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Composition of total sterols (4-desmethyl-sterols) in extravirgin olive oils obtained with different extraction technologies and their influence on the oil oxidative stability

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Abstract

The composition and antioxidant activity of total sterols in extravirgin olive oils obtained with different extraction technologies from olives harvested at two ripening stages, were studied. The antioxidant activity was evaluated with an oxidative stability instrument (OSI), by using a model system (made of a mixture of treated/untreated commercial refined peanut oil) enriched with the total sterol fractions of the extravirgin olive oils. No correlation was found between the OSI time and the extraction technologies, the ripening stages or the actual amount of sterols added. No significant differences were observed in the percent composition of sterols of extravirgin olive oils produced with different technologies during the same harvesting period. The latter, however, had a significant effect on the percent of β-sitosterol and 5-avenasterol in extravirgin olive oils produced with the same technology.

Keywords: Sterols; Oxidative stability; Extravirgin olive oil; Gas chromatography; Olive oil extraction technology

1. Introduction

The high nutritional value of extravirgin olive oils is related to the presence of many components with interesting chemical and nutritional properties, including antioxidants and sterols. These compounds, together with other minor components, contribute to the oxidative stability of the oil. However, the reaction mechanisms of some of these substances are not completely known. The prooxidant or antioxidant effects of these compounds are usually evaluated in model systems, which are not fully representative of the structure and composition of the food matrix; this type of experimental set sometimes does not consider the synergic or antagonistic effects with other compounds naturally present in food and, therefore, it could give rise to misleading results relative to the prooxidant or antioxidant activity of certain substances. In olive oil, there is a series of compounds with asserted antioxidant power, such as polyphenols, carotenoids and tocopherols (Aparicio, Roda, Albi, & Gutiérrez, 1999; Baldioli, Servili, Perreti, & Montedoro, 1996; Cerretani, Bendini, Biguzzi, Lercker, & Gallina Toschi, 2003; Nakatani, Tachibana, & Kokuzaki, 2001). Other oil components can, on the other hand, promote lipid oxidation, such as highly unsaturated triacylglycerols, free fatty acids, chlorophyll pigments and metals (Angerosa & Di Giacinto, 1993; Frega, Mozzon, & Lercker, 1999; Gutiérrez-Rosales, Garrido-Fernández, Gallardo-Guerrero, Gandul Rojas, & Mingez-Mosquera, 1992). However, there is little available information on the effects of other components of the unsaponifiable fraction on the oxidative stability of oil. Some experiments carried out with the Rancimat test have shown that squalene, the most representative component of the olive oil unsaponifiable fraction, does not significantly affect the oil

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stability (Psomiadou & Tsimidou, 1999), despite its positive effects on human health (Rao, Newmark, & Reddy, 1998; Smith, Yang, Seril, Liao, & Kim, 1998). On the other hand, other studies have proven that triterpenic alcohols, hydrocarbons (squalene) and some sterols can delay oil degradation when subjected to prolonged heating (Boskou & Katsikas, 1979; Boskou & Morton, 1976; Sims, Fioriti, & Kanuk, 1972). White and Armstrong (1986) monitored the linoleic acid content and the peroxide value of soybean oil added with (24Z)-24-ethylidencholest-5-en-3β-ol (5-avenasterol or avenasterol) and heated at high temperatures, confirming the antioxidant activity of 5-avenasterol. Other studies reported that (24Z)-24-ethylidencholest-8,14-dien-3β-ol (vernosterol), 5-avenasterol, (24E)-24-ethylidencholest-5-en-3β-ol (fucosterol) and (24Z)-4-methyl-24-ethylidencholest-7-en-3P-ol (citrostadienol) behaved as antioxidants in oils kept at 180 °C, whereas (24S)-24-ethylcholest-5,22-dien-3β-ol (stigmasterol) and cholest-5-en-3β-ol (cholesterol) did not exhibit any antioxidant activity (Gordon & Magos, 1983). Wang, Hicks, and Moreau (2002) tested the antioxidant capacities of different amounts of free and esterified stanols, γ-oryzanol and rice bran oil by using the OSI; they proved that only rice bran oil led to an increase of the oxidative stability of soybean oil and distilled fatty acid methyl esters. In some cases, it has been observed that a higher content of sterols with an ethylidene group in position 24 (24′) of the side chain, leads to an increase in the antioxidant activity (Gordon & Magos, 1983); (24R)-ethylcholest-5-en-3β-ol (β-sitosterol), (24R)-24-methylcholest-5-en-3β-ol (campesterol) and stigmasterol, the most representative phytosterols in nature, do not exhibit this trend, though. It has been hypothesized that the first group of sterols react more rapidly with free radicals, leading to the formation of more stable radical compounds. The presence of a free hydrogen atom in the allylic carbon of the ethylidene group in the side chain is more prone to radical formation; this free radical can easily isomerize to a tertiary free radical, which is more stable. The intensity of this effect can also depend on the number and position of the endocyclic double bonds. In the particular case of stigmasterol, a tertiary free radical can be formed, but it does not display any antioxidant activity; this may be due to steric problems, which would reduce the velocity of formation of free radicals (Gordon & Magos, 1983).

However, it must be taken into account that many of the cited studies have been performed with considerable amounts of sterols, being significantly higher than those found in vegetable oils. On the other hand, it is well-known that due to the presence of oxidatively sensitive hydrophilic hydroxyl groups and double bonds in their chemical structure, phytosterols are susceptible to oxidation, and this phenomenon has been evaluated in model systems, as well as in some oils and food products (Bortolomeazzi, Cordano, Pizzale, & Conte, 2003; Dutta, 1997; Grandgirard, Martine, Joffre, Juaneda, & Berdeaux, 2004; Soupas, Juntunen, Lampi, & Piironen, 2004). Photoxidation can be the starting means of degradation for phytosterols, but the most common oxidation mechanism for sterols is autoxidation, which is a free radical reaction that generally starts with the abstraction of a reactive allylic hydrogen at C7 (Smith, 1981). Oxidation products can also be formed in the side-chain structure of the steroid molecule, but the latter is much less pronounced than oxidation in the ring-structure (Smith, 1992).

Since most of the reactive molecules (free radicals, triplet and singlet oxygen) that occur in the oxidation of phytosterols are the same as those involved in the oxidative degradation of lipids, this phenomenon would partially protect other lipid molecules (such as triacylglycerols and fatty acids) from oxidation. Considering these facts, it is evident that the influence of the different components of the unsaponifiable fraction on the oil oxidative stability, must be studied more in depth.

