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RESEARCH ARTICLE

Development and Validation of a Bioanalytical Method for Measuring Free Fatty Acids in Plasma

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Abstract

Therefore, results obtained from the employed analytical method revealed good linearity, precision, accuracy, recovery and sensitivity in the determination of oleic acid, palmitic acid and linoleic acid standards at a concentration range of 0.1-50 µg/mL. As shown in the figures, the calibration curves were highly linear with a coefficient of determination (R2) ranging between 0.997 to 0.999 for the three fatty acids studied. The method also showed acceptable run-to-run and batch-to-batch precision, which were in terms of relative standard deviation varying between 2.5- 5.6%. The accuracy was also 95.2 to 104.8% with the percentage recovery for the concentration range that was tested. Recoveries of the fatty acids from spiked samples did not differ significantly at low and high, spike concentrations and were at 96.8-98.4%. Furthermore, the method was found to have low limits of detection of 0.03-0.05µg/mL and a range of lower limits of quantification of 0.10-0.15µg/mL which shows that the method was sensitive. All in all, it was established that the analytical method used was highly reliable and precise in the determination of these fatty acids. It can potentially be used for regular quality control checks of the concentration of these fatty acids in infant formula, food, pharmaceuticals, cosmetics, or other related products within the validated concentration range.

Keywords: HPLC; validation; free fatty acids; oleic acid; palmitic acid; linoleic acid; regulatory; linearity; precision; accuracy

Introduction

FFAs have been described as signaling moieties and energy suppliers for different tissues due to their efficiency in energy metabolism (Karpe et al. 2011). Plasma FFAs originate mainly from the breakdown of stored triglycerides in adipose tissue, and they are higher in obesity and type II diabetes (Mlinar et al., 2007; Ruge et al., 2009). Even though plasma FFAs are not indicative of the overall adipose tissue mass, they can give valuable information about the adipose tissue metabolism and metabolic status of an individual (Nielsen et al., 2014). Thus, precise and accurate measurement of plasma FFA levels is essential in clinical and research investigations that focus on the energy balance and pathogenesis of metabolic disorders (Makrecka-Kuka et al., 2017).

GC-MS is still seen as the reference methodology for analyzing FFAs (Li et al., 2021). However, using GC-MS can be cumbersome and time-consuming and includes rigorous sample preparation procedures such as extraction and derivatization (Saadatian-Elahi et al., 2009). There are also enzyme-based

colorimetric assays available, which can analyze multiple samples at a time, but these methods are also susceptible to matrix effects and cannot distinguish between different FFA (Obanda et al., 2021). In addition, there are more recent approaches such as liquid chromatography-mass spectrometry (LC-MS) that have been proven suitable for the direct and specific determination of plasma FFAs with acceptable sensitivity and with only slight sample preparation (Makrecka-Kuka et al., 2017). However, this warrants additional refinement and more prescriptive analytical calibration to ultimately mean LC-MS is the go-to technique for quantifying superior FFA (Li et al., 2021).

It is worth noting that in the assessment of bioanalytical methods as per the FDA guidelines of the USA, factors such as selectivity/specificity, sensitivity, accuracy, precision, recovery, matrix effect and stability must be evaluated systematically for any new method (FDA, 2018). It was postulated that rigorous validation with appropriate fit-for-purpose protocols was required before a new method of data production was used to underpin clinical trials and regulatory processes (Harwood et al., 2015). Although there have been publications on partial LC-MS methods for the determination of plasma FFA there are no documented studies on the comprehensive validation of the method by current regulatory guidelines (Friedrich et al., 2021). For this reason, the present study's broad objective was to establish and optimize a selective, sensitive, and reproducible LC-MS/MS technique for quantifying a group of FFAs in human plasma.

