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Development and Validation of an LC-MS/MS Method for the Determination of Plasma and Red Blood Cell Omega Fatty Acids: A Useful Diagnostic Tool

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Abstract: Background: LC-MS is an ever-increasingly used methodology for clinical applications. Due to the superior selectivity and sensitivity, in certain situations, it can offer an advantage or be the only option for diagnostics and biomonitoring applications. Methods: A high selectivity sensitive LC-MS/MS method was developed for direct quantification of free plasma polyunsaturated fatty acids as well as conjugated membrane polyunsaturated fatty acids, using isocratic reverse phase elution. A quick and simple sample purification method was used in order to ensure high-throughput analysis of biological samples. The method was validated with regard to selectivity, sensitivity, linearity, accuracy, precision, carryover, and recovery, as well as other relevant parameters. Results and Conclusions: The method was developed and validated with respect to all relevant parameters and was successfully used in a number of clinical diagnostics and biomonitoring applications. The simple sample purification process allowed for an easy learning curve for analysts and other users, while ensuring a low chance of systematic or random errors and thus reliable results usable in a clinical setting.

Keywords: PUFA; LC-MS; metabolomics; diagnostic

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1. Introduction

Fatty acids (FAs) are molecules that have various roles in living cells including humans. Therefore, the analysis of FAs (free, total, or as omega index) in biological samples can provide useful information regarding the physiological and pathological conditions [1,2].

Free fatty acids (FFAs) can act as biomarkers, modulate gene expression, and control inflammation and immunity and they can be used in the clinical laboratory for the diagnosis of insulin resistance, diabetes, obesity, and metabolic syndrome. Total fatty acids (TFAs) (unbound and esterified forms) reveal the quality of dietary consumption and metabolic status of omega 3 and omega 6 fatty acids, which are essential for cell membrane structure, cognitive function, and cardiovascular health. Regarding the last implication, recently, the importance of monitoring saturated (myristic, palmitic, and stearic) and unsaturated fatty acids (including arachidonic—AA, eicosapentaenoic—EPA, and docosahexaenoic—DHA) in cardiovascular diseases (CVD) was summarized in a scientific research paper by Shramko et al. [3]. It is already well-established that the most important factor to take into consideration is the ratio between saturated and unsaturated fatty acids and its correlation with mortality due to CVD. A connection between short-chain fatty acids (SCFA) and unsaturated fatty acids (UFA) with lipid–lipoprotein parameters and markers of inflammation and oxidative stress was also demonstrated.

Taking into account these well-known facts, the demand for accurate and reliable methods for the quantification of FFAs and TFAs in plasma or other relevant biological matrices is high, both for clinical and research purposes.

There are different methods for the determination of FAs and TFAs, including chromatographic ones. Liquid chromatography coupled with mass spectrometry (LC-MS/MS) is nowadays the "gold standard" clinical technique, which allows not only the identification of biomarkers with high selectivity/specificity but also their quantification at low levels of concentration. Regarding its ability to quantify fatty acids, there are some challenges that have to be taken into consideration, such as poor ionization efficiency, isobaric interference, and complex sample preparation.

Different approaches have been proposed to overcome these issues and increase the performance of LC-MS/MS analysis of FAs and TFAs. These strategies refer to chemical derivatization, chromatographic optimization, and sample preparation techniques [4,5].

By chemical derivatization, the structure of FAs is modified to a new product with proper, desirable LC-MS/MS behavior [5]. Chemical derivatization improves their ionization efficiency and selectivity under ESI-MS conditions but also chromatographic separation and quantification acting on their polarity.

Because FAs have many isomers that can co-elute and interfere with each other in the mass spectrometer, leading to inaccurate quantification and identification, it is necessary to emphasize chromatographic separation optimization. Therefore, by improving the separation of isobaric fatty acids acting on column stationary phase type, mobile phase composition, flow rate, and gradient elution, the best resolution and peak shape for fatty acids can be obtained.

Nevertheless, FAs can exist in different forms in biological samples, such as free fatty acids, esterified fatty acids, or bound to proteins or lipoproteins. These forms need different approaches for extraction and derivatization making them suitable for LC-MS/MS analysis. Saponification is a sample preparation step to hydrolyze esterified fatty acids into free fatty acids, which can then be extracted and analyzed by LC-MS/MS. All these methods can be highly expensive, laborious, and time-consuming and predisposed to human errors in the case of lack of automatization.

The aim of this paper is to describe a complex analytical methodology for the quantification of PUFA in both human plasma, as well as red blood cell (RBC) membranes, which can be used as biomarkers or to calculate the omega index for patients. This method was proven to be useful in various fields of medicine for diagnostic or research purposes.

2. Materials and Methods

2.1. Reagents

HPLC grade Acetonitril (≥99%) was purchased from Scharlau, analytical grade ammonium formate (98%) was purchased from Fluka, and hydrochloric acid (37%) and hexane (≥97%) were both made by Riedel de Haen. Ultrapure water was produced in-house using a Millipore DQ-3 system, while analytical standards for arachidonic acid (100%, Cat No ASB-00001965-010), eicosapentaenoic acid (100%, Cat No ASB-00005053-025), and docosahexaenoic acid (100%, ASB-00004985-025) were acquired from ChromaDex (Los Angeles, USA). The internal standard, deuterated arachidonic-d11 acid, was purchased from Cayman Chemical (≥99%, Cat No 10006758). Blood and plasma were obtained from different studies (published separately), all of which were subject to Ethics Committee reviews and received approval.

2.2. Equipment

An LC-MS system consisting of an AB Sciex (Framingham, MA, USA) 4600 QTOF type mass spectrometer coupled with a Flexar FX-10 UHPLC liquid chromatograph from Perkin Elmer (Shelton, CT, USA) was used for bioanalysis. Vortex mixers used in the study were manufactured by Velp Scientifica (Usmate Velate, Italy) and Heidolph (Schwabach, Germany), while centrifugation was performed on an Eppendorf (Hamburg, Germany)

centrifuge. Other equipment used during the study included a Radwag (Radom, Poland) analytical balance, JP Selecta (Barcelona, Spain) ultrasonic bath, VWR (Radnor, PA, USA) heated magnetic stirrer, Eppendorf (Hamburg, Germany) automatic pipettes, and a Millipore (Burlington, VT, USA) DQ3 water ultrapurifying system.

