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Optimization and application of methods of triacylglycerol evaluation for characterization of olive oil adulteration by soybean oil with HPLC–APCI-MS–MS

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ABSTRACT

Triacylglycerols (TAGs) are the main constituents of vegetable oils where they occur in complex mixtures with characteristic distributions. Mass spectrometry using an atmospheric pressure chemical ionization interface (APCI-MS) run in positive mode and an Ion Trap mass analyser were applied in the study of olive and soybean oils and their mixtures. Direct injections of soybean and olive oil solutions allowed the identification of ions derived from the main TAGs of both oils. This procedure showed to be a simple and powerful tool to evaluate mixtures or addition of soybean to olive oil. TAG separation was optimized by high performance liquid chromatography (HPLC) using an octadecylsilica LiChrospher column (250 mm × 3 mm; 5 μm) and a gradient composed of acetonitrile and 2-propanol allowed the separation of the main TAGs of the studied oils. APCI vaporization temperature was optimized and best signals were obtained at 370 °C. Multiple reaction monitoring (MRM) employing the transition of the protonated TAG molecules ($[M+H]^+$) to the protonated diacylglycerol fragments ($[M+H-R]^+$) improved the selectivity of TAG detection and was used in quantitative studies. Different strategies were developed to evaluate oil composition following TAG analysis by MRM. The external standard calibration and standard additions methods were compared for triolein quantification but the former showed to be biased. Further quantitative studies were based on the estimates of soybean and olive oil proportions in mixtures by comparison of TAG areas found in mixtures of known and unknown composition of both oils. Good agreement with expected or labeled values was found for a commercial blend containing 15% (w/w) of olive oil in soybean oil and to a 1:1 mixture of both oils, showing the potential of this method in characterizing oil mixtures and estimating oil proportions. Olive oils of different origins were also evaluated by mass spectra data obtained after direct injections of oil solutions and principal component analysis (PCA). Argentinean olive oils were clustered in a different area of the principal components plot (PC2 × PC1) in comparison with European olive oils. The commercial blend containing 15% (w/w) of olive oil in soybean oil appeared in a completely different area of the graphic, showing the potential of this method to screen out for olive oil adulterations.

1. Introduction

Vegetable oils are complex mixtures of lipids that contain triacylglycerols (TAGs) as major components. Complex TAG distribution is found among edible oils as TAG acyl radicals may contain 8–22 carbon atoms and up to 3 double bonds per radical. An additional complicating factor is the occurrence of TAG isomers [1,2]. Besides, several families of compounds such as diacylglycerols (DAGs), free fatty acids and their esters, wax esters, phospholipids, phytosterols, tocoferols and long chain hydrocarbons are also present in edible vegetable oils [3,4]. The vegetable seed or fruit from which the oil is extracted determine most of its characteristics and composition [1,5], that also depends on several factors such as soil, climate, processing, harvesting and chemical process occurring during storage [4].

Among edible oils, olive oil shows important and outstanding characteristics due to its differentiated sensorial qualities (taste and flavor) and higher nutritional value. Several health benefits associated with its consumption were initially observed among

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Mediterranean people and its dietary consumption is nowadays considered to provide many benefits to human health [6,7]. In this way, although olive oil shows a relatively modest world production (~2%) [8,9], there are several regulations concerning its characteristics, production and composition, and also international regulations and trademarks, such as those established by the International Olive Oils Council [7,10–12]. Furthermore, olive oils are classified in different types according to their characteristics [13].

However, the relatively low production of olive oil, its high prices and unique characteristics make olive oil a target for adulteration that results in frequent problems for regulatory agencies, oil suppliers and consumers [3,4]. The addition of vegetable oils of low commercial and nutritional values to olive oils represents common adulterations that are reflected in the final oil composition and quality.

TAGs represent up to 95–98% (weight to weight – w/w) of olive oil composition [12] and show a characteristic distribution. As a consequence, the addition of other edible oils to olive oils, modifies TAG distribution. The characterization of TAG molecules distribution by their direct determination and/or determination of fatty acid esters by high resolution gas chromatography (HRGC), using flame ionization detector (FID) or mass spectrometry (MS) detection, following TAG transterification can be employed to evaluate olive oil composition and to detect the addition of other oils to olive oil [5,14–17].

High performance liquid chromatography (HPLC) is an alternative for TAG analysis. Normal-phase (NP) or reverse-phase (RP) solvent systems were used for TAG separation by HPLC [2,9,12,18–20]. The elution order of TAGs in RP systems depends on the equivalent carbon number (ECN) that is calculated by subtracting twice the number of double bonds from the total number of carbon atoms of the acyl radicals of each TAG. UV [9,16] or MS detection (HPLC–MS) were used in these studies.

HPLC–MS depends on the interface of ionization. Protonated TAGs ($[M+H]^+$) result after ionization in atmospheric pressure chemical ionization (APCI) interfaces [12,18–22] and their characterization was recently reviewed [22]. The fragmentation of TAG ions after ionization in APCI interfaces is well known and the main fragments are protonated DAG ions, which result from the loss of one acyl radical [19–21,23]. Electrospray ionization (ESI) interfaces after direct infusion of oil solutions or of oil extracts were also used [13,24–26] for oil characterization. Atmospheric pressure photoionization (APPI) interfaces were also employed in the study of olive oils [27,28]. APPI and ESI interfaces were considered to be complementary, because the former led to fragments due to the loss of one or two acyl radicals, while TAG ions or their adducts were mainly observed after ionization in the ESI interface [27]. In NP systems, APCI and APPI interfaces showed comparable linear ranges of 4–5 decades, but APPI was 2–4 times more sensitive than APCI. Both were more sensitive than the ESI interface without addition of modifiers, although their use increased ESI sensitivity [28].

Mass analysers of different characteristics were used for HPLC–MS analysis of olive oil, including high resolution systems such as Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometers [25] or tandem mass spectrometers composed of hybrid mass analysers, such as the Quadrupole Time-of-Flight (QqTOF) [24,26,27]. Besides, single Quadrupoles [23,28] and Ion-Traps (IT) [13] mass spectrometers were also employed to characterize oil samples. At least, in principle, high resolution and/or tandem mass spectrometers are advantageous over single low resolution instruments because chromatographic separation may be not necessary. Significant reduction of sample preparation time and, consequently, analysis time associated with a large selectivity can be obtained in several MS systems such as QqTOF-MS and IT-MS. Fingerprinting of oils can be obtained, providing information about all the components of the oil sample.

Multiple stages of mass spectrometry can also be used. Multiple reaction monitoring (MRM) allows improved selectivity and sensitivity of the analytical methods used for TAG identification and quantification in oils. The transitions $[M+H]^+$ to $[M+H-R]^+$, where R represents an acyl radical can be used with this purpose. TAG profiles and distribution obtained by direct infusion or TAG percentages obtained after HPLC separation, with or without the aid of principal component analysis (PCA) were used to evaluate and characterize olive oil samples and to detect adulterated oils [13,17,19,23–26]. Adulteration of olive oils with hazelnut oils that was considered a challenge [23], has been studied and properly solved elsewhere [23,26].

