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Determination of Nonprotein Amino Acids and Betaines in Vegetable Oils by Flow Injection Triple-Quadrupole Tandem Mass Spectrometry: A Screening Method for the Detection of Adulterations of Olive Oils

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ABSTRACT: A novel screening method using an automated flow injection electrospray ionization tandem mass spectrometry system is proposed for the simultaneous determination of five nonprotein amino acids (*β*-alanine, alloisoleucine, ornithine, citrulline, pyroglutamic acid) and three betaines (glycine betaine, trigonelline, proline betaine) after derivatization with butanolic HCl. MS/MS experiments were carried out in a triple-quadrupole instrument using multiple reaction monitoring mode in <2 min. The proposed method provided high fingerprinting power to identify the presence of five of the studied compounds in different types of vegetable oils (soybean, sunflower, corn, olive) with LODs at parts per billion levels. The method was validated, and different mixtures of extra virgin olive oil with seed oils were analyzed, achieving the typification for the detection of adulterations in extra virgin olive oils up to 2% w/w. The nonprotein amino acid ornithine was confirmed as a marker for adulteration in the olive oils analyzed.

KEYWORDS: *adulteration, betaines, nonprotein amino acids, screening, tandem mass spectrometry, vegetable oils*

INTRODUCTION

Olive oil authentication is an issue of great interest in the manufacturing countries of this food product. Authenticity covers many aspects, including adulteration, mislabeling, characterization, and misleading origin.[1] Owing to its higher price, extra virgin olive oil is susceptible to fraudulent practices concerning its authenticity, being a frequent problem for regulatory agencies, oil suppliers, and consumers. The most common process is the addition of cheaper vegetable oils with less commercial value such as seed oils, which is not allowed by European regulations.[2] For this reason, there is a need for detecting lower adulteration levels in the food industry, which requires the development of new analytical methodologies with suitable sensitivity, accuracy, and straightforward automation.

A wide number of powerful techniques have been employed for the authentication of olive oils and can be classified into chromatographic and spectrometric techniques. Separative methods for identifying adulterated olive oils based on HPLC or GC[1,3−5] and, more recently, capillary electrophoresis (CE),[6−8] with and without mass spectrometry (MS) detection, have widely been employed. Spectrometric methods without separation, such as MS, nuclear magnetic resonance, Raman spectroscopy, infrared spectroscopy, fluorescence, and chemiluminiscence, among others, have also broadly been reported.[5,9]

Among the spectrometric techniques, MS has become a powerful technique to characterize vegetable oils. This is because soft ionization methods, such as electrospray ionization mass spectrometry (ESI-MS), combine sensitivity, selectivity, and versatility being capable of gently ionizing a wide range of molecules, especially polar ones.[10,11] In fact, they allow fingerprinting in complex food matrices, such as vegetable oils, and provide structural information on their components. In addition, the injection of the sample can easily be automated, particularly when using robotized sample injection systems such as direct infusion or flow injection analysis (FIA), permitting very high throughput. The most commonly used mass analyzers for this purpose are tandem mass analyzers. Thus, for the analysis of several vegetable oils, the use of direct infusion in MS has been reported employing ion trap (IT)[12−16] or quadrupole time-of-flight (QTOF)[17,18] mass spectrometers, whereas FIA has been used with triple quadrupole (QqQ)[19] or QTOF.[20,21] The target compounds studied in these works were free fatty acids, triacylglycerols, diacylglycerols, and monoacylglycerols as major components and phenolic compounds as minor components. The minimum adulteration limits found in blended vegetable oils were 10% w/w for mixtures of olive oil with soybean oil,[18] 10% w/w for olive oil with hazelnut oil,[21] and 20% w/w[14] or even 1% w/w[16] for extra virgin olive oils with ordinary olive oils. In these works, the need to use complex chemometric techniques and/or multivariate analysis was described. Thus, chemometric treatments of the spectral information by principal component analysis,[16−18,20,21] linear discriminant analysis,[12−15,21] and/or hierarchical clustering analysis[16] were reported.

On the other hand, screening methods present the capability of a high sample throughput and are used to sift a large number of samples, being specifically designed to avoid false-compliant results. Accordingly, the objective of this work was the

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development of a new sensitive screening methodology by FIA-ESI-MS/MS with a QqQ mass spectrometer based on multiple reaction monitoring (MRM) mode to detect adulterations of olive oils with other vegetable oils using free-minor components as markers. Thus, the simultaneous determination of three betaines and five nonprotein amino acids in vegetable oils (soybean, sunflower, corn, and olive oils) was carried out to find at least one of them that can serve as a screening marker for the adulteration of olive oils.