2.3. LC-MS Analysis Method

Chromatographic separation of the three polyunsaturated fatty acids, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, was performed using a Phenomenex Kinetex XB-C18 (size 3.0 × 100 mm, particle diameter 2.6 µm) column and mobile phase composed of 10 mM ammonium formate and acetonitrile in a ratio of 15:85 (*v*/*v*) with a constant flowrate of 0.4 mL/min, in isocratic elution. The analytical column was thermostatted at 25 °C, while the samples were kept in the autosampler at 20 °C. All analytes were chromatographically separated after a total runtime of 6 min per sample. The injection volume was 5 µL per sample for analysis of plasma samples, while for analysis from the membrane of red blood cells, the injection volume was 10 µL per sample, the same for all types of solutions (standard solutions, quality control solutions, and biological samples) and dependent only on the nature of biological samples analyzed.

After chromatographic separation, the analytes and internal standard were ionized using negative electrospray ionization mode (ESI-) and detected in the mass spectrometer using MS/MS MRM detection mode, by monitoring specific fragmentation patterns for each analyte and the internal standard. The parameters for the ionization source were as follows: spray voltage = −3300 V, vaporizer temperature = 580 °C, ion gas source 1 = 29 bar, ion gas source 2 = 30 bar, and curtain gas = 19 bar. For the quantification of analytes, a number of specific fragments were used for each and were summed in order to increase peak intensity and thus sensitivity of the method, as described in Table 1.

Table 1. Mass spectrometric fragments were monitored for analyte quantification.

Polyunsaturated Fatty Acid	Molecular Weight (g/mol)	Parent Ion (m/z)	Fragment Ions (m/z)	Collision Energy (V)
Arachidonic acid	304.5	303.25	234.94; 259.27	−16
Eicosapentaenoic acid	302.5	301.15	203.19; 257.25	−16
Docosahexaenoic acid	328.5	327.25	229.22; 249.21; 283.26	−13
Arachidonic-d11 acid	315.5	314.26	270.35	−16

2.4. Calibration and Quality Control Standard Solutions for Plasma Sample Analysis

For the quantification of the analytes, plasma calibration curves were prepared using certified reference substances and the internal standard method was applied. In the first step, stock solutions were prepared in acetonitrile with concentrations of 10 mg/mL for AA and 250 µg/mL for both EPA and docosahexaenoic acid, as well as a solution of internal standard in acetonitrile with 1 µg/mL arachidonic-d11 acid. Stock solutions of the three analytes were then further diluted with 0.2% formic acid to prepare a working solution, five for each analyte, which was further used for the calibration curve standard solutions. The working solutions had a concentration range of 2.5–125 µg/mL for AA and a range of 50–2500 ng/mL for EPA and DHA. Using these working solutions the calibration curve standard solutions were prepared by mixing 200 µL of each working solution with 100 µL internal standard solution and 500 µL of acetonitrile. The nominal concentration range for the calibration curves was 2.5–125 µg/mL for AA and a range of 50–2500 ng/mL for EPA and DHA. Separately, working solutions for quality control samples were prepared at three different concentration levels, 12.5 µg/mL (QCA), 25 µg/mL (QCB), and 62.5 µg/mL (QCC) for AA and 250 ng/mL (QCA), 500 ng/mL (QCB), and 1250 ng/mL (QCC) for both EPA and DHA. These were processed similarly to calibration curve standards and used during the validation of the method and for quality control of biological sample analysis.

Calibration curves were constructed automatically by Analyst 5.0 software with linear fit and $1/y^2$ weighing.

2.5. Calibration and Quality Control Standard Solutions for Red Blood Cell Membrane Analysis

For the semi-quantitation of the analytes from red blood cell membranes, calibration curves were prepared using certified reference substances and the external standard method was applied. In the first step, stock solutions in acetonitrile were prepared with concentrations of 10 mg/mL for AA and 50 μg/mL for both EPA and DHA. Stock solutions of the three analytes were then used to prepare standard solutions, five for each analyte, which were further used for the calibration curve standard solutions. The standard solutions had a concentration range of 1–50 μg/mL for AA and a range of 10–500 ng/mL for EPA and DHA. Separately, quality control solutions were prepared at three different concentration levels (similarly called QCA, QCB, and QCC), 5 μg/mL, 10 μg/mL, and 25 μg/mL for AA and 50 ng/mL, 100 ng/mL, 250 ng/mL for both EPA and DHA. These were used during the validation of the method and for quality control of biological sample analysis. Calibration curves were constructed automatically by Analyst 5.0 software with linear fit and $1/y^2$ weighing.

2.6. Preparation of Biological Samples

For the determination of free plasma PUFA, 200 μL of plasma sample are precipitated with 100 μL ST-ISTD-1 internal standard solution and 500 μL of acetonitrile. The mixture is vortexed for 2 min at 2000 rpm and then centrifuged at 9900 g rcf for 10 min. The supernatant is transferred to HPLC vials and injected into the LC-MS/MS system.

For the determination of total plasma PUFA (including conjugated PUFA), 200 μL of plasma are added to a 5 mL tube and hydrolyzed with 1 mL 10% HCl solution at 70 °C for 17 h. After 17 h, 1 mL of hexane is added to the mixture and the tubes are vortexed for 20 min at 2000 rpm, after which they are centrifuged at 8000 g rcf for 10 min. In total, 900 μL of supernatant (hexane) is transferred to a clean tube and evaporated, after which the residue is dissolved in 0.2 mL acetonitrile by vortexing for 20 min at 2000 rpm. Finally, 100 μL of ST-ISTD-1 internal solution, 300 μL of acetonitrile, and 200 μL of 0.2% formic acid are added and the mixed together. This sample is transferred to HPLC vials and injected into the LC-MS/MS system.

