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DETERMINATION OF BETAINES IN VEGETABLE OILS BY CAPILLARY ELECTROPHORESIS TANDEM MASS SPECTROMETRY. APPLICATION TO THE DETECTION OF OLIVE OIL ADULTERATION WITH SEED OILS

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Running title: determination of betaines in vegetable oils by CE-MS/MS

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Abstract

A capillary electrophoresis-tandem mass spectrometry methodology enabling the simultaneous determination of betaines (glycine betaine, trigonelline, proline betaine and total content of carnitines) in vegetable oils was developed. Betaines were derivatized with butanol previous to their baseline separation in 10 min using a 0.1 M formic acid buffer at pH 2.0. Ion trap conditions were optimized in order to maximize selectivity and sensitivity. Analytical characteristics of the proposed method were established by evaluating its selectivity, linearity, precision (RSDs ranged from 4.8% to 10.7% for corrected peak areas), accuracy by means of recovery studies (from 80% to 99%) and LODs and LOQs at 0.1 ppb level. The method was applied to the determination of the selected betaines in seed oils and extra virgin olive oils. MS^2 experiments provided the fingerprint fragmentation for all betaines studied in seed oils. In extra virgin olive oils, carnitines were not detected being possible to propose them as a feasible novel marker for the detection of adulterations of olive oils. Application of the developed method to the analysis of different mixtures of extra virgin olive oil with seed oil (between 2-10 %) enabled the detection and quantitation of the total content of carnitines. The results obtained show the high potential of the developed method for the authentication and quality control of olive oils.

1. Introduction

Olive oil shows outstanding characteristics due to its differentiated sensorial qualities and its high nutritional value [1]. Different studies have shown the relationship between the consumption of olive oil and its protective effect against cancer (especially colorectum and breast cancer) and cardiovascular disease [2-5]. Because of its high price, extra virgin olive oil is often illegally adulterated by fraudulent producers with cheaper oils such as sunflower, corn or soybean oils [6, 7]. However, the definition of virgin oils established in the European regulations excludes the mixture with oils of other kinds [8]. For these reason, the fight against olive oil adulteration is a relevant aspect to determine the authenticity and quality of edible oils.

Despite that different spectrometric techniques in conjunction with multivariate parametric analysis have been applied to establish oil authenticity [9], most of the current works on edible oil adulteration employ chromatographic analysis [6]. Coupling these techniques with MS detection provides a powerful technique for the unequivocal determination of particular compounds in oils. Thus, different methodologies by GC-MS [10, 11] and HPLC-MS [1, 12-14] have been reported in the literature for the authentication of olive oils using as markers triacylglycerols or fatty acids (the main compounds of any edible oil). Although the unsaponifiable fraction, which makes up around 2-5 % of all oils, has been less studied, there are also different methods by GC-MS and HPLC-MS described in the literature, that consider volatile compounds [15], aliphatic and triterpene alcohols [16], or tocopherols and sterols [13, 17] as markers for the detection of adulterations. However, most of these methodologies need sophisticated chemometric tools to interpret the data and differentiate the adulterated samples from pure olive oils. Recently, our research group proposed for the first time the use of non-protein amino acids as novel markers for the detection of adulterations of olive oils with

seed oils [18]. In fact, the development of a CE-MS^2 methodology enabling the identification and determination of six non-protein amino acids in vegetable oils allowed to propose ornithine and alloisoleucine as markers for the detection of adulterations of olive oils.

Betaines could also be included among the variety of substances of different structure that makes up the unsaponifiable fraction of oils. They are highly polar zwitterionic molecules possessing a quaternary ammonium group with a permanent positive charge and a carboxylic group. These compounds are known to be one of the major osmoregulating compounds accumulated in many plants in response to environmental stress [19]. In mammals, betaines act as osmolytes in most tissues regulation [20-22] and as source of methyl groups for methylation of homocysteine to originate methionine. The glycine betaine, proline betaine and trigonelline concentration of a wide range of foods has been surveyed by HPLC-UV [23], LC-MS [24] and CE-UV [25]. However, only the presence of glycine betaine was detected in a concentration of 0.11 mg/100 g olive oil [24] whereas proline betaine and trigonelline were not detected [23, 25]. On the other hand, trigonelline has been identified in sunflower seeds and soybean seeds by CE-UV [25], UV-vis spectrophotometry [26] and HPLC-UV [27], while in corn seeds, both trigonelline and glycine betaine have been identified by HPLC-UV [23] and MS [28-30]. With respect to carnitine, it has been radioisotopic determined in olives, showing a concentration of 0.5 mg/100 g [31]. Based on the results obtained by our research group using CE with UV detection on the presence of trigonelline in soybean and sunflower oils, but not in olive oils, trigonelline was proposed as a marker for the detection of adulterations of olive oils with seed oils [25].