The aim of this work, was to evaluate the total composition of sterols (4-desmethyl-sterols) in extravirgin olive oils obtained with different extraction technologies from olives harvested at two. ripening stages and to assess their influence on the oil oxidative stability, using a similar sterol concentration to those usually found in the extravirgin olive oil. Another objective was to evaluate the possible analytical interferences in the sterols identification; this part of the study was focused on the correct identification of the most common oil unsaponifiable compounds, as well as the recognition of artifacts produced along the extraction and purification steps. A vegetable model system enriched with the total sterol fractions of the extravirgin olive oils was used, considering the synergistic effect of the amount and ratio of total sterols usually found in the extravirgin olive oil. The qualitative and quantitative determination of the total sterols was performed by GC and GC—MS. The antioxidant activity of the total sterol fraction was evaluated by measuring its forced oxidative stability with the OSI at 98 °C. Although several authors do not support the use of OSI for the evaluation of the oxidative stability of oils (Frankel, 1993), it has been proven that it is a valid method for comparing the oxidative behavior of lipid matrices when only one parameter, such as the sterols content, is changed (Lercker & Caboni, 1998).

2. Experimental

2.1. Reagents and solvents

Chloroform, *n*-hexane, methanol, ethanol and silica TLC plates (20 cm × 20 cm × 0.25 mm of film thickness), were purchased from Merck (Darmstadt, Germany) Silylating agents (pyridine, hexamethyldisilazane and trimethylchlorosilane), diethyl ether (stabilized and not stabilized with butylated hydroxytoluene (BHT)), double distilled water and 2′,7′-dichlorofluorescein (sodium salt) were supplied by Carlo Erba (Milan, Italy). β-Sitosterol (60% β-sitosterol and 40% campesterol), 5α-cholestan-3β-ol (cholestanol) (>95% purity), *N*-methyl-*N*-nitroso-*p*-toluenesolfonamide

(diazald®) and diethyl-glycol-monomethyl-ether and aluminum oxide type WB-2, were purchased from Sigma (St. Louis, USA). Anhydrous sodium sulfate and potassium hydroxide were supplied by BDH (Poole, England) and Prolabo (Fontenay, France), respectively. No. 1 filters (70 mm diameter) were used (Whatmann, Maidstone, England).

2.2. Samples

Non-filtered, extravirgin olive oils produced according to the Aprutino Pescarese (Pescara-Abruzzo, Italy) protected origin denomination (DOP) (European Commission Regulation No. 1263/96 of July 1st, 1996), were analyzed. The olives were sampled the first week of October and the last week of November, which corresponded to two different ripening stages (beginning and end of the harvest season). The olives were 95% of the cultivar Dritta and 5% of the cultivar Leccino. Five processing plants, located in the Aprutino Pescarese area, were employed for the oil extraction, which used three different extraction systems: by pressure extraction (PE1 and PE2) (traditional system), by centrifugation (continuous system) without mill waste water recycling (continuous extraction (CE1 and CE2)) and by centrifugation (continuous system) with mill waste water recycling (called as ecological-extraction (EE)). A Spanish extravirgin olive oil of known high oxidative stability (OSI time = 45 h), obtained in the 2001/2002 campaign from the Toledo region, was also analyzed.

2.3. Extraction of the total sterol fraction

Five grams (±0.02 g) of oil sample were subjected to cold saponification (Sander, Addis, Park, & Smith, 1989). The unsaponifiable fraction was dried under nitrogen flow, added with 0.5 ml of diazomethane (for free fatty acid methylation) and dried under nitrogen flow; this step was repeated twice (total volume of diazomethane = 1.5 ml). The treated unsaponifiable fraction was then dissolved in *n*-hexane:isopropanol (4:1, v/v) and of the latter solution was loaded on 15 cm of a TLC plate, so as to prepare a total of 4 TLC plates per unsaponifiable fraction; a spot containing sterol standards (β-sitosterol and campesterol) was loaded on each TLC plate, in order to correctly identify the sterol band. The mobile phase was a mixture of *n*-hexane/diethyl ether (65:35, v/v); the TLC was run at 4 °C. The sterol TLC band was visualized under UV light (254 nm), after being sprayed with a 0.2% ethanolic solution of 2′,7′-dichlorofluorescein sodium salt. The sterol bands of the TLC plates (the 2nd band from the TLC sample loading line, as shown in Fig. 4) were then scraped off, joined and extracted three times with 4, 2 and 2 ml of chloroform. To eliminate silica residues, the solution was centrifuged at 395*g*/3 min and then transferred to another vial. The solvent was evaporated under nitrogen flow at 40 °C; the sterols were weighed and then dissolved in 1000 μl of *n*-hexane:isopropanol (4:1, v/v).

2.4. Determination of the oxidative stability by OSI

2.4.1. Preparation of the model system for OSI measurements

Commercial refined peanut oil from the same production batch, was used for the preparation of the model system. A treatment with aluminum oxide was performed, in order to reduce the sterol content naturally present in the peanut oil. One hundred grams of peanut oil were mixed with *n*-hexane and aluminum oxide at a 2:2:1 (w/v/w) ratio, respectively, and the flask was shaken for 45 min at room temperature. The mixture was loaded into a chromatographic glass column (19 mm × 400 mm), which contained 30 g of aluminum oxide in *n*-hexane. The eluate was collected in a 500-ml flask and taken to dryness in a rotovapor at 60 °C for 45 min. A mixture of refined peanut oil and the oil obtained with treatment above mentioned (1:4, w/w) was prepared; this modified oil (called model system from this moment onwards) was used for the subsequent tests.

To evaluate the suitability of the model system, different tests were performed with various antioxidants, metal chelators and substances (as briefly described in Section 3). Two replicates of each test and the model system alone were then subjected to forced oxidation at 98 ± 0.1 °C, by using the OSI.