MATERIALS AND METHODS

Reagents and Standards. Figure 1 shows the five nonprotein amino acids (β-alanine, pyroglutamic acid, alloisoleucine, ornithine, and citrulline) and the three betaines (glycine betaine, trigonelline, and proline betaine) studied in this work and selected according to the possibilities shown with previous methodologies.[6−8]

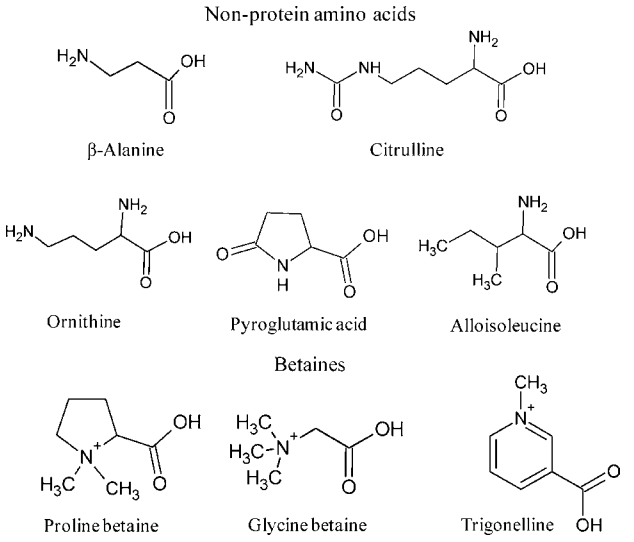

Figure 1. Structures of the nonprotein amino acids and betaines studied.

Trigonelline, β-alanine, and glycine betaine were supplied from Sigma (St. Louis, MO). Proline betaine was from Hallochem Pharma (Chongqing, China). Pyroglutamic acid, alloisoleucine, ornithine, citrulline, and hydrogen chloride/1-butanol solution were from Fluka (Buchs, Switzerland). Methanol, chloroform, and acetonitrile were supplied from Scharlau Chemie (Barcelona, Spain). Formic acid was from Riedel-de Häen (Seelze, Germany). Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA).

Stock standard solutions (1 mg/mL) were prepared in acetonitrile/water (40:60, v/v). Standard solutions were further obtained by serial dilutions of each stock standard solution with the same acetonitrile/water solution. The standard solutions were evaporated to dryness under vacuum at 15 mbar and 80 °C using a concentrator (Labconco, Barcelona, Spain). Finally, derivatization with butanolic HCl was accomplished before injection in the MS system.

FIA-ESI-MS Analysis. Analyses were accomplished by using a liquid chromatography system (model 1200 from Agilent Technologies, Palo Alto, CA) coupled through an orthogonal electrospray interface (model G1948B from Agilent Technologies) to a triple-quadrupole mass spectrometer (model 6410A from Agilent Technologies). Ultrapure nitrogen (99.999%) was used as collision gas, whereas pure nitrogen (97.0%) was used as nebulizer gas. Data acquisition was performed using the Mass Hunter software (version B.01.03 from Agilent Technologies). Flow injection analyses were achieved using a binary pump and introducing as the carrier solvent 60% of component A (0.1% v/v formic acid in water) and 40% of component B (0.1% v/v formic acid in acetonitrile) at a flow rate of 0.1 mL/min. A 40 cm long PEEK capillary of $^1/_{16}$ in. outer diameter and 0.13 mm inner diameter (Agilent Technologies) was used to connect the flow from the autosampler to the ion source. Injection volumes of 20 μL were used for samples and standards, and injections of 20 μL of solvent blanks were performed between samples for injector needle rinsing. The samples and standards were maintained at 4 °C in the thermostated autosampler during analysis.

The ESI source conditions were as follows: positive mode; gas temperature, 325 °C; gas flow, 10 L/min; nebulizer pressure, 20 psi; and capillary voltage, 3500 V. The tandem mass spectrometric detection was operated in the MRM mode for the highest possible selectivity and sensitivity. The dwell time was adjusted to a time of 100 ms for each mass transition between the precursor and product ion to obtain maximum sensitivity with a sufficient number of points to define the peak to quantify (≈15 points per peak). Collision-induced dissociation was performed using ultrapure nitrogen as the collision gas in the second quadrupole (Q_2) collision cell. The first quadrupole (Q1) and third quadrupole (Q3) were tuned at unit mass resolution. A summary of the optimized mass spectrometric conditions for MRM mode is provided in Table 1.

Table 1. Optimal MRM Conditions for the Studied Compounds: Transitions, Type of Transition, Percentage of Relative Ion Abundance, Fragmentor Voltage (fv), and Collision Energy (ce)

compound	transitions (m/z)	type[a]	relative ion abundance[b]	fv (V)	ce (V)
β-alanine	146.2 → 90.1	Q	100	80	5
	146.2 → 72.0	q_1	99	80	5
	146.2 → 57.1	q_2	38	80	15
proline betaine	200.2 → 144.1	Q	100	120	20
	200.2 → 58.0	q_1	20	120	25
	200.2 → 84.0	q_2	19	120	25
citrulline	232.2 → 70.0	Q	100	90	25
	232.2 → 215.2	q_1	76	90	5
	232.2 → 113.1	q_2	50	90	15
ornithine	189.3 → 70.1	Q	100	70	25
	189.3 → 172.2	q_1	39	70	5
	189.3 → 116.1	q_2	16	70	10
pyroglutamic acid	260.2 → 84.0	Q	100	80	25
	260.2 → 186.1	q_1	30	80	10
	260.2 → 130.1	q_2	23	80	15
glycine betaine	174.2 → 118.1	Q	100	120	10
	174.2 → 59.1	q_1	57	120	25
trigonelline	194.2 → 138.1	Q	100	100	15
	194.2 → 92.0	q_1	27	100	25
alloisoleucine	188.2 → 86.1	Q	100	80	5
	188.2 → 69.1	q_1	17	80	20

[a]Type of transition: Q, quantifier transition; q_1, first qualifier transition; q_2, second qualifier transition. [b]Relative ion abundance with respect to the base peak

Vegetable Oils. Nine extra virgin olive oils, three Arbequina (AEVOO), three Picual (PEVOO), and three Hojiblanca (HEVOO), and nine seed oils, three refined sunflower oils (RSO), three refined corn oils (RCO), and three refined soybean oils (RSYO), were acquired in different supermarkets (Madrid, Spain). For each variety

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and botanical origin, the trademarks were different, those that are the most consumed and marketed in Spain being selected.

