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Determination of Squalene in Organic Extra Virgin Olive Oils (EVOOs) by UPLC/PDA Using a Single-Step SPE Sample Preparation

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Abstract In this paper, a simple extraction and fast detection procedure was used to determine squalene (SQ) in extra virgin olive oils (EVOOs). SQ was purified from EVOOs by an efficient single-step solid phase extraction (SPE), and its content was determined using an UPLC-PDA instrument. The adopted technique was evaluated for accuracy, linearity, sensitivity, and repeatability. The precision of the SPE extraction was satisfactory and the mean recoveries were 91.9 ± 0.4 and 96.3 ± 0.3 % for 25 and 50 mg L^{-1} level of addition, respectively. The selected chromatographic conditions allowed a very fast SQ determination; in fact, it was well separated in ~0.54 min with good resolution. The UPLC method showed a good linearity in the range 50–500 mg L^{-1} (R^2 = 0.9998). Method sensitivity was evaluated by measuring the limits of detection (LOD) that was 0.3 mg L^{-1}. The method was utilized for SQ determination in 33 different organic EVOO samples, coming from different countries (Turkey, Tunisia, Spain, Portugal, Greece, USA, Slovenia, Albania, Israel, Italy) and the data were statistically evaluated.

Keywords Squalene · Extra virgin olive oil · UPLC/PDA · Food analysis

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Introduction

Extra virgin olive oil (EVOO) plays a crucial role within the Mediterranean diet, and several studies pointed out the beneficial effects of the daily uptake of EVOO because of its ʙperfect balance in the fat fractions.ˆ Specifically, these effects are mainly due to four chemical sources: polyphenols, carotenoids, oleic acid (mono-unsatured fatty acid, MUFA), and squalene (Giuffrida et al. 2007; Giuffrida et al. 2011). EVOO polyphenols are considered to be potent antioxidant compounds, able to control the free radicals in blood, preventing both aging and cardiovascular misfunctions as it reduces oxidation of the cholesterol transporting proteins (Clodoveo et al. 2015). A great amount of data are reported and summarized in wide studies evidencing the EVOO ability to enhance the plasma antioxidant features, preventing DNA damage and simultaneously reducing inflammatory markers; therefore, an anti-inflammatory effect is also suggested (Cárdeno et al. 2013; Killeen et al. 2014; Bulotta et al. 2014). Oleic acid is a MUFA; it is considered a ʙgoodˆ fat as it has been shown to prevent colon-rectal cancer (Yumi et al. 2005). On another hand, it regulates the blood fluidity reducing the low density lipoproteins (LDL) levels (ʙbad cholesterolˆ) (Narayan Bhilwade et al. 2010).

Last, but not least, squalene (SQ) seems to hamper photocarcinogenesis (skin tumors) by blocking the photo-oxidative damage of skin DNA (Kelly 1999). SQ is the third main component of the skin lipidic film secreted by sebaceous glands. This layer is the main skin protector, and it has been demonstrated that SQ is not very susceptible to per-oxidation and appears to function, on the skin surface, as a quencher of singlet oxygen (Tsujimoto 1916); this was recently confirmed by in vitro studies on SQ detoxification activity (Se-Kwon and Fatih 2012). Moreover, it is a main component of the lipid film

and is thought to preserve skin and retinas allowing also a stable cyto-architecture (Auffray 2007). Actually, since SQ is essential for biosynthesis of steroids and triterpenes and is also considered an intermediate in endogenous cholesterol synthesis, there was a concern that high SQ intake from the diet could lead to cholesterol increase, which translates into a greater risk for development of atherosclerosis (Chan et al. 1996). On the contrary, though, it has been also reported that SQ might lead to several beneficial effects reducing cholesterol and triglyceride levels in serum and protecting against a variety of cancers (Smith et al. 1998; Storm et al. 1993; Tsujimoto 1916). This theory is supported by several trials run over animals; indeed, sharks, whose liver oil is very rich in squalene, are thought to face a lower cancer occurrence (Mathews 1992; Newmark 1997).