The purpose of the present study was to investigate the potential of betaines as novel markers of adulterations of olive oils with seeds oils through the development of a

sensitive CE-MS methodology enabling the simultaneous determination of betaines in vegetable oils (seeds and olive oils).

2. Materials and method

2.1 Chemicals and samples

All reagents were of analytical grade. Methanol and chloroform (used for sample extraction) and isopropanol (used for sheath liquid preparation) were supplied from Scharlau Chemie (Barcelona, Spain). Sodium hydroxide and hydrochloric acid from Merck (Darmstadt, Germany) were used to rinse the capillary. Formic acid from Riedel-de Häen (Seelze, Germany) was used to prepare CE running buffer whose pH was adjusted with 25 % ammonium hydroxide solution from Merck. Hydrogen chloride/1-butanol solution from Fluka (Buchs, Switzerland) was used for betaines derivatization. Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

Trigonelline, glycine betaine, carnitine, acetylcarnitine and palmitoylcarnitine were supplied from Sigma (St. Louis, MO, USA). Proline Betaine was from Hallochem Pharma (Chongqing, China). Arbequina, Picual, and Hojiblanca extra virgin olive oils, refined sunflower oils, refined corn oils and refined soybean oils were acquired in different supermarkets (Madrid, Spain) from different trademarks.

2.2 Preparation of solutions

The separation buffer was 0.1 M formic acid adjusted with 25% (v/v) ammonium hydroxide solution to reach pH 2.0.

Stock standard solutions were prepared by dissolving 1 mg/mL of each betaine in acetonitrile/water (40:60, v/v) and diluting them to get a mixture of betaines at the desired

concentration. These solutions were stored at room temperature before use and 500 µL were evaporated at 80 ºC and 15 mbar in epperdorf tubes before their derivatization.

Sample preparation was carried out considering our previous method [25]. Briefly, 40 g of vegetable oils were weighed and extracted with 160 mL of methanol:chloroform (2:1, v/v) and left at -20ºC overnight. After centrifugation (4000g, 15 min, 4 ºC) the upper phase was collected in a new tube and the bottom phase was washed with 100 mL of methanol/chloroform/water (2:1:0.8, v/v/v), obtaining a new upper phase which was combined with the previous one. Then, the mixed fractions were washed with 40 mL of chloroform and 100 mL of water and centrifuged (4000g, 15 min, 4 ºC). The aqueous phase was separated for its evaporation at 80 ºC to dryness and finally it was derivatized with butanol before injection in the CE system.

2.3 Derivatization procedure

Butyl ester derivatization of betaines was carried out following a reported procedure which was slightly modified [32]. Thus, 0.5 ml or 1 ml of the butanol derivatizing agent (3 N HCl in butanol) was added to the evaporated extract of standards or samples, respectively and shaken in a vortex. The reaction was carried out in an oven at 80 ºC during 30 min. The derivatization process was stopped keeping the solution in the freezer during 5 min. Then, the excess of the derivatizing agent was evaporated in a concentrator at 80 ºC. Finally, the analytes were reconstituted in 500 µl of acetonitrile/water (40:60, v/v).

2.4 CE-MS conditions

A HP^{3D}CE instrument (Agilent Technologies, Palo Alto, CA, USA) coupled through an orthogonal electrospray interface (ESI, model G1607A from Agilent

Technologies) to a 3D Ion Trap mass spectrometer (model 1100 from Agilent Technologies) was employed. MS control and data analysis were carried out using LC/MSD Trap Software 5.2. Separations were performed using uncoated fused-silica capillaries of 50 µm id with a total length of 60 cm, which were purchased from Composite Metal Services (Worcester, England). Before first use, the capillary was conditioned with 1 M NaOH for 20 min, followed by water for 5 min, 0.1 M HCl for 5 min and finally the separation buffer for 30 min, in all cases at a 1 bar pressure. Between injections, the capillary was rinsed with the buffer solution for 2 min. The capillary temperature was 25 ºC, the injections were made at the anodic end by pressure (50 mbar for 50 s), and the applied voltage was 25 kV.

Electrical contact at the electrospray needle tip was established via a sheath liquid which consisted of isopropanol:water (50:50, v:v) containing 0.1 % formic acid and delivered at a flow rate of 3.3 µL/min by a syringe pump (model 100, Holliston, USA) with SGE syringe of 10 mL from Supelco (Bellefonte, PA, USA). The nebulizer pressure and flow, and the drying gas temperature were 2 psi N_2 and 3 L/min N_2 at 300 ºC, respectively. The mass spectrometer operated with the ESI source in the positive ion mode (4.5 kV) and it scanned at 50-280 *m/z* range. The trap parameters were programmed in smart mode using values of compound stability and trap drive level of 50 and 100 %, respectively. The ion charge control mode operated to accumulate 100000 ions for a maximum accumulation time of 300 ms with one scan. The fragmentation in the ion trap was performed for 10 ms with fragmentation amplitude of 1.00 V and isolation width of 4.0 m/z to obtain MS^2 spectra during the run in Multiple Reaction Monitoring (MRM) mode. Extracted ion electropherograms (EIEs) were obtained using the smoothed option of the software (Gauss at 1 point).

