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RESEARCH ARTICLE

Tagging Fatty Acids *Via* Choline Coupling for the Detection of Carboxylic Acid Metabolites in Biological Samples

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Abstract: ***Background*:** Fatty acids and other metabolites containing a carboxyl group are of high interest in biomedicine because of their major role in many metabolic pathways and, particularly in the case of oxidised fatty acids, their high biological activity. Tagging carboxylic acid compounds with a permanent positive charge such as a quaternary ammonium compound could increase the LC-MS detection sensitivity and selectivity. This paper describes a new and novel strategy for analysing carboxyl-containing compounds in biological samples by ESI-MS through coupling to choline.

***Methods*:** Coupling of carboxylic acid derivatives in biological samples was performed by coupling to 2-Fluoro-1, 3 dimethyl –pyridinium (FDMP). The variation in the fatty acid profile of five different plasma samples was studied and was illustrated by using principal components analysis (PCA) to group the samples. Orthogonal partial least squares discriminant analysis (OPLS-DA) modelling was then applied to identify the fatty acids that were responsible for the variation.

***Results*:** The test results showed that choline coupling reactions were successful in detecting fatty acids, oxidised fatty acids and other compounds containing carboxylic acid groups in biological samples. The PCA results showed loadings of different fatty acids according to the plasma sample allowing identification of the fatty acids responsible for the observed variation.

***Conclusion*:** A new and easy tagging method was developed to detect carboxylic acids in plasma samples. The method proved to be precise and reproducible and can quantify fatty acid compounds to 50 ng/ml.

Keywords: Fatty acids, choline, plasma, mass spectrometry, LC-MS, principal components analysis (PCA).

1. INTRODUCTION

There are many of the organic and pharmaceutical compounds that possess a carboxyl group. In biomedicine, fatty acids and other metabolites containing a carboxyl group are of high interest because of their major role in metabolic pathways [1]. Analysis of carboxylic compounds such as fatty acids is gaining importance for understanding the aetiology of many diseases and prevention of chronic disease such as atherosclerosis, obesity-induced insulin resistance and for improving cardiovascular health [2-4].

Many instrumental techniques have been applied in the analysis of carboxylic acids which include gas chromatography (GC) and High-Performance Liquid Chromatography (HPLC). However, these methods can lack sensitivity and selectivity [5, 6]. Sample derivatisation is widely used in analytical chemistry and instrumental analysis to improve analytical capabilities. The chemical structure of the sample can be modified into a chemical form that provides an enhanced response in terms of either improved selectivity or sensitivity. Derivatisation reactions used in MS are designed to enhance ionisation or introduce a specific mass shift to the sample ions that become evident in the mass spectrum. There are many MS derivatisation methods reported in the literature [7, 8].

Traditional derivatisation by silylation or methylation in GC/MS methods has been employed in the analysis of fatty acids [9, 10]. These days with the invention of soft ionization techniques such as electrospray ionization HPLC/ESI-MS has become a powerful technique in the analysis of such compounds [11, 12]. The negative ionization of carboxylic acids in LC/MS is best achieved in a basic mobile phase. However, chromatographic resolution with reversed-phase columns is better achieved at acidic pH mobile phases where the ionization of carboxyl groups is suppressed [13]. An alternative method is to tag carboxylic acids with a permanent positive charge such as by using a quaternary ammonium compound. This will cause targeted carboxylic acid com-

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pounds to have a positive charge at all pH values. This strategy has been very successfully applied in the HPLC/ESI-MS analysis of amino acids and peptides [14, 15].

Choline is chemically known as 2-hydroxyethyl(trimethyl)azanium. It is obtained as a quaternary ammonium salt containing the N, N,N-trimethylethanolammonium cation. It is chemically stable and commercially available in a reasonable price.

