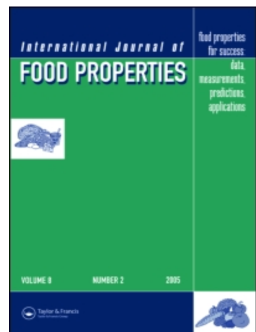

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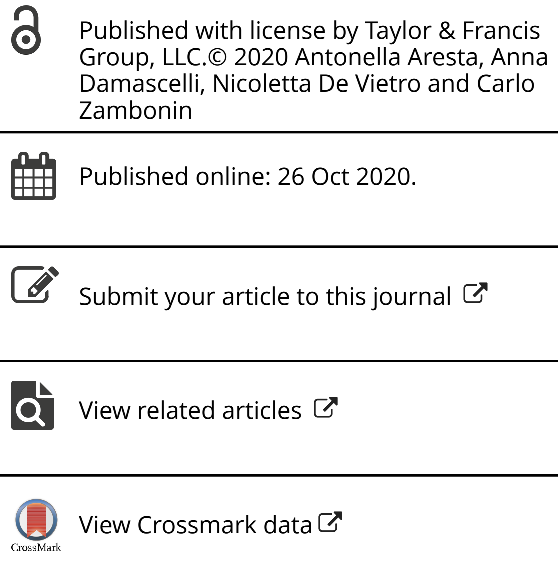

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Measurement of squalene in olive oil by fractional crystallization or headspace solid phase microextraction coupled with gas chromatography

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ABSTRACT

Squalene is the most abundant component in the unsaponifiable fraction of olive oil with strong antioxidant properties. Its concentration in olive oils varies between 0.2 and 16.2 g/kg depending on the cultivar(s) used. The propose of this work was to determine the effectiveness of two different extraction methods for squalene determination by gas chromatography (GC) coupled to a flame ionization detector (FID) or to mass spectrometry (MS). In a first approach, oil samples were dissolved in methanol/acetone mixture 7:3 (*v/v*) and triglycerides separated by fractional crystallization at −20°C. The organic layer was removed, reduced to dryness and the residue reconstituted in n-heptane (containing squalane as external standard) and analyzed by GC-FID. A headspace (HS) solid phase microextraction (SPME) GC-MS method has been also developed in order to have an environmentally friendly (*i.e.* solventless) extraction procedure. The linear range investigated with both methods was 1.0-10 g/kg. Within-day and between days precision values, expressed as RSD%, were 4 and 7% (GC-FID), and 3 and 6% (GC-MS), respectively. The limit of detection (LOD) at a signal-to-noise (S/N) ratio of 3 were 0.019 (GC-FID) and 0.003 (GC-MS) g/kg; the limit of quantification (LOQ) calculated at S/N = 10 were 0.063 (GC-FID) and 0.008 (GC-MS) g/kg, well below the typical squalene concentration levels found in olive oils. The obtained percentage recoveries were 70 ± 2 (GC-FID) and 98 ± 3 (GC-MS), and were not concentration dependent. The potential of the method has been demonstrated by the analysis of several different olive oil samples produced from different cultivars and different locations.

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Squalene; olive oil; fractional crystallization; solid phase microextraction; gas chromatography; mass spectrometry

Introduction

Squalene is a natural triterpenoid that serves as a precursor for the biosynthesis of steroids and, in particular, of cholesterol. It has antioxidant properties, protective effects on the skin and preventative effects in cardiovascular disease and cancer.[1–5] Squalene is the most abundant component in the unsaponifiable fraction of olive oil and occurs at concentrations in the range 0.2 ÷ 16.2 g/kg,[6,7] depending on cultivars, fruit ripening stage, oil extraction technique and storage conditions,[8–11] and plays an important role in shelf life and advantages to human health of extra virgin olive oil.[12–16] Based on the above considerations, analytical methods for squalene determination in olive oil could be very important to meet future needs for many purposes.

