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Applicability of MTBE-based lipid extraction methods assisted by microwave in food analysis. Statistical comparison and greenness evaluation with Soxhlet and Matyash

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ABSTRACT

This research investigates the applicability of a microwave-assisted extraction (MAE) methodology using methyl-tert-butyl ether (MTBE) as a one-step organic solvent extraction or in mixture with methanol and water compared to conventional extractions for lipid analysis in food samples. MAE methods were compared in terms of extraction yields and fatty acid (FA) composition to Soxhlet (using n-hexane, MTBE, and diethyl ether) and Matyash (MTBE/CH_3OH/H_2O, 10/3/2.5, v/v/v), considered gold-standard methodologies for lipid extraction. FAs, derivatized into methyl esters (MEs), were analyzed through a gas chromatography-flame ionization detector. One-way ANOVA and post-hoc Tukey tests were used to identify statistical differences in yields and FAMEs composition, resulting in <10 % with a $p < 0.05$. Methods using MTBE/CH_3OH/H_2O showed higher yields but also higher FAMEs variability compared to one-step solvent extraction methods. The extraction techniques showed comparable performance in terms of FAMEs composition. The greenness of the extractions were also evaluated using the AGREEprep metric.

1. Introduction

Lipid extraction methods using chloroform as main extraction solvent [1,2], developed in the 1950s for the extraction of lipids from various biological matrices, have been used tens of thousands of times and are considered, along with Soxhlet extraction, "gold standard methods" for the extraction of the lipid fraction in matrices of different origins (food, microbiological, petrochemical, etc.). Despite miniaturized alternatives have been proposed over the years, the proven toxicity of chloroform does not recommend its use [3,4]. The use of methyl-tert-butyl ether (MTBE) as solvent in lipid extraction methods was optimized in 2008 by Matyash et al. to improve the high-throughput capabilities of lipidomics investigations by reducing the toxicity of extraction solvents [3]. The method itself was inspired by the classical methods of Folch and Bligh & Dyer using similar principles [5] and involves the combination of MTBE/methanol/water solvents in a 10/3/2.5 (v/v/v) ratio. Different comparisons with Folch and Bligh & Dyer have been done over time, assessing the effectiveness of a comparable extraction [5–7]. In addition, due to the lower density of MTBE compared to the aqueous phase, the lipid-containing organic phase forms a higher layer during phase separation, simplifying its recovery compared to chloroform that instead lays on the bottom [3]. MTBE is less toxic and less harmful than most of the organic solvents used in traditional methods (e.g., chloroform), with a lower environmental impact, making it a greener alternative for lipid extraction. Since its first introduction, Matyash method has been extensively used in the extraction of lipids from biological and clinical samples [8,9], proving its efficiency and wide coverage of the different lipid classes, but its

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application in food and agricultural analysis is still rather limited to a few publications for qualitative investigations [10–12].

The aim of this study is to prove the applicability of the use of MTBE-based as extraction solvent in routine food analysis applications, such as the profiling of fatty acid methyl esters (FAMEs). To strengthen the applicability and the throughput, the extraction was also performed using microwave-assisted extraction (MAE). Indeed, MAE presents several benefits compared to traditional extraction methods, including higher extraction efficiency, significantly shorter extraction times, lower solvent consumption, and improved analyte recovery. Historically, microwaves have been employed in a range of extraction processes [13]. For instance, Dai et al. (2014) successfully used MAE to extract lipids from microalgae for biodiesel production [14]. In 2015, González-Arrojo et al. implemented a combined MAE and transmethylation procedure using hexane and methanolic hydrochloric acid to investigate fatty acids in dairy products [15], while Brunton et al. in the same year, adopted a comparable approach to assess fatty-acid composition in additional food samples such as chicken uncooked sausages, spaghetti bolognese and lasagne [16]. Other notable applications include the work of Fina et al., in 2022, who utilised microwave irradiation to isolate fatty acids from food matrices such as bacon chips and milk in order to characterise their fatty acid composition and product quality [17] Similarly, Ferrara et al. applied MAE to the extraction of fatty acids from a variety of food products, including cheese, infant formula, cordon bleu, pastry, and spreadable creams, and compared the resulting fatty acid profiles with those obtained using official reference methods [18]. Another example is the work of Campaniello et al., in which MAE was proposed as an alternative and environmentally friendly technique in lipidomics to study the lipid fingerprint of mozzarella samples [19]. Collectively, these studies underscore the versatility of MAE across heterogeneous matrices. As a case study, unroasted, unsalted pistachio nuts from Bronte (Sicily, Italy), recognized as a Protected Designation of Origin (PDO) product by the European Union since 2010, were analyzed [20–22]. In general, pistachios contain approximately 40 % fat (w/w), primarily composed of monounsaturated (MU), polyunsaturated (PU), and saturated fatty acids (SFAs), with oleic (O, C18:1n9) and linoleic (L, C18:2n6) acids comprising more than half of the total fat content. Research highlights pistachio consumption's potential cardiovascular benefits, with studies showing significant reductions in plasma total cholesterol levels among pistachio-supplemented groups compared to controls [23].

In this study, MTBE in combination with MAE was used either as a one-step organic solvent or mixed with methanol and water, with the same composition of traditional Matyash. The MAE methods were compared to Soxhlet using different solvents (*n*-hexane, MTBE, and diethyl ether) and traditional Matyash, both gold standards for lipid extraction [3,24]. The methods were evaluated in terms of total fat yields and FAs composition after their derivatization in methyl esters (MEs). FAMEs were analyzed using two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC-TOFMS) for a qualitative investigation and a GC system coupled with a flame ionization detector (FID) for quantification [25,26].

In order to assess the efficiency of the extraction methods and variability of FAME profile among the different lipid extractions, an one-way analysis of variance (ANOVA) and a *post-hoc* comparisons (Tukey's HSD) were used to test statistical differences in the yield of extracted lipids and FAME composition.

A greenness assessment using the AGREEPrep metric of the extraction methods applied were also performed [27].

2. Material and methods

2.1. Chemical and reagents

n-Hexane (99 %), methanol (≥99 %), methyl *tert*-butyl ether (≥99 %), potassium hydroxide, *n*-alkanes (from *n*-C7 to *n*-C30) and SUPELCO C37 mixture of fatty acids methyl ester (FAMEs) were obtained from Merck (Darmstadt, Germany) and diethyl ether (99.8 %) was obtained from Carlo Erba (Milan, Italy).