3. Results and discussion

3.1 Optimization of derivatization conditions for betaines

The derivatization of compounds that contain mono- and dicarboxylic acid groups using butanol as derivatizing agent not only greatly improves ionization efficiencies and hence analytical sensitivity [33], but also improves the mass differentiation among the analytes increasing the selectivity. As a consequence, butanol was employed in this work as derivatizing agent for betaines in order to develop a CE-MS^2 methodology enabling their sensitive determination in vegetable oils.

The efficiency of the derivatization reaction was optimized monitoring by UV detection the percentage of the derivatization achieved for trigonelline (betaine with a chromophore group). To obtain the maximum percentage, the temperature and the derivatization time were varied from 60 to 100 ºC (butanol boiling point, 117.73 ºC) and from 10 to 30 min, respectively. A percentage of 100 % for the derivatization reaction was obtained when the temperature increased up to 80 ºC. Figure 1 shows that when a percentage of derivatization of 62 % was achieved (at 60 ºC), two peaks corresponding to derivatized and underivatized trigonelline appeared in the electropherogram (Figure 1a). However, at 80 ºC (100 % derivatization reaction), only one peak, corresponding to derivatized trigonelline was observed (Figure 1b). On the other hand, the variation of the reaction time from 30 to 10 min decreased the percentage up to 55 %. As a consequence, 80 ºC and 30 min were chosen as optimal conditions enabling to reach values of 100% derivatization for trigonelline.

3.2 Identification of betaines

First, the individual identification of the different derivatized betaines in MS and MS^2 modes was carried out. Six different betaines (glycine betaine, trigonelline, proline

betaine, carnitine, acetylcarnitine and palmitoylcarnitine) were chosen since they are present in a high number of foods as mentioned in the introduction. According to our previous work [25] and taking into account the cationic nature of betaines, the running buffer was 0.1 M formic acid at pH 2.0 which makes negligible the electroosmotic flow and the interaction of these analytes with the capillary wall. Mass spectra obtained using MS experiments enabled to identify all the derivatized betaines. However, the derivatization of acetylcarnitine and palmytoilcarnitine produced non-expected molecular ions at *m/z* 218 due to their degradation to carnitine. For this reason, the differentiation between carnitine and other acylcarnitines was not possible, and a total content of carnitines was determined.

The fragmentation of the molecular ions determined previously in the MS mode was carried out. Figure 2 depicts the MS^2 spectra and the proposed structures of the derivatized betaines. Neutral losses of m/z 56 corresponding to the derivatizing agent ($CH_3CH_2CH=CH_2$) took place for all betaines. In addition, in the case of carnitines neutral losses of m/z 59 of the quaternary ammonium group and m/z 18 of a water molecule were observed. It should be noted that the molecular ions for all the studied compounds have sizes $\geq$ 150 *m/z*, due to the formation of the butyl derivative of the betaines, where the MS background noise is usually lower [34].

3.3 Simultaneous separation of betaines by CE-MS

Using the CE-MS mode and a capillary with a total length of 85 cm, the separation of the studied compounds was achieved in 18 min showing the following order of elution: glycine betaine, trigonelline, proline betaine and carnitines. The values of resolution between adjacent peaks were 4, 3 and 15, respectively. Then, the capillary length was reduced to 60 cm, which is the minimum length needed for CE-MS hyphenation, in order

to decrease the analysis time. Figure 3a shows the base-peak electropherogram (BPE) obtained for the mixture of betaines using the short capillary. The analysis time was reduced up to 10 min with all peaks at least baseline resolved.

To carry out the simultaneous identification of betaines in one run, MS^2 experiment in MRM mode was employed, which also allowed to improve the selectivity and sensitivity. Thus, to obtain good sensitivity (S/N ratio) with enough precision, the ion trap parameters were optimized to achieve at least 10-15 points per peak. Different parameters, such as the maximum accumulation time (ranging from 50 to 300 ms), the number of scans averaged (from 1 to 3), and the fragmentation time (from 10 to 40 ms) were investigated. The optimal conditions enabling to increase the S/N ratio and precision were set at 300 ms for the maximum accumulation time, one scan, and 10 ms for the fragmentation time. Using these values, at least 12 points per peak with a precision about 10 % were obtained.

Finally, a stacking sample preconcentration following our previous work [25] was carried out to increase the sensitivity for sample analysis. Thus, the reconstitution of the samples in acetonitrile/water (40:60, v/v) after the derivatization reaction, and a hydrodynamic injection of 50 s were employed. Figure 3b shows the MS^2 electropherograms for glycine betaine, trigonelline, proline betaine, and total content of carnitines in MRM mode under the optimal conditions.

3.4 Study of the analytical characteristics of the developed CE-MS^2 method

Selectivity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ) of the developed method were established in order to show its suitability for the determination of betaines in vegetables oils.

