

Determination of n-3 index and arachidonic acid/eicosapentaenoic acid ratio in dried blood spot by gas chromatography

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ABSTRACT

Background: Clinical and epidemiological studies suggest that analysis of the polyunsaturated fatty acids (PUFAs) is essential to evaluate nutritional requirements and disease risk. We describe a simple, sensitive and non-invasive method for estimating the n-3 index and arachidonic acid (AA)/eicosapentaenoic acid (EPA) ratio in dried blood spots (DBSs). **Experimental:** After obtaining DBSs on a spot card, PUFAs were transesterified (direct, acidic transesterification) and subsequently extracted with n-hexane. Gas chromatography with flame ionization detection (GC-FID) was used to analyze the extracted PUFAs, and then n-3 index and AA/EPA ratio were calculated. Method validation showed satisfactory precision and linearity. **Conclusion:** This analysis is simple and reliable to estimate PUFA status, and it was successfully applied to samples from 20 subjects, demonstrating its applicability.

METHOD SUMMARY

Dried blood spots (DBSs) were obtained by fingerstick. Polyunsaturated fatty acids (PUFAs) were transesterified by an acidic reagent and extracted with n-hexane. Gas chromatography with flame ionization detection (GC-FID) was used to analyze the extracted PUFAs. The n-3 index and arachidonic acid (AA)/eicosapentaenoic acid (EPA) ratio were calculated.

KEYWORDS:

AA/EPA ratio (arachidonic acid) (eicosapentaenoic acid) • dried blood spot • gas chromatography • n-3 index (fatty acids, omega-3) • polyunsaturated fatty acids (fatty acids, unsaturated)

Polyunsaturated fatty acids (PUFAs) are important constituents of the membranes of cells involved in the immune response. The PUFA composition of cell membranes also affects fluidity and signaling pathways [1]. Based on the location of the double bonds from the methyl terminal position, the PUFAs are classified as n-3 and n-6 PUFAs. The alpha-linolenic acid (ALA; 18:3n-3) of the n-3 series and linoleic acid (LA; 18:2n-6) of the n-6 series are considered as essential fatty acids (EFAs) because our body cannot synthesize them. Therefore, they should be obtained from the diet. The long-chain PUFAs are either absorbed from the diet or synthesized endogenously from the two EFAs [2]. The physiological effects of n-3 and n-6 PUFAs are described in many studies, indicating that their metabolism may affect clinical conditions associated with metabolic, inflammatory and oxidative processes [3]. The n-6 PUFA-derived lipid mediators enhance inflammation, platelet aggregation and vasoconstriction, while those of n-3 inhibit inflammation and platelet aggregation and enhance vasodilation. The high amount of n-6 PUFAs and the very high ratio of n-6/n-3 in the Western diet triggers the pathogenesis of various conditions, including obesity, cancer, cardiovascular and neurodegenerative diseases [2]. Therefore, the analytical determination of PUFAs may provide valuable information on nutritional needs, health outcomes and disease risk.

To date, several biomarkers have been suggested to determine PUFA status; among them, the n-3 index and arachidonic acid (AA; 20:4n-6)/eicosapentaenoic acid (EPA; 20:5n-3) ratio are the most reliable biomarkers [3,4]. The n-3 index is the amount of EPA and docosahexaenoic acid (DHA; 22:6n-3) in erythrocyte membranes expressed as the percentage of total erythrocyte membrane fatty acids (FAs). Evidence supports the n-3 index as a risk factor for several diseases [5]. The EPA and DHA play beneficial roles in lowering cardiovascular disease risk factors, such as hypertension, hypercholesterolemia and platelet hyperaggregation. Although there are contradictory results about their impact on oxidative stress, it is suggested that the DHA administration raises the levels of antioxidative enzymes, including glutathione peroxidase and catalase. Likewise, DHA and EPA positively affect skeletal muscle by increasing endurance exercise performance, enhancing mitochondrial function and improving vascular conductance and blood flow. Furthermore, DHA is essential

for the growth and development of the brain, and its deficiency is associated with neurodegeneration and cognitive decline [2]. Therefore, the estimation of the n-3 index may be considered beneficial for health promotion and disease prevention.

The AA/EPA ratio determines the ratio between two molecules competing to convert to bioactive eicosanoids. The AA produces 2-series prostaglandins and thromboxanes by COX-1/COX-2 enzymes. By the action of the 5-LOX enzyme, AA also produces 4-series leukotrienes. These compounds are inflammatory mediators with strong proinflammatory and vasoconstriction effects. On the other hand, by the action of COX and LOX, EPA generates three-series prostaglandins and thromboxanes and 5-series leukotrienes that exert anti-inflammatory effects compared to the lipid mediators derived from AA [2]. Therefore, the balance between AA and EPA is essential to regulate the production of inflammatory mediators. Furthermore, the AA/EPA ratio is a sensitive marker of cardiovascular disease risk factors, such as total cholesterol, low-density lipoprotein and triglyceride levels [6].

