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Paper Spray Tandem Mass Spectrometry for Assessing Oleic, Linoleic and Linolenic Acid Content in Edible Vegetable Oils

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Abstract: Oleic, linoleic and linolenic acids exert several beneficial effects on human health, some of which are also certified by recent European and U.S. regulations. The goal of the presented work was to develop an innovative methodology to evaluate their content in edible vegetable oils, in order to increase the value of oils from a nutraceutical perspective. The protocol is based on the use of paper spray ionization coupled with tandem mass spectrometry experiments, which allowed the recording of data very quickly and with high specificity. All investigated compounds gained a good linear relation (r^2 higher than 0.98). Accuracy values are near 100% for all concentration levels examined, and the repeatability and reproducibility data result lower than 15%, highlighting the consistence of the methodology. The developed approach was successfully applied for the analysis of different real samples, and its robustness was confirmed by comparing the results obtained with those coming from the classical and official methodology.

Keywords: fatty acid methyl esters; vegetable oils; paper spray ionization; tandem mass spectrometry

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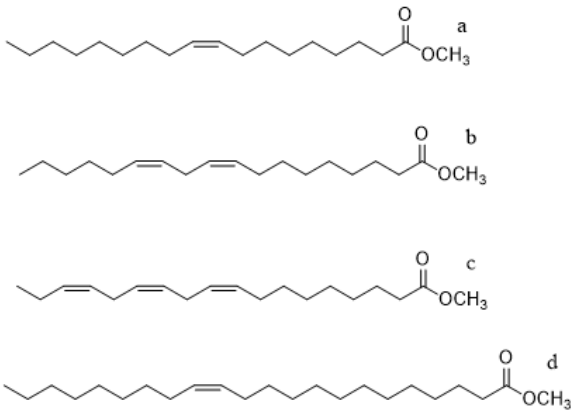
1. Introduction

Vegetable oils are one of the main fat constituents of the human diet, and represent an important daily energy source. These fat food matrices are composed, for the most part, by triacylglycerols (TAGs), which consist of two or three fatty acid chains esterified to glycerol molecules [1,2]. Fatty acid composition both affects the chemical and physical characteristics of oils and their health properties. For example, high levels of unsaturated fatty acids are positively associated with a reduction of cardiovascular disease by influencing the concentration of blood lipids (HDL, LDL, and triglycerides); in this regard, unsaturated fatty acids such as oleic, linoleic, and linolenic are the most noteworthy [3–5]. Oleic acid (C18:1), a monounsaturated (MUFA) ω-9 fatty acid, is the main component of many vegetable oils, especially olive oil, where it represents 70–80% of the composition. The beneficial effect of olive oil intake is partly related to its high oleic acid content, which has also shown to have anti-inflammatory, breast cancer protective and immune system function-enhancing effects [6–8]. Linoleic acid (C18:2) and α-linolenic acid (C18:3) are among the most important polyunsaturated fatty acids (PUFAs). They belong to the omega-6 and omega-3 fatty acids family, respectively, and are found mainly in soybean, sunflower, corn, and olive oils [9,10]. Several studies have reported that appropriate PUFAs intake may prevent cardiovascular and inflammatory diseases [11–13]. In addition, for both fatty acids, the recent Regulation 432/2012 of European Union allows the following health claim: "linoleic acid (LA)/α-linolenic (ALA) acid contributes to the maintenance of normal blood cholesterol levels". For LA, the sentence may be used only for a food containing at least 1.5 g of linoleic acid per 100 g and per 100 kcal; while for ALA, the nutraceutical declaration may be used for food which is a source of ALA (0.3 g of ALA per 100 g and per 100 kcal) as referred to in the claim regarding the source of ω-3 fatty acids listed in

