JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stability of Virgin Olive Oil. 2. Photo-oxidation Studies

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Virgin olive oil samples with similar oxidative stabilities and fatty acid compositions were exposed to 12100 lx (25 ± 1 °C) in closed bottles until bleached. The observed low changes in the substrate and polar phenols were related to oxygen availability. HPLC monitoring showed that pheophytin *a* gradual degradation (>90%) was accompanied by a considerable α-tocopherol loss (22−35%) due to the reaction of the latter with singlet oxygen. No changes were recorded for carotenoids, which acted as physical quenchers and light filters. Squalene loss was confined (4−12%). Complementary experiments on the activity of pheophytin *a*, using olive oil models, indicated a concentration dependence, enhanced by oxygen availability. In closed bottles, the degradation rate constant was higher at low amounts of pheophytin *a*. Squalene was preferentially consumed to protect α-tocopherol. An urgent change in the practice of packaging is needed to preserve the precious characteristics of the product during commercialization.

KEYWORDS: Virgin olive oil photo-oxidation; polar phenols; α-tocopherol; squalene; *β*-carotene; lutein; pheophytin *a*

INTRODUCTION

Precautions for the storage of virgin olive oil (VOO) in the dark do not necessarily ensure the stability of the oil under conditions promoting photo-oxidation. Thus, the VOO photo-oxidation rate is not expected to depend on the high level of oleic acid to the extent observed in autoxidation (*1*) but on the presence of photosensitizers and singlet oxygen quenchers. On the other hand, polar phenols, which act as chain-breaking antioxidants, play a rather limited role during exposure to light (*2*).

Among all of the vegetable oils, VOO is consumed containing high amounts of chlorophyll pigments (*3*). Therefore, it is notably vulnerable to light exposure on the shelf, which leads to inferior quality products. Chlorophyll pigments act as photosensitizers due to the ability to transfer energy from light to triplet oxygen, producing thus singlet oxygen, which then reacts with the unsaturated fatty acids. Endo et al. (*4*), who examined the pro-oxidative effect of some chlorophyll derivatives, found that pheophytins had a higher photosensitizing effect than chlorophylls but lower than pheophorbides. The activity of pheophytin *a* (Pheo *a*), the major olive oil pigment (*3*), is reported to be lower than that of Pheo *b*. The photosensitizing effect of Pheo *a* according to certain researchers (*5*) is not entirely due to singlet oxygen formation, but a hydrogen abstraction from the lipid substrate by the activated carbonyl group of the isocyclic ring may also occur.

Studies on photo-oxidation of VOO are rather limited (*6*−*8*). Some investigation on the role of chlorophyll pigments at levels lower than 10 mg/kg using olive oil models has been also carried out under various artificial light exposure conditions (*9*−*11*). In most cases changes in the lipid substrate were measured to follow the effect of photosensitizers. Changes in the content of minor components, in particular of those participating in autoxidation or of nutritional importance, such as carotenoids, α-tocopherol, or squalene, are not examined in depth.

Carotenoids are effective inhibitors of photo-oxidation by quenching singlet oxygen and triplet excited states of photosensitizers. The physical quenching mechanism of carotenoids is based on their low singlet energy state, which facilitates the acceptance of energy from singlet oxygen (*12*). Additionally, the antioxidant activity of carotenoids is related to a light-filtering effect due to the extended conjugation system (*10*). *β*-Carotene is the most studied carotenoid. Not only antioxidant but also pro-oxidant effects of *β*-carotene have been reported for purified olive oil models containing high or low levels of it, stored at various light exposure conditions. A pro-oxidant activity has also been reported for *β*-carotene during photosensitized oxidation of soybean or rapeseed oil, which was reversed when tocopherols were present (*12*, *13*). The synergistic effect of the combination of *β*-carotene and α-tocopherol was attributed to the protection of the former from autoxidation by α-tocopherol. In the above-mentioned cases, the oxidation took place in opened vessels.

α-Tocopherol efficiently quenches singlet oxygen by a charge transfer mechanism (*14*). A rapid α-tocopherol degradation during light exposure of olive oil models (*9*, *11*) or VOO (*8*, *15*, *16*) has been reported. Rahmani and Saad (*11*) also reported

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10.1021/jf010847u CCC: $22.00
Published on Web 01/18/2002

a concentration-dependent activity restricted to the initial stages of oxidation as a result of the kinetics of degradation.

