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INVESTIGACIÓN

Characterization and seasonal variation of the quality of virgin olive oil of the Throumbolia and Koroneiki varieties from Southern Greece

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RESUMEN

Caracterización y variación estacional de la calidad del aceite de oliva virgen de las variedades Throumbolia y Koroneiki del Sur de Creta.

Aceite de oliva virgen extra fue producida con aceitunas de las dos principales variedades cultivadas en la región de Rethymnon en la isla de Creta denominadas "Throumbolia" y "Koroneiki". La primera es una variedad muy famosa debido a la falta de amargor natural de su fruto, mientras que la última es la variedad más común de aceitunas cultivada en el norte de Grecia. La aceitunas fueron cosechadas en tres estados sucesivos de maduración de acuerdo al color de su piel y el aceite de oliva virgen extra fue extraído en una almazara experimental a 30ºC. El indice de peróxides, la absorción UV, la acidez, el contenido en ácidos grasos y los polifenoles totales fueron medidos y los contenidos de tyrosol, hydroxytyrosol 3,4- DHPEA-EDA, p-HPEA-EDA y 3,4-DHPEA-EA fueron determinados por HPLC. La fracción de esteroles y el perfil de los componentes volátiles fueron analizados por GC y SPME GC/MS, respectivamente. El aceite de olovas de Throumbolia presentó un contenido extremadamente más alto de β-sitosterol y ácido linoleico (n6) que la variedad koroneiki. La concentración del ácido linoleico decrecio en ambas variedades, a diferencia del ácido oleico que presentó un incremento al mismo tiempo. Además, el contenidode OH-tyrosol fue más alto, mientras que el contenido de 3,4-DHPEA-EDA y los fenoles totales fueron más bajo, en aceite de oliva de Throumbolia que en el aceite de oliva producido por la variedad Koroneiki. En general, diferencias significativas fueron observadas en todos los parámetros entre los aceites de oliva producidos por las dos variedades durante las diferentes etapas de maduración.

PALABRAS CLAVE: Calidad del aceite de oliva virgen – Características químicas – Olea europaea var. microcarpa alba – Olea europaea var. media oblonga.

SUMMARY

Characterization and seasonal variation of the quality of virgin olive oil of the Throumbolia and Koroneiki varieties from southern Greece.

Extra virgin olive oil was produced from olives of the two main varieties cultivated in the region of Rethymnon in the Greek island of Crete named "Throumbolia", and "Koroneiki". The former is very famous due to the natural way of fruit debittering, while the latter is the most common olive variety cultivated in Northern Greece. The olives were harvested at three successive stages of ripening according to their skin color and the extra virgin olive oil was extracted using an experimental olive oil extraction mill at 30ºC. Peroxide value, UV absorption, acidity, fatty acid content and total polyphenols were measured and the contents of tyrosol, hydroxytyrosol 3,4- DHPEA-EDA, p-HPEA-EDA and 3,4-DHPEA-EA were determined by HPLC. The sterol fraction and the volatile component profile were determined by GC and SPME GC/MS, respectively. Throumbolia olive oil presented an extremely higher content of β-sitosterol and linoleic acid (n6) in comparison to the Koroneiki variety. The concentration of linoleic acid decreased in olive oils produced from both varieties in contrast to oleic acid which increased at the same time. Furthermore, the content of OH-tyrosol was higher, while the content of 3, 4-DHPEA-EDA and the total polyphenols was lower in Throumbolia olive oil than in olive oil produced from the Koroneiki variety. In general, significant differences were observed in all parameters between the olive oils produced from the two varieties during different stages of maturation.

KEY-WORDS: Chemical characteristics – Olea europaea var. microcarpa alba – Olea europaea var. media oblonga – Virgin olive oil quality.

1. INTRODUCTION

Olive oil is a natural fruit juice with excellent nutritional characteristics. It represents a typical lipid source of the Mediterranean diet and its consumption has been associated with a low incidence of cardiovascular diseases, neurological disorders, breast and colon cancers, as well as with hipolipidemic and antioxidant properties. Due to its fatty acid composition and content of other functional food components, such as polyphenols, an upsurge of interest in olive oil as a healthy food has been observed lately in regions other than the Mediterranean countries (Matos *et al.,* 2007; Temine *et al.,* 2008).

Olive cultivation is exceptionally spread in the island of Crete, in Greece, where almost 65% of the area is covered by olive groves. Among the best varieties of olives cultivated all over Crete are Koroneiki (*Olea europaea* var. microcarpa alba) (Therios, 2006) and Throumbolia (*Olea europaea* var. media oblonga), which are mainly cultivated in the Rethymnon region. Koroneiki is more resistant than Throumbolia and thrives at altitudes of over 500m. Its fruits are rather small, but they have a distinctive aromatic character. Its trees take up almost 60% of the total Greek olive tree growing area and it is unquestionably the most important in the production of olive oil.

The Throumbolia variety has been cultivated longer than the Koroneiki variety in the island of Crete. It grows at altitudes of up to 700m and its fruits are medium-size. It is characterized by the fact that under special conditions of temperature and moisture, the bitter taste which is evident in the olives while they are still on the tree disappears due to the hydrolysis of oleuropein by the action of the fungus *Phoma oleae* (Alygizakis, 1982). Due to the height of the olive trees, it is important that olives are harvested as soon as they fall from the tree and pressed immediately to produce sweet oil, rich in aromatic substances. Otherwise, a deterioration of olive quality usually occurs resulting in unpleasant tasting oil. Especially in the mountainous area of Rethymnon region in Crete, the presence of the typical variety Throumbolia, the peculiar microclimatic conditions (e.g. the type of soil) and precise olive orchard management led to the production of olive oils with a distinctive but not highly appreciated taste characterized by high acidity. Although part of the traditional Throumbolia culture has been replaced by the Koroneiki variety in many regions, the former continues to grow across the Crete Island.

