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A validated method for analyzing polyunsaturated free fatty acids from dried blood spots using LC–MS/MS☆

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ABSTRACT

Omega–3 and omega–6 polyunsaturated free fatty acids (PUFA–FFA) are precursors to potent downstream lipid mediators that are regulators of inflammation. We describe the development and validation of a novel and sensitive method for quantification of individual PUFA–FFA in a dried blood spot using liquid chromatography tandem mass spectrometry (LC–MS/MS). Lipids were extracted from dried blood spot and six individual PUFA–FFA were quantified by LC–MS/MS using stable isotope dilution analysis with deuterated internal standards. PUFA–FFA concentrations in blood samples from 30 subjects were measured using the new method and compared to the traditional approach of thin layer chromatography followed by gas chromatography with flame ionization detection (TLC–GC). Responses for each PUFA–FFA were linear throughout a range of concentrations expected in clinical samples. Intra–day and inter–day variations for all PUFA–FFA were ≤ 16%. The concentrations of all PUFA–FFA measured by LC–MS/MS were positively correlated with measures of the same PUFA–FFA obtained by a traditional TLC–GC method. This novel method for the quantification of PUFA–FFA extracted from dried blood is sensitive and precise, and accurately measures levels of biologically important PUFA–FFA in blood.

1. Introduction

Omega–3 and omega–6 polyunsaturated fatty acids (PUFA) have been implicated in a number of health conditions [1] and their effects are thought to be due to inflammatory and immune modulating activities [2–4]. The biological actions of PUFA are mediated by a series of downstream metabolites, collectively known as oxylipins, which are enzymatically derived from the parent fatty acids [5]. However, in order for esterified fatty acids to be converted into bioactive oxylipins they must be released from being esterified in triglycerides and phospholipids by cytoplasmic lipases into non–esterified or free fatty acid (FFA) pool [6,7]. It is also well–documented that total FFA levels are closely related to clinical conditions and high levels in human biological tissues have been associated with trauma [8], heart attacks [9] and rheumatoid arthritis [10]. However, individual FFA have distinct biological properties, and thus determining the concentrations of individual PUFA–FFA in human blood samples is likely to provide a more accurate indication of the relationship between specific fatty acids and disease state than measuring esterified fatty acids by traditional means.

Clinically, plasma FFA are most often measured using enzymatic kits that quantitate the total amount of FFA in plasma samples, but are unable to measure individual FFA [11–14]. Although it is possible to measure individual FFA using TLC followed by gas chromatography with flame ionization detection (TLC–GC) [15,16], the process is tedious and the accuracy of these measures has been questioned [17,18]. More recent methods using GC–MS [19] and LC–MS [20,21] provide greater speed and sensitivity. The increased availability of LC–MS/MS to investigators has meant the publication of many methods for directly measuring individual PUFA–FFA in a range of tissues including the brain [7] and plasma from patients with diabetes [22] and high throughput systems will make the method more widely used [23]. However, these methods still require invasive venous blood collection and complex sample extraction methods. In addition, venous blood samples require cold–chain transportation which is costly.

Rather than collecting and working with venous blood, dried blood spot technology offers a simple and convenient sample collection

Abbreviations: PUFA–FFA, polyunsaturated free fatty acids; TLC–GC, TLC followed by gas chromatography with flame ionization detection; AR, analytical reagent; BHT, 3,5–di–*tert*–4–butylhydroxytoluene; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantitation; FAME, fatty acid methyl esters; SEM, standard error of the mean; IS, internal standard; DP, declustering potential; CE, collision energy; and CXP, cell exit potential

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option. A modified dried blood spot system called PUFAcoat™ paper has been recently developed for the collection of lipids in blood and transportation at room temperature that ensures stability of PUFA for periods up to 3 months [24].

This study reports on a robust method that utilizes blood collected as dried blood spot on PUFAcoat™ paper and when combined with LC–MS/MS can quantify individual PUFA–FFA with greater accuracy than TLC–GC approaches.

