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ORIGINAL ARTICLE

Discrimination between vegetable oil and animal fat by a metabolomics approach using gas chromatography–mass spectrometry combined with chemometrics

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Abstract Adulteration of olive oil with the other cheap oils and fats plays an important role in economics and has nutritional benefits. In this work, metabolite profiling was performed using gas chromatography–mass spectrometry to identify and quantify animal fat (lard) adulteration in vegetable oil (olive oil). Principal component analysis could correctly identify and clustering olive oil, sunflower oil, sesame oil, lard, and adulterated samples through the changes in their fatty acid methyl esters (FAMEs) profile. A targeted metabolomics method was then optimized and validated through construction of calibration curves of known FAMSs in olive oil and lard. The method was presented high linearity ($R^2 > 0.96$) and good intra and inter day accuracy and precision (79–101 and 86–102% and 2–7 and 3–7, respectively) for determination of FAMEs. Afterwards the absolute concentration and relative percentage of FAMEs were successfully determined in 12 commercial olive oils and 3 lards samples. Methyl myristate, methyl palmitate, methyl oleate, and methyl stearate were selected as discriminant markers to identify and quantify lard adulteration even at a low level of lard (5%w/w), with errors less than 2% in the comparison of the absolute or relative concentrations of FAMEs using several statistical methods. The proposed methodology allowed us to quantify the FAMEs simultaneously and also could predict small amount of lard in the adulterated olive oil samples.

Keywords Olive oil · Lard · Fatty acid methyl ester · Adulteration · Principal component analysis · Gas chromatography–mass spectrometry

Introduction

Food is a vital part of human life, and given its importance, any activity that compromises food safety and quality can cause health and economic problems in the society. Given that food is a highly complex mixture, it is important to apply reliable and powerful methods for the assessment of food quality and authenticity. Foodomics has been defined as a discipline that studies "the food and nutrition domains through the application of omics technologies" (Cifuentes 2009; García-Cañas et al. 2012; Del Castillo et al. 2013), such as metabolomics. In metabolomics, several metabolites (low-molecular-weight compounds < 1 kDa) are qualitatively and quantitatively investigated (Khakimov et al. 2016). Given that the analysis of metabolites provides a large set of raw data, it is essential to utilize chemometrics for data processing (Maggio et al. 2010) and extract the most relevant information. The most commonly used methods in chemometrics have been divided into supervised and unsupervised methods. Principal

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components analysis (PCA) is one of the unsupervised methods that has been used to distinguish the most relevant linear combination of variables and used them to demonstrate the relationships between the samples (Bro and Smilde 2014). After the reduction of variables dimension, some clustering analyses such as PCA score plot can be utilized for samples clustering (Anderson et al. 2017). Currently, metabolite profiling with chemometrics is commonly performed to investigate food authenticity, particularly in the area of adulteration (Nunes 2014).

The most common type of food fraud is replacement, which happens when some adulterants have been added to foods and food ingredients (Moore et al. 2012). Fats and oils are some of the food ingredients that undergo adulteration more than other foods (Moore et al. 2012). Olive oils have the highest percentage of adulteration compared with other kinds of fats and oils; thus, olive oils are a high-value product (Valli et al. 2016). Moreover, different kinds of olive oils do not have the same quality and price; for instance, extra virgin olive oil (EVOO), which has the highest percentage of adulteration, is the most expensive olive oil compared with other low-price or low-quality vegetable oils (Delfino et al. 2019). Animal fats, such as lard, are the most common adulterants of olive oil. Lard is pig fat, which is widely used as an adulterant in edible oils because of its low cost. Lard contains high levels of cholesterol and saturated fatty acids (SFA), which are known to be detrimental to human health (Man et al. 2005). Moreover, lard has been prohibited in several religions, such as Islam, Judaism, and Hinduism (Che Man et al. 2005). Thus, lard consumption is a matter of concern for religious groups and other consumers, necessitating the detection of even small amounts of lard in various food products through robust approaches and powerful analytical techniques (Fadzillah et al. 2017; Upadhyay et al. 2018). For instance, lard in sunflower oil (SFO) was detected by investigating the thermal profile of blends and pure SFO using differential scanning calorimetry (Marikkar et al. 2012). Moreover, the authentication of chicken fat and virgin coconut oil that underwent lard adulteration was performed on the basis of their aroma compounds (Mansor et al. 2011; Nurjuliana et al. 2011), and triacylglycerols (TAGs) were analyzed in adulterated butter through high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (Fadzillah et al. 2017).

The International Olive Council (IOC) has provisionally adopted a method based on the determination of the theoretical and experimental values of TAGs as a reliable technique for detecting extraneous oil adulterants in olive oils (COI/T.20/Doc. No. 25; International Olive Council (IOC): Madrid, Spain, 2006). However, according to the IOC, the recommended method has some limitations; for instance, the identification of oil adulterants is impossible. Moreover, this method is unsuitable for the detection of low levels of adulteration (i.e., 5% and lower). Hence, new analytical methods in combination with chemometrics are proposed and developed to enhance the usual methods for the detection of olive oil adulteration. For example, EVOO adulteration with corn, soybean, and sunflower oils was determined by synchronous front-face fluorescence and visible spectroscopies (Tan et al. 2018). The presence of lard in combination with EVOO in cream cosmetic was also investigated using Fourier transform infrared spectroscopy and chemometrics (Rohman et al. 2014). These spectroscopic methods are rapid, nondestructive, and easy to use in sample preparation. However, these methods only provide information about fingerprinting characteristics.

