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Solid phase extraction in the analysis of squalene and tocopherols in olive oil

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Abstract

Solid phase extraction (SPE) is only marginally employed in the official EU methods on the characteristics of olive oil. In the present study a SPE procedure was optimised for the subsequent extraction of squalene and α-tocopherol prior to HPLC of triacylglycerols. Squalene elution from a silica cartridge was achieved with 10 ml of *n*-hexane whereas α-tocopherol was isolated using 2×10 ml *n*-hexane/diethylether, 99:1, v/v. For both constituents, the precision of the SPE procedure (squalene: CV % = 4.4–6.4, $n = 5$; α-tocopherol: CV% = 5.3, $n = 7$) and the mean recovery (squalene: $88 \pm 9\%$ and $85 \pm 4\%$ for 700 and 4000 mg/kg levels of addition; α-tocopherol: $88 \pm 6\%$ and $91 \pm 3\%$ for 70 and 225 mg/kg levels of addition, respectively) were satisfactory with regards to those reported using other HPLC procedures with or without sample pretreatment. The results of the present study can be proved useful in the official control of virgin olive oil.
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Keywords: SPE; Squalene; α-Tocopherol; Virgin olive oil; HPLC analysis

1. Introduction

Solid phase extraction (SPE) is considered as a convenient approach for sample preparation in food analysis. However, in the official EU methods on the characteristics of olive oil, SPE is only marginally employed (EEC Regulation, 2568/91 and later Amendments) (EEC, 1991). Thus, silica cartridges are simply used for the clean up of olive oils prior to the analysis of methylesters by GC and triacylglycerols by RP-HPLC when the removal of free fatty acids is necessary (e.g. in crude and lampante olive oils). In the literature SPE applications can be found in both the analysis of olive oil constituents included in the above regulations and also in the analysis of minor components for which there is no provision in the current legislation (polar phenols, pigments – carotenoids and chlorophylls, hydrocarbons, fatty acid esters, tocopherols, diglycerols, etc.) (Angerosa, Campestre, & Giassante, 2006; Cert, Moreda, & Pérez-Camino, 2000; Panagiotopoulou & Tsimidou, 2002; Pérez-Camino, Moreda, Mateos, & Cert, 2002).

The aim of this study was to extend the potential of SPE in the official control of virgin olive oil (VOO). Thus, silica cartridges were employed for the isolation of constituents that could be eluted earlier than triacylglycerols. Our effort was focused on squalene and tocopherols, constituents of high nutritional importance.

Triterpenoid squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is the main hydrocarbon in the non-saponifiable fraction of olive oil occurring in high concentrations (60–75%). Squalene is reported to occur in concentrations between 0.8 and 12 g/kg in virgin olive oil and is characterized by high stability under autoxidation conditions while it was found to contribute to virgin olive oil stability under light exposure (Lanzón, Albi, Cert, & Gracian, 1994; Nenadis & Tsimidou, 2002; Psomiadou & Tsimidou, 2002a, 2002b). The presence of squalene has been allegedly related to various health benefits of virgin olive oil, still concrete proof is limited (Newmark, 1997;

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Rao, Newmark, & Reddy, 1998; Scolastici, Ong, & Moreno, 2004). Squalene is determined by titrimetric or chromatographic procedures (HPLC, GC, hyphenated chromatographic techniques). The only official method for the determination of squalene is the one proposed by AOAC that involves sample saponification, extraction of the non-saponifiable matter with large quantities of solvents, fractionation through column chromatography, and other treatments just before titration (AOAC, 1999).

Vitamin E is comprised of tocopherols along with tocotrienols. The fat-soluble α-tocopherol, the analogue having the highest biological activity, is the predominant representative of Vitamin E in virgin olive oil. It is the main chain breaking antioxidant of the oil. The concentration of α-tocopherol, reported in the literature for good-quality VOO's, is usually in the range 100–300 mg/kg, β- and γ-tocopherols are found in smaller amounts, and δ-tocopherol only in traces (Boskou, Blekas, & Tsimidou, 2006; Psomiadou, Tsimidou, & Boskou, 2000). Tocopherols in oils are determined either by direct normal phase HPLC or by reversed phase after saponification. Fluorescence or UV detection is then employed for identification and quantification. The method currently recommended by IUPAC (1988) for the determination of tocopherols in VOO, employs the direct analysis of the oil sample using normal phase HPLC and fluorescence or UV detection.