It is well known that the differences in olive oil quality from various regions are attributed to olive variety, environmental factors, harvesting methods, time of harvest, and extraction techniques. Therefore, two of the most important factors that influence olive oil quality are the type of cultivar and the olive ripening stage (Matos, *et al.,* 2007; Boskou, 2006). There are several studies concerning the olive oil quality of the Koroneiki variety (Stefanoudaki, *et al.,* 1999; Koutsaftakis, *et al.,* 1999; Vekiari and Koutsaftakis, 2002), but, to our knowledge, there is little information on the olive oil quality of the Throumbolia variety nor is there a comparison between the two varieties, Koroneiki and Throumbolia, especially during fruit development. Hence, the strategy adopted in the present study was to characterize the main characteristics of the two varieties cultivated in the same area (mountainous Rethymnon region in island Crete in Greece). This is expected to be useful, especially because of the peculiarity the Throumbolia variety to loose its bitter taste naturally on the tree under specified conditions. To avoid the effect of other factors, the selected olive trees were cultivated under similar climatic conditions and olive fruits were picked at the same maturity stage, while olive oil was extracted using the same processing system.

The aim of the present work is a first approach to quantify the chemical composition of extra virgin olive oil produced by the Throumbolia variety in comparison to Koroneiki, the most extended variety in the area of southern Greece, harvested manually directly from the tree during fruit development and extracted in an experimental olive oil mill.

2. MATERIALS AND METHODS

Olive fruits from both Throumbolia (*Olea europaea* var. media oblonga) and Koroneiki (*Olea europaea* var. microcarpa alba) varieties from the Rethymnon area at a height of about 500 m from the sea level were used for the olive oil production. Samples were collected at three different growth stages on the basis of their skin color (green, purple and black). The olives were harvested by hand from three non irrigated trees in each case. The infected olives were removed and the rest were washed with tap water. A representative sample of 2,5 kg was collected in duplicate for each variety and ripening stage. The extraction of the virgin olive oil was carried out in a laboratory scale olive mill as follows: The crushing of olives was carried out using a hammer mill operating at 3000 rpm. A sieve with 5mm mesh size was used at the exit. The paste (*ca.* 800g) obtained from the hammer mill was malaxed at 50 rpm for 30 min at 30±1°C. After malaxation, the paste was centrifuged at 1400 x g for 5 min to remove solid residues, without any addition of water. The liquid phase (olive oil plus water) was centrifuged again at 3000 x g for 10 min and the olive oil layer was carefully recovered.

Free acidity (g/100 g oleic acid), peroxide value (meq O_2/kg of olive oil), and the UV absorption for the determination of the extinction coefficients K_{232} and K_{270} were measured following the analytical methods described in Regulation EEC/2568/91 (1991).

Total polyphenols (expressed in mg equivalent of gallic acid /kg of olive oil) were quantified colorimetrically according to the method of Servili *et al* (2004). A Varian Cary 50 Conc. UV / VIS spectrophotometer (Varian, Middelburg, the Netherlands) was used at 725 nm.

The polyphenolic fraction was separated and analyzed using HPLC according to Montedoro *et al.* (1993). The HPLC system consisted of a Thermo-

Separation chromatograph model 980 equipped with a 250 mm x 4.6 mm C18 NovaPak column, coupled with a UV detector. The flow rate was 1 ml/min and the elution were detected at 278nm. The mobile phase consisted of 0,2% acetic acid in water (A) and methanol (B) for a total running time of 60 min, and the gradient changed as follows: 95%A/ 5% B for 2 min, to 80% A/ 20% B in 10 min, 70% A / 30% B in 10 min, 60% A / 40% B in 10 min, 40% A / 60% B in 10 min, 100% A / 0% B in 10 min until the end of running. The samples were dissolved in methanol and introduced through a loop of 20 ml capacity. The main polyphenols, tyrosol, hydroxytyrosol, 3,4- DHPEA-EDA, p-HPEA-EDA and 3,4-DHPEA-EA were estimated, through comparison of retention times with standards. The compounds used as standards were: tyrosol (98%) purchased from Aldrich, (Germany), OH- tyrosol from Extrasynthese (Genay, France) and the rest of the polyphenols were kindly offered by our collaborators in Italy.

The fatty acid composition of the oil was determined by gas chromatography (GC) as fatty acid methyl esters (FAME). FAME were prepared using saponification / methylation with sodium methylate according to the European Union Commission modified Regulation EEC/2568/91 method (1991). The samples were analyzed using a Varian CP-3800 gas chromatograph, equipped with a Stabilwax-type analytical column, (30 m length x 0.32 mm i.d x.0.25 μm) (J&W Scientific Inc. Rancho Cordova, CA, USA), and coupled with an FID detector (Varian, Middelburg, The Netherlands). Helium was used as the carrier gas. Then, the oxidative susceptibility of olive oil was calculated according to Torres and Maestri (2006) by means of the formula: OS = (monounsaturated fatty acids) + (45 x linoleic acid) + (100 x linolenic acid).

The sterol content was determined according to the official method of Regulation EEC/2568/91(1991). The oil sample was saponified with an ethanolic potassium hydroxide solution. The unsaponifiable fraction was removed with ethyl ether. The sterols were isolated and analyzed using a Varian CP-3800 gas chromatograph, equipped with a capillary column J & W Scientific, DB-S (30 m length x 0.25 mm i.d.) coated with a 0.25 μm film thickness, coupled with an FID detector (Varian, Middelburg, The Netherlands). The analytical conditions were as follows: carrier gas, helium: 1.3ml/min flow rate; injector temperature, 280°C; detector temperature, 300°C; oven temperature, 270°C; injection volume; 1μl. The sterols in the non-saponifiable fraction of olive oil were identified by the comparison of retention time with those obtained for corresponding standards. Quantification was made by the addition of an internal standard (alfa-cholestanol).