For red blood cell membrane PUFA determination, the whole blood sample is centrifuged at 1300 g rcf for 5 min and the plasma is discarded. In total, 200 μL of the red blood cells are carefully transferred to a 5 mL tube and washed with 1 mL purified water. The mixture is vortexed for 5 min to achieve cell lysis at 2000 rpm, after which it is centrifuged at 8000 g rcf for 10 min. Overall, 800 μL of supernatant (purified water containing hemoglobin) are discarded with care taken to not discard any of the red blood cell membrane. The remaining mixture of cell membrane is hydrolyzed with 1 mL 10% HCl solution at 70 °C for 17 h. After 17 h, 1 mL of hexane is added to the mixture and the tubes are vortexed for 20 min at 2000 rpm, after which they are centrifuged at 8000 g rcf for 10 min. In total, 900 μL of supernatant (hexane) is transferred to a clean tube and evaporated, after which the residue is dissolved in 0.5 mL acetonitrile by vortexing for 20 min at 2000 rpm. This sample is transferred to HPLC vials and injected into the LC-MS/MS system.

2.7. Validation of the Analytical Method for Plasma Sample Analysis

The method for total free PUFA quantification from plasma was validated with regard to all relevant performance parameters in order to ensure reproducible and reliable results. The method was validated according to international guidelines [6,7] with regard to all relevant parameters, taking into consideration the scope and possible future applications of the method.

The selectivity and sensitivity of the method were tested and validated by comparing the peak area at the lower limit of quantification for each analyte with possible interfering peaks appearing for blank solutions using 0.2% formic acid (instead of standard solution),

prepared with the same method as standard solutions, on mass-specific extracted chromatograms at the retentions times of the analytes. Carryover of the method was tested by reinjecting these same blank solutions used for sensitivity/selectivity testing after the highest concentration calibration standard solution and verifying if there were any interfering peaks at the retention times of analytes due to cross-contamination from one injected solution to the next.

The linearity of the method was verified by plotting calibration curves for each analyte, composed of the calibration standards described, and by using a linear fit with $1/y^2$ weighing. Calibration curves were then checked for correlation coefficient (R) and accuracy of recalculated calibrations standard solution.

The accuracy and precision of the method were validated by injecting multiple quality control solutions (five of each of the three different concentration levels) both within the same analytical run as well as during multiple analytical sequences for between-run accuracy and precision determination. The accuracy of each quality control standard was calculated as the bias (%) of the calculated concentration compared to the theoretical concentration, while precision was calculated as the coefficient of variation (CV, %) of the calculated concentration of the QC standards.

Recovery of analytes from plasma was determined by comparing standard solutions prepared in 0.2% formic acid solution with calibration standards prepared in plasma. As such, three types of solutions were prepared in order to determine the total analyte recovery: plasma samples (mixture of 150 µL acid formic 0.2% and 50 µL plasma, precipitated with 100 µL ST-ISTD-1 internal standard solution and 500 µL acetonitrile); aqueous standard solution (mixture of 50 µL arachidonic acid solution with a concentration of 62.5 µg/mL, 50 µL docosahexaenoic acid solution with a concentration of 1250 ng/mL, 50 µL eicosapentaenoic acid solution with a concentration of 1250 ng/mL, 50 µL 0.2% formic acid solution precipitated with 100 µL ST-ISTD-1 internal standard solution, and 500 µL acetonitril); and plasma spiked with standard solution (mixture of 50 µL arachidonic acid solution with a concentration of 62.5 µg/mL, 50 µL docosahexaenoic acid solution with a concentration of 1250 ng/mL, 50 µL eicosapentaenoic acid solution with a concentration of 1250 ng/mL, and 50 µL plasma precipitated with 100 µL ST-ISTD-1 internal standard solution and 500 µL acetonitril). The recovery was calculated as the bias (%) of the concentration difference between the spiked and unspiked plasma compared to the concentration of analyte in the aqueous standard solution.

The method was also validated with regard to the reproducibility of the sample preparation method of the total PUFA determination (using hydrolysis with HCl solution); given the increased complexity of this method, in order to ensure that the results are reliable, six samples of the same plasma sample were prepared in parallel. The percentage ratio of PUFA was calculated for each sample as the ratio between the sum of DHA and EPA as well as AA, by using the concentrations determined by the method. For the coefficient of variation between the six different samples, it was calculated in order to determine the reproducibility of the same preparation method.

2.8. Validation of the Method for Red Blood Cell Membrane Analysis

The method for red blood cell membrane PUFA semi-quantification for the determination of the omega index was validated similarly with regard to all relevant performance parameters in order to ensure reproducible and reliable results. The method was validated according to international guidelines [6,7]. With regard to selectivity, sensitivity, linearity, accuracy, precision, and carryover, by the same working method as described for plasma sample analysis, the only difference is the calibration concentration range and method of preparing solutions.

Similar to the plasma analysis method, this method was validated with regard to the reproducibility of the sample preparation process in order to ensure that, although semi-quantitative, the results are reliable and result in the same omega index being calculated. Six samples of the same biological matrix are in parallel. The percentage ratio of PUFA was

calculated for each sample as the ratio between the sum of DHA and EPA as well as AA by using the concentrations determined by the method. The coefficient of variation between the six different samples was calculated in order to determine the reproducibility of the same preparation method.

In order to accommodate real-life scenarios, where often a lengthy and complex sample preparation method needs to be interrupted, the effect of pausing sample preparation and continuing later on the accuracy of the determinations was investigated. Ten red blood cell (RBC) samples were prepared starting at the same time, by the method described. For one-half of the samples, the sample preparation was stopped after the washing step with water and the discarding of the hemoglobin and these samples were stored at 5 °C for approximately 48 h. For the other half of the samples, the sample preparation was continued until the extraction step with hexane (after the hydrolysis) and the sample preparation was stopped after the hexane supernatant was separated. These samples were also stored at 5 °C for approximately 48 h. After the storage time elapsed for each batch of samples, the sample preparation was continued accordingly. The samples were then analyzed and the results were compared to freshly prepared samples of the same biological matrix. The effect of the interruption of the sample preparation was calculated as the bias (%) of the (DHA + EPA)/AA ratio between freshly prepared samples compared to samples with paused preparation.

3. Results

All three fatty acids were separated chromatographically (Figure 1) as well as based on the specific m/z mass fragmentation patterns. The method, for both free and total plasma fatty acids, as well as red blood cell membrane PUFA determination, was validated according to the methodology described. All validation parameters were within acceptance criteria described by both European and American guidelines [6,7].