To our knowledge the effect of squalene during the photo-oxidation of VOO or any possible interaction with the above-mentioned compounds has not been examined yet. Squalene, which acts as a quencher of singlet oxygen (*17*), plays a vital role in skin lipid protection so that its participation in the photo-oxidation mechanism of other lipids cannot be a priori excluded.

The present study was undertaken to examine the resistance of VOO to photo-oxidation with regard to the presence of certain minor components. In the experiments the same samples used in autoxidation studies (*18*) were exposed to light (12100 lx, 25 ± 1 °C). Changes in the lipid substrate and the evolution of Pheo *a*, *β*-carotene, lutein, α-tocopherol, and squalene as well as of polar phenols were followed to obtain a better understanding of oil stability. Complementary experiments on olive oil stripped of antioxidants and pro-oxidants were carried out to elucidate the contribution of squalene and Pheo *a* in photo-oxidation. Interactions that possibly take place among components were also examined.

MATERIALS AND METHODS

Samples. Extra virgin olive oil samples and refined olive oil were kindly donated by ELAIS SA (Piraeus, Greece). Olive oil stripped of antioxidants and pro-oxidants was prepared from refined oil as previously described (*19*).

Solvents and standards. The solvents, of HPLC grade, were used without further purification. *n*-Hexane was a Baker Analyzed HPLC 95% reagent (Deventer, Holland); 2-propanol, acetone and acetonitrile (Chromasolv) and methanol (pro-analysis) were from Riedel de-Häen (Seelze, Germany); and diethyl ether was from Labscan, Analytical Science (Dublin, Ireland). *dl*-α-Tocopherol (99% for biochemistry) (α-T), a set of four tocopherol species (α-, *β*-, *γ*-, and *δ*-T, for biochemistry), and *β*-carotene (for biochemistry) were purchased from Merck (Darmstadt, Germany). Chlorophylls *a* and *b* (Chl *a* and *b*), caffeic acid, and squalene were from Sigma Chemical Co. (St. Louis, MO). Pheophytins *a* and *b* were prepared from the respective chlorophylls by acidification (HCl, 1 N). Folin−Ciocalteu reagent was from Merck.

Apparatus. The solvent delivery system consisted of two Marathon IV series HPLC pumps (Rigas Labs, Thessaloniki, Greece) and a Rheodyne injection valve (model 7125) with a 20 μL fixed loop (Rheodyne, Cotati, CA). The liquid chromatograph was equipped with a UV−vis spectrophotometric detector SPD-10AV (dual wavelength) from Shimadzu (Kyoto, Japan) connected in series with a diode array linear UVIS-206 multiple wavelength system (Linear Instruments, Fermont, CA). Computer software, Linear UV−vis-206 (Linear Instruments), was used to obtain and store absorption spectral chromatographic data. The data from the UV−vis SPD-10AV detector were stored and processed with the chromatographic software EZChrom (Scientific Software, Inc., San Ramon, CA). Absorbance measurements were recorded by a Hitachi, U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) in 1 cm quartz cells. Oxidative stability was evaluated using a Rancimat 617 apparatus (Metrohm, Herisau, Switzerland). Photo-oxidation experiments were performed in a light chamber (GRW-500D, Chrisagis, Athens, Greece) (615 × 600 × 1330 mm) equipped with 12 fluorescence tubes (6 on each side) (Sylvania Luxline, F36W/840 (184), coolwhite deluxe).

HPLC Analysis of Tocopherols and Pigments of VOO. A gradient elution was used with *n*-hexane/2-propanol (99:1 v/v) (A) and 2-propanol (B) as eluents. The gradient was as follows: 0% B for 10 min; 0−5% B in 4 min; 5% B for 6 min; 5−0% B in 4 min; 0% B for 6 min. Separation was achieved on a 250 × 4 mm i.d., LiChrospher-Si, 5 μm column (MZ Analysentechnik, Mainz, Germany) at a 1.2 mL/min flow rate. The injection volume was 20 μL. Detection of tocopherols was performed using a UV detector at 294 nm, whereas pigments were monitored using a diode array detector. Detailed information for the identification and quantification of individual compounds is given in a previous paper (*16*).

