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A High-Throughput Method for the Analysis of Erythrocyte Fatty Acids and the Omega-3 Index

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Abstract
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A high throughput method for the analysis of erythrocyte fatty acids and the omega-3 index

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Abstract

Omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) have several health benefits. In particular, low n-3 LCPUFA status is associated with cardiovascular disease (CVD) and led to the development of the omega-3 index which is the proportion of eicosapentaenoic acid and docosahexaenoic acid in the erythrocyte membranes, as a marker of CVD risk. Most methods used to measure the omega-3 index are laborious and time consuming. Therefore, the aim of this study was to develop a high-throughput method for the extraction and measurement of erythrocyte fatty acids and the omega-3 index. For sample extraction and quantification two methods were used; a single-step extraction, degradation and derivatisation method by Lepage and Roy, followed by gas chromatography flame ionization (GC-FID) which is commonly used and a high throughput method using an automated methyl tert-butyl ether extraction followed by electrospray ionization mass spectrometry. Both methods were first applied to the analysis of known concentrations of synthetic phospholipid mixtures to determine recovery and precision prior to their application in the analysis of human erythrocytes. The range of recoveries over five synthetic PL mixtures were 86.4% - 108.9% and the coefficient of variation was < 10% (within-run) and ≤ 15.2 % (between-run). Both methods showed high correlation (R=0.993) for the omega-3 index and there was no systematic bias in the detection of omega-3 index using either method. The new high throughput method described here offers considerable advantages in terms of simplicity and throughput compared to GC-FID method and provides additional information on molecular phospholipid concentrations.

Abbreviations CerPCho Sphingomyelin, PtdSer Phosphatidylserine, PtdEtn Phosphatidylethanolamine, PtdCho Phosphatidylcholine

Key words: Mass spectrometry, Omega-3 index, Gas chromatography, Docosahexaenoic acid, Eicosapentaenoic acid.

Introduction

Omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), particularly eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), provide several health benefits including enhanced cognitive function, prevention and treatment of CVD, arthritis and other inflammatory and autoimmune disorders (Salem, 1989; Simopoulos,1999; Bakker et al., 2003; Hibbeln et al., 2007; Marchioli et al., 2002). Conversely, insufficient n-3 LCPUFA intake (Meyer, 2016) has been implicated in increased risk of CVD (Mozaffarian, 2008), suboptimal neural development (Salem, Kim, & Yergy, 1986), psychiatric disorders (Hibbeln & Salem, 1995) and neurodegenerative diseases (Calon et al., 2005).

The 'omega-3 index' is defined as the sum of EPA and DHA levels in erythrocyte membranes expressed as percent of total fatty acids (Harris & Von Schacky, 2004; Harris, 2008; Von Schacky & Harris, 2007; Block et al., 2008). The omega-3 Index in the western countries, the Middle East, Africa and Southeast Asia is believed to be ≤ 4%. In countries where fish consumption is high such as Korea, Japan and Scandinavia the average omega-3 Index is ≥ 8% and coronary artery disease is less common (Stark et al., 2016). Most notably, it has been observed that an omega-3 index of 5% or more was associated with a 70% reduction in the risk of primary cardiac arrest (Siscovick et al., 1995). Also, similar results suggest that whole blood EPA and DHA concentration is strongly associated with a reduced risk of sudden death among men without evidence of prior cardiovascular disease (Albert et al., 2002). Based on this foundation, the omega-3 index was proposed in 2004 as a marker of risk of death from CVD (Harris & Von Schacky, 2004; Harris, 2008) and the omega-3 index has now been widely measured in different studies that examined not only CVD but also schizophrenia and depression (Parletta et al., 2016) .

Erythrocytes have advantages over plasma since the former reflects the last few months of food intake (Sullivan, Williams, & Meyer, 2006; Katan et al., 1997), while plasma EPA and DHA status is representative of food intake over the previous few days (Arab, 2003).

Therefore, erythrocyte fatty acids are a more reliable biomarker of long-term EPA and DHA status (Arab, 2003; Harris & Thomas, 2010). Specialized methods for extraction of lipids from erythrocyte membranes have been developed in the past (Reed, Swisher, Marinetti, & Enen, 1960; Rose & Oklander, 1965) and these two methods have noted the difficulty of extracting lipids from erythrocytes. A common method used for measuring erythrocyte membranes fatty acid concentrations including EPA and DHA (Ridges et al., 2001; Rodriguez-Palmero et al., 1998; Stewart et al., 2007; Goozee et al., 2017) is the gas chromatography-based method of Lepage & Roy (1986). The development of efficient analytical techniques, to improve recovery of all lipid species, to reduce the time and amount of labour that is involved in the estimation of fatty acids would improve the utility of measuring the omega-3 index. Accordingly, several recent studies have improved the throughput of gas chromatography-based fatty acid analysis (Masood, Salem, & Stark, 2005; Masood & Salem 2008; Glaser et al., 2010)

The aims of this study were 1) to develop high throughput method for measuring erythrocyte fatty acids and omega-3 index utilizing an electrospray ionization mass spectrometry (ESI-MS)-based method and 2) to compare this new method to the commonly used Lepage and Roy method (GC-FID method) in terms of time, sample volume and overall cost.

Materials and Methods

Chloroform, methanol and methyl tert-butyl ether (MTBE), were purchased from VWR International (QLD, Australia) and were high performance liquid chromatography grade or higher. Analytical grade ammonium acetate was obtained from Crown Scientific (New South Wales, Australia). Ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), acetyl chloride, toluene and potassium carbonate (K_2CO_3) were all purchased from Sigma Aldrich (NSW, Australia). Synthetic phospholipids (PL): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PtdCho 16:0/18:1), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PtdCho 16:0/18:2), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PtdCho 16:0/20:4), 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (PtdCho 16:0/22:6), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (PtdEtn 18:0/20:4), 1-stearoyl-2- docosahexaenoyl –sn-glycero-3-phosphoethanolamine (PtdEtn 18:0/22:6), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PtdEtn 16:0/18:1) were purchased from Avanti Polar Lipids through Auspep (Victoria, Australia).

Disposable tips used on the Hamilton-star liquid handling workstation were purchased from Hamilton Robotics (Reno NV, USA). 50 uL and 300 uL sizes of conductive CO-RE tips (without filter) were used. Twin-tec PCR (Polycarbonate) clean plates and 96-well plates were purchased from Eppendorf (POCD scientific, Australia).