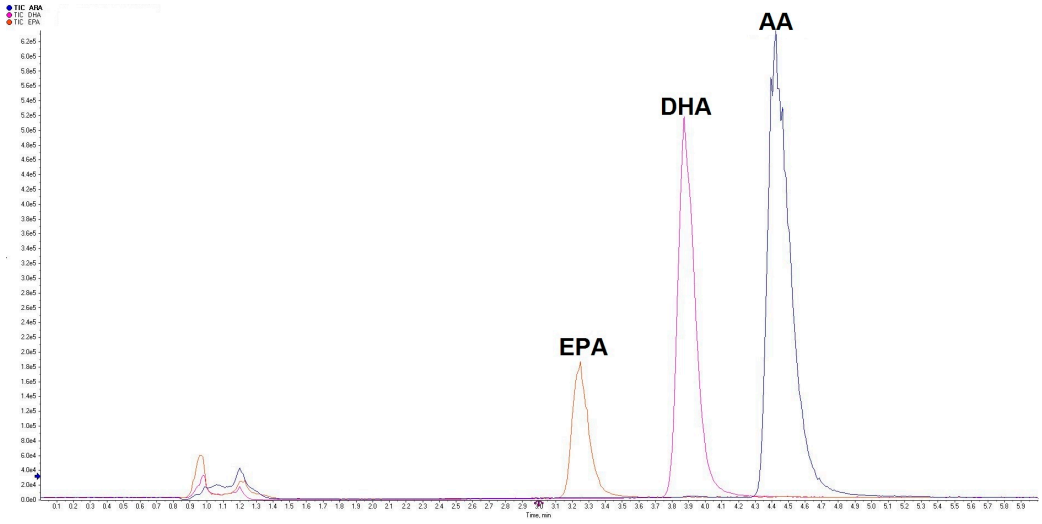

Figure 1. Overlayed total ion chromatograms (TIC) of AA, DHA, and EPA standard solutions.

3.1. Sensitivity and Selectivity

No interfering peaks were detected for any of the analytes in the blank samples. Thus, as guidelines consider a method sensitive and selective enough if no peaks are detected in blank samples having peak areas larger than 20% of the peak area at the lower limit of quantification (LLOQ), the method can be considered sensitive and selective with regard to the blank samples tested and for the purpose it was developed for.

3.2. Carryover

Similarly to the sensitivity testing, after reinjecting the blank samples after the calibration standard with the largest concentration (at the upper limit of quantification—ULOQ), there were no peaks detected at the retention times of the analytes. As such, there is no detectable carryover effect when using the method. This can also be attributed to the needle-washing step that the UHPLC system is set to carry out after each sample injection.

3.3. Linearity

3.3.1. Linearity of the Calibration Curves for Plasma Analysis

For all calibration curves injected during the method validation, the mean curve was plotted using a linear fit and $1/y^2$ weighing. The coefficient of correlation (R) was larger than 0.99 for all calibration curves and thus all calibration curves for each analyte are considered linear (Table 2).

Table 2. Value of the coefficient of correlation for the calibration curves.

Batch	AA	EPA	DHA
Batch 1	0.9981	0.9937	0.9994
Batch 2	0.9991	0.9992	0.9990
Batch 3	0.9966	0.9961	0.9990
Batch 4	0.9995	0.9996	0.9995
Batch 5	0.9976	0.9995	0.9996

For each of the analytes, the calibration curves had no calibration standards that needed to be excluded from the final curve due to accuracy issues. All calibration standards had a recalculated concentration with accuracy within the ±15% limit described in the guidelines (Table 3).

Table 3. Accuracy interval for calibration curve standard solutions, slope, and intercept.

	AA	EPA	DHA
Accuracy (%)	−10.0 ÷ 10.4	−11.5 ÷ 14.7	−4.9 ÷ 5.6
Slope	0.059 ÷ 0.065	0.002 ÷ 0.002	0.008 ÷ 0.009
Intercept	0.003 ÷ 0.035	0.010 ÷ 0.019	0.005 ÷ 0.048

3.3.2. Linearity of the Calibration Curves for RBC Membrane Analysis

For all calibration curves injected during the method validation, the mean curve was plotted using a linear fit and $1/y^2$ weighing. The coefficient of correlation (R) was larger than 0.99 for all calibration curves and thus all calibration curves for each analyte are considered linear (Table 4).

Table 4. Value of the coefficient of correlation for the calibration curves.

Batch	AA	EPA	DHA
Batch 1	0.9983	0.9957	0.9991
Batch 2	0.9991	0.9953	0.9974
Batch 3	0.9960	0.9975	0.9991
Batch 4	0.9982	0.9961	0.9998
Batch 5	0.9935	0.9959	0.9970

For each of the analytes, the calibration curves had no calibration standards that needed to be excluded from the final curve due to accuracy issues. All calibration standards had a recalculated concentration with accuracy within the ±15% limit described in the guidelines (Table 5).

Table 5. Accuracy interval for calibration curve standard solutions.

	AA	EPA	DHA
Accuracy (%)	−10.8 ÷ 14.8	−8.2 ÷ 13.6	−12.6 ÷ 9.6
Slope	5509.2 ÷ 10260.3	197.4 ÷ 438.8	624.4 ÷ 1489.3
Intercept	−831.9 ÷ −44.7	352.5 ÷ 1091.3	−196.3 ÷ 2135.0

3.4. Accuracy and Precision

The accuracy and precision of the method were determined at three different concentration levels, which cover the calibration range. All mean accuracy values and precision values for quality control standard solutions were within the ±15% acceptance criteria described in the guidelines for all analytes and for both within run, as well as between run accuracy and precision. Thus, the method can be considered accurate and precise for the applications it has been developed for (Tables 6–9).

Table 6. Within run accuracy and precision for plasma analysis.

QC Solution	AA		EPA		DHA	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
QCA	−1.0	7.7	5.4	3.4	−5.9	6.8
QCB	1.4	5.4	−1.0	8.1	−2.7	5.9
QCC	6.1	4.3	−4.0	7.4	−7.2	1.4

Table 7. Between run accuracy and precision for plasma analysis.