Sample preparation was carried out according to the method described by Sánchez-Hernández et al.[6] Forty grams of each oil was placed in a centrifuge tube, dissolved with 160 mL of methanol/chloroform (2:1, v/v), and left at −20 °C overnight. Then, the tubes were centrifuged (4000*g*, 15 min, 4 °C). The upper phase obtained was collected in a new tube, and the rest of the sample was dissolved with 100 mL of methanol/chloroform/water (2:1:0.8, v/v/v). After its centrifugation (4000*g*, 15 min, 4 °C), the new upper phase obtained was combined with the previous one. The two combined fractions were mixed with 40 mL of chloroform and 100 mL of water, and after centrifugation (4000*g*, 15 min, 4 °C) an aqueous phase and an organic phase were obtained. The aqueous phase, containing the compounds of interest, was collected and evaporated to dryness under vacuum at 15 mbar and 80 °C in a concentrator. Finally, derivatization with butanolic HCl was accomplished before injection in the MS system.

Derivatization. The procedure consisted of a butyl ester derivatization using 3 M HCl in butanol as derivatizing agent.[7,8] Thus, 0.5 mL of derivatizing agent was added to the evaporated extract of standards, whereas up to 1 mL was added to the evaporated extract of samples to have enough agent excess. After shaking in a vortex, the reaction was performed in an oven at 80 °C during 30 min. The derivatization process was stopped, keeping the solution in the freezer during 5 min. Then, the solutions were evaporated to dryness under vacuum at 15 mbar and 80 °C in a concentrator. Finally, the analytes were reconstituted in 500 μL of acetonitrile/water (40:60, v/v).

Validation Study. The MS/MS screening method for the quantitation of nonprotein amino acids and betaines was validated for vegetable oils analysis by determining the performance characteristics required by the Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results.[22] During method validation the considered characteristics were selectivity, linearity, accuracy, precision, and detection capability (CCβ), also known as limit of detection (LOD).

The transitions from precursor to product ions of the target compounds were used to verify the selectivity. Thus, the identification of the compounds was possible using at least two MS/MS transitions to achieve the four identification points required in accordance with the Commission Decision 2002/657/EC.[22]

Linearity was tested in a range of 5−500 ng/mL by analyzing six concentration levels of standard solutions. Each point of the calibration graph corresponded to the mean value from three individual injections of the standard mixture. The obtained data were used to build the calibration curves by taking the peak area for each compound versus standard concentrations using a least-squares linear regression model.

CCβ was determined by injecting low amounts of each standard compound and considering 6 times the signal-to-noise ratio as the LOD.

The precision of the method (repeatability and intermediate precision) was assessed for the quantification transition of each compound by analyzing the oil samples. Instrumental repeatability was determined from six replicate injections in the same day; the method repeatability was established from three individual samples injected in triplicate in the same day; and the intermediate precision of the method was evaluated from three individual samples injected in triplicate in two consecutive days. Precision was expressed as relative standard deviation (RSD in percent) of the peak area.

The accuracy of the method was evaluated as the recovery obtained for each compound when samples of HEVOO were spiked with standard solutions at concentration levels covering low (0.1 ng/g of each compound) and high concentrations (10 ng/g of each compound). Three injections of each sample were analyzed. The percentage of recovery was determined from

$$\text{recovery}\ (\%) = \frac{C_{\text{spiked}} - C_{\text{initial}}}{C_{\text{added}}} \times 100$$

where C_{spiked} is the obtained concentration of the spiked samples, C_{initial} is the concentration of the sample without spiking, and C_{added} is the concentration of the added standards.

Quantitative Method. Because the presence of matrix interferences in vegetable oils was demonstrated previously by CE-UV,[6] the quantification of the samples was carried out using single-point standard addition method calibration. With this aim, two solutions were injected for each vegetable oil: (i) the sample solution and (ii) the spiked sample with a known amount of nonprotein amino acids and betaines. Seed oil samples were spiked with 100 ng/mL of each nonprotein amino acid (except in the case of citrulline, for which 50 ng/mL was added) and 50 ng/mL of glycine betaine, 25 ng/mL of trigonelline, and 25 ng/mL of proline betaine. Extra virgin olive oil samples were spiked with 20 ng/mL of each nonprotein amino acid (except for citrulline, for which 10 ng/mL was added), 10 ng/mL of glycine betaine, 5 ng/mL of trigonelline, and 5 ng/mL of proline betaine.