The samples were studied for volatile by-product composition using Solid Phase Microextraction Gas Chromatography/Mass Spectrometry (SPME GC/MS) analysis. A quantity of 10 gr of the sample were introduced into a 20 mL headspace vial fitted with a teflon-lined septum sealed with an aluminum crimp seal, through which the SPME syringe needle (bearing a 2-cm fibre coated with 50/30 mm Divinylbenzene/Carboxen on poly-dimethyl-siloxane bonded to a flexible fused silica core, Supelco, Bellefonte, PA, USA). The container was then thermostatted at 40°C for 40 min. The absorbed volatile analytes were analyzed by GC/MS (Shimadzu GC-17A, MS QP5050, capillary column Supelco CO Wax-10 60 m, 0.32 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.8 mL/min. Oven temperature was set at 40°C for 5 min, followed by a temperature gradient of 4°C/min to 75°C, and then raised to 250°C at 5°C/min with a final isothermal period of 10 min. The injector was operated in splitless mode. Injector and detector temperatures were 260°C and 280°C, respectively. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70 eV and mass range *m/z* 29-400. The identification was effected by comparing the retention times with those of standard compounds, by mass spectra of these standards generated in the laboratory, by mass spectra obtained from NIST107, NIST21 and SZTERP libraries, and by determining kovats' retention indexes and comparing them with those reported in the literature (Vichi *et al*., 2003; Vichi *et al*., 2007; Bianchi *et al*., 2007, 2008; Kandylis and Koutinas, 2008). Kovat's retention indexes were determined from the injection of a standard mixture containing the homologous series of normal alkanes (C_8-C_{22}) in pure hexane under exactly the same experimental conditions as described above. Quantification of volatile compounds was carried out as described recently (Mallouchos *et al.,* 2003; Kourkoutas *et al.,* 2006) with some modifications. Specifically, methyl octanoate (Sigma-Aldrich, Poole, UK) diluted in pure hexane (Sigma-Aldrich) was used as an internal standard (IS) at various concentrations (0.88, 8.75, 87.5, and 875 μg/kg). The volatile compounds were quantified by dividing the peak areas of the compounds of interest by the peak area of the IS and multiplying this ratio by the initial concentration of the IS (expressed as μg/kg). The peak areas were measured from the full scan chromatograph using total ion current (TIC). Each determination was carried out in triplicate and the mean data are presented (standard deviation for all values was less than ± 10% in most cases).

All measurements were conducted in duplicate and the data presented are the mean values and standard deviations. The obtained data were statistically analyzed with two way ANOVA using Statistica 7.0 software (StatSoft Inc., Tulsa, USA) and significant differences ($p<0.05$) were determined by Duncan's multiple range test.

3. RESULTS AND DISCUSSION

The free acidity of the studied oils did not exceed the limit of 0.8% which is established for the best commercial quality olive oil, designated as extra virgin. Some significant differences, indicated in Table 1, could be attributed to oil recovery and

Table 1
Variation of the main quality characteristics of extra virgin olive oil from Throumbolia and Koroneiki varieties of mountainous Crete (Rethymnon)

Variety	Growth stages	Acidity (%)	S.D	PV	S.D.	K_{232}	S.D.	K_{270}	S.D.
Throumbolia	1st	0,24^{ab}	0,02	5,0^{a}	0,07	2,4882^{c}	0,0000	0, 1843^{c}	0,0001
	2nd	0,25^{b}	0,00	11,1^{b}	0,49	2,8937^{e}	0,0001	0,1357^{a}	0,0002
	3rd	0,33^{c}	0,02	13,9^{d}	0,14	2,3330^{b}	0,0120	0,1386^{a}	0,0002
Koroneiki.	1st	0,21^{a}	0,00	13,0^{c}	0,07	2,4080^{bc}	0,0008	0,1658^{b}	0,0005
	2nd	0,25^{b}	0,00	13,0^{c}	0,14	2,7892^{d}	0,0765	0,1918^{d}	0,0055
	3rd	0,25^{b}	0,00	11,3^{b}	0,07	2,2451^{a}	0,0368	0,2175^{e}	0,0012

Mean values in the same column followed by different letters differ significantly (P<0.05).

handling procedures. Peroxide values (PV) were rather high and increased with harvesting period for the Throumbolia variety, while the same trend was not observed in olive oils produced from the Koroneiki variety (Table 1). However, PVs were below 20 meq O_2/kg of oil, which is established as the limit for "extra virgin" olive oil quality. Likewise, K_{232} and K_{270} did not exceed the limit of 2.5 and 0.22 units respectively as also established for extra virgin olive oil. The values of K_{232} and K_{270} were significantly affected (P<0.05) by ripening degree although a clear trend was not evident. Similar behavior was previously described by other researchers (Salvador *et al.,* 2001). In general, the differences cannot be related to olive maturity and seem to depend rather on the handling of the oil after recovery. This is in agreement with the literature (Boskou, 1996) where it is reported that qualitative characteristics of olive oil, such as free acidity, peroxide value and spectrophotometric absorption in the UV are rather unaffected during the ripening; however, these parameters mainly determine the quality of the oils.

Virgin olive oil contains phenolic substances, which affect the stability and flavor. Polyphenols, the most bioactive components of virgin olive oils, are the basis of the nutritional quality of olive oil (Boskou, 1996; Vekiari and Koutsaftakis, 2000; Servilli, 2002; Kiritsakis, 2007). Throughout the ripening of the fruit or during processing, several metabolic processes take place, such as chemical and enzymatic reactions resulting in the formation of free phenols and the subsequent variations in the profiles of several compounds. These changes are reflected on the quality grade, sensorial characteristics, oxidative stability and/or the nutritional value of the obtained product (Matos *et al.,* 2007; Boskou, 1996).