This study was focused on the application of different strategies to evaluate the addition of soybean oil to olive oil that is a relatively frequent problem of economical and nutritional consequences. It is also a common problem of olive oils bottled in Brazil, a major soybean oil producer. Anyway, many insights and developments presented here allow the study of olive oil adulteration with other edible oils. Emphasis was put into the characterization of oil TAG profiles without the determination of all TAGs, which can be a difficult and/or expensive task due to standards cost and reduced availability. TAG profiles obtained by APCI-MS, following direct injection of oil solutions, were used to characterize olive oils of different origins and their mixtures with soybean oil. This procedure was evaluated as a diagnostic tool of olive oil adulteration. TAG distributions of different oils were characterized by HPLC–APCI-MS and HPLC–APCI-MS–MS. TAG areas obtained by integration of TIC and MRM signals the proportions among areas were used to support the conclusions obtained from the former profiles. PCA was also applied to compare TAG areas of olive oils of different origins obtained by APCI-MS following direct injection of oil solutions.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile and 2-propanol (HPLC grade, J.T. Baker, NJ, USA) and hexane (HPLC grade, TediaBrazil, RJ, Brazil) were employed. Triolein standard (≥99%) was purchased from Sigma Chemical Co. (WI, USA).

2.2. Samples

Extra virgin olive oil (EVOO) or refined extra virgin olive oil from different countries (Spain, Portugal, Italy, Greece and Argentine), a Brazilian soybean and a commercial oil blend labeled as 15% (w/w) of olive oil in soybean oil were studied. All oils, including corn, sunflower and rapeseed oils, were purchased in supermarkets of Rio de Janeiro and Niterói cities, Brazil, and represent products usually sold there.

2.3. Sample preparation procedures

The studied oils were previously homogenized in their own packings (cans or plastic bottles) before sampling. Oil aliquots of 8–12 mg were precisely weighted, dissolved in hexane at room temperature and diluted up to 25.0 mL in this solvent. When necessary, aliquots of these solutions were further diluted in hexane.

2.4. Chromatographic analysis

The HPLC system consisted of a quaternary pump, an automated injector and a column oven (all Agilent 1100 Series, USA) interfaced to an Ion Trap SL mass spectrometer (Agilent 1100 Series, USA). Chromatographic conditions (mobile phase composition and flow-rates) were optimized using a reverse-phase LiChrospher octadecyl

(ODS) column (250 mm × 3 mm; 5 μm) and a guard column of similar characteristics (Vydac ODS, 201TP54, 2.1 mm; 10 mm). A constant flow-rate of 0.7 mL min^{-1} was used and the column temperature was kept at 35 °C. Injected volumes were always 5 μL. TAG separation was achieved using a step-wise binary elution gradient consisting of acetonitrile (A) and 2-propanol (B). The gradient was as follows: 100% of A held for 2 min, with a linear increase of B up to 70% until 30 min, held for 5 min at 70% B and a linear increase of A up to 100% during 5 min to allow for equilibration before the next injection.

2.5. Mass spectrometric detection

The Ion Trap mass spectrometer was interfaced to the HPLC system through an atmospheric pressure chemical ionization (APCI) interface operated in positive mode. The mass spectrometer was weekly calibrated with direct infusion of the Tune Mix solution (APCI/APPI Calibrant Solution, G2432A, Agilent) in a flow-rate of 0.3 mL h^{-1} in positive mode APCI. The drying gas was set at 5 L min^{-1} and the nebulizer nitrogen gas pressure was 60 psi. Smart target was set at 30,000, the maximum accumulation time was 200 ms and the number of average scans was set at 7. Expert Parameters Settings was used and the other MS parameters were obtained using the calibrant solution.

2.6. TAG identification

TAGs were identified by their mass spectra considering m/z values of $[M+H]^+$ and $[M+H-R]^+$ ions that were previously calculated using a homemade C^{++} program. The chromatographic and mass spectrometric conditions did not allow neither separation nor identification of isomeric TAGs that is, those differing in acyl radical positions. Thus, abbreviations used in this text correspond to the acyl radicals present in each TAG and not to their positions in these molecules. Acyl radical abbreviations are shown in Tables 2 and 3.

2.7. Evaluation of the fragmentation pattern of TAGs

The first step of this work consisted in the evaluation of the fragmentation pattern of TAGs in positive mode. With this purpose 3 μL of hexane solutions containing around 20 mg L^{-1} of EVOO or of commercial blend of olive oil in soybean oil was directly injected (without column) from the HPLC onto the APCI interface. The carrier consisted of 2-propanol in a flow-rate of 200 μL min^{-1}. Mass spectra of positive ions were scanned between m/z 200 and 1000.

2.8. MRM detection of TAGs

Multiple reactions monitoring (MRM) was also employed for TAG detection. Oil solutions were injected using the chromatographic conditions described in Section 2.4. Time segments were employed to allow the selective detection of the main TAGs present in the studied samples. Transitions $[M+H]^+$ to $[M+H-R]^+$, where R represents an acyl radical, were monitored. For TAGs producing more than one $[M+H-R]^+$ fragment, due to different acyl radical losses, the most intense one was considered. For coeluting TAGs, the transition of the most abundant TAG was explored.

2.9. Optimization of vaporization temperature of the APCI interface

Vaporization temperature of APCI interface was studied and optimized between 300 and 420 °C. Due to the main interest of this study, a solution containing 50:50% (w/w) of soybean oil and olive oil and a total oil concentration of 40 mg L^{-1} was used. This solution was injected (5 μL) under the optimized chromatographic conditions and signal was recorded in MRM. Areas of the most abundant TAGs were integrated and compared in different temperatures.

The drying temperature of the APCI interface was kept at 350 °C during vaporization temperature optimization and in the following work. Drying gas flow-rate was kept at 5 L min^{-1} with a nebulizer pressure of 60 psi. These conditions represent the default conditions for a mobile phase flow-rate of 0.700 mL min^{-1}.

2.10. Quantitative analysis

For the quantitative evaluation of TAGs, oil solutions were always analysed in triplicates, using the optimized chromatographic conditions and vaporization temperature. Signal areas were recorded under MRM conditions considering the most intense transitions and time segments. Different quantification strategies were used.

The first quantitative strategy consisted in triolein (OOO) quantification by the external standard method. With this purpose, calibration curves were obtained using OOO solutions in hexane, with concentrations ranging between 1 and 50 mg L^{-1}.

The standard additions method was the second strategy employed for triolein quantification. Three to five addition levels corresponding to 2–20% of the expected OOO concentrations of the studied oils were evaluated. This was accomplished by the addition of aliquots of a solution containing 130 mg L^{-1} of OOO to the oil solutions.

The third strategy was based on the evaluation of mixtures of EVOO and soybean oil that contained known w/w proportions (0–100% of each one) with a final constant total oil concentration. Areas of selected TAGs, which were considered as olive oil or soybean oil markers, were determined under MRM and plotted against the concentrations of both oils in the mixture.

2.11. Data analysis

Final treatments of HPLC–MS data were performed using Microsoft Excel® spreadsheets. Principal component analysis (PCA) performed using the package Statistica® 7.0, was employed to evaluate scan data obtained after triplicate and direct injections of selected oil solutions. Prior to PCA, scan data were auto-scaled using also a Microsoft Excel® spreadsheet. The mean intensity of each ion was divided by the oil concentration resulting in ion intensity expressed as counts (a.u.) per unit of concentration (mg L^{-1}). These values were auto-scaled by subtracting the intensity of each ion in each oil from the mean intensity of each ion in all oils, followed by the division of the difference by the standard deviation of the intensity of each ion in all oils [29].