This paper describes a new and novel strategy for analysing carboxyl-containing compounds in biological samples by ESI LC-MS using hydrophilic interaction chromatography (HILIC) after being coupling the fatty acids to choline. The quaternary ammonium part of the coupled choline makes the derivatised compound permanently ionized in both acid and basic mobile phases and the positive charge allows the derivatives to retain in HILIC columns promoting an orthogonal selectivity to the commonly used reversed phase chromatography.

2. EXPERIMENTAL

2.1. Chemical and Materials

Acetonitrile (ACN), Tetrahydrofuran (THF) and formic acid were HPLC, 2-Fluoro-1, 3 dimethyl –pyridinium p-toluene sulfonate (FDMP), triethyl amine (TEA), *N,N′*-icyclohexylcarbodiimide (DCC), 4-(dimethyl amino)pyridine (DMAP), fatty acid standards : Palmitic acid, dodecanoic acid, myristic acid, oleic acid, cis-vaccenic acid and choline chloride were purchased from Sigma-Aldrich Dorset UK. HPLC grade water was produced by Direct-Q 3 Ultrapure Water System from Millipore, UK.

2.2. Plasma Sample Collection

Samples of pooled plasma were obtained from the Blood Transfusion Service to support a previous study.

2.3. LC-MS Analysis

Measurements of samples and standards were carried out on an Accela HPLC system combined with an Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK), resolution. 60,000 at m/z 400 at a scan rate of 1 Hz over the range 75-1200 amu. The column used was ZIC -HILIC column (150x 4.6mm, 5µm Hichrom, Reading, UK).

Mobile phase A consisted of 0.1% v/v formic acid in water and mobile phase B consisted of 0.1% v/v formic acid in ACN. The gradient used was as follows: 0 min 10% A, 10 min 20% A, 16 min 80% B, 21min 90% A followed by column re-equilibration for 5 min before the next injection. The flow rate was 300µl/min. ESI interface was operated in positive ion mode with a spray voltage of 4.5kv. The temperature in ion transfer capillary was 275°C and the flow rate of the sheath and auxiliary gases were 50 and 17 arbitrary units. Some of the samples were also run on an LTQ Orbitrap under the same conditions in order to obtain MS/MS spectra using a collision energy of 35 V. The acquired data was processed by Xcalibur™ Software.

2.4. Preparation of Plasma Solution, Reagents and Standard Fatty Acid Solutions

An aliquot of plasma (100 µl) was transferred to an Eppendorf tube. Protein precipitation (PP) was performed by addition of ACN (1ml) to the sample which was then vortexed for two minutes and centrifuged at 9000 rpm for 10 minutes. After centrifuging, the supernatant solution was taken to perform the coupling reaction [16].

Fatty acid standard stock solutions were prepared at a concentration of 1µg/ml in THF and stored at room temperature. Choline chloride solution was prepared in ACN at a final concentration of 0.12mg/ml. DMAP and DCC coupling reagents were prepared using ACN at a final concentration of 0.1mg/ml and 0.2mg/ml respectively.

2.5. Coupling of Choline to Fatty Acids:

2.5.1. Reaction Procedure using Steglich Esterification

The coupling of choline to the fatty acid was performed by using the Steglich esterification procedure with little modification [17]. Fatty acid solution (100µL) was taken and DMAP solution (100 µL) was added to it. Choline chloride solution (100µL) was then added to the mixture and this was vortexed for 1 minute. DCC solution (100 µl) was then added to the vortexed solution and the volume was complete to 1ml with ACN. The final solution was shaken at room temperature for 3 hours. The reaction is shown in Fig. (**1**).

2.5.2. Reaction Procedure Using FDMP Coupling Reagents

Choline esterification to the fatty acid was performed using FDMP coupling agent [18]. The fatty acid sample (100µl) was mixed with 100µL of choline solution (0.1mg/ml). 100µL of the FDMP solution (1mg/ml in ACN) were added to the mixture. Then 6µL of TEA was added to the mixture and it was heated to 40° for 30 minutes. The final volume was adjusted to 1 ml with ACN before it was injected into the LC-MS.