2. Materials and methods

2.1. Standards and reagents

LC–MS/MS grade methanol, water and acetonitrile were purchased from Merck, Darmstadt, Germany. A.C.S grade formic acid, acetic acid and sulfuric acid, and analytical reagent (AR) grade 3,5–di–*tert*–4–butylhydroxytoluene (BHT) and ammonium formate, were obtained from Sigma–Aldrich, MO, USA. Omega–3, alpha–linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA) and omega–6, linoleic acid (LA) and arachidonic acid (AA) standards (≥ 98% purity) and deuterated internal standards d_5–ALA, d_5–EPA, d_5–DHA, d_4–LA and d_8–AA (≥ 99% purity) were purchased from Cayman Chemical Company, MI, USA. Saline was purchased from Baxter Healthcare, NSW, Australia. AR grade chloroform, diethyl ether, and petroleum ether (40–60 °C), and laboratory reagent grade isopropanol, were obtained from Chem–Supply Pty Ltd, SA, Australia. AR grade methanol and heptane were acquired from Rowe Scientific, SA, Australia. $C_{17:0}$ internal standard, TLC reference standards and GLC–463 fatty acid methyl ester standards were purchased from Nu–Check, MN, USA.

2.2. Subjects and sampling

Ethics approval for the collection of venous and capillary blood for this study was obtained from The University of Adelaide Human Research Ethics Committee (H–2016–088). All participants provided written informed consent prior to blood collection.

Whole blood (~4–6 mL) was collected through the antecubital vein into dipotassium EDTA vacutainers (Becton, Dickinson and Company, SA, Australia) from each of 30 subjects (25 females and 5 males) aged between 22 and 70 years. At the same appointment, ~40 μL of venous blood was spotted onto PUFAcoat™ paper (Xerion Limited, VIC, Australia) and air–dried at room temperature for 3 h in the dark.

2.3. LC–MS/MS method for analysis of PUFA–FFA from dried blood spot

2.3.1. Instrumentation and optimized conditions

Analyses were conducted using an Agilent 1290 Infinity ultra–high performance liquid chromatography system (Agilent Technologies, Germany) equipped with a binary pump and thermostated autosampler held at 4 °C and connected to a 5500 triple quadrupole mass spectrometer (AB Sciex, USA), using electrospray ionization in negative mode. The mass spectrometer had the following conditions: nitrogen was used as the source gas; curtain gas and collision gas were set at 20 and 6 arbitrary units, respectively; capillary voltage was −4500 V; entrance potential was set at −10 V and temperature was set at 200 °C. An Eclipse plus C8 column (2.1 × 100 mm, 1.8 μm, Agilent Technologies, Germany) was used for chromatographic separation of the compounds and maintained at 40 °C. The injection volume was 20 μL, and the flow rate was 400 μL/min. Gradient elution was carried out with 50% aqueous acetonitrile containing 0.05% formic acid (mobile phase A), and 100% acetonitrile containing 0.05% formic acid (mobile phase B). The linear gradient for solvent B was as follows: 0 min, 10%; 7 min, 80%; 9 min, 10%; followed by 1 min of column equilibration with 10% B. Compound identification was achieved by comparing the retention times and multiple reaction monitoring (MRM) transitions of signals from samples to those of commercial PUFA–FFA standards. Quantification of the analytes was carried out by calculating the analyte to internal standard peak area ratios. Analyst software (version 1.6.2) was used for instrument control and data analysis. The MRM parameters for each analyte were optimized with infusion MS/MS experiments, using PUFA–FFA standards diluted in methanol (0.01 μM) and a syringe pump operating at 5 μL/min. Optimized parameters for each analyte are presented in Table 1.

Table 1
MS/MS parameters of PUFA–FFA in dried blood spot.

Analyte	MRM[a] transition	IS[b]	DP[c]	CE[d]	CXP[e]
ALA	277.1→259.1	d_5–ALA	−150	−23	−21
EPA	301.2→257.2	d_5–EPA	−120	−16	−20
DPA	329.2→285.2	d_5–DHA	−150	−19	−28
DHA	327.2→283.2	d_5–DHA	−82	−14	−20
LA	279.1→261.1	d_4–LA	−150	−25	−21
AA	303.1→259.2	d_8–AA	−135	−16	−18
d_5-ALA	282.2→238.1		−100	−20	−20
d_5-EPA	306.2→262.2		−100	−17	−20
d_5-DHA	332.3→288.2		−100	−16	−20
d_4-LA	283.1→265.2		−150	−27	−15
d_8-AA	311.1→267.1		−100	−16	−22

[a] MRM, Multiple reaction monitoring.
[b] IS, Internal standard.
[c] DP, Declustering potential, V.
[d] CE, Collision energy, eV.
[e] CXP, Collision cell exit potential, V.