In none of the above-recommended protocols of squalene or tocopherol analysis, SPE is used for sample preparation.

In our study, the subsequent elution of squalene and tocopherols from silica cartridges was attempted. The compounds were then determined by suitable HPLC methods. Recovery and repeatability studies as well as analysis of real samples were performed in order to examine the applicability of the procedure to virgin olive oil.

2. Materials and methods

2.1. Samples

Virgin olive oil samples were donated by ELAIS SA, (Piraeus, Greece). For recovery studies purified olive oil was used. The latter was prepared from refined olive oil by column chromatography in order to remove anti-/prooxidant components using a procedure that has been repeatedly used in our laboratory (Psomiadou & Tsimidou, 1999).

2.2. Solvents and standards

The solvents of HPLC grade were used without further purification. *n*-Hexane 95%, 2-propanol, acetonitrile and methanol were from Panreac Quimica SA. (Barcelona, Spain). Acetone (Chromasolv) and diethylether were from Riedel de-Haën AG (Seelze, Germany). Squalene of 98–100% purity was from Sigma Chemical Co., (St. Louis, MO, USA) and DL-α-tocopherol 98% pure for HPLC from Fluka Chemie GmbH (Buchs, Switzerland).

2.3. Apparatus

Squalene determination was carried out using a solvent delivery system that consisted of a SSI liquid chromatography pump (Model 300LC; Scientific System Inc.) equipped with a SSI pulse dumper (Model LP-21 LO pulse), a Rheodyne injection valve (Model 7125) with a 20 μl loop and a UV–Vis detector (SPD-10AV; Shimadzu Co.). Column temperature was controlled with a SSI (Model 207) column oven. A Hewlett Packard model HP 3396, Series II (Avondale, PA, USA) electronic integrator was used for recording and quantifying the chromatographic peaks. Triacylglycerols were determined with the same solvent delivery system as squalene, using a refractive index detector (RID-6A, Shimadzu Co., Kyoto, Japan).

The solvent delivery system for the determination of α-tocopherol consisted of two Marathon IV series HPLC pumps (Rigas Labs, Thessaloniki, Greece) and a Rheodyne injection valve (model 7125) with a 20 μl fixed loop (Rheodyne, Cotati, CA, USA). The liquid chromatograph was equipped with a SSI 502 programmable fluorescence detector (Scientific Systems, Inc., State College, PA, USA) set at 294 nm (excitation) and 330 nm (emission), and a SPD-10AV diode array detector (Shimadzu Co., Kyoto, Japan). The data were stored and processed using the chromatographic software EZChrom (Scientific Software, Inc., San Ramon, CA, USA).

The SPE apparatus used in sample preparation was from Supelco (Bellefonte, USA).

2.4. Solid phase extraction

The optimised procedure was as follows. VOO (0.12 g) was weighed and dissolved in 0.5 ml *n*-hexane. The silica cartridge (1000 mg/8 ml reservoir volume, Rigas Labs, Thessaloniki, Greece) was conditioned with 6 ml of *n*-hexane before the application of oil solution. The examined compounds were obtained in two different fractions (*A* and *B*) by subsequent elution using mixtures of *n*-hexane–diethylether of different polarities. At first, squalene was eluted with 10 ml of *n*-hexane (fraction *A*), while α-tocopherol was eluted with 2×10 ml of *n*-hexane/diethylether (99:1 v/v) (fraction *B*). The collected fractions were evaporated under reduced pressure at room temperature. The dry residues were dissolved in the appropriate solvent for HPLC analysis. Method validation was performed according to Eurachem Guidelines (Eurachem, 1998).

2.5. RP-HPLC of squalene

Squalene determination was carried out on a reversed phase Nucleosil C_{18} column (particle size 5 μm, 125×4.0 mm i.d.) (Macherey-Nagel, Duren, Germany) maintained at 26 °C. The elution solvent was 100% acetonitrile, the flow rate 1.2 ml/min and the injection volume 10 μl. Detection was achieved with a UV–Vis detector at

208 nm. Quantification was accomplished with the use of standard curves calculated by linear regression analysis.