Statistical Analysis. The data sets for ANOVA test and multivariate statistical analysis were processed using Statgraphics 5.1 software. The ANOVA test allows evaluation of the linearity of the experimental data. It was carried out from the data of calibration curves of each studied compound. Multivariate statistical analysis was used for the statistical classification of the botanical oil origin ,where the variability of data values was tested by discriminant function analysis. With this aim, the data employed were the type of each sample according to its botanical origin (RSYO, RSO, RCO, HEVOO, AEVOO, PEVOO, or adulterated olive oils) and the concentration, in ng/g, of the identified compounds (citrulline, ornithine, pyroglutamic acid, glycine betaine, and trigonelline).

RESULTS AND DISCUSSION

Development of the Screening Method. A method based on a strategy of screening and quantitation was developed in this work. In this sense, the screening method provides full information, enabling the analyte to be identified at the level of interest.[22]

In conformity with Directive 96/23/EC,[23] only those analytical techniques having a false-compliant rate of <5% (β-error) at the level of interest shall be used for screening purposes. If no permitted limit has been established for a substance (substances with "zero tolerance"), as in the case of the compounds to be considered as markers of adulteration, it is necessary to develop methods enabling the identification of these compounds at the lowest levels possible. For this reason, we have decided to develop a quantitative screening method based on a FIA-ESI-MS/MS system with QqQ working in MRM mode, as it is possibly one of the most sensitive and selective analytical methods.

MS experiments were first achieved to obtain the maximum intensity of the precursor ions to work in the MRM mode. Standard solutions of each compound (1 ng/mL) were individually injected to optimize some MS parameters in MS mode. Thus, dry gas temperature and the capillary voltage were modified between 250 and 325 °C and between 2500 and 4000 V, respectively. With higher values of temperature and voltage, better ionizations were achieved. being 325 °C and 3500 V the optimized values as a compromise among the different compounds studied. Second, the fragmentor voltage (between 60 and 140 V in steps of 10 V) was adjusted for each compound. All full-scan mass spectra were dominated by the protonated molecule (MH^+) of each derivatized amino acid (amino acid butyl ester) or molecular ion (M^+) of each derivatized betaine (betaine butyl ester). Third, the collision energies (between 5 and 25 V in steps of 5 V) were optimized for each precursor/product transition from product ions spectra using the MS/MS mode.