In the present study the evolution of the main polyphenols in the studied oils as affected by the ripening degree and olive variety is shown in Table 2, while in Figure 1 the variations of total polyphenol content of extra virgin olive oil in both varieties are also given. The tested samples were relatively rich in total phenols. As it has been reported by previous authors (Boskou, 1996; Temime *et al.,* 2006), the amount of total phenols shows a great variability (from 50 to ~ 1000μg/g) depending on various factors, such as variety, climate and environmental factors, ripeness, processing and storage. In the present study, total polyphenols varied from *ca.* 500μg/g to 1000 μg/g. This high content could probably be attributed to limited water availability, as a water deficit tends to generate a stress situation that induces the production of phenolics. Consequently, olive oils from the Rethymnon area, which is characterized by a low rainfall accumulation in the last years, presented high phenol content. Of note, no water was added during the extraction of olive oil and thus, water soluble polyphenols and especially anthocyannins deriving from the ripe olives may have remained in the olive oil (Di Giovacchino *et al.,* 1994). The addition of water to the paste may alter the partition equilibrium between liquid phases and may cause a reduction of phenol concentration through dilution to the aqueous phase.

The polyphenols OH-tyrosol, tyrosol and 3, 4- DHPEA-EDA were also identified in the tested samples (Table 2). These results are in accordance with other previously reported (Servili and Montedoro, 2002; Montedoro *et al.,* 2007). Their content varied from 3.42 to 4.90 for tyrosol, from 8.02 to 17.27 for OH-tyrosol and from 1.17 to 4.69 mg of gallic acid / Kg of olive oil for 3,4-DHPEA-EDA. The olive oil of the Throumbolia variety presented significantly higher contents of OH - tyrosol compared to the oil from Koroneiki in the majority of cases, with lower contents of 3,4-DHPEA-EDA and total phenols. Generally, the content of total polyphenols and of each phenol separately increased during ripening. However, variation in polyphenol concentration during ripening is complex and is influenced by many factors, such as the size of the fruit, the extraction process and the variety. Specifically, total polyphenol content is greatly affected by the absolute disposability and distribution of water during the vegetative cycle of the olive tree (Servili *et al.,* 2004; Di Giovacchino *et al.,* 1994). Since the extraction process, the olive

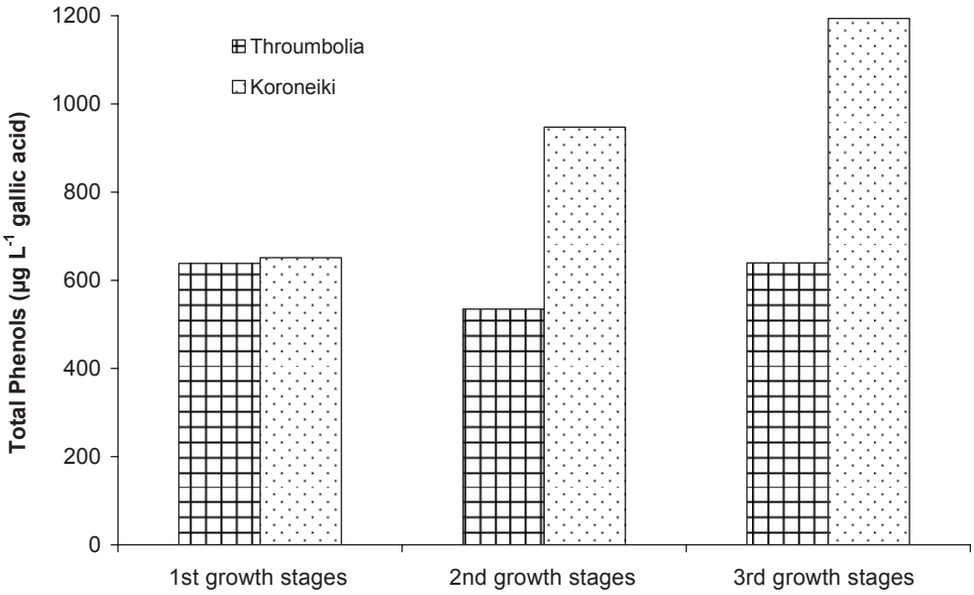

Figure 1.
The evolution of total polyphenols (mg equivalent of gallic acid /kg of olive oil) of extra virgin olive oil from the Throumbolia and Koroneiki varieties of mountainous Rethymnon (Crete) through one period harvesting.

Table 2
The variation of polyphenols OH – tyrosol, tyrosol, 3, 4- DHPEA-EDA, p-HPEA-EDA and 3,4-DHPEA-EA (expressed in mg equivalent of gallic acid /kg of olive oil) contents of extra virgin olive oil from Throumbolia and Koroneiki varieties of mountainous Crete (Rethymnon)

Variety	Growth stages	OH – tyrosol	Tyrosol	3,4-DHPEA -EDA	p-HPEA-EDA	3,4-DHPEA-EA
		M.V.	M.V.	M.V.	M.V.	M.V.
Throumbolia	1st	8,02±0,25[a]	3,87± 0,04[b]	1,17±0,05[a]	9.68±0.07[a]	3.39±0.02[a]
	2nd	13,55±0,87[c]	4,46±0,52[c]	1,17±0,03[a]	8.86±0.04[a]	1.37±0.05[b]
	3rd	17,27±0,80[d]	2,80±0,11[a]	2,28±0,14[c]	3.81±0.05[b]	2.04±0.01[c]
Koroneiki	1st	8,04±0,44[a]	3,42±0,10[b]	1,46±0,07[b]	11.86±0.1[c]	3.59±0.15[a]
	2nd	10,91±0,25[b]	4,90±0,11[c]	3,47±0,16[d]	5.93±0.05[d]	7.02±0.35[d]
	3rd	13,23±0,28[c]	5,55±0,01[d]	4,69±0,06[e]	7.13±0.03[a]	9.43±0.05[e]

Mean values in the same column followed by different letters differ significantly.

ripeness, and the other conditions were the same for both cultivars and differences in polyphenol contents indicate a genotype effect (Zarrouk *et al.,* 2008).