2.6. Coupling of the Fatty Acids and its Oxidised Derivatives in Plasma Samples

Choline chloride solution (100µl) was added to the plasma prepared solution as described above. Then 100 µL of the coupling agent FDMP was added. The combined solution was mixed well and 6 µL of TEA was added to it. The mixture was vortexed for two minutes and heated using a water bath at 50° C for 30 minutes. The heated mixture was then evaporated using nitrogen gas. ACN (1ml) was then added and mixed well-using vortex before the solution was ready for LCMS analysis.

2.7. Chemometrics

MS data were processed using MZmine software version 2.21 [19]. Ion features were detected with a minimal signal of 10^4 for the Mass spectrometry data, followed by retention time alignment with the random sample consensus (RANSAC) aligner, gap-filling was performed using the "peak finder" routine with retention time correction and a retention time tolerance of 0.5 min. FA and oxidised fatty acids were identified using a self-generated library of 155 possible fatty

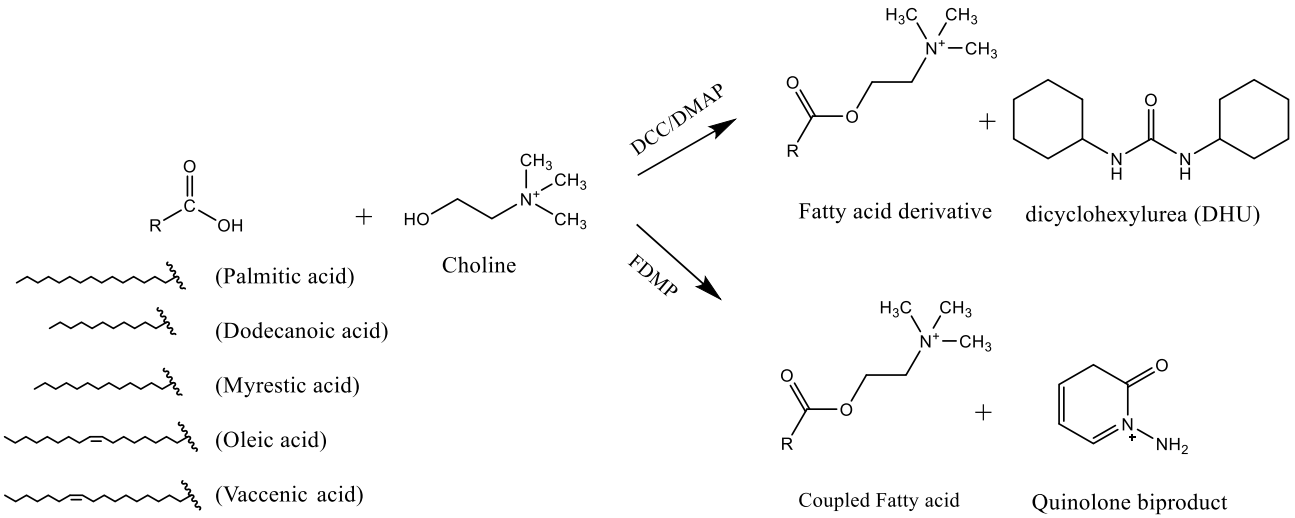

Fig. (1). Choline coupling of fatty acids.

acids and their oxidised derivatives. The final data tables consisting of the columns and rows representing respectively different samples and areas of each detected component were exported to Excel spreadsheets and then to SIMCA -14 software (Umetrics, Umea, Sweden) for multivariate statistical analysis. All variables were Pareto scaled to induce normality and standardize the range of independent X-variables (detected components). In order to detect sample clustering in the measured data, as a dimensional reduction technique, principle component analysis (PCA) was firstly applied for non-supervised visualization of all the three groups based on linear combinations of shared features [20]. Afterwards, a discriminant model using the orthogonal partial least squares-discriminant analysis (OPLS-DA) was implemented in order to extract the systematic variation and find the potential biomarkers which were selected according to Variable Importance in the Project (VIP) value, the loading plot and the S-plot [21].

