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Application of selected ion monitoring to the analysis of triacylglycerols in olive oil by high temperature-gas chromatography/mass spectrometry

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ABSTRACT

The analysis of the triacylglycerol (TAG) composition of oils is a very challenging task, since the TAGs have very similar physico-chemical properties. In this work, a high temperature-gas chromatographic method coupled to electron ionization-mass spectrometry (HT-GC/EI-MS), in the Selected Ion Monitoring (SIM) mode, method was developed for the analysis of TAGs in the olive oil; this is a method suitable for routine analysis. This method was developed using commercially available standard TAGs. The TAGs studied were separated according to their equivalent carbon number and degree of unsaturation. The peak assignment was carried out by locating the characteristic fragment ions having the same retention time on the SIM profile such as $[RCO+74]^+$ and $[RCO+128]^+$ ions, due to the fatty acyl residues on *sn*-1, *sn*-2 and *sn*-3 positions of the TAG molecule and the $[M-OCOR]^+$ ions corresponding to the acyl ions. The developed method was very useful to eliminate the interferences that appeared in the mass spectrum since electron ionization can prevent satisfactory interpretation of spectra.

1. Introduction

Edible oils are complex mixtures containing a wide range of compounds. They are mainly composed of triacylglycerols (TAGs) and other minor components, such as diacylglycerols (DAGs), free fatty acids (FFAs), sterols, wax ester, phospholipids, hydrocarbons, tocopherols and phenolic compounds. Every TAG molecule contains three fatty acids (similar or different) esterified to a glycerol backbone, which can vary in chain length, degree of unsaturation and position of double bonds [1]. The edible vegetable oil samples are usually mixtures of TAG. The quantity and the type of the minor components depend mainly on the variety of the oil [2].

Gas chromatography (GC) is an important analytical technique for qualitative and quantitative analysis of organic chemicals in a wide range of application areas. GC is fast and provides a high peak capacity; also it is sensitive and allows combination with a wide range of selective detection methods including mass spectrometry. However, the application of GC is limited because the molecules to be analysed have to be thermally stable and sufficiently volatile. A common approach to the analysis of TAGs is to release free fatty acid and perform GC after derivatization, for example, methylation. However, this approach does not identify actual intact TAG molecular species, but only determines the percentage of individual FA present in the total TAG fraction [3].

Whereas complete separation of the fatty acid methyl esters (FAMEs) can be achieved in one way, TAG analysis has to be performed in two ways: the analysis can either focus on the intact TAGs or target the fatty acids after hydrolysis of the TAGs. An enormous number of individual TAG species are possible, due to the large number of possible FA combinations on the glycerol backbone. The analysis of the TAG composition of oils is a very challenging task, since the TAGs have very similar physico-chemical properties [4].

Recent research has resulted in better chromatographic columns that allow the most detailed analysis of TAGs. High temperature GC (HT-GC) on non-polar stationary phases separates them according to increasing acyl carbon number (ACN). The main problem is to separate TAGs according to the degree of unsaturation and molecular weight simultaneously. Recently, high temperature (up to 370 °C) polar-phase capillary GC has been demonstrated as a powerful tool for separating acylglycerols according to the number of both double bonds and carbon atoms [5–7].

Usually chromatographic methods are necessary to identify TAGs. Chromatography–mass spectrometry combinations are the most efficient techniques nowadays to analyse them [8]. The recent developments of ionization techniques (electron impact EI, or chemical ionization CI, in either positive or negative ion mode) that are readily interfaced with mass spectrometers, have greatly simplified the sample preparation and could largely eliminate the need of derivatization [9–11].

TAGs presented in vegetable oils have been analysed by chromatographic methods for identification and quantification. Several methods that have been used for the analysis of oils and fats

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are reviewed. Among them high-performance liquid chromatographic (HPLC) [12,13], performed in several detection modes (UV, evaporative light scattering detector (ELSD) [14], charged aerosol detector (CAD) [15], flame ionization detector (FID), and mass spectrometry with atmospheric pressure chemical ionization (APCI)MS and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)) [16,17]. In addition, thin layer chromatographic (TLC) [18] and supercritical fluid chromatographic (SFC) [19] methods have been applied effectively for the identification of different TAG species present in a majority of common vegetable edible oils (and fats).