2.6. NP-HPLC of tocopherols

The separation of tocopherols was achieved on a LiChrospher 1 0 0 Si (particle size 5 μm column, 250 × 4.0 mm i.d.) (Analyzentechnik, Mainz, Germany) maintained at 30 °C, isocratically, using a mixture of *n*-hexane/2-propanol, 98:2, v/v at a flow rate of 1.0 ml/min. The injection volume was 10 μl. Tocopherols were quantified with the use of standard curves calculated by linear regression analysis.

2.7. RP-HPLC of triacylglycerols

The analysis was carried out according to the E.C. Regulation No. 2568/91, on a Nucleosil C_{18} column (particle size 5 μm, 125 × 4.0 mm i.d.) (Macherey-Nagel, Duren, Germany) maintained at 26 °C, under isocratic conditions. The elution solvent was acetone/acetonitrile (60:40 v/v), the flow rate 1.0 ml/min and the injection volume 10 μl.

2.8. Other methods

Squalene analysis by HPLC was also performed for oil samples pretreated as follows.

(a) Fractional crystallization.
About 0.5 g of oil sample was vortexed for 1.5 min in the presence of a mixture of methanol/acetone (7:3, v/v) (20 ml) in a 25 ml ground–glass stoppered test tube and stored at −22 ± 1 °C for 24 h. The supernatant was then rapidly filtered through a coarse filter paper. The solvent was evaporated under vacuum at 40 °C and the residue was dissolved in acetone (5 ml) for further chromatographic analysis (Nenadis & Tsimidou, 2002).
(b) Saponification.
About 0.1 g of oil sample was weighed in a 25-ml ground –glass stoppered test tube. Then, 3 ml potassium hydroxide (600 g/l), 2 ml of ethanol and 5 ml of an ethanolic pyrogallol solution (60 g/l) were added in the glass tube. The tube was flushed with nitrogen. After alkaline digestion at 70 °C for 30 min, 15 ml of NaCl (10 g/l) were added and the suspension extracted twice with 15 ml of a mixture of *n*-hexane/ethyl acetate (9:1, v/v). The organic phase was evaporated to dryness and the residue was dissolved in acetone (Manzi, Panfili, Esti, & Pizzoferrato, 1998).

3. Results and discussion

3.1. Squalene isolation

3.1.1. Preliminary work

In the work on the determination of esters of fatty acids with low molecular weight alcohols in olive oil, Pérez-Camino et al. (2002) reported the elution of squalene from silica SPE cartridges (1000 mg) using hexane. In the present study, after some initial trials to adjust required solvent volume, isolation of squalene on silica SPE cartridges, as described in the experimental part, was performed for a number of virgin olive oil samples. For comparison, both saponification (Manzi et al., 1998) and fractional crystallization (Nenadis & Tsimidou, 2002) were also used for sample pretreatment prior to HPLC analysis. The results of this preliminary work are shown in Table 1. Squalene levels among the three methods of pretreatment were found comparable. The above promising findings directed us to further validate the whole analytical procedure, i.e. SPE-HPLC.

3.1.2. Optimisation of elution conditions

The optimum isolation of squalene was achieved using 10 ml of *n*-hexane. The repeatability of the optimized isolation method, checked for a VOO sample ($n = 5$) at two random working days, was found satisfactory (day 1: CV% = 4.4, day 2: CV% = 6.4). Coefficient of variation values were similar to those found by Pérez-Camino et al. (2002) (CV% = 3.5–6.0) for SPE-GC and by Nenadis and Tsimidou (2002) (CV% = 3.8) for fractional crystallization-HPLC. Recovery was examined at 700 mg/kg and

Table 1
Squalene level of olive oil samples determined by HPLC after isolation with three different methods

Sample	Sample pre-treatment					
	Squalene content[a]					
	Method 1 fractional crystallization		Method 2 saponification		Method 3 SPE	
	Mean value[b] ± sd	CV%	Mean value[b] ± sd	CV%	Mean value[b] ± sd	CV%
1	2192 ± 182	8.31	2642 ± 52	7.40	2694 ± 355	14.00
2	4677 ± 238	5.08	4166 ± 265	3.84	3937 ± 24	0.62
3	4589 ± 146	3.15	4549 ± 233	5.12	4657 ± 156	3.36
4	3839 ± 75	1.95	3173 ± 89	2.81	3379 ± 96	2.85
5	4140 ± 62	1.49	4992 ± 65	1.31	4108 ± 108	2.64
6	2763 ± 328	11.88	1718 ± 95	5.54	2037 ± 157	7.69

[a] Expressed as mg/kg.
[b] Mean value of three measurements.