Despite its known biological benefits, medicinal and cosmetic use of SQ took place in the first years of this century. Besides, because of the chemical structure, SQ and its derivative squalene are easily absorbed at the local/topical level. These features account for their uses as the following: (a) emollients, to recover the lipid film; (b) demulcents for delicate skin treatment; (c) anti-aging agent against the oxygen derived free radicals; and (d) antioxidant ointments able to protect cutis and the entire body from microbiological and oxidative burden (Se-Kwon and Fatih 2012).

In conclusion, generally, the regular uptake of EVOO is considered beneficial against cardiovascular threat, blood-pressure disorders, cancer challenges as well as microbial diseases and inflammatory events; this is the reason why it is suggested the consumption of about 30 g of SQ per day (especially from raw EVOO) and it is possible to find many medical ointments containing SQ (Reddy and Couvreur 2009).

Chemically, SQ is an isoprenoid compound with 30 carbon atoms, containing six double bonds and it is present mainly in the cod liver oil. For the first time, it was isolated from the shark liver oil but is widely distributed in nature in both vegetal and animal tissues (Smith 2000). In humans, about 60 % of SQ is absorbed from food and main source is EVOO which contains about 0.2–0.7 % of SQ (Saitta et al. 2014). SQ is transported through the serum in association with very low density lipoproteins and is distributed ubiquitously in human tissues, especially in the skin where it is a major component of lipid body surface area (Kohno et al. 1995). Our biological system is able to use SQ as a simple ʙbrickˆ toward the synthesis of steroidal hormones or other lipid substances. The average intake of SQ is 30 mg/ day; however, in Mediterranean areas, where the consumption of olive oil is higher and combined with other SQ containing food-stuff, SQ intake can reach 200–400 mg/day (Reddy and Couvreur 2009; Grigoriadou et al. 2007).

One of the first studies related to the SQ determination involved colorimetric method (Rothblat et al. 1962). Traditionally, SQ in foods, oils, and fats was determined by titrimetric (AOAC 1999) or chromatographic procedures (Cert et al. 2000; Bondioli et al. 1993; De Leonardis et al. 1998) and some studies recommended the solid phase extraction (SPE) for sample preparation (Grigoriadou et al. 2007; Sagratini et al. 2013; Popa et al. 2015). The up-to-date analytical techniques widely used for the SQ quantification in several food matrices are the following: (a) the gas-chromatography (GC-FID) by itself (Bueno et al. 2005) or coupled online with high-performance liquid chromatography (LC-GC-FID) (Esche et al. 2013; Grob et al. 1992; Villén et al. 1998) or (b) the bare HPLC (Nenadis et al. 2002; Sagrantini et al. 2013) or coupled to mass spectrometry (HPLC/MS) (Di Stefano et al. 2012; Mountfort et al. 2007; Russo et al. 2010). In the existing HPLC or HPLC/MS methods, both normal and reversed phase are employed (Manzi et al. 1998; Mountfort et al. 2007) and several authors proposed the use of UV, DAD, and RI detectors for SQ determination in different natural sources (Cortesi et al. 1996; He et al. 2002; Sun et al. 1997). The direct SQ determination is complicated, and generally, the SQ extraction methods from foods involve sample pre-treatment, to eliminate the interfering substances. Commonly, it consists in saponification and extraction of unsaponifiable, followed by chromatographic separation by column with different fillers (Popa et al. 2015) or a preliminary fractional crystallization (Nenadis et al. 2002). However, as observed by Nenadis et al. (2002), most of the existing methodologies have been developed for the determination of other compounds and SQ was simply co-determined. In the last years for the SQ isolation, the supercritical fluid extraction has been preferred (AOAC, Official method of analysis 1999), but this method is still expensive at the industrial level. The most accredited method for the determination of SQ involves sample saponification, extraction of the non-saponifiable matter with large volume of solvent, fractionation through chromatographic column, and other treatment just before titration (Nenadis et al. 2002). In the existing methods, the quantification of SQ by direct analysis is difficult and requires a pre-treatment step to eliminate the interfering substances. Usually, pre-treatment involves relatively high volumes of organic solvent, high capital cost and long elution time.