Preparation Synthetic PL mixtures

The seven synthetic PL listed above were mixed in concentrations designed to mimic human erythrocyte membranes lipid levels (additional information is available in supplementary materials). The concentrations of PtdCho (16:0/22:6) and PtdEtn (18:0/22:6) were varied in order to create five synthetic PL mixtures with a range of omega-3 indexes (2, 4, 6, 8 and 10 %). The synthetic PL stock solutions were stored at -80^{o}C in 10 mL glass vials containing 2:1 methanol: chloroform with 0.01 % BHT as an antioxidant. To the best of the authors' knowledge, there are currently no commercially available synthetic PL species containing EPA and hence EPA was not used in these synthetic PL mixtures. Nevertheless, EPA is present at < 1% of total erythrocyte fatty acids. In comparison, DHA is usually present at ≥ 4

% (Jackson et al., 1997) and therefore in the synthetic PL mixtures DHA was used to represent the n-3 fatty acids in the omega-3 index. Synthetic PL mixtures were first analysed in order to a determine recovery and accuracy of both methods. Analysing erythrocytes with unknown fatty acid concentrations does not allow these parameters to be determined accurately.

Human Erythrocytes

This study was approved by the human research ethics committee at the University of Wollongong (HE16/016) and written consent was obtained from all participants. Participants were recruited from the University of Wollongong staff and students. Blood samples were collected into vacutainers coated with EDTA to prevent clotting and platelet clumping. This followed by centrifugation at 3000 rpm at 4 °C for 10 minutes and the remaining plasma and white blood cells removed. Aliquots 1.5 ml of erythrocytes were stored at -80 °C until required for analysis.

Lipid extraction & direct transesterification

The method for fatty acid extraction has been described previously (Ridges et al., 2001). Briefly, frozen (-80℃) packed erythrocytes (n=25) were thawed and 400 uL resuspended at room temperature in a Tris buffer, which contained 10 mM Trizma Base and 2 mM EDTA Na2 (pH 7.2) in polycarbonate ultracentrifuge tubes (16 × 76mm). The samples were capped, gently mixed and left at room temperature for 30 minutes to induce erythrolysis. Erythrocyte membranes were pelleted by ultracentrifugation at 49000 rpm at 4℃ for 30 minutes using a 70.1 TI rotor in a Beckman L-8 ultracentrifuge (Beckman, USA). The supernatant was discarded and removed, and the pelleted erythrocyte membranes were resuspended in 200 µL of distilled water. Aliquots (150 µL) of resuspended erythrocytes or synthetic PL mixtures were subjected to direct transesterification according to the method of Lepage and Roy (1986). Briefly, 2.2 mL of a stock solution methanol: toluene (4:1) and 0.01% BHT with the internal standard heneicosaenoic acid 0.2 mg/ml) was added to the 150 µL aliquots of erythrocyte membranes resuspension or synthetic PL mixtures. Following this,

200 uL of acetyl chloride was added while vortexing at low speed. The tubes containing this solution were tightly sealed and heated on a heating block at 100°C for a period of 60 minutes. The tubes were cooled to room temperature in a water bath for 15 min and 6% K_2CO_3 was added prior to centrifugation for 10 min at 3000 rpm at 4 °C. The extracted upper toluene phase containing fatty acid methyl esters (FAME) was then transferred into glass GC vials for analysis by GC-FID.

Gas chromatography-flame ionization detection (GC-FID)

The gas chromatograph (GC-2010 plus, Shimadzu, Rydalmere, NSW, Australia) was fitted with a fused silica capillary column with 50 m x 0.25 mm internal diameter x 0.25 um film thickness from Agilent J & W GC Columns with the following temperature program: 150°C initial temperature, 10°C/min to 162.5°C, 0.4°C/min to 172.5°C and 10°C/min to 211°C, the injector and detector temperature were set at 250°C. The carrier gas was ultra-high purity hydrogen and the column flow rate was 2.0 mL/min. Column pressure was maintained at 157.1 kPa. The split ratio was set at 20:1. One microliter of each sample was injected onto the column using an auto injecting unit (Shimadzu AOC-20i) and the fatty acids were detected using FID. The total analysis time was approximately 30 minutes per sample with fatty acids eluting between 2.5 and 25 minutes. The fatty acids were identified by comparing their retention time with known fatty acid standards (Nu-chek and Sigma, Sydney, NSW, Australia). After analysis, each fatty acid was quantified by comparing their peak area to the peak area of internal standard.

Adaptation of a lipid extraction protocol to a robotic platform

To assess the suitability of existing protocols to a robotic platform several key properties were evaluated. They were: 1) the ability of the homogeising solvent to lyse erythrocytes, 2) the impact of the solvent on erythrocyte clotting, 3) the ability to convert the protocol to small volumes (<2000 µL) to fit a 96-well plate configuration, 4) an organic phase with a lower desity than water to allow removal of lipids from the top layer of the two-phase system, and 5) spontaneous phase separation (removing the need to centrifuge samples). The protocol

described by Matyash et al., (2008) appeared to fulfil many of these criteria and was chosen as the method used in the current investigation.

The MTBE extraction protocol was initially tested manually using packed erythrocytes. The first observation made was the difficulty of pipetting packed erythrocytes from the eppendorf tubes in which they were stored. The second notable observation was the tendency of erythrocytes to form an insoluble mass upon the addition of combined MTBE and methanol. This was quite different from what occurs with the Lepage & Roy (1986) method where the addition of Tris buffer to the packed erythrocyte causes the cellular membranes to immediately lyse with no sedimentation of the celluar membranes upon standing. The clumping suggested that the membrane bound proteins had become denatured in the presence of the organic solvents used. Furthermore, it was also unclear if the erythrocyte membranes had been effectively lysed by the addition of solvents. This was an important consideration as without the membrane being properly lysed, the lipids contained within the membrane bi-layer would not be exposed to the solvents and it would not be possible to solubilise the lipids in the following steps thus making the protocol ineffective for its proposed means. The focus of refining the MTBE protocol thus turned to how the erythrocyte membranes could be effectively lysed as well as preventing the “clotting”.