the Annex to Regulation (EC) No 1924/2006 [14]. In the case of oleic acid, the European regulation provides no specific health claim, but the U.S. Food and Drug Administration (FDA) has established that there is evidence to support a claim stating that "consumption of $1^{1/2}$ tablespoons, about 20 g of oil containing high levels of oleic acid is able to reduce the incidence of coronary heart disease" [15]. Oleic, linoleic, and linolenic acids are, therefore, quite relevant for their beneficial effects and, in this context, the use of fast, accurate and reliable methodologies is essential for their quantitative determination; also, in order to address the above-mentioned regulations. Commonly, fatty acids in vegetable oils are quantified as methyl esters after transesterification reaction, while the instrumental analysis is performed by gas chromatography (GC) coupled with flame ionization detection (FID), as described by the EU official method (regulation 2015/1833, Annex X) [16], or mass spectrometry (MS) [16–20]. To date, mass spectrometry is certainly the most widely used instrument for both structural investigations and quantitative determinations, due to its high specificity and sensitivity [21–24].

The current study aimed to find a new methodology for a fast and accurate quantification of the total oleic, linoleic, and linolenic acid content in vegetable oils typically consumed in our daily diet, such as extra virgin olive oil, sunflower, soybean, and corn oil. For this purpose, we employed paper spray mass spectrometry (PS-MS), a methodology belonging to the ambient ionization techniques which has quickly become popular, due to its operative easiness [25–27]. The paper spray source is composed of a metallic alligator clip which holds a paper triangle in front of the mass spectrometer inlet. To perform this type of experiment, a small volume of sample solution is deposited on the thin paper triangle. The paper spray ion generation is gathered by applying a high voltage (4–5 kV) to the metal clip and, at the same time, by dropping few microliters of an extraction/spray solvent to the paper; in this way, charged droplets are accumulated on the paper tip and a Taylor cone is generated, providing ions through an electrospray-like process [28,29]. This technique has already been used for quantitative determination of other bioactive compounds in foods and beverages and also for molecular fingerprinting used for quality control purposes [30–37]. Multiple reaction monitoring (MRM) is used as a scanning mode to quantify the fatty acids, with the aid of erucic acid methyl ester as an internal standard. The sample preparation is based on a transesterification reaction which provides the methyl esters (Scheme 1), directly analyzed via PS-MSMS. The innovation of the proposed procedure stands on the simplicity and specificity of analysis, underlined by the absence of any chromatographic step and by the use of the tandem mass spectrometry. The entire experiment is very timesaving; in fact, after the transesterification reaction, the internal standard is added to the mixture, properly diluted, and directly submitted to the PS-MSMS analysis, whose scanning time is two minutes. Furthermore, in order to demonstrate the robustness of the presented methodology, the values obtained from the analysis of the investigated edible oils have been matched with those obtained by the application of the official EU method.



Scheme 1. Chemical structures of oleic acid methyl ester (**a**), linoleic acid methyl ester (**b**), linolenic acid methyl ester (**c**) and internal standard (**d**).

2. Materials and Methods

2.1. Chemicals and Reagents

All solvents used were HPLC grade and commercially available from Sigma-Aldrich (St. Louis, MO, USA). Pure compounds, methyl oleate (methyl *cis*-9-octadecenoate), methyl linoleate (methyl *cis*,*cis*-9,12-octadecadienoate), methyl linolenate (methyl *cis*,*cis*,*cis*-9,12,15-octadecatrienoate), methyl erucate (methyl *cis*-13-docosenoate), triolein (oleic acid triglyceride) and trilinolein (linoleic acid triglyceride) were also purchased from Sigma-Aldrich.

2.2. Standard Solutions

Standard solutions of fatty acid methyl esters were achieved at a concentration of 3000 mg/L by solubilizing the standard compounds in *n*-hexane. The calibration curves were obtained by analyzing five standard solutions of methyl oleate, methyl linoleate and methyl linolenate at increasing concentrations, in the range from 2.5 to 20 mg/L, and internal standard (methyl ester of erucic acid) at fixed concentration of 10 mg/L.