3. RESULTS

Fatty acids at a concentration of 1µg/ml were coupled using both of the coupling procedures and the results showed that both saturated and unsaturated long fatty acid were successfully coupled with choline and the exact mass of the coupling was detected as shown in *(full data is shown in supporting data file 1).*

The results showed that the peak area and shape were comparable when the coupling was performed using the FDMP or when using DCC/DMAP coupling agents. However, using FDMP requires only 30 minutes to complete the reaction while DCC/DMAP required 3 hours to get the same result and the DCC creates more of a reagent residue in the sample, thus all further couplings were performed by using FDMP coupling agent.

A calibration curve was constructed by performing serial dilution of palmitic acid in the range 0.25-1µg/ml, the area of the peak was plotted against its concentration and the calibration curve was constructed. The calibration curve showed linearity with $R^2 = 0.9983$ and the regression equation was y = 2e+07x + 329368.

The limit of quantification (LOQ) was found to 50 ng/ml, LOQ was calculated based on the standard deviation of the response (Sy) and the slope of the calibration curve (S) according to the formula: LOQ = 10(Sy/S). The standard deviation of the response was determined based on the standard deviation of y-intercepts of regression lines. The values of Sy and slope was obtained form the LINEST function [22]

Plasma test results showed that choline coupling reactions were successful in detecting fatty acids and their oxidised metabolites. The MS data of aliquots of plasma samples were processed using the MZmine program. The results showed an identification of 56 fatty acids and their oxidised derivatives out of 155 compounds in the generated library. Some of the identified compounds have the same molecular weight but in different oxidation structure form *(full data is shown in supporting data file 2).*

In order to overview the variation of fatty acids and its metabolites abundance between plasma samples; PCA plot was produced as illustrated in Fig. (**2**). The pooled plasma samples group into three clear clusters pooled plasma samples represented by different coloured dots.

The s-plot combined the modelled covariance and modelled correlation from the OPLS-DA model in a scatter plot, this plot looks like an S (Fig. **3**). All the dots represent fatty acids and their oxidative metabolites which look like the S shape. The S-plot was used to identify the chemical markers according to their contributions to the separation of clustering. The variable of importance in the projection (VIP) values reflected variable the importance of a particular value to the model, a variable with VIP >1 was considered as an important loading in the model [23]. The potential chemical makers were selected according to VIP value mentioned. To ensure the accuracy of the final result, the established OPLS-DA model was evaluated and all the models were proper and all the analysis for its fitness and prediction of the model. According to the criterion established above, 9 fatty acids were identified and selected as markers to distinguish significant differences among two plasma samples (p24 and p28) as an example. (*full data is shown in supporting data file 3)*