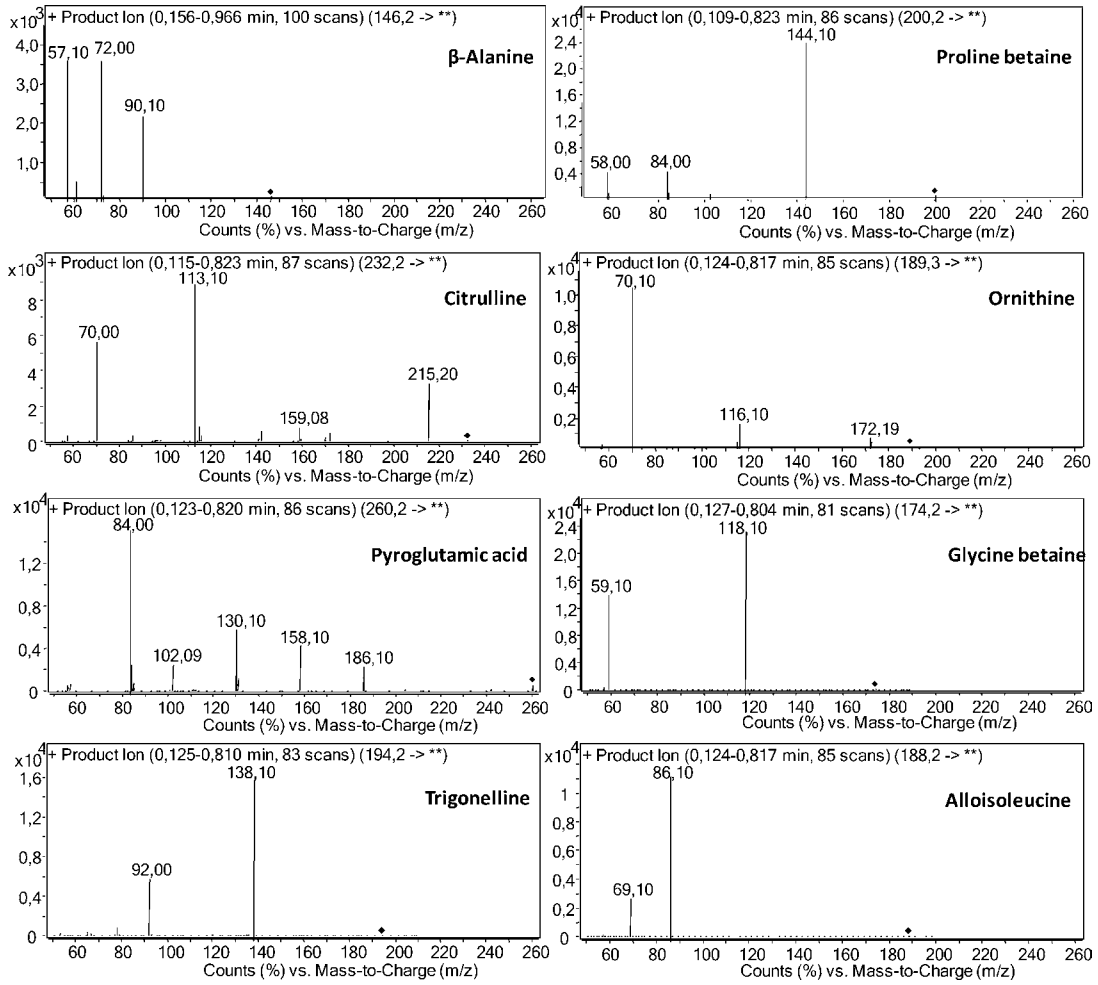

Figure 2. Product ion mass spectra (positive mode) of individual standard solutions of 1 μg/mL of β-alanine (fv, 80; ce, 15 V), proline betaine (fv, 120; ce, 25 V), citrulline (fv, 90; ce, 15 V), ornithine (fv, 70; ce, 15 V), pyroglutamic acid (fv, 80; ce, 15 V), glycine betaine (fv, 120; ce, 25 V), trigonelline (fv, 100; ce, 25 V), and alloisoleucine (fv, 80; ce, 20 V). Other conditions are indicated under Materials and Methods. fv, fragmentor voltage; ce, collision energy.

Figure 2 shows the product ion mass spectra of the studied compounds for the optimal values of fragmentor voltage and the most representative values of collision energy to illustrate a characteristic product ions mass spectrum for each compound. Note that although the precursor ions were the same as those obtained in our previous works using an IT as analyzer,[7,8] the product ions were in some cases different. Thus, for β-alanine, three transitions were obtained with a new product ion of m/z 57. For proline betaine and citrulline, better selectivity was reached because two product ions (m/z 58 and 84) were new for proline betaine and three product ions (m/z 70, 113, and 159) were new for citrulline. In addition, with this methodology, the most abundant ion for citrulline corresponded to m/z 70 instead of m/z 215. Similar results were observed for ornithine and pyroglutamic acid, for which the most abundant transitions were those with the lowest product ions (at m/z 70 for ornithine instead of m/z 172 and at m/z 84 for pyroglutamic acid instead of m/z 186). Also, although pyroglutamic acid showed again five product ions, the product ion m/z 102 was obtained instead of m/z 242. Finally, in the case of glycine betaine, trigonelline, and alloisoleucine two transitions were obtained, the product ions of m/z 59 and 92 being new for glycine betaine and trigonelline, respectively. In conclusion, all MS/MS spectra obtained with the QqQ were richer in product ions than those obtained by IT, providing more information of the studied compounds. This could be explained because the energy of fragmentation in a QqQ MS is higher than in an IT MS.

From these results, three transitions of precursor ion to product ion were selected and monitored for each compound to optimize the analysis by MRM and to verify the presence of the compounds. The exceptions were alloisoleucine, trigonelline, and glycine betaine, for which only two transitions could be monitored. In this sense, the transition with the most abundant product ion was designated the quantifier transition and was used to calculate the concentration of the compounds. Meanwhile, the other transitions corresponding to the other product ions were used to verify the presence of the compounds (qualifier transitions). Thus, the identification of compounds in the samples was achieved only when the two aspects were corroborated. First, both transitions (quantifiers and qualifiers) were detected. Second, between the transitions, the same relative intensities of ions obtained by standard solutions analyzed on the same day were shown, within a margin of tolerance in accordance with the requirements set by Commission Decision 2002/657/EC for mass spectrometric techniques.[22] The optimized mass parameters in MRM mode for each transition monitored are reported in Table 1.

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In the validation study, as described under Materials and Methods, excellent selectivity was obtained in MRM mode for all compounds because their identification was possible using at least two MS/MS transitions to achieve the four identification points required in accordance with Commission Decision 2002/657/EC.[22]

Taking the peak area for each compound versus standard concentrations, good linearity for calibration curves was observed for all studied compounds at the concentrations checked with adequate determination coefficients ($R^2 > 0.99$) as can be seen in Table 2. ANOVA tests enabled confirmation that experimental data fit properly to linear models for each compound. Thus, the test obtained from the calibration curves by the Statgraphics software showed that the lack of fit was always statistically smaller than the pure error with p values of ≤ 0.05. Therefore, the intercept was considered not different from 0 with a confidence level of 95%.

Table 2. Linearity and LODs for the MRM Developed Method

	linearity			LOD (S/N = 6)	
compound	slope[a]	Δintercept[b]	R^2	ng/mL	pg/g
β-alanine	283	3184 ± 3453	0.996	5.0	62.5
proline betaine	866	3036 ± 4811	0.990	1.0	12.5
citrulline	580	2583 ± 6706	0.996	5.0	62.5
ornithine	405	4975 ± 5339	0.996	5.0	62.5
pyroglutamic	598	12956 ± 14233	0.992	2.0	25.0
glycine betaine	1309	8390 ± 13334	0.997	1.0	12.5
trigonelline	1165	3699 ± 9374	0.998	1.0	12.5
alloisoleucine	1311	3087 ± 7171	0.995	2.0	25.0

[a]Counts mL/ng × min. [b]Confidence intervals at 95%: $\pm t \times s_{intercept}$.