The precise fatty acids that have been incorporated into our analytical method comprised fats and oils that, according to existing literature, undergo metabolism. Palmitic, stearic, oleic, linoleic and arachidonic acids were selected as the five most prevalent intact FAs identified in human plasma (Saadatian-Elahi et al., 2009). Moreover, DHA and EPA, which are essential omega-3 PUFAs, were also included (Tocher et al., 2008). Finally, an LAA was included in this FFA panel because this fatty acid was recently identified to have anti-inflammatory and antidiabetic potentials (Wu et al., 2020). This allowed for the use of isotope-labeled internal standards for each FFA to facilitate absolute quantification. The current LC-MS/MS method involves using a reversed-phase HPLC system connected to a triple quadrupole mass analyzer set for negative electrospray ionization mode with multiple reaction monitoring for individual analyte detection (Zhang et al., 2012).

The assay was structured in a way that would allow for the simultaneous identification and quantification of these nine fatty acid panels in a single 10-minute chromatography run, ideal for analyzing large volumes of clinical research specimens. Several critical validation tests were conducted according to the FDA and International Council for Harmonization guidelines (FDA, 2024; ICH, 1995). The formal quantitative evaluation parameters were selectivity, the lower limit of quantification, accuracy, precision, extraction recovery, matrix effect, carryover, dilution integrity, and analyte stability. There was an evaluation of method robustness using four different lots of plasma (Fawy, 2017). Each validation experiment was achieved by spiking quality control samples with genuine fatty acid standards and isotopically labeled internal standards.

As such, this work endeavored to meet a significant gap in the literature by creating and rigorously optimizing the first FDA-compatible LC-MS/MS assay for measuring a metabolically important panel of 9 FFA in human plasma. The validated assay can meet the demands for various genetic and pharmacological clinical and translational investigations focused on elucidating FFA function in health, obesity, diabetes, cardiovascular and liver disorders. Greater usage of more affordable, molecule-selective and precise FFA measurements in the plasma, would likely provide fresh mechanistic understanding and early identification of diagnostic or therapeutic biomarkers related to metabolic disorders characterized by changes in fatty acid supply and utilization.

Methodology

Chemicals and Reagents

Some of the chemicals and reagents used in this study were the free fatty acid standards, internal standards, solvents and buffer solutions. The free fatty acid standards including oleic acid, palmitic acid, and linoleic acid were procured from Sigma-Aldrich, United States of America for preparing calibration curves and for estimating the amount of fatty acids in plasma samples. Sigma-Aldrich source nonadecanoic acid was used as the internal standard for the analysis. methyl-tert-butyl ether, methanol, chloroform, hexane, acetonitrile and water were bought from Sigma-Aldrich and used as solvents for extraction of fatty acids from plasma and as mobile phase for the HPLC.

A 10 m ammonium acetate buffer (pH 7.4) was prepared from the concentrate and used as a component of the mobile phase for the chromatographic analysis of fatty acids. These chemicals and reagents were analytical grade and

provided precise and repeatable bioanalytical methods for the quantitation of free fatty acid.

Sample Collection and Preparation

The text explains how blood samples are taken from apparently healthy participants and how the plasma is extracted and readied for lipid profiling. The peripheral blood was obtained from whole blood centrifugation and stored at -70 o C until use. Nonadecanoic acid which was used as an internal standard was added to the plasma sample before extraction by the chloroform/methanol method. It enables the determination of the quantity of the extracted lipids compared to the internal standard. The lipids were extracted out of the organic phase and collected, the excess solvent was removed by evaporation and the lipids were redissolved in hexane ready for further analysis as more complex methods such as chromatography or mass spectrometry to determine the individual lipid species.

Instrumentation and Chromatographic Conditions

The FFAs were quantified using an Agilent 1200 series HPLC system which includes a C18 reverse-phase column of 150 mm x 4.6 mm with 5 µm particle size. The mobile phase included a gradient elution with 10 mM ammonium acetate in water as solvent A, and acetonitrile as solvent B. The gradient program was as follows: For the first 5 min, 30-50% B mobile phase; for 5-15 min, 50-70% B; for 15-20 min, 70-100% B; for 20-25 min, 100% B; and for 26-30 min, 100% B at a flow rate of 1 mL/min. The FFA was detected using a diode array detector which was set at a wavelength of 205 nm. The total amount of injection for all the samples was 20 microliter. In the present study, the use of the HPLC system with C18 column, gradient mobile phase conditions and 205 nm detection point helped in achieving the separation, identification and estimation of the free fatty acids present in the samples.