2.3.2. Evaluation of extraction solvents, their stability and gradient elution

A sub–set of 4 PUFA–FFA (EPA, DHA, LA and AA) were selected for initial optimization of extraction solvents and assessment of compound stability. This selection was based on the inclusion of fatty acids with chain lengths between 18 and 22, and between 2 and 6 double–bonds and which were therefore representative of the full range of omega–3 and omega–6 fatty acid structures. Each of these PUFA–FFA were assessed from triplicate 6 mm discs punched from dried blood spot using 150 μL of five different extraction solvents with deuterated internal standards (80% aqueous methanol; 70% aqueous acetonitrile + 12 mM ammonium formate + 0.02% acetic acid; 80% aqueous acetonitrile; 80% aqueous acetonitrile + 0.02% formic acid; 80% aqueous acetonitrile + 0.05% formic acid). The extracts were analyzed immediately and after 1 week in the autosampler at 4 °C to assess the stability of the PUFA–FFA post extraction in the different extraction solvents. In addition, the gradient elution was optimized with acetonitrile and water as has been used previously [25] to enable the complete separation of all six PUFA–FFA within a short run time.

2.3.3. Optimized dried blood spot extraction procedure

A 6 mm disc (estimated to contain ~ 9 μL of blood) was obtained from each dried blood spot sample using a card punch (EK tools) and each disc placed into a separate well in a 96–well plate (Greiner Bio-One, Australia). Extraction solvent (150 μL of 80% aqueous methanol) containing deuterated internal standard mix (0.1 ng/μL of d_5–DHA and d_8–AA; 0.05 ng/μL d_5–EPA; 0.7 ng/μL of d_5–ALA; and 1 ng/μL of d_4–LA) was added and the plate was covered with heat sealing foil (Eppendorf, Hamburg, Germany) and gently shaken for 30 min at room temperature. The extract from each well was then transferred to a fresh well and sealed with heat sealing foil using a MicroSeal manual plate sealer (Pathtech, VIC, Australia) in readiness for immediate analysis.

2.4. LC–MS/MS method validation

2.4.1. Preparation of calibration curves for validation and quantifying batches of samples

All solutions were prepared with methanol containing 0.005% BHT.

A stock solution of mixed PUFA–FFA standards (consisting of ALA, EPA, DPA, DHA, LA and AA) was diluted volumetrically to prepare five working solutions, which were further diluted to obtain nine calibration standards to achieve concentrations expected in human blood [26,27]. All prepared standards were stored at −80 °C in glass vials with screw caps (Adelab Scientific, SA, Australia) sealed with Parafilm.

Each calibration standard solution (10 μL) was spiked to 490 μL of human blood and incubated at room temperature for 10 min to enable the binding of PUFA–FFA to albumin. Then, 40 μL of PUFA–FFA standard spiked blood was spotted onto PUFAcoat™ paper and air–dried at room temperature for 3 h in the dark. After drying, the paper was placed inside an aluminium zip lock bag (VWR International, QLD, Australia) containing silica gel desiccant (Silica Gel, NSW, Australia) and stored at −80 °C until use. To evaluate the matrix effect of blood, PUFA–FFA standard spiked extraction solutions without sample matrices (10 μL calibration standards spiked into 490 μL extraction solvent) were prepared as a reference and stored at −80 °C in glass vials with screw caps sealed with Parafilm. Final concentrations of standards are summarized in Supplemental Table 1.

2.4.2. Linearity

Method validation was conducted according to guidelines [28] with nine–point calibration curves (Supplemental Table 1) prepared from triplicates of 6 mm discs obtained from dried blood spot samples from three separate individuals in which the blood had been spiked with PUFA–FFA standards prior to spotting. For comparison, calibration curves were also produced from 9 μL (estimated volume in a 6 mm dried blood spot) of spiked extraction solvent. In each case, the sample was extracted according to the optimized method and analyzed by LC–MS/MS.

2.4.3. Precision and recovery

Intra–day and inter–day precision was determined by using a low (QC 3, Supplemental Table 1), medium (QC 2, Supplemental, Table 1) and high (QC 1, Supplemental Table 1) concentrations of PUFA–FFA standards spiked in blood and spotted on paper. Intra–day variation was determined by analyzing discs from the dried blood spot from 20 different locations on the same day. Inter–assay variation was determined by measuring the dried blood spot in duplicates 2 times each day for 20 days over a month [29]. Recoveries were determined [28].