Chromatography-based techniques are some of the most widely used and most reliable methods for quality control (QC) of olive oils. These methods exhibit high sensitivity and accuracy and can be used to separate different components from complex mixture. Thus, these methods obtain signals with a considerable amount of analytical information that can be used to authenticate fats and oils (Esteki et al. 2018). These methods analyze a special fraction of olive oils (Jabeur et al. 2016), either saponifiable or unsaponifiable matters, such as fatty acids (FA) (Kenar et al. 2019), TAGs (Wang et al. 2019), and sterols (Günç Ergönül and Aksoylu Özbek 2018). Moreover, chromatography in combination with mass spectrometry is actually usable in metabolomics profiling and targeted analysis.

This study aimed to investigate the changes in the fatty acid methyl esters (FAMEs) profile and detect markers to quantify lard adulteration in olive oil using gas chromatography–mass spectrometry (GC–MS). To the best of our knowledge, this type of quantification has not yet been conducted. Moreover, PCA has been utilized to identify olive oils, SFO, sesame oil (SeO), lards, and adulterated samples. The developed model had been able to detect and quantify the amount of lard in unknown samples. Finally, lard adulteration, even at a low percentage, was quantitatively determined using specific discriminant markers, such as methyl myristate and methyl stearate.

Materials and methods

Standards and chemicals

The standards of olive oil (SOO), lard (SL), and seven component FAME mixtures (C_{14}, C_{16}, $C_{18:2(9Z,12Z)}$, $C_{18:1(9Z)}$, $C_{18:1(9E)}$, C_{18}, and C_{20}) (SM-FAME), were purchased from Supelco (Bellefonte, PA, USA). The standard mixture of HPLC-grade *n*-alkanes (C_{10}–C_{24}), potassium

hydroxide, anhydrous magnesium sulfate, methanol, and hexane was obtained from Merck (Darmstadt, Germany).

Vegetable oil samples

The vegetable oil samples, namely, EVOO (10 samples; EVOO1 to EVOO10), virgin olive oil (1 sample) (VOO), olive pomace oil (1 sample) (OPO), SFO (1 sample), and SeO (1 sample), were purchased from local markets. The detailed information about these vegetable oils is provided in Supplementary Table 1. The samples were stored in dark bottles at room temperature for further use.

Lard samples

Lard oils (L1 and L2) were purchased from England and France, and some pig fats were bought from Armenia. Lard (L3) was extracted from pig fat according to the method previously described by Marikkar et al. (2001). Lard samples (Supplementary Table 1) were stored at 4 °C and melted in an oven before use.

Sample preparation steps

FAs have to be converted into FAMEs for GC–MS analysis. This procedure is called transmethylation, which could be applied in three standard methods (ISO 12966–2:2012). Alkaline transmethylation by methanolic potassium hydroxide was selected in this study. The preparation of olive oils, lards, and standard adulterated samples was performed according to the International Olive Oil Council (IOOC) method with some modifications (COI/T, 20/Doc. No. 24. In: Int. Olive Oil Council (IOC) 2001). This specific extraction procedure limited the targeted analysis to specific compounds (i.e., FAMEs). Briefly, 50 mg of fats and oils was weighed in a glass vial with a screw cap. After the addition of 2 mL of hexane, the mixture was vortexed for a few seconds. Then, 200 µL of the 2.0 M solution of methanolic potassium hydroxide was added to the mixture and vigorously vortexed for 1 min. The prepared sample was kept in a dark place and given sufficient time to separate into two phases. After the upper hexane layer became transparent, 1 µL was injected into the GC–MS column.

Fatty acid methyl ester analysis

FAMEs were separated and quantified using an Agilent model 6890 N GC instrument (Santa Clara, CA, USA) equipped with a nonpolar capillary column (length = 30 m, i.d. = 0.25 mm, and film thickness = 0.25 µm) of HP-5MS (Agilent, Santa Clara, CA, USA). Helium was used as carrier gas with a flow rate of 1 mL min^{-1}. FAMEs in hexane (1 µL) were injected in split mode, 30:1 (v/v), when the injection temperature was 270 °C. The initial oven temperature was 40 °C with a 1 min holding time, increased by 30 °C min^{-1} to 150 °C with a 3 min holding time, increased by 2 °C min^{-1} to 190 °C with a 5 min holding time, and increased by 50 °C min^{-1} to 280 °C with a 10 min holding time. FAMEs were identified using a 5973 Agilent mass spectrometer with quadruple analyzer and electron energy of 70 eV. Mass spectra were recorded in the range of 30–800 m/z with a scanning frequency of 1.95 scans s^{-1}. The ion source temperature was set at 230 °C. The identification of target metabolites (i.e., FAMEs) was performed using the WILEY 2007 library. Moreover, the comparison of the FAME retention times in both real samples and standards and the computation of the Kovats retention indices (KI) of *n*-alkanes ($C_{10} - C_{24}$) were utilized to confirm the identified FAMEs.