A good selectivity was obtained since the analysis of betaines was possible monitoring one precursor-product ion transition by MS^2 experiments for all compounds (except in the case of carnitine, where three transitions were obtained). In addition, a resolution higher than base-line separation was achieved (see Figure 3a).

Linearity was established by plotting the corrected peak areas (Ac, peak area to migration time ratio) from the EIEs as a function of the concentration for each betaine using the external standard calibration method. The correlation coefficient (r), the intercept, and the slope are grouped in Table 1. Satisfactory results were obtained in terms of linearity with a correlation coefficient > 0.99 for the average calibration plot, and with all the confidence intervals at 95% for intercept, including the zero value.

Precision was evaluated considering the instrumental and method repeatability as well as the intermediate precision (see Table 1) for a sample of a seed oil (sunflower oil (RSO-1)). The values of relative standard deviations (RSDs in %) for corrected peak areas were always lower than 8 % for repeatability and lower than 11 % for intermediate precision, except for proline betaine which was not detected in the samples.

To test the accuracy, a recovery study was carried out by spiking a representative extra virgin olive oil sample (HEVOO-1) with 5 and 50 ng of each betaine and injecting the samples in triplicate. Values of recovery ranging from 80% to 99% with RSDs $\leq$ 5% were obtained as shown in Table 1.

Finally, LODs and LOQs for betaines were calculated as the minimum analyte concentration yielding a S/N ratio equal to 6 and 10 times, respectively [35]. Using this definition for the LOD, the "α-error" (deciding that the component is present when it is not) and "β-error" (deciding that the component is absent when it is present) are well

balanced (only 5 %). According to this definition, Table 1 groups the values for LOD and LOQ obtained for the solutions previous to their derivatization.

3.5 Quantitation of betaines in vegetable oils.

The determination of the studied betaines in vegetable oils (three different samples of each kind of seed oils and nine different samples of three different varieties of extra virgin olive oil) was performed using the MRM which enabled to obtain an improvement in the sensitivity and selectivity of the method.

To carry out the quantification of the samples, the single-point standard addition calibration was employed. Using this methodology, it is only necessary the injection of two samples solutions for each vegetable oil sample, i.e. the sample solution and the spiked sample containing a known amount of betaines (0.1 µg/mL of each one). The content of each betaine determined in the different samples is presented in Table 2**.** Although the content of proline betaine was not detected in any of the analyzed samples, it is important to highlight that is the first time that glycine betaine and total content of carnitines have been determined in soybean, corn, and sunflower oils. As Table 2 shows, the highest content was obtained for glycine betaine in all seed oils. The values obtained for the content of trigonelline and carnitines were similar, except in sunflower oils where trigonelline was about 2 times higher than the content of total carnitines. Regarding extra virgin olive oils, the amount of glycine betaine and trigonelline was around 40 and 30 times lower than the amount obtained in seed oils, respectively. The results obtained for trigonelline, differ from those obtained previously where this compound was not detected in olive oils [25]. This is because with MS the sensitivity was 20 times better than with UV detection (LOD = 1 ng/g) and it was possible to detect low quantities of trigonelline in olive oils. However, note that, although in all olive oils trigonelline peaks were

detected, contents were smaller than LOQ in all cases except for two Arbequina extra virgin olive oils (see Table 2). Finally, the content of carnitines was not detected or not quantifiable in extra virgin olive oils being a feasible novel marker for the detection of adulterations of olive oils by this methodology.

To demonstrate the method suitability for detecting adulterations using carnitines as markers, different mixtures of olive oils with seed oil (soybean oil) were analyzed. Adding percentages of 2, 5 and 10 % (w/w) of soybean oil in olive oil not only increases the quantity of glycine betaine and trigonelline in the sample, but also enables to quantify a certain content of carnitines (see Table 2). These results can be observed in Figure 4 which shows the EIEs obtained by CE-MS^2 for glycine betaine, trigonelline and total content of carnitines in a soybean oil sample (RSYO-3), an extra virgin olive oil sample (HEVOO-1), and the oil mixture of HEVOO-1 with a 5% (w/w) of RSYO-3. This figure also shows the MS^2 spectra employed to carry out the unequivocal identification of each compounds in the oil mixtures. Taking into account the results obtained in this work, the detection of adulterations of olive oils with other vegetable oils can be performed using as marker the total content of carnitines. In addition, the presence of glycine betaine or trigonelline at concentrations higher than 0.7 ng/g or 0.1 ng/g, respectively, would suppose their adulteration with other vegetable oils. Therefore, better results for oil mixtures were achieved by this methodology than those previously obtained by UV detection where percentages $\geq$ 20 % (w/w) of refined soybean oil in olive oil could be detected. These data confirm the high potential of the developed method to easily determine the authenticity and quality of olive oils.

