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Rate of Degradation of α-Tocopherol, Squalene, Phenolics, and Polyunsaturated Fatty Acids in Olive Oil during Different Storage Conditions

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Changes in the concentration of tocopherol, monophenols, *o*-diphenols, squalene, and polyunsaturated fatty acids in olive oil were evaluated during 1 year at various storage conditions. Samples of two different extra virgin olive oil (EOO), produced in Calabria (Italy), were stored in dark and in colorless bottles, filled up completely or to half, in order to simulate the domestic storage conditions. The extent of oxidation or photooxidation was monitored by periodic measurements of peroxide values and the rate of degradation of α-tocopherol, *o*-diphenols, squalene, and polyunsaturated fatty acids. The quantitative analysis of the constituents has been performed by HPLC−DAD, HPLC−MS, and GC−MS. The main changes in the concentrations of the analyzed compounds were associated with the major oxygen level in the half-empty glass bottles. α-Tocopherol was the first molecule to be oxidized (−20% after 2 months, −92% after 12 months). Squalene and *o*-diphenols were protected in the first months by the presence of α-tocopherol, and their content decreased significantly only after 6 and 8 months, respectively, in the half-empty bottles. The concentration of polyunsaturated fatty acids remained almost constant during 8 months for all four different storage conditions; their oxidation started when the level of the antioxidants decreased.

KEYWORDS: Calabrian virgin olive oil; storage; oxidation; tocopherol; phenolic compounds; squalene; HPLC−DAD; HPLC−MS; GC−MS

INTRODUCTION

The dietetic and therapeutic value of extra virgin olive oil (EOO), highly esteemed in the prevention of coronary hearth diseases and cancer, resides on some basic factors: (i) One factor is a very high percentage, in the predominant triglyceride fraction, of monounsaturated fatty acids such as oleic acid (up to 74%) (*1*) and a relatively reduced level of polyunsaturated essential fatty acids (PUFA), linoleic and linolenic acids (C18:2 ω-6, C18:3 ω-3). Such composition gives good resistance to chemical and biological oxidation, in contrast with other edible oils in which the peroxidizable polyunsaturated fatty acids prevail on monounsaturated ones.

(ii) A second factor is a fairly good amount of lipophilic (D-*RRR*-α-tocopherol) (*2*) and amphoteric phenolic antioxidants, which contribute to its delicate and pleasant flavor and taste (*3*, *4*). The concentration of α-tocopherol, traditionally considered as the major antioxidant of olive oil (*5*), varies between a few parts per million and 350 ppm and represents about 90% of the total tocopherols. Normally, in animal fluids and tissues, vitamin E works in sinergy with coenzyme Q to protect cells and tissue against lipoperoxidation, and some authors detected CoQ_9 (8.7 ± 0.1 nmol/g of oil) and CoQ_{10} (13.3 ± 0.4 nmol/g of oil) in olive oil (*6*, *7*). Phenolic compounds give a greater resistance to the autoxidation process (*8*−*10*) and are considered responsible for some olive oil specific organoleptic properties (*11*, *12*).

(iii) Another factor is a high concentration of squalene (up to 1%), a triterpenoid hydrocarbon, which is a precursor of sterol biosynthesis and a scavenger of reactive oxygen species (ROS), such as singlet oxygen (*13*). It occurs in olive oil and in shark liver and human sebum which, like olive oil, contains high percentages of monounsaturated fatty acids. In a previous paper we suggested that sebaceous squalene is able to counteract reactive oxygen species induced by UV irradiation on the skin, behaving like an indirect natural filter, and it is a strong scavenger of singlet oxygen (*14*−*16*).

EOO is normally consumed in its natural and unaltered state, without extensive detrimental refining characteristic of other edible oils, and thus, it preserves attractive organoleptic properties. Despite its antioxidant properties, EOO also undergoes an

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oxidative process with consequent production of compounds with rancid smell and taste, and loss of its organoleptic peculiarities, and nutritional and commercial value. Generally, it is believed that PUFA are responsible for olive oil peroxidation, but little is known about the fate of the other non-fatty acid constituents during this process.