3. Results and discussion

3.1. Evaluation of mass spectra obtained after direct injection of oil samples

TAGs were identified by their mass spectra considering m/z values of $[M+H]^+$ and $[M+H-R]^+$ ions calculated by the homemade C^{++} program. This program was very useful because the occurrence of isomeric TAGs leads to a very large number of structural possibilities even if only even acyl chains are considered. As an example, the structural possibilities of a TAG $[M+H]^+$ ion of m/z = 885.8, considering only C18 acyl radicals (and consequently 3 double bonds), together with the expected $[M+H-R]^+$ fragments are shown in Table 1. Six fragments can be obtained from the 3 possible protonated species and, in addition, different TAGs can lead to fragments of the same m/z. Of course, the number of possibilities quickly increases if acyl radical positions in TAGs or different chain

Table 1
Structures of isomeric $[M+H]^+$ ions of m/z 885.8 and fragments corresponding to one acyl radical loss ($[M+H-R]^+$). Only acids containing 18 carbon atoms were considered.

Protonated TAGs	Fragments
$[OOO+H]^+$	$[OO+H]^+$ (m/z 603.6)
$[SOL+H]^+$	$[SO+H]^+$ (m/z 605.6); $[OL+H]^+$ (m/z 601.6); $[SL+H]^+$ (m/z 603.6)
$[SSLn+H]^+$	$[SS+H]^+$ (m/z 607.6); $[SLn+H]^+$ (m/z 603.6)

Acid radicals: O = oleic acid (18:1); S = stearic acid (18:0); L = linoleic acid (18:2); Ln = linolenic acid (18:3).

lengths (for example, C16 and C20) are considered [20]. Through this text, TAG abbreviations only indicate acyl radicals present in each molecule and not their positions in these molecules.

Mass spectra (m/z = 300–900) were obtained in the initial steps of this study by direct injection of 3 μL of hexane solutions of edible oils of different origins. Mass spectra showed protonated ions derived from TAGs ($[M+H]^+$) and DAG-like [21] fragments ($[M+H-R]^+$). Isotopic clusters were also clearly identified in these mass spectra (Fig. 1).

Fig. 1a shows part of the mass spectrum (m/z = 550–900) of an Italian olive oil diluted in hexane (28.5 mg L^{-1}). The predominant ion corresponded to the fragment of m/z 603.6 ($[OOH]^+$), whose isotopic cluster is clearly observed in Fig. 1b. Another ion cluster is found around m/z 885.8 ($[OOO+H]^+$). This ion corresponds to the protonation of triolein (OOO), which is the predominant olive oil TAG, and of its possible isomers (Fig. 1c). The continuous decrease of ion abundance in the isotopic cluster indicates the presence of only one fragment ($[OOH]^+$). However, a low contribution of $[SOH]^+$ (m/z = 605.6) is found in this cluster, as expected, due to the occurrence of SOO and SSO in olive oil.

In fact, direct injection of oil solutions possibly represents the simplest way to screen out the addition of soybean oil to olive oil. This is illustrated by the mass spectrum of a commercial oil blend labeled as 15% (w/w) of olive oil in soybean oil (Fig. 1d). Fig. 1e shows an ion cluster with a predominant ion of m/z 601.6 ($[OL+H]^+$), a large peak of m/z 599.6 ($[LLn+H]^+$) originated from soybean oil and a small peak of m/z 603.6 ($[OO+H]^+$). Fig. 1f shows a cluster with three predominant ions of m/z 881.8 ($[OLL+H]^+$), m/z 879.8 ($[LLL+H]^+$) and m/z 877.8 ($[LLLn+H]^+$) originated from soybean oil and peak of m/z 885.8 ($[OOOH^+]$), derived of the predominant olive oil TAG. As a consequence of data presented in Fig. 1d and e, a large signal of ion of m/z 601.6 indicates a fragment ($[OL+H]^+$) that is formed by the fragmentation of TAGs that are important components of soybean oil, such as POL, OOL and SOL. The addition of other edible oils to olive oils can be possibly screened out in a sim-

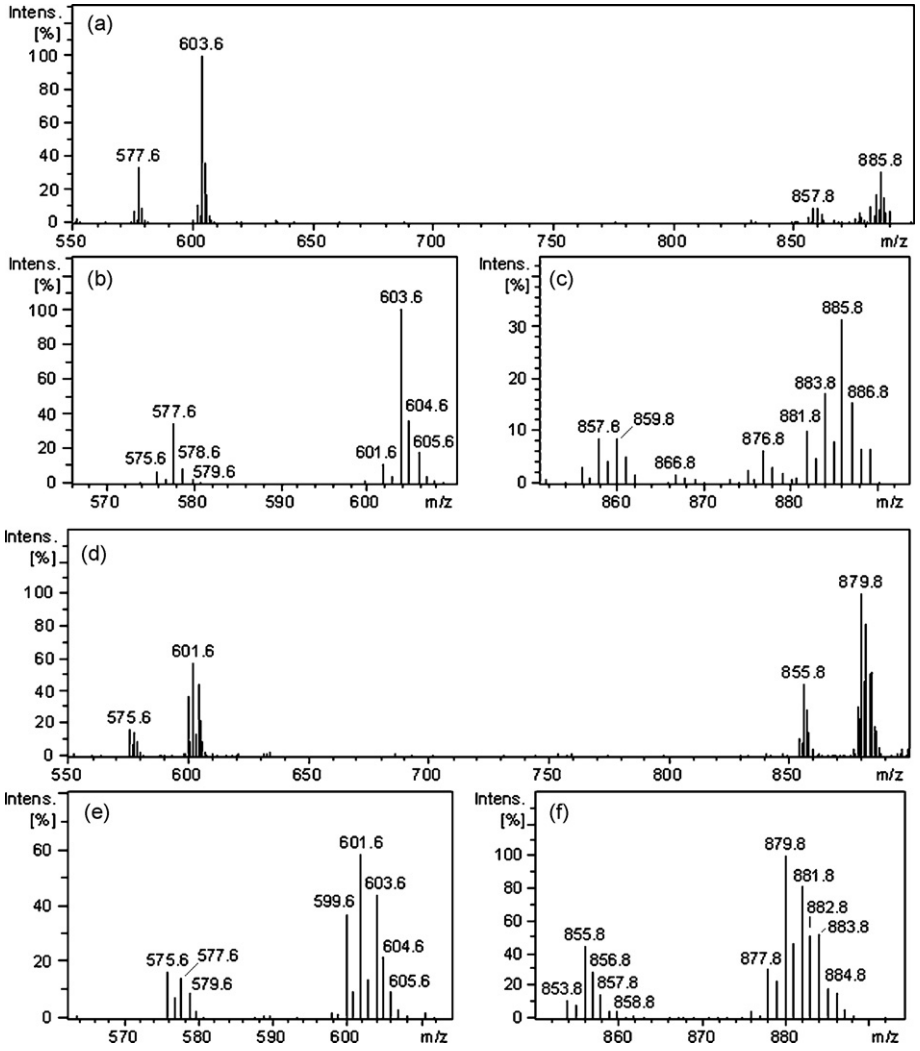

Fig. 1. Mass spectra obtained by APCI-MS after direct injection of oil solutions in hexane. (a) Solution of Italian EVOO showing the correspondent isotopic clusters of ions of $[OOO+H]^+$ (b) and $[OO+H]^+$ (c). (d) Commercial blend of olive and soybean oils showing the cluster of the fragments of $[OL+H]^+$, ($[OO+H]^+$) and $[LLn+H]^+$ (e) and the cluster of the predominant protonated TAGs ($[LLL+H]^+$, $[LLLn+H]^+$ and $[OOOH^+]$) (f).

ilar way, but this study emphasized the adulteration of olive oil by soybean oil due to the incidence of this problem.