Although it is a demanding nutritional analytic procedure, the measurement of PUFAs is challenging, mainly because of their biological diversity and physicochemical similarity. PUFA analysis should be adjusted for sample collection, storage conditions, extraction and derivatization, and instrument and data analysis. There are several biological samples to estimate PUFA levels. In general, the erythrocyte membrane is preferred because it provides information on the long-term dietary intake of PUFAs and their biosynthetic conversion to eicosanoids [7]. However, the analysis of PUFAs in blood requires a few specific sample preparation steps that need equipment, reagents and time. In addition, it is essential to develop techniques that use a small sample volume. Dried blood spots (DBSs) could be an attractive sample source because some sample preparation steps can be avoided. DBS from fingertip blood has been validated to effectively determine whole blood FA profile changes associated with lifestyle and dietary factors [8]. In addition, DBS offers a rapid, minimally invasive and accessible sample collection and can be easily applied to studies of large populations [9].

Gas chromatography (GC) is one of the essential analytical techniques that have been applied for the characterization of various compounds. The optimal qualitative and quantitative GC analysis generally presumes a good resolution, high repeatability and reproducibility of retention times, precise and accurate quantification of peak area measurements and lowest sample degradation [10]. A commonly used detector in GC analysis is a flame-ionization detector (FID) that is easy to manage and offers a wide linear range and rapid response [11]. Under optimized conditions, ordinary GC-FID analysis determines the changes in human tissue FAs. Furthermore, by carefully choosing column and instrument conditions, GC-FID allows the estimations of PUFAs and PUFA-derived lipid mediators from various biological samples to study disease states [3].

This study describes a sensitive and reliable method based on GC-FID technique to analyze 11 PUFAs from DBS and subsequently estimate the n-3 index and AA/EPA ratio. The method is based on a one-step transesterification process from DBS without sample preparation before the derivatization reaction. The method is further applied to compare the n-3 index and AA/EPA ratio in uninjured and injured runners. The results will help us better understand the predictive values of these PUFA biomarkers.

Materials & methods

Chemicals and reagents

The 22 fatty acid methyl ester (FAME) mixture was purchased from Nu-Chek-Prep (GLC727, MN, USA). Boron trifluoride-methanol (BF₃-MeOH) (12% w/v, 1.5 M) reagent was purchased from Acros Organics (Geel, Belgium), and n-hexane was purchased from CARLO ERBA Reagents S.r.l. (France).

GC-FID analysis

A GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan), equipped with an SP[®]-2560 capillary GC column (L × I.D. 100 m × 0.25 mm × 0.2 μm) with a poly (biscyanopropyl siloxane) phase, one FID, an automatic injection system (AOC-20i), and an electronic flow and pressure control system, was used for the GC analysis. The GC column was connected to the injector and the FID. The electronic flow and pressure controller supplied carrier gas (helium) at constant pressure. Samples (1 μl) were introduced by the injector heated to 250°C with a split ratio of 10:0. The initial temperature was 170°C with a 1 min hold followed by 7.00°C/min ramp to 200°C with a 1 min hold and 1.00°C/min ramp to 220°C with a 20 min hold. The FID temperature was 280°C with hydrogen, air, and nitrogen make-up gas flow rates of 40 ml/min, 400.0 ml/min and 30 ml/min, respectively. The configuration and condition of this system are described in Table 1.

Method validation

The calibration of the proposed method was produced from average response factor (RF) and linear regression equations. According to the US FDA guideline for biological methods, the procedure was also validated for linearity and precision. Five batches of the 22 FAMES standard mixture were prepared to determine the average RF, the limit of quantitation (LOQ), and linearity range. The average RF was calculated as the average of each calibration factor of the external standard batches, and the linear regression technique was used for generating calibration lines. The average RF was then evaluated from the calculated relative standard deviation (RSD). To calculate the RSD, the standard deviation of the calibration factors was first determined. Then the standard deviation was divided by the mean of the calibration factors to give the RSD. The precision of the method was determined from the calculated relative standard deviation (RSD). Generally, an RSD of <15% or <20% is utilized as a criterion for accepting the precision of the method. Additionally, the regression analysis formed an association equation between the instrument response (peak area) and the concentration of individual FAMES in the

Table 1. The configuration and condition of the gas chromatography system.

Column	Configuration
Gas chromatography column	SP-2560 (100 m × 0.25 mm, df 0.20 μm)
Gas chromatography flow	2.24 ml/min
Autoinjector	
Temperature	250.0° C
Pressure	457.3 kPa
Injection size	1.0 μl
Total flow	29.6 ml/min
Purge flow	5.0 ml/min
Flame-ionization detector	
Temperature	280.0° C
Hydrogen	40.0 ml/min
Air	400.0 ml/min
Make-up (Nitrogen)	30.0 ml/min
Column oven temperature program	
Initial temperature	170.0° C for 1 min
Ramp number 1	7.00° C/min to 200.0° C for 1 min
Ramp number 2	1.00° C/min to 220.0° C for 20 min

analyte. The linearity of the calibration curve was assessed by the coefficient of determination (r^2). The r^2 permits the determination of the percent of data nearest to the line, and a value ≥ 0.99 is considered acceptable. A repeatability test was carried out through ten consecutive injections. A reproducibility test was then performed, in which the whole blood sample was recovered on ten different DBS cards and analyzed.