The aim of this study was the development of a rapid, simple, and efficient method for the determination of SQ in EVOO samples. In the present work, the SQ determination was performed in reversed phase, and in the UPLC analytical method, the mobile phase was optimized with respect to short-time analysis and maximizing chromatographic resolution of the analyte. Also, considering that the chemical composition of EVOO may vary depending on the cultivar, maturity at harvest, ecological conditions, and growing areas, the validated method was successfully applied on 33 organic EVOO samples provided by a worldwide competition from several nationalities, to determine and compare the SQ contained, trying to exploit this parameter as geographical indicator.

Material and Methods

Samples, Chemicals, and Reagents

The organic EVOO samples were donated by the President of the C.I. Bi. Cooperative Society, general coordinator of the Biol award 2015 (Andria, Italy). The study was carried out on 33 samples, coming from different countries, and the cultivars were genetically identified for both monovarietal samples and blends. Belonging country, cultivar and registry numbers for the prize competition are shown in Table 1. All samples were kept at −20 °C until analysis.

Acetone and acetonitrile were Optima UHPLC/MS and were from Fisher Chemical products (Milan, Italy). Squalene (SQ) standard 98 % and *n*-hexane were purchased from Sigma-Aldrich (Milan, Italy). Stock solution of SQ was prepared in *n*-hexane at concentration of 10 mg mL^{-1} and stored at −18 °C; more dilute solutions were then prepared by appropriate dilution with *n*-hexane immediately before use. All the solutions were filtered through a non-sterile PTFE syringe filter 0.2 μm, purchased from Phenomenex (Bologna, Italy), before UPLC/PDA analysis. Cartridges Discovery DSC-Si Silica SPE (6 mL, 500 mg) were supplied from Supelco (Milan, Italy).

Table 1 Squalene content in 33 organic EVOO samples selected for the BIOL 20° International Price 2015

ID	Country	Region	Cultivar	Mean (mg/kg)	SD (%)[a]
1	Turkey	Balikesir	Adremittion	2550	4.39
2	Turkey	Izmir	Domat	2625	2.06
3	Tunisia	Tunisia	Chemiali	1534	1.86
4	Tunisia	Tunisia	Chemiali	1448	4.07
5	Spain	Andalucia	Hojiablanca	4044	1.68
6	Spain	Extremadura	Manzanilla Cacerena	4445	0.56
7	Spain	Jaen	Picual	4918	0.15
8	Spain	Murcia	Picual	4207	3.53
9	Spain	Navarra	Arbequina	4046	1.48
10	Portugal	Alentejo	Calega Vulgar	4094	1.50
11	Portugal	Alentejo	Cobrancosa Frantoio	4976	1.71
12	Portugal	Douro Tras Os Montes	Verderal Madural blend	4151	4.61
13	Portugal	Porto	Cobrancosa/Madural/Verdeal/Cordovil	4073	1.00
14	Portugal	Tras Os Montes	Cobrancosa/Madural/Verdeal/Cordovil	4513	0.64
15	Greece	Atene	Koroneiki	3179	2.65
16	Greece	Grecia	Koroneiki/Athinoelia	3465	8.52
17	Greece	Lesvos	Kolovi	3288	4.26
18	Greece	Messinia	Koroneiki	3360	6.50
19	Greece	Peloponnese	Koroneiki	3135	7.43
20	USA	California	Ascolana/Tagiasca/Missio	2194	10.43
21	USA	California	Picholine/Columella	2689	5.31
22	USA	California	Arbequina/Manzillo	2709	2.58
23	USA	California	Lungiana/Pendolino/Frantoio	2581	1.20
24	Slovenia	Koper	Bianchera Istriana/Leccino/Maurino	1730	19.80
25	Slovenia	Slovenska Istra	Istrska Belica/Leccinino/Buga/Ascolana	2060	3.93
26	Albania	Tirana	Oliva di Tirana	1675	18.43
27	Albania	Vlore	Kalinjot	1543	2.22
28	Israel	Galilee	Barneia	4554	6.90
29	Italy (Sicily)	Sciacca (Ag)	Biancolilla/Cerasuola/Ogliarola/ Carolea/Coratina	6485	3.85
30	Italy (Sicily)	Carlentini (Ct)	Nocellara Etnea/Tonda Iblea	6203	4.22
31	Italy (Sicily)	Ferla (Sr)	Tonda Iblea	6548	6.43
32	Italy (Sicily)	Chiaramonte Gulfi (Rg)	Tonda Iblea	7474	4.89
33	Italy (Sicily)	Buccheri (Sr)	Tonda Iblea	6371	2.80