4. Concluding remarks

The sensitive and simultaneous determination of betaines previous derivatization with butanol was performed using a CE-ESI-MS^2 method. Ion trap variables were optimized and MS^2 experiments in MRM mode were carried out to improve the sensitivity and selectivity. Satisfactory separation among the betaines with short analysis times (10 min) was obtained. The analytical characteristics of the developed method were studied achieving good sensitivity and adequate precision and accuracy for all the analytes. The optimized method was applied to the analysis of different commercial vegetable oils (extra virgin olive oils, soybean oils, sunflower oils and corn oils). The results revealed the presence of glycine betaine, trigonelline and carnitines in seed oils while proline betaine was not detected in any sample. Moreover, the absence of carnitines in olive oils enabled to propose them as novel markers for detecting adulterations of extra virgin olive oils with seed oils. These results show that the proposed methodology is a promising alternative offering a sensitive and rapid fingerprint of olive oils for quality control purposes.

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Figure Captions

Figure 1. Electropherograms showing the effect of derivatization temperature for trigonelline at **a)** 60 ºC and **b)** 80 ºC. CE conditions: BGE, 0.1 M formic buffer (pH 2.0); uncoated fused-silica capillary, 50 µm ID×68.5 cm; injection by pressure at 50 mbar×15 s; applied voltage, 25 kV; temperature, 25 ºC; detection at 195 nm. Standard of 50 µg/mL in water.

Figure 2. MS^2 spectra and structure of precursor ions for each betaine are shown. CE conditions: BGE, 0.1 M formic buffer (pH 2.0); uncoated fused-silica capillary, 50 µm ID×85 cm; injection by pressure at 50 mbar×15 s; applied voltage, 25 kV; temperature, 25 ºC. ESI conditions: positive ion mode; spray voltage, 4.5 kV; sheath liquid, isopropanol/water (50/50 v/v) with 0.1% formic acid at 3.3 µL/min; drying gas flow, 3 L/min; drying temperature, 300 ºC; nebulizer pressure, 2 psi. Ion trap conditions: maximum accumulation time, 300 ms; averages, 3; scan, 50-350 m/z; MS^2 transitions with width, 4 m/z; fragmentation amplitude, 1.00 V; fragmentation time, 40 ms.

Figure 3 a) CE-MS base peak electropherogram (BPE) for standard betaines mixture of 5 µg/mL each one (injection by pressure at 50 mbar×15 s) and **b)** simultaneous CE-MS^2 EIE for a standard betaines mixture of 5 µg/mL each one (injection by pressure at 50 mbar×50 s). CE Conditions: uncoated fused-silica capillary, 50 µm ID×60 cm; Other CE conditions and ESI conditions as in Fig. 2. Ion trap conditions: maximum accumulation time, 300 ms; averages, 1; scan, 50-280 m/z. MS^2 transitions in MRM mode with width, 4 m/z; fragmentation amplitude, 1.00 and fragmentation time, 10 ms. Peak 1. Glycine betaine, 2. Trigonelline, 3. Proline betaine, 4. Carnitine and Acetylcarnitine. Standards dissolved in acetonitrile/water (40:60, v/v).

Figure 4. CE-MS^2 EIE for glycine betaine, trigonelline and total content of carnitines in a) soybean oil sample (RSYO-3), b) extra virgin olive oil sample (HEVOO-1), c) oil mixture

of HEVOO-1 with a 5% (w/w) of RSYO-3, and d) MS^2 spectra for the peaks obtained in c) of glycine betaine, trigonelline or carnitines in the oil mixtures (HEVOO-1 with a 5% (w/w) of RSYO-3). All other experimental conditions were as in Fig. 3.

Table 1. Analytical characteristics of the method developed for the determination of betaines by CE-MS^{2} [a)]

Compound	Linearity[b)]			Precision (Ac and RT, RSD(%))[c)]						Recovery[g)]		LOD	LOQ
				Instrumental Repeatability[d)]		Method Repeatability[e)]		Intermediate Precision[f)]		Low level	High level	(ng/g)	(ng/g)
	r	Intercept	Slope	Ac	RT	Ac	RT	Ac	RT				
Glycine betaine	0.996	7.5 (±7.6) x 10^3	109.9 (±8.3)	4.8	5.5	7.7	6.8	10.7	11.2	99±2	94±1	0.075	0.125
Trigonelline	0.994	12.8 (±15.8) x 10^3	148.3 (±30.3)	5.8	5.9	7.4	8.9	9.5	10.2	92±5	88±2	0.050	0.083
Proline betaine	0.997	1.9 (±9.4) x 10^3	75.6 (±10.6)	-		-		-		80±5	88±2	0.075	0.125
Carnitines	0.994	8.2 (±12.0) x 10^3	241.9 (±22.8)	5.4	7.7	7.9	8.9	9.1	12.5	96±1	99±1	0.050	0.083

[a)] Experimental conditions as in Fig. 3.