Blood sample collection and FA extraction

DBSs from six male and four female runners were obtained with individual consent from a single medical clinic – Sports Medicine Varese Medical Campus – in Varese, Italy. These ten subjects were self-reported to have running-related injuries. In addition, DBSs were also collected from six uninjured males and four uninjured female runners. DBS samples were obtained using lancet finger sticks. After sterilizing the participant's finger and applying the lancet, the first drop of blood was discarded, and subsequent drops were placed onto the spot card (BioSample TFN cards, Ahlstrom, Bärenstein, Germany). The spot cards were dried for 12 h in the dark at room temperature. The DBS cards were stored in a fridge at 4° C until analysis.

The sample preparation was based on an earlier report [8]. As depicted in Figure 1, the DBSs were placed inside a 10 ml headspace vial with a sealing cap, and 2 ml of BF₃-MeOH reagent (12%, w/v) was added. The vials were sealed and heated at 100° C for 60 mins using a convectional block heater. This leads to the direct transesterification of the FAs in the mixture into FAMES. After cooling to 25° C, 2 ml of n-hexane was added, and the mixture was then vortexed for 1 min, resulting in the formation of a transparent n-hexane top layer containing the FAMES. This organic layer was removed into a fresh 2-ml glass GC vial. The aliquot was air-dried in the darkness and subsequently redissolved in 400 μl of n-hexane and injected into GC-FID for analysis.

Identification and quantification of the PUFAs

Individual FAMES were identified by the standard mixture (Nu-Chek-Prep, MN, USA), which was dissolved in n-hexane at various concentrations before injection into GC-FID. The data were analyzed using Shimadzu system GC Solutions software, designed for this system. The retention times of the 22 FAMES mixture on the GC column were used to identify the FAs, as shown in Table 2. The retention time used for calibration was representative of five concentrations of the standard. A peak represented each FAME (Figure 2). The surface of the peaks is proportional to the amounts of the corresponding FAs. The percentage of each PUFA was calculated starting from the surface of the peak which represent it on the chromatogram:

$$\text{PUFA\%} = \left(\frac{\text{surface peak corresponding to the PUFA}}{\text{sum of all the peak surfaces corresponding to the total mixture of the FAs}} \right) \times 100$$

The n-3 index, defined as the amount of EPA and DHA content in erythrocyte membrane as a percentage of the total amount of FAs in the membrane, and the AA/EPA ratio were then calculated.

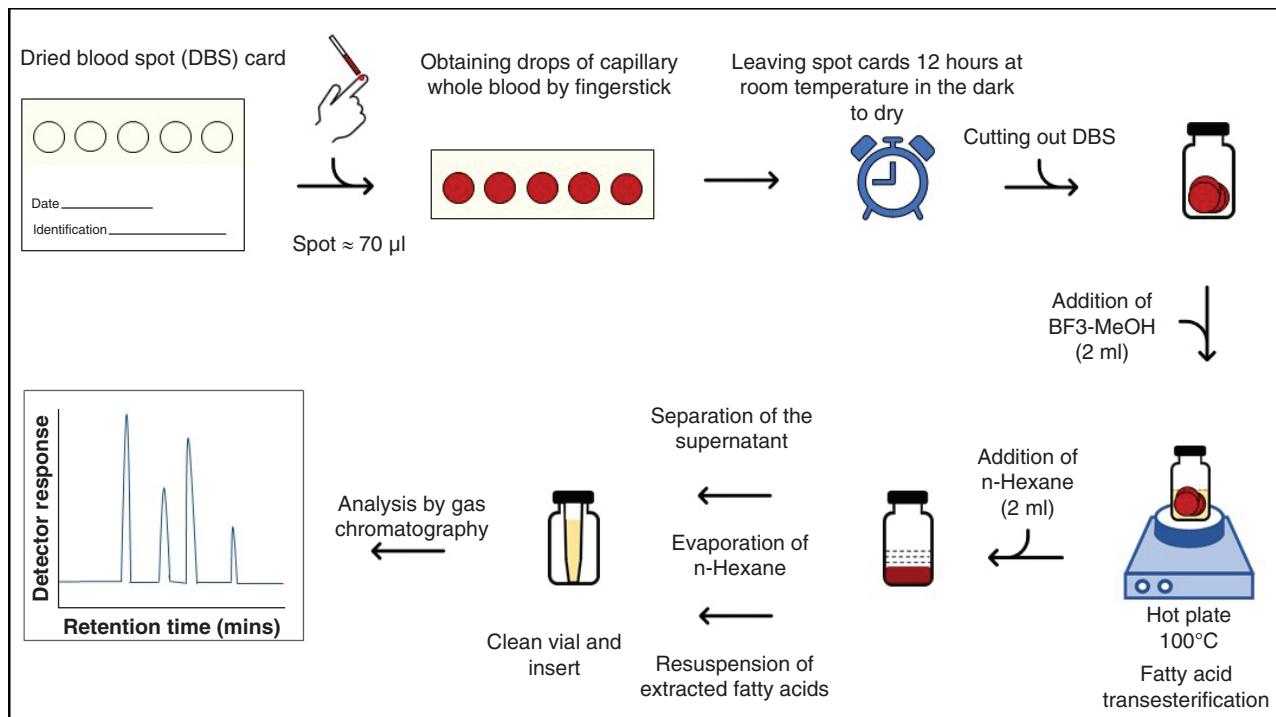


Figure 1. Extraction of fatty acids from dried blood spots and their analysis by gas chromatography with flame ionization detector (GC-FID).