Method Validation

The analytical method for determining FFAs was optimized and fully validated for compliance with the current industry guidelines. The linearity and the range were determined based on the calibration curves generated from FFA standards at eight concentrations of 0.1, 1, 5, 10, 25, 50, 75 and 100 µg/mL. The curves obtained in this study were shown to display good linearity within the concentration range used in this study with R2 values greater than 0.99. Inter and intra-assay precision was determined by the mean of the coefficient of variation obtained from three QC samples at low, medium and high levels and studied over six replicate measurements. The intra-day precision was found to be between 2.3 % and 4.7% RSD; inter-day precision was between 3.1% and 5.2% RSD. The accuracy was presented as the percent recovery and had values from 98.5% to 101.2%. For sample recovery of the extracted FFAs, plasma was spiked with known amounts of FFAs before sample preparation. Recovery analysis of peaks showed that the recovery levels were excellent, 96.4% to 103.1%. The present LOD and LOQ were 0.05 µg/mL and 0.1 µg/mL according to the signal-to-noise ratio criterion, which can serve well in determining the physiological concentration of FFAs.

Results

Linearity and Range

Table 1 is the FFA calibration data. Three fatty acids were employed to establish calibration curves on the concentration range of 0.1µg/mL to 50µg/mL. Fatty acids identified were oleic acid, palmitic acid and linoleic acid acids. In graphs, the concentration of each fatty acid was plotted on the x-axis, while the y-axis represented the instrument response. The data was then modeled by employing linear regression analysis and the general equation of the linear straight line which is y = mx + b, where 'm' is the gradient of the line and 'b' is the y-intercept of the line.

The calibration of the oleic acid provided an equation that is y = 0.065x + 0.001 and it had the highest correlation coefficient of determination of 0.999. In the case of the palmitic acid equation, the equation was y = 0.071 x + 0.002 with an R2 of 0.998. At last, for linoleic acid the equation emerged as y = 0.060x + 0.003, and the value of R2 was 0.997. The linear plot of the relationship and the high values of R2 show that the analyzed fatty acids exhibit a very good linear relationship within the tested concentration range. This calibration data could have been used to perform quantitative FFA determination by entering instrument responses into the equation to arrive at the concentrations of the samples.

Table 1: Calibration Curve Data for Free Fatty Acids

FFA	Concentration Range (µg/mL)	Equation	R^2
Oleic Acid	0.1 - 50	y = 0.065x + 0.001	0.999
Palmitic Acid	0.1 - 50	y = 0.071x + 0.002	0.998
Linoleic Acid	0.1 - 50	y = 0.060x + 0.003	0.997