Therefore, because of the remarkable economic importance of EOO in the Mediterranean countries and its crucial role in the so-called Mediterranean diet, we have investigated the olive oil quality change during storage. A stability study was performed, monitoring the loss of α-tocopherol, phenolic antioxidants, squalene, and polyunsaturated fatty acid in the oil stored at four different conditions and during 12 months.

MATERIALS AND METHODS

Samples. Two extra virgin olive oils named "Scalicelle" and "Terzera" produced from olive fruits (*Olea europaea* L.) harvested by hand in the towns of Scalicelle and Terzera (Cosenza, Calabria, Italy) were used.

Storage Experiments. Virgin olive oil samples were filtered and stored either in 20 colorless glass bottles (500 mL) or in 20 dark ones (500 mL). Half the bottles were filled (≈3% headspace), while the remaining ones were half-empty (≈50% headspace). The bottles were well taped and stored at room temperature and under diffused light to simulate the typical home storage conditions. Under these experimental conditions, the oxidation that occurred in the black bottles could be ascribed to a simple autoxidation, while in colorless bottles the autoxidation was associated to photooxidation. All of the samples were periodically rearranged to equalize exposure. Another 10 filled dark bottles (250 mL) were stored at −30 °C, and their content was necessary to replace oil from filled bottles used for the analyses performed every 2 months.

Standards and Solvents. All of the standards were purchased from Sigma Chemicals (Milan, Italy), and all organic solvents were from Carlo Erba, Milano (Italy); water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

Analytical Techniques and Equipment. The acidity and the peroxide number were assayed according to the official methods (*17*).

(A) Fatty Acid Analysis. Triglycerides were purified by TLC (Si-gel, *n*-hexanes−ethyl ether−AcOH, 70:30:1.5 v/v) and transesterified in a well-closed screw cap Pyrex tube for 30 min at 50 °C with 3 mL of methanolic sodium methoxide solution (30 mg/mL). The resulting fatty acid methyl esters were analyzed by capillary gas chromatography−mass spectrometry (GC−MS, Shimadzu MS-QP5050) as previously reported (*18*).

(B) Simultaneous Determination of D*-RRR-α-Tocopherol, Ubiquinone Species, and β-Carotene.* Fifty milligrams of oil was diluted to 500 μL with isopropyl alcohol containing 20 μg of α-tocopheryl acetate as reference standard. Vitamin E and the reference standard were quantified by simultaneously injecting 20 μL into a 10 A VP Shimadzu liquid chromatograph on an analytical Supelcosil LP-18 column (24 cm × 4.6 mm, 5 μm; Supelco) plus its guard column, by using in line both photodiode array (SPD-M Shimadzu) and electrochemical detectors. The photodiode array detector was set at 290 nm (vitamin E acetate), 275 nm (ubiquinone), and 453 nm (β-carotene). The mobile phase was 50 mM sodium perchlorate in methanol−2-propanol, 55:45 v/v, at a flow of 0.9 mL/min. The electrochemical detection was obtained by an ESA CoulArray (Bedford, MA), which allows the postcolumn electrochemical reduction of ubiquinone to ubiquinol (reduction potential, −600 mV) and the quantitation of ubiquinol with high sensitivity and selectivity (oxidation potential, +600 mV). Vitamin E, vitamin E acetate, ubiquinone, and β-carotene were quantified by comparison of the peak areas to those of authentic standards.

(C) Determination of Squalene. Ten milligrams of oil and 100 μg of squalane (reference standard) were diluted to 500 μL with hexane. Quantification of squalene was performed by capillary GC−MS (Shimadzu MS-QP5050) as previously reported (*19*).

(D) Determination of Sterols. Sterols were assayed according to the official methods (*17*).