HPLC Determination of Squalene. Squalene was separated from the triacylglycerols using fractional crystallization. The separation was performed on a Nucleosil C_{18}, 250 × 4 mm, 5 μm column (Macherey-Nagel, Düren, Germany), at 30 °C and a flow rate of 1 mL/min. The elution solvent was acetone/acetonitrile (40:60 v/v). Squalene was detected at 208 nm, and the chromatograms were integrated using an HP series 3665 integrator (attenuation, AT × 10; and paper rate, 0.5 cm/min) (*20*).

Colorimetric Determination of Total Phenol Content. Total phenols were measured in the polar fraction extracted from 2.5 g of oil using methanol/water (60:40 v/v), and the determination was based on the Folin−Ciocalteu method (*21*).

Spectrometric Estimation of Chlorophyll Content. The content of chlorophyll pigments was estimated from the equation C (mg of Pheo *a*/kg of oil) = $345.3[A_{670} - (A_{630} + A_{710})/2]/L$, where A_λ = the absorbance of the oil at the respective wavelength and L = cell thickness (mm) (*16*).

Other Quality Characteristics of Olive Oil Samples. Acidity, peroxide value (PV), and absorbance at 232 and 270 nm (K_{232} and K_{270}) were measured according to official methods (*22*). Oxidative stability indices (OSI) were determined by the Rancimat test (120 °C, 20 L/h).

Photo-oxidation Studies. Oil samples (10 mL) were poured into 15 mL transparent glass bottles (7% headspace). The closed bottles were placed in the center of a metallic shelf in the light chamber. The distance of the samples from the fluorescence lamps was 30 cm, and between the samples adequate space was left to ensure equalized exposure. The temperature of the chamber was kept at 25 ± 1 °C, and the light intensity at sample level was 12100 lx as measured by a pyranometer (model CM21, Kipp and Zonene, Delft, Holland). Photo-oxidation was followed by measuring chlorophyll and Pheo *a* contents. Photo-oxidation experiments were also carried out for olive oil stripped of antioxidants and pro-oxidants, which was used as a model system. The samples were prepared in conical flasks to which the antioxidants/pro-oxidants were added dissolved in an appropriate solvent. The solvent was then evaporated by nitrogen flushing and magnetic agitation of the sample in the dark, at room temperature.

Squalene (200 and 7000 mg/kg) and α-tocopherol (100 mg/kg) were added in the lipid substrate dissolved in isooctane and ethanol, respectively, and Chl *a* (5 mg/kg) dissolved in diethyl ether was used as a photosensitizer. A series of opened transparent glass bottles, containing 2 g of oil each, were then exposed to 12100 lx at 25 ± 1 °C. Samples were periodically withdrawn for PV and HPLC analysis.

Pheo *a*, dissolved in diethyl ether, was added at three levels (5, 20, and 40 mg/kg) in opened and closed transparent glass bottles. The filled bottles were exposed to light until their Pheo *a* content destruction was >90%. Samples were periodically withdrawn for PV and HPLC analysis.

RESULTS AND DISCUSSION

The quality characteristics of the four VOO samples and the initial levels of photosensitizers and singlet oxygen quenchers examined in this study are given in **Table 1**. Discussion of the characteristics of the samples is presented in a previous paper (*18*). In this paper emphasis is given to those factors participating in photosensitized oxidation. The four samples differed slightly in their content in singlet oxygen quenchers. More characteristic were the differences in the chlorophyll content, which is the important factor in the photo-oxidation of VOO. Therefore, the behavior of the oil samples during exposure to light is expected to relate to their chlorophyll content. The samples were exposed to fluorescent light, which is reported to comprise visible light (∼2%), fluorescence (∼18%), heat radiation (∼26%), and heat by conduction (∼54%) (*23*). The influence of heat was controlled in the chamber (25 ± 1 °C). A similar light intensity has been used in other photo-oxidation studies (*13*). The samples were kept in the chamber until the destruction of Pheo *a* was

Table 1. Physicochemical Characteristics and Levels of Photosensitizers and Singlet Oxygen Quenchers of VOO Samples

quality characteristic	sample 1	2	3	4
acidity, % oleic acid	0.39	0.41	0.28	0.54
PV, mequiv of O_2/kg	6.4	10.4	12.9	8.7
K_{232}	1.56	1.88	2.11	1.63
C18:1, % peak area	75.5	75.8	76.7	77.0
C18:2, % peak area	9.2	8.2	6.7	5.8
OSI, h (120 °C, 20 L/h)	6.7	6.4	6.5	6.5
total polar phenol content, mg of caffeic acid/kg	98	209	130	120
α-tocopherol, mg/kg	210	169	182	202
squalene, mg/kg	5801	3882	4571	5858
β-carotene, mg/kg	1.5	1.0	1.4	2.7
lutein, mg/kg	2.3	0.9	1.1	1.2
chlorophyll content, mg of Pheo *a*/kg	15.1	9.1	19.2	23.0
Pheo *a*, mg/kg	9.3	4.1	10.0	15.1