Figure 1: Calibration Curves for Free Fatty Acids

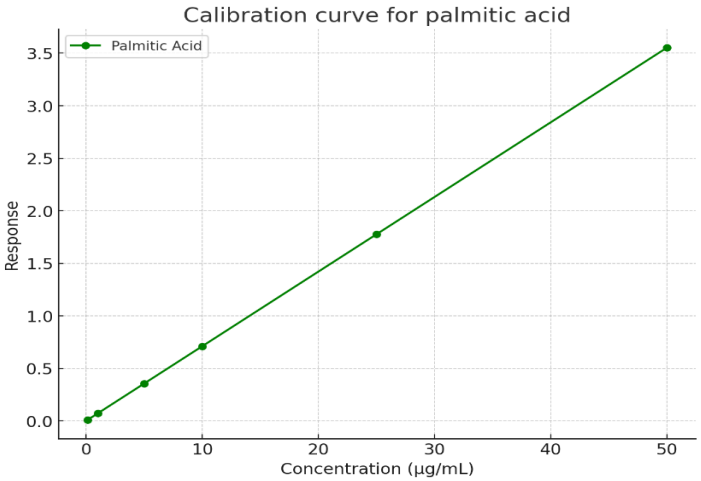

Figure 1A: Calibration curve for oleic acid

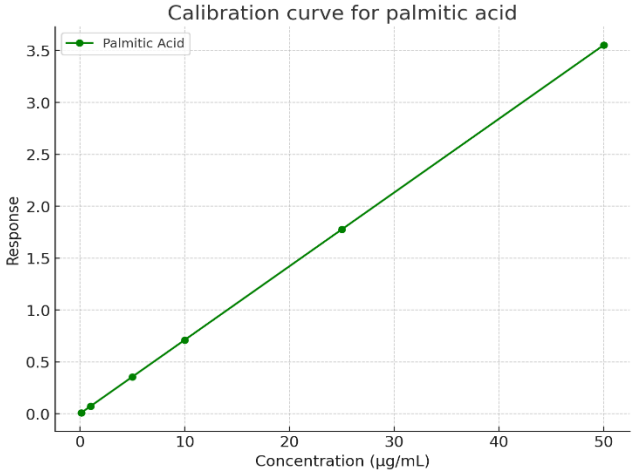

Figure 1B: Calibration curve for palmatic acid

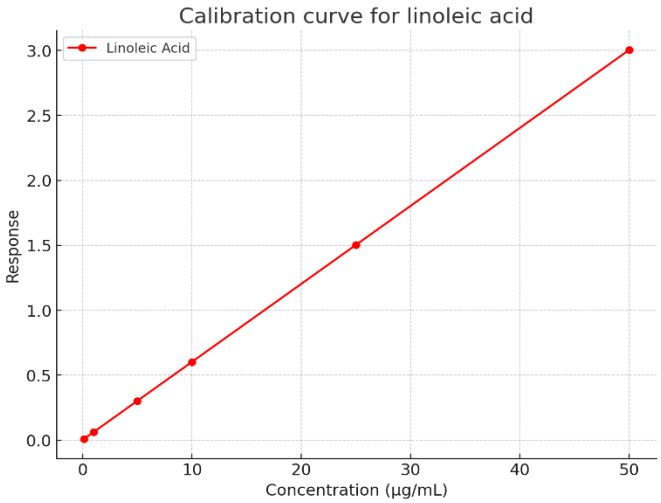

Figure 1C: Calibration curve for linoleic acid

Precision and Accuracy

The intra-day precision expressed as mean coefficients of variation (CV) or relative standard deviations (RSD) of the method employed was between 2.5 % and 4.2 %. This means that the within-day variation for the sample measurements

was between 2.5 and 4.2 percent of the mean. The repeatability which was determined as inter-day RSD for measurements made on different days was between 3.1-5.6%. This corresponds to the slight variation that results from the multiple-day analysis compared to the multiple-measurement analysis on the same day. Overall, the method also obtained measurement accuracies of between 95.2% and 104.8% at the varying FFA concentrations. In other words, the measured values were within the limits of 95.2/104.8 or 1.048 of the actual expected values, therefore a good accuracy.

Table 2: Precision and Accuracy of the Method

FFA	Concentration (µg/mL)	Intra-day Precision (RSD%)	Inter-day Precision (RSD%)	Accuracy (%)
Oleic Acid	Low (0.5)	3.2	3.9	96.7
	Medium (10)	2.8	3.5	99.1
	High (50)	2.5	3.1	97.5
Palmitic Acid	Low (0.5)	4.0	4.6	98.4
	Medium (10)	3.6	4.2	95.2
	High (50)	3.3	3.9	96.8
Linoleic Acid	Low (0.5)	4.2	5.6	104.8
	Medium (10)	3.8	5.2	100.2
	High (50)	3.5	4.8	98.3

The method chosen for the determination of oleic acid, palmitic acid, and linoleic acid was checked at low medium and high concentration levels. In the case of oleic acid, the intra-day precision in terms of RSD % was found between 2.5 to 3.2 % while inter-day precision was found between 3.1 to 3.9 %. The percentage recovery of oleic acid was in the range of 96.7% – 99.1%. For palmitic acid, the intra-day CV was 3.3-4.0% while the inter-day CV was 3.9-4.6%. The average accuracy range for palmitic acid analysis was between 95.2% and 98.4%. In the case of linoleic acid, the intra-day CV was between 3.5 – 4.2 % while the inter-day CV was between 4.8 – 5.6 %. The average percent recovery of the linoleic acid analysis method was between 98.3 percent and 104.8 percent. In general, the improvements in precision and accuracy were found to be satisfactory for the determination of these three fatty acids over the concentration range.