The chromatographic analysis of fatty acid methylesters is useful for the characterization and the quality of olive oil (Boskou, 1996; Zarrouk *et al.,* 2008). The fatty acid composition and especially the oleic / linoleic acid ratio affects the taste of virgin olive oil and is related to its health effects (Boskou, 1996). In the present study, 14 fatty acids were identified, the majority of which were oleic, palmitic, linoleic, stearic and palmitoleic (Table 3). The oleic acid percentage varied from 62.92 (Throumbolia) to 76.33% (Koroneiki) of total fatty acid contents in full maturity stage. The current biochemical evidence indicates that the polyunsaturated fatty acids are produced by the consecutive desaturation of oleic acid in olives and other plant species. Accordingly, Throumbolia and Koroneiki oils had the highest and the lowest linoleic acid levels, respectively. The oleic / linoleic ratio of Koroneiki olive oil was indeed 3.5 times higher than that of Throumbolia. This ratio varied with ripening and is in agreement with the observation of Matos *et al.* (2007). As it is also shown in Table III, the values of C16:0, C16:1, C17:0, C17:1 (10c), and C18:0 differ significantly in relation to the olive oil variety. Similar results

Table 3
Mean values ± std of extra virgin olive oil fatty acids (%) from the Throumbolia and Koroneiki varieties of mountainous Crete (Rethymnon)

	Throumbolia			Koroneiki		
	1st growth stage	2nd growth stage	3rd growth stage	1st growth stage	2nd growth stage	3rd growth stage
C16:0	12.41±0. 85^{d}	12.31±1.50^{d}	11.294±1.40^{b}	10.83±0.81^{a}	11.89±1.87^{c}	10.64±1.75^{a}
C16:1	0.58±0.00^{b}	0.57±0. 00^{ab}	0.512±0.15^{a}	0.73±0.24^{c}	0.87±0.042^{d}	0.86±0.039^{d}
C17:0	0.13±0.01^{c}	0.12±0. 11^{c}	0.134±0. 07^{c}	0.06±0.03^{b}	0.05±0.005^{ab}	0.04±0.003^{a}
C17:1 (10c)	0.01±0.008^{a}	0.19±0. 11^{d}	0.208±0. 02^{e}	0.10±0.010^{c}	0.08±0.005^{b}	0.08±0.008^{b}
C18:0	2.12±0.12^{b}	2.05±0.04^{a}	2.178±0. 01^{c}	2.82±0. 06^{e}	2.82±0.04^{e}	2.67±0.16^{d}
C18:1(9c)	61.07±2,97^{a}	61.92±1.05^{b}	62.92±2.73^{c}	75.86±2.63^{e}	74.97±1.16^{d}	76.33±0.63^{e}
C18:2n6c (omega 6)	20.97±0. 24^{d}	19.53±1.17^{c}	19.68±1.1^{c}	6.72±0.40^{b}	6.12±0.36^{a}	6.56±0. 07^{b}
C18:3n3 (omega 3)	0.79±0. 01^{c}	0.72±0.073^{bc}	0.69±0.14^{ab}	0.68±0.30^{ab}	0.75±0.045^{bc}	0.61±0.015^{a}
C20:0	0.35±0. 01^{a}	0.34±0.19^{a}	0.34±0.035^{a}	0.50±0.027^{b}	0.45±0.053^{b}	0.37±0. 03^{a}
C20:1n9c	0.29±0. 01^{ab}	0.28±0. 18ab	0.32±0.042^{b}	0.32±0.034^{b}	0.26±0.035^{ab}	0.23±0.020^{a}
C20:2(c11, 14)	0.04±0.00^{c}	0.07±0.006^{d}	0.09±0.03^{e}	0.01±0. 00^{a}	0.03±0.01^{b}	0.02±0.003^{b}
C22:0	0.11±0. 01^{a}	0.11±0.014^{a}	0.10±0.06^{a}	0.15±0.016^{b}	0.13±0.015^{ab}	0.11±0.015^{a}
C24:0	0.06±0. 00^{c}	0.03±0. 01^{b}	0.03±0.003^{b}	0.06±0.006^{c}	0.05±0.004^{c}	0.02±a0.00^{2}
Polyun/sat	1.440	1.359	1.453	0.518	0.447	0.518
Oleic/linoleic	2.91	3.17	3.19	11.28	11.13	11.63
Oxidat.suscesib.	1084.97	1014.05	1018.73	447.56	426.3	433.57
Omega6/omega3	26.338	27.092	28.350	9.852	8.166	10.728

Mean values in the same row followed by different letters differ significantly at P<0.05.

reporting that the olive oil fatty acid composition is influenced by the olive's variety among other factors were previously published (Tsimidou and Karakostas, 1993). The oil from both varieties was rich in C16:0, with the Throumbolia variety oil presenting significantly higher values than the Koroneiki variety oil. The values of most fatty acid contents were not significantly different in relation to the ripening stage, except C18:1(9c) content which showed an increasing trend during harvesting time, while C18:2n (6c) and C16:0 contents presented a decreasing trend in both varieties. These results are in agreement with previous researchers (Poiana and Mincione, 2004). In contrast, the values of C20:0, C20:1(9c), C22:0, C22:4 and C24:0 did not show any clear tendency in relation to the ripening stage.

The ratio of polyunsaturated to saturated fatty acids was much higher in oil of the Throumbolia variety than in oil of Koroneiki and the oxidative susceptibility index was also higher in the same varieties. The latter could also be attributed to the lower amount of polyphenols present in the Throumbolia variety. This could result in an olive oil which is more sensitive to oxidation under certain storage conditions.