Most of the existing methods on this topic are based on chromatographic techniques, involving the removal of triglycerides and the fractionation of the unsaponifiable compounds into several classes[17–19] or oil samples transmethylation to allow direct instrumental analysis.[7,20,21] Sample pre-treatment approaches such as alkaline digestion, extraction and distillation,[6,22] solid-phase extraction (SPE)[23]

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and CO_2 supercritical fluid extraction (SFE) has also been reported.[24,25] Non-chromatographic methods for squalene determination in olive oils include nuclear magnetic resonance (NMR),[26] near-infrared spectroscopy (NIRS)[27] and laser desorption ionization time of flight mass spectrometry (LDI-TOFMS).[28]

In this work, a traditional sample pre-treatment approach, fractional crystallization, and a solventless extraction technique, headspace solid phase microextraction (HS-SPME), were developed for squalene determination by gas chromatography coupled to flame ionization detector (FID) or to mass spectrometry (MS), respectively. Both methods were eventually evaluated to determine the quantitative determination of squalene in the same olive oil samples produced from different cultivars and different locations.

Material and methods

Olive oil samples

Monocultivar olive oils were obtained from: *Cellina di Nardò, Coratina, Nociara, Cima di Bitonto, Peranzana* (cultivated in Apulia), *Cima di Melfi* (Basilicata), *Leccino* (Central Italy), *Moraiolo* (Tuscany, Lazio and Umbria), *Frantoio* (Umbria, Veneto, Tuscany and Marche), *Grignano* (Veneto and Lombardy) and *Picholine* (France). All samples were obtained from a local olive mill. The olive trees of the different cultivars were grown in the same olive plant and olive oil was obtained and stored under strictly controlled conditions so that the squalene content should be indicative of the cultivars.

Squalene ((all-E)-2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene, ≥98%, CAS 111–02-04) and squalane (2,6,10,15,19,23-Hexamethyltetracosane, ≥95%, CAS number 111–01-3) were obtained from Sigma–Aldrich (St. Louis, Missouri, USA) and Fluka (Buchs, Switzerland), respectively. All solvents used were HPLC grade.

SPME device

The SPME device consisted of a manual holder (57330-U, Supelco, Bellefonte, PA, USA) and a polydimethylsiloxane (PDMS) fiber (partially crosslinked, 100 µm thickness, GC 57300-U Supelco, Bellefonte, PA, USA). The PDMS fiber was conditioned in a GC injector at 250°C, according to the indications of the manufacturer.

GC-FID analysis

GC-FID analyses were carried out on a Shimadzu GC-17 AF Ver 3 gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID). Nitrogen was used as carrier gas. Separation was carried out on a Supelco (Bellefonte, PA, USA) SPB-5 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). The temperature programme was: 50°C isothermal for 3 min, 25°C/min to 280°C, isothermal for 30 min. The carrier linear flow rate was 15 cm/s and the injector operated in splitless mode at 250°C. Detector temperature was set at 290°C.

GC-MS analysis

GC-MS analyses were performed using a Trace GC Ultra (Thermo-Finningan) coupled to Polaris Q ion trap mass spectrometer (Thermo-Finningan). GC was provided with a PTV injector fitted with a 0.75 mm i.d. liner; helium was used as carrier. GC separation was carried out with a Supelco SPB-5 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). In scan acquisition mode, employed for the analysis of the volatile component, the oven temperature programme was: 40°C isothermal for 3 min, 5°C/min to 250°C, isothermal for 20 min. In SIM acquisition mode, used for the analysis of squalene, due to the increased selectivity, a faster temperature programming was used: 80°C

isothermal for 3 min, 25°C/min to 280°C, isothermal for 10 min. The carrier flow rate was 1 ml/min and the injector operated in splitless mode at 250°C. The temperatures of the transfer line and the ion source were set at 280°C and 250°C, respectively. Mass spectra were recorded at 70 eV in the 50–800 amu mass range (acquisition rate: 3 scans/s). Unless otherwise specified, quantification was performed in selected ion monitoring (SIM) mode and the monitored m/z ions were 85 and 125 for squalane and 107, 121 and 149 for squalene. SIM acquisition was activated in a restricted time window (from 12.3 to 21 min) encompassing the retention time of the external standard (squalane) and the analyte.

