the refrigerator (maximum loss of 6%), for up to 3 years at -20°C (maximum loss (at 2.5 years) 13%), and for at least 4 years at -80°C (maximum loss 5.6%) **(Table 1)**.
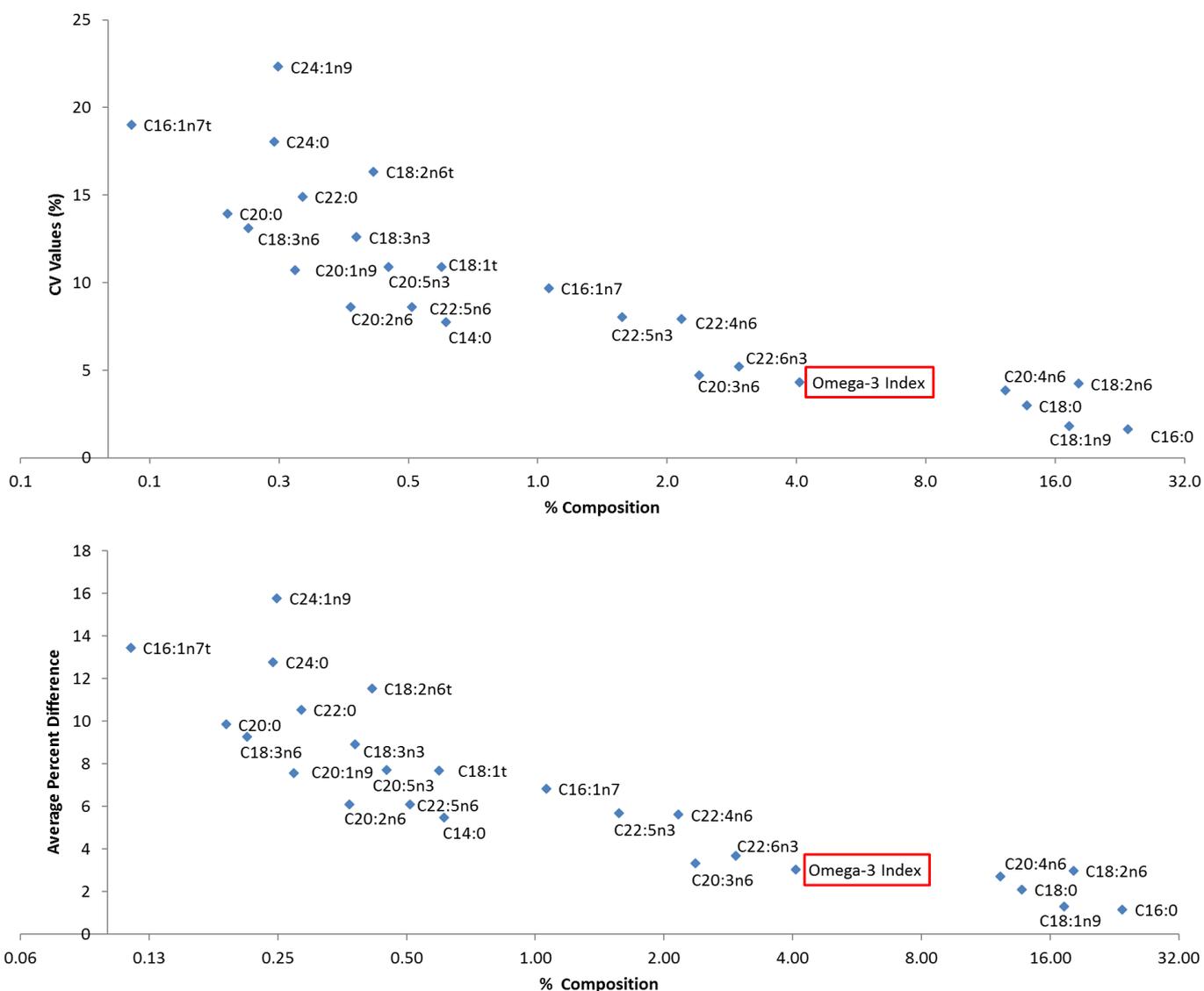
Figure 2 Coefficients of variation (CVs; top) and average percent differences (bottom) for DBS fatty acids from 128 paired blind duplicate samples analyzed blindly from a study in Mexican women [6]. The Omega-3 index is highlighted.

