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Stability of Virgin Olive Oil. 1. Autoxidation Studies

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Virgin olive oil samples with similar oxidative stabilities and fatty acid compositions were stored for 24 months. Changes in the lipid substrate were followed by peroxide value and K_{232} measurements. HPLC was used to evaluate changes in the α-tocopherol, pigment, and squalene contents. Total polar phenol content was measured colorimetrically. The loss of α-tocopherol and carotenoids was comparable with that of polar phenol content, suggesting an active participation in autoxidation. The limited role of squalene in autoxidation was further confirmed using an olive oil model and in the presence of α-tocopherol. Pheophytin *a* degradation was high, although spectrometric estimation of chlorophyll content did not indicate so. Evaluation of pheophytin *a* activity at three different levels of addition on the oil model indicated a concentration-dependent antioxidant role more pronounced at elevated temperatures, which could be partially due to the activity of certain degradation products.

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

INTRODUCTION

The high oxidative stability of virgin olive oil (VOO) is mainly due to its fatty acid composition, but as recognized by all investigators the presence of certain minor components is equally significant. Some of these minor components have biological and nutritional functions and endow added value to virgin olive oil such as antioxidant activity, vitamin E value, and anticancer properties (*1*).

In autoxidation VOO stability has been correlated with the polar phenol content (*2*, *3*). The active phenols in VOO are the *o*-diphenol hydroxytyrosol and its oleosidic forms. Less attention has been paid to the contribution of other components, such as tocopherols, carotenoids, chlorophylls, and squalene, to its stability.

α-Tocopherol, the most important antioxidant in vivo, is found at considerable levels in VOO (*4*). Tocopherols are known to act as antioxidants by donating a hydrogen atom to chain-propagating peroxyl radicals (*5*). Addition of α-tocopherol in purified olive oil stripped of antioxidants and pro-oxidants conferred protection up to the level of 1000 mg/kg, although the activity was higher at lower levels (*6*).

The major carotenoids of VOO are *β*-carotene and lutein. Their levels do not exceed 10 mg/kg as a sum (*7*). Apart from contributing to color, they protect the oil from photo-oxidation by quenching singlet oxygen and acting as light filters (*8*). The activity during autoxidation is complex due to their susceptibility to oxidation (*9*). The overall experimental conditions and the presence of tocopherols seem to define the antioxidant or pro-oxidant behavior of carotenoids. The latter interrupt the free radical propagation through several parallel mechanisms that lead to the formation of an adduct or a radical cation.

VOO is unique among edible oils because it is consumed containing chlorophyll pigments, mainly pheophytin *a*, at levels up to 40 mg/kg (*10*). These pigments, responsible for the characteristic color of the oil, are well-known to act as photosensitizers during light exposure. However, information about their role during autoxidation is extremely limited. As reported, chlorophylls exert an antioxidant activity dependent on the derivative present, the lipid substrate, and storage temperature (*11*−*14*).

The high content of squalene in VOO (200−12000 mg/kg of oil) would justify an interest in the participation of this unsaturated hydrocarbon to the autoxidation mechanism as indicated by a recent publication (*15*).

In the present study the stability of VOO was examined with regard to the combined effect of different minor components. Two series of bottles for each sample were stored in the dark at ambient temperature. One was opened periodically, and oil aliquots were withdrawn for analyses; the other remained closed throughout the storage period. Changes in the lipid substrate and the evolution of the minor components during storage were followed to obtain a better understanding of oil stability and the interactions that possibly take place among components. Complementary experiments using standard compounds on olive oil stripped of antioxidants and pro-oxidants were also carried out to further investigate interactions where this was considered beneficial.

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

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antioxidants and pro-oxidants was prepared from refined oil as previously described (*15*).

Solvents and Standards. The solvents, of HPLC grade, were used without further purification. *n*-Hexane was 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* (Pheo *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).

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 Analyzentechnik, 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; paper rate, 0.5 cm/min) (*17*).

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 (*2*).

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 (*18*). Induction periods were determined by the Rancimat test (120 °C, 20 L/h).