Preparation of oil free samples

For methods calibration, oil samples (extracted from *"Cima di Bitonto"* cultivar) were made free from squalene with a series of repeated extractions. The squalene-free oil samples were then divided into aliquots that were added with known amounts of squalene in order to prepare the calibration standards that were subsequently subjected to sample pre-treatment according to the selected procedure. Three replicates for each squalene concentration level were performed.

Fractional crystallization

Olive oil (0.125 g) was dissolved in 10 ml of methanol/acetone mixture 7:3 (*v/v*) by vortex-mixing for 2 min and subsequently kept at −20°C for 24 hours allowing solidification of triglycerides. After centrifugation (30 s), the organic phase was separated and evaporated in a rotary evaporator under vacuum; the residue was reconstituted in 2.5 mL of n-heptane containing squalane (0.2 g/kg) as external standard and analyzed by GC-FID.

HS-SPME

Olive oil samples (2 g) were spiked with 0.2 g/kg of squalane (internal standard) in a 4 ml vial hermetically closed with a silicone septum. Before extraction, stabilization of the headspace was obtained by equilibrating the vial in a water bath at 80°C for 60 min. Then a polydimethylsiloxane (PDMS) fiber was exposed in the headspace for 30 min. The extracted volatile compounds were thermally desorbed for 5 min into the GC injector maintained at 250°C.

Method validation

Calibration curves for both methods were prepared using "squalene free" olive oil samples added with 0.2 g/kg of squalane and variable amounts of squalene in order to cover the concentration range 1.0–10 g/kg. Three replicates for each concentration level were performed. The within-day (n = 6) and between-days (n = 6 over 5 days) coefficient of variation in oil were calculated on analyte free oil samples added with variable amounts of the target analytes in order to obtain the following concentration levels: 1, 5 and 10 g/kg. Recoveries were calculated at the same concentration levels as the ratio between analyte/squalane (spiked olive oil samples) and analyte/squalane (standard) peak area ratios.

Results and discussion

Determination of squalene by GC-FID analysis

Figure 1 reports typical chromatograms obtained for oils produced from "*Leccino*", "*Moraiolo*" and "*Peranzana*" cultivars; squalane (I.S.) and squalene (S.Q.) were detected at retention times of 28.1 and 33.0 min, respectively. The FID response was linear in the range 1.0‾10 g/kg (one decade is more than adequate to cover the squalene concentration range observed in real samples – see later). A typical