Table 2. Retention time of the polyunsaturated fatty acids.

Polyunsaturated fatty acid	Retention time (min)
18:3n-3	16.217
20:5n-3	28.606
22:5n-3	23.009
22:6n-3	30.235
18:2n-6	15.578
18:3n-6	15.578
20:2n-6	17.627
20:3n-6	19.081
20:4n-6	20.287
22:4n-6	25.279
22:5n-6	26.602

The retention time is representative of five concentrations of the standard used for calibration.

18:2n-6: Linoleic acid; 18:3n-3: Alpha linolenic acid; 18:3n-6: Gamma-linolenic acid; 20:5n-3: Eicosapentaenoic acid; 20:2n-6: Eicosadienoic acid; 20:3n-6: Dihomo-gamma-linolenic acid; 20:4n-6: Arachidonic acid; 22:5n-3: Docosapentaenoic acid; 22:4n-6: Docosatetraenoic acid; 22:5n-6: Docosapentaenoic acid; 22:6n-3: Docosahexaenoic acid.

Method application

The proposed method was applied to estimate the n-3 index and AA/EPA ratio in recreational runners with running-related injuries (n = 10) and uninjured runners (n = 10). The levels of the PUFA biomarkers were compared between the injured and uninjured subjects to examine the association between the n-3 index and AA/EPA ratio and the prevalence of running-related injuries. Wilcoxon-Mann-Whitney test was used to compare the n-3 index and AA/EPA ratio between the two groups. The data were analyzed using IBM SPSS Statistics version 26 (IBM Corporation, NY, USA). Statistical significance was set at $p < 0.05$.

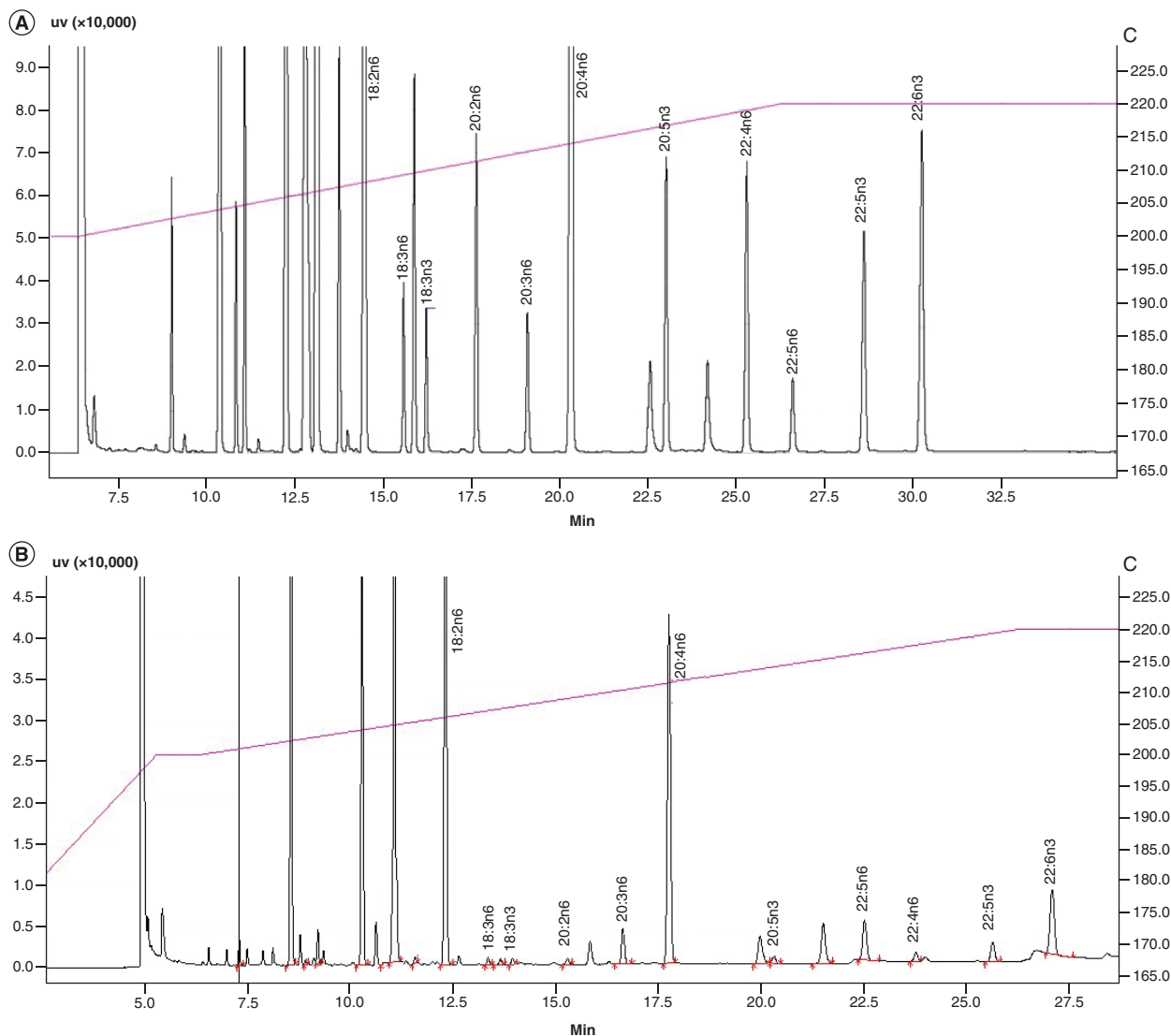


Figure 2. Gas chromatography-flame-ionization detector chromatogram. (A) Chromatogram of a standard mixture of fatty acid methyl esters. (B) Chromatogram of a test sample.