>90% of the initial level. As a result, the duration of exposure was different for each sample. Oxidation of the lipid substrate was followed by PV and K_{232} measurements. Changes in the content of α-tocopherol and pigments were followed by HPLC, whereas total polar phenol content changes were measured colorimetrically. Squalene was determined by reversed-phase HPLC in the end of the exposure period.

Changes in the Lipid Substrate and the Total Polar Phenol Content. In all of the samples PV increased during the first hours of exposure and then remained constant for the rest of the exposure period (**Figure 1**). No increase (or even decrease) in PV during light exposure has been related to peroxide decomposition (*24*). Our results support the assumption that the decisive factor should be oxygen availability. In this view the continuous increase in PV during photo-oxidation of a VOO sample at 2 °C and 5340 lx reported by Rahmani and Csallany (*8*) was possibly the result of the enhanced oxygen availability in the opened Petri dishes rather than the result of photosensitized oxidation. Sample 2, which showed the smallest overall increase in PV, had also the lowest chlorophyll content. This is in agreement with the observation made by Rahmani and Saad (*11*) that between samples of identical degree of unsaturation and similar α-tocopherol contents, the sample with a lower chlorophyll content had a greater stability. The change in K_{232} values was almost insignificant in all samples as expected (*2*), and it is not further discussed.

As seen in **Figure 1** total polar phenol loss is rather limited and occurs during the first hours of photo-oxidation. Sample 2 was an exception (almost 50% reduction). The same sample had also a high total polar phenol loss during storage in the dark accompanied by rather small changes in the lipid substrate (*18*). This finding is in agreement with the limited role of chain-breaking antioxidants expected in photosensitized oxidation. High polar phenol loss reported in the literature should be ascribed to the overall experimental conditions, for example, oxygen availability (*8*).

Changes in the Content of Photosensitizers. Total chlorophylls were gradually lost during exposure, and the same was observed for Pheo *a*, as illustrated in the chromatographic profiles of **Figure 2** for sample 4. Pheo *a* destruction was faster in the case of sample 2, which contained the lowest chlorophyll pigment content. Pheo *a* level is much higher in VOO than that found in refined vegetable oils, so that it is interesting to investigate its effect to the pro-oxidant activity. Studies on the role of Pheo *a* on photo-oxidation of olive oil triacylglycerols reported in the literature are concerned with very low levels of Pheo *a*, much lower than that naturally occurring in VOO (*9*−*11*). For this reason the effect of Pheo *a* at three levels of addition on the stability of olive oil stripped of antioxidants and pro-oxidants was examined. Samples with and without Pheo