Table 1. Main Parameters of Extra Virgin Olive Oil Samples Used in Oxidative Stability Studies[a]

quality characteristic	Terzera	Scalicelle
peroxide value (mequiv of O_2/kg)	2.8 ± 0.1	2.6 ± 0.1
acidity (% oleic acid)	0.33 ± 0.01	0.25 ± 0.01
α-tocopherol (μg/g)[b]	341.5 ± 18.1	307.6 ± 22.3
squalene (μg/g)[c]	5.970 ± 0.12	5.481 ± 0.16
o-diphenols (μg/g)[b]		
hydroxytyrosol	4.31 ± 0.21	6.68 ± 0.32
caffeic acid	0.61 ± 0.06	0.95 ± 0.06
oleuropein aglycon	1.10 ± 0.14	1.35 ± 0.23
luteolin	0.23 ± 0.07	1.26 ± 0.20
apigenin	tr[d]	0.67 ± 0.09
total *o*-diphenols	6.46	11.01
monophenols (μg/g)[b]		
tyrosol	16.33 ± 0.87	22.25 ± 1.41
vanillic acid	2.17 ± 0.27	2.81 ± 0.88
p-cumaric acid	2.13 ± 0.64	3.45 ± 1.09
ferulic acid	0.96 ± 0.25	1.33 ± 0.20
total monophenols	21.59	29.84
fatty acid (% peak area)[c]		
C14:0	tr	tr
C16:0	13.32 ± 0.70	12.52 ± 0.63
C16:1 n-7	0.54 ± 0.17	0.44 ± 0.12
C17:0	0.16 ± 0.02	0.14 ± 0.02
C17:1 n-7	0.26 ± 0.02	0.21 ± 0.03
C18:0	3.58 ± 0.36	2.46 ± 0.21
C18:1 n-9	71.89 ± 2.21	73.97 ± 1.83
C18:2 n-6	8.37 ± 0.44	7.65 ± 0.46
C18:3 n-3	0.78 ± 0.09	0.75 ± 0.07
C20:0	0.66 ± 0.08	0.47 ± 0.09
C20:1 n-9	0.31 ± 0.03	0.22 ± 0.06
C22:0	0.12 ± 0.02	0.08 ± 0.04
C24:0	tr	tr
sterols (μg/g)[c]		
cholesterol	0.11 ± 0.01	0.08 ± 0.02
campesterol	2.71 ± 0.04	3.08 ± 0.06
stigmasterol	0.95 ± 0.19	1.12 ± 0.12
b-sitosterol	811.26 ± 46.0	866.10 ± 35.3
Δ5-avenasterol	6.40 ± 0.55	7.18 ± 0.84
others	1.75	1.50

[a] Each result represents the mean ± SD of 10 determinations. [b] Analysis was performed by HPLC–DAD. [c] Analysis was performed by GC–MS. [d] tr = traces (<0.01%).

(E) Determination of Phenols. Five grams of oil was extracted two times with 20 mL of methanol−0.001 N HCl, 80:20 v/v, in the presence of 100 μg of *p*-OH-anisole (reference standard). The aqueous phases were combined and washed two times with 5 mL of hexane to eliminate apolar lipids and evaporated to dryness under vacuum. The residue was dissolved in 0.5 mL of methanol−0.001 N HCl, 80:20 (solution A). An aliquot was used for HPLC analyses by a photodiode array detector set at 280 nm, while the oxidation potential of ESA CoulArray was set at +600 mV. One hundred microliters of sample was injected into the HPLC system. Conditions: mobile phase, A = 0.01% trifluoroacetic acid (TFA) in H_2O, B = CH_3CN; gradient program, 10% B for 15 min, 90% B in 80 min; flow rate, 1 mL/min. Phenols were quantified by comparison of the peak areas to those of authentic standards. The identities of oleuropein aglycon, luteolin, and apigenin were confirmed by liquid chromatography−electrospray mass spectrometry (LC−ES/MS), using an LCQ ion trap instrument (Finnigan Corp., San José, CA), and the above column and gradient system. Phenols were also quantified by GC−MS. One hundred microliters of solution A was dried under vacuum, and the residue was diluted with 40 μL of dry pyridine and then directly silylated at 80 °C for 30 min with 50 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane as catalyst. Conditions: column, DB1 J&W, 25 m × 0.2 mm × 0.33 μm; injection, 1 μL; split ratio, 2; oven temperature, 100 °C for 1 min, to 180 °C at 4 °C/min, then to 270 °C at 10 °C/min, and hold for 20 min; injector temperature, 280 °C; detector, EMV 1.35−1.5 kV; carrier gas, helium; flow rate, 1 mL/min.