18:2n-6: Linoleic acid; 18:3n-3: Alpha linolenic acid; 18:3n-6: Gamma-linoleic acid; 20:2n-6: Eicosadienoic acid; 20:3n-6: Dihomo-gamma-linolenic acid; 20:4n-6: Arachidonic acid; 20:5n-3: Eicosapentaenoic acid; 22:5n-3: Docosapentaenoic acid; 22:4n-6: Docosatetraenoic acid; 22:5n-6: Docosapentaenoic acid; 22:6n-3: Docosahexaenoic acid.

Results & discussion

Method development

In the present study, we describe a practical and economical method involving the collection of a low volume of whole blood from the finger prick on a DBS card that provides compositional data within 1-2 days. The GC could separate 22 FAs, including 11 PUFAs of interest in 1 μ l of the analyte within 40 mins, indicating that the method is sensitive and satisfactory. Additionally, the n-3 index and AA/EPA ratio calculation utilize qualitative FA values; therefore, the DBS analysis is suitable as blood volumes are often unknown.

Most FAs in the body are in their bound forms as phospholipids, triglycerides and cholesteryl esters, and only a small amount of FAs are in free form. In FA analysis, the bound FAs can be released in the presence of a base or an acid and then transesterified to FAMES before chromatographic separation [12]. GC requires FAs to be derivatized so that they may become volatile to be eluted at sufficient temperatures without thermal decomposition [13]. There are several reagents for the transesterification of FAs. In our method, BF₃-MeOH is beneficial because BF₃ is a strong acid catalyst for the transesterification of FAs that can transform both bound and free fatty acids (FFAs) concurrently to FAMES. In addition, a direct transesterification reaction allows simultaneous hydrolysis and methylation of FAs without prior extraction of FAs. Conventionally, sample preparation for FA analysis by GC involves initial lipid extraction and

Table 3. Reproducibility test.

PUFA	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
18:3n-3 (%)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08
20:5n-3 (%)	0.23	0.24	0.23	0.24	0.24	0.24	0.24	0.23	0.23	0.24
22:5n-3 (%)	0.77	0.77	0.78	0.77	0.78	0.77	0.77	0.77	0.77	0.77
22:6n-3 (%)	1.92	1.89	1.87	1.89	1.90	1.89	1.88	1.87	1.90	1.89
18:2n-6 (%)	21.43	21.40	21.42	21.43	21.41	21.37	21.38	21.43	21.41	21.40
18:3n-6 (%)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
20:2n-6 (%)	0.28	0.29	0.29	0.28	0.29	0.29	0.29	0.29	0.29	0.29
20:3n-6 (%)	0.88	0.86	0.88	0.86	0.86	0.86	0.88	0.90	0.86	0.87
20:4n-6 (%)	7.77	7.79	7.78	7.80	7.83	7.80	7.78	7.79	7.78	7.83
22:4n-6 (%)	1.01	1.03	1.02	1.01	1.02	1.01	1.03	1.01	1.05	1.03
22:5n-6 (%)	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23

1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th and 10th represent replicates of one sample recovered on 10 different DBS cards.

18:2n-6: Linoleic acid; 18:3n-3: Alpha linolenic acid; 18:3n-6: Gamma-linolenic acid; 20:2n-6: Eicosadienoic acid; 20:3n-6: Dihomo-gamma-linolenic acid; 20:4n-6: Arachidonic acid; 20:5n-3: Eicosapentaenoic acid; 22:4n-6: Docosatetraenoic acid; 22:5n-3: Docosapentaenoic acid; 22:5n-6: Docosapentaenoic acid; 22:6n-3: Docosahexaenoic acid; PUFA: Polyunsaturated fatty acid.