2.2. Lipid extraction

Lipid extraction from raw pistachios was performed using different methods, including Matyash method; Soxhlet method; and microwave-assisted extraction (MAE) as described in the following sub-sections. Each type of extraction was performed in triplicate. Before extractions, 100 g of pistachios were ground with a commercial coffee grinder to obtain a homogeneous powder [28].

2.2.1. Matyash lipid extraction

Differently from the original Matyash method [3], the total volume of sample/solvents was scaled-up three times, maintaining their ratio. In this way, the comparison with the other methods was more straightforward. Briefly, 600 mg of ground pistachio sample was mixed with 4.5 mL of methanol and 15 mL of MTBE in 20 mL vials. The vials were incubated on a shaker at room temperature for 60 min. To induce phase separation, 3.75 mL of water was added. The sample was then incubated at room temperature for 10 min and centrifuged at 6000 rpm for 20 min. The upper (organic) phase was collected, and the lower aqueous phase was subjected to a re-extraction step using 6 mL of a solvent mixture of MTBE/CH_3OH/H_2O (10/3/2.5, v/v/v). The combined organic extracts were dried using nitrogen (N_2) and the extracted lipids were weighed and then dissolved in MTBE for storage [3].

2.2.2. Soxhlet extractions

Lipid extraction was performed using a 3-position semi-automated solvent extractor (SER 148/3, VELP Scientifica, Usmate, MB, Italy) according to the Randall modification of the conventional Soxhlet extraction, with slight modifications in accordance with the official method for the determination of oil content in oilseeds [29], which uses *n*-hexane or petroleum ether as extraction solvent. Randall method is an improved version of traditional Soxhlet extraction that uses hot solvents directly in contact with the sample, ensuring a considerable reduction of the extraction time. Three different solvents with different polarity were used: MTBE, *n*-hexane (hex), and diethyl ether (Et_2O). According to the specifications of the apparatus, a total of 2 g of ground pistachio sample was weighed into a thimble, with each thimble placed into three corresponding beakers each containing 50 mL of an extraction solvent, hex, Et_2O, and MTBE, respectively. The extraction process consisted of three phases: immersion (30 min), washing (30 min), and recovery (10 min). The extracted lipids were dried, weighed and then dissolved in the same solvent used for extraction for storage before the analysis.

2.2.3. MAE methods

Three approaches using MTBE as the principal solvent were applied using MAE. The applied microwave (MW) parameters were adapted with slight modifications from a previous study which used MW irradiation at 65 °C for 5 min in combination with various lipid extraction methods [30]. The first approach aimed to evaluate whether the conventional Matyash method, which uses a solvent mixture of MTBE/CH_3OH/H_2O (10/3/2.5, v/v/v) could be improved by using MW energy instead of the conventional shaking employed in the traditional Matyash method. Briefly, a total of 600 mg of ground pistachio sample was weighed and transferred into a glass tube. Then, the same solvent ratio and composition as the original Matyash method (10/3/2.5 v/v/v) was used. 4.5 mL of CH_3OH and 15 mL of MTBE were added into the glass tube containing the weighted sample, followed by a MW extraction under a program of ramp to 65 °C in 2 min and hold 65 °C for 10 min in a system ETHOS UP, equipped with a FastEX-12 rotor from Milestone srl (Sorisole, Bergamo, Italy). After the extraction, 3.75 mL of water was added to induce phase separation. After incubating for 10 min at room temperature, the sample was centrifuged at 6000 rpm for 20 min. To

investigate the impact of the re-extraction step in the procedure, six replicates were performed. In three of them, the upper (organic) phase was collected, dried using a flux of nitrogen, weighed, and then dissolved in MTBE for storage prior to the GC analysis. The remaining three replicates were subjected to the additional re-extraction step as described in 2.2.1, that was performed on the aqueous phase using 6 mL of a mixture of MTBE, CH_3OH, and H_2O (10/3/2.5 v/v/v). The vials were placed on a shaker at room temperature for 60 min, allowed to rest for 10 min, and then centrifuged for 20 min at 6000 rpm. The upper organic phase was subsequently collected and combined with the organic phase previously obtained, dried, weighed and dissolved in MTBE.

A third approach aimed to evaluate if MTBE as single extraction solvent could achieve similar yields compared to the solvent mixture used in the Matyash method. MTBE is part of the alkyl ethers chemical class which is more polar than hydrocarbons but less polar than alcohols, allowing it to extract both polar and non-polar lipids. Specifically, 19.5 mL of MTBE was added to 600 mg of ground pistachio sample and extracted using the MW under the same program previously described. The procedure followed was the same as the one described earlier, without the re-extraction step with the MTBE/MeOH/H_2O mixture.

Finally, the organic phase containing lipids was dried using a gentle flux of nitrogen, weighed and dissolved in MTBE for storage before the GC analysis.

2.3. Derivatization process and GC parameters

FAMEs from pistachio oil were prepared according to the procedure reported in the Regulation EC/2568/91 [31]. Briefly, an aliquot of each lipid stock solution containing about 10 mg of extracted sample was transferred into 2 mL screw-cap vials. To each sample, 500 μL of 0.4 M potassium hydroxide (KOH) in methanol solution, was added. Subsequently, the samples were vortexed for 30 s and kept at room temperature for 15 min. Then, 500 μL of hex was added and vortex-mixing for 30 s each. Finally, the upper organic layer was transferred to a new vial, and used for GC analysis [32].

The GC analyses of FAMEs were carried out on a GC system 8860 (Agilent Technologies, Milan, Italy) equipped with a J&W HP-5 GC Column (stationary phase: 5 % phenyl-methylpolysiloxane and 95 % dimethylpolysiloxane (Agilent, Santa Clara, CA, USA) 30 m × 0.32 mm i.d. × 0.25 μm film df. 1 μL was injected in split mode (split ratio 1:10), oven temperature program was from 60 °C to 300 °C at 5 °C/min and to 320 °C at 20 °C/min. Injection temperature was set at 280 °C, FID temperature was set at 350 °C. Carrier gas was Helium, at a constant linear velocity of 27.081 cm/s and an initial head pressure of 7.8 psi. FID conditions: sampling frequency: 20 Hz. Data were processed through the Agilent OpenLab CDS (Agilent Technologies, Milan, Italy) software (ver. 2.5).