After testing several solvent sytems (see supplementary material), we decided to use a 150 mM ammonium acetate solution due to its ability to lyse erythrocytes, prevent clotting and is compatible with MS analysis. The Matyash et al., (2008) method reports the use of large volumes of both the sample to be analysed and the reagents used in the protocol (matyash et al., 2008). The starting volume of plasma used was 200 uL to which 1.5 mL of methanol is added. Upon the addition of 5mL of MTBE this far exceeds the 2mL of the standard format 96-well plates. Hence, the first amendment made to the protocol was to reduce the volumes used. To begin with, 10 uL was arbitrarily assigned as the volume of packed erythrocytes on which the extraction would be performed. It was then calculated that the ratio of specimen to methanol used by matyash et al., (2008) was 1:7 (sample: methanol v/v) and that the solvent system used comprised of MTBE: methanol: aqueous reagents in the ratio 10:3:2.9 (v/v/v).

Applying these ratios to the new starting amount of 10 uL packed erythrocytes created a protocol where the highest volume reached was 1600 µL. This is in line with the proposed requirement for robot extraction that the highest volume reached is ≤2000 µL. This protocol was then applied to the automated extraction as decribed below.

Automated Lipid Extraction

For ESI-MS analysis, lipids where extracted from erythrocytes (n=25) and synthetic PL mixtures (n=5) using the method of Matyash et al., (2008), with some modifications. In the automated extraction method, 290 uL of an aqueous mixture containing 150 mM Ammonium acetate and 2 mM EDTA was added to the wells of a 2.0 mL 96-well plate on ice. Aliquots of 10 µL taken from the synthetic PL mixtures or erythrocytes were then added to the wells prior to vortexing at 800 rpm for 10 minutes. Robotic procedures were then started with all plates, solvents and reagents loaded onto the automated liquid handling workstation (Hamilton, Nevada, USA). A 300 µL aliquot of an internal standard mixture in methanol [500 uM (PtdCho 19:0_19:0); 166.7 uM (PtdEtn 17:0_17:0); 100 uM (PtdSer 17:0_17:0); 500 uM (CerPCho 18:0_12:0)] was added to the wells prior to mixing and standing for 10 minutes. Then 1 mL of MTBE was added to the wells and mixed for 30 minutes before the mixture was allowed to stand for 15 minutes to allow phase separation. Following this, 500 µL of the MTBE top phase was removed to the extraction plate (containing the extracted PL) and 40 µL of this extract was transferred to another 96-well plate and diluted with 760 µL of a 2:1 (v:v) methanol: chloroform solution containing 5 mM Ammonium acetate. A 40 µL aliquot of the diluted extract was transferred to another 96-well plate, which was sealed with aluminum foil prior to analysis by mass spectrometry.

Electrospray ionization mass spectrometry (ESI-MS)

Mass spectrometry was performed utilizing a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP 5500 ,Sciex, MA, USA) with an automated chip-based nanoelectrospray source (Triversa NanoMate, Advion Biosciences, New York, USA) as

previously described (Brown et al., 2013; Norris et al., 2015). A 10 uL aliquot of lipid extract diluted in methanol: chloroform (2:1 v/v) containing 5mM ammonium acetate (approximately 10 uM) was infused into the nano-electrospray ion source. Spray parameters were a voltage of 1.1 kV for negative ion acquisition and 1.2 kV for positive ion acquisition and a nitrogen gas pressure of 0.40 psi (Brown et al., 2013; Norris et al., 2015). Lipids were identified using precursor ion and neutral loss scans as show in Supplementary Table S7. Specifically, precursor ion scans for phosphocholine cations (m/z 184.1) were used to detect PtdCho and CerPCho species while neutral loss scans were used to detect PtdEtn species (phosphoethanolamine loss, 141.0 Da) and PtdSer species (phosphoserine loss, 185.0 Da) (Brugger et al. 1997). The identitiy of the FA component of each PL was determined using precursor ion scanning for each FA in negative ion mode (Supplementary Table S7).

Ionized lipids identified with a minimum signal-to-noise ratio of 10 were included in the analysis. Identification and quantification was accomplished using LipidView software (version 1.2, Sciex, MA, USA) this included smoothing, identification, removal of isotope contribution from lower mass species, and correction for isotope distribution, as described (Brown et al., 2013; Norris et al., 2015). Quantification was performed using LipidView software by comparing the spectral peak area of individual lipids to their class specific internal standards following isotope correction. Fatty acid concentrations were then determined directly from PL concentration i.e. the concentration of each fatty acid is equivalent to the parent. For example if PtdCho 16:0_18:0 is 1.5 nmol/ml then the fatty acid 16:0 and fatty acid 18:0 are both 1.5 nmol/ml. Isobaric phospholipids e.g. PtdCho 16:0/22:6 and PtdCho 18:1/20:5 were resolved using fatty acid precursor ion scans. The concentration obtained from the head group scan was multiplied by the ratio of the relative abundance of each fatty acid pair to obtain the concentration of each molecular PL as described previously (Nealon, Blanksby, Mitchell, & Else, 2008). An excerpt (plus explanatory comments) from the spreadsheet where these calculations were performed is show in Supplementary Figure S1.

A quality control (QC) sample was produced using pooled erythrocytes from healthy volunteers (n = 10, 5 male and 5 female) aged between 13 and 40 years. The QC aliquots were stored frozen at (−80 ℃). With each full plate prepared (96 well plate) in the high-throughput ESI-MS method 3 QC samples distributed in positions (2, 49 and 95) to monitor instrument performance and assay precision. To assess contamination and interference 3 blank samples were processed side-by-side with analytical samples and placed in positions (1, 48 and 96) of each 96-well plate. Additionally, blanks were used to correct the data by subtracting blank readings from sample readings.

Statistics

Statistical analyses were performed using Microsoft excel and SPSS statistics. Pearson's correlation coefficient *(r)* was determined and tested for significance via two-tailed analysis. A Bland-Altman plot was produced to assess the agreement between the two methods (Bland & Altman, 1986; Altman & Bland, 2002; Ludbrook, 2010).

Results and Discussion

Recovery

Extraction recovery was tested to evaluate the accuracy of the method. As is evident in table 1 recovery and detection in both the GC-FID and the high-throughput ESI-MS methods was greater than 86% for most fatty acids.