2.4.2. Evaluation of the influence of sterols on the oxidative stability of the model system by means of OSI

In order to evaluate the antioxidant properties of sterols, each OSI run was simultaneously performed on:

(a) 2 model systems alone.
(b) 2 model systems added with a TLC blank. To prepare this, a silica TLC was subjected to the same development conditions described for the extraction of the total sterol fraction and an area of the TLC with similar dimensions to that of the extravirgin olive oil sterol band, was scraped off, extracted (refer to point 2.3), dissolved in 1 ml of *n*-hexane:isopropanol solution (4:1, v/v) and 975 μl of this solution were placed in the OSI tube.
(c) 4 model systems added with the sterol extract from extravirgin olive oils (2 replicates per sample). To prepare the latter, 975 μl of the *n*-hexane:isopropanol solution (4:1, v/v) that contained the sterol fraction of each extravirgin olive oil sample, were transferred in OSI tubes and taken to dryness under nitrogen flow ($T < 40$ °C). About 5 g (±0.02 g) of the model system were then added to these OSI tubes and were subjected to ultrasonic bath for 1 min to ensure complete solubilization of sterols in the model system.

2.4.3. Determination of the oxidative stability

The evaluation of the oxidative stability was performed by using an eight-channel OSI (Omnion, Decatur, IL, USA). The conductibility was measured in polycarbonate tubes using double distilled water. The air flow was set at

120 ml/min. The OSI of the treated oils were performed at 110 ± 0.1 °C and 98 ± 0.1 °C. The OSI tests carried out with the selected model system, were run at 98 ± 0.1 °C. Two replicates were analyzed per each sample.

2.5. Determination of the qualitative and quantitative sterol composition

Twenty-five microliters of the *n*-hexane:isopropanol solution (4:1, v/v) that contained the sterol fraction of each extravirgin olive oil sample, were added with 20 μl of the internal standard solution (1.046 mg/ml of 5α-cholestanol in hexane) and taken to dryness under nitrogen flow. The sterols were then silylated with 200 μl of a pyridine:hexamethyldisilazane:trimethylchlorosilane mixture (5:2:1, v/v/v) at 40 °C for 20 min (Sweeley, Bentley, Makita, & Wells, 1963); the sample was then evaporated to dryness under nitrogen flow, dissolved in 50 μl of *n*-hexane and centrifuged at 395*g*/3 min. One microliter of this solution was then injected into the GC and GC–MS. Sterol identification was performed by comparing the peak retention times with those of the sterol standards, as well as by injecting into GC–MS. The internal standard method was used for quantitation of sterols. The GC response factor of sterols were calculated by using the internal standard (0.97). Method repeatability was calculated for an extravirgin olive oil, by analyzing eight independent replicates, which were injected twice each.

The relative percentage of each sterol was calculated as the percent ratio of the chromatographic area of the each sterol peak to the total area of sterols peaks. The amount of sterols added to the model system for the OSI experiments, were calculated as follows:

$$\text{Sterols (mg)} = \text{Ai} \times \text{Cis} \times 1000/\text{Ais} \times 25$$

where Ai was the area of the corresponding sterol, Cis was the amount of the internal standard added (mg), Ais was the area of the internal standard and 1000/25 was a dilution factor.

2.6. GC and GC–MS

Capillary gas chromatography (GC) was performed with a Carlo Erba Instruments HRGC 5300 (Rodano, Milan, Italy), equipped with a split–splitless injector and a flame-ionization detector, and coupled to a computerized system for data acquisition (Turbochrom Navigator 6.1.1.0.0:K20, Perkin–Elmer, Norwalk, USA). The column used was a fused-silica SE52 (30 m × 0.25 mm i.d. × 0.10 μm film thickness) (Mega, Milan, Italy), coated with 5% diphenyl–95% dimethylpolysiloxane. The oven temperature was programmed from 240 to 325 °C at a rate of 1.5 °C/min and kept at 325 °C for 10 min. The injector and detector temperatures were both set at 325 °C. Helium was used as carrier gas at a flow rate of 2.7 ml/min. The split ratio was 1:30.

GC–MS analysis for sterol identification were performed using a Varian (Walnut Creek, CA, USA) gas chromatograph mod. 3300/3400, equipped with a split–splitless injector and coupled to a mass spectrometric detector mod. Finnigan MAT ITS40 (San Jose, CA, USA). The analyses were performed by electron impact (El) and a ZB-5 low bleed fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) coated with 5% diphenyl–95% dimethylpolysiloxane (Phenomenex, Torrance, CA, USA) was used. The temperature was programmed from 240 to 300 °C at a rate of 3 °C/min. The injector, transfer line and manifold temperatures were set at 300, 300 and 200 °C, respectively. Helium was used as carrier gas. The filament emission current was 10 μA, the electron energy was 70 eV and the scanning speed was 1500 amu/s. The split ratio was 1:30.

2.7. Data analysis

Mean values of the relative percent of the sterols present in extravirgin olive oil, extracted with different technologies at two olive ripening stages, are shown in Table 4. One-way analysis of variance (ANOVA) with a posteriori Tukey *b* test was carried out, in order to determine statistical differences among samples ($p \leqslant 0.05$). A Statistica r. 6.0 software (2001) was utilized (StatSoft srl Vicenza, PD, Italy).

3. Results and discussion

A refined peanut oil was used for the preparation of the model system, because it presents a degree of unsaturation similar to that of olive oil. Different tests were performed in order to obtain a suitable model system for the evaluation of the antioxidant activity of sterols extracted from extravirgin olive oils. Table 1 shows the effect of the treatments on the stability and the reduction of the sterols in the model system. To increase the oxidative stability of the model system, 20% (w/w) of the refined peanut oil and 80% (w/w) of the oil treated with aluminum oxide were used, and the OSI tests were run at 98 ± 0.1 °C. Although this model system was in part prepared with the untreated refined peanut

Table 1
Effect of different treatments on the reduction of sterols and the OSI time of refined peanut oil

	Crude peanut oil[a]	Aluminum oxide at 30 °C[b]	Model system[c]
Total sterols (mg/Kg)	2160	1048	1156
Total sterols (%)[d]	100	49	53
OSI time at 110 °C (h)	8.90	1.75	4.30

For details, please refer to Section 2.

[a] Untreated peanut oil.

[b] Batch plus column chromatography treatments (as described in Section 2.3).

[c] Mixture of crude peanut oil and the oil obtained after aluminum oxide treatment (Section 2.3), at a 1:4 ratio (w/w).