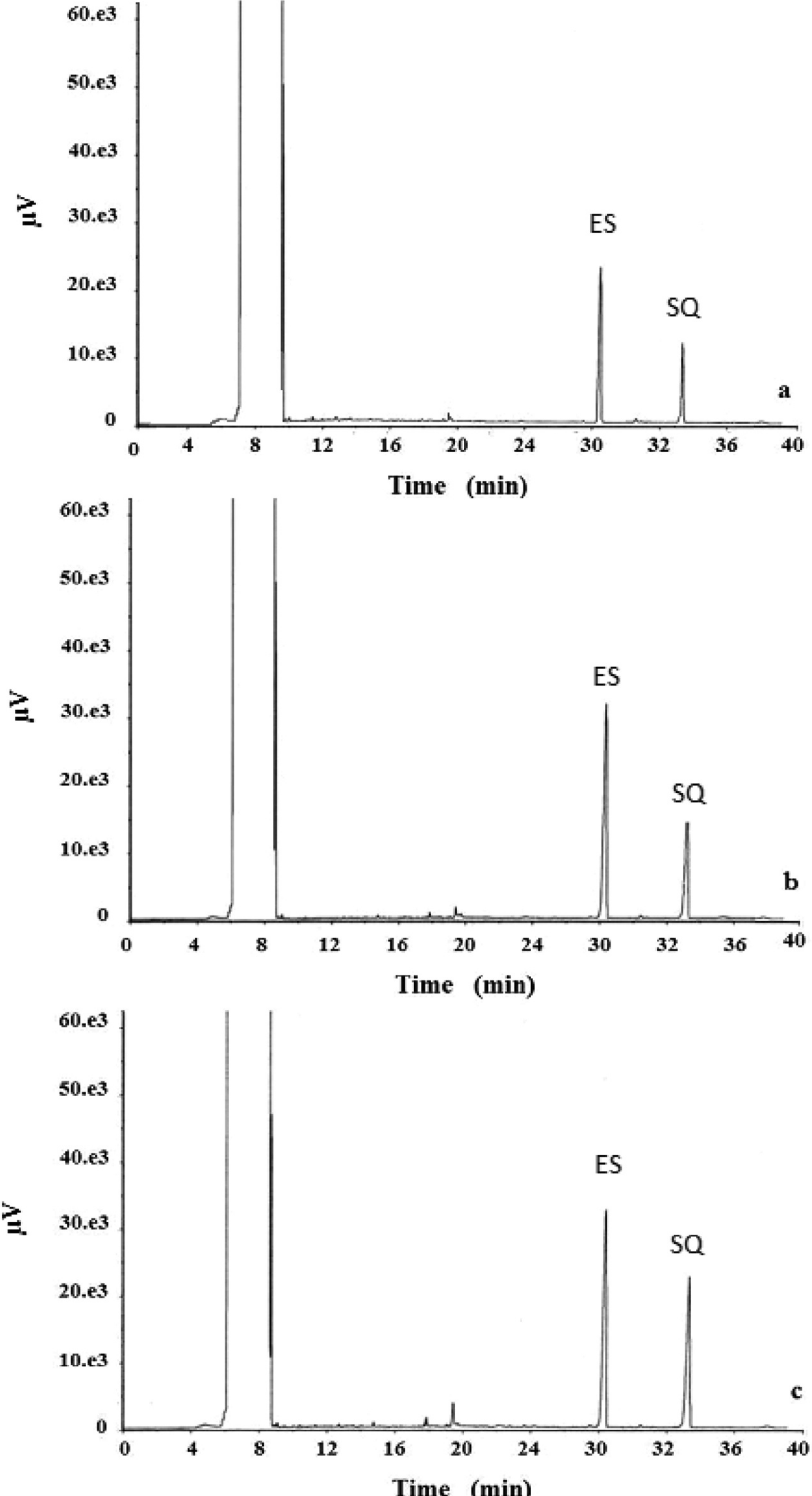

Figure 1. GC-FID chromatograms obtained from the analysis of the extracts of olive oil samples produced from: (**a**)"*Moraiolo*", (**b**) "*Leccino*" and (**c**) "*Peranzana*" cultivars. ES = external standard (squalane); SQ = squalene.

calibration line (squalene/squalane peak area ratio (A), versus squalene concentration (C), expressed in g/kg, was described by the following equation:

$$A = (0.007 \pm 0.008) + (9.64 \pm 0.17) \times 10^{-5}C$$

with R^2 = 0.999 and a standard error of regression of 9.8×10^{-6}. The calibration line presented an intercept not significantly different from zero (according to a *t-test* at 95% confidence level). The obtained within-day and between-days RSD values were 4 and 7%, respectively, and were not concentration dependent. The obtained limits of detection (LOD) and quantitation (LOQ), calculated at a signal-to-noise (S/N) ratio of 3 and 10 (noise calculated peak to peak on a blank chromatogram at the squalene retention time), were 0.019 and 0.063 g/kg, respectively, largely below the lowest squalene concentration in olive oil (0.2 g/kg). The obtained percentage recovery was 70 ± 2 and remained practically unchanged at 1, 5 and 10 g/kg levels.

Finally, olive oil samples, extracted from different cultivars of various Italian and foreign regions, were analyzed by the described method and squalene was quantified. Figure 1 reports, for instance, the GC-FID chromatograms obtained from the analysis of olive oil samples extracts produced from "*Moraiolo*", "*Leccino*" and "*Peranzana*" cultivars. The difference between the intensities of the squalene peaks suggested that squalene concentration in the analyzed samples could be different between the cultivar. As can be seen in Table 1, that reports squalene concentrations estimated in oil samples in the present work, the analyte concentration varied in the range 4–7 g/kg. The oil extracted from "*Peranzana*" (a typical Apulian cultivar), was characterized by the highest concentration of squalene while the "*Moraiolo*" oil, typical of Tuscany, Umbria and Lazio, presented the lowest amount. Therefore, the squalene content could be potentially used as a marker for oil traceability.