Excellent values of LOD were reached at ng/mL levels in the measured solutions and at pg/g levels in the samples. The results for each compound are reported in Table 2. Thus, LODs ranging from 1.0 to 5.0 ng/mL or from 12.5 to 62.5 pg/g were achieved. According to the LOD definitions, if the concentrations of the studied compounds are lower than their LODs, this method has a remaining risk of making a false decision (false compliant or β-error) of <5%, which is a requirement that must meet any screening method in conformity with Council Directive 96/23/EC.[23]

Good precision was obtained, with values of RSD for peak areas which were <3% for those compounds that were detected in the oils (Table 3). The values of β-alanine, alloisoleucine, and proline betaine were not considered because the relative ion intensities of their qualifier ions were out of the confidence intervals set by Commission Decision 2002/657/EC for mass spectrometric techniques.[22]

Finally, values of recovery ranging from 81 to 95% with standard deviations of ≤1% were obtained for samples of olive oil spiked at low (0.1 ng/g of each compound) and high levels (10 ng/g of each compound) as can be seen in Table 3 for HEVOO-1. Again the values of β-alanine, alloisoleucine, and proline betaine were not included in the table because their qualifier ions were outside the limit.

Quantitation of Nonprotein Amino Acids and Betaines in Vegetable Oils. The developed method in MRM mode was applied to the quantitation of the selected nonprotein amino acids and betaines in vegetable oils. Once all of the samples had been analyzed by the screening method, it was observed that the first qualifier ion for β-alanine, the qualifier ion for alloisoleucine, and both qualifier ions for proline betaine were not verifiable because they were present but their relative ion intensities were outside the confidence interval limits. Therefore, only the verified compounds (citrulline, ornithine, pyroglutamic acid, glycine betaine, and trigonelline) were quantified in the samples (Table 4) as indicated under Materials and Methods.

The highest contents were obtained for pyroglutamic acid and glycine betaine and the lowest for citrulline, this being about 40 times lower. Citrulline was first detected in these oils due to the excellent sensitivity of the screening method developed. These results allowed us to confirm the traceability of citrulline from seeds to their corresponding oils, which was not possible previously.[7] For all other compounds, their traceability was reiterated, except for alloisoleucine and β-alanine, the presence of which was not verified.

In extra virgin olive oils, pyroglutamic acid, glycine betaine, and trigonelline were determined, obtaining much lower amounts than in seeds oils (about 18, 55, and 40 times lower, respectively). Moreover, the amount of trigonelline was significantly lower than the other two compounds. On the basis of the results obtained from seed oils, it is expected that the presence of these compounds in extra virgin olive oils comes from olives. However, arguably they are in very low concentration despite the fact that HEVOO extraction is performed by mechanical processes rather than by refining as seeds. Note that the contents of ornithine and citrulline were lower than the LOD in the analyzed olive oils. Therefore, the presence of one of these two compounds could serve as a marker of adulteration of olive oil with seed oils, but taking into account the higher concentration of ornithine in the seed oil (about 10 times higher than citrulline), this nonprotein amino acid could be an ideal candidate to detect smaller amounts of adulteration.

Table 3. Precision in RSD (Percent) in Peak Area for Samples of Sunflower Oil (RSO-1) by the MRM Method and Recovery (Average ± Standard Deviation, $n = 3$) for Samples of Olive Oil (HEVOO-1) Spiked at Low Level (0.1 ng/g of Each Compound) and at High Level (10 ng/g of Each Compound)

	citrulline	ornithine	pyroglutamic acid	glycine betaine	trigonelline
precision					
instrumental repeatability ($n = 6$)	1.9	1.9	1.0	1.7	1.3
method repeatability ($n = 3$)	2.0	2.5	1.3	1.5	2.1
intermediate precision ($n = 6$)	2.7	2.6	1.6	1.5	2.9
recovery at					
low level	84.3 ± 0.7	87.9 ± 0.9	90.1 ± 0.5	85.8 ± 0.9	83.2 ± 0.8
high level	85.1 ± 0.7	81.4 ± 0.3	85.1 ± 0.5	94.9 ± 0.9	82.8 ± 0.4

Table 4. Botanical Origin, Names of the Vegetable Oil Samples Analyzed in This Work, and Their Quantitation (Average in ng/g ± Standard Deviation, n = 3) Obtained by FIA-ESI-MS/MS