3.2. TAG separation by HPLC

The optimization of TAG separation by HPLC led to the conditions described in Section 2.4. Solutions of olive and soybean oils and of a 50:50% (w/w) mixture of both were studied with this purpose. This mixture was very useful in the optimization of HPLC conditions because it contained all the major TAGs of both oils. It was observed that an increase of 2-propanol percentage in the mobile phase tended to reduce peak widths and retention times of the more retained TAGs and consequently, analysis time. However, the high viscosity of 2-propanol and maximum pressure of the chromatographic pump (400 bar) prevented the use of 2-propanol proportions larger than 70%. The applicability of the optimized chromatographic method was further evaluated by the analysis of other edible oils of significant worldwide production [8] and wide consumption in Brazil (namely rapeseed, corn and sunflower oils). Total ion current (TIC) chromatograms of rapeseed oil, corn oil, sunflower oil, (Portuguese) EVOO, soybean oil and a mixture of the last two oils obtained by HPLC–APCI-MS in the optimized gradient are shown in respectively Fig. 2a–f.

TAG separation followed ECNs. For example LLL (ECN = 42), OLL (ECN = 44), OOL (ECN = 46) and OOO (ECN = 48) were eluted in this order. Some isomeric TAGs or TAGs of identical ECNs were not well resolved. However, the application of the Dissect Function, available in the Data Analysis Program, which allows peak separation processing without any user interaction or prior information, in connection with mass spectra, allowed the identification of superimposed or low area TAGs present in these oils. Retention times,

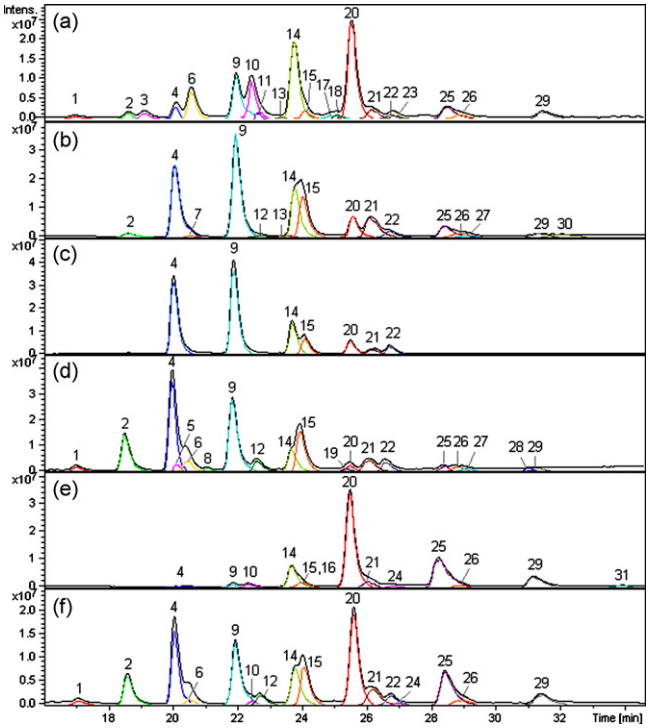

Fig. 2. Total ion current (TIC) chromatograms obtained using the optimized chromatographic conditions: (a) rapeseed oil, (b) corn oil, (c) and sunflower oil, (d), Portuguese EVOO, (e) soybean oil and (f) a mixture of these two oils.

Table 2
Names, retention times (min), ECNs and $[M+H]^+$ and main $[M+H-R]^+$ ions of the studied TAGs.

Peak numbers	Retention times (min)	Rapeseed oil	Corn oil	Sunflower oil	Soybean oil	Olive oil	Protonated molecules $[M+H]^+$	Fragments $[M+H-R]^+$	Possible TAG structures	ECNs
1	17.0	0.6	–	–	1.4	–	875.8	595.6; 597.6	LLnLn	38
2	18.5	1.1	1.2		10.9		887.8	597.6; 599.6	LLLn	40
3	19.0	1.3	–	–	–	–	877.8	595.6; 599.6	OLnLn	40
4	19.9	2.1	20.3	30.0	23.0	0.3	879.8	599.6	LLL	42
5	20.1	–	–	–	1.3	–	853.8	573.6; 599.6	PoLL	42
6	20.3	6.7	–	–	3.4	–	879.8	597.6; 599.6. 601.6	OLLn	42
7	20.4	–	0.2	–	–	–	853.7	597.6; 601.6; 679.6	NI	–
8	21.2	–	–	–	0.2	–	851.7	573.6; 585.6; 599.7	NI	–
9	21.8	12.1	26.6	36.7	22.4	0.9	881.8	599.6; 601.6	OLL	44
10	22.2	8.9				1.0	881.8	599.6; 603.6	OOLn	44
11	22.4	1.1	–	–	–	–	881.8	573.6; 575.6; 853.6; 601.6	NI	–
12	22.6	–	0.5	–	3.6	–	853.7	573.6; 575.6; 597.6	PLLn	42
13	23.3	0.25	0.4	–	–	–	909.8	599.6; 629.6	GLL	46
14	23.7	21.1	13.9	13.3	6.8	11.0	883.8	601.6; 603.6	OOL	46
15	23.9	2.0	11.9	7.2	12.9	2.6	855.7	575.6; 599.6	PLL	44
16	23.9	–	–	–	–	ND	857.7	575.6; 603.6	PoOO	46
17	24.5	1.0	–	–	–	–	855.7	573.5; 577.6; 599.6	POLn	44
18	24.6	0.8	–	–	–	–	911.8	601.6; 629.6; 631.6	GOL	48
19	25.3	–	–	–	0.8	–	881.8	579.6; 601.6; 603.6	SLLn	44
20	25.5	26.7	5.7	6.2	1.7	48.1	885.8	603.6	OOO	48
21	26.1	3.0	7.8	2.8	4.7	3.0	857.7	575.6; 577.6; 601.6	POL	46
22	26.6	1.0	1.8	3.9	3.2	–	883.8	599.6; 603.6	SLL	46
23	26.6	0.9	–	–	–	–	913.8	603.6; 631.6	GOO	50
24	26.8	–	–	–	–	0.4	947.8	577.6; 603.6; 631.6	NI	–
25	28.3	4.8	4.5	–	1.1	23.2	859.8	577.6; 603.6	POO	48
26	28.8	1.5	1.8	–	1.7	0.7	885.8	601.6; 603.6; 605.6	SOL	48
27	29.0	–	0.5	–	0.2	–	911.8	599.6; 631.6	ALL	48
28	31.0	–	–	–	0.3	–	939.8	599.6; 659.6	BLL	50
29	31.2	2.8	1.3	–	0.5	7.9	887.8	603.6; 605.6	SOO	50
30	31.9	–	1.6	–	–	–	887.8	551.6; 575.6; 603.6; 605.6	NI	–
31	33.9	–	–	–	–	0.7	ND	603.6; 633.6	NI	–

ND = not detected; NI = not identified. Acid radicals: Po = palmitoleic acid (16:1); P = palmitic acid (16:0); A = arachidic acid (20:0); G = gadoleic acid (20:1); B = behenic acid (22:0). See also Table 1 for the other radicals.

ECNs and m/z values of $[M+H]^+$ and of the main $[M+H-R]^+$ ions of the identified TAGs are shown in Table 2.