2.3. Sample Preparation

Each oil sample was submitted to transesterification reaction following the EU official procedure 2015/1833, Annex X [16], with minor amendments. Briefly, 50 mg of oil were weighted, added of 50 µL of 2 M potassium hydroxide methanolic solution and 2 mL of *n*-hexane. The resulting solution was vigorously shaken for 30 s and allowed to stratify until the supernatant was clear. Before the PS-MS/MS analysis, the supernatant was diluted by using *n*-hexane and the appropriate amount of internal standard was added. Olive oil samples supernatant was diluted 1:1000 for the quantification of methyl oleate; for methyl linoleate and linolenate, it was diluted 1:200 and 1:10, respectively. For seeds oil samples, the supernatant was diluted 1:1000 for the assay of methyl oleate and linoleate, and 1:100 for the quantification of methyl linolenate.

2.4. Paper Spray Mass Spectrometry

PS-MS determinations were carried out in positive polarity by using a TSQ Quantum Vantage (Thermo Fischer Scientific, San José, CA, USA) triple-stage quadrupole mass spectrometer coupled with an in-house implemented paper spray source. The latter is composed of a small triangular shaped piece of paper (qualitative Whatman filter paper n° 1) which supports the sample and held in front of the triple quadrupole inlet using a metallic clip. Up to 15 µL of sample solution were deposited onto the paper and left to dry for 1 min. After this time, a high voltage, set using the power supply enclosed in the mass spectrometer for the electrospray source, was applied to the triangle through the clump, and 15 µL of methanol was added every 30 s to permit the spray desorption. The total scan time was 2 min. The MS working conditions were set as follows: applied voltage +5000 V, vaporizer temperature 280 °C and capillary temperature 290 °C. The gas used for CID experiments was argon, with a pressure in the collision cell (Q2) of 1.5 mTorr. Mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum. The scan time was set at 0.4 s while the number of micro scans was set at 2. The collision energy (CE) was optimized for each compound and ranged from 12 to 20 eV; S-lens values was set at 120 V for all investigated compounds. The quantitative determination was conducted under multiple reaction monitoring (MRM) conditions, using the ion current generated by two gas phase transitions from the protonated compounds $[M+H]^+$, the first one for the quantitative assay, and second for confirmation (Table 1). For each quantitative transition, the ion current was averaged over the total scanning time. Instrument control was carried out by means of Xcalibur 2.1 software.

2.5. GC-FID

The gas chromatographic analyses were carried out following the EU official method 2015/1833, Annex X [16] by using a GC-FID instrument from Varian (Palo Alto, CA, USA) The detector temperature was set at 225 °C and the flame of detector was kept at 250 °C

with 30 mL/min H_2 and 300 mL/min air. The chromatography was performed with a 100 m × 0.25 mm DB-23 capillary column with a film thickness of 0.25 µm from Agilent Technologies. Sample injection volume was 1 µL and the oven temperature started from 100 to 240 °C with a speed rate of 3 °C/min and 5 min of plateau. The total time of analysis was 43 min.

Table 1. Selected MRM transitions, instrumental parameters and calibration curve equations.

Compound	Transition	CE (eV)	S-Lens (eV)	Linearity
Oleic acid	m/z 297 → m/z 265 (quan)	12	120	y = 0.356x + 0.2888
methyl ester	m/z 297 → m/z 247	15	120	R^2 = 0.9852
Linoleic acid	m/z 295 → m/z 263 (quan)	12	120	y = 1.6759x + 0.8368
methyl ester	m/z 295 → m/z 245	15	120	R^2 = 0.9913
Linolenic acid	m/z 293 → m/z 261 (quan)	15	120	y = 1.1231x + 0.1458
methyl ester	m/z 293 → m/z 243	18	120	R^2 = 0.9953
Erucic acid	m/z 353 → m/z 321 (quan)	18	120	
methyl ester (IS)	m/z 353 → m/z 303	20	120	