Determination of squalene by HS SPME GC-MS analysis

Preliminary experiments were performed in order to compare the extraction efficiency of PDMS and PA coated fibers. The PDMS coating was capable of the most efficient extraction of squalene and was then chosen for further experiments. Adsorption times ranging from 5 to 180 min were investigated in order to establish the equilibration time for analytes partition between the aqueous and the polymer phase. Equilibrium conditions were reached after 60 min; anyway, an adsorption time of 30 min revealed a good compromise between sample throughput and peak response. After each adsorption step, the fiber was immediately transferred into the injector port where it was thermally desorbed at 250°C for 5 min. A quantitative desorption could be obtained under these conditions as demonstrated by the absence of memory effects of the fiber.

Figure 2a shows a typical HS-SPME GC-MS chromatogram relevant to the analysis of an olive oil sample produced from the "*Cima di Bitonto*" cultivar, pre-conditioned for 60 min at 50°C to enrich the

Table 1. Squalene concentrations estimated in different monocultivar oils.

		[Squalene] g/kg (n = 3)	
Region	*Cultivar*	GC-FID	HS-SPME GC-MS
Apulia	*Cellina di Nardò*	6.62 ± 0.04	6.60 ± 0.02
	Coratina	5.21 ± 0.04	5.26 ± 0.04
	Nociara	6.03 ± 0.03	6.05 ± 0.05
	Cima di Bitonto	5.05 ± 0.03	5.01 ± 0.03
	Peranzana	7.05 ± 0.04	7.10 ± 0.06
Basilicata	*Cima di Melfi*	5.75 ± 0.03	5.70 ± 0.02
Central Italy	*Leccino*	5.34 ± 0.03	5.38 ± 0.05
Tuscany, Lazio and Umbria	*Moraiolo*	4.14 ± 0.02	4.12 ± 0.04
Umbria, Veneto, Tuscany and Marche	*Frantoio*	4.83 ± 0.03	4.79 ± 0.03
Veneto and Lombardy	*Grignano*	6.74 ± 0.04	6.71 ± 0.05
France	*Picholine*	5.22 ± 0.03	5.18 ± 0.04