[d] Percent residual sterols with respect to the total sterols originally present in the crude peanut oil.

oil, a significant reduction of the sterol content was still obtained (about 47%, w/w). Moreover, due to their lower affinity with aluminum oxide, the remaining sterols were likely to be present in their esterified form. The quantitative distribution of the sterol fraction of the model system was different from that of the refined peanut oil (Fig. 1). In fact, it had a higher content of (24R)-24-ethylcholest-7-en-3β-ol (7-stigmastenol) (0.27% instead of 0.18%) and (24Z)-24-ethylidencholest-7-en-3β-ol (7-avenasterol) (1.33% instead of 0.95%), but it also displayed a significant decrease in the amount of stigmasterol (from the original 8.7% to 6.1%) ($P \leqslant 0.05$); this last result confirmed that aluminum oxide mainly removes free sterols, as already described in a previous study (Cercaci & Lercker, 2000). This study, Cercaci and Lercker (2000) shown that stigmasterol was mainly present in its free form in the extravirgin olive oil, whereas 7-sterols (7-stigmastenol and 7-avenasterol) were mostly esterified. The total phenol content of the model system and the untreated peanut oil, determined according to Bendini et al. (2003), were lower than the quantification limit (LOQ) of the analytical system (LOQ = 0.5 mg of each polyphenol/kg of oil). The total tocopherol content of the model system and the untreated peanut oil, determined as suggested by LoCurto, Dugo, Mondello, Errante, and Russo (2001), were 15 and 45 mg/kg of oil (expressed as α-tochopherol), respectively. Since the quantity of oil used for the OSI test is equal to 5 g, the total amount of tocopherol contained therein is less than 0.1 mg; such a low level of tocopherols cannot invalidate the effect of the added substances with an eventual antioxidant activity. In addition, it should be considered that tocopherols, at the temperature used in these experiments, have a low or negligible effect on the oxidative stability of oil (Aparicio et al., 1999). Another important fact to be taken into account is that the content of tocopherol was constant in all tests performed, so no significant effects on the oil stability can be attributed to the tocopherol content.

To further evaluate the suitability of the model system, different antioxidants and metal chelators were separately added to the model system and then subjected to accelerated oxidation; the compounds tested are listed as follows: polyphenol extracts obtained from 5 g of extravirgin olive oils (~350 mg of polyphenols/kg of oil expressed as caffeic acid), 0.5 g of the Spanish extravirgin olive oil, 3 mg of ethylenediaminotetraacetic acid ($EDTA(Na)_2$) and 3 mg of citric acid. Fig. 2 shows the average OSI times of the model system, as well as those obtained with the different antioxidant and chelator mixtures. The addition of metal chelators did not produce significant differences with respect to the model system. On the contrary, the addition of the polyphenol extracts and the extravirgin olive oil to the model system significantly increased the oxidative stability, which means that the model system positively reacted to the presence of compounds of known antioxidant activity at concentration levels similar to those usually found in extravirgin olive oils. This suggested that the system could be used also for the evaluation of the antioxidant activity of other compounds that are naturally present in these oils, such as sterols.

Further tests were performed to evaluate the possible interferences on the OSI time. As shown in Fig. 2, the silica TLC blank resulted in a slightly lower OSI time as compared to that of the model system, showing a slight prooxidant effect of the substances present in the silica powder. Therefore, it was important to simultaneously run a silica TLC blank test when evaluating the oxidative stability with the OSI, as well as to centrifuge the chloroform extract that

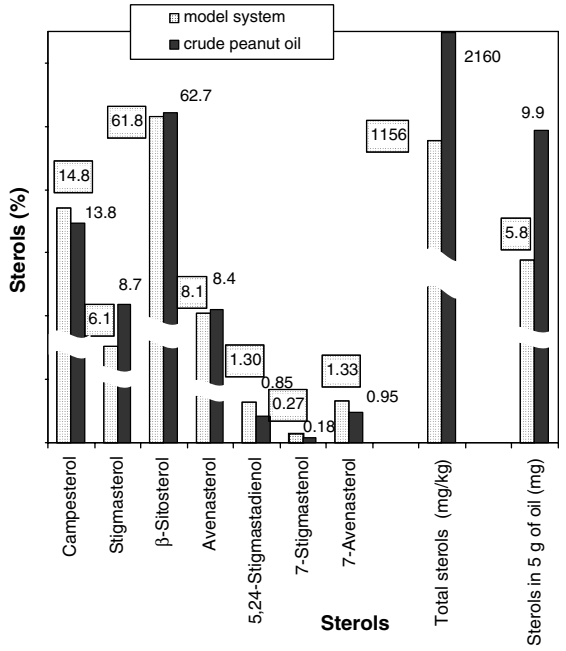

Fig. 1. Comparison between total sterols and the percentage of the main sterols present in crude peanut oil and the model system. The reported values are the mean of two replicates.

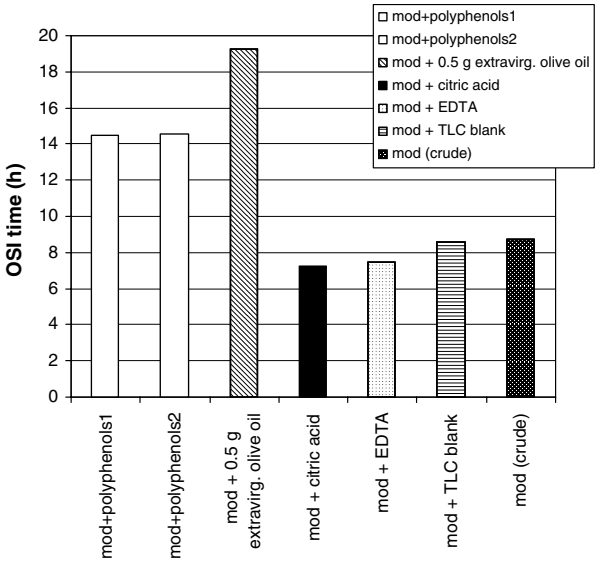

Fig. 2. Effects of various substances on the OSI time (98 °C) of the model system. For experimental details, please refer to Section 2.

contained the sterol TLC band to avoid introduction of silica powder into the model system. In addition, it was also verified that the non-stabilized diethyl ether (used for the cold saponification and TLC development), *n*-hexane and aluminum oxide did not contain peroxides or other substances that could affect the OSI time.