Simopoulos (2003) has indicated that the ingested ratio of n-6 to n-3 (especially linoleic vs α-linolenic), which are both essential fatty acids, is important to maintain cardiovascular health. Healthy ratios of *n*-6 to *n*-3 range from 1:1 to 4:1. In our study, this ratio was beyond these limits, 28.35:1 and 10.72:1 for oil of the Throumbolia and Koroneiki variety respectively (Table 3). However, there is a contradiction about the role of the high content of linoleic fatty acid in olive oil which has not been yet clarified especially in the determination of the upper limit of this acid that could act beneficially to human health (Kiritsakis, 2007).

The composition of the sterol fraction of olive oil is a very useful tool for detecting adulteration or to check authenticity, since it can be considered similar to a fingerprint for olive oil. Additionally, the determination of sterols is of major interest due to their health benefits (Temime *et al.,* 2008). Several

factors are known to affect the quantitative sterolic profiles of olive fruits, such as the ripening cycle of the fruit and the nature of the variety, oil extraction and refining procedures, storage conditions, agronomic and climatic conditions (Temime *et al.,* 2008; Vekiari and Koutsaftakis, 2002).

Eight compounds were identified and characterized (Table 4) in the present study. As expected for virgin olive oil, the main sterols in both varieties were β–sitosterol, Δ_5-avenasterol and campesterol. The health aspects of β-sitosterol have recently been reported by Temime *et al.,* (2008). They mainly referred to the reduction of cholesterol levels by opposing its absorption in the intestinal tract, and the prevention of many diseases including various types of cancer. It is noteworthy that in the present study the β-sitosterol presented a higher amount (two-fold) in the Throumbolia than in the Koroneiki oil variety, while Δ_5–avenasterol content was double in the Koroneiki oil variety. Another point is that the campesterol content is below the threshold established by EE Regulations (4%) in all the samples studied, indicating a peculiarity of this olive oil variety. Cholesterol was not found in the studied oils. Stigmasterol was found in traces, which indicates that the oil originated from healthy fruits as this sterol is related to the quality of virgin olive oil. More specifically, high levels of this sterol correlate with high acidity and low organoleptic quality (Temime *et al.,* 2008). Concerning the variation of sterol content during ripening (Table 4), the content of β – sitosterol generally presented a decrease while Δ_5-avenasterol increased and this was in accordance with a previous study (Salvador *et al.,* 2001). However, the change in the campesterol content was not so clear. In the Throumbolia oil variety it increased, while in Koroneiki it decreased slightly.

Volatile components of olive oil are of great interest since they are related to its quality and are also used to detect adulteration (Temime *et al.,* 2006). For the evaluation of the aromatic profile, the oil samples were analyzed using the SPME GC/MS technique. From the results of the semiquantitavive analysis, 73 compounds were detected (Table 5) in total. The most important were esters, alcohols, carbonyl compounds and hydrocarbons, which contribute to the aroma of olive oil (Kalua *et al.,* 2007). Significant differences in the concentration of alcohols, esters, hexanal and 2-hexenal between the two varieties were found.

Esters consisted primarily of hexyl and [Z]-3-hexenyl acetates deriving from the lipoxygenase pathway. The above esters are usually present in olive oils (Haddada *et al.,* 2007), while methyl salicylate detected in both Koroneiki and Throumbolia oils was recently identified in virgin olive oil (Haddada *et al.,* 2007; Vichi *et al.,* 2007). However, esters are considered minor components compared to aldehydes and alcohols.

The alcohols identified included mainly C5 and C6 compounds, such as 1-penten-3-ol, [Z]- and [E]-2-penten-1-ol, [Z]- and [E]-3-hexen-1-ol, [E]-2-hexen-1-ol and 1-hexanol deriving from the lipoxygenase pathway (Angerosa *et al.,* 2004). Alcohols such as ethanol, 2-ethyl-1-hexanol, benzyl alcohol and phenylethyl alcohol are common in olive oil (Vichi *et al.,* 2007; Haddada *et al.,* 2007; Baccouri *et al.,* 2007).

The carbonyl compounds identified included mainly aldehydes and ketones. 1-penten-3-one, which was detected in all samples, is usually found in oils produced principally from unripe olives and is positively correlated to pungency (Kalua *et al.,* 2007). Compounds such as 2-pentenal, octanal,

Table 4
The sterol composition (mg/100g olive oil) in extra virgin olive oil from the Throumbolia and Koroneiki varieties of mountainous Crete (Rethymnon)

Variety	*Ripening stage*	campesterol	clerosterol	β- sitosterol	sitostanol	Δ5–avenasterol	Δ5,24-stigmastadienol	Δ7-stigmastenol	Δ7-avenasterol
Throumbolia	1st	2.99[a]	0.06[a]	175.37[a]	0.07[a]	7.46[a]	0.05[a]	2.74[a]	3.47[a]
	2nd	4.76[b]	1.89[b]	212.06[b]	0.93[b]	8.95[b]	0.09[a]	2.84[a]	4.16[b]
	3rd	5.19[b]	1.71[b]	188.88[c]	3.15[c]	8.45[b]	1.69[b]	2.58[a]	2.76[c]
Koroneiki	1st	3.66[c]	1.47[c]	97.94[d]	0.08[a]	17.58[c]	0.07[a]	0.10[b]	0.09[d]
	2nd	3.65[c]	1.00[a]	94.12[d]	0.10[a]	10.21[d]	0.08[a]	0.14[b]	0.12[d]
	3rd	2.93[a]	0.09[a]	81.51[e]	0.11[a]	23.91[e]	0.11[a]	0.09[b]	0.10[d]

Mean values in the same column followed by different letters differ significantly (P<0.05)

Table 5
Relative amounts (µg/kg) of volatile compounds determined by SPME GC/MS analysis in extra virgin olive oil from the Throumbolia and Koroneiki varieties in mountainous Crete (Rethymnon)