A Pegasus BT 4D (LECO Corporation, Mönchengladbach, Germany) was used as MS detector in combination with GC × GC, equipped with an Agilent 8890 GC and an automated PAL System device (CTC Analytics AG, Zwingen, Switzerland) with the following set of columns, a Rxi-5ms (5 % phenyl-polysiloxane and 95 % methyl-polysiloxane), 30 m × 0.25 mm i.d. × 0.25 μm d_f as the first dimension (^{1}D) and Rxi- 17 Sil MS (50 % phenyl-polysiloxane 50 % methyl-polysiloxane), 1.45 m × 0.25 mm i.d. × 0.25 μm d_f as the second dimension(^{2}D) both from Restek Corporation (Bellefonte, PA, USA). The oven temperature program was from 45 °C (held 1 min), then ramped at 6 °C/min to 335 °C. Temperature offsets for the secondary oven and for the modulator oven were set at +5 °C and +15 °C, respectively. The injector temperature was set at 300 °C. Carrier gas was He, at 1.5 mL/min. A modulation period of 2 s was used (hot jet: 0.70 s, cold jet: 0.30 s). MS conditions: a time-of-flight mass analyzer was used with electron ionization (70 eV) at 100 Hz of acquisition rate with a mass range from 40 to 500 m/z; The ion source temperature was held at 250 °C and the acquisition delay was 190 s. Data was processed through ChromaTOF software (LECO Corporation, ver. 5.56.53).

2.4. Statistical analysis

Bar plots, principal component analysis (PCA), ANOVA test and post-hoc Tukey's test were performed using Excel® (Microsoft Office, version 16.75.2) and Python (version 3.12.5).

3. Results and discussion

In this contribution, for the first time, the Matyash method was employed with the assistance of MWs for lipid extraction. In addition, a simplified version of Matyash method and an MTBE one-solvent extraction method were also performed assisted by MWs. Matyash-MAE, simplified Matyash-MAE and MTBE-MAE were compared to Soxhlet extraction using organic solvents with different polarities (hex, Et_2O, MTBE) and traditional Matyash as conventional lipid extraction methods. The different methods were compared in terms of total lipid extraction yields and FAMEs profile.

3.1. Total lipid extraction yield

The efficiency of different lipid extraction methods was assessed by evaluating the lipid yield obtained using various solvents and techniques. A one-way ANOVA was implemented to test statistically significant differences between the extraction yields of different methods ($\alpha = 0.05$). To attribute which specific pair of methods differed significantly, a Tukey's HSD Test was used ($\alpha = 0.05$). Details regarding the yield% and statistical evaluations are reported in the Supplementary Material Table S1.

Soxhlet extraction using hex resulted in a lipid yield of 32.66 % (±1.28), while replacing hex with MTBE led to a slightly lower yield of 31.47 % (±1.77). Notably, Soxhlet extraction with Et_2O yielded the highest recovery among the Soxhlet conditions, reaching 33.67 % (±1.26).

In contrast, the traditional Matyash liquid-liquid extraction method, employing a biphasic MTBE/MeOH/H_2O system (10/3/2.5, v/v/v), provided a yield of 37.08 % (±1.98). MAE methodology, applying the condition of traditional Matyash, resulted in the highest yield among all the extraction methods applied (39.73 %, ±1.91). This higher yield value compared to the conventional Matyash probably comes from the contribution of MWs during the extraction process. Indeed, the oscillating movement of the polar molecules within the matrix, generated by the MWs, may possibly contribute to disrupt the matrix structure and thus favouring the lipid extraction [13,33]. In addition to achieving a higher yield with the MAE Matyash method, it is worth highlighting the significant reduction in total processing time compared to the traditional method. In the latter, the procedure required a first step of weighting the pistachio sample, then solvents addition, and finally incubating at room temperature for 60 min in a shaker. After recovering the organic phase, it followed a subsequent re-extraction step, which takes another hour. This two-steps procedure resulted in a total processing time of approximately 3 h. With the proposed MAE Matyash methodology the overall processing time was also significantly reduced. Specifically, after weighing the pistachio sample, the initial extraction step time was reduced to approximately 30 min, including 12 min of MAE, followed by a cooling step to room temperature, before proceeding with the subsequent re-extraction step. To further reduce the time required for the procedure, a simplified version of the MAE Matyash was proposed. While in the MAE Matyash the two steps of extraction were conducted, the first taking advantage of the contribution of the MWs, while the second step of re-extraction was performed as reported in the traditional Matyash method by using the shaker, the proposed simplified Matyash approach did not include the re-extraction step, resulting in a slightly reduced lipid yield of 35.04 % (±0.91), with no statistical differences observed ($p = 0.0676$) compared to other extraction methods. This

simplification allowed the reduction of the overall processing time to approximately 30 min, as well as lowering the amount of solvents needed. However, even if this simplified approach showed a lower yield compared to the MAE-Matyash, both methods were higher in terms of yields compared to the ones obtained using the Soxhlet apparatus.

To further simplify the procedure by using only one solvent, overall time needed, while maintaining the advantages of the MWs, the use of MTBE as a single extraction solvent was investigated. This approach resulted in a yield of 36.85 % (±2.67), which is a comparable result with the traditional Matyash method, and an intermediate result between the highest yield obtained with the use of the MAE-traditional Matyash, and the slightly lower yield of the MAE-simplified Matyash approach.

These findings indicate that while Soxhlet extraction remains a viable approach, the Matyash-based liquid-liquid extraction method demonstrates superior lipid recovery, suggesting its enhanced efficiency in extracting lipids from the tested sample matrix.

Only 5 out of 21 pair of methods showed statistical differences ($p < 0.05$). All the Soxhlet methods resulted significantly different in comparison with MAE-Matyash method ($p < 0.01$), and among them, Soxhlet-MTBE was found statistically different with both traditional Matyash ($p = 0.0216$) and MAE-MTBE ($p = 0.0287$). Details of the extraction techniques applied and pair of methods showing statistical differences are reported in Table 1.

Nevertheless, when dealing with routine analysis in food, different aspects need to be considered, as not necessarily the highest result is the most accurate. The most common analysis done in lipid extract is the analysis of the profile of FAs. Different extraction methods of the lipid fraction can lead to different compositions of the kind of lipids extracted, leading to differences in the FA profile. Therefore, the latter was further investigated for comparison purposes.