QC Solution	AA		EPA		DHA	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
QCA	3.6	3.7	−2.2	7.3	−6.8	7.8
QCB	−0.2	5.6	−7.7	6.3	−4.3	4.2
QCC	0.7	6.3	−4.4	6.0	−10.6	4.8

Table 8. Within run accuracy and precision for RBC membrane analysis.

QC Solution	AA		EPA		DHA	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
QCA	−3.0	5.4	3.4	10.3	−4.1	8.4
QCB	5.7	8.9	6.0	13.7	−8.5	3.5
QCC	−0.8	7.4	4.0	13.4	−2.2	9.5

Table 9. Between run accuracy and precision for RBC membrane analysis.

QC Solution	AA		EPA		DHA	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
QCA	−6.3	4.8	13.0	12.5	10.8	9.1
QCB	2.6	8.0	7.2	12.0	−0.2	7.7
QCC	5.8	5.3	2.6	10.9	−3.4	12.7

3.5. Recovery for Plasma Sample Analysis

After analyzing aqueous standard solutions, plasma samples, and the same plasma spike with standard solutions, the recovery was calculated as the bias (%) of the concentration difference between the spiked and unspiked plasma compared to the concentration of analyte in the aqueous standard solution. The results proved that recovery of all the analytes is above 90% after plasma protein precipitation and, thus, aqueous standards can be used for calibration purposes, as a truly blank matrix is not obtainable for this application. The results of the recovery testing are shown in Tables 10–12.

Table 10. Recovery of AA.

Conc. Aq. Standard Solution (µg/mL)	Accuracy of Aq. Standard Solution (%)	Avg. Conc. of Aq. Standard Solution (µg/mL)	Conc. of Plasma Solution (µg/mL)	Conc. of Spiked Plasma Solution (µg/mL)	Average Recovery (%)
14.7	94.1		20.4	33.5	
14.8	94.7		19.9	34.7	
13.9	89.1	14.7	20.6	33.2	92.6
14.7	93.9		20.1	32.3	
15.3	97.8		20.3	35.7	

Table 11. Recovery of DHA.

Conc. Aq. Standard Solution (ng/mL)	Accuracy of Aq. Standard Solution (%)	Avg. Conc. of Aq. Standard Solution (ng/mL)	Conc. of Plasma Solution (ng/mL)	Conc. of Spiked Plasma Solution (ng/mL)	Average Recovery (%)
295.3	94.5		176.6	465.9	
297.2	95.1		181.3	445.2	
281.0	89.9	296.4	183.0	465.5	97.0
300.2	96.1		175.2	473.7	
308.1	98.6		171.3	474.8	

Table 12. Recovery of EPA.

Conc. Aq. Standard Solution (ng/mL)	Accuracy of Aq. Standard Solution (%)	Avg. Conc. of Aq. Standard Solution (ng/mL)	Conc. of Plasma Solution (ng/mL)	Conc. of Spiked Plasma Solution (ng/mL)	Average Recovery (%)
311.5	99.7		81.2	380.6	
296.3	94.8		77.1	363.4	
284.4	91.0	297.3	82.8	351.2	95.8
303.0	97.0		84.4	362.8	
291.1	93.2		85.6	376.7	

3.6. Reproducibility of Plasma Sample Preparation for Total PUFA Analysis

The validation of the reproducibility of the sample preparation method of total PUFA determination (using hydrolysis with HCl solution) was performed by determining the percentage ratio of PUFA, which was calculated for each sample as the ratio between the sum of DHA and EPA, AA, and the coefficient of variation (CV, %); for six samples, the same plasma sample was prepared in parallel. The results showed that the method yields consistent and reliable results and there is no significant variation during sample preparation (Table 13).

Table 13. Reproducibility of the sample preparation method.

Conc. of AA (µg/mL)	Conc. of DHA (ng/mL)	Conc. of EPA (ng/mL)	Omega Index (C_{DHA} + C_{EPA})/C_{ARA} (%)	Average Index (%)	CV (%)
30.8	145.9	109.9	0.83	0.87	5.5
36.1	172.5	115.5	0.80		
25.5	117.6	102.4	0.86		
17.8	85.5	81.2	0.93		
26.5	128.0	107.2	0.89		
28.2	134.0	115.2	0.88		

3.7. Reproducibility of RBC Sample Preparation for PUFA Analysis

The validation of the reproducibility of the sample preparation method of RBC membrane PUFA determination (using hydrolysis with HCl solution) was performed by determining the percentage ratio of PUFA and was calculated for each sample as the ratio between the sum of DHA and EPA, AA, and the coefficient of variation (CV, %); for six samples, the same plasma sample was prepared in parallel. The results showed that the method yields consistent and reliable results and there is no significant variation during sample preparation (Table 14).

Table 14. Reproducibility of sample preparation method.

Conc. of AA (µg/mL)	Conc. of DHA (ng/mL)	Conc. of EPA (ng/mL)	Omega Index (C_{DHA} + C_{EPA})/C_{ARA} (%)	Average Index (%)	CV (%)
12.4	57.1	9.0.	0.53	0.52	4.6
11.1	49.7	7.2	0.51		
23.8	113.3	17.2	0.55		
12.6	57.4	7.4	0.51		
12.9	54.3	7.5	0.48		
11.7	52.7	8.0	0.52		

3.8. Accuracy of RBC Sample Preparation in Case of Pausing and Resuming

The validation of the effect of pausing and resuming the sample preparation after 48 h on the accuracy of the determination of the omega index highlighted that the process cannot be stopped at any moment due to the possibility of PUFA oxidation and degradation influencing the result. For the five samples paused after the washing step (samples 1–5), the accuracy of discarding the hemoglobin is impacted much less and the results are more accurate and reliable, compared to the five samples paused after the extraction with hexane (samples 6–10). Thus, due to the much more difficult sample collection and preparation for RBC membrane determination (samples need to be processed freshly and cannot be frozen, compared to plasma where freezing is possible), this type of analysis needs extra care and time. The sample preparation can be taken to the point of RBC washing and paused for continuation on a different day but from this point must be continued without interruption, even if it takes over 17 h. The results for the accuracy of RBC sample preparation in case of pausing and resuming the process are presented in Tables 15 and 16.