TIC chromatograms of Fig. 2a–f show characteristic fingerprintings and predominant TAGs of the studied oils. For example, OOL and OOO predominated in rapeseed oil (Fig. 2a), while LLL and OLL predominated in both corn (Fig. 2b) and sunflower (Fig. 2c) oils, which showed similar profiles, although heavier TAGs such as ALL, SOL and SOO were found in corn oil. These results show good agreement with previous data [2]. The chromatograms of a soybean oil and a (Portuguese) EVOO are shown respectively in Fig. 2d and e. OOO that predominates in olive oil showed lower signals in soybean oil, whereas the more unsaturated TAGs (LLL, OLL and OOL) predominated in this oil. Peaks corresponding to coeluted or minor oil TAGs were clearly identified. For example, three TAGs (OOL, PoOO and PLL) contributed to peaks 4 and 5 of olive oil (Fig. 2e), with the later two coeluting in peak 5. The main TAGs identified in the chromatograms of Fig. 2a–e agree well with previously reviewed and compiled data of edible oil TAGs [2]. Table 2 also presents estimates of TAG area percentages. Although different TAGs show different response factors under our analytical conditions, those results led to rough estimates of TAG composition (% w/w) that agree well with previous data [1,2].

Fig. 2f shows a chromatogram of a 50:50% (w/w) mixture of olive and soybean oils. Predominant TAGs of both oils (LLLn, LLL, OLL, OOL, OOO and POO) can be easily identified in this chromatogram. Reduction of peak areas is observed when compared with that of the original oils (Fig. 2d and e). As a consequence, the proportions of the areas of TAGs originated from olive or soybean oils in the mixture were similar to those found in the original oils, while the proportions of the areas of TAGs originated from both oils tended to show different values due to oil mixture. Fig. 2f highlights the possibility of a qualitative preliminary evaluation of olive oil adulteration by soybean oil by the determination of the TIC areas of selected TAGs using HPLC–APCI-MS. This would also be a powerful tool to verify olive oil adulteration by other oils whose TAG distributions differ from that of olive oil.

Chromatograms of Fig. 2d–f corroborate the conclusions obtained from Fig. 1d–f: mass spectra obtained after direct injection of olive oil solutions that show unexpected TAG ions indicate the addition of other oils to this olive oil. This fact is illustrated in the chromatogram depicted in Fig. 2f that show important peaks of $[LLLn+H]^+$, $[LLnLn+H]^+$ and $[OLL+H]^+$ and in the mass spectra showed in Fig. 1d–f that show the ions of these TAGs. As a consequence, mass spectra obtained after direct injection of olive oil solutions, represent an "easy to use" powerful screening tool to evaluate the addition of other oils to olive oil.

3.3. Quantitative evaluation of TAGs in mixtures of soybean and olive oils by MRM

MRM was employed due its improved selectivity that allows an almost complete elimination of interferences in connection with HPLC separations. For example, OOO and POL that partially coelute in olive oil (Fig. 2e), can be selectively detected by monitoring distinct transitions ($[OOO+H]^+$ to $[OO+H]^+$ and $[POL+H]^+$ to $[PO+H]^+$, $[PL+H]^+$ or $[OL+H]^+$).

Time segments allowed selective TAG detection using MRM transitions corresponding to the loss of an acyl radical. For type I TAGs that contain three identical acyl radicals there was only one possible transition. For type II or type III TAGs that contain respectively 2 or 3 different acyl radicals, the most intense $[M+H-R]^+$ ion was monitored. For example, the fragmentation of $[OOO+H]^+$ (m/z 885.8) leads only to $[OO+H]^+$ fragment (m/z 603.6), while $[OLL+H]^+$ (m/z 881.8) leads to two fragments $[OL+H]^+$ (m/z 601.6) and $[LL+H]^+$ (m/z 599.6). Time segments and monitored transitions are shown in Table 3 and TAG identification followed data of Fig. 2 and Table 2.

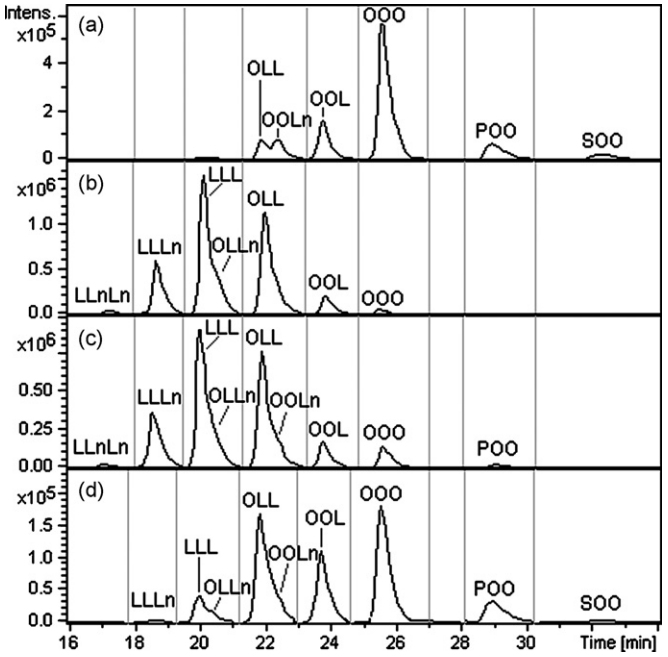

Fig. 3. MRM chromatograms obtained using the optimized chromatographic conditions and vaporization temperature (T_{vap} = 370 °C). (a) Spanish EVOO, (b) soybean oil, (c) commercial blend of olive and soybean oils and (d) Argentinean EVOO.

To obtain quantitative TAG data, the vaporization temperature of the APCI interface was previously optimized with triplicate injections (5 μL) of a solution of a mixture 50:50% (w/w) of soybean and olive oils and a total oil concentration of 40 mg L^{-1}. This equal concentration was chosen because the main TAGs of both oils showed comparable signal areas. MRM was employed for TAG detection.

Ionization processes in the APCI interface occur in vapor phase and aerosol formation, that is critical in this interface, is strongly influenced by T_{vap} and also by the drying temperature of the interface (T_{dry}). Approximated bell-shaped curves of area versus temperature were obtained for all TAGs and maximum signal areas of all TAGs were obtained at 370 °C. This temperature and a drying temperature of 350 °C, which represents the maximum value available in the employed equipment, were used in the continuity of this study.

MRM chromatograms of two EVOOs, of a soybean oil and of a mixture of soybean and olive oils obtained by the optimized conditions are shown in Fig. 3. These chromatograms can be interpreted in a similar way of those of Fig. 2d–f: they show that TAG peak areas vary according to oil composition. Chromatograms of a Spanish and an Argentinean olive oils, assigned as EVOO by their producers, are depicted in Fig. 3a and d, respectively. OOO, OOL, OLL, OOLn, POO and SOO showed the main peaks in both EVOO samples. OOO predominated in the Spanish EVOO, but in contrast, comparable areas of OOO, OOL and OLL + OOLn were found in the Argentinean EVOO. This different composition may be due to a combination of several factors such as olive variety and environmental factors, including climate, temperature and soil [4].

The chromatogram of soybean oil (Fig. 3b) shows predominant peaks of LLL + OLLn, OLL + OOLn and lower peaks of LLnLn, OOL and OOO, whereas the chromatogram of the oil mixture (Fig. 3c) shows characteristics of both oils with predominant peaks of LLL + OLLn and OLL + OOL and lower peaks of comparable areas of OOL and OOO. Of course, comparable areas of OOO and OOL peaks in connection with high areas of LLL + OLLn and OLL + OOLn peaks indicate an olive oil containing an important concentration of soybean oil.

A quantitative interpretation of the differences depicted in Fig. 3 was carried out by the evaluation of OOO concentration in soybean,

Table 3
Time segments and transitions employed for MRM detection and quantification of selected TAGs.