3.2. Fatty acid composition

Following extraction and derivatization by KOH/MeOH, initial gas chromatographic analyses were performed using a GC-FID system and GC × GC-TOFMS. GC × GC-TOFMS analysis was performed to exclude the presence of interferent compounds co-extracted differently in the tested extraction methods and to confirm the identification of the peaks quantified in the GC-FID. A total of 15 FAMEs were tentatively identified by comparing with the data in the mass library (≥800/1000) and considering the FAMEs structure on the 2D-GC plot (Fig. S1). However, only FAMEs ≥0.2 % (n = 8) were considered for comparison purposes. FAME profile and saturated (S), monounsaturated (MU), and polysaturated (PU) FAs with their standard deviations among the different extraction methods as well as their statistical evaluation are reported in Supplementary Material Table S2, wherein the most abundant FAMEs were oleic acid (Me.C18:1n9, average of 71.04 %) followed by linoleic acid (Me.C18:2n6, average of 14.55 %) and palmitic acid (Me.C16:0, average of 9.01 %), which together account for approximately 95 % of the total FAs. SFA (average 11.45 %) and PUFA (average 16.31 %) showed higher percentages in Matyash and MAE methods, while MUFA (average 72.24 %) in Soxhlet methods.

Fig. 1 shows a bar plot of FAME composition and the percentage of SFA, MUFA, and PUFA in pistachio sample according to the different extraction methods. Statistical differences of FAME composition, SFA, MUFA and PUFA in the different extraction methods are discussed in section 3.4. MW-based methods showed a slightly higher ratio of PUFA, which can be attributed to the reduced thermal degradation due to shorter extraction times and milder heating conditions compared to Soxhlet methods. These findings are in line with what has been previously published by Guckert et al. [34], who reported that harsh extraction conditions lead to preferential recovery of SFA and MUFA, while reducing PUFA. The authors attributed this to the fact that PUFAs are the most easily degraded FAs, with potential artifacts caused by prolonged high-temperature exposure in Soxhlet extractions [35]. In addition, extraction efficiency is dependent on both the solvent or solvent mixture used, as different solvents have varying affinities for specific lipid classes, and on the extraction method employed [35]. The traditional Matyash method showed only slightly higher PUFA recovery compared to Soxhlet with hexane as extraction solvent, and lower than all other tested extraction conditions. These results suggest that while liquid-liquid extraction methods are gentler than Soxhlet, the combination of microwaves with the Matyash protocol provides optimal conditions for PUFA preservation. Thus, this may support the use of this rapid and energy-efficient extraction method, especially when accurate quantification and preservation of PUFA is of particular interest.

To compare fatty acid profiles under standardized conditions, minimizing the yield and instrumental variability, FAMEs were expressed as normalized percentages.

Table 1
For each extraction method, solvent(s), amount of sample, amount of total lipid extracted, and yield% are reported. Each extraction method is indicated with a letter (from *a* to *g*) in the solvent column. In the yield% column, each letter reported indicates the pair with a statistical difference ($p < 0.05$) after Tukey's test.

Extraction method	Solvent(s) (n = 3)	g of sample (n = 3)	g of lipid extract (n = 3)	Yield % (n = 3)
Matyash	MTBE/MeOH/water[(a)] 10/3/2.5 (v/v/v)	0.601 (±0.001)	0.223 (±0.012)	37.08[(c)] (±1.98)
Soxhlet	Et_2O[(b)]	2.02 (±0.021)	0.68 (±0.030)	33.67[(e)] (±1.26)
	MTBE [(c)]	2.004 (±0.002)	0.631 (±0.036)	31.47[(a), (e), (g)] (±1.77)
	n-hexane [(d)]	2.005 (±0.003)	0.655 (±0.025)	32.66[(e)] (±1.28)
Microwave-assisted extraction (MAE)	Matyash [(e)]MTBE/MeOH/water 10/3/2.5 (v/v/v)	0.610 (±0.008)	0.242 (±0.012)	39.73[(b), (c), (d)] (±1.91)
	Simplified Matyash[(f)] without re-extraction step MTBE/MeOH/water 10/3/2.5 (v/v/v)	0.606 (±0.005)	0.212 (±0.007)	35.04 (±0.91)
	MTBE [(g)]	0.606 (±0.004)	0.223 (±0.015)	36.85[(c)] (±2.67)

3.3. Principal component analysis (PCA) of the different extraction methods

Fig. 2a shows a 2D-PCA based on Euclidean distance, where the first two principal components collectively explain 59 % of the total variance (PC1: 38.46 %; PC2: 20.59 %), providing a meaningful representation of the total variance in the dataset. The remaining 40.95 % variance is distributed across additional components, with PC3 contributing 17.28 % and PC4-PC8 accounting for 23.67 %. Seven extraction methods, each with three replicates, are represented by different colors in the PCA plot. Confidence ellipses are designed to show the 95 % confidence region (equivalent to 2 standard deviations), highlighting the variance within each method. Larger ellipses indicate greater variability among replicates (i.e., lower repeatability), whereas smaller, more compact ellipses suggest higher repeatability. The positions of the ellipse centers, along with the degree of their overlap, illustrate the relationships between different extraction techniques. Ellipses that are close together and overlap substantially suggest similar FAME profiles, while those that are more distant and do not overlap indicate distinct extraction characteristics. The Soxhlet-based methods cluster relatively close together in the PC space in Fig. 2 (Et_2O: light green square; MTBE: orange square; hex: yellow square), indicating that they produce similar chemical compositions. Notably, Soxhlet-MTBE (orange square) and Soxhlet-Hexane