Keepability Tests. VOO samples were selected on the basis of their stability as determined by the Rancimat test and their fatty acid composition. The samples were stored in the dark at ambient temperature (23 ± 5 °C). Each sample was put in two screw-capped transparent glass bottles (30 mL), completely filled with oil. No headspace was left in the bottles. The bottles were screwed, covered with aluminum foil, and kept in a carton box. For one series (a) aliquots were withdrawn periodically for analyses, whereas the other series of bottles (b) were stored without opening for 24 months. Peroxide values and K_{232} were measured to follow the extent of oxidation. All analyses were performed in duplicate. The repeatability of PV measurements was satisfactory (CV% = ±3.5 for a mean value of 7.5 mequiv of O_2/kg and $n = 7$; CV% = ±10 for a mean value of 0.3 mequiv of O_2/kg and $n = 7$). The repeatability of K_{232} was CV% = ±2.3, $n = 6$.

Table 1. Physicochemical Characteristics and Pro-oxidant and Antioxidant Factors of Virgin Olive Oil 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

Oven Tests. Two extra virgin olive oil samples (VOO1 and VOO2) selected on the basis of their chlorophyll content were stored in the oven at 40 and 60 °C. The oil samples (∼10 g) were put in open transparent glass bottles of pharmacopeia quality (18 mm i.d.). The process of oxidation was followed by PV and K_{232} measurements, whereas the evolution of chlorophyll pigments was monitored by HPLC. Oven tests were also carried out for standard compounds in olive oil stripped of antioxidants and pro-oxidants, which was used as a model system in the subsequent experiments. The samples were prepared in conical flasks, to which the standard compounds 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. A series of clear glass bottles, containing 2 g of oil each, were prepared.

Evaluation of Squalene Activity in the Presence of α-Tocopherol. Squalene (7000 mg/kg) and α-tocopherol (100 mg/kg) were added in the lipid substrate dissolved in isooctane and ethanol, respectively. The filled bottles were stored at 60 °C. Samples were withdrawn for PV and HPLC analysis.

Evaluation of Pheo a Activity. Pheo *a*, dissolved in diethyl ether, was added at three levels (5, 20, and 40 mg/kg). The filled bottles were stored at 60 °C. Samples were withdrawn for PV and HPLC analysis.

RESULTS AND DISCUSSION

The virgin olive oils used in autoxidation studies were selected on the basis of the oxidative stability index (OSI) as presented in **Table 1**. No differences were observed in the fatty acid composition, PV, K_{232} values, and acidity. The similar induction periods of the four samples are the combined result of the fatty acid composition and the simultaneous activity of various pro-oxidant and antioxidant factors endogenous in the oils. Polar phenol content itself could not support this finding unless possibly other pro-oxidant and antioxidant factors are taken into consideration.

The olive oil samples were stored in the dark for a period of 24 months, a date beyond that considered as the maximum storage period from bottling to consumption (18 months). Two bottles (a and b) were stored for each sample. One (a) was opened periodically and oil (∼2 g) was withdrawn from it. This procedure imitates the domestic use of olive oil and the transfer procedure from tanks to smaller containers. In both cases oil removal increases the headspace, resulting, thus, in renewal of

Table 2. Changes in the Lipid Substrate and Total Polar Phenol Content of Virgin Olive Oil Samples Stored in the Dark at Room Temperature

storage period[a]	sample 1	2	3	4
	PV, mequiv of O_2/kg			
8 months (a)	15.6	12.8	21.9	16.7
24 months (a)	31.5	24.1	31.0	36.8
24 months (b)	7.9	10.1	13.7	11.6
	K_{232}			
8 months (a)	2.65	2.11	3.12	2.48
24 months (a)	4.67	3.64	4.17	5.14
24 months (b)	2.03	2.24	2.56	2.25
	Total Polar Phenol Content, mg of Caffeic Acid/kg			
8 months (a)[b]				
24 months (a)	61	144	98	96
24 months (b)	84	148	120	100

[a] (a) opened and (b) closed bottles. [b] Not measured.

Table 3. Changes in α-Tocopherol, β-Carotene, Lutein, Chlorophyll, and Squalene Contents of Oil Samples Stored in the Dark at Room Temperature

storage period[a]	sample 1	2	3	4
	α-Tocopherol, mg/kg			
8 months (a)	186	165	183	167
24 months (a)	140	98	145	84
24 months (b)	204	148	172	162
	Squalene, mg/kg			
24 months (a)	5458	3800	3970	5800
24 months (b)	5735	3850	4097	5860
	β-Carotene, mg/kg			
8 months (a)	1.3	1.0	1.4	1.8
24 months (a)	1.0	0.8	1.1	1.7
24 months (b)	1.3	1.0	1.4	2.6
	Lutein, mg/kg			
8 months (a)	1.9	0.8	1.0	1.1
24 months (a)	1.5	0.6	0.8	0.8
24 months (b)	1.7	0.7	0.8	1.0
	Chlorophyll, mg of Pheo *a*/kg			
8 months (a)	14.3	8.6	16.6	22.0
24 months (a)	13.5	7.7	14.8	20.4
24 months (b)	14.4	8.3	17.5	21.5
	Pheo *a*, mg/kg			
8 months (a)	6.0	3.8	7.0	8.1
24 months (a)	2.0	0.9	2.2	1.7
24 months (b)	4.2	2.0	5.2	6.3
	Total Pheo *a*,[b] mg/kg			
8 months (a)	6.7	3.8	7.9	13.0
24 months (a)	5.5	3.0	7.3	10.3
24 months (b)	6.3	2.8	9.4	12.6