Segment	Time interval (min)	Monitored TAGs	MRM detection	
			Transitions	Ions (*m*/*z*)
1	0–17.9	LLnLn	$[LLnLn+H]^+ \rightarrow [LLn+H]^+$	875.8–597.5
2	17.9–19.5	LLLn	$[LLLn+H]^+ \rightarrow [LLn+H]^+$	877.8–597.5
3	19.5–21.3	OLLn	$[OLLn+H]^+ \rightarrow [OLn+H]^+$	879.8–599.5
		LLL	$[LLL+H]^+ \rightarrow [LL+H]^+$	
4	21.3–23.2	OLL	$[OLL+H]^+ \rightarrow [OL+H]^+$	881.8–601.6
		OOLn	$[OOLn+H]^+ \rightarrow [OLn+H]^+$	
5	23.2–24.8	OOL	$[OOL+H]^+ \rightarrow [OL+H]^+$	883.8–601.6
6	24.8–26.9	OOO	$[OOO+H]^+ \rightarrow [OO+H]^+$	885.8–603.6
7	26.9–28.0	SLL	$[SLL+H]^+ \rightarrow [SL+H]^+$	883.8–603.6
8	28.0–30.2	POO	$[POO+H]^+ \rightarrow [PO+H]^+$	859.8–577.5
9	30.2–40.0	SOO	$[SOO+H]^+ \rightarrow [SO+H]^+$	887.8–605.6

Table 4
Concentrations (% w/w) of OOO in olive oil, soybean oil and in a blend oil obtained by the standard additions method ($n = 3$; at 3 different levels) and estimated mean recoveries (%).

Sample	Origin	% OOO (mean ± std. dev.)	Mean recoveries (%)
Extra virgin olive oil	Spain	48.4 ± 2.4	101
Extra virgin olive oil	Argentine	23.8 ± 1.2	100
Soybean oil	Brazil	3.8 ± 0.6	102
Blend oil (15%)	Brazil	15.9 ± 1.4	102

olive oil and their mixtures. OOO was chosen as an EVOO marker because it is the most abundant TAG of olive oils (Figs. 2e and 3a, d) and in good agreement with well known nutritional facts. As a consequence, important additions of soybean oil to olive oil markedly reduce OOO contents of these mixtures (Figs. 2f and 3c) leading to OOO concentrations outside its expected range of values. Thus, the determination of OOO concentrations allows an indication of olive oil adulteration by soybean oil, without the need of quantification of all TAGs or all acyl radicals by HRGC. With purpose of determining OOO in olive oils, three quantitative strategies were evaluated and compared.

Firstly, OOO concentrations were determined by the application of the external standard method. Olive oils of different origins, soybean oils and a commercial blend containing 15% (w/w) of olive oil in soybean oil were analysed in triplicates. Recovery studies run in parallel indicated a clear positive bias in the determination of OOO. This fact may be due to the different ionization environments that oils and standards solutions represented in the APCI interface.

The second strategy consisted in applying the standard additions method to overcome this bias in OOO quantification. OOO additions were performed in at least 3 different levels, with triplicate injections. A comparison of the angular coefficients of the straight lines obtained by both methods showed that the angular coefficient of the standard additions line was around 64% larger than that of the external standard method due to the different OOO responses of both methods. The standard additions method was used to evaluate the percentages of OOO in the studied commercial oil blend, in Spanish and Argentinean EVOOs and in soybean oil (Table 4). The concentrations of OOO in EVOOs and soybean oil agree well with previous data [2]. Moreover Table 4 shows that EVOOs of different origins (Spain and Argentine) contain different concentrations of OOO, in agreement with Fig. 3a and d. Data of Table 4 confirms that soybean oils contain low concentrations of OOO (3–4%) [2], which is also evident in the chromatogram of soybean oil (Fig. 3b). Of course, the relatively low concentration of OOO found in the oil blend (Table 4) indicates a not pure olive oil.

However, the application of the standard additions method is known to be very laborious. In this study, at least 6 h were necessary to accomplish a complete quantitative analysis of only one sample (3 addition levels and 9 chromatographic runs). Moreover, the determination of only one TAG affects the possibility of detecting low additions of soybean oil to olive oil, because these mixtures lead to OOO concentrations comparable with those of certain EVOOs that contain low concentrations of OOO, such as the Argentinean ones (Fig. 3a, d and Table 4).

The third quantitative strategy also represents an alternative to the usual determination of all TAGs and/or acyl radicals present in oil samples [14–17]. It considered the comparison of selected TAG areas obtained under MRM conditions after the analysis of oil mixtures, as exemplified below with data obtained by the analysis of known mixtures of a Spanish EVOO and soybean oil. To implement this strategy, TAGs that are potential markers of both oils were selected. Selection criteria considered: (a) the absence or relatively low TAG concentration in the other oil, (b) an intense signal area with low or without interference of coeluted TAGs and (c) a good response factor.

TAG selections become evident in the superimposed TIC chromatograms of Fig. 4. The dashed line represents the chromatographic signal of the Spanish EVOO solution (44.5 mg L^{-1}) and continuous line represents the chromatographic signal of the soybean oil solution (44.5 mg L^{-1}). Two regions dependent on ECN values can be observed in the chromatograms of Fig. 4. One of them corresponds to the more saturated TAGs (OOO, POO and SOO), of higher ECNs and retention times, which predominate in olive

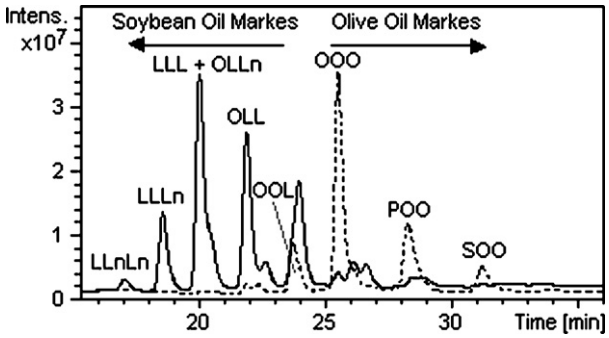

Fig. 4. Superimposed TIC chromatograms of EVOO and of soybean oil showing two distinct groups of TAG markers.

Table 5
Peak areas (a.u.) obtained by MRM and area ratios of selected TAGs in Spanish EVOO and soybean oil.

TAG	Peak areas (a.u.)		Area ratios		Marker of oil
	Soybean oil	Spanish EVOlive oil	Olive oil to soybean oil	Soybean oil to olive oil	
LLnLn	7.96×10^5	ND	0	–	Soybean
LLLn	1.06×10^7	ND	0	–	Soybean
LLL + OLLn	3.14×10^7	2.76×10^5	0.009	114	Soybean
OLL + OOLn	2.49×10^7	3.46×10^6	0.139	7.19	Soybean
OOL	3.66×10^6	4.15×10^6	1.13	0.88	–
OOO	1.03×10^6	1.83×10^7	17.8	0.056	Olive
POO	3.28×10^5	2.92×10^6	8.88	0.113	Olive
SOO	ND	1.24×10^6	–	0	Olive

ND = not detected.

oil. The other corresponds to less saturated TAGs (LLnLn, LLLn, LLL + OLLn and OLL + OOLn) of lower retention times and ECNs, mainly originated in soybean oil. At least in principle, these TAGs represent potential markers of these oils. Their areas observed in EVOO and soybean oil are shown in Table 5. Area ratios are good indicators of soybean oil addition to olive oil. For example, after the addition of soybean oil, LLL areas become higher than those usually found in pure EVOOs. As a consequence, the ratio of LLL areas found in adulterated olive oil and in soybean oil is lower than that found when LLL areas found in olive oil and soybean oil are compared. The same is true for other TAGs originated mainly from one of these oils. This fact has been used to assess olive oil adulteration [9,16] and it can possibly be used to detect the addition of other lower priced oils, of different TAG distribution, to olive oils.