Compounds	Identification[a] method	KI	KIref	Throumbolia			Koroneiki		
				Growth stages					
				1	2	3	1	2	3
Esters									
ethyl acetate	RT, KI, MS	895	892[a]	nd	79.8	60.7	nd	36.0	16.3
ethyl 2-methyl butanoate	MS	1063	-	nd	19.8	19.8	nd	nd	nd
methyl 3-methyl-2-butenoate	MS	1148	-	nd	83.1	44.1	nd	nd	29.3
ethyl hexanoate	RT, KI, MS	1258	1258[c]	5.5	nd	38.0	4.2	9.3	8.8
hexyl acetate	RT, KI, MS	1283	1274[a], 1281[b]	6.2	46.4	47.4	46.0	108.5	163.3
[Z]-3-hexenyl acetate	RT, KI, MS	1325	1316[a]	12.5	37.4	69.2	11.5	100.9	131.6
ethyl octanoate	RT, KI, MS	1449	1451[c]	113.1	nd	nd	64.3	42.1	nd
methyl 3-hydroxy-butanoate	MS	1484	-	nd	nd	nd	nd	nd	5.2
ethyl decanoate	RT, KI, MS	1652	1652[c], 1647[d]	258.2	nd	nd	210.7	27.8	nd
diethyl butanedioate	MS	1683	1700[c]	11.1	nd	nd	6.0	nd	nd
ethyl 9-decenoate	KI, MS	1709	1709[c]	24.9	nd	nd	23.9	9.5	nd
methyl salicylate	KI, MS	1798	1798[b]	5.9	30.4	53.0	0.7	6.9	48.6
2-phenylethyl acetate	RT, KI, MS	1845	1847[c]	19.1	nd	nd	20.3	1.3	nd
ethyl dodecanoate	MS	1850	1850[c]	40.4	nd	nd	54.0	nd	nd
ethyl 3-hydroxy-hexanoate	MS	2001	-	1.9	nd	nd	2.2	nd	nd
ethyl tetradecanoate	KI, MS	2094	2094[c]	9.8	nd	nd	nd	nd	nd
ethyl hexadecanoate	KI, MS	2271	2271[c]	13.0	nd	nd	9.4	nd	nd
ethyl 9-hexadecenoate	KI, MS	2292	2292[c]	11.6	nd	nd	8.1	nd	nd
diethyl phthalate	KI, MS	2365	2365[c]	nd	nd	34.2	nd	nd	54.4
Organic acids									
octanoic acid	RT, KI, MS	2152	2156[c]	25.7	nd	nd	15.2	nd	nd
decanoic acid	RT, KI, MS	2313	2336[c]	18.1	nd	nd	19.6	nd	nd
Alcohols									
Ethanol	RT, KI, MS	935	932[a]	67.1	190.5	189.6	71.9	90.3	109.5
3-methyl-2-butanol	MS	1150	-	nd	nd	8.0	nd	65.4	nd
1-penten-3-ol	KI, MS	1163	1164[a], 1166[b]	3.5	30.5	73.8	6.4	40.7	89.8
dihydro-citronellol	MS	1196	-	119.3	nd	208.4	43.2	nd	nd
[Z]-2-penten-1-ol	KI, MS	1321	1320[a]	nd	82.7	108.1	nd	86.1	38.7
[E]-2-penten-1-ol	KI, MS	1333	1335[d]	nd	65.3	59.9	nd	71.4	45.9
1-hexanol	RT, KI, MS	1360	1357[a], 1362[b]	29.2	85.5	60.0	13.0	29.2	42.9
[E]-3-hexen-1-ol	RT, KI, MS	1371	1372[b], 1371[d]	24.4	105.4	48.3	29.1	122.6	147.0
[Z]-3-hexen-1-ol	RT, KI, MS	1385	1385[a]	32.9	55.8	49.7	33.3	59.0	89.2
[E]-2-hexen-1-ol	RT, KI, MS	1407	1414[b]	nd	168.1	109.8	nd	88.7	nd
2-butoxy-ethanol	MS	1433		nd	109.1	87.5	nd	nd	130.4
2-ethyl-1-hexanol	RT, KI, MS	1504	1492[d]	5.6	33.7	nd	nd	nd	25.9