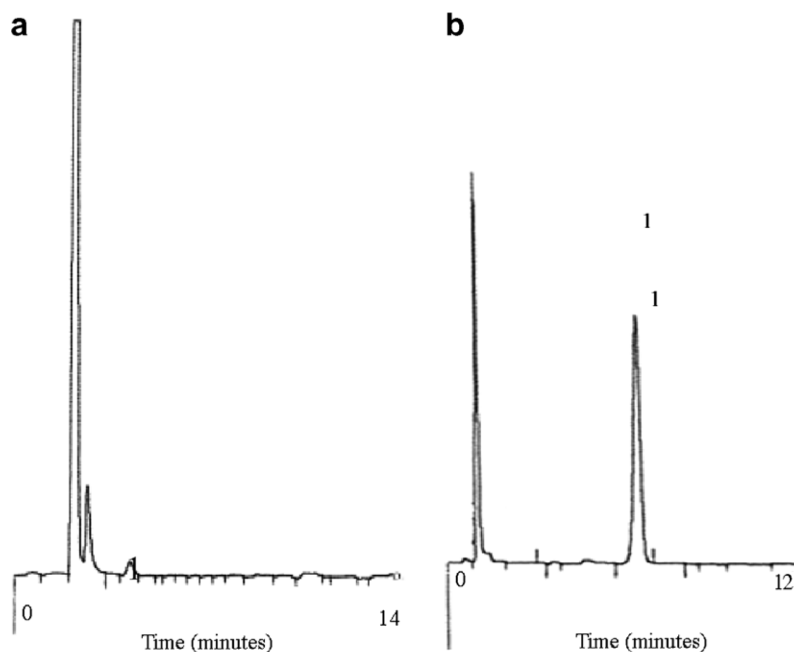

Fig. 1. Chromatographic profile of the hexane fraction: (a) elution system as in 2.7; RI detection, no triacylglycerol species present; (b) elution system as in 2.5; UV detection (208 nm), Peak (1): squalene.

4000 mg/kg levels of addition, characteristic for the content of squalene in virgin olive oil. Purified olive oil was used as a substrate and the recovery found from five replications was $88 \pm 9\%$ and $85 \pm 4\%$, respectively.

Hexane elution of olive oil from silica cartridges permitted the isolation of a fraction free of triacylglycerols (Fig. 1a) and rich in squalene. This made possible the modification of the HPLC procedure for squalene analysis suggested by Nenadis and Tsimidou (2002) and the use of 100% acetonitrile as elution solvent (Fig. 1b). We underline that if triacylglycerols are present in the analytical sample the use of a mixture of acetone/acetonitrile or, alternatively, careful washings with such mixtures become necessary in squalene analysis. The respective consequences are, then, loss in sensitivity or considerable increase in overall analysis time. Using only acetonitrile the detection limit was 6.2 ng/10 μl and the quantification limit was 7.8 ng/10 μl as calculated according to Eurachem Guidelines. Both limits were approximately three times lower than those reported in our previous work (Nenadis & Tsimidou, 2002).

3.2. Tocopherol isolation

3.2.1. Preliminary work

HPLC examination of the hexane fraction for the presence of tocopherols showed that it was free of them. Subsequent elution with 90:10 v/v mixture of *n*-hexane/diethylether gave rise to a fraction containing both tocopherols and triacylglycerols. This fraction contained the majority of tocopherols (~90% of total). Given that HPLC separation of tocopherols on silica columns is realized using a mixture of *n*-hexane/2-propanol, 98:2, v/v, it was examined whether the quantitative elution of tocopherol fraction could be feasible using a less polar mixture to the one applied before. Literature survey indicated that mixtures of *n*-hexane/diethylether 99:1, v/v give fractions free of triacylglycerols (Reiter, Lechner, & Lorbeer, 1999). In such a fraction Pérez-Camino et al. (2002) had recovered fatty acid alkyl esters and waxes. This system was, thus, investigated further for the presence of tocopherols.