Table 15. PUFA ratios when pausing and resuming sample preparation.

Samples without Interruption			Samples Paused and Resumed		
DHA/ARA (%)	**EPA/ARA (%)**	**DHA + EPA/ARA (%)**	**DHA/ARA (%)**	**EPA/ARA (%)**	**DHA + EPA/ARA (%)**
0.39	0.07	0.46	0.44	0.07	0.51
0.55	0.09	0.64	0.46	0.07	0.53
1.05	0.06	1.11	0.99	0.06	1.04
0.50	0.06	0.56	0.49	0.06	0.55
0.28	0.03	0.32	0.33	0.04	0.36
0.29	0.03	0.32	0.34	0.03	0.37
0.19	0.09	0.28	0.23	0.08	0.30
0.38	0.05	0.43	0.51	0.05	0.56
0.41	0.10	0.51	0.55	0.11	0.65
0.44	0.09	0.53	0.54	0.10	0.64

Table 16. Accuracy when pausing and resuming sample preparation.

Sample	Samples without Interruption	Samples Paused and Resumed	Bias (%)	Average Bias (%)
1	0.46	0.51	11.85	
2	0.64	0.53	−17.55	
3	1.11	1.04	−6.30	−0.02
4	0.56	0.55	−2.02	
5	0.32	0.36	13.91	
6	0.32	0.37	17.84	
7	0.28	0.30	10.26	
8	0.43	0.56	28.94	21.21
9	0.51	0.65	28.38	
10	0.53	0.64	20.65	

4. Discussions

There are a number of published analytical methods that demonstrate the potential of LC-MS/MS as a reliable and robust technique for the determination of FAs and omega index in various biological matrices. The studies show different strategies regarding derivatization, chromatographic optimization, and various sample preparation. Some of them compare their analytical performances with other techniques such as GC-MS, a standard technique for FAs analysis, and prove their applicability in different matrices and for various intends. Regarding the type of mass spectrometer used for determinations, it ranges from triple quadrupole (QQQ) mainly to ion-trap (IT), triple-quadrupole linear ion trap (Q-Trap), or orbitrap mass spectrometer.

Koch et al. [4] proposed an optimized chromatographic separation of isobaric fatty acids on a C8 reversed-phase analytical column with core-shell particles. The method allows the determination of 41 saturated and unsaturated fatty acids by LC-MS/MS with a QQQ mass spectrometric detection, with low limits of detection, and high accuracy and precision. The method proves a high-throughput approach with a run-time of 15 min and a simple sample preparation by hydrolysis and dilution. The method can also be combined with the analysis of fatty acid oxidation from the same sample, which provides a comprehensive view of lipid metabolism. The method was applied to quantify fatty acids in human plasma and edible oils and showed consistent results with gas chromatography-flame ionization detection analysis used as a reference method. While this method is more versatile and has the capacity to quantify a larger number of analytes, the more focused method described in this manuscript, with a much shorter runtime, can allow for an even higher throughput analysis of PUFA of interest on a larger number of samples in a much shorter time span.

In another study [5], a method for the profiling of free fatty acids using LC/ESI-MS/MS after methylation with trimethylsilyldiazomethane (TMSD) is reported. The chemical derivatization enhanced the ionization efficiency and selectivity of fatty acids when using electrospray ionization (ESI), after which the analytes were detected by a QQQ mass spectrometer. This method also allows high-throughput analysis with short analysis time (5 min) and derivatization time (10 min). Methylated FFAs were analyzed in positive ionization mode, showing good analytical performances and un-derivatized FFAs in the negative mode of the ESI ion source. The method was applied in animal models to compare the fatty acid profiles of hepatitis B virus (HBV) and mock mice tissues and showed that the methylation reaction improved the discrimination of the samples and could be used for discovering other FFA as biomarkers. Compared to the method developed by our team, this method only quantifies FFAs, since it lacks an extra hydrolysis step. Derivatization of analytes, at the same time, has the disadvantage of increasing the cost of analysis and introducing a further source for the possibility of lab errors, compared to methods that quantify FFAs underivatized.

Chen et al. [8] propose a simple and rapid LC-MS/MS method for the determination of 22 FFAs in human colon tumor tissues. By combining two types of sample preparation, liquid–liquid (LLE) and solid-phase extractions (SPE), the final sample was injected and analytes were separated on a C18 column with the aid of a mobile phase consisting of ammonium acetate in acetonitrile and isopropranol. The mass spectrometer was a triple-quadrupole linear ion trap (Q-Trap) mass spectrometer and ionization was performed in a TurboIonSpray interface. This method also only quantifies FFAs due to the lack of a hydrolysis reaction and has a longer total runtime at 15 min per sample. At the same time, the combination of both LLE and SPE can greatly increase the analysis time and cost.

In a short communication by Thermo Scientific, a method for the quantitation of 22 omega fatty acids in serum by LC-MS/MS using protein crash and liquid–liquid extraction for the sample preparation of free and total fatty acids in plasma is described [9]. The method uses a triple quadrupole mass spectrometer under negative ESI MRM mode and achieves good linearity, recovery, and reproducibility in most cases. However, the results are incomplete, as the results do not cover all 22 FA profiles. This method also has a very long sample preparation time of over 2 h and a runtime of 10 min for each sample. These aspects make this method unpractical to use in a high-throughput situation, such as for diagnostics or studies with high numbers of samples. While useful as a guide and proof of concept, a simpler sample preparation time and shorter sample runtime, such as for the method developed by our team, ensure the practicality of a bioanalytical method.

Five FAs are determined without derivatization by using a proper LC-MS/MS method with a QQQ detection and a relatively simple sample preparation protocol [10]. In this methodology, after liquid–liquid extraction from plasma with hexane/isopropanol, the FFAs in the reconstituted samples were analyzed on a reversed-phase C18 column with a mobile phase containing ammonium acetate solution and acetonitrile and FAs were determined in negative electrospray ionization mode. For TFAs, an alkaline hydrolysis of plasma samples is applied. The method is simple, it takes less than 7 min for five FAs to be quantified and there is no need for chemical derivatization. Thus, this method is suitable for high-throughput bioanalysis. The biggest disadvantage of this method is the use of liquid–liquid extraction for FFA analysis, which is much more time and resource-consuming compared to the protein precipitation method. At the same time, the use of multiple isotopically marked internal standards increases the cost of analysis significantly.