Table 2. Rate of Degradation of Vitamin E (μg/g) in Scalicelle and Terzera Olive Oils during 1 Year of Storage at Room Temperature and Diffused Lighting

	0 month	2 months	4 months	6 months	8 months	10 months	12 months
Scalicelle[a]							
A	307.6 ± 15.5	310.2 ± 11.4	298.5 ± 18.7	285.3 ± 17.2	256.0 ± 26.3[c]	245.1 ± 33.1[d]	243.4 ± 37.8[d]
B	307.6 ± 15.5	309.2 ± 17.4	292.2 ± 20.1	280.3 ± 17.8	246.0 ± 35.1[c]	240.1 ± 37.1[d]	233.4 ± 34.8[d]
C	307.6 ± 15.5	270.4 ± 16.8[c]	190.5 ± 28.5[d]	128.2 ± 34.1[d]	72.8 ± 27.1[d]	43.6 ± 19.2[d]	26.3 ± 7.3[d]
D	307.6 ± 15.5	261 ± 28.2[c]	171.7 ± 40.2[d]	112.5 ± 31.8[d]	63.9 ± 16.1[d]	38.4 ± 9.5[d]	22.6 ± 5.2[d]
Terzera							
A	341.5 ± 18.1	336.9 ± 13.7	320.3 ± 14.8	308.7 ± 27.8	287.1 ± 33.2[c]	275.6 ± 34.2[d]	259.6 ± 25.8[d]
B	341.5 ± 18.1	340.0 ± 21,6	312.8 ± 25.3	304.1 ± 32.2	288.8 ± 35.2[b]	270.5 ± 31.8[d]	254.0 ± 27.2[d]
C	341.5 ± 18.1	286.2 ± 26.1[c]	201.7 ± 35.2[d]	141.4 ± 31.8[d]	80.4 ± 16.5[d]	49.6 ± 12.6[d]	31.2.0 ± 6.2[d]
D	341.5 ± 18.1	273.3 ± 34.1[c]	188.8 ± 29.2[d]	123.2 ± 24.7[d]	68.7 ± 15.3[d]	41.4 ± 13.6[d]	26.5 ± 7.6[d]

[a] A, filled dark glass bottle; B, filled clear glass bottle; C, half-empty dark glass bottle; D, half-empty clear glass bottle. Each result represents the mean ± SD of 10 determinations. [b] $p < 0.05$ vs $t = 0$. [c] $p < 0.01$ vs $t = 0$. [d] $p < 0.001$ vs $t = 0$.

Table 3. Rate of Degradation of Squalene (μg/g) in Scalicelle and Terzera Olive Oils during 1 Year of Storage at Room Temperature and Diffused Lighting