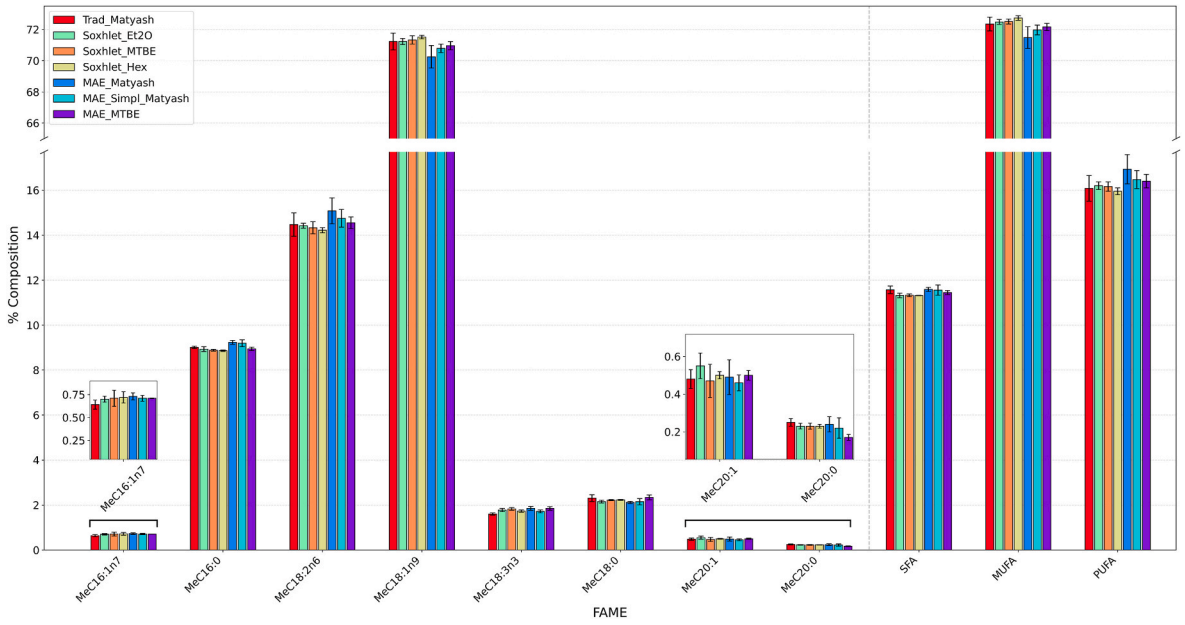

Fig. 1. Bar plot of the average of FAME composition, SFA, MUFA, and PUFA in pistachio oil from different extraction methods. Error bar indicates the standard deviation of three replicate. For the details of the extraction methods, refer to Table 1.

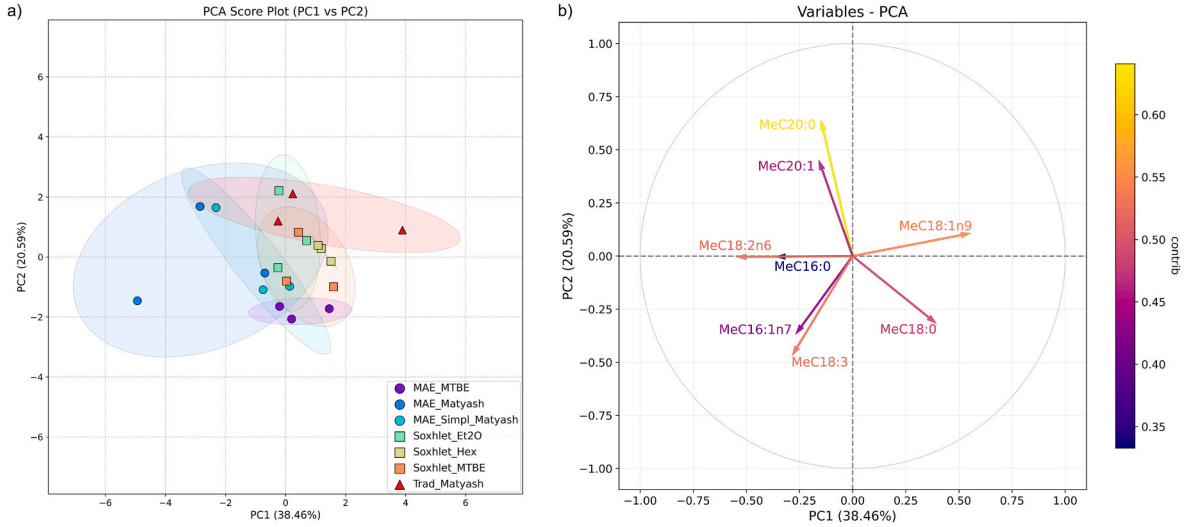

Fig. 2. Principal Component Analysis (PCA): (a) Score Plot of the different extraction methods, displaying the first two principal components (PC1 and PC2), (b) Loading Plot of the different FAMEs contributing to PC1 and PC2. FAMEs are color-coded based on their contribution magnitude, with a colorbar indicating the strength of contribution.

(yellow square) clusters overlap substantially, suggesting similarities in extraction selectivity, while Soxhlet-Et_2O (light green square) shows slight divergence. In contrast, MAE-MTBE (purple circle) forms a separate cluster, and it is highly distinct from other methods. However, a cluster overlap between Soxhlet-MTBE (orange square) and MAE-MTBE (purple circle) can be observed, suggesting also an effect of MTBE to drive extraction regardless of the technique. Overall, one-solvent extraction methods (Soxhlet methods and MAE-MTBE) show tight clustering, indicating high repeatability, whereas Matyash-based extractions (traditional Matyash: red triangle; MAE-Matyash; blue circle, and MAE-Simplified Matyash; cyan circle) appear more distinct, indicating greater variability compared to one-solvent extraction methods. Traditional Matyash (red triangle) and MAE-Matyash (blue circle) show the higher variability, likely due to the additional manipulation required during the re-extraction step. In fact, the MAE-Simplified Matyash (cyan circle) showed a lower variability compared to the other two previously mentioned. The main FAs driving the differences between extraction methods are Me.C18:1n9, Me.C18:2n6, Me.C18:0 (PC1) and Me.C20:0, Me.C18:3n3, Me.C20:1 (PC2), as reported in Fig. 2b, while PC3 is primarily driven by Me.C16:0 (loading +0.617), Me.C18:3 (loading −0.406) and Me.C20:1 (loading −0.510).

3.4. Statistically significant differences of FAMEs composition and SFA, MUFA and PUFA among different extraction methods

ANOVA was conducted to evaluate statistical differences in FAME compositions among different extraction methods. The independent variable was the extraction method, which consisted of seven different techniques, namely traditional Matyash, Soxhlet using Et_2O, MTBE, and hex; MAE with Matyash method, with the simplified Matyash approach,

and with MTBE as one-solvent extraction. Comparison of means for the individual extractions was done at 5 % significance level (α = 0.05) based on the *F*-test of the analysis of variance. The statistical analysis revealed significant differences: four FAMEs at $p < 0.05$, indicating that at least one extraction method differs from the others. Me.C16:0 showed the most significant variation ($p = 0.0001$), followed by Me.C18:3n3 ($p = 0.033$), Me.C20:0 ($p = 0.0153$), and Me.C18:1n9 ($p = 0.0278$).