Method validation

All experiments were performed in three replicates. During data processing, the weight correction and detector response factors affected the peak areas. Afterward, method validation was conducted using the calibration method. For this purpose, calibration solutions with wide concentration ranges were prepared. Given that FAMEs have different concentration levels in olive oil and lard, the investigation of linearity was performed in different concentration levels. Thus, various linear ranges were obtained by the linear least square regression method. The limits of detection (LOD) and quantification (LOQ) were calculated using the concentration of each FAME at threefold and tenfold of the standard deviation of six injections of the blank sample (hexane) to the slope of the calibration curve, respectively. Accuracy and precision were determined by calculating the recoveries and relative standard deviations (RSD; %) of FAMEs in a standard solution of SM-FAME at 7000 mg L^{-1} in intraday (n = 3) and interday (n = 3) analyses.

Real sample analysis

Real samples were identified using PCA. The retention times and peak intensities every 0.008 min were recorded and imported into MATLAB (version R.2013a). Prior to PCA, the metabolite data were pre-processed. Baseline correction and peak alignment of the retention times were performed using the airPLS 2.0 MATLAB software and correlation optimized warping algorithm, respectively.

The quantification of FAMEs in real samples was performed using the constructed calibration curve of the standard FAME mixture of eight FAMEs and the internal normalization technique, which calculated the percentage

of all FAMEs as the ratio of the peak area of each FAME to the total peak area. Univariate statistics was applied to compare FAMEs in olive oil and lard to identify some markers of lard adulteration using both the Student's *t*-test and box plots. To qualify and quantify lard adulteration in olive oil, binary admixtures of lard and olive oils in five different levels, i.e., 5%, 10%, 25%, 50%, and 75% (w/w) were prepared. Adulterated samples were clustered using PCA score plots, and distance differences between pure olive oil, lard, and adulterated samples were calculated. Three other blends (i.e., 12%, 35%, and 60% w/w of lard in olive oil), which were different from the calibration samples, were also made as the QC samples. The percentages of selected markers were obtained from the QC samples to construct the calibration curve. Moreover, recoveries were determined by dividing the experimental value of selected markers of the QC samples by the actual value to evaluate the proposed method for the detection of lard in olive oil.

Results and discussion

Fatty acid methyl ester analysis

To analyze the differences between the metabolite profiles (i.e., FAMEs) of olive oil and lard to select the appropriate markers, the levels of various parameters in sample preparation and GC–MS analysis were optimized to achieve the best total ion current (TIC) with a large number of metabolites extracted at a level detectable by the mass spectrometer ion detector. These parameters were methylation method, volume of the extraction solvent, weight of the sample, split ratio, and temperature program (Data not shown). As a typical example, the optimized TICs of the SOO and one real sample of olive oil (EVOO1) and that of the SL and one real sample of lard (L1) are shown in Supplementary Figures 1 and 2, respectively, under optimum conditions (see Sects. 2.4 and 2.5). Although long polar columns are typically used in FAMEs analysis (Schober et al. 2017), their thermal stability is low. Thus, a nonpolar column with high thermal stability was utilized in this study. This column is compatible with the FAMEs of lard and EVOO, which have high molecular weights. The selection of the nonpolar column significantly reduced the runtime of the analysis in comparison with similar works that used polar columns (Park et al. 2014). The identification of metabolites was performed using the WILEY 2007 library. The structure was selected from the first three suggestions of the library, with a match percentage of more than 95% for most of them. The match percentages between obtained and suggested structures are shown in Supplementary Table 2. The obtained results indicated that the FAMEs retention times in the standards were highly consistent with those in the real samples. Moreover, the comparison of the KI with the reference values confirmed the identified FAMEs.

Validation of the proposed method for fatty acid methyl ester determination

To verify the practicality of the method, serial calibration solutions of seven component FAME mixtures (SM-FAME) were analyzed by GC–MS for validation. The investigation of peak areas in SM-FAME and real samples showed that the concentration of some FAMEs in oils were low and that of other FAMEs were high. Thus, the calibration curves of various concentration ranges were constructed using the linear least square regression method. The linear range for each FAME in SM-FAME is shown in Supplementary Table 3. Moreover, the correlation coefficients (R^2) of FAMEs in SM-FAME were higher than 0.9691. To assess the validity of the quantification of FAMEs, the LOD and LOQ were determined. The results are shown in Supplementary Table 3. The LODs (1–10 mg L^{-1}) and LOQs (3–34 mg L^{-1}) obtained in this study were lower than the LODs (9–18 mg L^{-1}) and LOQs (27–55 mg L^{-1}) determined in the work of Truzzi et al. (Truzzi et al. 2017). Moreover, the linear range obtained in this study was wider (3–9400 mg L^{-1}) than the linear range (24–324 mg L^{-1}) determined in the work of Truzzi et al. (Truzzi et al. 2017). To determine the intraday and interday precisions and accuracies, a standard solution of SM-FAME at 7000 mg L^{-1} was analyzed three times within one day and three different days. The experimental values of FAMEs were calculated using calibration models. Then, accuracy and precision were determined by calculating the recoveries using the percentages of the ratio of the experimental value to the real value and their RSDs, respectively. As shown in Supplementary Table 3, the obtained recoveries were 79–102 and the intraday and interday precisions (RSDs) were less than 7% for the investigated FAMEs.