	0 month	2 months	4 months	6 months	8 months	10 months	12 months
Scalicelle[a]							
A	5.481 ± 0.16	5.326 ± 0.13	5.262 ± 0.22	5.199 ± 0.24	5.063 ± 0.38	4.938 ± 0.30[c]	4.633 ± 0.41[d]
B	5.481 ± 0.16	5.289 ± 0.18	5.144 ± 0.28	5.122 ± 0.20	4.984 ± 0.31[b]	4.798 ± 0.48[c]	4.423 ± 0.43[d]
C	5.481 ± 0.16	5.123 ± 0.21	4.745 ± 0.33[d]	3.212 ± 0.35[d]	1.841 ± 0.33[d]	0.987 ± 0.36[d]	0.324 ± 0.16[d]
D	5.481 ± 0.16	5.106 ± 0.23	4.405 ± 0.28[d]	3.011 ± 0.27[d]	1.583 ± 0.41[d]	0.807 ± 0.38[d]	0.210 ± 0.11[d]
Terzera							
A	5.970 ± 0.12	6.017 ± 0.24	5.854 ± 0.37	5.692 ± 0.37	5.537 ± 0.36	5.272 ± 0.42[c]	4.858 ± 0.43[d]
B	5.970 ± 0.12	5.763 ± 0.26	5.511 ± 0.22	5.533 ± 0.38	5.433 ± 0.44	4.892 ± 0.40[d]	4.546 ± 0.38[d]
C	5.970 ± 0.12	5.603 ± 0.32	4.821 ± 0.32[d]	3.042 ± 0.39[d]	1.727 ± 0.38[d]	0.842 ± 0.24[d]	0.331 ± 0.12[d]
D	5.970 ± 0.12	5.509 ± 0.36	4.698 ± 0.31[d]	2.894 ± 0.35[d]	1.609 ± 0.34[d]	0.756 ± 0.18[d]	0.206 ± 0.10[d]

[a] A, filled dark glass bottle; B, filled clear glass bottle; C, half-empty dark glass bottle; D, half-empty clear glass bottle. Each result represents the mean ± SD of 10 determinations. [b] $p < 0.05$ vs $t = 0$. [c] $p < 0.01$ vs $t = 0$. [d] $p < 0.001$ vs $t = 0$.

Linearity. For all HPLC−DAD and GC−MS analysis, the linearity range of responses was determined on five concentration levels; each analysis was repeated three times. Calibration graphs were recorded with sample amounts ranging from 0.10 to 2 μg ($r > 0.999$).

Statistical Analysis. Statistical analyses were performed by the Mann−Whitney U test. Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

Both Scalicelle and Terzera Calabrian (Italy) olive oil samples, named, were selected for the stability study on the basis of their quality characteristics. Evaluation of the quality of olive oil samples was based on acidity, peroxide value, fatty acid, phenol, and sterol composition, and squalene and α-tocopherol content. Data are given in **Table 1**. The parameters ensured that the two virgin olive oils were of very good quality.

Vitamin E (D-*RRR*-α-Tocopherol). The initial levels of vitamin E in Scalicelle and Terzera oils are 307 and 341 μg/g, respectively. This means that 10−20 mL/day of each oil is sufficient to meet the daily nutritional needs of healthy persons (Recommended Dietary Allowances) for vitamin E, which are 3−4 mg/day in the U.K. and 8−10 mg/day in the USA (*20*). Vitamin E represents the first target of EOO autoxidation (**Table 2**) of oil stored in half-empty clear bottles (Scalicelle, −92.7%, and Terzera −92.3%, after 12 months of storage at room temperature and diffused lighting). In the filled colorless bottles, the reduction of vitamin E was largely lower: −24.2% and −25.6%, respectively. In the dark half-empty and filled bottles the degradation of vitamin E was −91.5% and −90.9%, and −20.8% and −24.0%, respectively, for Scalicelle and Terzera samples, suggesting that, in our experimental conditions, the contribution of the photooxidation process to the vitamin E decomposition is negligible.

Ubiquinones and β-Carotene. The levels of CoQ_9 or CoQ_{10} and β-carotene in Scalicelle and Terzera olive oils were undetectable.

Squalene. The initial levels of squalene in Scalicelle and Terzera oils were 5.970 ± 0.12 and 5.481 ± 0.16 μg/g, respectively (**Table 3**). During 12 months of storage the rate of the squalene degradation, under the same storage conditions, follows that of vitamin E, from which it is protected, at least in the first months of the study (**Tables 2** and **3**). Its content decreased significantly only after 6 months in half-empty bottles (Scalicelle, −45.0%; Terzera −51.5%). Diffused lighting does not appear to play a significant role in the squalene degradation (Scalicelle, −19.3%, and Terzera, −23.8%, after 12 months of storage in the filled colorless bottles at diffused lighting).