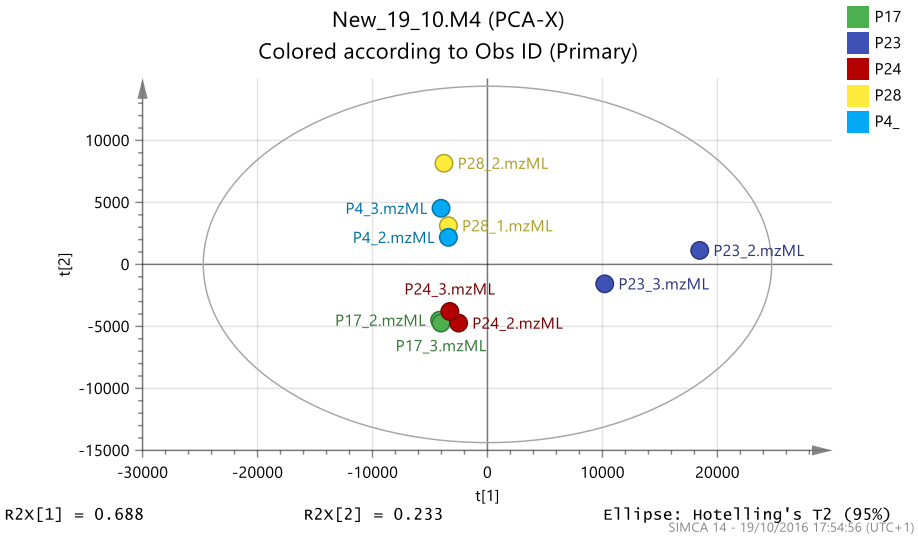

Fig. (2). PCA plot of detected fatty acids and its metabolites from duplicate analysis five different pooled plasma samples. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

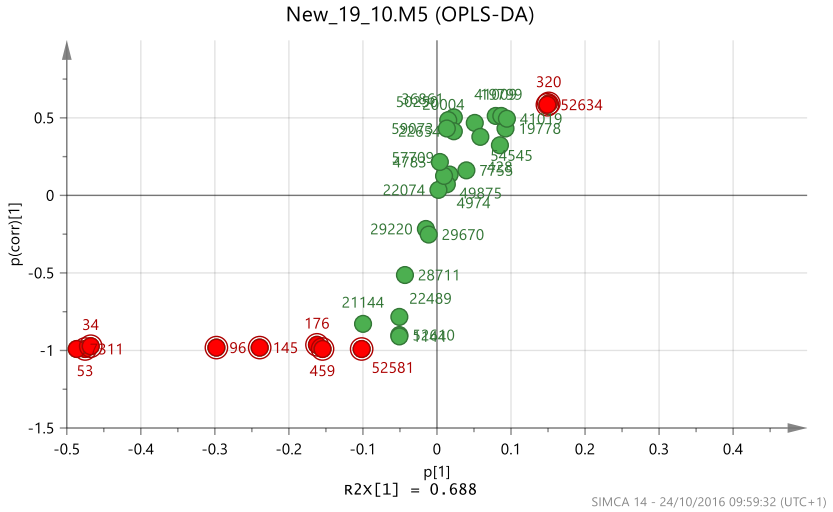

Fig. (3). The S-plot plot of fatty acids and their oxidation products in the tested plasma samples. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

MS/MS study was also done for marker fatty acids and the results of MS fragmentation give the main peak at m/z of the coupled fatty acid losing the tertiary methyl nitrogen (-59) and a very minor peak representing the molecular ion of the non-fragmented fatty acid. Fig. (**4**) shows the MS^2 data of stearic acid coupled to choline showing the main fragment at m/z of 311.3310 and a minor peak for the non-fragmented coupled stearic acid at m/z of 370.481. The Proposed fragmentation pattern is illustrated in *supplementary file 4*. From the MS^2 spectrum, it can be seen that the tagged derivative has good potential for producing a high sensitivity MS/MS with much of the ion current being carried by the dominant fragment ion.

4. DISCUSSION

The coupling procedure using FDMP coupling reagent was selected as-as it took less time (30 min) compared to the procedure using DCC which required at least three hours for the reaction to complete and produced more reagent residue. Moreover, there is a common problem associated with DCC residue in LC-MS as it sticks to the MS electrospray capillary [24]. The method has the potential to increase the sensitivity of the detection of fatty acids and other carboxylic acid containing compounds since detection limits of compounds in negative ion mode tend to be higher than in positive ion mode [18]. For the current procedure, the method was linear in the range of 0.25µg /ml to 1µg/ml and the derivatives were detectable to at least 50 ng/ml. The coupling can be applied to many other metabolites that have carboxylic acid moiety such as prostaglandin. The prostaglandins (9,11-Dideoxy-11α,9α-epoxy methano prostaglandin F2α) and ($F_{2\alpha}$ Tromethamine) were coupled with choline before being tested by the LC-MS. The masses of these prostaglandins were detected which prove the capability of our method to detect other metabolites with bearing carboxylic acid (Fig. **5**) in addition the method would be applicable to the determination of bile acids.