origin	sample	citrulline	ornithine	pyroglutamic acid	glycine betaine	trigonelline
sunflower oil	RSO-1	0.15 ± 0.01	2.9 ± 0.2	5.9 ± 0.3	9 ± 1	5 ± 1
	RSO-2	0.16 ± 0.01	1.1 ± 0.1	4.9 ± 0.9	10 ± 2	6 ± 1
	RSO-3	0.16 ± 0.01	1.4 ± 0.2	6.8 ± 0.1	13 ± 1	7 ± 1
corn oil	RCO-1	0.17 ± 0.01	1.4 ± 0.3	5.3 ± 0.4	4.1 ± 0.5	0.3 ± 0.1
	RCO-2	0.19 ± 0.02	1.2 ± 0.8	7.2 ± 0.1	8.6 ± 0.2	1.2 ± 0.2
	RCO-3	0.20 ± 0.01	1.6 ± 0.1	5.4 ± 0.1	5.0 ± 0.5	0.5 ± 0.1
soybean oil	RSYO-1	0.23 ± 0.01	2.2 ± 0.1	13.6 ± 0.7	4.7 ± 0.9	0.5 ± 0.1
	RSYO-2	0.24 ± 0.01	1.1 ± 0.2	10.1 ± 0.3	10.2 ± 0.3	1.0 ± 0.1
	RSYO-3	0.23 ± 0.02	1.2 ± 0.1	12.7 ± 0.9	5.2 ± 0.4	0.7 ± 0.1
Hojiblanca extra virgin olive oil	HEVOO-1	nd[a]	nd	0.54 ± 0.02	0.13 ± 0.01	0.051 ± 0.005
	HEVOO-2			0.71 ± 0.03	0.11 ± 0.01	0.049 ± 0.005
	HEVOO-3			0.18 ± 0.02	0.10 ± 0.03	0.051 ± 0.005
Arbequina extra virgin olive oil	AEVOO-1	nd	nd	0.86 ± 0.03	0.14 ± 0.02	0.09 ± 0.01
	AEVOO-2			0.82 ± 0.01	0.21 ± 0.02	0.059 ± 0.005
	AEVOO-3			0.15 ± 0.01	0.20 ± 0.01	0.088 ± 0.001
Picual extra virgin olive oil	PEVOO-1	nd	nd	0.39 ± 0.01	0.11 ± 0.01	0.053 ± 0.001
	PEVOO-2			0.14 ± 0.02	0.12 ± 0.07	0.073 ± 0.009
	PEVOO-3			0.18 ± 0.03	0.13 ± 0.01	0.055 ± 0.005
mixtures of HEVOO-1 with RSYO-3	10%	nd	0.21 ± 0.01	1.80 ± 0.08	0.65 ± 0.02	0.14 ± 0.01
	5%		0.15 ± 0.02	1.09 ± 0.05	0.44 ± 0.02	0.10 ± 0.02
	2%		0.10 ± 0.06	0.68 ± 0.02	0.25 ± 0.01	0.067 ± 0.002

[a]nd, not detected.

Thus, the usefulness of the developed method was evaluated by analyzing mixtures of olive oil samples with seed oils such as soybean oil. These samples were selected as representative samples to compare the results with those achieved by a previous separation step.[7,8] Percentages of 2, 5, and 10% w/w corroborated the presence of ornithine in the olive oil mixtures and the increase of pyroglutamic acid, glycine betaine, and trigonelline concentrations (Table 4). However, citrulline was not detected in the mixtures due to its low concentration in the samples, a higher percentage of mixture (around 50% w/w) being necessary to be quantified. According to the different contents of ornithine in the seed oils studied (between 1.1 and 2.9 ng/g), the minimum amount of seed oil that it is possible to detect could be between about 2 and 6%. These percentages could even be lower if it is considered that the content of ornithine in olive oils may not be zero, just below the LOD, and therefore could contribute to the total content of ornithine when an adulteration exists. Thus, note that the concentrations of ornithine in the blended samples (between HEVOO-1 and RSYO-3 in Table 4) were higher than expected. This higher concentration than that expected by simple dilution may be due to the contribution of ornithine in the oil at a concentration below the LOD.

In any case, ornithine was corroborated as a marker for olive oil adulterations with other vegetable oils at percentages below 10%, which is adequate because, in the case of fraud, adulterations of >10% would be made to obtain some profit. In addition, identification of ornithine was confirmed through its three precursor/product transitions in real samples (Figure 3). From these results, it can be stated that the developed

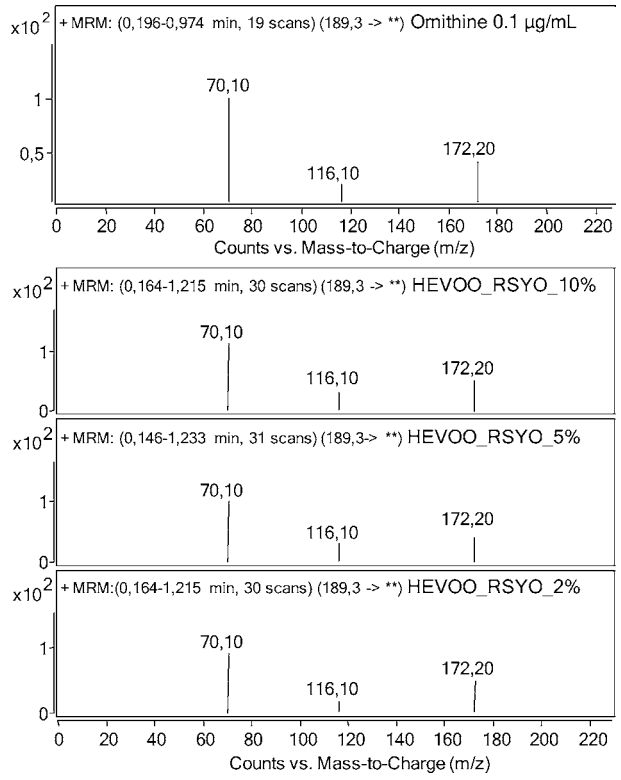

Figure 3. MRM spectra for ornithine in a standard solution (0.1 µg/mL) and in the oil mixtures of HEVOO-1 with 2, 5, and 10% w/w of RSYO-3. Other conditions are indicated under Materials and Methods.