2.4.4. QC samples

Three QC samples, consisting of PUFA–FFA standard mix (Supplemental Table 1) spiked into human blood, then spotted and dried on PUFAcoat™ paper, were prepared in triplicate and included with every batch of samples along with a calibration curve to confirm the accuracy of the analysis.

2.4.5. Limit of detection and limit of quantitation

The limit of detection (LOD) was determined by examining the chromatograms visually and establishing the minimum level at which the analytes could be reliably detected from the analysis of samples with known concentration of the analyte (signal–to–noise ratio of 3). The limit of quantitation (LOQ) was calculated by multiplying LOD by 3.3 (signal–to–noise ratio of 10) [30].

2.4.6. Determination of PUFA–FFA stability in dried blood spot

The PUFA–FFA standards mix (standard 1, Supplemental Table 1) was added to blood and spotted on to a total of 12 pre–punched 6 mm discs of PUFAcoat™ paper. Blood spots were dried for ~ 30 min under nitrogen at room temperature (~ 25 °C). Lipids were extracted from three dried blood spot discs immediately after drying and analyzed by LC–MS/MS to quantify ALA, EPA, DPA, DHA, LA and AA at baseline. The remaining dried blood spot discs were stored inside aluminium zip lock bags containing desiccant at room temperature and FFA concentrations were measured after 1 week, 2 weeks and 1 month of storage.

2.5. TLC–GC analysis of PUFA–FFA

2.5.1. Total lipid extraction and FFA separation

Total lipids from whole blood were extracted using a modified Folch method [15]. Briefly, 1 mL of whole blood sample containing $C_{17:0}$ internal standard (10 mg/mL) was extracted with 6 mL chloroform/methanol (2:1 v/v, containing 0.05% BHT as antioxidant) using vigorous shaking. The mixture was allowed to stand at room temperature for 5 mins before being centrifuged at 1560 × *g* for 10 mins. The organic phase containing total lipid extract was withdrawn and dried under a steady stream of nitrogen at room temperature. The FFA in total lipid extract was separated from other lipid classes by one dimensional TLC using silica gel 60 H plates (Merck) and petroleum ether/diethyl ether/acetic acid (82:18:1, v/v) as the mobile phase [16]. The band corresponding to FFA was visualised under ultraviolet light and scraped into a scintillation vial for fatty acid transesterification.

2.5.2. Fatty acid transesterification

The band corresponding to the FFA from the TLC plate was mixed with 2 mL of 1% (v/v) H_2SO_4 in anhydrous methanol (containing 0.05% BHT as antioxidant) in a 5 mL sealed scintillation vial and heated at 70 °C for 3 h. The resultant fatty acid methyl esters (FAME) were extracted into *n*–heptane for GC analysis [24].

2.5.3. GC analysis

FAME were separated and quantified [24] using a 6890 gas chromatograph (Hewlett-Packard, CA, USA) equipped with a BPX70 capillary column (30 m × 0.32 mm, film thickness 0.25 μm; SGE Analytical Science, VIC, Australia), programmed temperature vaporisation injector and flame ionization detector. The injector temperature was set at 250 °C with a split ratio of 20:1, the flame ionization detector temperature was maintained at 300 °C, and a programmed temperature ramp (140 – 240 °C) was used. Ultra–high purity helium (BOC) was utilised as carrier gas at a flow rate of 35 cm/second. The identification and quantification of FAME were achieved by comparing the retention indices and peak area values of unknown samples to those of commercial GLC–463 lipid standards and internal standards (Nu–Chek Prep, MN, USA). Instrument control and data analysis were undertaken with Chemstation software (rev.b.01.03).

2.6. Statistical analysis

Unless otherwise indicated, all data are reported as the mean ± standard error of the mean (SEM) of triplicate measurements. Least–squares regression analysis was used to obtain calibration data. Linearity was assessed [31] using the D'Agostino K^2 test [32]. Slope of the lines were compared between the calibration curves generated from three different individuals' blood. The stability of individual PUFA–FFA at various time points was compared by using one–way repeated measures ANOVA with comparison of individual means by Tukey's post–hoc test. Similarly, different extraction solvents were compared to each other by using one–way ANOVA with comparison of individual means by Tukey's post–hoc. Extraction solvent stability post extraction was compared between time zero and 1 week using paired t–tests. Bland–Altman test was performed to compare LC–MS/MS and TLC–GC methods. All tests were performed using GraphPad Prism 7.02.