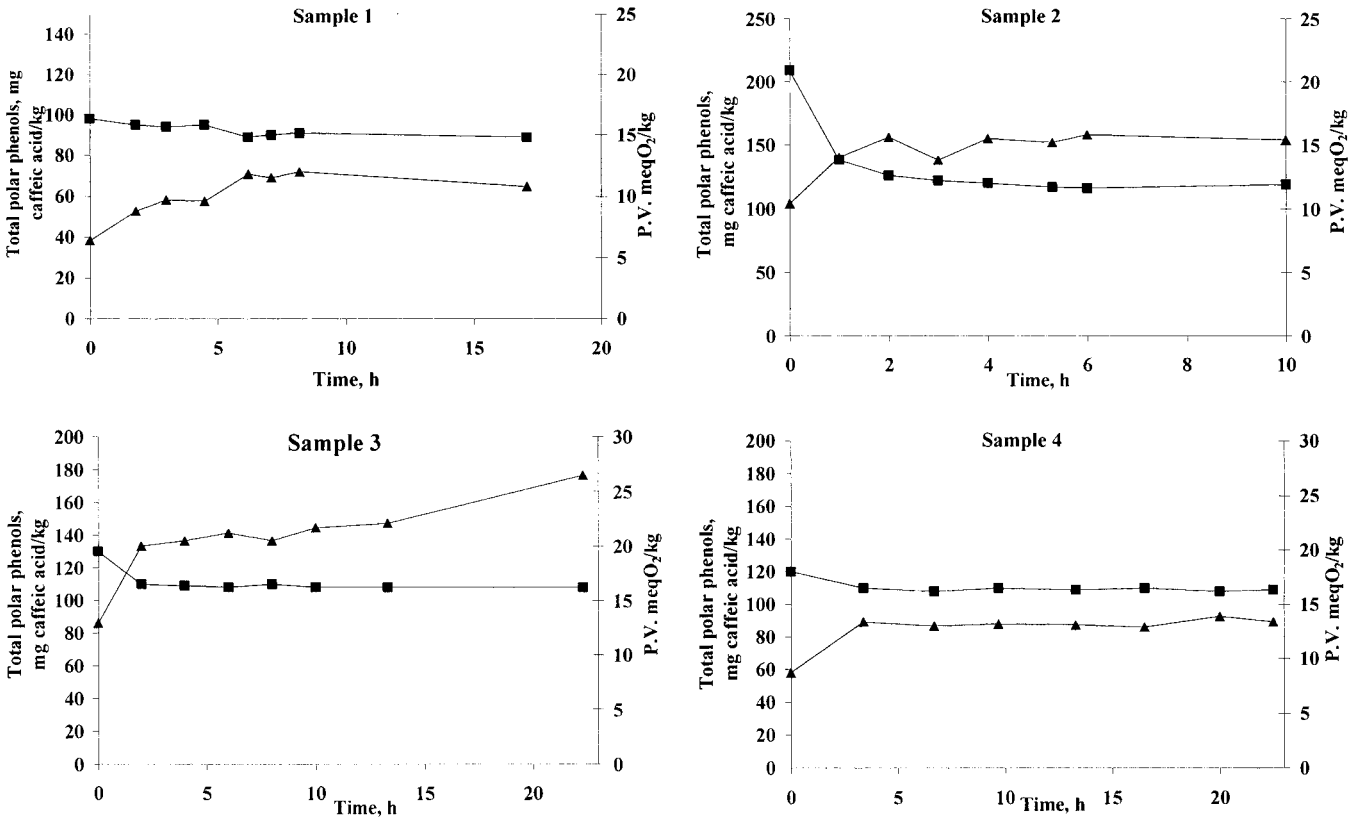

Figure 1. Changes in the total polar phenol content (■) and PV (▲) during exposure of virgin olive oil samples to 12100 lx at 25 ± 1 °C.

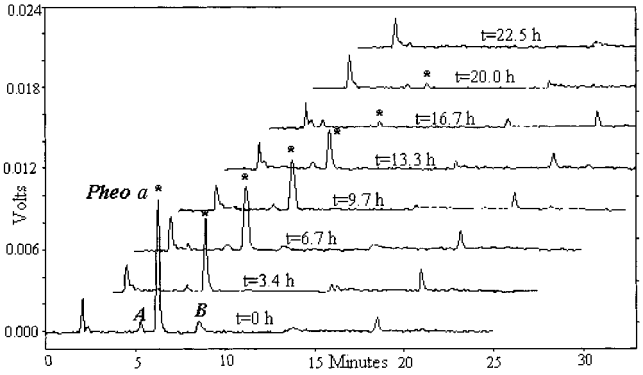

Figure 2. Changes in the chromatographic profile at 410 nm of virgin olive oil sample 4 exposed to 12100 lx at 25 ± 1 °C. Peaks A and B are pheophytin *a* derivatives; *t* = exposure time (h).

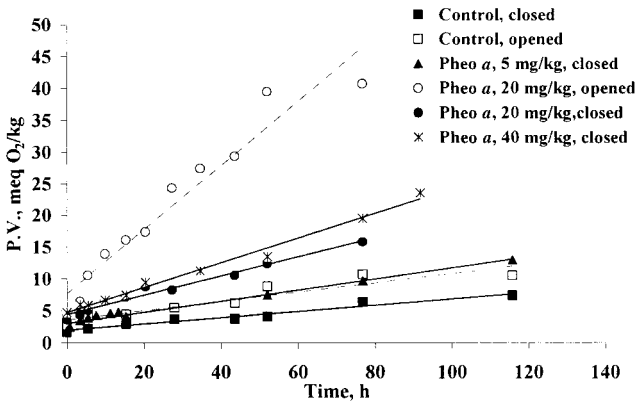

Figure 3. Effect of Pheo *a* at various levels of addition on the photo-oxidation of olive oil stripped of antioxidants and pro-oxidants.