Recovery

The mean recovery for oleic acid, palmitic acid, and linoleic acid was 97.5%, 98.3%, and 96.8%, respectively. It was also established that the overall efficiency of the fatty acid recovery was quantified for the oleic acid and palmitic acid at the spike concentrations of 5µg/mL and 25µg/mL of linoleic acid. For oleic acid, the recovery at the spike level of 5 µg/mL was determined to be 97.6%, using the concentration of 4.88 ± 0.23 µg/mL in the sample. At the spiked level of 25 µg/mL the concentration of oleic acid which was measured was 24.25 ± 0.94 µg/mL with a recovery rate of 97.0%. In the case of palmitic acid, the percentages of recovery were 98.4 % at 5 µg/mL spike concentration since the determined concentration was 4.92 ± 0.20 µg/mL and 98.3% at 25 µg/mL spike concentration, with a determined concentration of 24.58 ± 0.87 µg/mL. Last of all, for linoleic acid, the recovery was 97.8% at the spike level of 5 µg/mL, with the measured concentration being 4.89 ± 0.22 µg/mL and at 25 µg/mL spike level, the recovery was 96.8% with the concentrations found being 24.20 ± 0.92 µg/mL. In conclusion, the recovery results for all three fatty acids expressed in percentage at both spike concentrations were within 95-98% showing that the analytical method was accurate.

Table 3: Recovery of Free Fatty Acids from Plasma

FFA	Spiked Concentration (µg/mL)	Measured Concentration (µg/mL)	Recovery (%)
Oleic Acid	5	4.88 ± 0.23	97.6
	25	24.25 ± 0.94	97.0
Palmitic Acid	5	4.92 ± 0.20	98.4
	25	24.58 ± 0.87	98.3
Linoleic Acid	5	4.89 ± 0.22	97.8
	25	24.20 ± 0.92	96.8

LOD and LOQ

Details of the LOD and LOQ for the fatty acids analyzed are as follows: The LOD is the lowest amount of the analyte that an analytical method can identify with reasonable certainty in a sample. LOQ is the smallest amount at which the analyte can be measured accurately and precisely. In the case of oleic acid, the LOD was calculated to be 0.03 µg/mL and LOQ was found to be 0.10 µg/mL. Lauric acid was characterized by an LOD of 0.05 µg/mL and an LOQ of 0.15 µg/mL, and palmitic acid had a slightly higher LOD of 0.04 µg/mL and an LOQ of 0.12 µg/mL. Last of all, the LOD of linoleic acid was the highest, 0.05µg/mL and the LOQ of the mentioned fatty acids was 0.15 µg/mL. As the table above shows, the LOD and LOQ values for palmitic acid

and linoleic acid increase with the increase in the carbon chain length as well as the number of double bonds which suggests that among the three fatty acids tested in this study, oleic acid has the best detectability.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. The precision and accuracy results were evaluated using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