An unexpected result was the low recovery of 18:0 using the MS-based method (50.9-61.3%) and 22:6 using both methods (66.9-85.1% for GC-FID method and 63.5-75.3% for ESI-MS method). Interestingly, PtdEtn (18:0/22:6), was noticeably yellow in colour inside the 2.5 mL ampule (starting material), suggesting oxidation had occurred and may explain these findings. The GC-FID method measures only the fatty acids and not the whole PL molecule. This means that only the data for the oxidized fatty acid is altered; i.e. a lower than expected 22:6 level was detected. This is in contrast to the high-throughput ESI-MS method, which measures the intact PL. Therefore, when one fatty acid is oxidized the data from both fatty acids in that particulalr PL species is lost. In the case of oxidized PtdEtn (18:0/22:6) both 22:6 and 18:0 would be reduced as observed. If the concentration of PtdEtn (18:0/22:6) add to the synthetic mixtures is removed from the expected values, the recovery of 18:0 of using the ESI-MS method increases to (70.3-99.3%) and 22:6 is improved for both methods (83.7-106.4% for the GC-FID method and 81.2-94.1% for the ESI-MS method).

Precision

Precision was assessed by estimating within-run and between-run %CV for both methods as shown in Table 2.

The within-run %CV of each fatty acid in each synthetic PL mixture for both the GC-FID method and the high-throughput ESI-MS methods were calculated for n=3 (Table 2). The within-run precision for high-throughput ESI-MS method was less than 10% and this is comparable to the GC-FID method. The high-throughput ESI-MS method also shows comparable results for within-run precision with previously published work using gas

chromatography-flame ionization detection (GC-FID) fatty acid analysis (CV < 8% and < 14%) (Glaser et al., 2010; Lin et al., 2012) and gas chromatography/mass spectrometry analysis GC-MS (CV <14%) (Quehenberger, Armando, & Dennis, 2011).

Measurement of between-run precision was calculated for samples of synthetic PL mixtures (n=3/day) over one month (Table 2). The between-run %CV in high-throughput ESI-MS method was slightly higher (≤15.2%) compared to the between-run coefficient of variation (≤13.9%) observed in the GC-FID method. Nevertheless, the between-run coefficient of variation observed in the high-throughput ESI-MS method was comparable to that of previous GC-FID and GC-MS fatty acid analyses (CV <16% and <17%) (Lin et al., 2012; Quehenberger, Armando, & Dennis, 2011). The QC samples intra-assay (within batch) precision ranged from 0.90% to 17.4% of 19 batches and the average inter-assay (batch-to-batch) precision ranged from 6.9% to 20% of 19 batches. (See supplementary material table S9 for QC sample fatty acid concentration)

Measurement of erythrocyte fatty acids and omega-3 index using two methods

There is a strong correlation (R= 0.993) between the GC-FID method and the high throughput ESI-MS method when assessing omega-3 index over a range of 2-10% (figure 1).

There was good agreement between the two methods across the range of omega-3 index (2-10%) with all points plotted falling within both the upper and lower limits of agreement (95% CI) as shown in figure 2. There was no systematic bias in the detection of omega-3 index extracted using either method. To the best of the authors' knowledge, this is the first validation study using Bland-Altman plot in comparing the difference between the two methods measuring fatty acids. The reporting of correlation coefficients can be misleading as correlations measure the strength of a relationship between two methods rather than the level of agreement between the results yielded by two methods (Bland & Altman, 1986). As described by Bland and Altman (1986) a significant correlation is expected if two measures

are measuring same thing. As such, studies validating a novel method against a reference method should be interpreted with caution where validity is measured and reported by way of correlation.

The concentration of erythrocytes fatty acids as determined by the GC-FID method and the high throughput ESI-MS method are presented in Table 3. The difference between the two methods was less than 10% with exception for fatty acid 22:4, where the difference was 37.5%. The abundance of fatty acid 22:4 is relatively low. The amount of 22:4 measured by the GC-FID method detects the total fatty acid amount obtained from all PL species in a single GC peak. This is advantageous as it improves signal -to-noise and therefore sensitivity of fatty acid with low relative abundance. Because 22:4 is of relatively low total abundance and was bound to several different PL species, the signal obtained by ESI-MS was further diluted. This results in a decreased signal-to-noise and therefore, some 22:4 containing PLs were below the limit of quantification and are not included in the MS totals (but would be included in the GC analysis).

Sample derivatization is required to improve fatty acid volatility (Brondz, 2002). Nowadays, procedures allowing simultaneous hydrolysis and methylation of bound fatty acids are becoming more and more popular. Lepage & Roy (1986) have proposed the one-step direct transesterification procedure suitable for fatty acid classes. The analysis of fatty acid methyl esters (FAME) from erythrocyte membranes may be differentially affected by the method of lipid extraction. Rodríguez-Palmero coworkers compared direct (Lepage & Roy) and conventional esterification (Folch) methods and found that the direct method has substantial advantages over the conventional method in terms of reduce contamination and increased throughput. However, the transesterification of amides from sphingomyelin (CerPCho) is less efficient than that of esters from glycerophospholipids (Christie & Han, 2012); this results in an underestimation of fatty acids bound to CerPCho when using the GC-FID method described here.

To determine the transesterification efficiency of sphingomyelin using the Lepage and Roy method CerPCho 24:1 was added to the synthetic PL mixture shown in table S5. This mixture was then subjected to both the GC and MS methods described earlier and the results shown in the supplementary material (Table S6). A very low recovery (45%) was obtained using the GC-FID method compared to 98% recovery using the high-throughput ESI-MS method. In order to account for this underestimation a correction factor was determined and applied as follows. Five erythrocytes samples were selected at random and the concentration of fatty acid 24:1 obtained from the high-throughput ESI-MS method (123 nmol/ml blood) was divided by the fatty acid 24:1 concentration determined by the GC-FID method (49.5 nmol/ml blood). This produced a correction factor of 2.48. Fatty acid 24:1 was chosen to calculate the correction factor as it was found exclusively bound to CerPCho in erythrocytes. To correct for fatty acids exclusive to CerPCho, i.e. 24:1 and 24:0 the GC-FID data was simply multiplied by 2.48 (the correction factor).

For fatty acids bound to both phospholipids and CerPCho (16:0, 18:0 and 18:1) further calculations were required to determine the corrected fatty acid concentrations. They were; 1) dividing the concentration of CerPCho bound fatty acid by the total fatty acid concentration (both determined by ESI-MS) for 16:0, 18:0 and 18:1 in every sample to obtain the proportion of CerPCho fatty acid, 2) multiplying the GC data by this proportion to obtain the approximate CerPCho fatty acid concentration, 3) multiplying the calculated CerPCho fatty acid concentration by the correction factor (2.48), and 4) adding the corrected CerPCho fatty acid concentration to the PL fatty acid concentration. A step-by step example of this calculation is provided in the supplementary material. The result of applying the correction factor to 16:0, 18:0, 18:1, 24:0 and 24:1 concentrations determined by GC-FID are shown in Table 3.