Table 2. Rate Constants of Pheo *a* Photodegradation for Various Levels of Addition at 25 ± 1 °C

Pheo *a*, mg/kg	$k \times 10^2$ (h^{-1})	Pheo *a*, mg/kg	$k \times 10^2$ (h^{-1})
5, closed	11.9	20, closed	5.5
20, opened	5.6	40, closed	3.2

a were exposed in closed transparent glass bottles kept in the light chamber at 25 ± 1 °C. In the case of the addition of 20 mg/kg another series of bottles were kept opened. From the results shown in **Figure 3** it is obvious that the pro-oxidant activity depends on the level of the photosensitizer. The increase in the conjugated diene concentration was minimal due to the mechanism of photo-oxidation, which mainly favors the formation of monounsaturated hydroperoxides (*11*). PV changes were higher in the case of samples exposed in opened bottles. After 80 h of light exposure, the PV in the sample containing 20 mg of Pheo *a*/kg of oil was higher than that of the sample with 40 mg/kg in closed bottles. It seems that oxygen availability accentuates the effect of the level of addition of Pheo *a*. The concentration of Pheo *a* was gradually decreased during exposure, and no new peaks were observed in the chromatographic profile at 410 nm.

Pheo *a* photodegradation has been described as a first-order reaction (*25*). Rate constants given in **Table 2** were calculated from the relevant graphs of ln[Pheo *a*] versus time. The rate of Pheo *a* degradation was dependent on the level of addition. The rate constants were higher at the lowest level of the pigment addition. This fact may be attributed to the higher amount of oxygen in relation to pigment concentration as suggested for the behavior of other pigments (*26*, *27*). However, in the case

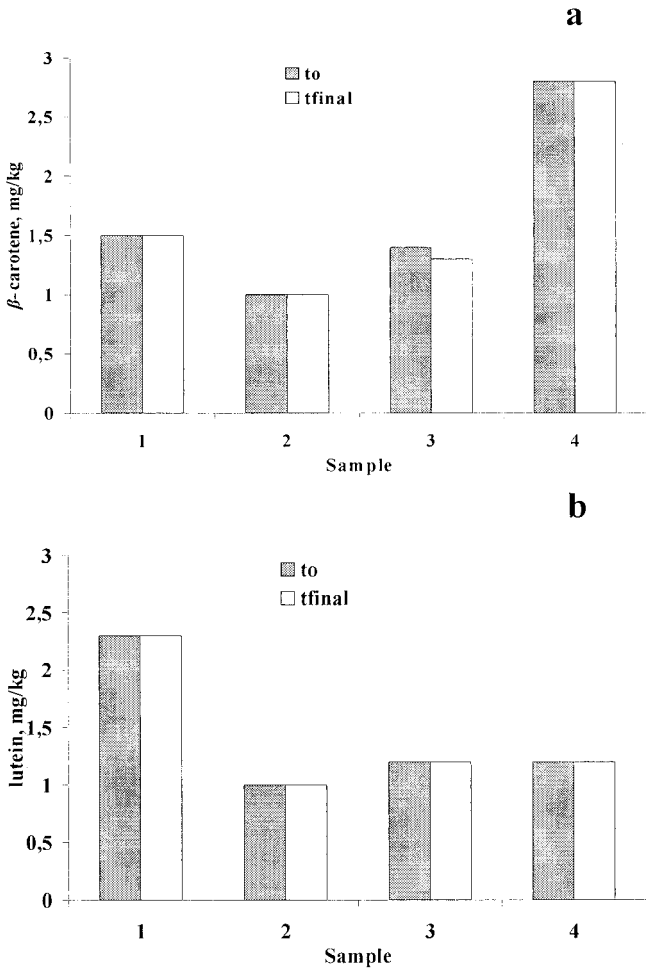

Figure 4. Changes in the carotenoid content of virgin olive oil samples exposed to 12100 lx at 25 ± 1 °C; β-carotene (a), lutein (b); t_{final} = end of exposure time. Sample 1, 17.1 h; sample 2, 10 h; sample 3, 22.3 h; sample 4, 22.5 h.

of the study of the photodegradation of Pheo *a* in the opened and closed bottles, no difference in the rate constants was observed. Although the PVs of the substrate were higher in the opened bottles, it could be argued that no further bleaching of Pheo *a* was taking place due to its co-oxidation with the oxidation products (*24*).