3. Results

3.1. Method development and validation

3.1.1. Evaluation of extraction solvents, their stability and instrument conditions

Comparing the five different extraction solvents trialled, 80%

Table 2
Precision and recovery of PUFA–FFA at 3 concentration levels (low, medium and high) from dried blood spot.

Analyte	LOD[a]	LOQ[b]	Range	R^2	Intra–day precision (%CV)			Inter–day precision (%CV)			Recovery %
	ng/μL				Low	Medium	High	Low	Medium	High	
ALA	0.1	0.3	0.3 – 10	0.9929	13	12	16	16	16	14	80–83
EPA	0.05	0.2	0.2 – 10	0.9916	11	15	14	16	14	10	81–83
DPA	0.05	0.2	0.2 – 5	0.9892	7	12	14	15	16	14	89–98
DHA	0.05	0.2	0.2 – 20	0.9909	6	14	11	16	11	12	84–103
LA	1	3	3 – 150	0.9746	12	8	14	16	15	16	84–93
AA	0.05	0.2	0.2 – 20	0.9779	11	14	14	10	12	12	82–89

[a] LOD, Limit of detection.
[b] LOQ, Limit of quantitation.

aqueous methanol resulted in significantly higher extraction of EPA, DHA, LA and AA from the dried blood spot (Supplemental Fig. 1). There was no significant change in PUFA–FFA extracted into this extraction solvent up to 1 week of storage inside the autosampler (Supplemental Fig. 2) and this solvent was adopted for the lipid extraction. In addition, optimization of the gradient elution resulted in good separation and easy identification of the individual PUFA–FFA within 10 min with detection by MRM (Supplemental Fig. 3).

3.1.2. Linearity

There was a linear relationship between LC–MS/MS responses and concentration for the six PUFA–FFA extracted from dried blood spot, with coefficients of determination (r^2) ranging from 0.9746 to 0.9929 (Table 2) and normally distributed regression residuals. A matrix effect was observed, such that the concentrations of PUFA–FFA measured in dried blood spot samples spiked with standards were consistently lower than those measured when the PUFA–FFA standards were spiked directly into extraction solvent (Fig. 1). However, the blood of several different individuals did not yield significantly different results when spiked with any of the six PUFA–FFA (Fig. 2). Although a blood matrix must be used for calibration, these results imply that any individuals' blood sample (or pooled blood) can be used to generate the calibration curve and QC samples required when determining PUFA–FFA in batches of unknown samples.

LOD and LOQ ranged from 0.05 to 1 ng/μL and 0.2 – 3 ng/μL in blood, respectively (Table 2). Although our method has a higher limit of detection in comparison to others [25,33], it is of sufficient sensitivity to accurately quantify the levels of the PUFA–FFA typically observed in human blood samples and is therefore fit for purpose.

3.1.3. Precision and recovery

Intra–day and inter–day CV for each PUFA–FFA at each of the three concentrations (low, medium, high) were all acceptable at ≤ 16% (Table 2), highlighting the reproducibility of the assay [34]. Recoveries of PUFA–FFA ranged from 80% to 103% (Table 2) and were within accepted ranges [35], however recoveries of ALA, EPA and AA appeared to be lower than the other FFA.