As shown in the table 4 the new method is cheaper than the GC-FID method with several other advantages e.g. throughput is increased by almost 8 times; sample and reagent

volumes can be reduced four-fold and additional information, i.e. phospholipid class and species information is obtained.

The throughput ESI-MS method does however, have limitations. First the diluting of signal from low relative abundance fatty acids across several PL species can hinder their detection. Second, the double bond location cannot be determined, e.g., this method is unable to determine whether 22:5 docosapentaenoic acid is 22:5n-3 or 22:5n-6 or if 18:1 is 18:1n-9 or 18:1n-7. While several MS-based methods for determining double bond position have been developed they are not yet amenable to high-throughput, quantitative anlysis (Hancock et al. 2017). Nevertheless, future addition of such techniques will further enhance the analysis of FAs and the omega-3 index. Additionally, pipetting of 10 uL of packed erythrocytes introduced variation between samples (data not shown), as pipetting this volume of packed cells is difficult. Given that sample volume is generally not limited in this process, the initial starting volume of erythrocytes could easily be increased to further reduce variability between sample measurements. This could possibly be achieved using the robotic system to initially pipette a larger cell volume and perform hemolysis prior to aliquoting a small volume of the lyzed cell mixture for extraction.

In conclusion, the high throughput ESI-MS method described here allows the determination of human erythrocyte fatty acid concentration and omega-3 index from low sample volumes. The method is convenient, simple, low in cost and significantly increases throughput compared to the reference GC-FID method. Consequently, the method is well suited for application in epidemiological studies and large clinical trials.

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References

Albert, C.M., Campos, H., Stampfer, M.J., Ridker, P.M., Manson, J.E., Willett, W.C., MA, J. (2002) Blood levels of long-chain n-3 fatty acids and the risk of sudden death. New England Journal of Medicine, 346, 1113-8.

Altman, D.G., Bland, J.M. (2002) Commentary on quantifying agreement between two methods of measurement. Clinical Chemistry, 48**,** 801-802.

Arab, L. (2003) Biomarkers of fat and fatty acid intake. Journal of Nutrition, 133**,** 925S-932S.

Bakker, E., Ghys, A., Kester, A., Vles, J., Dubas, J., Blanco, C., Hornstra, G. (2003) Long-chain polyunsaturated fatty acids at birth and cognitive function at 7y of age. European Journal of Clinical Nutrition, 57**,** 89-95.

Bland, J.M., Altman, D.G. (1986) Statistical methods for assessing agreement between two methods of clinical measurement. Lancet, 327, 307-310.

Block, R.C., Harris, W.S., Reid, K.J., Sands, S.A., Spertus, J.A. (2008) EPA and DHA in blood cell membranes from acute coronary syndrome patients and controls. Atherosclerosis, 197**,** 821-828.

Brondz, I., (2002) Development of fatty acid analysis by high-performance liquid chromatography, gas chromatography, and related techniques. Analytica Chimica Acta, 465, 1-37.

Brown, S.H., Kunnen, C.M., Duchoslav, E., Dolla, N.K., Kelso, M.J., Papas, E.B., Lazon de la jara, P., Willcox, M.D., Blanksby, S.J., Mitchell, T.W. (2013) A comparison of patient matched meibum and tear lipidomes. Investigative Ophthalmology & Visual Science, 54**,** 7417-7424.

Brugger B., Erben G., Sandhoff R., Wieland F.T., Lehmann W.D. (1997) Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proceedings of the National Academy of Sciences of the United States of America, 94, 2339-2344

Calon, F., Lim, G.P., Morihara, T., Yang, F., Ubeda, O., Salem, N., Frautschy, S.A., Cole, G.M. (2005) Dietary n-3 polyunsaturated fatty acid depletion activates caspases and

decreases NMDA receptors in the brain of a transgenic mouse model of Alzheimer's disease. European Journal of Neuroscience, 22**,** 617-626.

Christie, W.W., Han, X. (2012) Lipid Analysis, Separation, Identification and Lipidomic Analysis. (Fourth edition). Woodhead Publishing.

Folch, J., Lees, M., Sloane-Stanley, G. (1957) A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226, 497-509.

Glaser, C., Demmelmair, H., Koletzko, B. (2010) High-throughput analysis of total plasma fatty acid composition with direct in situ transesterification. PloS one, 5**,** e12045.

Goozee, K., Chatterjee, P., James, I., Shen, K., Sohrabi, H.R., Asih, P.R., Dave, P., Ball, B., Manyan, C., Taddei, K., Chung, R., Garg, M.L., Martins, R.N. (2017) Alterations in erythrocyte fatty acid composition in preclinical Alzheimer's disease. Scientific Reports, 7**,** 676.

Hancock S.E., Poad B.L.J., Batarseh A., Abbott S.K., Mitchell T.W. (2017) Advances and unresolved challenges in the structural characterization of isomeric lipids. Analytical Biochemistry, 524, 45-55.

Harris W.S., Von Schacky, C. (2004) The omega-3 Index: A new risk factor for Death from Coronary Artery disease? *Preventive* Medicine, 39, 212-220.

Harris, W.S. (2008) The omega-3 index as a risk factor for coronary heart disease. The American Journal of Clinical Nutrition, 87**,** 1997S-2002S.

Harris, W.S., Thomas, R.M. (2010) Biological variability of blood omega-3 biomarkers. Clinical Biochemistry, 43**,** 338-340.

Hibbeln, J.R., Davis, J.M., Steer, C., Emmett, P., Rogers, I., Williams, C., Golding, J. (2007) Maternal seafood consumption in pregnancy and neurodevelopmental outcomes in childhood (ALSPAC study): an observational cohort study. Lancet, 369**,** 578-585.

Hibbeln, J.R., Salem, N. (1995) Dietary polyunsaturated fatty acids and depression: when cholesterol does not satisfy. The American Journal of Clinical Nutrition, 62**,** 1-9.

Jackson, J.A., Riordan, H.D., Hunninghake, R., Meng, X., Sarwar, Y. (1997) Red blood cell membrane fatty acids as a diagnostic test. Journal of Orthomolecular Medicine, 12, 20-22.

Katan, M.B., Deslypere, J.P., Van birgelen, A.P., Penders, M., Zegwaard, M. (1997) Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: an 18-month controlled study. Journal of Lipid Research, 38**,** 2012-2022.