While the ANOVA test identified statistical differences among the different extraction techniques applied, it did not specify which particular extraction method differs from each other. To determine these specific differences, a post-hoc Tukey HSD (Honestly Significant Difference) test was conducted. Tukey test computes the mean difference for each pair of groups, calculating the standard error using the mean square error from ANOVA. The statistical evaluation of each FAME and SFA, MUFA and PUFA is reported in the Supplementary file Table S2. Table 2 shows the *p* value of FAME profile and SFA, MUFA, and PUFA calculated by Tukey test comparing each extraction to other ones applied (n = 21). Regarding FAME profile, out of 168 FAME comparisons (representing the 21 possible extraction method combinations for each of the 8 different FAMEs analyzed), only 16 were statistically different at α = 0.05 (<10 %).

Palmitic acid (Me.C16:0, 9.01 %) showed the highest statistical difference among the extraction methods, resulting statistically different in 9 out of 21 pair comparisons. In particular, MAE-Matyash resulted statistically different in 5 out of 6 of group comparisons while MAE-simplified Matyash in 4 out of 6, although they did not turn out statistically different to each other (MAE-Matyash *vs* MAE-Simpl. Mat.: $p = 0.9966$).

Oleic acid (Me.C18:1n9, 71.04 %) resulted to be statistically different for the same groups comparison.

Among different extraction methods, linolenic acid (Me.18:3n3, average 1.74 %) presents a lower percentage in traditional Matyash (1.60 %), resulting statistically different in the comparison of 3 out of 6 pair groups (Soxhlet-MTBE, MAE-Matyash, and MAE-MTBE).

Eicosanoic acid (Me.C20:0) resulted statistically different in MAE-MTBE in comparison with Soxhlet-Et_2O, MAE-Matyash, and traditional Matyash.

A one-way ANOVA test was also conducted to evaluate whether the different extraction methods led to significant variations in the composition SFA, MUFA, and PUFA. For SFA, ANOVA indicated slightly significant differences among extraction methods ($F(6,14) = 3.0177$, $p = 0.0415$, $\alpha = 0.05$). However, subsequent Tukey's HSD post-hoc tests (critical $q = 4.829$, $df = 14$) failed to identify specific pairwise differences among the 21 possible method combinations. This apparent contradiction exemplifies a known statistical phenomenon in multiple comparison analyses. ANOVA is designed to detect overall variation among group means and tends to have greater statistical power in identifying global effects, especially when the sample size is limited. In contrast, post-hoc tests like Tukey's HSD are more conservative by design, adjusting for the increased risk of Type I errors (false positive) across multiple comparisons. As a result, they require larger differences between group means to achieve statistical significance. This means that while ANOVA may reveal that not all group means are equal, Tukey's test might still fail to identify which specific groups differ, highlighting the importance of considering both global and pairwise statistical tests.

In general, MAE-Matyash based methods accounted for higher % of palmitic and linoleic acid, and, on the contrary, for a lower % of oleic acid, especially compared to Soxhlet methods. This suggested a more selective extraction of MAE-Matyash based methods of lipid classes containing higher % of palmitic and linoleic acids. Of course, to avoid any speculative claims, dedicated studies should be performed considering the absolute quantity (and not the percentage profile) and also to perform the quantification of the different lipid classes as intact lipids and of the FAMEs after transesterification.

Overall, the methods produced comparable results, allowing flexibility in selecting the most suitable approach based on specific laboratory needs and available resources.

3.5. Green analytical evaluation of the lipid extractions

All the extraction methods applied were evaluated for greenness using the PrepAGREE metric [36]. The software generates pictograms

Table 2
P value of FAMEs profile, SFA, MUFA, and PUFA calculated by Tukey test comparing each extraction to other ones applied (n = 21).

Group 1 *vs* Group 2		Me. C16:0	Me. C16:1n7	Me. C18:0	Me. C18:1n9	Me. C18:2n6	Me. C18:3n3	Me. C20:0	Me. C20:1	SFA	MUFA	PUFA
Matyash	Soxhlet-Et_2O	0.8349	0.7636	0.4237	1.0000	1.0000	0.0555	0.9681	0.8023	0.2361	0.9992	0.9996
Matyash	Soxhlet-MTBE	0.3652	0.6285	0.8978	0.9999	0.9989	***0.0106****	0.8948	1.0000	0.2803	0.9974	1.0000
Matyash	Soxhlet-Hex	0.2634	0.4736	0.9387	0.9745	0.9757	0.2698	0.8237	0.9996	0.2528	0.8478	0.9996
Matyash	MAE-Matyash	***0.0387****	0.3795	0.2628	0.0970	0.4352	***0.0046****	0.9991	0.9998	1.0000	0.1075	0.1879
Matyash	MAE-Simpl. Mat.	0.1060	0.5877	0.4408	0.8110	0.9575	0.3496	0.5424	0.9998	1.0000	0.8374	0.8730
Matyash	MAE-MTBE	0.8812	0.6893	0.9995	0.9729	1.0000	***0.0057****	***0.0094****	0.9995	0.8718	0.9934	0.9500
Soxhlet-Et_2O	Soxhlet-MTBE	0.9728	1.0000	0.9667	0.9999	0.9999	0.9634	0.9999	0.7679	1.0000	1.0000	1.0000
Soxhlet-Et_2O	Soxhlet-Hex	0.9191	0.9983	0.9379	0.9743	0.9918	0.9545	0.9992	0.9500	1.0000	0.9756	0.9836
Soxhlet-Et_2O	MAE-Matyash	***0.0036****	0.9912	0.9998	0.0973	0.3531	0.8030	0.9992	0.9351	0.1771	***0.0498****	0.3353
Soxhlet-Et_2O	MAE-Simpl. Mat.	***0.0101****	0.9999	1.0000	0.8116	0.9145	0.9024	0.9563	0.6083	0.2852	0.5971	0.9771
Soxhlet-Et_2O	MAE-MTBE	1.0000	1.0000	0.2403	0.9731	0.9994	0.8609	***0.0474****	0.9508	0.8605	0.9200	0.9962
Soxhlet-MTBE	Soxhlet-Hex	1.0000	1.0000	1.0000	0.9970	0.9997	0.5226	1.0000	0.9989	1.0000	0.9876	0.9938
Soxhlet-MTBE	MAE-Matyash	***0.0008****	0.9991	0.8622	0.0587	0.2271	0.9990	0.9891	0.9995	0.2125	***0.0411****	0.2777
Soxhlet-MTBE	MAE-Simpl. Mat.	***0.0021****	1.0000	0.9720	0.6536	0.7832	0.4209	0.9920	0.9999	0.3358	0.5348	0.9535
Soxhlet-MTBE	MAE-MTBE	0.9525	1.0000	0.7001	0.9012	0.9881	0.9998	0.0772	0.9989	0.9052	0.8827	0.9887
Soxhlet-Hex	MAE-Matyash	***0.0005****	1.0000	0.8016	***0.0214****	0.1260	0.2903	0.9689	1.0000	0.1904	***0.0108****	0.1001
Soxhlet-Hex	MAE-Simpl. Mat.	***0.0014****	1.0000	0.9461	0.3465	0.5742	1.0000	0.9983	0.9864	0.3044	0.1998	0.6794
Soxhlet-Hex	MAE-MTBE	0.8808	0.9997	0.7723	0.6200	0.9181	0.3457	0.1033	1.0000	0.8792	0.4903	0.8103
MAE-Matyash	MAE-Simpl. Mat.	0.9966	0.9997	0.9996	0.6440	0.9211	0.2209	0.7968	0.9911	0.9999	0.6463	0.7896
MAE-Matyash	MAE-MTBE	***0.0044****	0.9972	0.1380	0.3660	0.5798	1.0000	***0.0214****	1.0000	0.7860	0.2983	0.6554
MAE-Simpl. Mat.	MAE-MTBE	***0.0123****	1.0000	0.2521	0.9984	0.9911	0.2666	0.2335	0.9860	0.9181	0.9931	1.0000