The commercial availability of the stable-isotope choline chloride-(trimethyl-d9) would make it possible to better define metabolic shifts by using a standard pooled sample

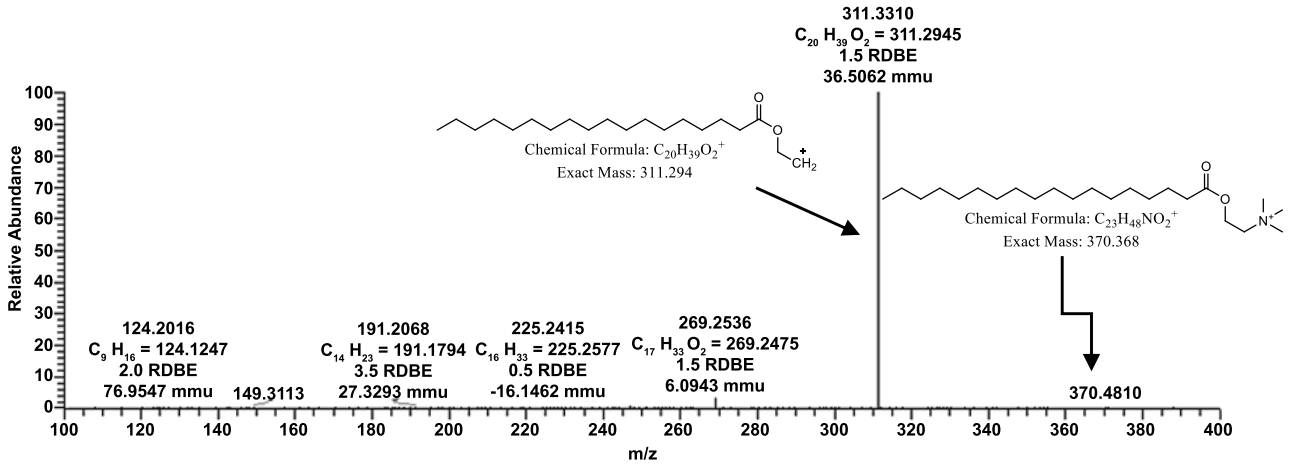

Fig. (4). MS^2 spectrum for stearic acid coupled to choline.