On the other hand, mass techniques offer an unequivocal identification of a component apart from the inspection of the overall chromatogram of the mixture in the total ion chromatogram (TIC) mode [20]. The identification of the components is achieved by the mass spectra of each component, the ionized overall molecule and from ionized fragments of the molecule; the sum of all these is almost exclusive for every chemical compound [21]. TAGs in oils and fats are formed more or less selectively. In some cases information about actual TAG compositions is lost because of this. The combination of mass spectral and GC retention data may also serve to eliminate alternative structures [22].

One problem, in the analysis of higher molecular weight, is the high level of chemical background that results from column bleed into the mass spectrometer during HT-GC/MS analyses that employ EI, which can prevent satisfactory interpretation of spectra [23]. Moreover, TAG molecules do not fragment in an arbitrary manner but tend to split at weaker bonds, such as those adjacent to specific functional groups. Standard EI mass spectra are achieved with 70 eV, which results in extensive fragmentation of the odd-electron molecular ions due to excess of their internal energy. The major disadvantage of EI mass spectra is the low abundance of ions providing molecular weight information, such as M^+ or $[M-18]^+$ ions. EI mass spectra of TAGs typically exhibit several fragment ions useful for structure elucidation.

A partial solution to the problem is to carry out GC/MS analyses in the single-ion mode. In selected ion monitoring (SIM) certain ion fragments are entered into the instrument method and only those mass fragments are detected by the mass spectrometer. The advantages of SIM are that the detection limit is lower since the instrument is only looking at a small number of fragments during each scan and more scans can take place each second. In addition, since only a few mass fragments of interest are being monitored, matrix interferences are typically lower. In the SIM profile of TAGs, the peak identification is accomplished by localizing certain fragment ions having the same retention times, such as RCO^+, $[M-RCO_2]^+$, $[M-RCO_2H_2]^+$, $[RCO+128]^+$, and $[RCO+74]^+$ (where R is an aliphatic hydrocarbon chain) [8,24]. The first two ions are most abundant, and the third one is formed only by cleavage from the *sn*-1 and *sn*-3 positions, permitting positional estimations [25,26]. Different applications of the SIM mode can be found on bibliography [27–29]. Actually, it has also been used with the aim of diagnosing if cross-contamination in olive oils by other vegetable oils [30].

The aim of this work is to use an alternative, highly sensitive and selective method for the identification of TAGs, the high sensitivity and selectivity of GC coupled with (EI)MS performed in the selected ion monitoring mode using selective fragment ions to the peak assignments. Three kinds of ions $[M-RCO_2]^+$, $[RCO+128]^+$ and $[RCO+74]^+$ corresponding to the fatty acyl residues on the glycerol moiety are examined, which is sufficient for peak identification instead of the molecular ion. Finally, satisfactory peak identification for olive oil was achieved by monitoring several characteristic fragments and with the ions derived from the electron impacted of the TAG molecules.

2. Materials and methods

2.1. Samples

TAGs are abbreviated by means of three letters corresponding to the fatty acids bound to glycerol. The following abbreviations are used: (P) palmitic acid (C16:0); (O) oleic acid (C18:1); (S) stearic acid (C18:0) and (L) linoleic acid (C18:2).

PPP (tripalmitin) and OOO (triolein) of >99% purity were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PPS (1,2-palmitin-3-stearin), PPO (1,2-palmitin-3-olein), PPL (1,2-palmitin-3-linolein), PSO (1-palmitin-2-stearin-3-olein), OOP (1,2-olein-3-palmitin), POL (1-palmitin-2-olein-3-linolein), SSO (1,2-stearin-3-olein), OOS (1,2-olein-3-stearin), OOL (1,2-olein-3-linolein), LLS (1,2-linolein-3-stearin), LLO (1,2-linolein-3-olein) and LLL (trilinolein) of >99% purity were purchased from Larodan Fine Chemicals AB (Sweden).

100 mg of each pure standard was accurately weighed into a clean and dry vial and then was diluted using chloroform (99%, reagent grade) to a final weight of 10 g to prepare the stock solution of reference working standard of 1% (w/w). Standard stock solutions were stored in the darkness at −15 °C until their use; under these conditions, the TAGs are stable for several months. Working solutions are freshly prepared from stock solutions, previously kept at room temperature, by chloroform dilution.