Table 2 reports the OSI times of the sterol fractions of extravirgin olive oils extracted with different technologies at two ripening stages. The effect of the sterol fraction on the oxidative stability of the model system, was evaluated by measuring, in the same set, the OSI times of the model system alone, the model system added with the sterol extract and the TLC blank. Although, in most cases, a minor increase was observed when the sterol extract from the extravirgin olive oil samples was added, these variations were not significant. Moreover, no correlation was found between the oxidative stability and the extraction technologies or ripening stages. Under the conditions here tested, there was no correlation between the OSI time and the actual amount of sterols added to the model system, as shown in Fig. 3. The amount of free sterols that was added ranged from 2.3 to 4.2 mg per each test, which corresponded to 460–840 mg of sterols/kg oil. This addition of sterols allowed to restore an amount of total sterols that was close to the one originally present in the peanut oil. Moreover, the esterified to free sterol ratio was roughly brought back to the values that were likely to be present in the peanut oil (Phillips, Ruggio, Toivo, Swank, & Simpkins, 2002).

Regarding the GC analysis of sterols, Fig. 4 shows the chromatograms of the different TLC bands of the unsaponifiable fraction of a Spanish extravirgin olive oil; the tocopherols band was enriched with the corresponding standards, because they are present at low concentrations in the virgin olive oils. It must be noted that, under the analytical conditions used here 4,24-dimethylcholest-7,24(24′)-dien-3β-ol (gramisterol) and 4,4′,19α-trimethylcholestan-3β-ol (cycloartanol) (peaks C and E of Fig. 4, respectively) overlapped with 7-stigmastenol; other 4-methylsterols and triterpenic alcohols GC peaks also had similar retention times to those of (24S)-24-ethylcholest-7,9(11)-dien-3β-ol (7,9(11)-stigmastadienol) and (24S)-24-ethylcholest-5,23-dien-3β-ol (5,23-stigmastadienol). It was confirmed that several steroid substances originally present in the TLC plate are coextracted with the unsaponifiable compounds, which can interfere with the correct identification of sterols (Cercaci, Rodriguez-Estrada, & Lercker, 2003). Attention must be also paid to the non-silylated compounds, which can be misidentified as sterols.

Sterol identification (Fig. 5) was performed by comparing the peak retention times with those of the sterol standards, as well as by GC–MS analysis.

Table 3 shows the internal percentage of the trimethyl silyl derivatives of sterols of an extravirgin olive oil. The

Table 2
Comparison of the OSI times (h) of the model system alone, added with TLC blank and added with sterol fractions of extravirgin olive oils extracted with different technologies at two ripening stages

Samples	Model system mean[a]	TLC blank mean[b]	Sample mean[a]	Absolute sample mean[b]	Model system vs. sample[c]
EE Oct rep1	8.77	8.17	8.87	9.47	>
EE Oct rep2	8.77	8.17	8.82	9.42	>
EE Nov rep1	8.72	8.17	8.55	9.10	>
EE Nov rep2	8.72	8.17	8.62	9.17	>
CE1 Oct rep1	8.87	8.40	8.47	8.94	=
CE1 Oct rep2	8.87	8.40	8.47	8.94	=
CE1 Nov rep1	8.85	8.55	6.85	7.15	≪[d]
CE1 Nov rep2	8.85	8.55	8.72	9.02	=
CE2 Oct rep1	8.80	8.55	8.87	9.12	>
CE2 Oct rep2	8.80	9.30	9.27	8.77	=
CE2 Nov rep1	8.90	9.30	8.97	8.57	<
CE2 Nov rep2	8.90	9.30	9.40	9.00	=
PE1 Oct rep1	8.95	8.50	9.27	9.72	>
PE1 Oct rep2	8.95	8.50	8.75	9.20	>
PE1 Nov rep1	9.00	8.97	8.95	8.98	=
PE1 Nov rep2	9.00	8.97	8.97	9.00	=
PE2 Oct rep1	8.57	8.52	8.40	8.45	=
PE2 Oct rep2	8.57	8.52	8.32	8.37	<
PE2 Nov rep2	8.37	8.30	8.65	8.72	>
PE2 Nov rep1	8.37	8.30	8.57	8.64	>
Spanish oil	8.45	7.92	8.32	8.85	>

Abbreviations: Oct, October; Nov, November; rep, replicate; EE, centrifugation with mill waste water recycling; CE, centrifugation; PE, pressure.

[a] These values are the average of two replicates per sample.

[b] Absolute sample mean = model system mean + ($\Delta^1 - \Delta^2$), where Δ^1 = model system mean − TLC blank mean and Δ^2 = model system mean − sample mean.

[c] These symbols represent the actual trends and correspond to the comparison between the OSI times of the absolute sample mean and those of the model system.

[d] This value is an outlier ($p \leqslant 0.05$), probably due to the presence of residual silica powder in the sterol extract.

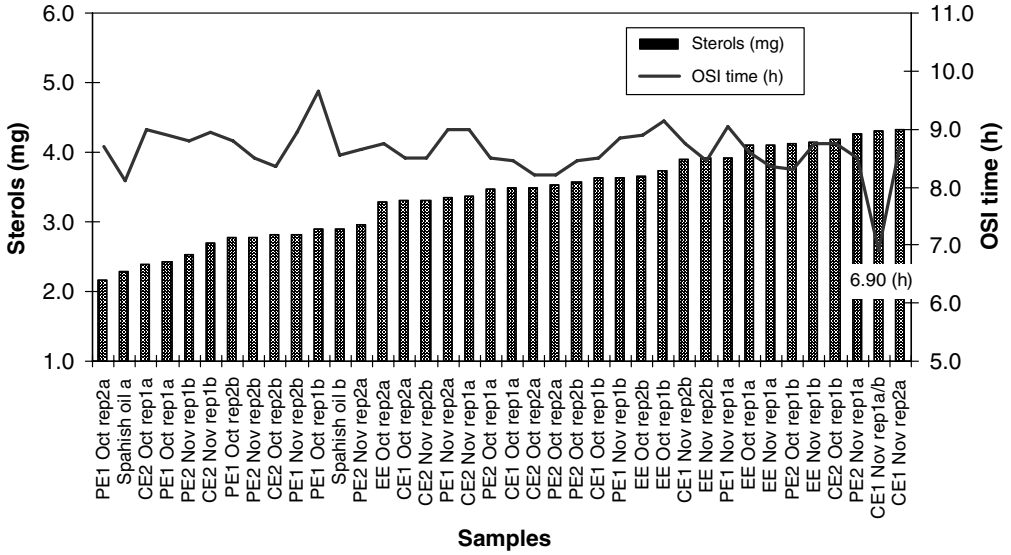

Fig. 3. Correlation between OSI time and increasing amount of sterols added to model system. For experimental details, please refer to Section 2. The value indicated in the lower part of the plot is an outlier ($p \leqslant 0.05$), probably due to the presence of residual silica powder in the sterol extract.