Lepage G, Roy C, (1986) Direct transesterification of all classes of lipids in a one-step reaction. Journal of Lipid Research, vol. 27, pp. 114-120.

Lin, Y.H., Salem, N., Wells, E.M., Zhou, W., Loewke, J.D., Brown, J.A., Lands, W.E., Goldman, L.R., Hibbeln, J.R., (2012) Automated high-throughput fatty acid analysis of umbilical cord serum and application to an epidemiological study. Lipids, 47**,** 527-539.

Ludbrook, J. (2010) Confidence in Altman–Bland plots: a critical review of the method of differences. Clinical and Experimental Pharmacology and Physiology, 37**,** 143-149.

Marchioli, R., Barzi, F., Bomba, E., Chieffo, C., DI gregorio, D., DI mascio, R., Franzosi, M.G., Geraci, E., Levantesi, G., Maggioni, A.P., Mantini, L., Marfisi, R.M., Mastrogiuseppe, G., Mininni, N., Nicolosi, G.L., Santini, M., Schweiger, C., Tavazzi, L., Tognoni, G., Tucci, C., Valagussa, F. (2002) Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. Circulation, 105**,** 1897-903.

Masood, A., Salem, N. (2008) High-throughput Analysis of Plasma Fatty Acid Methyl Esters Employing Robotic Transesterification and Fast Gas Chromatography, Journal of Lipid Research, vol. 43, 171-180.

Masood, A., Salem, N., Stark, K. (2005) A simplified and efficient method for the Analysis of fatty acid methyl esters suitable for large clinical studies, Journal of Lipid Research, vol. 46, pp 2299-2305.

Matyash, V., Liebisch, G., Kurzchalia, T.V., Shevchenko, A., Schwudke, D. (2008) Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. Journal of Lipid Research, 49**,** 1137-1146.

Meyer, B.J. (2016) Australians are not Meeting the Recommended Intakes for Omega-3 Long Chain Polyunsaturated Fatty Acids: Results of an Analysis from the 2011–2012 National Nutrition and Physical Activity Survey. Nutrients, 8**,** 111.

Mozaffarian, D. (2008) Fish and n– 3 fatty acids for the prevention of fatal coronary heart disease and sudden cardiac death. The American Journal of Clinical Nutrition, 87**,** 1991S-1996S.

Nealon, J.R., Blanksby, S.J., Mitchell, T.W., Else, P.L. (2008) Systematic differences in membrane acyl composition associated with varying body mass in mammals occur in all phospholipid classes: an analysis of kidney and brain. The Journal of Experimental Biology, 211**,** 3195-3204

Norris, S.E., Friedrich, M.G., Mitchell, T.W., Truscott, R.J.W., Else, P.L. (2015) Human prefrontal cortex phospholipids containing docosahexaenoic acid increase during normal adult aging, whereas those containing arachidonic acid decrease. Neurobiology of Aging, 36**,** 1659-1669.

Parletta, N., Zarnowiecki, D., Cho, J., Wilson, A., Procter, N., Gordon, A., Bogomolova, S., O'dea, K., Strachan, J., Ballestrin, M., Champion, A., Meyer, B.J. (2016) People with schizophrenia and depression have a low omega-3 index. Prostaglandins*,* Leukotrienes *and* Essential Fatty Acids*,* 110, 42-47

Quehenberger, O., Armando, A.M., Dennis, E.A. (2011) High sensitivity quantitative lipidomics analysis of fatty acids in biological samples by gas chromatography–mass spectrometry. Biochim Biophys Acta - Molecular *and* Cell Biology *of* Lipids*, 1811, 648-656.*

Reed, C.F., Swisher, S.N., Marinetti, G.V., Enen, E.G. (1960) Studies of the lipids of the erythrocyte. I. Quantitative analysis of the lipids of normal human red blood cells. Journal of Laboratory and Clinical Medicine, 56**,** 281-289.

Ridges, L., Sunderland, R., Moerman, K., Meyer, B., Astheimer, L., Howe, P. (2001) Cholesterol lowering benefits of soy and linseed enriched foods. Asia Pacific *Journal of* Clinical Nutrition*,* 10, 204-211.

Rodríguez-Palmero, M., Lopez-Sabater, M.C., Castellote-Bargallo, A.I., De la torre-Boronat, M.C., Rivero-urgell, M. (1998) Comparison of two methods for the determination of fatty acid profiles in plasma and erythrocytes. Journal of Chromatography A, 793, 420-425.

Rose, H.G., Oklander, M. (1965) Improved procedure for the extraction of lipids from human erythrocytes. The Journal of Lipid Research, 6**,** 428-431.

Salem, N. (1989) Omega-3 fatty acids: molecular and biochemical aspects. In: Current Topics in Nutrition and Disease: New Protective Roles for Selected Nutrients. (Spiller GA, ed), pp 109–228.

Salem, N., Kim, H.Y., Yergy, J.A. (1986) Docosahexaenoic acid: membrane function and metabolism. In: health effects of polyunsaturated fatty acids in seafoods (Simopoulos AP, Kifer RR, Martin RE.,eds), pp 263-317

Simopoulos, A.P. (1999) Essential fatty acids in health and chronic disease. The American Journal of Clinical Nutrition, 70**,** 560s-569s.

Siscovick, D.S., Raghunathan, T.E., King, I., Weinmann, S., Wicklund, K.G., Albright, J., Bovbjerg, V., Arbogast, P., Smith, H., Kushi, L.H., Cobb, L.A., Copass, M.K., Psaty, B.M., Lemaitre, R., Retzlaff, B., Childs, M., Knopp, R.H. (1995) Dietary Intake and Cell Membrane Levels of Long-Chain n-3 Polyunsaturated Fatty Acids and the Risk of Primary Cardiac Arrest. The Journal of the American Medical Association, 274, 1363-1367.

Stark, K.D., Van elswyk, M.E., Higgins, M.R., Weatherford, C.A., Salem, N. (2016) Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Progress* in *Lipid* Research, 63, 132-152.

Stewart, F., Rodie, V.A., Ramsay, J.E., Greer, I.A., Freeman, D.J., Meyer, B.J. (2007) Longitudinal assessment of erythrocyte fatty acid composition throughout pregnancy and post partum. Lipids, 42**,** 335-344.

Sullivan, B.L., Williams, P.G., Meyer, B.J. (2006) Biomarker validation of a long-chain omega-3 polyunsaturated fatty acid food frequency questionnaire. Lipids, 41**,** 845-850.