A simulated olive oil was prepared from a mixture of fourteen TAG standard species, an aliquot of 0.05 g was taken from each standard solution and it was taken to a final concentration of 700 ppm (w/w) of each TAG.

For optimisation purpose, a commercial extra virgin olive oil (Spain) was used. It was stored at 4 °C until its analysis. Olive oil was dissolved in chloroform to a final concentration of 0.2% (w/w). Any further sample preparation was not needed prior to analysis.

2.2. Instrumentation

All separations were performed with a VARIAN GC 3800 gas chromatograph (PA, USA) equipped with a split/splitless injector coupled to a mass spectrometer (ion trap). A split injection with a ratio of 1:10 was used. The samples were introduced using a robotized autosampler module (Combipal, CTC ANALYTICS, Switzerland). The sample volume injection was 2 μl. The analytical column was a capillary column coated with 65% diphenyl-35% dimethyl-polysiloxane stationary phase (Restek Rtx-65TG; 30 m × 0.32 mm i.d. × 0.1 μm film thickness, maximum temperature 370 °C; Restek Corp., Bellefonte, PA, USA).

The GC oven temperature was programmed from 315 °C to 350 °C at 1 °C/min. The injection port was held isothermally at 370 °C. Helium (99.995%) was used as the carrier gas and its flow rate was 1.5 ml/min.

A VARIAN 4000 ion trap mass spectrometer (PA, USA) equipped with an electron impact (EI) source was used to perform the mass analyses. The mass spectrometric conditions were as follows. The ion source temperature was held at 250 °C during the GC/MS runs. The transfer-line temperature was maintained at 350 °C throughout the analyses. The electron energy was 70 eV and the emission current 10 μA. In full-scan mode, average spectra were acquired in the m/z range of 200–1000 m/z and were recorded at a scan speed of 1.20 s. Scan control, data acquisition, and processing were performed by a MS Workstation software (VARIAN) data system.

Selected Ion Monitoring (SIM) mode was performed for the following ions $[RCO+74]^+$, $[RCO+128]^+$ and $[M-RCO_2]^+$, which were the most abundant peaks in the EI mass spectra and they could be used for TAG identification.

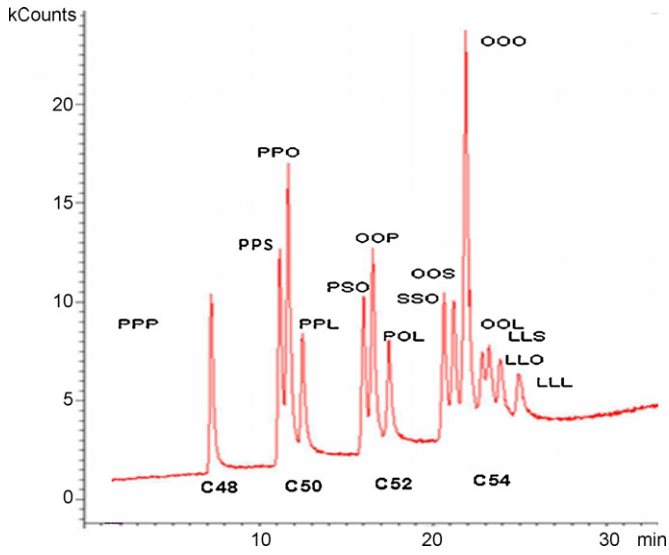

Fig. 1. TIC profile for the high temperature of GC/MS analysis of standard triacylglycerols. Temperature programming was at 350 °C (for further practical details see experimental section).

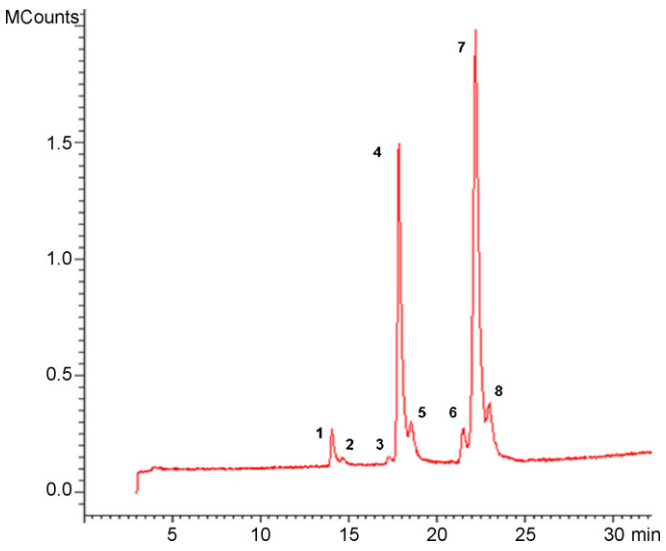

Fig. 2. Total ion chromatogram (TIC) profile for the high temperature of GC/MS analysis of triacylglycerols in the olive oil. The peaks are numbered from 1 to 8 (for peak identification, see Table 2 and Section 3).