[b)] Six standard solutions at different concentration levels (LOQ-100LOQ) injected in triplicate during three days. The calibration plot was represented using the average of the triplicate injections for each day. Values in parentheses are confidence intervals at 95%: $\pm t \times s_{intercept}$, $\pm t \times s_{slope}$.

[c)] Ac means corrected peak area (peak area divided by migration time) and RT means retention time.

[d)] Obtained from six consecutive injections of RSO-1 in the same day (n=6).

[e)] Obtained from three individual RSO-1 samples injected by triplicate in the same day (n=3).

[f)] Assessed from three individual RSO-1 samples injected in triplicate in three consecutive days (n=9).

[g)] Recovery for HEVOO-1 samples spiked at low level (5 ng of each compound) and at high level (50 ng of each compound). Average ± standard deviation (n=3).

Table 2. Quantitation of betaines (using the single point standard addition) in vegetable oils from different botanical origin. ND: not detected (< LOD).

		Quantitation (ng/g)			
Origin	*Sample Name*	*Glycine betaine*	*Trigonelline*	*Proline betaine*	*Carnitine and Acylcarnitines*
Sunflower oil	*RSO-1*	9 ± 1	5.5 ± 0.1	ND	2.1 ± 0.5
	RSO-2	12 ± 1	6.7 ± 0.1	ND	4.4 ± 0.3
	RSO-3	11 ± 1	7.7 ± 0.6	ND	2.2 ± 0.3
Corn oil	*RCO-1*	4 ± 1	0.3 ± 0.1	ND	0.3 ± 0.1
	RCO-2	8 ± 1	1.1 ± 0.7	ND	0.8 ± 0.2
	RCO-3	5 ± 2	0.5 ± 0.1	ND	1.0 ± 0.3
Soybean oil	*RSYO-1*	4 ± 1	0.7 ± 0.1	ND	1.1 ± 0.2
	RSYO-2	9.7 ± 0.8	1.2 ± 0.3	ND	1.0 ± 0.3
	RSYO-3	5.4 ± 0.4	0.6 ± 0.2	ND	0.8 ± 0.1
Hojiblanca extra virgin olive oil	*HEVOO-1*	0.16 ± 0.01	< LOQ	ND	< LOQ
	HEVOO-2	0.12 ± 0.01	< LOQ	ND	ND
	HEVOO-3	< LOQ	< LOQ	ND	ND
Arbequina extra virgin olive oil	*AEVOO-1*	0.13 ± 0.02	0.085 ± 0.007	ND	ND
	AEVOO-2	0.24 ± 0.01	< LOQ	ND	< LOQ
	AEVOO-3	0.19 ± 0.01	0.089 ± 0.001	ND	ND
Picual extra virgin olive oil	*PEVOO-1*	< LOQ	< LOQ	ND	< LOQ
	PEVOO-2	0.12 ± 0.02	< LOQ	ND	ND
	PEVOO-3	0.14 ± 0.01	< LOQ	ND	ND
Mixtures of HEVOO-1 with RSYO-3	*10%*	0.68 ± 0.01	0.12 ± 0.04	ND	0.14 ± 0.01
	5%	0.45 ± 0.03	0.09 ± 0.02	ND	0.096 ± 0.006
	2%	0.36 ± 0.02	< LOQ	ND	< LOQ

Figure 1.

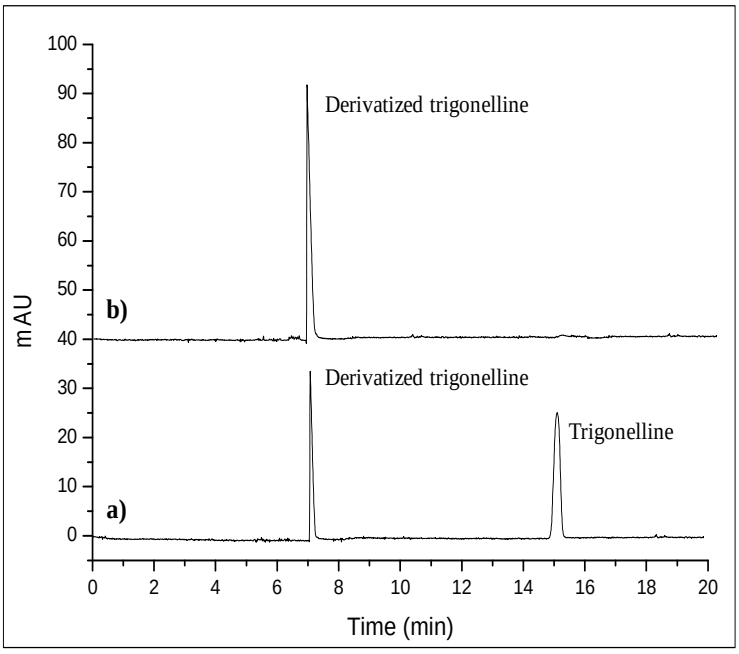

Derivatized trigonelline
b)
Derivatized trigonelline
Trigonelline
a)
mAU
Time (min)
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14
16
18
20

Figure 2.