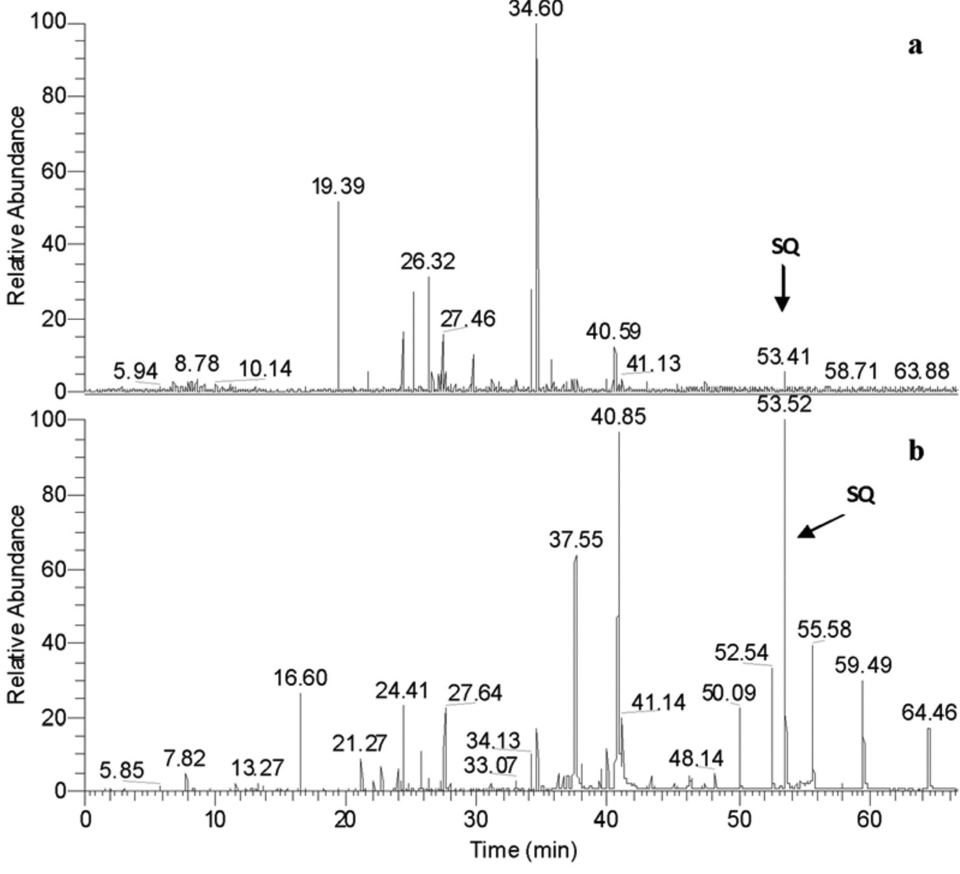

Figure 2. HS-SPME GC-MS (TIC) chromatograms obtained from the analysis of the volatile fraction of an olive oil sample produced from the *"Cima di Bitonto"* cultivar. Headspace temperature: (a) 50 and (b) 80°C. SQ = squalene.

Table 2. Compounds identified in an olive oil sample produced from the "*Cima di Bitonto*" cultivar analyzed by HS-SPME GC-MS.

Retention time (min)	Analyte
7.82	2-Hexyn-1-ol
13.02	4-Decyne
14.68	1,3,5-Cycloheptatriene
15.16	2-Octyn-1-ol
19.95	2,4-Dimethyl 1,4-pentadiene
21.27	2-Decyn-1-ol
22.79	2,4-Decadienal, (E,E)
25.67	p-Thyrosol
26.31	Cyclopentaneacetaldehyde, 2-formyl-3-methyl-α-methylene
32.32	9,12-Octadecadienal
35.70	(Z,Z,Z)-8,11,14-Eicosatrienoic Acid
36.97	Z,Z-10,12-Hexadecadienal
53.52	Squalene

headspace. The chromatographic profile appeared rich of matrix components; some of them were identified by the comparison of their mass spectra with those reported in the NIST library and the attributions reported in Table 2.

As apparent, squalene was identified as the peak at the retention time of 53.41 min, based on the comparison with mass spectrum and retention time of an authentic standard. In order to increase squalene concentration in the headspace, further experiments were conducted raising the extraction temperature at 80°C. As reported in Figure 2b, following these extraction conditions, squalene was detected with a high signal-to-noise (S/N) ratio that could be further improved operating in *selected ion monitoring* (SIM) mode (see later). Due to the increased selectivity achievable in SIM, mode a faster temperature programming could be used (see Experimental) that considerably shortened (a threefold factor) the GC analysis time (squalene retention time was correspondingly reduced from *ca.* 53 to *ca.* 14 min). Figure 3 shows, for instance, the HS-SPME GC-MS (SIM) chromatograms obtained for three representative olive oils extracted, respectively, from "*Coratina*", "*Moraiolo*" and "*Picholine*" cultivars typical of different geographical regions. The high selectivity achieved in SIM mode is evident since only peaks of squalane and squalene at the retention time 12.98 and 14.35 min, respectively, could be observed. The possibility to differentiate the three monovarietal olive oils according to the different relative intensities of squalene/squalane peaks is also apparent.