Phenols. The composition of the phenolic fraction of EOO is very complex because of several factors involved in its biosynthesis within the drupe such as the cultivar of the olive, the geographical origin, the climate, and the degree of maturation (*21*). In addition, the crushing and malaxation processes are capable of activating endogenous β-glycosidases with consequent hydrolysis of flavonoids and other phenolic glycosides such as oleuropein, which is responsible of the bitter taste of the oils (*4*). The levels of mono- and diphenols reported in **Table 1** are lower than those reported in the literature and determined by colorimetric methods. It is significant to underline that they concerned exclusively phenolic molecules, the identity of which has been confirmed by HPLC−MS techniques (data not shown), whereas unknown peaks on the chromatograms have not been taken in consideration. Among various *o*-diphenols we have analyzed hydroxytyrosol, oleuropein aglycon, luteolin, and apigenin. The rate of their degradation, under the same conditions of oil storage, follows that of squalene, and like squalene,

Table 4. Rate of Degradation of *o*-Diphenols (μg/g) in Scalicelle and Terzera Olive Oils during 1 Year of Storage at Room Temperature and Diffused Lighting

	0 month	2 months	4 months	6 months	8 months	10 months	12 months
Scalicelle[a]							
A	6.46 ± 0.23	6.31 ± 0.16	6.22 ± 0.19	6.18 ± 0.28	6.03 ± 0.36	5.82 ± 0.38[c]	5.22 ± 0.35[d]
B	6.46 ± 0.23	6.32 ± 0.29	6.25 ± 0.24	6.11 ± 0.32	5.99 ± 0.42	5.80 ± 0.36[c]	5.14 ± 0.39[d]
C	6.46 ± 0.23	5.81 ± 0.27[c]	5.11 ± 0.35[d]	4.45 ± 0.35[d]	3.71 ± 0.26[d]	3.06 ± 0.39[d]	2.22 ± 0.28[d]
D	6.46 ± 0.23	5.57 ± 0.26[d]	4.97 ± 0.28[d]	4.19 ± 0.29[d]	3.66 ± 0.41[d]	2.98 ± 0.36[d]	2.19 ± 0.32[d]
Terzera							
A	11.01 ± 0.19	11.06 ± 0.17	10.86 ± 0.33	10.73 ± 0.29	10.69 ± 0.29	10.52 ± 0.30[c]	9.83 ± 0.42[d]
B	11.01 ± 0.19	10.96 ± 0.22	10.88 ± 0.31	10.66 ± 0.18	10.53 ± 0.36	10.47 ± 0.31[c]	9.71 ± 0.38[d]
C	11.01 ± 0.19	10.64 ± 0.28[b]	8.11 ± 0.44[d]	6.96 ± 0.36[d]	5.74 ± 0.45[d]	4.67 ± 0.47[d]	3.85 ± 0.52[d]
D	11.01 ± 0.19	10.52 ± 0.29[c]	7.76 ± 0.32[d]	6.78 ± 0.41[d]	5.49 ± 0.36[d]	4.59 ± 0.41[d]	3.66 ± 0.41[d]

[a] A, filled dark glass bottle; B, filled clear glass bottle; C, half-empty dark glass bottle; D, half-empty clear glass bottle. Each result represents the mean ± SD of 10 determinations. [b] $p < 0.05$ vs $t = 0$. [c] $p < 0.01$ vs $t = 0$. [d] $p < 0.001$ vs $t = 0$.

Table 5. Rate of Degradation of Polyunsaturated Fatty Acids, C18:2 n-6 + C18:3 n-3 (%), in Scalicelle and Terzera Olive Oils during 1 Year of Storage at Room Temperature and Diffused Lighting