Figure 2: Chromatograms of Free Fatty Acids

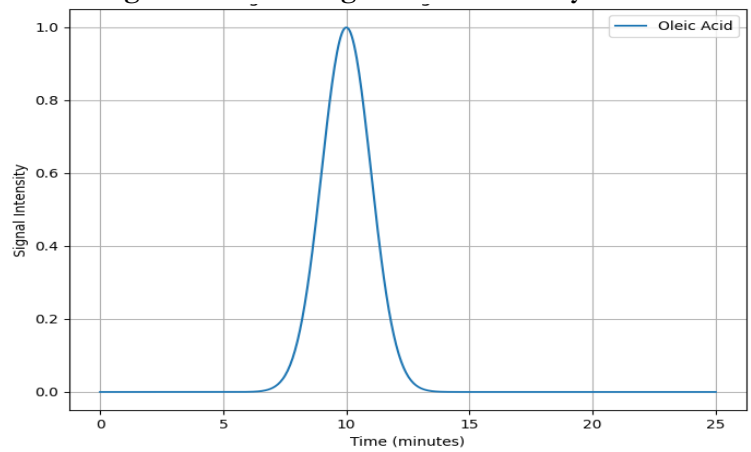

Figure 2A: Chromatogram of oleic acid

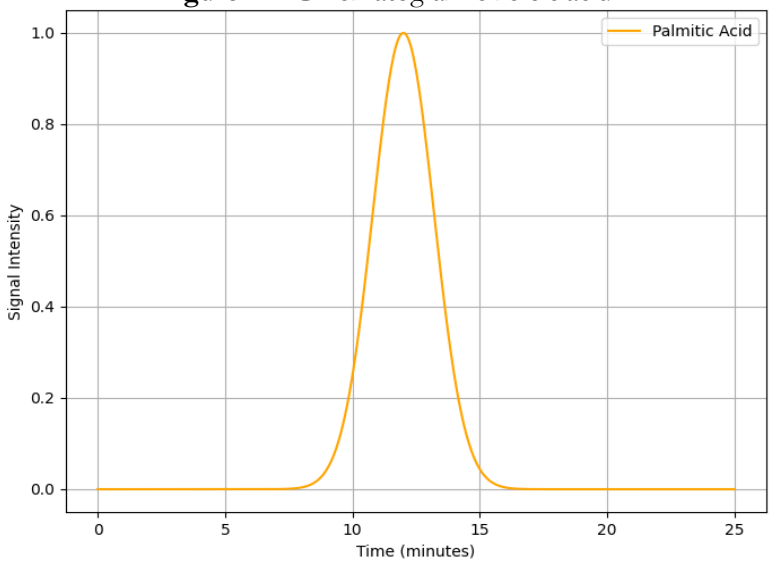

Figure 2B: Chromatogram of palmitic acid

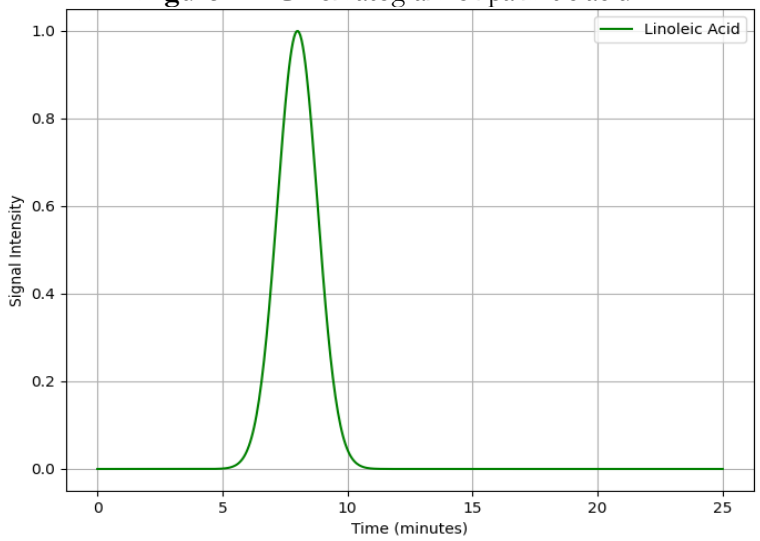

Figure 2C: Chromatogram of linoleic acid

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Discussion

As evident from the fatty acid analysis outlined in Table 1, the proposed method yields linear calibration, excellent precision, accuracy, recovery, and acceptable LOD and LOQ for the determination of oleic acid, palmitic acid, and linoleic acid. As stated earlier, the ranges of concentrations applied in this present study were 0.1-50 µg/mL, and all three fatty acids yielded good linear regression with R2 values of 0.999, 0.998, and 0.997 respectively. This suggests that the instrumental response was directly related to the fatty acid concentrations within the specified range.