[a] The standard deviation was estimated on the entire analysis, considering the extraction process

Squalene Clean-Up

Each EVOO sample was submitted to a solid phase extraction and the optimized procedure was as follows. The oil sample was weighted (~0.12 g), dissolved in 0.6 mL of *n*-hexane, and loaded on the top of a 500-mg Supelco Discovery DSC-Si Silica column, previously washed with 5 mL of *n*-hexane. After that, the SPE column was connected to vacuum manifold and SQ was eluted with 10 mL of *n*-hexane at a flow rate of about 1 drop/s. The eluate was dried under vacuum at room temperature, redissolved in 1 mL of mobile phase, filtered through a 0.2 μm PTFE membrane filter and transferred into vial for immediate and subsequent UPLC/PDA analysis.

Three extraction processes were carried out to each sample, and each extract injected three times.

UPLC-PDA Analysis

The SQ analysis was carried out using an *Acquity UPLC® Waters* liquid chromatography system equipped with a column heater, a photodiode array detector *ACQ-PDA*, a quaternary solvent manager *ACQ-QSM*, and a sample manager *ACQ-FTN*, controlled by *Waters® Empower™* chromatographic software. In all the analyses, an *Acquity UPLC® Waters BEH C18* column of 1.7 μm (2.1 × 50 mm), protected by 0.2 μm stainless steel In-*Line Filte*r with a *Holder Waters*, was used.

After analyzing different elution conditions and chromatographic profiles at various wavelengths, analyses were run at 40 °C, under isocratic condition, with a mobile phase composed of acetonitrile/acetone (60:40 *v*/*v*). The injection volume was 2 μl and the flow rate was 0.8 mL/min. SQ was detected and quantified using the PDA set at 217 nm wavelength.

Statistical Analyses

All the data were subjected to statistical analysis of variance (ANOVA) comparing the average with Tukey's test at the maximum significance, using the OriginPro 2016 program.

The results regarding the SQ contents of all the analyzed samples were compared in order to discriminate the EVOOs coming from different countries.

Results

SPE and UPLC-PDA Analysis

In a preliminary phase of this study, the extraction parameters were subjected to optimization.

First, the stage of SPE purification was studied. Taking as the starting point, the condition previously devised by other authors for the SQ determination in oil samples (Grigoriadou et al. 2007; Sagratini et al. 2013), the conditions were slightly modified to adapt the methods to the SPE column that we used. Different volumes of organic solvent were seep through to desorb SQ from SPE and the best result was achieved using 10 mL of *n*-hexane. Poor recoveries were obtained when smaller volumes were used whereas higher volumes ended up to just a sample dilution. The mixture extracted and purified, dried under vacuum at room temperature, and redissolved in the mobile phase, was injected into the UPLC/PDA system.

In the UPLC analytical method, detection at 217 nm was considered more suitable and selective since at this wavelength, as illustrated in Fig. 1, the other compounds eluting before SQ are not detected. The mobile phase was optimized with respect to short-time analysis and maximizing chromatographic resolution of the analyte. For this reason, various elution phases and chromatographic conditions were studied. Finally, acetonitrile/acetone (60:40 *v*/*v*) were selected as this system gave better resolution, peak shape, and stable baseline. The mobile phase velocity was also evaluated at 0.3, 0.5, 0.8, and 1 mL min^{-1} values, and 0.8 mL min^{-1} was chosen as an optimal flow velocity. The selected isocratic chromatographic conditions allowed a very fast SQ determination; in fact, this compound was well separated in ~0.54 min with good resolution. To test the kindness of the chromatographic separation, initially, the mobile phase was injected three times and no peak was detected at the same SQ retention time. Then, for assessing the matrix effect and in order to establish the exact SQ retention time, three aliquots of an EVOO sample, whose SQ content had been previously determined, were spiked with different increasing amounts of SQ standard. The chromatographic analyses of these samples revealed a peak with gradually increasing area, but no interference was observed. Moreover, the peak purity was confirmed by comparing the PDA data of SQ standard with the peak of the respective analyte. In our chromatographic conditions, impurity or overlapping were not found.