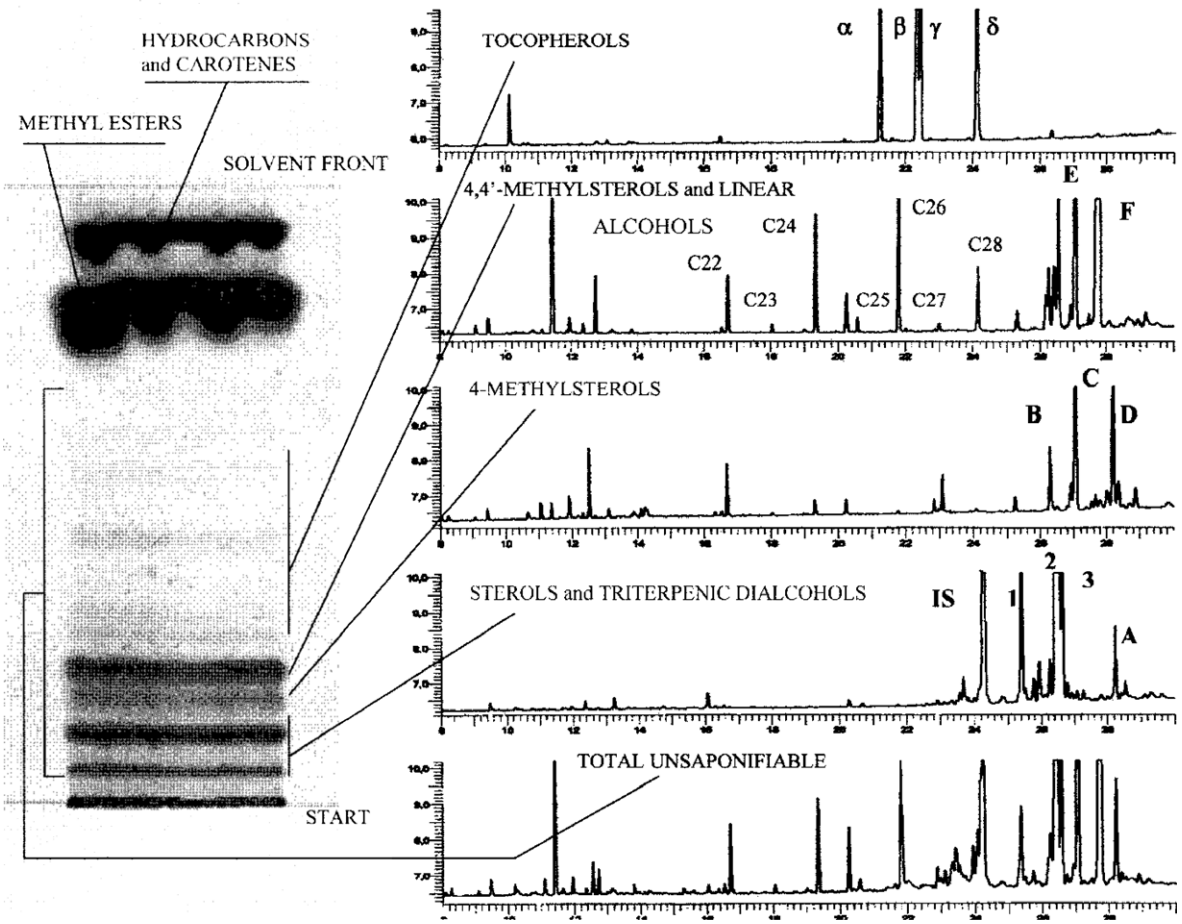

Fig. 4. GC trace of different TLC bands of the unsaponifiable fraction of an extravirgin olive oil. For TLC and GC conditions, please refer to Section 2. For correct identification, the tocopherols band was spiked with the corresponding standards. Peak identification: IS, cholestanol; 1, campesterol; 2, sitosterol; 3, avenasterol; A, erythrodiol; B, obtusifoliol; C, gramisterol; D, citrostadienol; E, cycloartanol; F, 24-methylencycloartenol; C22–C28, linear alcohols with different number of carbon atoms.

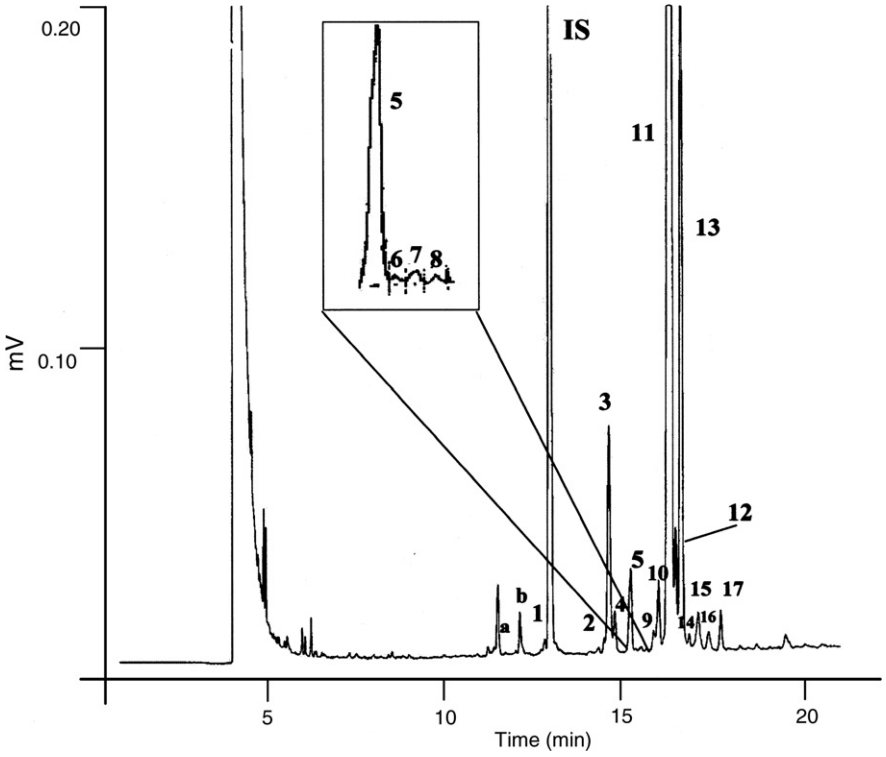

Fig. 5. GC trace of the sterol fraction of an extravirgin olive oil obtained with a SE52 GC column. F or GC conditions, please refer to the experimental section. Peak identification: 1, cholesterol; IS, 5α-cholestanol (internal standard); 2, 24-methylencholesterol; 3, campesterol; 4, campestanol; 5, stigmasterol; 6, 22-stigmastenol; 7, unknown peak at RRT 0.93 (with respect of β-sitosterol); 8, 7-campesterol; 9, 5,23-stigmastadienol; 10, clerosterol; 11, β-sitosterol; 12, sitostanol; 13, 5-avenasterol; 14, 7,9(11)-stigmastadienol; 15, 5,24-stigmastadienol; 16, 7-stigmastenol; 17, 7-avenasterol. (a, b) impurities of the internal standard.