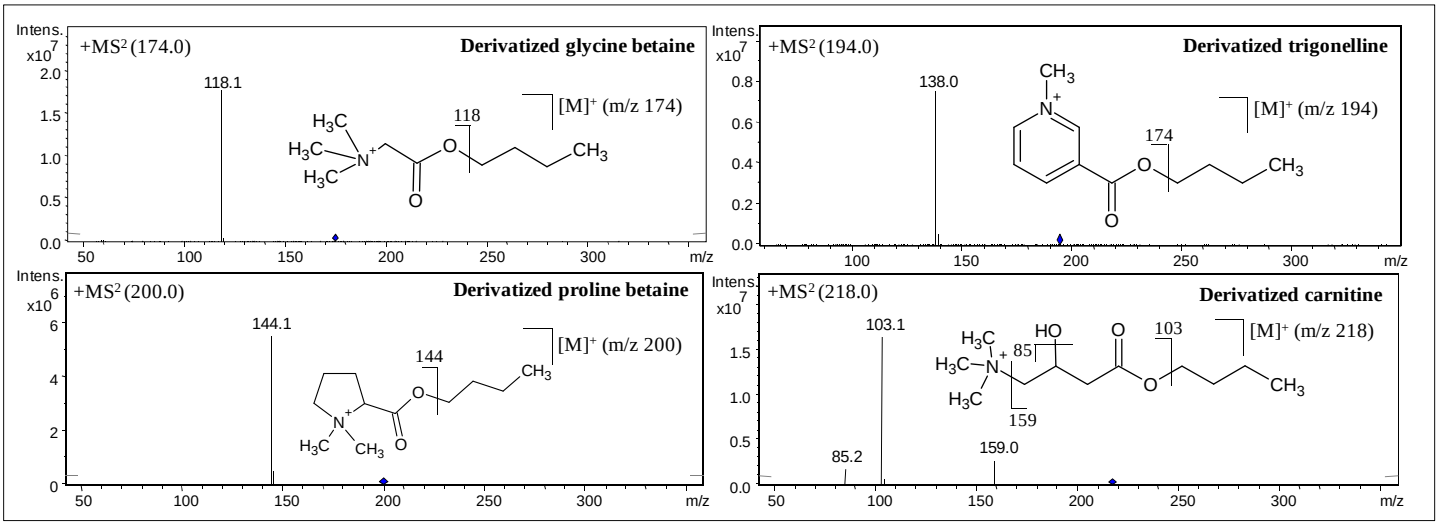

Intens.
x10^7
+MS²(174.0)
Derivatized glycine betaine
118.1
118
[M]⁺ (m/z 174)
+MS²(194.0)
Derivatized trigonelline
138.0
174
[M]⁺ (m/z 194)
x10^6
+MS²(200.0)
Derivatized proline betaine
144.1
144
[M]⁺ (m/z 200)
+MS²(218.0)
Derivatized carnitine
103.1
103
85
159
85.2
159.0
[M]⁺ (m/z 218)
m/z

Figure 3.