Two other series of samples containing 20 mg/kg Pheo *a* were set at 15 and 35 °C ($k = 4.1 \times 10^2$ and 10.5×10^2 h^{-1}, respectively). From the graph of the logarithm of the calculated rate constants versus reciprocal temperature a crude estimation of the size of activation energy was attempted. The apparent activation energy was found to be ~8.0 kcal/mol, which was near the range of activation energies of food constituents (*28*).

Changes in the Content of the Singlet Oxygen Quenchers. β-Carotene and lutein contents remained almost unchanged in the course of the photo-oxidation experiments (**Figure 4**). This supports the physical quenching mechanism through which they act as quenchers. More changes were found in the tocopherol content (**Figure 5**). Indeed, α-tocopherol loss was higher than that observed for the total polar phenol content except for sample 2. α-Tocopherol loss was gradual and increased with exposure time in contrast with total phenol content loss. The extent of reduction ranged from 20 to 35% and depended on the exposure period. The gradual destruction of α-tocopherol supports its active participation in photosensitized oxidation in contrast to polar phenols.

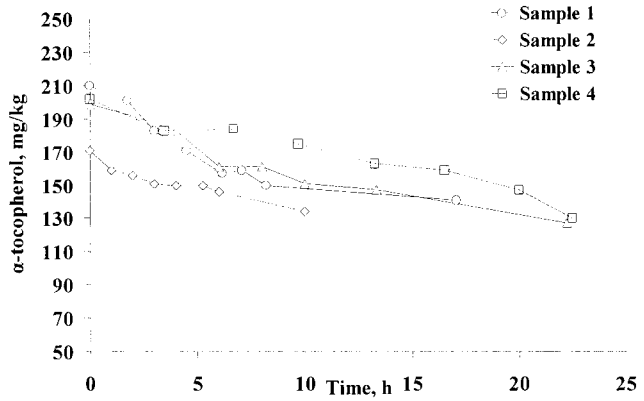

Figure 5. Changes in the α-tocopherol content during exposure of virgin olive oil samples to 12100 lx at 25 ± 1 °C.

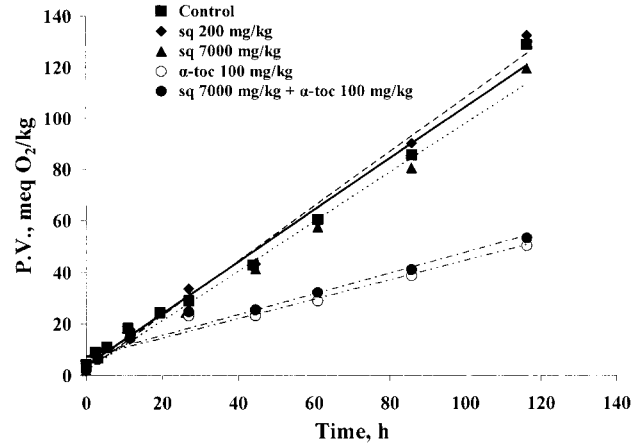

Figure 6. Effect of squalene, α-tocopherol, and their combination on the photo-oxidation of olive oil stripped of antioxidants and pro-oxidants.

Squalene has been reported to act as a singlet oxygen quencher, so an antioxidant activity during the photo-oxidation of VOO could be expected (*17*). A small squalene loss (4−12%) was observed in all samples after the exposure period. To examine further its role, additional experiments were carried out using the same model system as in the case of Pheo *a*. The activity was studied for two levels of addition (200 and 7000 mg/kg) and in the presence of α-tocopherol (100 mg/kg) for comparison with data found during storage in the dark (*18*, *19*). Chlorophyll *a* was used as a photosensitizer at a concentration of 5 mg/kg. **Figure 6** shows that squalene did not have a protective effect on the lipid substrate and the activity of α-tocopherol was not enhanced in its presence. However, HPLC monitoring of the evolution of α-tocopherol and squalene contents revealed that squalene participated in the oxidation process. α-Tocopherol consumption during light exposure was lower in the presence of squalene (**Figure 7a**). The result was further supported by the much higher squalene consumption found for these samples (**Figure 7b**) and the presence of a new peak eluted earlier than squalene observed in the HPLC chromatograms that may be ascribed to an oxidation product.