3. Results

The vegetable oils under investigation were submitted to transesterification to convert the triacylglycerols to fatty acid methyl esters. The procedure was very simple and required just few minutes. The reaction products were initially analyzed by direct injection ESI (+)-MS analysis to verify that the reaction conversion was quantitative. The positive mode was chosen for the paper spray MS experiments because a better ionization efficiency for the fatty acid methyl esters investigated was observed. After several trials involving different extraction spray solvents, we decided to use methanol for the ionization step which provided a good signal at m/z 297, m/z 295 and m/z 293 for the protonated fatty acid methyl esters $[M + H]^+$ of oleic, linoleic and linolenic acids, respectively. The quantitative determinations were performed using multiple reaction monitoring (MRM) scan mode by following specific transitions for the analytes. The latter were selected by observing the fragmentation behaviour of methyl esters in tandem mass spectrometry (MS/MS) experiments. For all fatty acid methyl esters, the MS/MS spectra were characterized by few diagnostic fragments; the base peak is relative to the formation of the acyl ion, generated by the formal loss of a methanol neutral molecule from the protonated molecule $[M + H]^+$, while the second most intense product ion is produced by a formal loss of one molecule of water from the latter, aided by the transfer of a proton in alpha position with respect to the carbonyl oxygen (Figure 1).

An internal calibration method was used to quantify the total fatty acids content, employing the methyl ester of erucic acid (methyl *cis*-13-docosenoate) as internal standard. The selected standard is a monounsaturated fatty acid methyl ester with a chemical structure similar to the investigated compounds and not present in the vegetable oils under study. Methyl *cis*-13-docosenoate exhibits the same fragmentation behaviour of the other analytes: the product ions provided by the loss of methanol molecule from the $[M + H]^+$ parent ion and the subsequent formal loss of water gave signals at m/z 321 and m/z 303, respectively. Table 1 summarizes the gas-phase reaction generated from the protonated species $[M + H]^+$ and monitored during the assay of the oils. In particular, the loss of the neutral methanol was used for quantitative analysis, while the subsequent dehydration was used to confirm the signal. The collision energy for each transition was optimized to achieve the highest signal.

The calibrations curves, built by analyzing in triplicate each of the five standard solutions at increasing concentrations of analytes, show a good linearity in the chosen range, with a correlation coefficient (r^2) higher than 0.98 for all analytes (Table 1). The range of concentrations of the fatty acid methyl esters in the standard solutions was selected from 2.5 to 20 mg/L, while the internal standard was maintained at 10 mg/L. The

calibration reproducibility (RSD%) was evaluated by preparing two standard solutions at concentrations corresponding to the linear dynamic range edges (2.5 and 20 mg/L) and analyzed three times over a period of a week. For both concentration levels, the percentage relative standard deviation (RSD%) was less than 15%, highlighting a good reproducibility for what concerns the instrumental response. Due to the lack of a blank matrix, the accuracy of the whole procedure was disclosed by submitting two mixtures of triolein and trilinolein to transesterification process and then analyzed. The mixtures were prepared at the following ratios: S1 80/20 and S2 20/80 (triolein/trilinolein), in order to mimic an oil with a high oleic and linoleic acid content, respectively. After transesterification, both samples were diluted 1:1000 and 1:100 using hexane and submitted to PS-MSMS analysis. In all cases, the accuracy values were around 100%. The same samples were also employed for evaluating the repeatability and reproducibility of the methodology, both expressed as RSD%. The first one was assessed by performing instrumental analyses for each sample in triplicate, while the reproducibility was calculated by analyzing the samples three times at one-week intervals. An RSD% below 15% was obtained for each experiment. Table 2 shows the value of analytical parameters discussed above.