Von schacky, C., Harris, W.S. (2007) Cardiovascular risk and the omega-3 index. Journal of Cardiovascular Medicine, 8**,** S46-S49.

Figure legends

Figure 1:- Correlation between the GC-FID method and the high throughput ESI-MS method using erythrocyte samples (n=25) over an omega-3 index range of 2-10%. The line of best fit is y = 1.01567x. R= 0.993.

Figure 2:- Bland-Altman plot of the difference between the two methods (the high throughput ESI-MS method minus GC-FID method) against their mean of omega-3 index (%) using erythrocytes (n=25). The line of best fit (dashed line) is y = 02 + -0.01x (R^2 = 0.015). {95 % confidence interval = Mean difference ± (STD of difference * 1.96)} STD = 0.235.

Table 1:- Average amount of synthetic PL mixtures (nmol/ml) across a range of omega-3 indexes recovered using the GC-FID and high-throughput ESI-MS methods (n=3 for all mixtures).

* Synthetic PL mixtures (%)	Fatty acids	Expected value	GC-FID method		The high-throughput ESI-MS method	
		nmol/ml	Mean ± SEM	(%) recovery of synthetic PL	Mean ± SEM	(%) recovery of synthetic PL
2	16:0	941.5	930.0 ± 24.3	98.8%	929.9 ± 10.1	98.8%
	18:0	58.5	57.8 ± 0.9	98.8%	35.5 ± 0.2	60.7%
	18:1	444.6	429.8 ± 15.9	96.7%	406.5 ± 11.5	91.4%
	18:2	363.8	344.9 ± 2.7	94.8%	396.0 ± 2.6	108.9%
	20:4	151.6	147.4 ± 7.5	97.2%	132.8 ± 5.5	87.6%
	22:6	40.0	27.8 ± 0.2	69.6%	30.1 ± 0.9	75.3%
4	16:0	935.6	927.0 ± 17.8	99.1%	890.1 ± 13.9	95.1%
	18:0	64.4	64.9 ± 1.9	100.8%	36.9 ± 2.0	57.3%
	18:1	426.1	423.1 ± 3.3	99.3%	400.3± 7.83	93.9%
	18:2	348.6	347.8 ± 10.8	99.8%	341.3 ± 3.1	97.9%
	20:4	145.3	141.9 ± 4.4	97.7%	125.5 ± 1.9	86.4%
	22:6	80.0	56.5 ± 1.9	70.6%	59.9 ± 1.7	74.8%
6	16:0	929.7	827.0 ± 7.8	88.9%	893.4 ± 3.0	96.1%
	18:0	70.3	67.9 ± 1.7	96.6%	40 ± 0.9	57.2%
	18:1	407.6	386.7 ± 3.2	94.9%	401.2 ± 4.5	98.4%
	18:2	333.5	316.7 ± 2.2	94.9%	329.1 ± 6.2	98.7%
	20:4	138.9	128 ± 1.6	92.4%	125.4 ± 2.8	90.3%
	22:6	120.0	80.3 ± 1.2	66.9%	77.9 ± 0.9	64.9%
8	16:0	923.8	907.4 ± 19.1	98.2%	855.3 ± 4.7	92.6%
	18:0	76.2	75.8 ± 3.1	99.4%	41.9 ± 0.4	54.9%
	18:1	389.1	384.2 ± 9.9	98.7%	374.2 ± 6.4	96.2%
	18:2	318.3	313.7 ± 1.6	98.6%	301.2 ± 1.7	94.6%
	20:4	132.6	121.0 ± 6.1	91.6%	120.1± 0.8	90.6%
	22:6	160.0	136.2 ± 9.3	85.1%	101.7±1.9	63.5%
10	16:0	917.9	896.3 ± 3.6	97.6%	879.3 ± 8.6	95.8%
	18:0	82.1	83.9 ± 0.4	102.3%	41.8 ± 1.6	50.9%
	18:1	370.5	367.9 ± 2.6	99.3%	360.2 ± 5.3	97.2%
	18:2	303.2	303.6 ± 1.4	100.1%	294.0 ± 4.6	96.9%
	20:4	126.6	125.6 ± 8.5	99.2%	118.5 ± 0.5	93.6%
	22:6	200.0	149.1 ± 1.7	74.5%	148.4 ± 5.2	74.2%

SEM, standard error of the mean. * See supplementary materials (Tables S1 to S5) for additional Synthetic PL mixtures information.

Table 2: Within–run and between-run precision for the GC-FID and high-throughput ESI-MS methods.