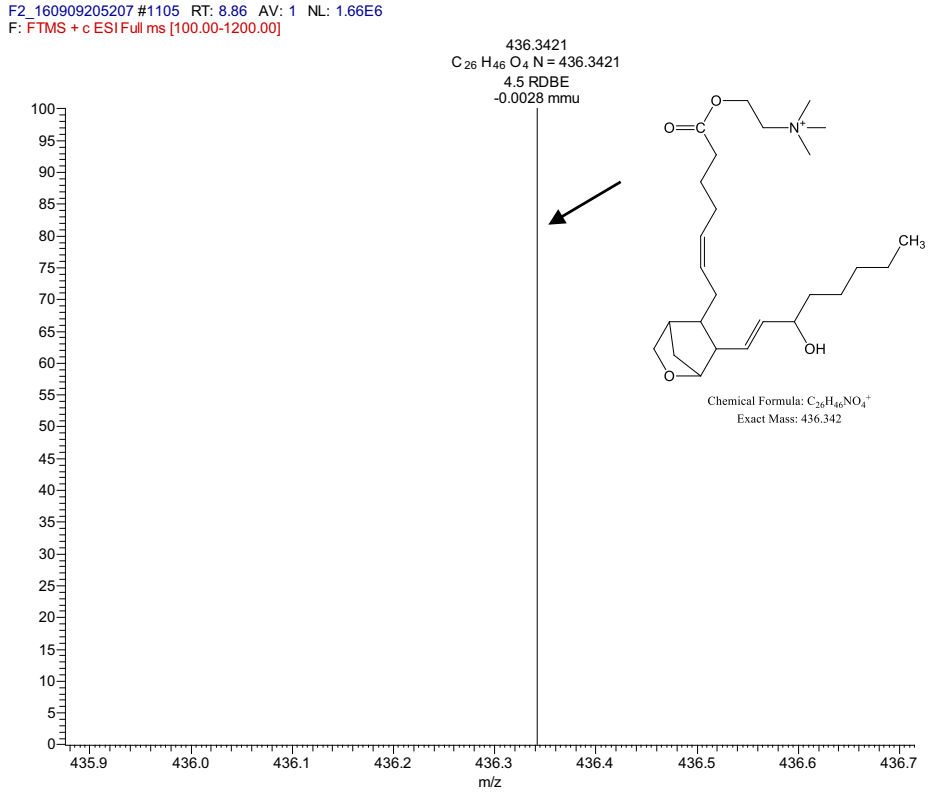

Fig. (5). The detected mass of the prostaglandin F2α coupled with choline. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

derivatised with the labelled tagging agent which could be spiked into a set of samples as an internal standard. In addition, commercially available mixtures of fatty acids could be tagged with the deuterated choline to act as internal standards. The current study is a proof of concept study and subsequent use of the method has proved that it is robust and the favourable fragmentation in MS^2 gives an indication that this method could be the basis of a highly sensitive MS/MS method using selected reaction monitoring for the determination of low level oxidised fatty acids such as prostaglandins or leukotrienes. Tagging the fatty acids with choline allows the use of HILIC chromatography which provides orthogonal selectivity which could be useful in the separation of complex mixtures of isomers.

CONCLUSION

A new derivatisation method was developed for detecting fatty acids and prostaglandins in biological samples. The method was found to be linear over the range 0.25 to 1µg/ml and was capable of detecting palmitic acid to 50 ng/ml. The PCA analysis results showed that the developed method was capable of showing variation in fatty acids and oxidised fatty acids between different plasma samples.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Authors confirm that all data explained can be found with in the paper.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Galli, C.; Simopoulos, A.P.; Tremoli, E. *Effects of fatty acids and lipids in health and disease*. Karger: Berlin, **1994**.

[2] Joffe, Y.; Collins, M.; Goedecke, J. The relationship between dietary fatty acids and inflammatory genes on the obese phenotype and serum lipids. *Nutrients,* **2013**, *5*(5), 1672.

[3] Soardo, G.; Donnini, D.; Domenis, L.; Catena, C.; De Silvestri, D.; Cappello, D.; Dibenedetto, A.; Carnelutti, A.; Bonasia, V.; Pagano, C.; Sechi, L.A. Oxidative stress is activated by free fatty acids in cultured human hepatocytes. *Metab. Syndr. Relat. Disord.,* **2011**, *9*(5), 397-401.

[4] Morgan, A.; Mooney, K.; Mc Auley, M. Obesity and the dysregulation of fatty acid metabolism: implications for healthy aging. *Exp. Rev. Endocrinol. Metabol.,* **2016**, *11*(6), 501-510.

[5] Eder, K. Gas chromatographic analysis of fatty acid methyl esters. *J. Chromatogr. B. Biomed. Appl.,* **1995**, *671*(1-2), 113-131.

[6] Chen, S.-H.; Chuang, Y.-J. Analysis of fatty acids by column liquid chromatography. *Anal. Chim. Acta.,* **2002**, *465*(1-2), 145-155.

[7] Zaikin, V.G.; Halket, J.M. Derivatization in mass spectrometry--8. Soft ionization mass spectrometry of small molecules. *Eur. J. Mass Spectrom. (Chichester),* **2006**, *12*(2), 79-115.

[8] Qi, B.-L.; Liu, P.; Wang, Q.-Y.; Cai, W.-J.; Yuan, B.-F.; Feng, Y.-Q. Derivatization for liquid chromatography-mass spectrometry. *TrAC Trend. Anal. Chem.,* **2014,** *59*, 121-132.