Compounds	Identification[a] method	KI	KIref	Throumbolia			Koroneiki		
				Growth stages					
				1	2	3	1	2	3
1-octanol	RT, KI, MS	1564	1562[a], 1563[b], 1561[d]	9.1	58.2	38.1	1.7	9.9	60.8
2-(2-methoxyethoxy) ethanol	MS	1576		nd	nd	nd	nd	nd	96.9
2-(2-ethoxyethoxy) ethanol	MS	1636	-	9.7	34.2	53.6	2.9	18.2	174.2
Nonanol	RT, KI, MS	1666	1665[a,b]	nd	nd	51.6	nd	Nd	nd
4-butoxy-1-butanol	MS	1701	-	nd	99.6	nd	nd	6.6	nd
2-(2-butoxyethoxy) ethanol	KI, MS	1823	1823[c]	nd	259.2	140.2	nd	Nd	731.5
benzyl alcohol	KI, MS	1890	1883[a]	13.5	113.8	110.3	4.1	13.1	530.8
2-phenylethanol	RT, KI, MS	1922	1919[a], 1933[c]	181.9	203.5	75.5	157.7	62.1	nd
Phenol	KI, MS	2022	2020[b]	nd	162.4	134.2	nd	12.5	274.2
3-phenoxy-1-propanol	MS	2063	-	nd	242.5	183.1	nd	14.1	287.8
2-phenoxy-1-propanol	MS	2131	-	nd	193.1	54.6	nd	Nd	195.3
Carbonyl compounds									
Acetone	RT, KI, MS	816	813[d]	nd	nd	31.3	nd	27.0	29.5
1-penten-3-one	KI,MS	1008	1016[a]	13.1	52.5	161.2	7.2	71.8	196.9
Hexanal	KI, MS	1073	1074[a], 1086[b]	8.9	152.7	138.9	nd	46.9	nd
[E]-2-pentenal	RT, KI, MS	1127	1127[a]	1.9	nd	55.9	nd	Nd	nd
[Z]-2-hexenal	RT, MS	1205	1189[b]	10.7	3.8	25.7	nd	Nd	35.4
Heptanal	MS	1207	1194[e], 1191[d]	nd	nd	35.5	nd	Nd	nd
[E]-2-hexenal	RT, KI, MS	1229	1228[b]	485.8	870.0	960.1	364.7	371.8	570.3
3-octanone	MS	1235	-	nd	9.3	nd	nd	Nd	nd
Octanal	MS	1297	1296[e], 1292[d]	nd	27.5	nd	nd	Nd	25.5
Nonanal	KI, MS	1399	1396[a,d], 1402[b]	24.3	128.7	nd	nd	36.8	136.2
Furfural	RT, KI, MS	1486	1486[c]	nd	nd	9.8	nd	13.8	39.1
Benzaldehyde	RT, KI, MS	1533	1540[b]	7.3	165.4	122.6	nd	25.0	113.7
5-methyl-furfural	MS	1578	1578[c]	nd	nd	nd	nd	3.6	6.1
Butyrolactone	MS	1660	-	nd	40.4	19.9	nd	Nd	35.3
β-damascenone	MS	1834	1834[c]	13.8	nd	nd	0.9	Nd	nd
1-phenoxy-2-propanone	MS	1956	-	nd	16.7	8.8	nd	Nd	nd
Hydrocarbons									
Benzene	MS	951	-	nd	nd	nd	nd	Nd	30.9
Trichloroethylene	MS	970	-	3.9	14.5	41.9	nd	16.2	26.2
Toluene	RT, KI, MS	1026	1030[a], 1043[b,d]	12.5	86.0	53.6	10.2	39.9	53.8
ethyl benzene	KI, MS	1122	1119[a], 1127[b]	nd	58.4	24.8	nd	19.5	28.4
1,3-dimethyl benzene (*m*-xylene)	KI, MS	1131	1133[a], 1135[b]	6.0	79.0	31.5	nd	37.6	47.0
1,4-dimethyl benzene (*p*-xylene)	KI, MS	1138	1142[b]	nd	102.52	71.2	nd	79.5	nd
1,2-dimethyl benzene (*o*-xylene)	KI, MS	1182	1187[b]	nd	54.0	nd	nd	34.4	nd
Limonene	KI, MS	1199	1190[a], 1198[b], 1197[d]	nd	94.1	50.4	nd	42.3	59.1
β-ocimene	MS	1250	1260[b]	nd	nd	60.1	nd	Nd	31.0
1,3,5-trimethyl benzene	MS	1254	-	3.5	61.9	29.8	nd	20.0	25.4
α-cubebene	MS	1446		nd	nd	nd	1.6	46.3	8.5

Compounds	Identification[a] method	KI	KIref	Throumbolia			Koroneiki		
				Growth stages					
				1	2	3	1	2	3
Naphthalene	MS	1765	-	14.3	107.9	86.4	2.0	102.8	91.9
1-methyl-naphthalene	MS	1860	-	nd	nd	27.0	nd	9.3	21.1

RT: Positive identification by retention times that agree with authentic compounds and by mass spectra of authentic compounds generated in the laboratory. KI: Tentative identification by kovats' retention index. MS: Positive identification by mass spectra obtained from NIST107. NIST21 and SZTERP libraries. Nd: Not detected.
[a]: Vichi et al., 2003, [b]: Vichi et al., 2007, [c]: Kandylis and Koutinas 2008, [d]: Bianchi et al., 2007, [e]: Bianchi et al., 2008

and nonanal, present in both Koroneiki and Throumbolia oil samples, have been associated with the oxidative status of virgin olive oil (Morales *et al.,* 1997; Vichi *et al.,* 2003). Hexanal, also identified in both Koroneiki and Throumbolia oil samples, is an oxidation product of linoleic acid (Kiritsakis, 1998) deriving from either lipoxygenase action or from chemical oxidation and it has been related with grassy, green-sweet and green-apple odors (Kalua *et al.,* 2007). Likewise, [E]-2-hexenal, a product of the lipoxygenase pathway, which is inversely related to the oxidation degree of virgin olive oil (Vichi *et al.,* 2003), was the dominant compound in both Koroneiki and Throumbolia oils. This is in accordance with a previous study on olive oil (Temime *et al.,* 2006).

Hydrocarbons mainly included alkylated benzenes, which are considered environmental pollutant, were previously found in virgin oil fractions (Bindermann *et al.,* 1995; Vichi *et al.,* 2005), although their origin has not yet been fully documented. Alpha cubebene, present only in Koroneiki oils, and β-ocimene, detected in all samples, were previously tentatively identified in olive oils (Vichi *et al.,* 2006). Likewise, toluene, ethyl benzene, *m*-, *p*- and *o*-xylene, present in both Koroneiki and Throumbolia oils, are usual constituents of olive oils (Vichi *et al.,* 2005; Vichi *et al.,* 2007). Trichloroethylene, detected in both Koroneiki and Throumbolia oil samples, was probably an environmental pollutant.

It is obvious from the results of the present study that the volatile composition of olive oil is greatly variable during maturation. These differences depend on enzymatic activity and a number of external parameters, such as climate, soil, harvesting and extraction conditions, which may alter the olive oil's volatile profile (Vichi *et al.,* 2003; Morales *et al.,* 2005; Kanavouras *et al.,* 2005).

4. CONCLUSIONS

In conclusion, the olive oils of the present study were of good quality according to the examined characteristics. In besides, the results allowed differentiation of the two studied varieties especially concerning their fatty acid composition, volatiles and sterol profile. Additionally, it was proved that the Throumbolia variety might result in olive oil of good quality if the fruits are collected immediately after they have dropped from the tree and the olive oil is extracted as soon as possible.

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