3. Results and discussion

A simulated olive oil, made in the laboratory, in which all the TAGs were in the same concentration, was used in order to optimise the chromatographic conditions (details are described in materials and methods section). Fig. 1 shows a total ion chromatogram of the GC/(EI)MS analysis (scan mode). An excellent chromatographic performance, under the GC/(EI)MS conditions employed, is evident from triacylglycerols containing up to fifty-four acyl carbon atoms. In preliminary studies, TAG species were well-separated even species with identical carbon number and number of double bonds (54:4) such as LLS and OOL. In order to achieve this chromatographic separation, it was necessary to raise the column temperature as high as 350 °C.

The mass spectra of these standard TAGs were obtained by using electron ionization (EI) mode and with an ion trap mass spectrometer.

Depending on the type of mass spectrometer detector used in the analysis (quadrupole or ion trap), the mass spectra will be different according to fragmentation and grouping of ions. The available spectra on the library, in the commercial equipments, are obtained with a quadrupole mass spectrometer and due to this, a new library of forty-five different TAGs, with spectra obtained from our ion trap mass spectrometer, was built. It was observed that there are much more fragmentation, at low mass range, on the spectra from the library. However, with the ion trap mass spectrometer, these fragments are loosening. So, the "space-charge effects", were too many ions in the trap distort the electric fields leading to significantly impaired performance, are avoided.

The total ion chromatogram of olive oil is shown in Fig. 2; with the same experimental condition that in the previous case, it is noticed that, the chromatographic resolution is not achieved. The separations between peaks were incomplete. Only eight peaks are obtained even when the chromatographic method is able to separate according to degree of unsaturation and molecular weight simultaneously. This could be due to the different amounts of each triacylglycerol in the olive oil [9] and it could take place some overlapping peaks of TAGs.

Fig. 3 shows the GC/(EI)MS spectra of the peaks that appear in Fig. 2, corresponding to the TAGs of the olive oil, they show characteristic pattern, mainly in the low mass range, which are different for saturated or unsaturated TAGs. Also it is observed that the degree of dissociation of the acylium ion from the glycerol backbone is clearly dependent on the number of double bonds in the acyl chain. GC/(EI)MS spectra of TAGs contain abundant fragment ions in the form of $[RCO+74]^+$, $[RCO+128]^+$, $[M-OCOR]^+$ as it can be observed. Our preliminary study shows that the ion $[RCO]^+$ was quite small to use it to identify.

Moreover, some of the spectra were formed by a mixture of different fragment ions, which increased the difficulty of the identification procedure and also some spectra look alike among them (for example peak 7 and peak 8). Owing to this, a methodology based on Selected Ion Monitoring (SIM) mode was used.

The peak assignment was accomplished by locating the fragment ions having the same retention time on the SIM profile that the ions due to the fatty acyl residues and then compared with the standard TAG. Table 1 shows different TAGs, ranging from the 48 to 54 in carbon number, those used in the simulation of olive oil and available commercially. They were previously studied from their fatty acid composition to simplify this peak assignment. The studied TAGs would be expected to yield individual fragment ions such as $[RCO+74]^+$, $[RCO+128]^+$ and $[M-RCO_2]^+$. All the fragments (eighteen chromatograms) that appear in Table 1 were studied individually by the SIM mode and used them for the identification of the TAGs in olive oil. These ions produce useful information about the structure of the triacylglycerol. The corresponding molecular weights are also summarised in Table 1.

Fig. 4 shows the profile of some of the fragment ions of the TAGs of olive oil. Monitoring was carried out at the m/z from the results of previous experiences (Table 1). The m/z 551 ion is due to the $[M-OCOR]^+$ yielded from $[PP]^+$ residue, the m/z 603 is due to the $[OO]^+$ residue and m/z 577 corresponds to $[PO]^+$ residue. The ions m/z 367 and m/z 313 are ascribed to the $[RCO+128]^+$ corresponding to the palmitic acid residue. This method was applied to every single fragment of the TAGs in order to obtain a combination of each fragment at the same retention time.