[9] Brooks, C.J.W.; Edmonds, C.G.; Gaskell, S.J.; Smith, A.G. Derivatives suitable for GC-MS. *Chem. Phys. Lipid.,* **1978**, *21*(4), 403-416.

[10] Amos, W.H.; Neal, R.A. Gas chromatography-mass spectrometry of the trimethylsilyl derivatives of various thiamine metabolites. *Anal. Biochem.,* **1970**, *36*(2), 332-337.

[11] Niessen, W.M. Advances in instrumentation in liquid chromatography-mass spectrometry and related liquid-introduction techniques. *J. Chromatogr. A.,* **1998**, *794*(1-2), 407-435.

[12] Ho, C.S.; Lam, C.W.; Chan, M.H.; Cheung, R.C.; Law, L.K.; Lit, L.C.; Ng, K.F.; Suen, M.W.; Tai, H.L. Electrospray ionisation mass spectrometry: principles and clinical applications. *Clin. Biochem. Rev.,* **2003**, *24*(1), 3-12.

[13] Jemal, M.; Ouyang, Z.; Teitz, D.S. High performance liquid chromatography mobile phase composition optimization for the quantitative determination of a carboxylic acid compound in human plasma by negative ion electrospray high performance liquid chromatography tandem mass spectrometry. *Rapid Commun. Mass Spectr.,* **1998**, *12*(8), 429-434.

[14] Ko, B.J.; Brodbelt, J.S. Enhanced electron transfer dissociation of peptides modified at C-terminus with fixed charges. *J. Am. Soc. Mass Spectrom.,* **2012**, *23*(11), 1991-2000.

[15] Frey, B.L.; Krusemark, C.J.; Ledvina, A.R.; Coon, J.J.; Belshaw, P. J.; Smith, L.M. Ion-ion reactions with fixed-charge modified proteins to produce ions in a single, very high charge state. *Int. J. Mass Spectrom.,* **2008**, *276*(2-3), 136-143.

[16] Chambers, E.; Wagrowski-Diehl, D.M.; Lu, Z.; Mazzeo, J.R. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J. Chromatogr. B.,* **2007**, *852*(1–2), 22-34.

[17] Neises, B.; Steglich, W. Simple method for the esterification of carboxylic acids. *Angewandte Chem. Int. Ed. Engl.,* **1978**, *17*(7), 522-524.

[18] Yang, W.C.; Adamec, J.; Regnier, F.E. Enhancement of the LC/MS analysis of fatty acids through derivatization and stable isotope coding. *Anal. Chem.,* **2007**, *79*(14), 5150-5157.

[19] Pluskal, T.; Castillo, S.; Villar-Briones, A.; Oresic, M. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics,* **2010**, *11*, 395.

[20] Yeung, K.Y.; Ruzzo, W.L. Principal component analysis for clustering gene expression data. *Bioinformatics,* **2001**, *17*(9), 763-774.

[21] Bylesjö, M.; Rantalainen, M.; Cloarec, O.; Nicholson, J.K.; Holmes, E.; Trygg, J. OPLS discriminant analysis: Combining the strengths of PLS-DA and SIMCA classification. *J. Chemometr.,* **2006**, *20*(8-10), 341-351.

[22] Ismail, R.; Lee, H.Y.; Mahyudin, N.A.; Abu Bakar, F. Linearity study on detection and quantification limits for the determination of avermectins using linear regression. *J. Food Drug Anal.,* **2014**, *22*(4), 407-412.

[23] Perez-Enciso, M.; Tenenhaus, M. Prediction of clinical outcome with microarray data: A partial least squares discriminant analysis (PLS-DA) approach. *Hum. Genet.,* **2003**, *112*(5-6), 581-92.

[24] Sem, D.S. *Spectral Techniques in Proteomics*. CRC Press: Florida **2007**.