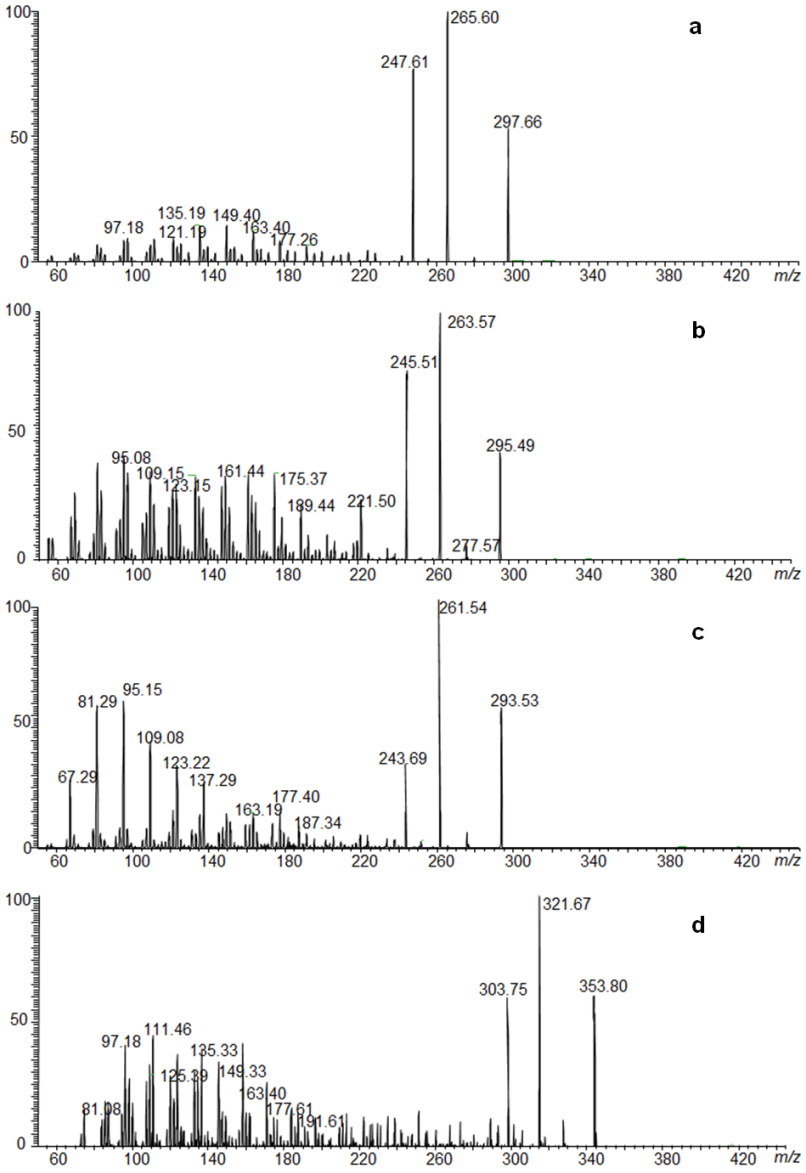


Figure 1. ESI(+)-MS/MS spectra of $[M+H]^+$ ions of oleic acid methyl ester (**a**), linoleic acid methyl ester (**b**), linolenic acid methyl ester (**c**), erucic acid methyl ester—IS (**d**).

Table 2. Accuracy, repeatability, and reproducibility values.

Sample			Calculated Amount (*w*/*w* %)	Accuracy (%)	Repeatability (RSD %)	Reproducibility (RSD %)
S1 (*w*/*w* %)	**Oleic acid**	**80%**	82 ± 7	102	8.5	9.2
	Linoleic acid	**20%**	18 ± 2	90	11.1	12.0
S2 (*w*/*w* %)	**Oleic acid**	**20%**	21 ± 2	105	9.5	10.2
	Linoleic acid	**80%**	77 ± 6	96	7.7	8.4

After evaluating the calibration linearity, accuracy and reproducibility, the developed protocol was applied to real samples of vegetables oils purchased from a local store. In particular, three extra virgin olive oils, three corn oils, two soybean oils and one sunflower oil were submitted to PS-MS/MS analysis after transesterification reaction. Table 3 shows the total fatty acid content (*w*/*w* %) found in the samples tested, which were also analysed in parallel by the classical GC method to corroborate the data obtained by PS-MS.