From the characteristic combination of the fragment ions obtained with the SIM mode at the same retention time, when monitoring, the particular species were identified.

The retention time (Rt) of peak #1 was 14.06 min and some of the fragment ions monitored at this time were m/z 577, m/z 551, m/z 367 and m/z 313. These fragments correspond to the $[RCO+74]^+$, $[RCO+128]^+$, $[M-OCOR]^+$ of the PPO component.

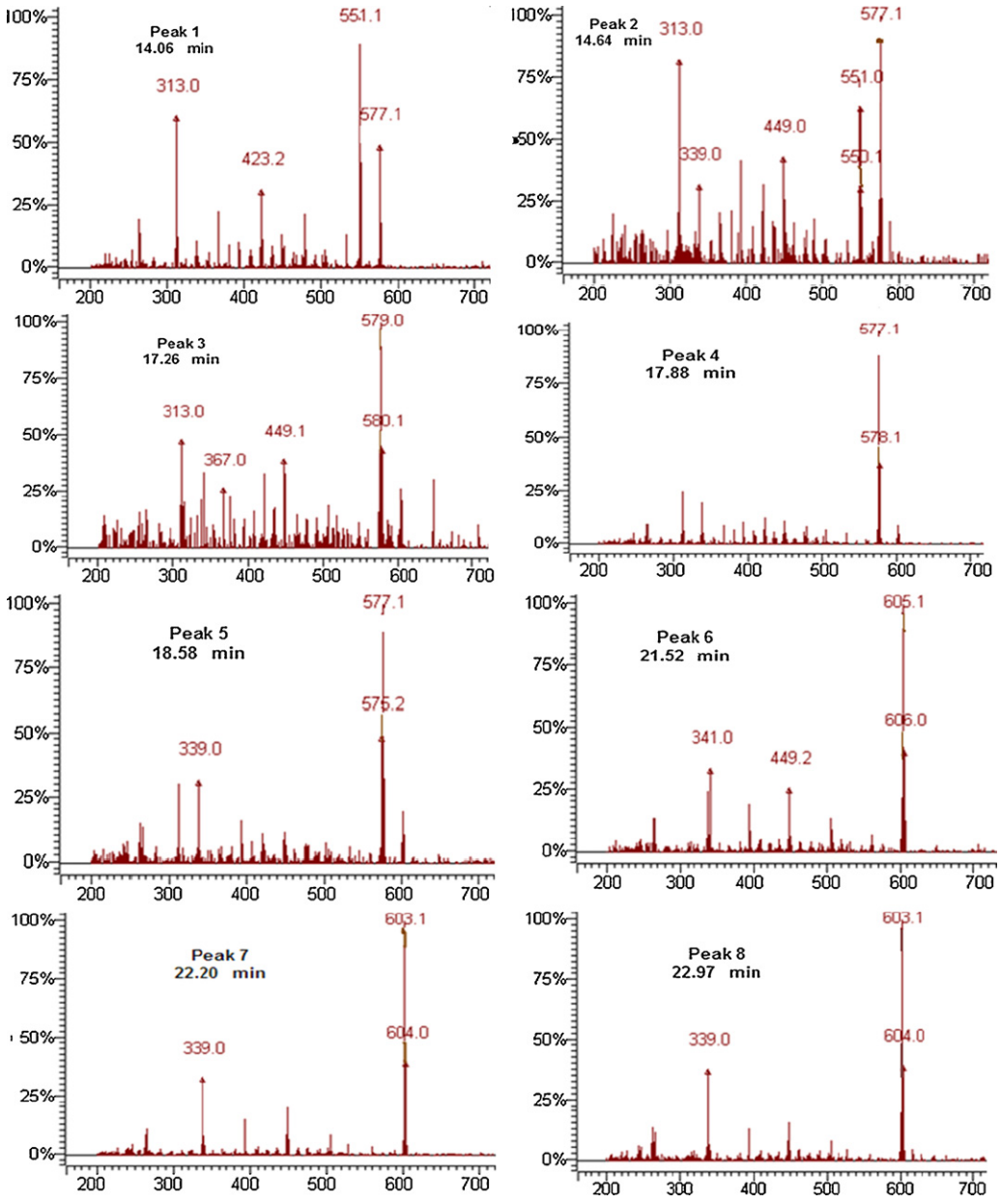

Fig. 3. Mass spectra of each peak in the chromatogram of olive oil.