*Synthetic PL mixtures (%)	Fatty acids	GC-FID method		The high-throughput ESI-MS method	
		Mean nmol/ml ± SEM (within-run %CV)	Mean nmol/ml ± SEM (between-run %CV)	Mean nmol/ml ± SEM (within-run %CV)	Mean nmol/ml ± SEM (between-run %CV)
2	16:0	930.0 ± 24.3 (4.4)	962.1 ± 36.4 (11.9)	929.9 ± 10.1 (1.8)	831.6 ± 22.2 (8.4)
	18:0	57.8 ± 0.9 (2.6)	59.4 ± 1.5 (7.9)	35.5 ± 0.2 (0.9)	36.5 ± 0.4 (3.5)
	18:1	429.8 ± 15.9 (6.3)	443.3 ± 16.5 (11.8)	406.5 ± 11.5 (4.8)	375.8 ± 8.9 (7.5)
	18:2	344.9 ± 2.7 (1.3)	362.9 ± 3.0 (2.6)	396.0 ± 2.6 (1.1)	335.8 ± 14.2 (13.4)
	20:4	147.4 ± 7.5 (8.7)	145.0 ± 5.5 (11.9)	132.8 ± 5.5 (7.0)	127.4 ± 3.4 (8.4)
	22:6	27.8 ± 0.2 (1.2)	27.6 ± 1.0 (11.4)	30.1 ± 0.9 (5.1)	29.1 ± 0.5 (5.4)
4	16:0	927.0 ± 17.8 (3.3)	924.8 ± 18.0 (6.2)	890.1 ± 13.9 (2.7)	843.4 ± 19.8 (7.5)
	18:0	64.9 ± 1.9 (4.9)	65.8 ± 2.5 (12.0)	36.9 ± 2.0 (9.2)	35.9 ± 1.3 (11.4)
	18:1	423.1 ± 3.3 (1.3)	420.7 ± 4.0 (3.0)	400.3± 7.8 (3.3)	379.8 ± 11.1 (9.2)
	18:2	347.8 ± 10.8 (5.3)	352.1 ± 10.0 (8.9)	341.3 ± 3.1 (1.5)	327.9 ± 10.4 (10.0)
	20:4	141.9 ± 4.4 (5.3)	136.7 ± 5.0 (11.6)	125.5 ± 1.9 (2.6)	113.4 ± 3.1 (8.6)
	22:6	56.5 ± 1.9 (5.7)	55.0 ± 1.5 (8.6)	59.9 ± 1.7 (4.8)	57.3 ± 1.4 (7.7)
6	16:0	827.0 ± 7.8 (1.6)	835.2 ± 8.9 (3.4)	893.4 ± 3.0 (0.6)	905.5 ± 5.5 (1.9)
	18:0	67.9 ± 1.7 (4.3)	70.9 ± 2.8 (12.5)	40.0 ± 0.9 (3.8)	41.7 ± 2.0 (15.2)
	18:1	386.7 ±3.2 (1.4)	397.5 ± 4.1 (3.3)	401.2 ± 4.5 (1.9)	395.1 ± 8.4 (6.7)
	18:2	316.7 ± 2.2 (2.6)	325.1 ± 3.7 (3.6)	329.1 ± 6.2 (3.2)	309.7 ± 9.5 (9.7)
	20:4	128 ± 1.6 (2.1)	130.1 ± 1.9 (4.6)	125.4 ± 2.8 (3.8)	119.0 ± 5.5 (14.6)
	22:6	80.3 ± 1.2 (2.5)	81.2 ± 3.1 (12.1)	77.9 ± 0.9 (1.9)	75.2 ± 1.1 (4.6)
8	16:0	907.4 ±19.1 (3.6)	929.4 ± 20.2 (6.9)	855.3 ± 4.7 (0.2)	778.9 ± 20.9 (8.5)
	18:0	75.8 ± 3.1 (6.9)	76.9 ± 3.4 (13.9)	41.9 ± 0.4 (1.6)	40.5 ± 1.9 (14.8)
	18:1	384.2 ± 9.9 (4.4)	379.6 ± 10.5 (8.7)	374.2 ± 6.4 (2.9)	334.5 ± 11.2 (10.6)
	18:2	313.7 ± 1.6 (0.9)	323.2 ± 1.9 (1.9)	301.2 ± 1.7 (0.9)	283.6 ± 6.6 (7.4)
	20:4	121.0 ± 6.1 (8.6)	118.3 ± 5.0 (13.4)	120.1± 0.8 (1.1)	105.8 ± 4.6 (13.7)
	22:6	136.2 ± 8.0 (10.0)	132.2 ± 5.8 (13.9)	101.7±1.9 (3.2)	95.6 ± 2.5 (8.3)
10	16:0	896.3 ± 3.6 (0.7)	900.9 ± 4.5 (1.6)	879.3 ± 8.6(1.7)	813.4 ± 21.5 (8.4)
	18:0	83.9 ± 0.4 (0.6)	84.8 ± 1.2 (4.5)	41.8 ± 1.6 (6.5)	42.6 ± 1.9 (14.0)
	18:1	367.9 ± 2.6 (1.2)	364.9 ± 2.9 (2.5)	360.2 ± 5.3 (2.5)	341.7 ± 14.9 (13.8)
	18:2	303.6 ± 1.4 (0.6)	307.9 ± 1.5 (1.5)	294.0 ± 4.6 (2.7)	261.7 ± 10.2 (12.3)
	20:4	125.6 ± 6.5 (8.8)	118.2 ± 5.1 (13.6)	118.5 ± 0.5 (0.7)	106.4 ± 4.1 (12.2)
	22:6	149.1 ± 1.7 (1.9)	144.3 ± 2.4 (5.3)	148.4 ± 5.2 (5.9)	146.2 ± 4.9 (10.6)

Coefficient of variation (%CV). SEM, standard error of the mean. Within run %CV (n=3); between run %CV (n= 3/day over one month).* See supplementary materials (Tables S1 to S5) for additional Synthetic PL mixtures information.

Table 3: comparison of erythrocytes fatty acids concentrations using GC-FID method and high throughput ESI-MS method. (nmol/ml blood) (n=25)

Fatty acids	GC-FID method (Mean ± SEM)	GC-FID method (Mean ± SEM) **corrected**	high throughput ESI-MS method (Mean ± SEM)	Difference %
16:0	331.8 ± 8.4	**460.3** ± 8.6	464.9 ± 7.3	N/A*
17:0	16.6 ± 0.8	-	18.1 ± 0.8	-8.6
18:0	232.9 ± 6.2	**263.8** ± 5.5	269.3 ± 7.1	N/A*
24:0	37.7 ± 2.1	**93.5** ± 2.9	85.0 ± 2.6	N/A*
16:1	4.9 ± 0.8	-	4.6 ± 0.7	6.3
18:1	186.5 ± 4.4	**198.0** ± 3.3	207.3 ± 3.9	N/A*
24:1	49.5 ± 1.8	**122.7** ± 3.6	122.7 ± 3.6	N/A*
18:2	155.1 ± 6.9	-	158.1 ± 6.4	-1.9
20:3	23.8 ± 1.6	-	22.9 ± 1.6	3.9
20:4	201.7 ± 5.2	-	195.7 ± 4.3	3.0
20:5	9.8 ± 1.1	-	9.9 ± 1.2	-1.0
22:4	37.6 ± 1.6	-	25.7 ± 1.9	37.5
22:5	31.5 ± 1.6	-	31.1 ± 1.5	1.3
22:6	67.6 ± 3.3	-	66.7 ± 3.2	1.3

Corrected data shown in **bold,** Values (V), Difference % = |V *1* – V 2| / (V 1 +V 2) / 2 × 100. (V1 GC-FID results) & V2 ESI-MS results). N/A* (the difference % was not calculated because the data has been corrected using the new method).

Table 4: comparison between methods using GC-FID method and high throughput ESI-MS method.

	GC-FID method	**high throughput ESI-MS method**
Number of samples per day	24 samples per day	186 samples per day
Time needed for sample preparation	Approximately 6 hours to prepare 24 samples	Approximately 6 hours to prepare 186 samples
Quantity of sample used	400 uL	10 uL
Data acquired	Only fatty acids	Fatty acids PL classes
Quantity of solvents used per sample	8 ml	Approximately 2 ml
Cost per sample	$ 50	$ 20