In another study, a new LC-MS/MS method for FA determination is proposed in order to test a hypothesis related to the effect of insulin analog initiation therapy on the arachidonic acid / eicosapentaenoic acid ratio [11]. This ratio, considered a biomarker of silent inflammation, proved to be reduced and the study underlined the role of insulin analog therapy in slowing the evolution of complications in diabetes. The mass spectrometer used in this study was also a QQQ type. This method has some limitations, however, as

the analytes are very poorly separated, there is a mobile phase gradient applied, which increases analysis time due to the need to reequilibrate the analytical column.

Another clinical application of LC-MS/MS analysis of FAs was recently published [12]. Five omega fatty acids on human dried serum spots were determined after extraction in a solvent containing the internal standard and centrifugation. The analysis was carried out using reverse-phase chromatography (C18 column) and gradient elution with a mobile phase composed of ammonium acetate solution, methanol, and 2-propanol. The detection was performed by a QQQ mass spectrometer and no chemical derivatization was performed. The samples analyzed proved the method could be used as preventive pre-diagnostic tests for asthma or other inflammatory disease. While very similar performance-wise to the method described in this manuscript, the method of Lee et al. does not manage to separate the five analytes chromatographically over the total run time of 7 min per sample.

The determination of FAs in red blood cell membranes, as pathological biomarkers, is of great importance as well. From this point of view, a method for the analysis of 14 saturated and unsaturated fatty acids in red blood cells (RBC) by LC-MS/MS analysis using a chemical derivatization approach with picolylamine was proposed by Li et al. [13]. The method uses a benchtop Orbitrap mass spectrometer with positive ESI ionization and full scan MS mode, achieving high sensitivity, accuracy, and precision. The method was applied to measure fatty acid concentrations in RBC from healthy subjects and patients with metabolic syndrome. The cumbersome sample preparation described in this methodology reduces the practicality of this method in high-throughput scenarios, while at the same time increasing the costs of analysis.

In another study [14], the AA/DHA, AA/(EPA + DHA), and AA/EPA ratio in plasma and red blood cells was determined by applying an LC-MS/MS method using an ion-trap mass spectrometric detection and ESI ionization. The chromatographic separation was in the reversed-phase mode and a mobile phase composed of acetonitrile and acetic acid in water was used. The sample preparation included hydrolysis and liquid–liquid extraction. The study focused on proving there are significant differences between the Omega index measured for plasma compared to red blood cell membranes. The results showed that AA/DHA and AA/(EPA + DHA) ratios in plasma are positively correlated to red blood cell membrane values, while there is no correlation between the AA/EPA ratios in plasma and red cell membranes. The LC-MS method described by Tero-Vescan et al. is capable of quantifying a total of ten FAs in 10 min; however, not all FAs are separated chromatographically during the analysis runtime.

Ogawa et al. studied different types of derivatization of FAs usable in LC-MS/MS analysis [6]. They managed to develop multiple derivatization methodologies and validate a method that can be used to determine EPA and AA from saliva. This method has a 10 min total runtime per sample and allows for complete chromatographic separation of derivatized AA and EPA.

In another study, PUFAs and their hydroxylated derivatives were studied as pro-resolving mediators in inflammation using LC-MS/MS [7]. The method uses a Qtrap mass spectrometer for detection and a 31-min gradient elution for the separation of 13 analytes. Plasma and serum samples and effluents from peritoneal dialysis were analyzed using this method after solid-phase extraction.

A pilot study on the impact of serum EPA/AA ratio on the survival rate of lung cancer patients after treatment with pembrolizumab by Tanaka et al. also makes use of LC-MS/MS for analyte quantification and used this method to determine that dietary supplementation of PUFAs could have a potentially beneficial effect on immune responses of lung cancer patients [15].

Salm et al. also developed an LC-MS/MS method for plasma TFA analysis, using a Qtrap mass spectrometric detection and reverse-phase chromatographic separation with isocratic elution [16]. The method developed manages to be very fast, with a runtime of under 4 min per sample, but has the downside of lacking almost any chromatographic separation of some of the analytes. The method was used in bioavailability studies on

PUFA supplementation using pharmaceutical products and the effects of nutritional supplementation on plasmatic levels of PUFA.

The method described in this manuscript was developed with ease of use in mind and was validated with regard to all relevant parameters outlined by analytical validation guidelines [17,18], as well as other parameters that are relevant and important in a real-life laboratory environment. Sample preparation was optimized in order to allow for the shortest analysis time and the possibility to optimize sample preparation in accordance with usual laboratory scenarios of equipment and personnel management. Cell lysis for RBC membrane analysis was tested and compared using a single-step approach, as in the final method, but also using multiple steps of washing RBCs with purified water. The optimal method proved to be the one using 1 mL of purified water, as increasing the volume of water did not yield improved results. The duration of the hydrolysis reaction was also optimized to allow for seamless continuation of the sample preparation after overnight hydrolysis. A shorter time for this reaction, of only 1–2 h, proved to be insufficient; however, the duration of 17 h allows for samples to be ready for analysis the next day after starting the sample preparation. The LC-MS/MS methodology, including sample preparation, proved to be simple to use and was successfully applied in a number of clinical studies, some already published [19,20]. The validation of the method, however, also highlighted some important aspects that need to be taken into consideration when using the method, such as assuring there is enough time and personnel/equipment availability to process RBC membrane samples immediately after collection and at least to the point of analyte liquid–liquid extraction with hexane, in order to assure the accuracy of the determinations.

5. Conclusions

The novel LC-MS/MS method developed was optimized to allow for simple sample preparation, while at the same time assuring sensitive, selective, accurate, and precise quantification of omega fatty acids. This allowed for the method to be successfully used in a wide variety of studies, where the method proved to be very robust and yielded solidly reliable results over the years. Furthermore, while the method can be used in metabolomics studies and for diagnostic purposes, it can also be used for bioavailability and biomonitoring studies of pharmaceutical formulations containing PUFA in prophylactic or therapeutic applications.