At Rt 14.64, peak #2, the fragment ions at *m/z* 577, *m/z* 551 and *m/z* 367 were detected between others. These ions result from the PPL.

The component of peak #3 (Rt 17.26 min) had three kinds of $[M-OCOR]^+$ ions, *m/z* 605, *m/z* 579 and *m/z* 577. The only TAG having this characteristic profile was regarded as PSO (see combination of characteristic ions in Table 1).

When monitored at *m/z* 603, an ion peak appeared at Rt 17.88, peak #4, and ions such as *m/z* 577 $[M-OCOR]^+$, *m/z* 313 $[RCO+74]^+$ and *m/z* 367 $[RCO+128]^+$, were also detected at the same Rt. These residues corresponded to the molecule of OOP.

At the retention time 18.58, peak #5, four important kinds of fragments $[M-OCOR]^+$, *m/z* 577, *m/z* 575, *m/z* 576, *m/z* 601 and *m/z* 603 appeared (see part a in Fig. 5). This is due to a mix of triacylglycerol molecules co-eluted with the same number of carbons and double bonds. The *m/z* 577, *m/z* 575 and *m/z* 601 correspond to $[PO]^+$, $[PL]^+$ and $[OL]^+$ fragments respectively. The first two fragments are due to the neutral loss of and linoleic acid and oleic acid respectively, of the POL molecule. However the other two ion fragments (*m/z* 576 and *m/z* 603), that appear at the same retention time, correspond to $[PoO]^+$ and $[OO]^+$ fragments. This strongly suggests that the triacylglycerol is PoOO, and it is co-eluted with POL (see part b in Fig. 5).

At the retention time of 21.52 min, peak #6, the ion peaks monitored were *m/z* 605, *m/z* 393, *m/z* 339 and *m/z* 265, this last one corresponding to $[RCO]^+$ of an oleic acid residue. The other fragments correspond to $[M-OCOR]^+$, $[RCO+128]^+$, $[RCO+74]^+$ fragment ions of the OOS.

The characteristic ion resulting from the unsaturated triacylglycerol OOO is *m/z* 603, which corresponds to [M−OCOR] due to cleavage of an oleic acid anion. With this information, and from other ion fragments such as *m/z* 339 and *m/z* 393, it was possible to identify peak #7, at retention time 22.20 min, as OOO definitely.

The last peak, peak #8, at 22.97 min (see part c in Fig. 5), was identified as OOL due to the fragments that appeared at this retention time such as *m/z* 603, *m/z* 601and *m/z* 339 (see part d in Fig. 5). Even when in the mass spectrum of this peak in Fig. 4 (peak #8), the fragment ion of *m/z* 601 did not come out because of the over-

Table 1
Calculated *m*/*z* of fragment ions from different molecular species of triacylglycerols used in the simulation of olive oil.

TAG	ACN:DB[a]	M^+	FA residue	*m*/*z* of fragment ions		
				$[RCO+74]^+$	$[RCO+128]^+$	$[M-OCOR]^+$
			P	313	367	551
PPP	48:0	860	P	313	367	551
			P	313	367	551
			P	313	367	579
PPS	50:0	834	P	313	367	579
			S	341	395	551
			P	313	367	577
PPO	50:1	832	P	313	367	577
			O	339	393	551
			P	313	367	575
PPL	50:2	830	P	313	367	575
			L	337	391	551
			P	313	367	605
PSO	52:1	860	S	341	395	577
			O	339	393	579
			O	339	393	577
OOP	52:2	858	O	339	393	577
			P	313	367	603
			P	313	367	601
POL	52:3	856	O	339	393	575
			L	337	391	577
			S	341	395	605
SSO	54:1	888	S	341	395	605
			O	339	393	607
			O	339	393	605
OOS	54:2	886	O	339	393	605
			S	341	395	603
			O	339	393	603
OOO	54:3	884	O	339	393	603
			O	339	393	603
			O	339	393	601
OOL	54:4	882	O	339	393	601
			L	337	391	603
			L	337	391	603
LLS	54:4	882	L	337	391	603
			S	341	395	599
			L	337	391	601
LLO	54:5	880	L	337	391	601
			O	339	393	599
			L	337	391	599
LLL	54:6	878	L	337	391	599
			L	337	391	599

[a] ACN: the number of total acyl carbons; DB: the number of double bonds.

lapping with the previous peak (OOO), with the SIM mode a right identification was possible.