Metabolite profiling of olive oil and lard samples

To compare the FAME profiles and determine the probability of the purity or authenticity of olive oils, some parts of the average TIC chromatograms for 13 pure olive oils (12 real samples and 1 standard) and 4 lards (3 real samples and 1 standard) are shown in Fig. 1. Notably, the FAME profile varied in pure olive oil and lard both in terms of numbers and abundances. Thus, lard adulteration could change the olive oil FAME profile in different ways. To analyze the discriminant markers, first PCA as a multivariate analysis technique was used to data pre-processing

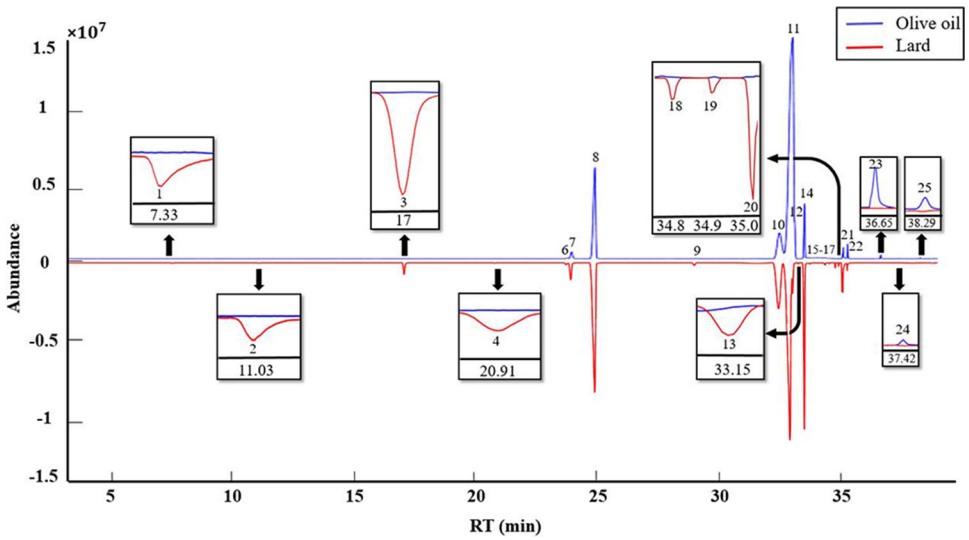

Fig. 1 The comparison of the average total TICs of olive oils and lards. The focused parts are FAMEs which are either in olive oil or lard: (1) methyl decanoate, (2) methyl laurate, (3) methyl myristate, (4) methyl pentadecanoate, (5) methyl (7Z, 10Z) hexadecadienoate, (6) methyl (7Z) hexadecanoate, (7) methyl palmitoleate, (8) methyl palmitate, (9) methyl margarat, (10) methyl linoleate, (11) Methyl oleate, (12) methyl elaidate, (13) methyl (11Z) octadecanoate, (14) methyl stearate, (15) methyl (7Z, 10Z) octadecadienoate, (16) methyl (10Z) nonadecanoate, (17) methyl nonadecanoate, (18) methyl arachidonate, (19) methyl (7Z, 10Z, 13Z) eicosatrienoate, (20) methyl (11Z, 14Z) eicosadienoate, (21) methyl (11Z) eicosanoate, (22) methyl arachidate, (23) methyl behenate, (24) methyl tricosanoate and (25) methyl lignocerate

by variable reduction. After data processing, the score plot was drawn for olive oils and lards with their replicates. In Fig. 2, the score plot showed a clear trend of group clustering between olive oils and lards. Moreover, two other vegetable oils (i.e., SeO and SFO) were utilized to confirm the model. Data differentiation was explained by approximately 92% of the variance in PC1. Thus, the detection of FAMEs, which are regarded as discriminant markers, will be useful for the determination of the type of adulterated oil and its quantification through the targeted metabolomics approach.

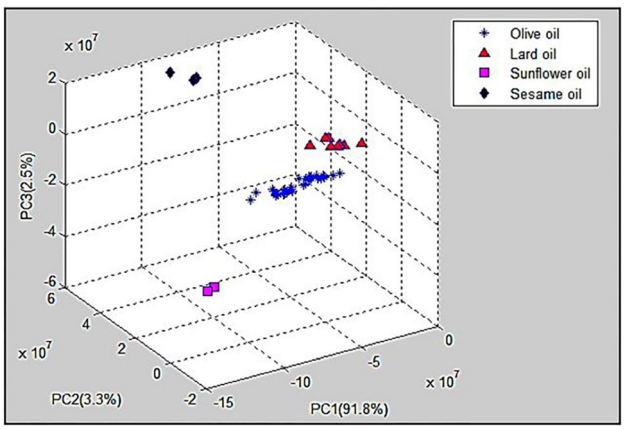

Fig. 2 The PCA 3-D scores plot of commercial three vegetable oils and lard. Olive oil (OO), sunflower oil (SFO), sesame oil (SeO) and lard (L) were clearly separated

Selection of discriminant markers

The selection of discriminant markers was conducted using two quantification methods, namely, calibration and normalization. The optimized GC–MS method was applied to all real samples of olive oils and lards to quantitatively determine the amount of eight FAMEs in 50 mg of the samples using the calibration method. The results are listed in Table 1. Moreover, Table 2 shows the normalized values of various FAME components of olive oil and lard using the normalization method. These data were averaged from three replicates of 13 olive oils (12 real samples and 1 standard) and 4 lards (3 real samples and 1 standard) and reported using their confidence intervals at the 95% confidence level. The confidence interval is a range of values, which likely includes the true value. On the basis of the obtained results, some FAMEs, such as methyl behenate, were only present in olive oil and other FAMEs, such as methyl myristate, were only present in lard. As shown in Tables 1 and 2, the four most abundant FAMEs in both olive oil and lard were methyl oleate, methyl palmitate, methyl linoleate, and methyl stearate.