Solutions of pure olive and soybean oils and of mixtures containing different proportions of both were analysed in triplicates by MRM. Total oil concentration was kept always constant (44.5 mg L^{-1}). It was observed that TAG marker areas increased linearly with the concentrations of its source oil in the mixture (Fig. 5a and b). The areas of OOO, POO and SOO increased with olive oil proportions (Fig. 5a), while the areas of LLnLn, LLLn, LLL + OLLn and OLL + OLLn increased with soybean oil proportions (Fig. 5b). Good linear relationships among TAG areas and oil concentrations, with high correlation coefficients (>0.998), were found. The areas of OOL that shows comparable concentrations in both oils, remained almost constant and independent of their concentrations.

Due to the high concentrations of OOO [2] found in olive oils, its area showed a larger sensitivity (expressed by a large angular coefficient) to the variation of olive oil concentrations than those shown by POO and SOO. Similarly, the sensitivity of soybean oil TAGs can be ordered as LLL + OLLn > OLL + OLLn > LLLn > LLnLn. Soybean oil contains low concentrations of OOO (up to 4%) and POO (up to 3%) as reviewed [2] and shown before (Table 5) hindering these TAG lines of crossing the zero even in the absence of olive oil (Fig. 5a). A similar fact is observed with OLL + OLLn line in soybean oil (Fig. 7a). The lines shown in Fig. 5a and b allowed the quantitative evaluation of soybean and olive oil concentrations in their mixtures (Table 6). SOO and LLnLn, although occurring almost exclusively in olive and soybean oil respectively, show low peak areas, leading consequently to poor sensitivity in the estimates of individual oil concentrations in their blends.

The concentrations of soybean oil and olive oil in a mixture containing 50:50% (w/w) were estimated respectively as 23.87 ± 0.47 and 22.67 ± 0.65 mg L^{-1}, with coefficients of variation below 3% (Table 6). These concentrations allowed estimates of oil percentages (w/w) in the mixture of 51.17% and of 48.83% respectively, which can be considered in good agreement with mixture composition, if the coefficients of variation of the estimated concentrations are considered. The application of the developed method to the commercial blend labeled as 15% (w/w) of olive oil in soybean oil led to an estimate of olive oil percentage of 15.22% (w/w), in very good agreement with the labeled value, although a coefficient of variation of 10.98% was found. This fact is certainly due to the evaluation of POO concentration that showed, as expected, a low concentration in this oil blend.

Another way of considering data of Fig. 5 is presented in Fig. 6. LLL + OLLn and LLLn were selected among soybean markers, while POO and OOO were selected among olive oil markers. OOO and LLL + OLLn represent the first choice TAG markers of respectively EVOO and soybean oil due to their sensitivities and because they occur in low concentrations in the other oil. POO and LLLn also show relatively high areas and can be used respectively as olive oil and soybean oil markers.

Areas of the above mentioned pairs of oil TAGs, OOO and LLL + OLLn and POO and LLLn, were plotted together in the same graphic against the percentages (w/w) of each oil in the mixtures. As shown in Fig. 6a and b, the areas of OOO and POO (olive oil markers) increased linearly with olive oil proportions (% w/w), but decreased with soybean oil proportions. In opposition, the areas of LLL + OLLn

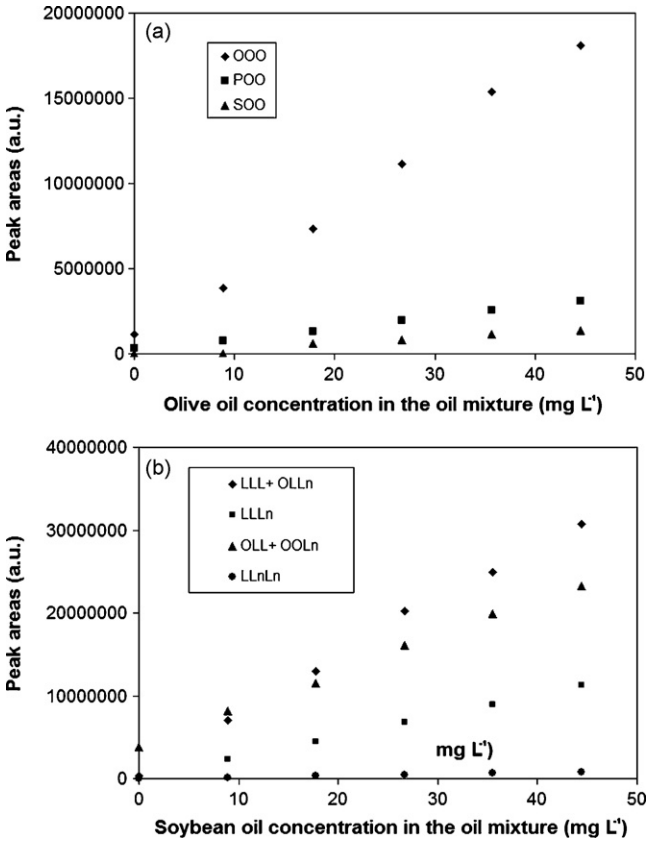

Fig. 5. Lines showing the variation of the areas of oil TAGs markers versus oil concentrations (mg L^{-1}) in mixtures containing 44.5 mg L^{-1} of total oil. (a) Olive oil; (b) soybean oil.

Table 6
Estimates of concentrations (mg L^{-1}) and of percentages (w/w) of olive and soybean oil in their mixtures.

TAGs	Estimated concentrations (mg L^{-1}) of oils in two mixtures			
	Mixture (50/50%)		Commercial blend	
	Soybean oil	Olive oil	Soybean oil	Olive oil
LLnLn	23.81	–	41.66	–
LLLn	23.23	–	40.26	–
LLL + OLLn	24.23	–	39.88	–
OLL + OOLn	24.21	–	39.41	–
OOO	–	22.32	–	7.80
POO	–	23.23	–	6.68
Means	23.87	22.67	40.33	7.24
Standard deviations	0.47	0.65	1.02	0.69
Coefficients of variation (%)	1.96	2.85	2.52	10.98
Mixtures composition (% w/w)	51.17	48.83	84.78	15.22

and LLLn (soybean markers) increased linearly with soybean oil proportions, but decreased with olive oil proportions. Curves of Fig. 6 show that the addition of soybean to olive oil changes the proportions among oil TAGs.

Olive oils do not contain appreciable concentrations of LLL or LLLn. As a consequence, MRM chromatograms of olive oils containing even low percentages of soybean oil show significant LLL and LLLn signal areas. This is the first and simplest indicative clue of soybean oil addition to olive oil as shown before (Fig. 1d–f). Simultaneously, after addition of soybean oil to olive oil, a reduction of OOO and POO areas is observed. In fact, this area reduction is less sensitive to characterize olive oil adulteration than the increase of LLL or LLLn areas, because a wide variation of OOO and POO concentrations is found among olive oils of different origins as shown in Fig. 3a and d. Besides, low concentrations of these two TAGs are found in soybean oils. Of course, there is only one composition capable of being assigned as a pure olive oil: that corresponding to the right axis of Fig. 6a and b and, as a consequence, all other sets of points of Fig. 6a and b correspond to olive oil containing soybean oil.