After the identification of each peak, with every single *m*/*z* at the same retention time, diagrams were made in order to simulate the mass spectrum of the previously identified triglyceride and then compared to the mass spectrum of the standard TAG (Fig. 5). This was used to validate the fragment ions obtained from the SIM profile at the same retention time.

As it can be seen the simulated spectra are slightly similar to the corresponding standard TAG. Furthermore with the SIM mode, it has been possible to reject fragments that are not useful for identification.

Although olive oil may contain the group acyl linolenic (Ln), no fragment ion peaks corresponding to this group were found in the SIM profile. This is probably due to the instability of the linolenic acid, during electron impact ionization and due to the high polarity of this compound [4]. Moreover it was observed that the signal intensity of the product, due to the neutral loss of an *sn*-2 fatty acid was smaller than that of the other two product ions, indicating that neutral loss of the *sn*-2 fatty acids was less favourable than loss of the fatty acid from the *sn*-1 or *sn*-3 position. In addition, the TAGs having unsaturated fatty acyl groups, these ions were relatively more intense than those from saturated triacylglycerols.

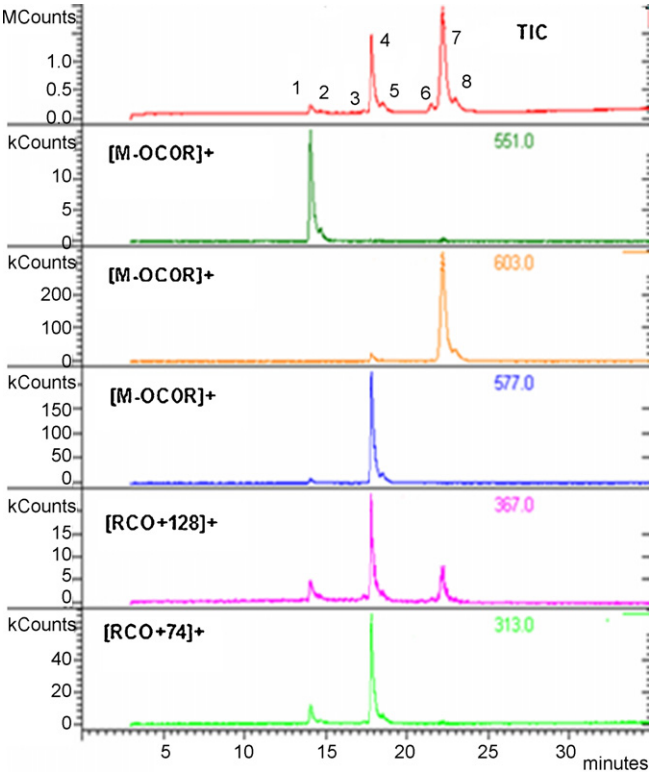

Fig. 4. Total ion chromatogram of the olive oil profile (top). Selected ion monitoring chromatogram of *m*/*z* 313, *m*/*z* 367, *m*/*z* 577, *m*/*z* 603 and *m*/*z* 551. Column Rtx-65TG, 65% diphenyl-35% dimethyl-polysiloxane (30 m × 0.32 mm i.d. × 0.1 μm) at 350 °C.

By using this method it was possible to identify the eight peaks of the chromatogram. Moreover it was possible to solve the problem of similar spectra because of overlapping between TAGs as well as to eliminate the interferences. The identified peaks that appeared in the total ion chromatogram profile for the high temperature GC–MS analysis of olive oil (Fig. 3) are shown in Table 2.

Although GC–MS offers much valuable information concerning the molecular species of TAGs, many applications of high polar column and high temperature in combination with mass spectrometer have not yet been achieved. The problem here may be that an unresolved peak leads to confusion in the peak assignment when using SIM profiles.