p < 0.05*. For the lipid extraction methods, refer to Table 1.

that display the score assigned to the method based on the 10 criteria of green sample preparation and taking into account the importance of criteria by assigning them specific weight. As suggested by Wojnowski et al. [36], the default weights for the calculation of each criterion were used [13,17,18,26].

Details about evaluation criteria as well as score value obtained in each criterium among different extraction methods is reported in Supplementary Material Table S3.

In summary, the MAE-Matyash and traditional Matyash methods were the least effective, with AGREEPrep scores of 0.19 and 0.20, respectively. These were followed by the Soxhlet-based one-step organic solvent extraction methods (hex: 0.22; MTBE and Et_2O: 0.27). MAE-Simplified Matyash achieved a slightly higher score (0.28), while MAE-MTBE recorded the highest score among all methods (0.33), as illustrated in Fig. 3. A notable distinction emerges in criterion 3 (target sustainable, reusable, and renewable materials), where Soxhlet-based methods scored higher due to the ability of our system to recover the solvents used during the evaporation process almost entirely (as reported on the manufacturer's website, the solvent recovery rate is up to 75 %) with an advantage not available in the other techniques. On the contrary, in criterion 5 (minimize sample, chemical, and material amounts) Soxhlet methods using 2 g of sample had a lower score compared to the others, where 0.6 g of sample was used, resulting in a higher amount of waste. Criterion 6 (maximize sample throughput) also varies across the investigated methods, except for MAE-Simplified Matyash and MAE-MTBE, where, due to reduced sample preparation steps and the availability of multiple positions in MW system, it is possible to prepare up to eight samples per hour. This contrasts with Soxhlet extraction, which only allows for 2.25 samples per hour. Closely linked to this, is criterion 8 (minimize energy consumption), where MAE-Simplified Matyash and MAE-MTBE achieve the highest score with an energy consumption of approximately 107 W/h per sample. This correlation is attributed to the fact that a greater number of samples prepared per hour results in lower energy consumption per individual sample. Although the multi-position capability of the microwave system offers a general advantage over other extraction methods, the lower score obtained by MAE-Matyash can be attributed to its requirement for more integrated steps (criteria 6 and 7) and higher energy consumption (criterion 8) compared to MAE-MTBE and MAE-Simplified Matyash. Criterion 10 (ensure safe procedures for the operator) plays a crucial role in determining overall score, evaluating according to the number of different hazard pictograms associated with the solvent used for sample preparation. The best result for this criterion was achieved by MAE-MTBE and Soxhlet methods with MTBE and Et_2O, as these solvents have a total of only two hazard pictograms compared to four for the other methods, demonstrating an increase in operator safety. In particular, according to Regulation (CE) No. 1272/2008, also known as the CLP Regulation (Classification, Labelling and Packaging), MTBE is classified with only two hazard pictograms: "flammable" (GHS02) and "irritant" (GHS07). This limited hazard classification highlights a relatively lower chemical risk for laboratory personnel compared to solvents with additional pictograms such as toxicity or environmental hazard, further supporting the safer profile of MTBE-based methods in terms of operator exposure.

Although AGREEPrep is a well-established metric tool that emphasizes sample preparation, some limitations may arise in evaluating individual criteria. For instance, criterion 2 (use of safer solvents and reagents), which carries the highest weight (5/5) among the default criteria, assigns scores based solely on a solvent consumption range of 0–8 mL, with any value at or above 8 mL receiving the lowest score (0). This approach fails to consider that the solvent consumption of MAE-MTBE and MAE-Simplified Matyash (19.5 mL) is significantly lower than other methods, representing a reduction of more than 50 % compared to the highest solvent usage (50.0 mL) required for Soxhlet extractions. Furthermore, even though all extraction methods received a score of 0 for criterion 2, reducing its weight from 5 to 1 (the lowest value) results in a notable increase of approximately 16 % (±3) in the overall greenness score for each method (Table S3).

Although not the primary focus of this study, it is worth to mention that a proper optimization of the steps to maximize the sample throughput (e.g., centrifugation, shaker, drying) along with the miniaturization of extraction systems could lead to more user-friendly and sustainable methods by reducing sample amounts, solvent use, and waste generation.