The last parameter examined was the column temperature. The values tested were 35, 40, and 45 °C. At higher values, the resolution of the chromatographic SQ peak did not improve and the column durability would decrease. The k' and the area versus temperature show that there were no differences among the temperature tested, although above 45 °C, there was a slight tendency to decrease. A symmetrical peak of SQ standard was obtained at all temperatures, and the peak area was slight higher at 40 °C. Thus, 40 °C was fixed for subsequent analysis.

Figure 1 shows the chromatograms representative of SQ standard and of an organic EVOO sample analyzed with the present UPLC-PDA acquisition mode.

Method Validation

The UPLC/PDA method was evaluated through validation parameters that included linearity, sensitivity, accuracy, and repeatability according to a protocol setup initially in our

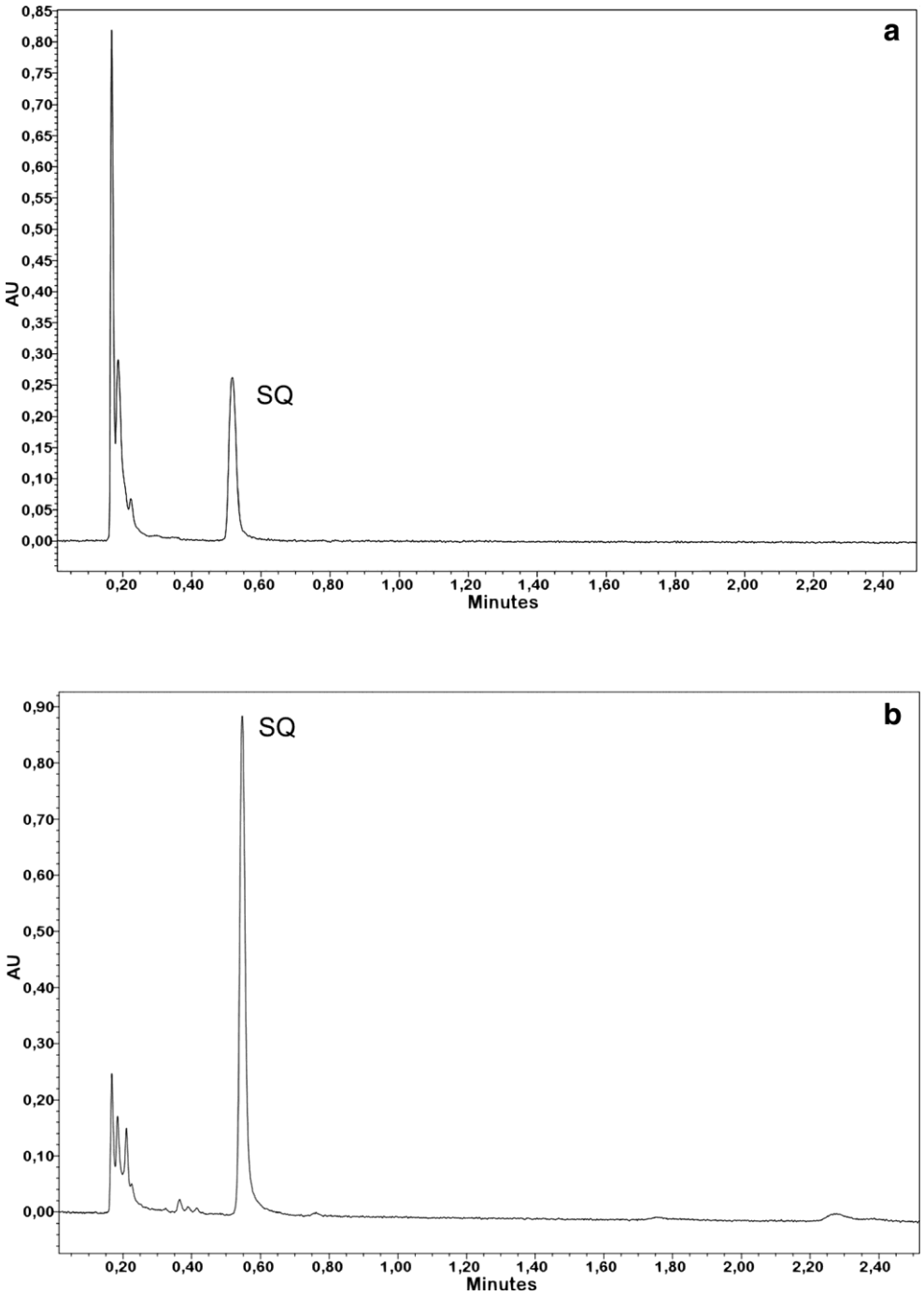

Fig. 1 UPLC-PDA chromatograms at 217 nm of a SQ standard solution and of b organic EVOO sample after SPE extraction

laboratories and already adopted for the development of other analytical methods (La Torre et al. 2010; Gentile et al. 2016).

SQ quantification was done by measuring peak areas at SQ retention time and by comparing them with a calibration curve. A five-point calibration graph was obtained with 50, 100, 250, 400, and 500 mg L^{-1} standards. Calibration graph was achieved using linear regression of the least squares method and the peak response of each standard injection plotted against SQ concentration. Linearity was evaluated by the determination of the least square regression coefficients (R^2). The correlation coefficient was 0.9998. Calibration solutions were freshly prepared each day before the measurement. Each solution at different concentration was prepared in duplicate and injected in triplicate; the mean was obtained from all measurements.

Method sensitivity was evaluated by measuring the limits of detection (LOD) and of quantification (LOQ). The limits of detection (LOD) and of quantification (LOQ) were calculated using a signal-to noise ratio equal to 3.3 and 10, respectively (EURACHEM/CITAC guide 2012), and were estimated with