The previous results suggest that the SIM of $[M-OCOR]^+$, $[RCO+128]^+$ and $[RCO+74]^+$ ions is useful for the identification of TAGs in the olive oil with saturated, monounsaturated, and diunsaturated acid groups, but may not be suitable for those with more highly unsaturated acyl chains. Moreover, the peak assignment is possible under no good conditions such as high background and column bleeding. In further studies, authors will go ahead with this method by using different ratios of TAGs to detect adulterations in olive oils.

Table 2
Correspondence between peak numbers in the chromatogram of olive oil profile and the identified triacylglycerols. The peak numbers refer to Fig. 2.

Peaks	TAG	Rt (min)
1	PPO	14.06
2	PPL	14.64
3	PSO	17.26
4	OOP	17.88
5	POL/PoOO	18.58
6	OOS	21.52
7	OOO	22.20
8	OOL	22.97

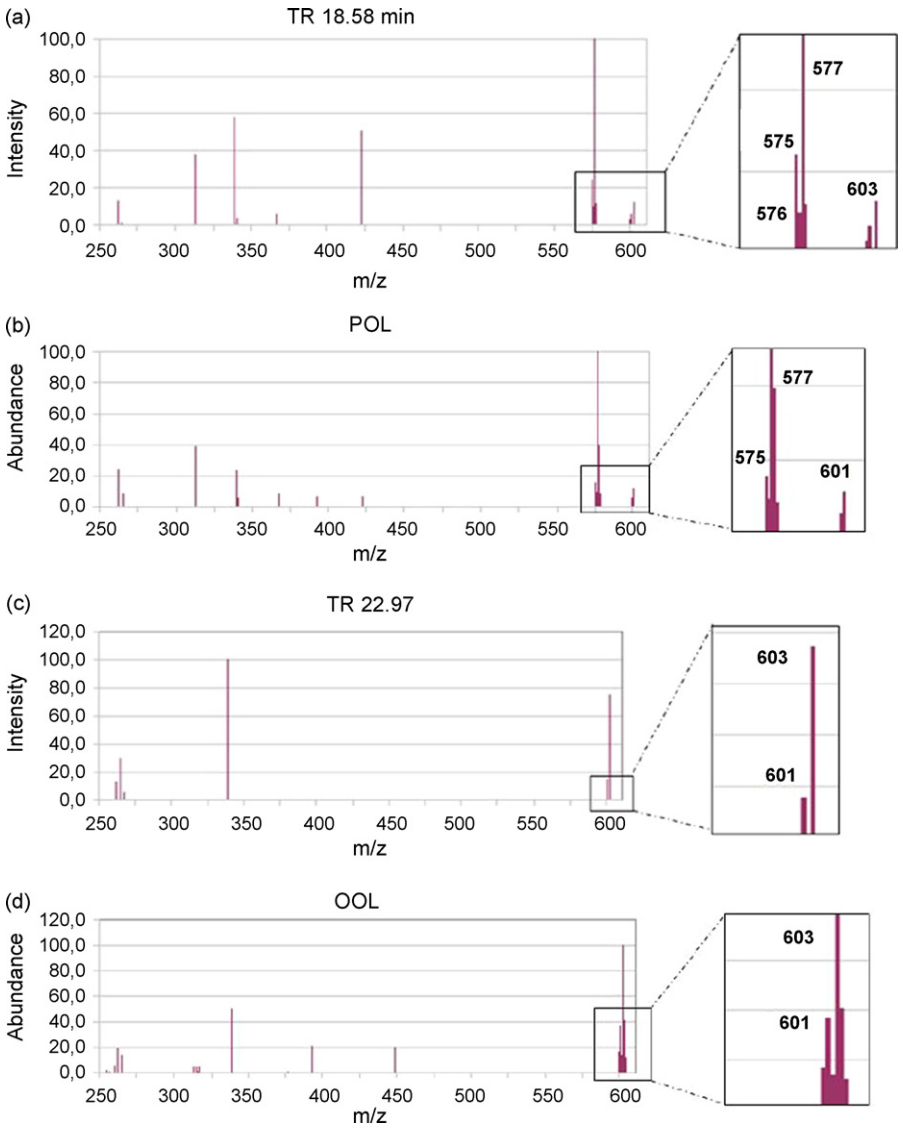

Fig. 5. Simulated spectra with the fragments obtained at the same retention times (parts a and c). Spectra of the corresponding standard triacylglycerol (parts b and d).

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