Although, under the experimental conditions, a singlet oxygen quenching effect could not be attributed to squalene, its protective effect to α-tocopherol was clearly seen. This is possibly owed to the regeneration of α-tocopherol radical by squalene as was also suggested by Kohno et al. (*17*). Additionally, it may be related to the ability of squalene to trap two molecules of O_2 forming stable cyclic hydroperoxides (*19*).

The monitoring of α-tocopherol decrease during photo-oxidation allowed the calculation of the rate constant of its

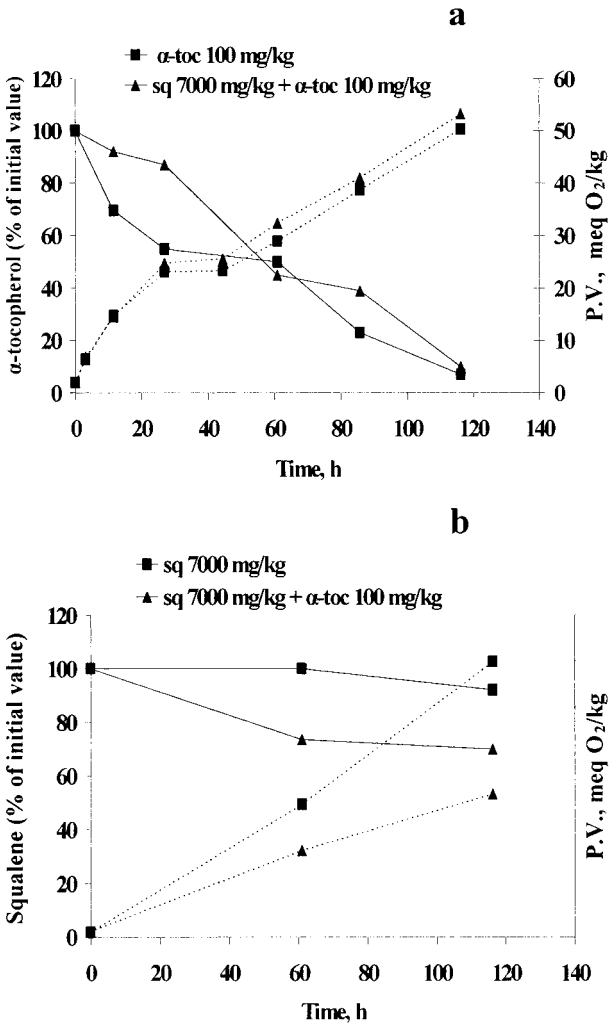

Figure 7. Changes (percent of initial value) in (a) α-tocopherol and (b) squalene during photo-oxidation of olive oil stripped of antioxidants and pro-oxidants. Comparison with the respective changes in PV values: —, changes in α-tocopherol and squalene contents; - - -, changes in PV.

decomposition. This reaction is reported to be of a pseudo-first-order with a rate constant of $(0.98-1.86) \times 10^{-4}$ s^{-1} in *n*-hexane models (*15*). The *k* value calculated from the graph of ln[α-tocopherol] versus time in the case of the olive oil model was significantly lower (0.0461×10^{-4} s^{-1}).

Under the experimental conditions used in this study olive oil bleaching as a result of chlorophyll destruction was complete, whereas the loss of tocopherols was not accompanied by a considerable increase in the peroxide value. Product deterioration is related to loss of organoleptic (color) and nutritional (α-tocopherol and squalene) characteristics and not with dramatic changes in the substrate. Because a high level of Pheo *a* in VOO is unavoidable, there is a need for careful handling not only during the extraction in the mills but also throughout commercialization. Packaging in dark glass bottles to avoid light exposure or even in transparent glass bottles in carton boxes or in paper bags appears to be appropriate to maintain the high quality of this precious commodity.

ACKNOWLEDGMENT

We express our sincere thanks to ELAIS SA (Piraeus, Greece) for providing the virgin olive oil samples and data for their fatty acid composition.

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Received for review July 2, 2001. Revised manuscript received October 23, 2001. Accepted October 23, 2001. E.P. thanks the Foundation of State Scholarships (I.K.Y., Athens, Greece) for financial support. This work has been partially financed by the General Secretariat for Research and Technology, Greek Ministry of Development (Program PAVE 99BE5 Virgin Olive Oil: Role of Pigments and α-Tocopherol in the Oxidative Stability and Development of a Method for the Assesment of the Oxidative Status of the Oil).

JF010847U