Table 3
Relative percent of trimethyl silyl derivatives of the sterols of the extravirgin olive oil

Sterols (%)	1	2	3	4	5	6	7	8	Mean[a]	SD[b]	RSD (%)[c]
Cholesterol	0.07	0.07	0.06	0.03	0.16	0.08	0.18	0.19	0.11	0.06	57.8
24-Methylencholesterol	0.12	0.12	0.12	0.13	0.12	0.12	0.13	0.12	0.12	0.00	3.1
Campesterol	2.88	2.89	2.88	2.90	2.92	2.91	2.92	2.92	2.90	0.02	0.6
Campestanol	0.07	0.11	0.09	0.05	0.09	0.08	0.10	0.09	0.08	0.02	19.2
Stigmasterol	0.30	0.36	0.33	0.34	0.34	0.33	0.37	0.33	0.34	0.02	6.1
Stigmastanol	0.05	0.08	0.04	0.06	0.10	0.05	0.05	0.04	0.06	0.02	39.3
X = RRT 0.93[d]	0.09	0.11	0.10	0.09	0.10	0.09	0.12	0.10	0.10	0.01	10.7
7-Campesterol	0.11	0.10	0.10	0.12	0.11	0.08	0.14	0.10	0.11	0.02	14.0
Clerosterol	1.00	1.03	1.00	0.98	0.99	1.01	1.05	0.97	1.00	0.03	2.6
β-Sitosterol	80.53	79.77	80.89	81.21	81.10	81.66	80.61	81.01	80.85	0.56	0.7
Sitostanol	1.37	1.43	1.33	1.32	1.31	1.11	1.51	1.35	1.34	0.11	8.6
Avenasterol	9.12	9.17	8.91	8.78	8.49	8.58	8.65	8.60	8.79	0.26	2.9
7,9(11)-Stigmastadienol	0.45	0.48	0.47	0.47	0.79	0.64	0.72	0.67	0.59	0.13	23.0
5,24-Stigmastadienol	1.27	1.40	1.16	1.03	1.04	1.02	1.04	1.04	1.13	0.14	12.5
7-Stigmastenol	0.96	1.17	0.98	0.94	1.01	0.92	1.05	1.06	1.01	0.08	8.1
7-Avenasterol	1.59	1.70	1.54	1.54	1.33	1.34	1.37	1.42	1.48	0.13	9.0

For details refer to Section 2.
[a] Mean value of eight independent replicates.
[b] Standard deviation.
[c] Percent relative standard deviation.
[d] Relative retention time (with respect of β-sitosterol) of the unknown peak.

analysis exhibited a good repeatability for sterols present at significant levels, whereas a larger variation was observed for those sterols present in small amounts or that partially overlapped with other peaks (such as cholesterol, stigmastenol and 7,9(11)-stigmastadienol). In any case, a good overall repeatability of the method was obtained.

In order to explain the results, it must be considered that olives were harvested from different plants located in differ-

ent places inside the production area of the DOP Aprutino Pescarese olive oil; although the two olive harvests (October and November) were processed in the same mills. Table 4 shows that there are no great differences in the percent composition of sterols in extravirgin olive oils produced with different technologies during the same harvesting period; apparently, the technological differences of grinding, kneading, oil extraction and amount of water used for the dilution of the olive paste, did not have a significant influence on the sterol composition. The amount of the total sterol remained roughly constant (1860 ± 140 mg/kg of oil) among the samples, and it was not significantly affected by the different technologies used or the harvesting period ($p \leqslant 0.05$).

The harvesting period, on the contrary, had a significant effect on the percent composition of the sterols present in the olive oils produced with the same processing technology. In particular, the relative percentage of 5-avenasterol exhibited a significant decrease in more than half of the oils analyzed. The content of 5-avenasterol in the CE1 oil remained practically unchanged, regardless of the olive ripening stage. On the contrary, in the CE2 sample, 5-avenasterol displayed a significant increase in the oil produced with olives harvested in November (from 11.2% to 16.1%), whereas β-sitosterol significantly decreased (from 79.5% to 75.0%). It must be noted that, in the CE2 oil, other sterols showed a particular behavior; in fact, a relevant rise of campesterol (from 3.00% to 3.22%) and a decrease of stigmasterol (from 1.79% to 0.87%) were registered in the oil produced with olives at a higher ripening stage. Nevertheless, it must be mentioned that, when compared with the other olive oil samples, the CE2 olive oil also exhibited a different behavior in terms of other analytical parameters, such as the content of polyphenols (data not shown). These differences in the sterol composition were not significantly correlated to the variations in the OSI times.

The actual decrease of 5-avenasterol observed in this study does not agree with the data reported by some researchers about the effect of ripening on the sterol composition of olive oils (Frega & Lercker, 1985; Gutiérrez, Jimenez, Ruíz, & Albi, 1999; Mariani, Fedeli, Grob, & Arthor, 1991), where an increase of 5-avenosterol was found in a longer ripening period than the one here considered. However, it must be underlined that the olive cultivars used in these works are different from the one here utilized (95% Dritta–5% Leccino) and that the initial content of 5-avenasterol of some of those cultivars (Picual and Hojiblanca) is lower (Gutiérrez et al., 1999) than the levels detected in the olives used in this study. It can be, thus, hypothesized that the 5-avenasterol destruction or transformation observed after one month of the first olive sampling, might be related to the natural impoverishment of natural antioxidants (especially polyphenols) during ripening. Due to its high susceptibility to isomerization, this would lead to a certain decrease of 5-avenasterol during ripening and/or olive processing. However, no increments were detected in the relative amounts of 5,23- and (24S)-24-ethylcholest-5,24-dien-3β-ol (5,24-stigmastadienols), which are known degradation products of 5-avenasterol (Meneghetti, Amelotti, & Griffini, 1987). Side-chain oxidation products of 5-avenasterol, as well as other phytosterol oxidation products, could have also been generated during ripening and/or olive processing, but they were not determined in this study.