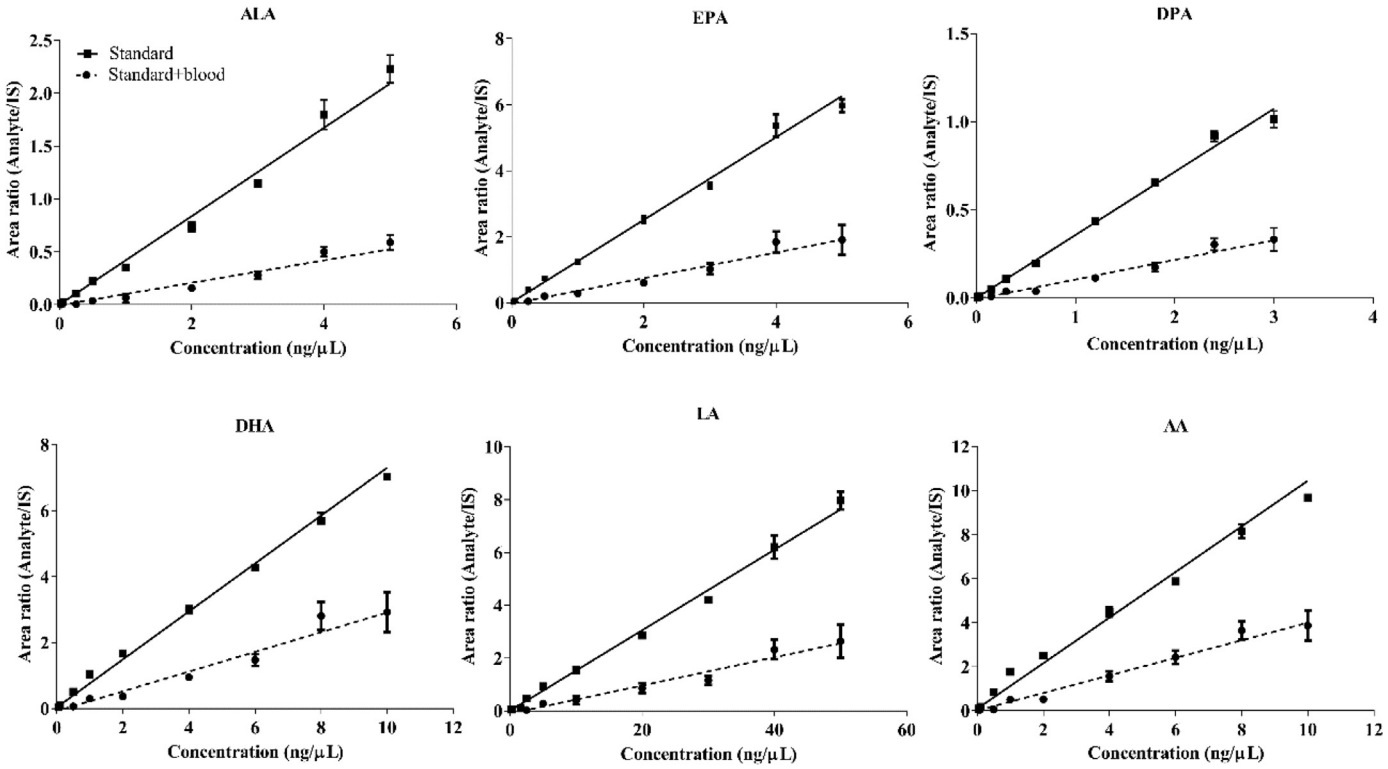

Fig. 1. Calibration curves for ALA, EPA, DPA, DHA, LA and AA as measured by analyte to internal standard (IS) area ratios from LC–MS/MS analysis. Plots show PUFA–FFA either added to blood then spotted on PUFAcoat™ paper and extracted (dashed lines) or added directly to extraction solvent without blood (solid lines). Data presented as mean ± SEM, n = 3.