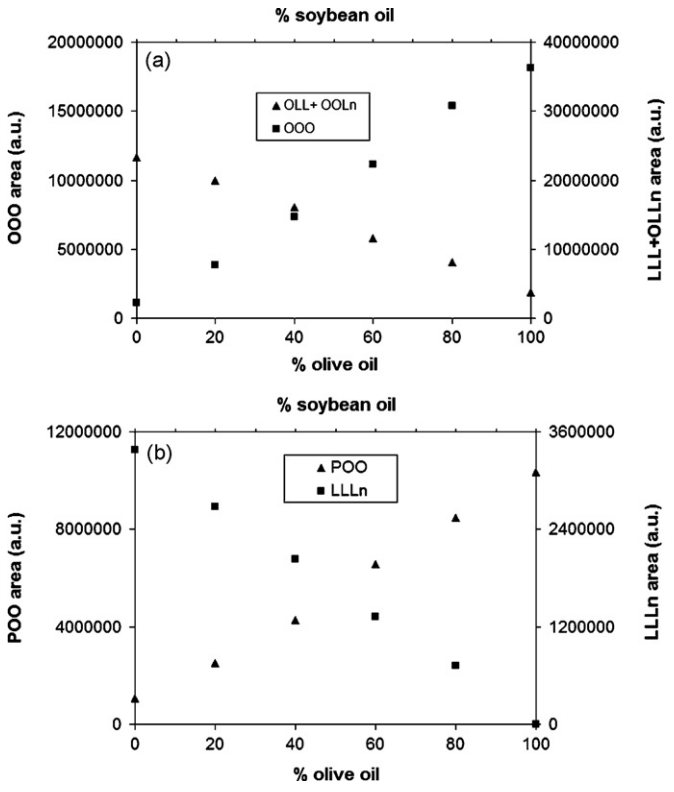

Fig. 6. Simultaneous plots of the areas of TAGs markers of olive oil and soybean oil versus oil concentrations (% w/w) in mixtures containing 44.5 mg L^{-1} of total oil. (a) OOO versus LLL + OLLn; (b) POO versus LLLn.

3.4. Application of PCA to oil characterization

The best way of constructing the curves shown in Figs. 5 and 6 is using (known) pure olive oils of different origins, which is a difficult task due to the variability of EVOO composition. In order to overcome this fact, PCA can be employed to evaluate olive oil variability and composition. With this purpose, triplicates of oil solutions were directly injected in the APCI interface. Their mass spectra were recorded, treated and auto-scaled, as described in Section 2.11 [29]. Samples of olive oils of different origins and the commercial blend containing 15% (w/w) of live oil in soybean oil were evaluated.

PC1 and PC2 were able to describe respectively 62.08% and 18.91% of total variance, while PC3 described 8.13%. They accounted for ~90% of total variance. PC1 loadings varied between −0.855 and +0.966 and TAGs were distributed along PC1. However, olive oil TAGs and their fragments (Table 3) showed negative PC1 loadings, while the most positive PC1 loadings were associated with soybean oil TAGs and their fragments. The plot of PC2 versus PC1 is shown in Fig. 7. Although a relatively low number of samples were evaluated, some conclusions can be obtained from Fig. 7. Clearly the oil blend (B15) appears in a different region when compared to the other samples. This sample has a very high PC1 value, with positive values that corresponded to soybean TAGs. This fact is, of course,

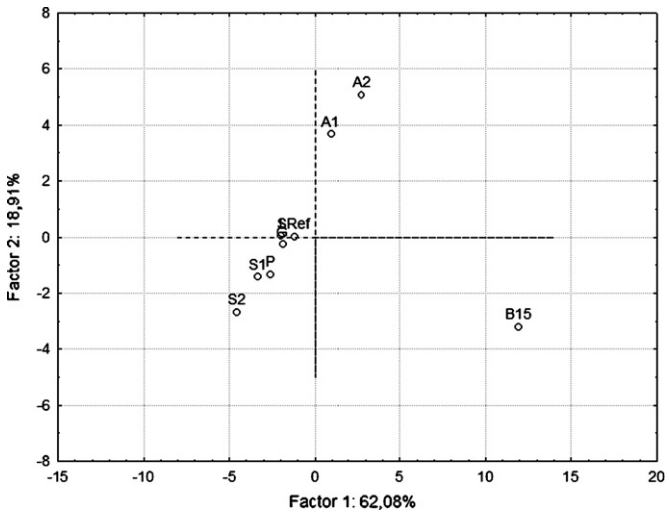

Fig. 7. Diagram of the loadings of the principal components (PC1 versus PC2) of olive oils and of a blend of olive and soybean oils.

expected from the composition of the oil blend that contains a large concentration of soybean oil.

Coordinates of all other samples (olive oils) seem to be superimposed in an imaginary axis (PC2 = 1.118, PC1 + 2.067; $R = 0.987$) that would be able to describe olive oil samples. Argentinean EVOOs (A1 and A2) appear to be clustered and showed comparatively larger PC2 values than the other 6 olive oils that seem to be clustered in another region of Fig. 7. Furthermore, Italian (I) and Greek (G) EVOOs were practically superimposed to a Spanish refined olive oil (SRef). Two Spanish (S1 and S2) and a Portuguese (P) EVOOs appear in the lowest PC1 values possibly due to their higher contents of OOO and POO, whose ions show important contribution to PC1.

This distribution of olive oils in the PC2 versus PC1 plot allowed a clustering related to their compositions and origins. This fact would indicate that the quantitative procedure discussed in Fig. 5a and b can be made more general even if the specific origin of a certain olive oil (suspect of being adulterated) is not exactly known. Of course, a more extensive evaluation, that means, evaluation of a higher number olive oils samples is necessary to further generalize these PCA findings.

Finally, other aspect of interest depicted in Fig. 7 is that PCA of ions obtained by APCI-MS after direct injection of oil solutions can lead to oil discrimination, without the need of chromatographic separation or extraction, with comparative advantages over HRGC [14–17] or HPLC–MS methods [18–21], because significant reductions of analysis time and solvent consumption and residues are obtained.

4. Conclusions

This paper presents and discusses several ways to assess the addition of soybean oil to olive oil. Mass spectra obtained by positive APCI-MS, after the direct injection of oil solutions allowed the identification of the main TAG ions present in these solutions and showed to be efficient to assess the addition of soybean oil to olive oil, through the detection of unexpected TAGs. This simple procedure is, at least, a powerful tool to screen out olive oil samples suspect of being adulterated by soybean oil and possibly, by other edible oils, but it can also be considered conclusive to classify olive oils as adulterated because certain TAGs (LLL, for example) do not occur in olive oils. Therefore, we suggest that any investigation of olive oil adulteration by HPLC–APCI-MS may start with the evaluation of the positive mode mass spectrum of the oil solution, because adulteration can be easily identified if unexpected TAGs are found in a suspected olive oil.

HPLC–APCI-MS allowed both TAG separation in different edible oils and estimates of their area percentages that were in good agreement with previous data. Unusual or discrepant TIC areas of certain TAGs, also allowed evaluation of soybean oil addition to olive oil and in a certain way, reinforced the conclusions obtained by direct injection of oil solutions.

MRM was employed for the quantitative determination of OOO in different oil solutions by the standard additions and external standard methods, but this method showed a clear positive bias. A comparison of TAG areas obtained by analysis of mixtures olive and soybean oil of known composition with those found in samples of these oils allowed estimates of EVOO concentration. Oil composition showed good agreement with known or expected concentrations and the potential of this quantitative method. This is an important result, because the evaluation of oil TAGs by the standard additions method is an expensive and time consuming task.

PCA performed using mass spectra data obtained by direct injections of oil solutions showed a potential to discriminate among Argentinean and European EVOOs and also to detect olive oil mixtures. These results highlight again the versatility of the method of direct injection to obtain mass spectra data capable of distinguishing different oils and possibly, detecting adulterations. The application of PCA in connection with mass spectra obtained after direct injection of oils is being studied now in our laboratory.

Acknowledgments

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