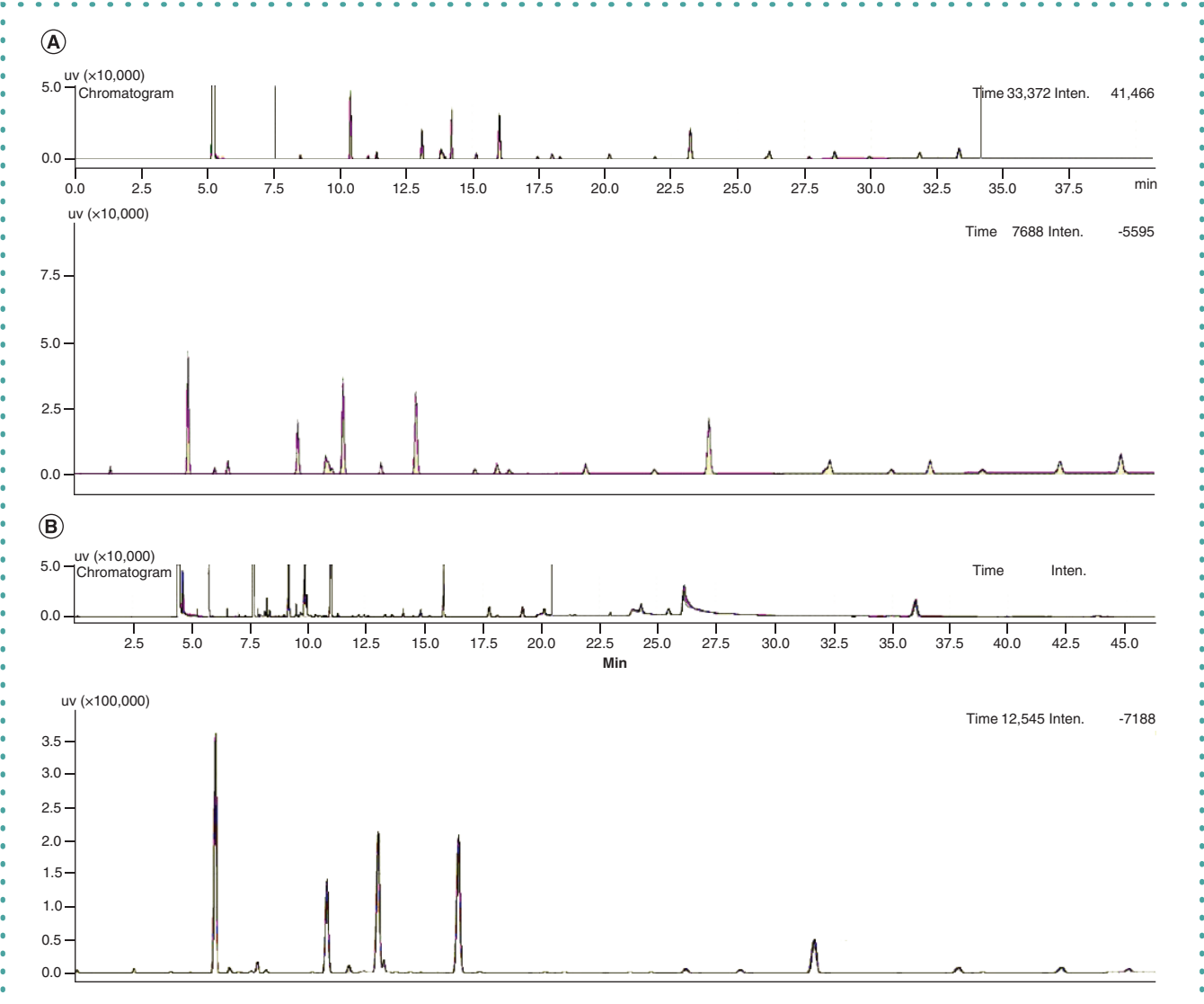


Figure 3. Instrumental response in repeatability and reproducibility tests. (A) Repeatability test. (B) Reproducibility test.

Table 4. Polyunsaturated fatty acid status among the study participants.

Variable	All (n = 20)	Uninjured (n = 10)	Injured (n = 10)	p-value
18:3n-3 (%)	0.35 ± 0.23	0.36 ± 0.27	0.34 ± 0.19	0.94
20:5n-3 (%)	0.45 ± 0.31	0.57 ± 0.38	0.32 ± 0.15	0.02
22:5n-3 (%)	0.67 ± 0.25	0.75 ± 0.23	0.59 ± 0.26	0.14
22:6n-3 (%)	1.89 ± 0.87	2.18 ± 1.01	1.60 ± 0.64	0.07
18:2n-6 (%)	18.82 ± 1.97	19.54 ± 2.06	18.10 ± 1.66	0.03
18:3n-6 (%)	0.20 ± 0.15	0.27 ± 0.19	0.13 ± 0.05	0.06
20:2n-6 (%)	0.23 ± 0.05	0.21 ± 0.06	0.24 ± 0.04	0.15
20:3n-6 (%)	1.26 ± 0.28	1.13 ± 0.26	1.39 ± 0.26	0.05
20:4n-6 (%)	7.09 ± 1.12	7.02 ± 0.85	7.16 ± 1.38	0.94
22:4n-6 (%)	0.84 ± 0.25	0.75 ± 0.16	0.93 ± 0.30	0.19
22:5n-6 (%)	0.23 ± 0.12	0.27 ± 0.16	0.19 ± 0.06	0.26
n-3 index (%)	3.20 ± 1.28	3.68 ± 1.43	2.73 ± 0.96	0.05
AA/EPA	20.33 ± 8.63	15.13 ± 4.94	25.54 ± 8.52	0.008

Values are presented as mean ± SD.