Table 4
Relative percent of the sterols present in extravirgin olive oil, extracted with different technologies at two olive ripening stages

Sterols (%)	Oct EE	Nov EE	Oct CE1	Nov CE1	Oct CE2	Nov CE2	Oct PE1	Nov PE1	Oct PE2	Nov PE2
Cholesterol	0.04^{c}	<0.01^{a,c}	0.01^{c}	0.02^{c}	<0.01^{a,c}	0.05^{c}	0.02^{c}	<0.01^{a,c}	0.01^{c}	0.02^{c}
24-Methylencholesterol	0.20^{d,e}	0.11^{c}	0.16^{c,d,e}	0.13^{c}	0.10^{c}	0.20^{e}	0.14^{c,d}	0.15^{c,d,e}	0.16^{c,d,e}	0.12^{c}
Campesterol	3.31^{d,e}	3.28^{d}	3.25^{d}	3.22^{d}	3.00^{c}	3.22^{d}	3.43^{e}	3.30^{d,e}	3.33^{d,e}	3.28^{d}
Campestanol	0.10^{c,d,e}	0.05^{c}	0.09^{c,d}	0.09^{c,d}	0.09^{c,d}	0.16^{d,e}	0.19^{e}	0.07^{c,d}	0.11^{c,d,e}	0.11^{c,d,e}
Stigmasterol	1.03^{c,d}	0.94^{c,d}	0.99^{c,d}	1.17^{d}	1.79^{e}	0.87^{c,d}	0.88^{c,d}	0.80^{c}	0.92^{c,d}	1.01^{c,d}
Stigmastanol	0.05^{c}	0.08^{c}	0.04^{c}	0.05^{c}	0.09^{c}	0.09^{c}	0.11^{c}	0.09^{c}	0.10^{c}	0.09^{c}
X = RRT 0.93[b]	0.08^{d,e}	0.08^{d,e}	0.05^{c}	0.07^{c,d,e}	0.09^{e}	0.06^{c,d}	0.06^{c,d}	0.07^{c,d}	0.07^{c,d}	0.07^{c,d}
7-Campesterol	0.02^{c}	<0.01^{a,c}	<0.01^{a,c}	<0.01^{a,c}	<0.01^{a,c}	<0.01^{a,c}	<0.01^{a,c}	0.03^{c}	<0.01^{a,c}	0.02^{c}
5,23-Stigmastadienol	0.15^{c}	0.15^{c}	0.10^{a,c}	0.08^{a,c}	0.12^{c}	0.22^{c}	0.21^{c}	0.22^{a,c}	0.05^{c}	0.08^{c}
Clerosterol	0.98^{c}	0.97^{c}	0.93^{c}	0.94^{c}	0.99^{c}	0.95^{c}	0.88^{c}	0.93^{c}	1.00^{c}	0.96^{c}
β-Sitosterol	76.3^{c}	81.1^{f}	78.7^{e}	80.2^{e,f}	79.5^{e,f}	75.0^{c}	75.5^{c}	78.5^{d,e}	76.6^{c,d}	80.1^{e,f}
Sitostanol	1.38^{c,d}	1.33^{c,d}	0.96^{c}	1.26^{c,d}	1.31^{c,d}	1.37^{c,d}	1.47^{c,d}	1.49^{c,d}	1.62^{d}	1.71^{d}
Avenasterol	14.5^{e,f}	10.4^{c}	13.3^{d,e}	11.2^{c,d}	11.2^{c,d}	16.1^{f}	15.2^{e,f}	12.8^{c,d,e}	14.5^{e,f}	10.9^{c,d}
7,9(11)-Stigmastadienol	0.31^{c}	0.35^{c}	0.21^{c}	0.13^{c}	0.11^{c}	0.24^{c}	0.25^{c}	0.23^{c}	0.17^{c}	0.34^{c}
5,24-Stigmastadienol	0.85^{c}	0.61^{c}	0.77^{c}	0.80^{c}	0.85^{c}	0.94^{c}	0.97^{c}	0.74^{c}	0.85^{c}	0.67^{c}
7-Stigmastenol	0.20^{c,d}	0.20^{c,d}	0.17^{c}	0.25^{c,d}	0.29^{d}	0.24^{c,d}	0.26^{c,d}	0.21^{c,d}	0.19^{c}	0.26^{c,d}
7-Avenasterol	0.30^{c,d}	0.42^{c,d}	0.24^{c}	0.43^{c,d}	0.56^{d}	0.37^{c,d}	0.47^{c,d}	0.31^{c,d}	0.31^{c,d}	0.26^{c}

The values are the average of two independent replicates injected twice in GC. Abbreviations: Oct, October; Nov, November; EE, centrifugation with mill waste water recycling, CE, centrifugation; PE, pressure.

[a] The limit of quantitation (LOQ) was 0.01%.

[b] Relative retention time (with respect of β-sitosterol) of the unknown peak.

[c–f] Different letters denote significant differences among different sterol ($p \leqslant 0.05$).

4. Conclusions

Under the analytical conditions here used, no correlation was found between the OSI time and the extraction technologies of the extravirgin olive oils, the olive ripening stages or the actual amount of sterols added to the model system.

On the other hand, no significant differences were observed in the percent composition of sterols of extravirgin olive oils produced with different technologies during the same harvesting period. The latter, however, had a significant effect on the percent of β-sitosterol and 5-avenasterol in extravirgin olive oils produced with the same technology.

Although previous works have proven the antioxidant activity of some sterols (Gordon & Magos, 1983; White & Armstrong, 1986), this study shows that, under the analytical conditions here used, there is a minor, non-significant effect of the total sterols obtained from extravirgin olive oil on the oxidative stability of a vegetable model system. The synergic or antagonistic effects of the various sterols were here considered, thus hiding their single antioxidant properties. This type of experimental approach provides a more realistic picture of the overall antioxidant capacities of sterols, which is one of the most abundant component of the olive oil unsaponifiable fraction. However, further studies are required to evaluate the synergic or antagonistic effects of the various components of the unsaponifiable fraction of olive oil, which can lead to a more complete knowledge of the chemical interactions and equilibrium that occur in the olive oil.

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