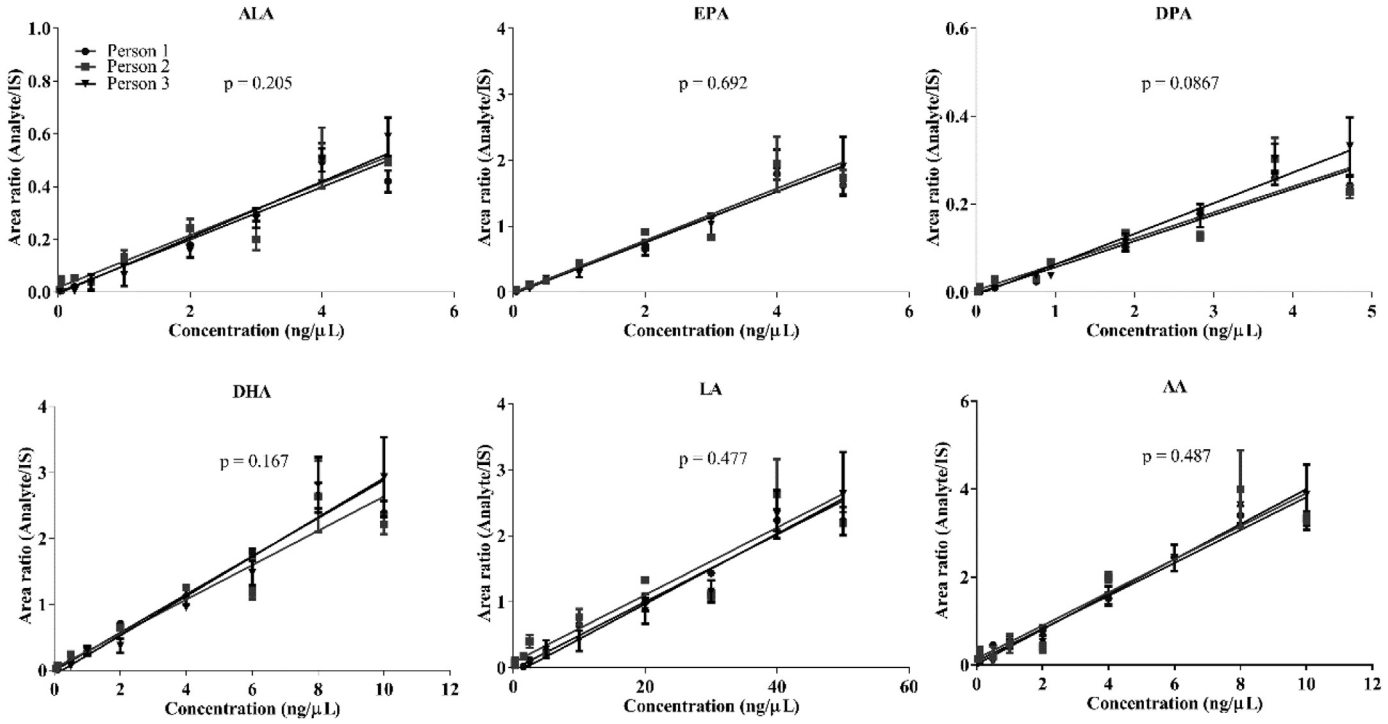

Fig. 2. Calibration curves from LC–MS/MS analysis of ALA, EPA, DPA, DHA, LA and AA in dried blood spot from three individuals, prepared by spiking PUFA–FFA standards into blood and spotting on dried blood spot cards. Results are expressed as mean ± SEM, n = 3 for each individual.

3.2. PUFA–FFA stability in dried blood spot

There were no statistically significant differences ($p > 0.05$) between levels of ALA, EPA, DPA, DHA, LA, and AA in dried blood spot samples extracted from PUFAcoat™ paper between measuring immediately after collection or after 1 week, 2 weeks or 1 month of storage at room temperature (Supplemental Fig. 4).

3.2.1. Comparison of PUFA–FFA measured using LC–MS/MS and TLC–GC

The newly developed LC–MS/MS method was compared to the traditional approach of TLC–GC. Concentrations of each PUFA–FFA measured by LC–MS/MS were positively correlated with PUFA–FFA measured by the traditional TLC–GC method for the same individual analytes (Table 3). Concentrations of AA as measured by TLC–GC were generally lower than the LC–MS/MS method. In addition, Bland–Altman test indicated a positive relationship between methods increased with increasing AA concentration (Supplemental Fig. 5). The reasons behind these apparent differences in the agreement between methods between different PUFA–FFA is unclear, however AA concentrations in the samples measured by TLC-GC were close to the limit of detection of the instrument. It is important to note, however, that contamination of one lipid class with another is during TLC separation is common and thus the traditional method is likely to be less accurate than the LC–MS/MS approach.

Table 3
Correlation between the LC–MS/MS method and TLC–GC method.

Fatty acid	r Value
Omega–3	
ALA	0.838
EPA	0.950
DPA	0.873
DHA	0.825
Omega–6	
LA	0.582
AA	0.563

4. Discussion and conclusions

There is increasing interest in the role of dietary fats in human health, particularly the balance between omega–3 and omega–6 PUFA as it relates to a wide range of clinical outcomes. Associations between measures of fatty acid status and clinical outcomes are often poor because most tests measure fatty acids incorporated into structural and storage lipids including membrane and plasma phospholipids. In order for fatty acids to act they must be initially liberated to a free or non–-esterified form. There is considerable confusion in the literature about the use of the term FFA and it is not uncommon for clinical investigators to claim that they are measuring FFA when in fact they are measuring the fatty acids in both esterified and free forms [36].