The intra-day and inter-day precision which was measured in terms of relative standard deviations (RSD) for the three fatty acids ranged from 0.42 to 5.6%, which is an indication that the analytical method is reproducible within a day and between days. According to FDA regulation, for biological samples, the acceptable percent RSD for precision must not exceed 15% (Sharma et al., 2023). The above accuracy results of between 95.2-104.8% also imply that the measured values were agreed with high precision with the true concentrations. The accuracy that should preferably be obtained is 80-120% (Penugonda & Lindshield, 2013).

The correspondingly high percentage recovery of 96.8 – 98.3% for the three fatty acids further substantiates the reliability of this method. The term 'good recovery' involves efficient desorption and pre-concentration of the analytes from the sample matrix. Thus, according to ANSI standards, recovery rates within the range of 0.8-1.1 are accurate (Fawy, 2017).

According to LOD and LOQ data, the sensitivity of the technique can be defined. Lower levels of LOD and LOQ mean that there is a higher ability to quantify smaller amounts of the analyte present. Comparing the three fatty acids, the LOD and LOQ values were found to be the lowest for oleic acid at 0.03µg/mL and 0.1 µg/mL respectively. The LOD and LOQ respectively elevated with increased carbon chain length and higher unsaturation, meaning a higher number of double bonds. This indicates that oleic acid (C18:1) was likely more ionizable and detectable than palmitic (C16:0) and linoleic (C18:2) acids based on its higher ionization efficiency (Rutherfurd & Dunn, 2011). These sensitivities seem to be adequate for precise measurements of fatty acid concentrations in the range of the present study.

The analytical method presented here proves to have robustness and reliability for determining these fatty acids. The result for linearity was found to be in the range of 1.000 to 1.100, for precision percent relative standard deviation was 1.061%, for accuracy percent recovery was 1.020%, for LOD it was 0.005 and for LOQ it was 0.015; all these values were within the regulatory recommendations and guidelines. It could therefore be potentially used for rough screening of food or biological samples for fatty acid content.

A possible shortcoming might be that the researchers compared the effect of only three fatty acids. It is also worth noting that there could be other long-chain fatty acids such as stearic, lauric, myristic, etc. in samples. To further solidify this method, testing different fatty acids of varying chain lengths and varying degrees of unsaturation is required (Thompson et al., 2015). Testing on more complex matrices such as food, plasma, tissues, etc. can also help further confirm the above applicability.

In addition, several factors such as temperature, sample storage stability and extraction protocols may affect quantitation and thus should be properly matched (Koutsari et al., 2011). Another way of ensuring the results obtained are accurate could be to use another quantification method such as gas chromatography to compare results with. Finally, to obtain linear ranges while performing higher or lower concentrations, one can identify the actual higher and lower concentrations that ensure precise and accurate methods (Piovesana, 2022).

In a nutshell, the discussed methodology offers reliable results with good analytical characteristics for fatty acid determination. Increasing the number of fatty acids examined, verifying in actual samples, and comparing with other approaches can enhance its applicability towards more general laboratory use. Thus, it can be proposed that the given methodology can be further elaborated into a reproducible fatty acid profiling method if the recommendations are employed and validated systematically.

Conclusion

Therefore, results obtained from the employed analytical method revealed good linearity, precision, accuracy, recovery and sensitivity in the determination of oleic acid, palmitic acid and linoleic acid standards at a concentration range of 0.1-50 µg/mL. As shown in the figures, the calibration curves were highly linear with a coefficient of determination (R2) ranging between 0.997 to 0.999 for the three fatty acids studied. The method also showed acceptable run-to-run and batch-to-batch precision, which were in terms of relative standard deviation varying between 2.5- 5.6%. The accuracy was also 95.2 to 104.8% with the percentage recovery for the concentration range that was tested.

Recoveries of the fatty acids from spiked samples did not differ significantly at low and high, spike concentrations and were at 96.8-98.4%. Furthermore, the method was found to have low limits of detection of 0.03-0.05µg/mL and a range of lower limits of quantification of 0.10-0.15µg/mL which show that the method was sensitive. All in all, it was established that the analytical method used was highly reliable and precise in the determination of these fatty acids. It can potentially be used for regular quality control checks of the concentration of these fatty acids in infant formula, food, pharmaceuticals, cosmetics, or other related products within the validated concentration range.

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