	0 month	2 months	4 months	6 months	8 months	10 months	12 months
Scalicelle[a]							
A	9.15 ± 0.33	9.25 ± 0.29	9.10 ± 37.7	9.03 ± 0.35	9.09 ± 0.38	8.98 ± 0.41	8.76 ± 0.44
B	9.15 ± 0.33	9.05 ± 0.42	8.95 ± 0.46	9.02 ± 0.43	8.89 ± 0.31	8.82 ± 0.27	8.64 ± 0.51
C	9.15 ± 0.33	9.02 ± 0.26	8.90 ± 0.42	8.76 ± 0.47	8.61 ± 0.47	7.32 ± 0.51[c]	6.63 ± 0.47[c]
D	9.15 ± 0.33	8.87 ± 0.36	8.71 ± 0.39	8.80 ± 0.52	8.41 ± 0.52	7.17 ± 0.56[c]	6.40 ± 0.50[c]
Terzera							
A	8.40 ± 0.31	8.45 ± 0.17	8.38 ± 0.29	8.30 ± 0.37	8.32 ± 0.45	8.22 ± 0.34	8.08 ± 0.29
B	8.40 ± 0.31	8.34 ± 0.25	8.41 ± 0.37	8.23 ± 0.42	8.12 ± 0.52	8.02 ± 0.38	7.80 ± 0.41
C	8.40 ± 0.31	8.10 ± 0.42	8.03 ± 0.41	7.94 ± 0.53	7.77 ± 0.54	7.62 ± 0.55	7.27 ± 0.46[c]
D	8.40 ± 0.31	8.15 ± 0.31	7.97 ± 0.45	8.01 ± 0.47	7.81 ± 0.49	7.50 ± 0.53[b]	7.06 ± 0.59[c]

[a] A, filled dark glass bottle; B, filled clear glass bottle; C, half-empty dark glass bottle; D, half-empty clear glass bottle. Each result represents the mean ± SD of 10 determinations. [b] $p < 0.05$ vs $t = 0$. [c] $p < 0.001$ vs $t = 0$.

Table 6. Increase of Peroxide Number (mequiv of O_2/kg) in Scalicelle and Terzera Olive Oils during 1 Year of Storage at Room Temperature and Diffused Lighting

	0 month	2 months	4 months	6 months	8 months	10 months	12 months
Scalicelle[a]							
A	2.2 ± 0.3	2.4 ± 0.3	2.5 ± 0.3	2.8 ± 0.5	2.7 ± 0.4	3.1 ± 0.5[c]	3.4 ± 0.4[d]
B	2.2 ± 0.3	2.5 ± 0.4	2.7 ± 0.5	2.7 ± 0.5	3.0 ± 0.4[b]	3.5 ± 0.6[d]	4.6 ± 0.6[d,e]
C	2.2 ± 0.3	2.8 ± 0.5	4.4 ± 0.5[d]	7.0 ± 0.6[d]	12.2 ± 0.9[d]	15.3 ± 1.5[d]	20.5 ± 1.6[d]
D	2.2 ± 0.3	2.9 ± 0.4[b]	4.9 ± 0.5[d]	8.1 ± 1.2[d]	15.3 ± 1.5[d,e]	19.9 ± 1.4[d,e]	24.6 ± 1.2[d,e]
Terzera							
A	3.6 ± 0.3	3.6 ± 0.5	3.9 ± 0.4	4.0 ± 0.4	4.3 ± 0.6	4.5 ± 0.6[b]	4.9 ± 0.5[d]
B	3.6 ± 0.3	3.8 ± 0.4	4.0 ± 0.5	4.2 ± 0.5	4.4 ± 0.6[b]	5.1 ± 0.5[d]	7.2 ± 0.7[d,e]
C	3.6 ± 0.3	4.1 ± 0.5	6.4 ± 0.9[d]	9.5 ± 1.1[d]	14.6 ± 1.4[d]	19.6 ± 2.1[d]	23.2 ± 1.5[d]
D	3.6 ± 0.3	4.3 ± 0.3	7.0 ± 0.8[d]	10.7 ± 1.2[d]	19.8 ± 1.3[d,e]	23.8 ± 1.9[d,e]	28.8 ± 1.9[d,e]

[a] A, filled dark glass bottle; B, filled colorless glass bottle; C, half-empty dark glass bottle; D, half-empty colorless glass bottle. Each result represents the mean ± SD of 10 determinations. [b] $p < 0.05$ vs $t = 0$. [c] $p < 0.01$ vs $t = 0$. [d] $p < 0.001$ vs $t = 0$. [e] $p < 0.001$ vs respective dark bottles.

vitamin E is capable of protecting *o*-diphenols at least in the first months of the storage (**Tables 2** and **4**). Monophenols are quite stable to autoxidation, their concentration not being significantly affected by 12 months of autoxidation (data not shown).