Although it is possible to measure individual PUFA–FFA using traditional TLC–GC approaches, these methods are labour–intensive and time consuming because lipids first need to be extracted and the different classes separated by TLC or solid phase extraction prior to transmethylation and analysis and this approach is thus not suited for high throughput analysis [16,37]. There is also the potential for both degradation of FFA when extracting these from the TLC plate [38], as well as interference from other lipid classes co–eluting with the FFA fraction during TLC. The LC–MS/MS method developed in this study overcomes many of the issues described above. The targeted analysis of specific compounds and the use of deuterated internal standards to help account for loss, degradation and interferences during the analysis in the LC–MS/MS methods are expected to contribute to improved accuracy and precision [21]. The described method enables the rapid quantification of individual PUFA–FFA extracted from a drop of blood with ease. This becomes particularly important when sample volumes are limited, such as in studies conducted with preterm infants [39]. The method was developed to measure those FFA that give rise to the metabolic regulators that are derived from PUFA–FFA, the oxylipins. The individual PUFA–FFA are reported in terms of the amount of FFA present in the sample injected which can then be related to the amount of

blood in the sample. For clinical purposes this is ideal as most metabolites are expressed in weight or moles per litre of blood.

The poor stability of blood PUFA has been overcome by collecting blood spots on PUFAcoat™. The use of silica impregnated paper and a chelating agent such as EDTA used in PUFAcoat™ prevent iron–mediated oxidation of fatty acids and drying prevents any enzymatic activity [24]. Combining dried blood spot sampling on PUFAcoat™ paper with LC–MS/MS analysis provides a powerful system to collect, store and subsequently determine concentrations of biologically relevant individual PUFA–FFA in small volumes of whole blood. In conjunction with the proposed analytical method, this is a new tool that will provide opportunities to measure PUFA–FFA efficiently and reliably across populations and relate them to clinical outcomes.

4.1. Strengths and limitations

The method is ideally suited for monitoring PUFA–FFA in clinical settings where continuous monitoring and repeat sampling is required since blood volume is small and collection can be done by finger–prick. It also lends itself to large scale population studies to estimate PUFA–FFA status because the PUFAcoat™ paper protects against changes post–sampling. One limitation is the assumption of the estimated volume in a 6 mm dried blood spot, which can vary depending on the individuals' haematocrit therefore changing the volume of blood in that disc [40]. The recoveries of some FFA, in particular ALA, EPA and AA, were lower than the others in this study. However, provided that the recoveries of each fatty acid is constant, they can be easily adjusted as is demonstrated on the strong linear relationships for all PUFA-FFA standard curves. While the CVs were acceptable for use of the assay as a research tool, it is important that the intra- and inter-day variation is taken into account when determining the appropriate sample size for detecting differences in FFA concentrations between population groups. The application of this assay to larger population groups will provide information on inter-individual variation in the concentrations of the individual FFA. Currently, there is very limited information on the extent of such variability, but this knowledge will be important for informing sample size calculations for research studies, and for establishing normative ranges of these compounds in different population groups.

5. Conclusions

A novel LC–MS/MS method for analyzing the PUFA–FFA contained in whole blood spotted on a special filter paper called PUFAcoat™ has been compared to the traditional TLC–GC approach. Responses were linear, did not vary between different donors and PUFA–FFA levels. The new method makes possible high throughput measurement of individual PUFA–FFA from dried blood spot samples.

Summary

Circulating free fatty acids (FFA) are measured clinically following traumatic events, commonly using enzymatic methods. However, these methods are unable to distinguish between individual FFA sub–types that are ultimately responsible for inflammatory and immune pathways. We have developed and validated a novel method for quantification of 6 polyunsaturated FFA from dried blood spot using LC–MS/MS. This new method is precise and correlates well with the traditional TLC-GC/FID method. Our work provides the opportunity to accurately determine biologically–important FFA in a small volume of blood and can be readily applied to large clinical trials.

Author contributions

RAG, DW, GL, BSM designed the study. EH, GL and DW conducted the experiments, collated and analyzed the data. All authors contributed to the interpretation of the results and drafting of the manuscript and have read and approved the final submitted version.

Conflicts of interest

RAG serves on the advisory board for the Fonterra group. All associated honoraria are paid directly to his host organisation and used to support travel and professional development for students and early career researchers. RAG is the inventor of PUFAcoat system, the patent for which is owned by the University of Adelaide. The licence for PUFAcoat has been granted to Xerion Ltd Melbourne. BSM serves on the advisory board for the Nestle Nutrition Institute and has given talks for Danone Nutricia and Aspen Nutrition. All associated honoraria are paid directly to her host organisation and used to support travel and professional development for students and early career researchers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2017.08.010.

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