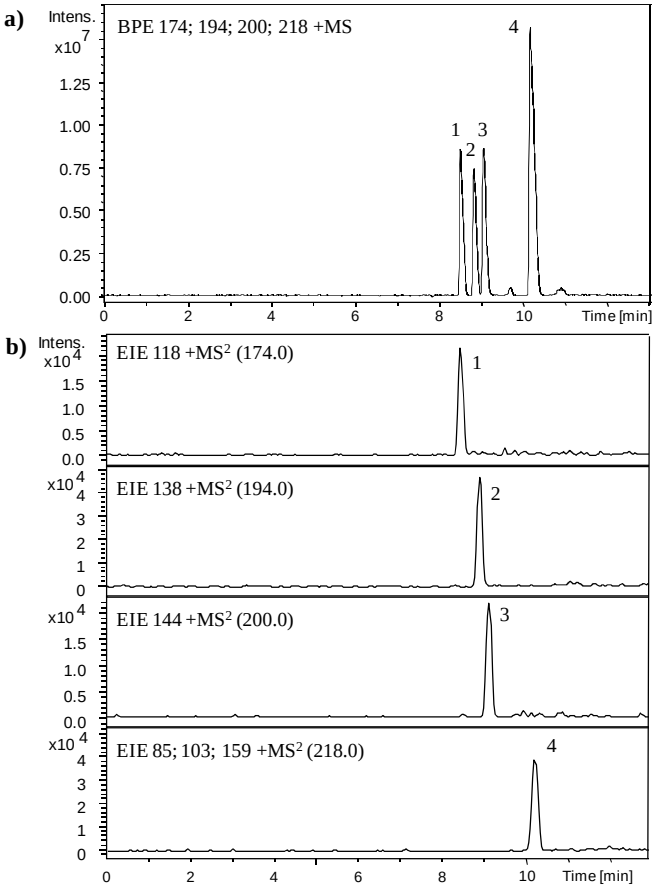
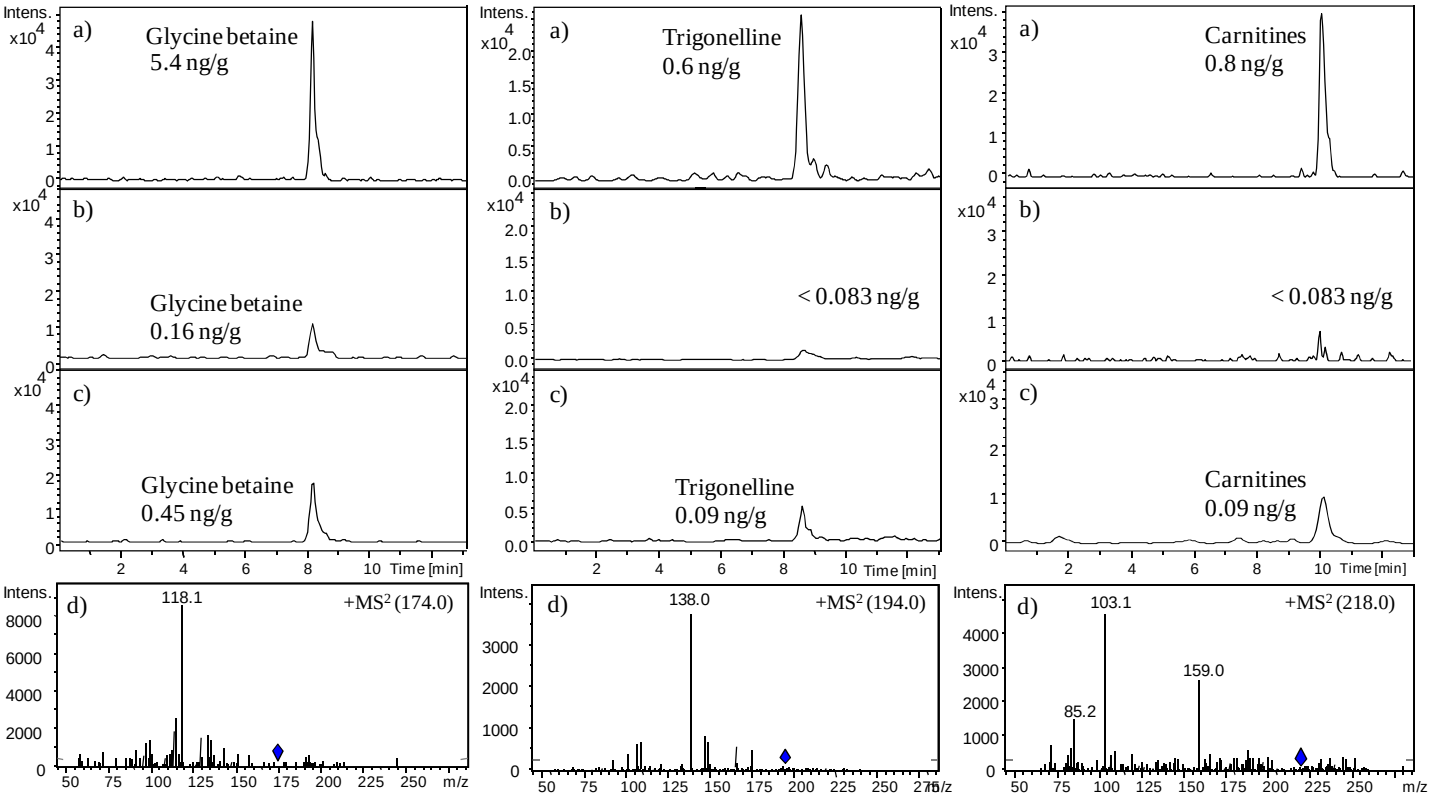

a)
Intens.
x10 7
BPE 174; 194; 200; 218 +MS
1
2
3
4
Time [min]
b)
Intens.
EIE 118 +MS2 (174.0)
EIE 138 +MS2 (194.0)
EIE 144 +MS2 (200.0)
EIE 85; 103; 159 +MS2 (218.0)
Time [min]

Figure 4


Intens.
a)
Glycine betaine
5.4 ng/g
b)
Glycine betaine
0.16 ng/g
c)
Glycine betaine
0.45 ng/g
Time [min]
d)
118.1
+MS² (174.0)
m/z
a)
Trigonelline
0.6 ng/g
b)
< 0.083 ng/g
c)
Trigonelline
0.09 ng/g
Time [min]
d)
138.0
+MS² (194.0)
m/z
a)
Carnitines
0.8 ng/g
b)
< 0.083 ng/g
c)
Carnitines
0.09 ng/g
Time [min]
d)
103.1
159.0
85.2
+MS² (218.0)
m/z