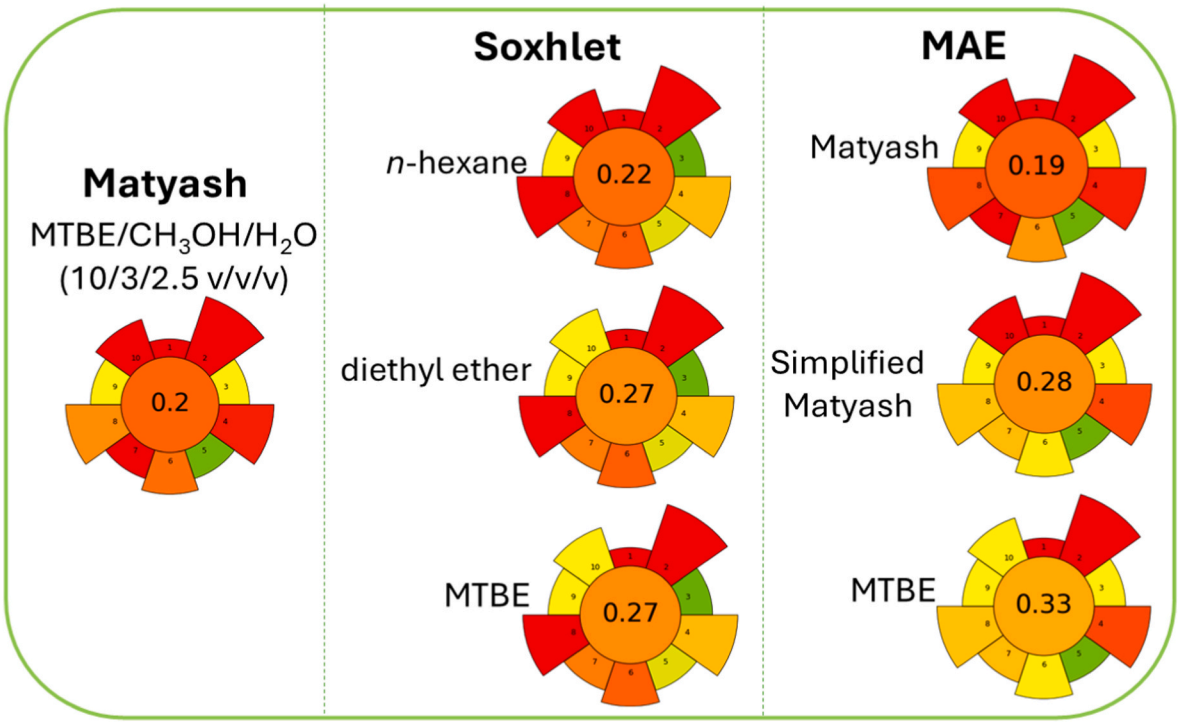

Fig. 3. Comparison of the greenness of traditional Matyash, Soxhlet with one-step organic solvent extraction, and MAE methods, using the AGREEPrep tool [36].

3.6. Limitation and future perspectives

This study focused exclusively on unroasted, unsalted pistachio nuts from Bronte (Sicily, Italy), selected as a suitable model matrix due to their high fat content. It should be noted, however, that different food products can exhibit varying lipid contents and structures, which may influence extraction efficiency and lipid profiles. Therefore, future investigations should extend the range of food products analyzed, including dairy, fish, and other seeds, to further evaluate the applicability of the proposed method in comparison with conventional lipid extraction techniques. With regard to the lipid molecules investigated, the present study is focused on FAME percentage profiles obtained after esterification of lipid extracts, without addressing intact lipid classes such as triacylglycerols and phospholipids. Future research will also aim to isolate and investigate individual lipid classes, assessing both their intra-class FAME variation and quantitative differences of individual lipid classes across extraction methods. Despite these limitations, the authors consider this work to address a significant knowledge gap in the field of lipid extraction, specifically the absence of MAE-Matyash applications in food matrices.

4. Conclusions

For the first time, the Matyash method was applied with the assistance of MWs for lipid extraction. Additionally, a simplified version of the Matyash method and a one-solvent MTBE extraction method were also performed with MWs assistance. The MAE methods were compared to Soxhlet extraction using organic solvents of varying polarities (hex, Et_2O, and MTBE) as well as the traditional Matyash method. The different extraction approaches were evaluated based on total lipid yields and FAME profiles. ANOVA identified statistically significant differences in the extractions yield and FAME compositions among the various extraction methods, post-hoc Tukey's HSD analysis revealed that only a limited number of pairwise comparisons were significantly different. MAE-Mathyash showed the highest yield (about 39 %), followed by the traditional Matyash (about 37 %), while MAE-MTBE and MAE-Simplified-Mathyash had slightly lower amount (35–36 %), with no statistical differences. The yields of Soxhlet extractions (hex, MTBE, and Et_2O) were statistically different from those of MAE-Matyash but were consistent among them (31–33 %). Among the FAMEs analyzed, palmitic acid (Me.C16:0) exhibited the greatest variability across methods, with MAE-Matyash and MAE-simplified Matyash frequently differing from Soxhlet-based methods. Despite these differences, the extraction techniques showed comparable performances in terms of FAME composition, supporting the reliability of MAE-based approaches in food analysis.

A greenness assessment of the seven extraction methods was also conducted, with MAE-MTBE achieving the highest AGREEPrep score (0.31) among all techniques, followed by MAE-Simplified Matyash (0.28) and Soxhlet methods using MTBE and Et_2O (0.27). Lower scores were observed for Soxhlet with hex (0.22), traditional Matyash (0.20), and MAE-Matyash (0.19). These findings emphasize the effectiveness of MAE combined with MTBE for lipid extraction, both as a one-step organic solvent method and in the simplified MAE-Matyash protocol involving CH_3OH and H_2O, offering advantages in both efficiency and environmental sustainability.

Although not explored in this study, MTBE extraction is also well-suited for shotgun lipidomics, where total lipid extracts can be directly infused into a mass spectrometer. Future research should focus on quantifying differences among individual lipid classes, including both the profiling of intact lipids and the determination of FA composition through transesterification, as well as validation across diverse food matrices to confirm the broader applicability of the proposed method.

CRediT authorship contribution statement

Carlo Bellinghieri: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Giulia Giacoppo:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Andrea Schincaglia:** Writing – review & editing, Software, Formal analysis, Data curation. **Donatella Ferrara:** Writing – review & editing, Software, Data curation. **Giorgia Purcaro:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Alberto Cavazzini:** Writing – review & editing, Visualization, Supervision. **Luisa Pasti:** Writing – review & editing, Supervision, Resources. **Claudia Stevanin:** Writing – review & editing, Visualization, Investigation. **Tatiana Chenet:** Writing – review & editing, Visualization, Investigation. **Nicola Marchetti:** Writing – review & editing, Resources, Investigation. **Annalisa Maietti:** Writing – review & editing, Visualization, Resources. **Flavio A. Franchina:** Writing – review & editing, Supervision, Conceptualization. **Marco Beccaria:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.129124.

Data availability

Data are reported in Tables S1-S3 of supplementary materials

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