3.2.2. Optimization of elution conditions

The elution steps are described in Fig. 2. Quantitative determination of tocopherols in fractions B_1, B_2 and B_3 indicated that almost 100% of the expected amount could be recovered in fractions B_1 and B_2. These fractions were not completely free of triacylglycerols, as it was shown using TLC on silica plates (*n*-hexane/diethlyether:acetic acid, 80:20:0.2, v/v/v). Repeatability of the procedure on three different cartridges was satisfactory (Table 2). Under the optimized conditions of elution (*n*-hexane:diethlyether, 99:1, v/v, 2×10 ml) validation of tocopherol isolation was then carried out. The repeatability of the procedure, checked again another random day for more replicates, was also found satisfactory ($CV = 5.3\%$, $n = 7$). The repeatability observed was similar to that reported by Cayuela, Garrido, Bañón, and Ros (2003) ($CV\% = 5.6\%$,) and Gimeno et al. (2000) ($CV\% = 3.0$). Recovery studies for α-tocopherol were carried out using purified olive oil as lipid substrate at two different levels of addition, 70 and 225 mg/kg, which represent the tocopherol content

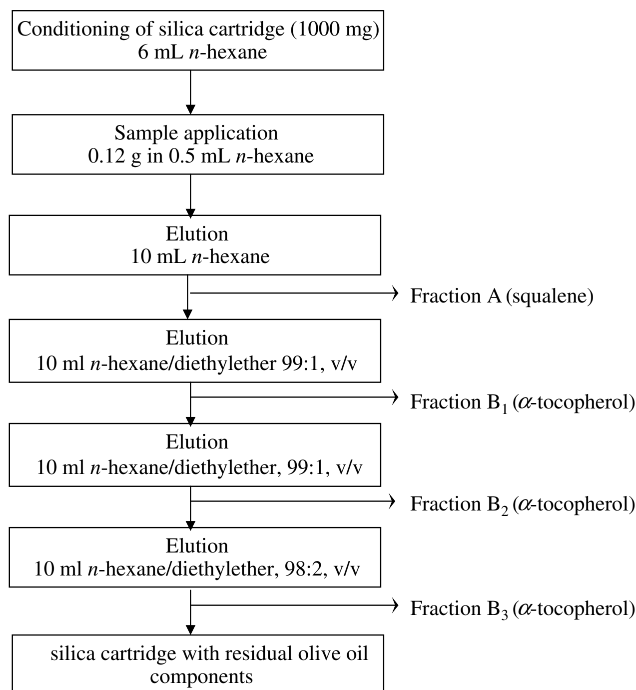

Fig. 2. Elution steps for the isolation of tocopherols using SPE.

of a refined and a virgin olive oil The recovery was 88 ± 6% and 91 ± 3% ($n = 5$), for 70 and 225 mg/kg level, respectively. To our knowledge, there were no methods mentioned for the isolation of tocopherols, mainly α-tocopherol, using SPE in olive oil. Similar studies for silylated α-tocopherol from silica cartridges and subsequent capillary gas chromatographic analysis in other vegetable oils showed recovery 75% for the 200 mg/kg level of addition (Lechner, Reiter, & Lorbeer, 1999). Detection limit (4.8 ng/10 ml, $n = 10$) and the respective quantitation limit (5.6 ng/10 ml, $n = 10$) were in agreement with those reported for normal phase HPLC-fluorescence detection without any sample pre-treatment (Eitenmiller & Landen, 1999).