standards containing SQ at low concentration levels. LOD was 0.3 mg L^{-1} and LOQ was 1.0 mg L^{-1}.

The accuracy of the present method was evaluated by means of a spiking and recovery study on EVOO samples. Considering that EVOOs could contain significant levels of endogenous SQ, the assessment of recoveries relied on the added amount with respect to the basal EVOO signals. Thus, the recovery of the full analytical procedure was carried out on three EVOO samples with a low SQ content and was calculated by adding known amounts of SQ standard to samples whose SQ content had been previously determined. The recovery studies were carried out for two levels of standard SQ addition (25 and 50 mg L^{-1}, respectively) and five replication ($n = 5$). After addition, the samples were subjected to whole analytical procedure and the concentration of SQ in the samples was measured. As a result, the peak area of SQ in the EVOO sample, tested before, was subtracted from that corresponding to spiked ones as a measure of net SQ signal in the EVOO. The recovery (%) at both levels was satisfactory (91.9 ± 0.4 and 96.3 ± 0.3, respectively). The SQ content in real EVOO samples (see Table 1) is given without recalculation for recovery.

The precision of the method was expressed as the relative standard deviation (RSD) and two quality parameter (repeatability as intra-day and inter-day of retention time and peak area measurement) were determined. The intra-day repeatability of the method was assessed by performing five consecutive injections of SQ standards at two different levels of concentration addition (25 and 50 mg L^{-1}, respectively) under the selected conditions and calculating the standard deviation. The same standards were also analyzed over a period of 12 successive days to determine the inter-day RSDs. The analytical precision assessed through the statistical results of the intra-day and inter-day determinations were 1.50 and 3.38 % for lower concentration and 1.00 and 2.12 % for high concentration, respectively. Retention time (RSD) values for intra-day repeatability ($n = 5$) was lower than 0.2 and 0.98 % for inter-day repeatability ($n = 12$).