18:2n-6: linoleic acid; 18:3n-3: alpha linolenic acid; 18:3n-6: gamma-linolenic acid; 20:2n-6: eicosadienoic acid; 20:3n-6: dihomo-gamma-linolenic acid; 20:4n-6: arachidonic acid; 20:5n-3: eicosapentaenoic acid; 22:4n-6: docosatetraenoic acid; 22:5n-3: n-3 docosapentaenoic acid; 22:5n-6: n-6 docosapentaenoic acid; 22:6n-3: docosahexaenoic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid.

methylation as two separate procedures. A study compared the conventional and simplified methods (excluding prior extraction) under the same chromatographic conditions. Interestingly, the recovery of long-chain PUFAs from biological samples in the simplified method was significantly higher than in the conventional method. Therefore, lipid extraction before methylation of long-chain PUFAs can be excluded without affecting the recovery of long-chain PUFAs and their composition. Furthermore, this one-step method is simple, time-saving, decreases the use of solvents and reduces the possibilities for sample loss and contamination [14].

DBSs from fingertip blood may be susceptible to lipid peroxidation during storage due to Fe²⁺-induced peroxidation mechanisms, light exposure, humidity and thermal degradation [8,15]. To avoid FA loss, it is recommended to pre-treat the spot card with an antioxidant, such as butylated hydroxytoluene (BHT). In a study by Metherel *et al.*, venous whole blood was saturated on paper strips pre-soaked with various concentrations of BHT and was compared to spot cards stored without BHT. The BHT delayed the decrease of PUFAs, such as EPA and DHA, from baseline to 28 days of storage. Furthermore, sealed storage with BHT tended to increase the stability of the PUFAs in DBSs. Therefore, it is recommended to store DBSs in sealed containers with BHT to potentially prevent PUFA degradation [8].

After derivatization and extraction with the organic solvent n-hexane, the FAMES were analyzed by GC. FAME separation is based on the number of carbon atoms, degree of unsaturation or conformation [16]. In capillary GC, the peak resolution, separation and retention time are mainly affected by the polarity of the stationary phase, column length and diameter and film thickness [10]. The use of a fused silica capillary column, such as SP-2560, with enhanced surface inertness, thermal stability and resolution achieves the requirement of FA analysis. Furthermore, the SP-2560 is highly polar biscyanopropyl, and it is primarily designed for detailed separation of FAME isomers. As shown in Figure 2, the SP-2560 column separated individual FAMES very well and overlapping was not observed in the chromatogram. Fused silica columns are efficient as they are flexible and easy to handle and connect to GC. Additionally, the efficiency of the column is affected by the nature and velocity of the carrier gas. Typical carrier gases used in the analysis of FAMES are helium, nitrogen and hydrogen. Hydrogen functions at higher linear speeds than helium and nitrogen, thus lowering the analysis time with no compromise separation. However, helium is frequently used at linear velocities higher than its optimum velocity to obtain the exact separation times as hydrogen [11].

Method validation

The calibration curves were created from five concentrations of each FAME in the standard mixture. According to US FDA guidelines, the lower limit of quantitation (LLOQ) was identified as the lowest point of the calibration curve, which can be determined with 80–120% accuracy and an imprecision <20% [17]. The standard curves of the PUFAs were linear ($r_2 \geq 0.99$), and the RSD was <20%. The repeatability test yielded perfectly overlapping chromatograms (Figure 3A). The results obtained from the reproducibility test, both in terms of instrumental response and FA percentages, were comparable (Figure 3B & Table 3). This indicated that the method is satisfactory.

Method application

This proposed method was further assessed by comparing PUFA levels and the n-3 index and AA/EPA ratio in DBSs collected from 10 injured and 10 uninjured non-elite runners (mean age: 41.95 ± 10.11 years). The aim of this assessment was also to examine whether the PUFA biomarkers are correlated with the prevalence of running-related injuries among the participants. The differences between each group were calculated using Wilcoxon–Mann–Whitney test. Among the 11 PUFAs, three PUFAs showed statistically significant