Table 2
Repeatability studies for α-tocopherol using SPE prior to HPLC analysis

Replicates	Fraction	α-Tocopherol[a] (MV[b] ± sd)	Total α-tocopherol[a] (MV[b] ± sd)
1	Fraction B_1	189 ± 1	216 ± 1
	Fraction B_2	27 ± 0	
2	Fraction B_1	173 ± 1	209 ± 1
	Fraction B_2	37 ± 0	
3	Fraction B_1	166 ± 3	223 ± 3
	Fraction B_2	57 ± 1	
Mean value (MV)[a]			216
Standard deviation (sd)/Coefficient of variation (CV%)			7/3.2%
α-Tocopherol content (IUPAC method) ± sd			203 ± 7

[a] Expressed as mg/kg.
[b] Mean value of three measurements.

3.3. Application to real samples

The above SPE methodology was then successfully applied to 20 virgin olive oil samples for the subsequent isolation of squalene and α-tocopherol. The results are given in Table 3. Squalene levels varied from 2000 to 5100 mg/kg and were similar to values reported in the literature (Nenadis & Tsimidou, 2002; Manzi et al., 1998; Pérez-Camino et al., 2002). α-Tocopherol levels varied from 86 to 222 mg/kg. The quantitative results obtained for α-tocopherol are consistent with data obtained by the recommended IUPAC method. As shown in Fig. 3, the results from the two methods correlated significantly ($R^2 = 0.887$, $y = a + bx$: $b = 0.941 \pm 0.166$, $a = 1.996 \pm 30.586$, degrees of freedom: 18, $P = 0.05$) according to Miller and Miller (1984).

Table 3
Squalene and α tocopherol levels in olive oil samples by HPLC after SPE

Sample	Squalene[a] (MV[b] ± sd)	α-Tocopherol[a] (MV[b] ± sd)	Sample	Squalene[a] (MV[b] ± sd)	α-Tocopherol[a] (MV[b] ± sd)
1	3152 ± 33	195 ± 6	11	4059 ± 27	190 ± 0
2	3612 ± 12	181 ± 2	12	3135 ± 35	172 ± 2
3	1964 ± 9	218 ± 3	13	3468 ± 24	169 ± 2
4	3565 ± 9	222 ± 1	14	3574 ± 20	167 ± 3
5	4337 ± 21	214 ± 3	15	3376 ± 1	164 ± 2
6	4421 ± 23	206 ± 2	16	3003 ± 1	117 ± 2
7	2963 ± 46	167 ± 4	17	3376 ± 5	124 ± 1
8	3671 ± 11	186 ± 2	18	3170 ± 20	140 ± 3
9	2918 ± 11	161 ± 3	19	3031 ± 23	158 ± 1
10	3123 ± 9	209 ± 0	20	2952 ± 5	86 ± 2

[a] Expressed as mg/kg.
[b] Mean value (MV) of 3 measurements.

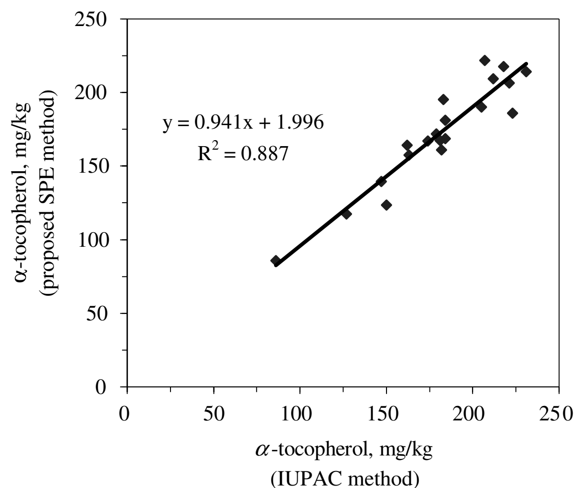

Fig. 3. Regression analysis on the HPLC determination of α-tocopherol between the proposed SPE method and the IUPAC method.

4. Conclusions

The results of the present study together with those of Pérez-Camino and coworkers can be proved of particular interest in the official control of virgin olive oil and it is expected to attract the attention of officials responsible for that. Using only one silica cartridge, it is possible to prepare fractions quantitatively rich in minor compounds with specific interest either as quality markers or as indices of nutritional value. The procedure was found to be reliable and fast, and allowed recovery of the compounds prior to the analysis of triacylglycerols. The advantages of the above solid phase extraction procedure is in line with current trend in food analysis.

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