Given that most FAMEs were common in olive oil and lard with different concentration levels, the selection of discriminant markers that exhibit significant differences is necessary. In this study, the *t*-test was conducted to compare the average levels of FAMEs. The obtained *p* values are presented in Tables 1 and 2. *p* value is a statistical concept which is represented the level of significance by

Table 1 Comparison the content of some FAMEs between olive oil and lard to determine discriminant markers

Fame	Olive oil Mean[a] ± CI[b] mg L^{-1} (mg g^{-1})	Lard Mean ± CI mg L^{-1} (mg g^{-1})	*p* value[c]
Methyl myristate	–	138 ± 12 (5 ± < 1)	–
Methyl palmitate	1200 ± 100 (47 ± 4)	4000 ± 1000 (150 ± 50)	< 0.05
Methyl linoleate	1100 ± 100 (44 ± 4)	1800 ± 600 (70 ± 20)	< 0.05
Methyl oleate	10,000 ± 1000 (390 ± 50)	2800 ± 500 (110 ± 20)	< 0.05
Methyl elaidate	260 ± 20 (10 ± 1)	280 ± 60 (11 ± 2)	0.48
Methyl stearate	340 ± 30 (14 ± 1)	1700 ± 400 (70 ± 10)	< 0.05
Methyl arachidate	73 ± 7 (3 ± < 1)	100 ± 107 (4 ± 4)	0.59
Methyl behenate	90 ± 20 (4 ± 1)	–	–

[a]Means were obtained by the average of 3 replicates of 13 olive oils (12 real sample + 1 standard) and 4 lards (3 real sample + 1 standard) concentrations

[b]*CI* confidence interval in 95%: $\frac{tS}{\sqrt{N}}$ (t for df = 38 is 2.024 and for df = 11 is 2.201, *S* standard deviation, *N* the number of samples with 3 replicates, 39 and 12 for olive oil and lard, respectively)

[c]A p value < 0.05 was considered statistically significant

Table 2 Comparison the relative percentages of FAMEs between olive oil and lard to determine discriminant markers

Fame	Olive oil Mean[a] ± CI[b] (%)	Lard Mean ± CI (%)	*p* value[c]
Methyl decanoate	–	0.14 ± 0.09	–
Methyl laurate	–	0.06 ± < 0.01	–
Methyl myristate	–	0.17 ± 0.05	–
Methyl pentadecanoate	–	0.07 ± 0.01	–
Methyl (7Z, 10Z) hexadecadienoate	–	0.03 ± < 0.01	–
Methyl (7Z) hexadecanoate	0.10 ± 0.01	0.29 ± 0.04	< 0.05
Methyl palmitoleate	1.01 ± 0.09	3.09 ± 1.11	< 0.05
Methyl palmitate	14.45 ± 0.46	23.84 ± 1.37	< 0.05
Methyl margarate	0.07 ± 0.02	0.31 ± 0.08	< 0.05
Methyl linoleate	10.85 ± 1.08	17.84 ± 4.12	< 0.05
Methyl oleate	63.01 ± 1.32	33.68 ± 1.49	< 0.05
Methyl elaidate	3.01 ± 0.11	3.08 ± 0.37	0.68
Methyl (11Z) octadecanoate	–	0.54 ± 0.01	–
Methyl stearate	3.96 ± 0.18	12.02 ± 2.44	< 0.05
Methyl (7Z, 10Z) octadecadienoate	0.06 ± 0.05	0.07 ± 0.04	0.88
Methyl (10Z) nonadecanoate	0.05 ± 0.01	0.11 ± 0.04	< 0.05
Methyl nonadecanoate	0.08 ± 0.04	0.13 ± 0.05	0.16
Methyl arachidonate	–	0.23 ± 0.04	–
Methyl (7Z, 10Z, 13Z) eicosatrienoate	–	0.18 ± 0.05	–
Methyl (11Z, 14Z) eicosadienoate	–	0.82 ± 0.17	–
Methyl (11Z) eicosanoate	0.72 ± 0.09	1.16 ± 0.40	< .05
Methyl arachidate	0.90 ± 0.05	1.00 ± 0.78	0.79
Methyl behenate	0.96 ± 0.09	–	–
Methyl tricosanoate	0.34 ± 0.03	–	–
Methyl lignocerate	0.42 ± 0.06	–	–

[a]Means were obtained by the averaging from the relative peak areas of 3 replicates of 13 olive oils (12 real sample + 1 standard) and 4 lards (3 real sample + 1 standard)