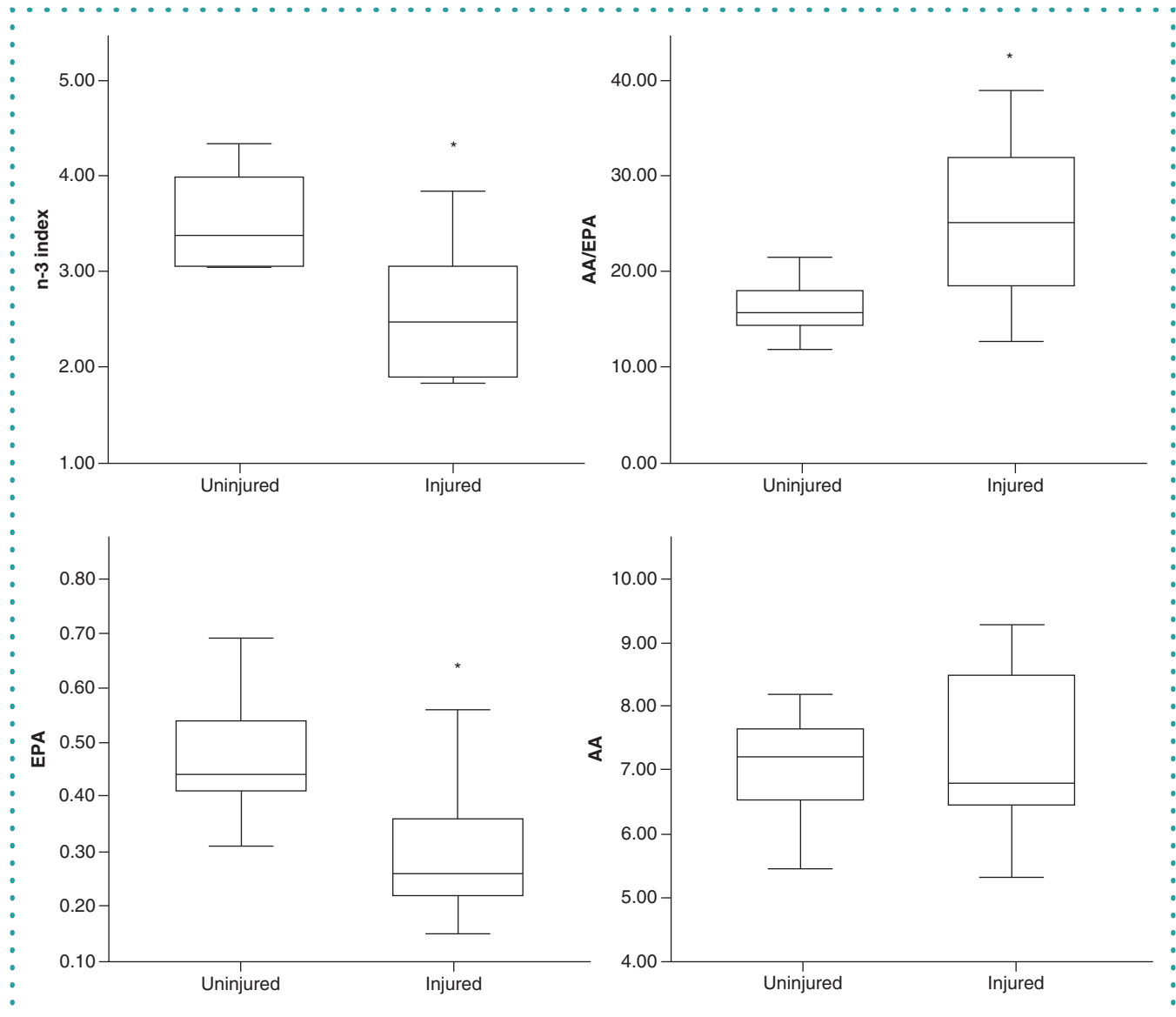


Figure 4. Polyunsaturated fatty acid biomarkers among the participants.
 *Significant difference in injured runners compared to uninjured runners; $p < 0.05$.
 AA: Arachidonic acid; EPA: Eicosapentaenoic acid.

differences ($p < 0.05$) between uninjured and injured subjects, including EPA, LA and dihomo-gamma-linolenic acid (DGLA; 20:3n-6) (Table 4). The results showed a statistically significant higher n-3 index and lower AA/EPA in the uninjured subjects than the injured ones ($p < 0.05$) (Figure 4). Our results agreed with an earlier study showing that lower daily fat intake is associated with increased injury risk among competitive runners [18]. Together, these findings suggest that the whole blood PUFA biomarkers may help monitor the risk of running-related injuries. Nevertheless, it is important to acknowledge that our evaluation is a preliminary study of 20 subjects. Several confounding factors are associated with injury risk. These DBS samples were used to assess the feasibility of the proposed method. Therefore, the statistical analysis is not very comprehensive. Although the study population is small and further confirmation is required, the presented approach can determine PUFA biomarkers in biological samples. Our findings suggest that the proposed method, from a translational healthcare point, is accurate and can be used as a diagnostic test for monitoring metabolic and nutritional status.

Conclusion

There is a growing interest in using PUFA indices to assess health status. However, methods to evaluate the PUFA status with high efficacy are required. The whole blood collection can be obtained on DBS cards and directly trans-esterified to allow an economical and

accurate PUFA profiling and consequently the estimation of the n-3 index and AA/EPA ratio. This provides a simple alternative to current labour-intensive methods that require a complex analysis of blood PUFA status.

Future perspective

Considering that several factors determine PUFA oxidation during DBS storage, future studies can improve storage methods for DBS samples, which would benefit population screening for PUFA biomarkers of disease risk and nutritional intake. In addition, this approach needs to be further assessed, incorporating rapid automated sample processing techniques combined with fast GC technology.

Executive summary

Background

- There is a growing interest in using polyunsaturated fatty acid (PUFA) biomarkers to assess health status. A dried blood spot (DBS) is a simple and non-invasive way to collect blood samples and allows accurate analysis of fatty acids.

Method development

- This study described a method to determine 22 fatty acid methyl esters, including 11 PUFAs, from DBS using gas chromatography. Subsequently, the n-3 index and arachidonic acid/eicosapentaenoic acid (AA/EPA) ratio were estimated.

Method performance

- A pilot study to determine the n-3 index and AA/EPA ratio in injured and uninjured runners was used to evaluate our method.

Author contributions

S Ali, A Pisanti and G Corbi: methodology, investigation and formal analysis; S Ali: writing – original draft preparation; S Davinelli and M Intrieri: writing-review and editing; G Scapagnini, G Corbi and S Davinelli: supervision.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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