The calibration curve resulted linear in the same range 1.0‾10 g/kg of GC-FID method with correlation coefficients better than 0.999 and intercept not significantly different from zero at 95% confidence level. The calibration line (squalene/squalane peak area ratio (A), versus squalene concentration (C), expressed in g/kg, was described by the following equation:

$$A = (0.0001 \pm 0.0001) + (1.23 \pm 0.01) \times 10^{-4}\,C$$

with R^2 = 0.999 and a standard error of regression of 6.8×10^{-6}. Within-day and between-days (six within-day replicates over five days) RSD values were 3 and 6%, respectively, showing a very satisfactory precision. Limits of detection (LOD) and quantitation (LOQ) calculated at a signal-to-noise (S/N) ratio

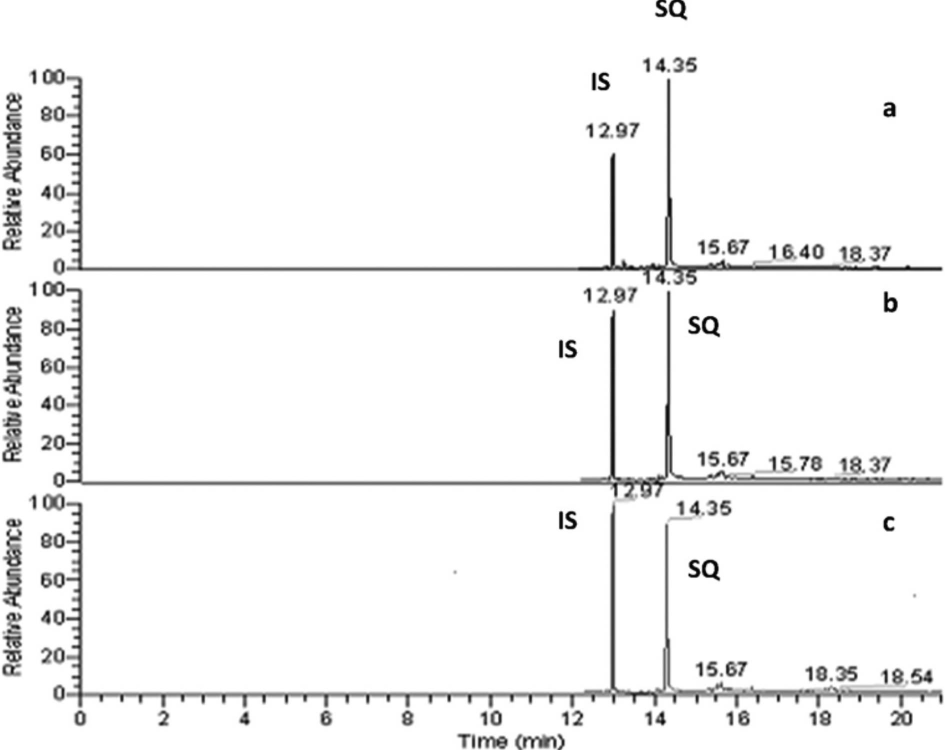

Figure 3. HS-SPME GC-MS (SIM) chromatograms obtained from the analysis of olive oil samples produced from: (a) "*Coratina*", (b) "*Moraiolo*" and (c) "*Picholine*" cultivars. IS = internal standard (squalane); SQ = squalene.

of 3 and 10 (noise calculated peak to peak on a blank chromatogram at the squalene retention time) were 0.003 and 0.008 g/kg, respectively, well below the typical squalene concentration range in olive oil (0.2 ÷ 16.2 g/kg). The obtained percentage recovery was 98 ± 3 and remained practically unchanged at 1, 5 and 10 g/kg levels. Different monovarietal oil samples were analyzed by HS SPME GC-MS and the relevant results were also reported in Table 1. As apparent, the results obtained for squalene determination in the analyzed olive oil samples were in good agreement (within the experimental errors).

Conclusion

Two different extraction methods, differing for complexity and organic solvent consumption, are described for the GC determination of squalene in olive oil samples. The first approach requires fractional crystallization (to remove triglycerides), the use of a limited amount of organic solvent and it runs on a readily available instrumental set-up (*i.e.* GC-FID). The second approach, consisting of a HS-SPME GC-MS method, requires a more expensive instrumental set-up but possesses distinctive advantages of being very simple (no sample pre-treatment), relatively fast and solventless.

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