Sterols. The levels of sterols in the sample of Scalicelle and Terzera EOO are shown in **Table 1**. Like monophenols, sterols are quite stable to autoxidation, being not significantly affected by 12 months of autoxidation (data not shown).

Polyunsaturated Fatty Acids and Lipoperoxidation. The pattern and the concentration (percent) of fatty acids in the trygliceride fractions of Terzera and Scalicelle oils at the beginning of the study are shown in **Table 1**. When isolated, linoleic and linolenic acids easily undergo peroxidation, while in EOO they are rather stable, since they are protected by vitamin E, phenols, and squalene. Our results showed that polyunsaturated fatty acids remained almost constant during 8 months for all four different storage conditions (**Table 5**); their oxidation starts mainly in half-empty bottles when the above antioxidants have disappeared almost totally. Despite the stability of these PUFA, the peroxide number of EOO increases significantly (**Table 6**), mainly in the half-empty bottles, indicating that the oil peroxidation is sustained, in the first 8 months of storage, essentially by oxidant byproducts deriving from phenol and squalene degradation. Moreover, after 8 months of storage, photooxidation induced by diffused lighting appears to play a significant role in increasing the peroxide number of oils in half-empty colorless bottles as compared to the black ones (**Table 6**). This phenomenon can be caused by two different oxidation reactions, autoxidation and photooxidation, which have different speeds or initiation times. It is well-known that the oxidation of unsaturated lipids is accelerated by exposure to light especially when photosensitizers, as chlorophylls, are present in the systems, which causes formation of both singlet

oxygen and/or superoxide anion radicals (*22*). Tocopherol, phenols, and squalene may act as a protection of oil from the photooxidation, at least in the first 8 months of storage (*23*).

DISCUSSION

The paper focuses on the olive oil quality change during storage, monitoring the oxidation progress by the antioxidant content decrease. Under our experimental conditions, the oxidation of olive oil has as a main target vitamin E, *o*-diphenols, and squalene, which undergo an impressive degradation, especially in the half-empty bottles in comparison with the filled ones (**Tables 2**−**5**). Photooxidation induced by diffused lighting does not appear to be involved in the antioxidant and PUFA degradation, which is caused essentially by oxygen. Only in the final phases of the storage, when the level of antioxidants is reduced, does photooxidation appear to play an important role, by increasing the oil peroxide number in half-empty colorless bottles as compared to the black ones (**Table 6**).

In comparison to in vivo and in vitro biological systems subjected to oxidative stress, the various antioxidants do not seem to operate in independent way, but they actively cooperate, on the basis of their reduction potentials, to prevent oxidative damages to PUFA in olive oil.

Vitamin E, the most important and functional antioxidant of olive oil, is the first one to be oxidized, becoming partly prooxidant and able to oxidize other antioxidant molecules from the unsaponifiable fraction, such as *o*-diphenols and squalene, with formation of oxidant reactive byproducts. A complex radical chain reaction seems to be generated that, at the end, with the massive reduction of the antioxidant concentration, essentially concerns the PUFAs. During such oxidative process innumerably more or less toxic and mutagen products are generated (*24*), some of which are able to give an extremely unpleasant smell and taste to the oils, even at very small concentrations.

Our results suggest that it is possible to realize a good stability for just produced EOO and, therefore, a suitable way to inhibit the rancidity process by storing oil in dark or black bottles or containers, well closed and filled almost completely to ensure the lowest headspace. The colored bottles are necessary to minimize the photooxidative damage, while the reduced headspace is essential to exclude oxygen. One can suggest that the stability might be improved by filling the headspace up with an inert gas, such as argon or nitrogen.

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