[b]*CI* confidence interval in 95%: $\frac{tS}{\sqrt{N}}$ (t for df = 38 is 2.024 and for df = 11 is 2.201, *S* standard deviation, *N* the number of samples with 3 replicates, 39 and 12 for olive oil and lard, respectively)

[c]A p value < 0.05 was considered statistically significant

acceptance or rejection of the null hypothesis and always compared with the cut-off value (α). If the *p* value is lower than α-value, the null hypothesis is rejected and results have significant difference (Greenland et al. 2016). In this study, α-value is 0.05. As shown in Tables 1 and 2, some FAMEs, such as methyl elaidate, did not exhibit significant differences in concentration levels between olive oil and lard. By contrast, other FAMEs exhibited significant differences in concentration levels between olive oil and lard. Notably, good consistency between the results obtained from the calibration and normalization methods, particularly for methyl oleate with the concentrations of 387 ± 47 and 113 ± 20 mg g^{-1} or normalized values of 63.01 ± 1.32% and 33.68 ± 1.49% in olive oil and lard, respectively. In addition, box plots were constructed using either absolute or relative concentration to denote the applicability of common markers in the differentiation of olive oil and lard. As shown in Fig. 3, the markers methyl palmitate, methyl oleate, and methyl stearate outperformed methyl linoleate in discriminating olive oil and lard in both the calibration and normalization methods. Methyl myristate was also selected from specific markers of lard to quantify lard adulteration as a discriminant marker.

Detection of lard adulteration in olive oil using the proposed markers

To differentiate and classify adulterated samples from pure olive oil, some samples of olive oil with different percentages of lard were prepared and investigated by analyzing the changes in their FAME profile and determining the percentage of the selected markers. The multivariate data matrix of pure olive oil, lard, and adulterated samples was subjected to PCA. Adulterated samples, even at the lowest percentage of adulteration (5% w/w of lard), created new classes, which were distinguished from pure classes (Fig. 4). Moreover, the average distance differences ($d_2 - d_1$) between adulterated samples and pure olive oil (d_2) and between adulterated samples and pure lard (d_1) were plotted. As shown in Fig. 4, the FAME profile of adulterated samples with low percentages of adulteration had low distance difference with pure olive oil ($d_2 < d_1$). Thus, the average distance differences had negative values. As adulteration increased, the distance also increased ($d_2 > d_1$). Thus, the average distance differences had shifted to positive values and the FAME profile of adulterated samples had changed to pure lard gradually.

Finally, the change trends of the percentage of each selected marker were analyzed. As shown in Fig. 5, when lard was added and increased in percentage, there was a gradual increased and decreased in the amount of specific markers in adulterated samples: methyl myristate (specific FAME of lard) increased and methyl palmitate and methyl stearate (as saturated FAMEs (SFA)) also increased, but methyl oleate (as unsaturated FAMEs (UFA)) decreased. Given that lard is animal fat and saturated FAMEs are the dominant FAMEs in animal fat, the ratio of SFA to UFA increased by increasing the adulteration in olive oil, as shown in Fig. 5. Therefore, it can be concluded that the discriminant markers shown in Fig. 5 can be used to separate the pure olive oils, lards, and adulterated samples shown in Fig. 4 through PCA. The calibration curves were constructed to quantify lard adulteration on the basis of the discriminant markers. Their equations and correlation coefficients ($R^2 > 0.9808$) are shown in Table 3. A series of QC samples with 12%, 35%, and 60% w/w of lard in olive oil was prepared and used to evaluate the predictive capability of the developed model. As shown in Table 3, the obtained recoveries indicate that the predicted values were consistent with the actual values. For instance, methyl stearate could predict a small amount of lard with a relative error less than 2%.

Conclusion

Metabolomics approach using GC–MS results in combination with principle component analysis provided a simple and accurate way to differentiate among edible oils through comparison of their FAMEs profiles. FAMEs as metabolites were quantitatively determined both in absolute and relative concentrations with the aid of the calibration and normalization methods, respectively. The figures of merit and relative errors obtained from the selected markers indicated that the proposed method has wide linear range and high precision and accuracy with low LOD and LOQ. Since lard may be a potential oil adulterant in olive oil, in this study application of the proposed method was highlighted to analyze the presence of lard in olive oil. Methyl myristate, methyl palmitate, methyl oleate, and methyl stearate were selected and their content were assayed in binary admixtures of lard in olive oil in various percentage concentrations ranging from 5–75%.