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method can be considered a sensitive method for the quality evaluation of extra virgin olive oils, permitting their authentication using a unique compound, such as ornithine, without the necessity of analyzing a high number of samples to use chemometric tools.

Comparison with the Results Obtained Using a Previous Separation Step. Results obtained by the FIA-MS/MS method with QqQ were in excellent agreement with those obtained by CE-MS/MS with IT for the samples analyzed in this work,[7,8] carrying out the same sample treatment in both cases. This corroborates the accuracy of both methods.

Analysis by CE-IT provided fundamental information about the studied nonprotein amino acids and betaines in vegetable oils and about the authenticity of the virgin olive oils analyzed.[7,8] However, the developed strategy by FIA-QqQ was more rapid, precis, and sensitive. Thus, without a separation step, analysis time was only 2 min for all compounds, whereas, with a separation step (CE), times of about 12 min for betaines[8] and 15 min for amino acids[7] were attained. A better precision was obtained with the FIA-QqQ system with values of <3% compared to almost 11% with CE-IT. Finally, lower LODs were obtained by FIA-QqQ (between 2 and 8 times) for all compounds studied except for ornithine and β-alanine, for which similar LODs were achieved. However, because FIA-QqQ is not a separative method, it presents the disadvantage of the worst selectivity, and some interference could not be avoided. Thus, in the case of nonprotein amino acids, whereas ornithine and alloisoleucine were proposed as markers by CE-IT,[7] ornithine was corroborated by FIA-QqQ as marker but alloisoleucine identification was not confirmed.

In conclusion, the screening methodology proposed allows checking the adulteration of the olive oils with the seed oils analyzed using ornithine as a marker, whereas the CE-MS/MS with IT methodology could be used as a confirmatory method in the case of a suspected noncompliant result.

Botanical Origin Classification of Vegetable Oils. Although the reported fingerprinting methods without any separation step are fast and simple, some plant oils have similar fingerprints, and the comparison of concentrations for the studied compounds in pure and adulterated samples is not often sufficient proof for their authentication because of the complexity of the data matrix, a statistical evaluation being necessary for the discrimination of different samples.

The developed method characterizes the studied compounds in vegetable oil samples and allows to detect adulterations in extra virgin olive oils without requiring chemometric tools. However, in order to mathematically test the feasibility and good possibilities of the developed method, it was evaluated, by multivariate analysis, for the statistical classification of the botanical origin of oils. Thus, a discriminant function analysis was applied to the compound data to distinguish the oil samples (seed oils, olive oils and mixtures of them) obtaining three discriminant functions for the five variables selected (citrulline, ornithine, pyroglutamic acid, glycine betaine, and trigonelline). As shown in Figure 4 a complete separation of four groups was achieved by functions 2 and 1, and the recognition ability was 100% for each class. Thus, the three kinds of seed oils (sunflower, corn, and soybean) were classified in three different groups, whereas the three varieties of olive oils were grouped. On the other hand, the mixtures of extra virgin olive oil with soybean oil were adequately characterized between the soybean oil and olive oil groups. Among 21 samples analyzed, no sample was incorrectly classified. confirming the usefulness of the developed methodology.

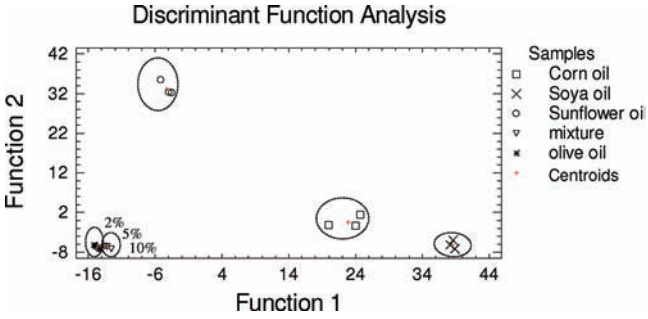

Figure 4. Discriminant function analysis on ESI-MS/MS data of the oil samples: sunflower oils, corn oils, soybean oils, olive oils, and oil mixtures with 2, 5, and 10% w/w of seed oils.

Conclusions. Results obtained in this work show the potential of tandem mass spectrometry with automated flow injection analysis in the quality control of olive oils. The presence of at least two transitions in MRM mode for each compound allowed the simultaneous characterization of the studied nonprotein amino acids and betaines. Validation data revealed that the method yielded reliable and reproducible results. Excellent LODs (at ppb level) were obtained. The developed method was applied to vegetable oils by determining glycine betaine, ornithine, trigonelline, citrulline, and pyroglutamic acid, whereas proline betaine, β-alanine, and alloisoleucine could not be verified by this method in samples. Concentrations of ornithine and citrulline in olive oils were below the LOD, whereas the analysis of mixtures of vegetable oils enabled ornithine to be corroborated as a marker for the detection of adulteration in the olive oils analyzed. Multivariate analysis evaluation showed the statistical reliability of the method, allowing us to classify the botanical origin for all sample oils reaching 100% of differentiation. The proposed method is selective and sensitive and enables the elimination of the separation step. Thus, the method proposed in this work has shown to be an interesting tool for olive oil authentication.

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