Discussion

The Omega-3 index has been used in a wide variety of research projects, from epidemiologic [10,20-29] to interventional [3,30-32], as a marker of omega-3 fatty acid biostatus. The index has been deduced from analysis of DBS samples in studies with U.S. soldiers deployed in Iraq [33], adolescents in the Netherlands [10], premature babies [34], vegans [3], young children in Tanzania [7], and cardiac patients in Norway [5]. Each of these studies included brief descriptions of the DBS method and alluded to its ability to derive the Omega-3 index, but none focused on the performance and validity of the test per se. This report is intended to provide that information.

Here we have shown that the Omega-3 index can be accurately derived from analysis of DBS EPA+DHA levels, with a 95% confidence band of 1 percentage point. As a storage and transport system, DBS cards (pretreated with an antioxidant cocktail) provide protection from degradation for blood EPA+DHA levels for at least 6 weeks at room temperature, 4 weeks in a refrigerator, 3 years at -20°C and 4 years at -80°C. Without the anti-oxidant, EPA+DHA degraded by more than 15% within 1 week at room temperature. (The Omega-3 index measured on untreated DBS cards was stable for at least 26 months when stored at -80°C; data not shown). The reasons why DBS samples are more stable at room temperature than they are at -20°C is unclear, but this has been seen previously with RBC samples which are even more unstable at -20°C than DBS samples are [35]. Since the inclusion of free iron scavengers and anti-oxidants in the stored samples can significantly mitigate this problem [12], it appears that lysis of the cells in the freezer releases pro-oxidants that are remain active at -20°C, but are inactivated at -80°C.

4

4°C			-20°C			-80°C		
Time	Omega-3 Index	% loss	Time	Omega-3 Index	% loss	Time	Omega-3 Index	% loss
Baseline	6.68%		Baseline	6.68%		Baseline	6.68%	
Day 4	6.51%	-2.62%	Day 4	6.64%	-0.66%	1 month	6.80%	1.74%
1 week	6.32%	-5.37%	1 week	6.60%	-1.22%	3 months	6.82%	2.15%
2 weeks	6.59%	-1.37%	2 weeks	6.75%	1.04%	6 months	6.78%	1.44%
3 weeks	6.24%	-6.62%	3 weeks	6.44%	-3.49%	9 months	6.74%	0.84%
4 weeks	6.28%	-5.93%	4 weeks	6.23%	-7.00%	1 year	7.02%	5.16%
			3 months	6.48%	-3.21%	1.5 years	6.90%	3.22%
			6 months	6.95%	4.15%	2 years	7.07%	5.78%
			9 months	6.77%	1.32%	2.5 years	6.71%	0.40%
			1 year	6.84%	2.29%	3 years	6.74%	0.87%
			1.5 years	6.24%	-6.37%	4 years	6.31%	-5.56%
			2 years	6.40%	-4.53%			
			2.5 years	5.84%	-13.10%			
			3 years	6.00%	-11.72%			
			4 years	5.45%	-20.45%			

Table 1 Stability of the Omega-3 index measured from dried blood spots at different temperatures and for different storage times (mean of n=5 samples analyzed at each time point).
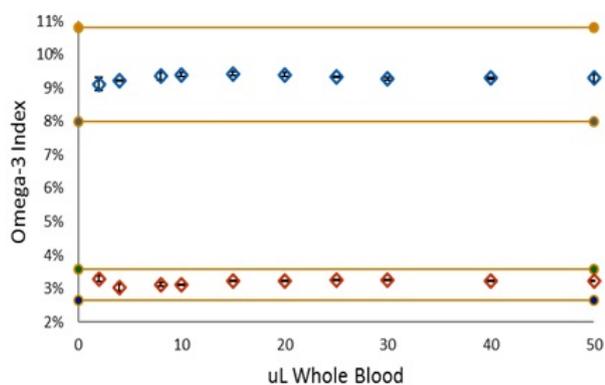

Figure 3 The Omega-3 index was measured in triplicate in one high (about 9%) and one low (about 3%) samples using volumes varying between 2 μL and 50 μL. The Omega-3 index can be measured in as little as 2 μL of blood.