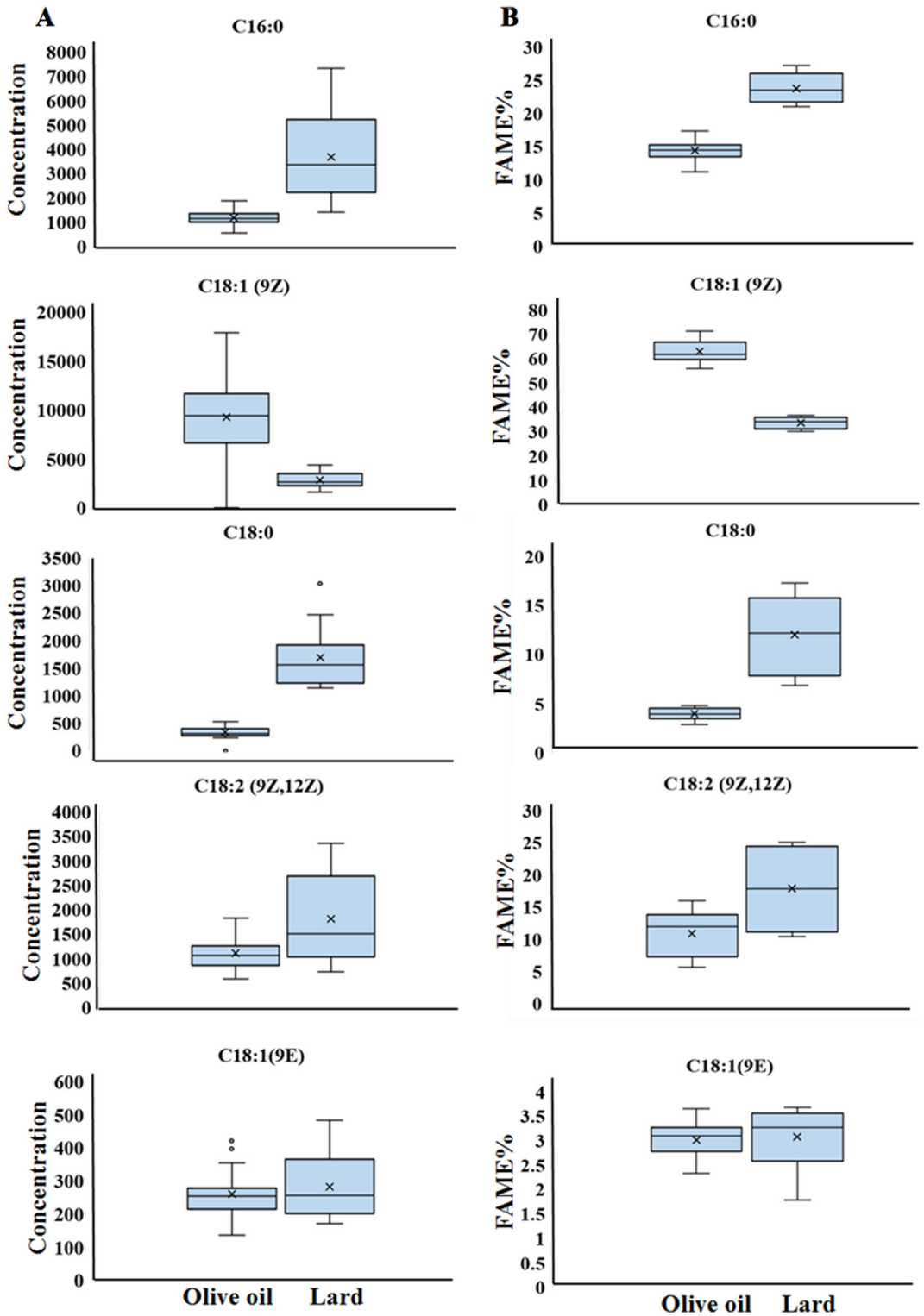

Fig. 3 Box plots of **a** concentrations and **b** relative percentages of some common FAMEs to detect discriminant markers (methyl palmitate (C16:0), methyl oleate (18:1(9Z)), methyl stearate (C18:0) and methyl linoleate (C18:2 (9Z, 12Z)) had significant difference both in **a** and **b**. However, methyl elaidate (C18:1 (9E)) had no significant difference)

Euclidean distance demonstrated that the process of moving from pure olive oil to pure lard as adulteration increased. It seems that, the proposed method can be used to investigate the authenticity of various animal fats and vegetable oils. Discriminant markers can also be used in the targeted metabolomics approach to examine the