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References

1. Wickramasinghe, M.; Weaver, J.U. *Chapter 10—Lipid Disorders in Obesity*; Weaver, J.U., Ed.; Practical Guide to Obesity Medicine; Elsevier: Amsterdam, The Netherlands, 2018; pp. 99–108. ISBN 9780323485593. [CrossRef]
2. Omega-3-6-9 Fatty Acids: A Complete Overview, Updated 19 May 2023. Available online: https://www.healthline.com/nutrition/omega-3-6-9-overview (accessed on 4 August 2023).
3. Shramko, V.S.; Polonskaya, Y.V.; Kashtanova, E.V.; Stakhneva, E.M.; Ragino, Y.I. The Short Overview on the Relevance of Fatty Acids for Human Cardiovascular Disorders. *Biomolecules* **2020**, *10*, 1127. [CrossRef] [PubMed]

4. Koch, E.; Wiebel, M.; Hopmann, C.; Kampschulte, N.; Schebb, N.H. Rapid quantification of fatty acids in plant oils and biological samples by LC-MS. *Anal. Bioanal. Chem.* **2021**, *413*, 5439–5451. [CrossRef] [PubMed]
5. Mok, H.J.; Lee, J.W.; Bandu, R.; Kang, H.S.; Kim, K.H.; Kim, K.P. A rapid and sensitive profiling of free fatty acids using liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS) after chemical derivatization. *RSC Adv.* **2016**, *6*, 32130–32139. [CrossRef]
6. Ogawa, S.; Tomaru, K.; Matsumoto, N.; Watanabe, S.; Higashi, T. LC/ESI-MS/MS method for determination of salivary eicosapentaenoic acid concentration to arachidonic acid concentration ratio. *Biomed. Chromatogr.* **2016**, *30*, 29–34. [CrossRef] [PubMed]
7. Kutzner, L.; Rund, K.M.; Ostermann, A.I.; Hartung, N.M.; Galano, J.-M.; Balas, L.; Durand, T.; Balzer, M.S.; David, S.; Schebb, N.H. Development of an Optimized LC-MS Method for the Detection of Specialized Pro-Resolving Mediators in Biological Samples. *Front. Pharmacol.* **2019**, *10*, 169. [CrossRef] [PubMed]
8. Chen, L.; Xie, B.; Li, L.; Jiang, W.; Zhang, Y.; Fu, J.; Guan, G.; Qiu, Y. Rapid and Sensitive LC–MS/MS Analysis of Fatty Acids in Clinical Samples. *Chromatographia* **2014**, *77*, 1241–1247. [CrossRef]
9. Doyle, R.M. LC-MS/MS Quantitative Analysis of Polyunsaturated Omega 3, 6, 7 and 9 Fatty Acids in Serum. Thermo Fisher Scientific: Waltham, USA, 2017. Available online: http://tools.thermofisher.com/content/sfs/posters/PO-64921-LC-MS-Fatty-Acids-Serum-MSACL2017-PO64921-EN.pdf (accessed on 15 March 2024).
10. Serafim, V.; Tiugan, D.A.; Andreescu, N.; Mihailescu, A.; Paul, C.; Velea, I.; Puiu, M.; Niculescu, M.D. Development and Validation of a LC-MS/MS-Based Assay for Quantification of Free and Total Omega 3 and 6 Fatty Acids from Human Plasma. *Molecules* **2019**, *24*, 360. [CrossRef] [PubMed]
11. Aslan, M.; Özcan, F.; Aslan, I.; Yücel, G. LC-MS/MS analysis of plasma polyunsaturated fatty acids in type 2 diabetic patients after insulin analog initiation therapy. *Lipids Health Dis.* **2013**, *12*, 169. [CrossRef] [PubMed]
12. Lee, H.; Yoon, H.R. Determination and Application of UHPLC-ESI-MS/MS Based Omega Fatty Acids on PUFA Filter Paper with Human Asthma Serum. *Chromatographia* **2022**, *85*, 281–294. [CrossRef]
13. Li, X.; Franke, A.A. Improved LC-MS method for the determination of fatty acids in red blood cells by LC-orbitrap MS. *Anal. Chem.* **2011**, *83*, 3192–3198. [CrossRef] [PubMed]
14. Tero-Vescan, A.; Vancea, S.; Huţanu, A.; Borka-Balás, R.; Dobreanu, M. Concordance and Controversy in Determining the Omega-3 Index in Plasma and Red Blood Cells Membrane. *Farmacia* **2015**, *63*, 504–509.
15. Tanaka, I.; Yano, Y.; Mori, M.; Manabe, S.; Fukuo, K. Impact of serum eicosapentaenoic acid/arachidonic acid ratio on overall survival in lung cancer patients treated with pembrolizumab: A pilot study. *Sci. Rep.* **2024**, *14*, 1384. [CrossRef] [PubMed]
16. Salm, P.; Taylor, P.J.; Kostner, K. Simultaneous quantification of total eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid in plasma by high-performance liquid chromatography-tandem mass spectrometry. *Biomed. Chromatogr.* **2011**, *25*, 652–659. [CrossRef] [PubMed]
17. *Bioanalytical Method Validation—Guidance for Industry*; Unites Stated Food and Drug Administration: Silver Spring, MD, USA, 2018.
18. *Guideline on Bioanalytical Method Validation*; European Medicines Agency: Amsterdam, The Netherlands, 2011.
19. Andone, S.; Farczádi, L.; Imre, S.; Bajko, Z.; Moțățăianu, A.; Maier, S.; Bărcuțean, L.; Bălașa, R. Serum Fatty Acids Are Associated with a Higher Risk of Ischemic Stroke. *Nutrients* **2023**, *15*, 585. [CrossRef] [PubMed]
20. Huțanu, A.; Zaharia, M.; Farczadi, L.; Pașcanu, I.M.; Pop, R.M.; Dobreanu, M. Evaluation of Plasma AA/DHA+EPA Ratio in Obese Romanian Children. *Rev. Romana Med. Lab.* **2021**, *29*, 165–178. [CrossRef]