Therefore, the analytical characteristic can be considered satisfactory for the aim of the analysis.

Analysis of Real EVOO Samples

After optimization of the analytical conditions, the method was applied for the SQ determination in 33 organic EVOO samples that had been selected for the BIOL 20° International Price 2015. The samples were from ten different countries (Turkey, Tunisia, Spain, Portugal, Greece, USA, Slovenia, Albania, Israel, and Italy). Particularly, the Italian samples were from Sicily only. As mentioned before, Table 1 summarizes the data regarding the origin and the varieties of each EVOO sample, together with the SQ quantitative results.

SQ was identified in all the analyzed EVOOS and, as Table 1 shows, the quantitative results indicated that great variability exist, particularly in SQ concentration from one country to another.

A trouble-free analysis of the data reported in the Table 1 highlights that the Italian organic EVOO samples, regardless of the cultivars, showed significantly higher SQ content when compared to the other organic EVOOs. In the five analyzed Italian EVOOs, the SQ concentration ranged from 6203 to 7474 mg kg^{-1} and these values were followed by those detected for the five Spanish (range between 4044 and 4918 mg kg^{-1}) and five Portuguese (range between 4073 and 4976 mg kg^{-1}) EVOOs which, on the other hand, have a very similar SQ content. What observed for these latter samples suggests that several factors may influence the SQ content in EVOOs and that, regardless of the genetic factor, other environmental and geographic factors could affect SQ content in EVOOs.

Our results indicate that the SQ content in organic EVOOs from Tunisia, Albania, and Slovenia were very comparable, and that in these samples, SQ displayed the lower levels; at the same time, within the four samples from the USA, the SQ concentration did not exceed 2709 mg kg^{-1} (mean and median value 2543 and 2635 mg kg^{-1}, respectively), while the five Greek EVOO samples showed a slightly higher content (from 3179 to 3465 mg kg^{-1}). The two EVOOs from Turkey showed comparable SQ contents with those from the USA, while the SQ level of the only Israeli EVOO that took part in the competition was close to those of the Spanish and Portuguese samples.

When ANOVA was performed, the significances of differences of the SQ concentrations, among the EVOO samples from different origins, were estimated. The results are presented in Fig. 2 where it is possible to have an idea of the differences and similarity among the EVOO samples. A careful analysis of the graph allowed four principal groupings to be identified. Group *a*, corresponding to EVOOs from Italy, shows that these samples had the highest SQ content; therefore, these are well distinguishable from the other EVOOs. In group *b*, the EVOO samples from Spain, Portugal, and Israel are grouped together, showing a clear overlapping of data. In order to analyze the plot in details, the results corresponding to each country are represented separately. A quantitative ʙsimilarity^ relationship among the EVOO samples of Turkey, USA, and Greece (group *c*) can be observed in Fig. 2, where EVOOs with similar SQ content are grouped in the same area of the plot. However, as the UPLC result evidenced, within this group, the samples relative to Greece gave values slightly highest in the plot area previously identified. The EVOO samples from Albania, Tunisia, and Slovenia are not similar to other EVOO samples and can be easily identified in group *d*, corresponding mainly to EVOO samples whose SQ content was lowest.