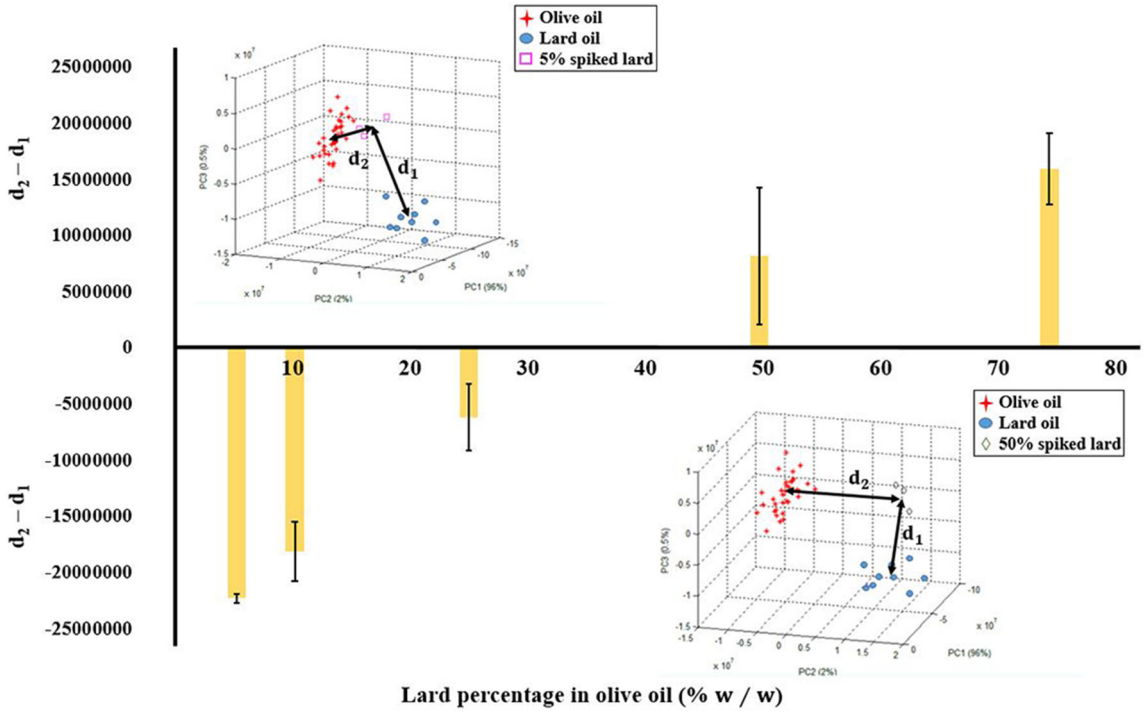

Fig. 4 PCA score plot and Euclidean distance plot for adulteration samples. (($d_2 - d_1$) was difference distances between adulterated samples-pure olive oil (d_2) and adulterated samples- pure lard (d_1))

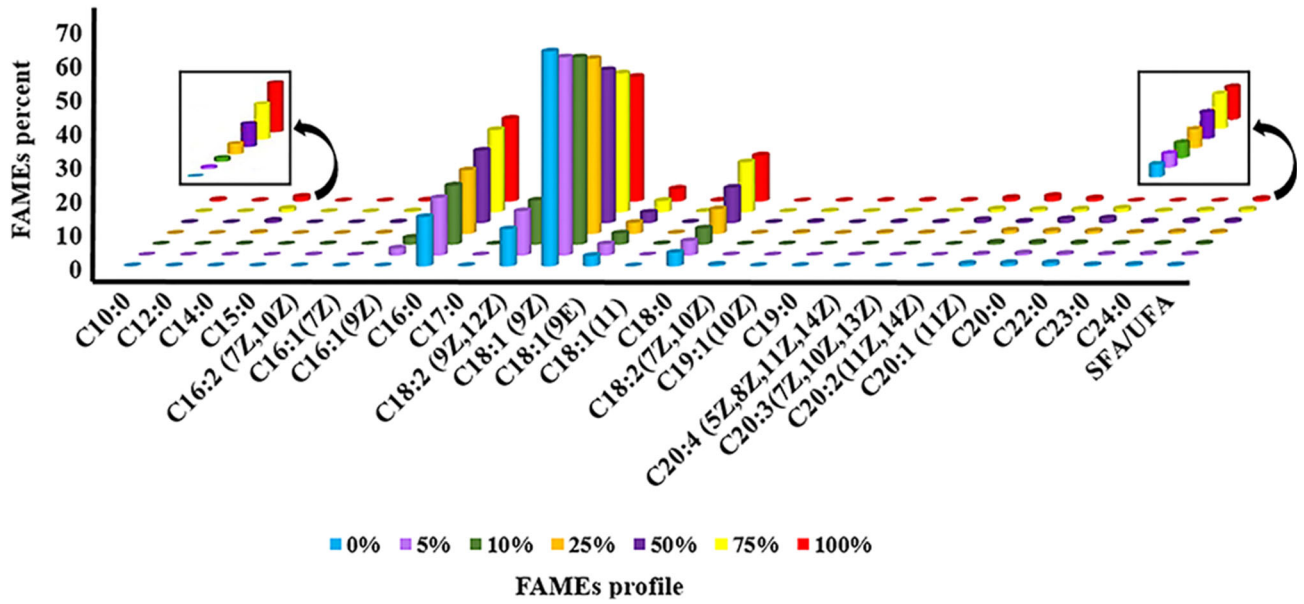

Fig. 5 The changes in FAME composition of olive oil adulterated with different levels of lard (Color figure available online)

Table 3 Correlation between main and predicted data based on discriminant markers to predict different levels of lard adulteration

Selected markers	Linear equation[a]	R^2	Recovery%[b]		
			12%	35%	60%
Methyl myristate[c]	y = 0.0132 (± 0.000247) x − 0.0227 (± 0.0104)	0.9990	105 ± 17	107 ± 3	106 ± 3
Methyl palmitate	y = 0.103 (± 0.00362) x + 16.193 (± 0.152)	0.9963	123 ± 35	99 ± 8	104 ± 5
Methyl oleate	y = -0.254 (± 0.0208) x + 58.769 (± 0.876)	0.9804	115 ± 9	120 ± 9	109 ± 8
Methyl stearate	y = 0.147 (± 0.00507) x + 3.331 (± 0.214)	0.9965	101 ± 4	102 ± 2	99 ± 4

[a]These correlations between main and predicted data were constructed in 5%, 10%, 25%, 50% and 75% (w/w) of lard in olive oil

[b]Recovery was determined by dividing of experimental value per actual one for quality control samples in 12%, 35% and 60% (w/w) of lard in olive oil

[c]Methyl myristate was a specific FAME of lard. The others were common FAMEs in both olive oil and lard

adulteration of other vegetable oils with lard in different food products and cosmetics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval his article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Publication has been approved by all individual participants.

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