The use of DBS samples for whole blood fatty acid analysis was first developed and validated by Marangoni et al. [2]. They noted that samples that were intended to be stored for more than 2 weeks should be pre-treated with butylated hydroxytoluene (BHT) to preserve the long-chain highly polyunsaturated fatty acids like EPA and DHA. Others [11,36] have also examined the use of DBS systems for fatty acid analysis and explored the use of BHT and other antioxidant approaches to protect these fatty acids from degradation. Care needs to be taken when using BHT to preserve fatty acids as it may interfere with certain fatty acids depending on chromatographic conditions (in our method, myristic acid). These perform adequately per FDA guidelines (i.e. <15% difference from baseline) as did the antioxidant cocktail used in this investigation.
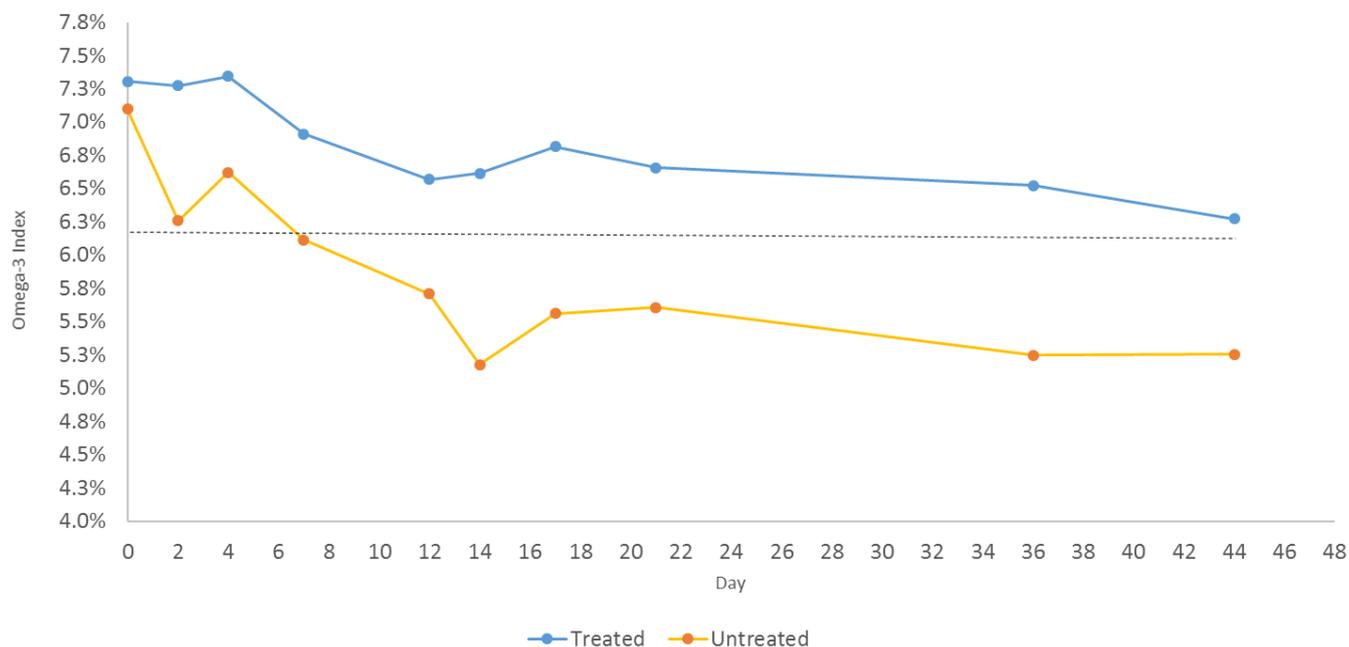
Figure 4 Three random blood samples with widely divergent Omega-3 index levels were spotted on DBS cards that had (blue line) or had not (orange line) been pre-treated with the antioxidant cocktail. The samples were stored at room temperature in a re-closable plastic bag in the dark and were removed periodically for fatty acid analysis. The dotted line represents a 15% loss from the baseline Omega-3 index value.

Conclusion

These data confirm the utility of this DBS system for generating the Omega-3 index. The use of DBS testing for the Omega-3 index will facilitate research into the biological roles that the omega-3 fatty acids play in health and disease.

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Conflicts of interest

Both authors are employed by OmegaQuant Analytics, LLC.

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