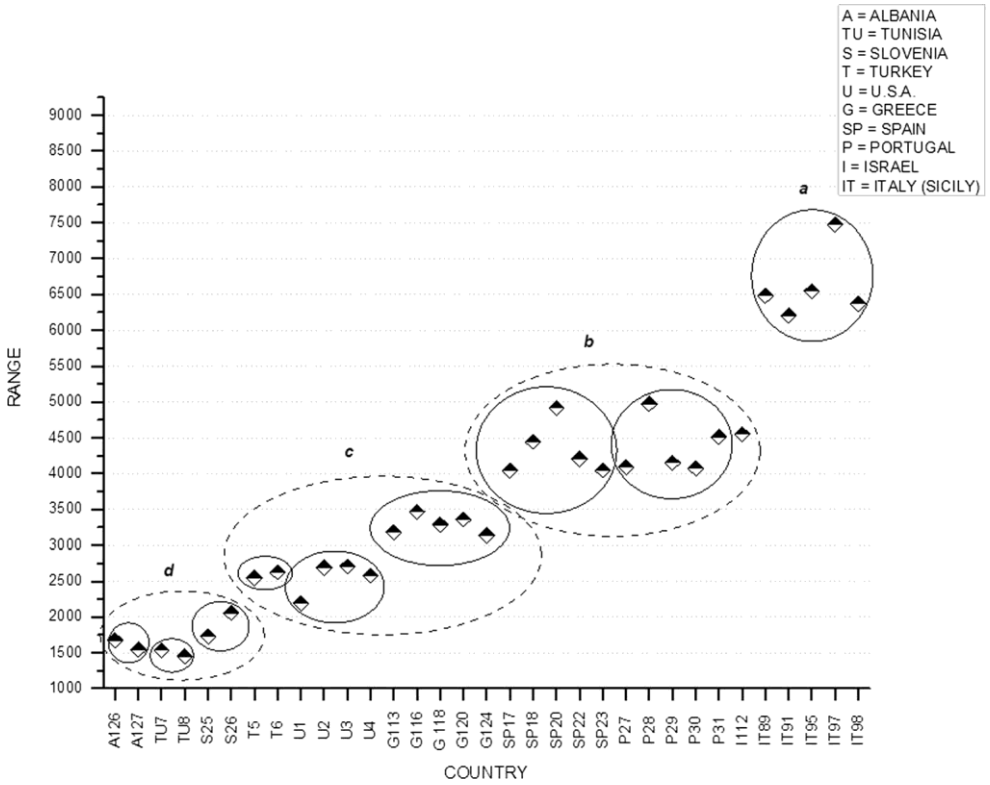

Fig. 2 Statistical analysis of variance performed on organic EVOO samples from different countries

Conclusion

The proposed method combines an efficient sample pre-treatment and a rapid UPLC quantification of SQ in complex matrices such as EVOO. It is a quick and reliable analytical procedure by-passing the saponification process. The sample pre-treatment procedure, based on SPE with C18 cartridges, has granted good extraction yields of SQ with satisfactory sample purification. The use of cartridge for clean-up followed by UPLC has shown to be a technique with good analytical performance for squalene determination in EVOO. Compared to other HPLC methods reported for the SQ analysis in food matrices (especially oil), good LOD was achieved (Nenadis et al. 2002; Sagratini et al. 2013).

Of course, it is not possible to directly compare our method with similar ones for SQ determination in extra virgin olive oils, since the work of Nenadis et al. (2002) uses a fractional crystallization method for the preparative part of the sample. However, the proposed SPE-UPLC/PDA method is surely less expensive and more simple and rapid than the methods which use on line LC-GC.

The differences in SQ content in the analyzed organic EVOO samples of this study could be explained by considering that the SQ occurrence and concentration could be explained by genetic factor (Beltrán et al. 2015), so the similarity relationship of SQ content among EVOO samples from Spain and Portugal, for example, would be better rationalized by a careful morphological and biogenetic characterization. However, as observed by D'Imperio et al. (2007), often ʙthe cultivars known with different names not necessarily have different genotype ˆ. Moreover, the data we obtained pointed that a possible explanation for the variation in the SQ contents from the different countries may be highly affected by a minor but well-defined geographic and climatic condition. So, in order to improve the knowledge and to understand the difference and similarity, it would be of great help to process many other EVOO samples, including also other typical monovarietal cultivars, according to the geographical areas.

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Compliance with Ethical Standards

Conflict of Interest Andrea Salvo declares that he/she has no conflict of interest. Giovanna Loredana La Torre declares that she has no conflict of interest. Archimede Rotondo declares that he has no conflict of interest. Valentina Mangano declares that she has no conflict of interest. Katia Erminia Casale declares that she has no conflict of interest. Vito Pellizzeri declares that he has no conflict of interest. Maria Lisa Clodoveo declares that she has no conflict of interest. Filomona Corbo declares that she has no conflict of interest. Nicola Cicero declares that he has no conflict of interest. Giacomo Dugo declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not applicable.

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