Table 3. Methyl esters amount (*w*/*w*%) found in the investigated oil samples by PS-MS/MS and GC-FID analysis.

	Methyl Oleate (*w*/*w* %)		Methyl Linoleate (*w*/*w* %)		Methyl Linolenate (*w*/*w* %)	
Sample	**PS-MS**	**GC-FID**	**PS-MS**	**GC-FID**	**PS-MS**	**GC-FID**
Olive oil 1	60 ± 8	69	9 ± 1	8	0.8 ± 0.1	1.0
Olive oil 2	55 ± 9	68	6 ± 1	6	0.6 ± 0.2	0.7
Olive oil 3	63 ± 9	70	7 ± 1	5	0.45 ± 0.05	0.8
Corn oil 1	28 ± 4	31	60 ± 11	53	0.8 ± 0.2	1.2
Corn oil 2	24.5 ± 4.0	32	47 ± 6	52	1.6 ± 0.2	1.6
Corn oil 3	25 ± 5	31	46 ± 3	51	1.5 ± 0.3	1.3
Sunflower oil 1	29 ± 4	25	57 ± 6	63	1.1 ± 0.1	0.8
Soybean oil 1	20 ± 4	24	55 ± 8	53	3.7 ± 0.5	5.3
Soybean oil 2	23.5 ± 4.5	23	51 ± 11	51	6.5 ± 0.5	6.1

4. Discussion

The assay of three fatty acids, important from a nutraceutical point of view, were performed in the present work. Numerous studies have highlighted the health properties of oleic, linoleic and linolenic acids; for the last two, a claim is also included in EU Regulation 432/2012, while for oleic acid, the FDA has also expressed an indication about its beneficial effects. The main purpose of our study was to develop a methodology that could have been a suitable alternative to the official method used, which involves gas chromatographic separation coupled to FID detection. The disadvantages of the latter rely on the long analysis times; furthermore, it is based on a relative quantitative determination calculated on the areas of the chromatographic peaks without the use of calibration curves. The proposed protocol takes advantages of the specificity of tandem mass spectrometry MRM scanning mode, but the most important advancement concerns the use of the paper spray ionization source which provides high-throughput determinations. The application of the paper-spray-based method allows accurate results within minutes, through a direct sample ionization, without the need for chromatographic separation steps and with minimal solvent consumption. Furthermore, the use of internal standard improves the accuracy and precision of analyses. The results obtained by PSMS were compared to GC data using a standard *t*-test (α = 0.05). For all vegetable oils under investigation, a p value $\geq$ 0.05 was obtained. This emphasizes there are no significant differences between the data and shows the reliability of the proposed method (see Table 3). This cross-validation emphasises also that the PS-MS approach is not affected by sample preparation and/or by matrix composition. Regarding the analytical parameters, accuracy values near 100% and

precision (repeatability and reproducibility) always below 15%, highlighted the reliability of methodology. Recovery is quantitative for all compounds, since no extraction procedure is involved. Some considerations may be provided around the fatty acid composition of the oil samples analyzed and its relation to the health claim regulation reported in the introduction. The amount of linoleic acid in the samples of corn, soybean and sunflowers oil satisfies the EU regulation 432/2012 for what regards the claims on the "maintenance of normal blood cholesterol levels"; for what concerns the nutraceutical linolenic acid, it appears from the data, that all the samples may be considered as source of ω-3 fatty acids, while no olive oil may be considered as high-grade oleic acid content, under the statements of the FDA regulation [15].

5. Conclusions

Paper spray mass spectrometry was first employed for determining the total content of important fatty acids in vegetable oils, such as oleic, linoleic and linolenic acids. The comparability between the results gained by classical GC-FID and PS-MS analysis demonstrates that developed protocol may be applied for very rapid screening of these compounds in oils and similar matrices, as an alternative to the determination employing chromatographic separation. The relevance of the presented methodology lies in the potential of its use to address recent EU and US regulations on the health claims for these nutraceutical compounds, enabling important health indications to be directly reported on the food labels, enhancing the products' value.

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