[a] (a) opened and (b) closed bottles. [b] Total Pheo *a* = Pheo *a* + peak A + peak B, where peaks A and B are pheophytin *a* derivatives.

oxygen in the container. The second bottle (b) remained sealed during the storage period. The room temperature where the samples were stored varied from 18 to 28 °C during the 24 months of storage. The oxidation process was monitored by PV and K_{232} measurements. Changes in the α-tocopherol, pigment, and squalene contents were followed by HPLC, whereas total polar phenol content changes were measured colorimetrically. All of the data concerning these changes are given in **Tables 2** and **3**.

Changes in the Lipid Substrate and the Total Polar Phenol Content. Peroxide values after storage for 8 months were below the limit of 20 mequiv of O_2/kg set by the EU. After 24 months of storage, the PVs of oil samples of series a were all >20 mequiv of O_2/kg. On the contrary, in the samples of series b only a slight increase was observed. The same applies for K_{232} values.

Because total polar phenol content has been successfully correlated with olive oil stability (*3*), total polar phenol content changes were also measured. Reduction in the total polar phenol content in the oils during storage is a result of oxidation and hydrolytic activity. Phenol loss was appreciable in samples that were opened periodically during storage. Similar behavior was observed in the two series (a, b) of bottles for samples 2 and 4, whereas the reduction in series b for samples 1 and 3 was comparatively lower. Polar phenol reduction data were in accordance with changes in the lipid substrate.

Changes in the α-Tocopherol Content. Considerable loss in the α-tocopherol content was observed in samples opened periodically within the period of two years due to the renewal of oxygen supply. α-Tocopherol loss in samples of series b was <20%. The size of tocopherol loss was similar to that found for polar phenols, indicating that tocopherol participation in autoxidation is equally important to that of polar phenols. In a previous study (*19*), by applying multivariate statistical techniques, it was found that olive oil stability depends on both tocopherol and polar phenol contents. Those findings are in agreement with the present experimental results as well as with findings of other recent publications (*3*, *20*). Moderate loss in the α-tocopherol content (14−32%) has been also reported for oils stored for a much shorter period (6 months) (*21*). In that study α-tocopherol loss was accompanied by an even higher loss of squalene (26−47%), leading the researchers to the assumption that squalene is preferentially consumed to protect α-tocopherol.

Changes in the Squalene Content. In our previous study on the contribution of squalene to olive oil stability (*15*) it was found that this hydrocarbon has a slight antioxidant activity, which was concentration dependent. This activity, associated with competitive oxidation phenomena, was not found to influence the activity of caffeic acid or α-tocopherol present at realistic concentrations. Measurement of changes in the content of squalene by HPLC in the present study revealed an insignificant decrease in all samples with the exception of sample 3 (10% loss). Squalene, a compound characterized by the presence of six nonconjugated double bonds, was rather stable at the experimental conditions despite the long storage period. This could be of particular importance if one takes into consideration recent findings for the beneficial role of squalene against certain cancers and the fact that olive oil is a major dietary source for it. The results are also in conformity with those indicating a squalene loss below 10% for virgin olive oils stored under similar conditions (*22*) but not with the much higher reduction referred to by Manzi et al. (*21*).

To support our finding a complementary experiment was carried out, using a model system. Squalene and α-tocopherol were added separately and as a mixture in olive oil stripped of antioxidants and pro-oxidants, and the samples were stored in the dark at 60 °C. Changes in the content of the two compounds as well as changes in the lipid substrate are given in **Figure 1**. The mixture of α-tocopherol and squalene had a slightly better antioxidant activity than α-tocopherol alone (**Figure 1a**), which was related to a gradual loss of α-tocopherol content until its complete destruction. No squalene loss was observed even in the presence of α-tocopherol (**Figure 1b**). One may assume that the presence of squalene did not protect α-tocopherol during

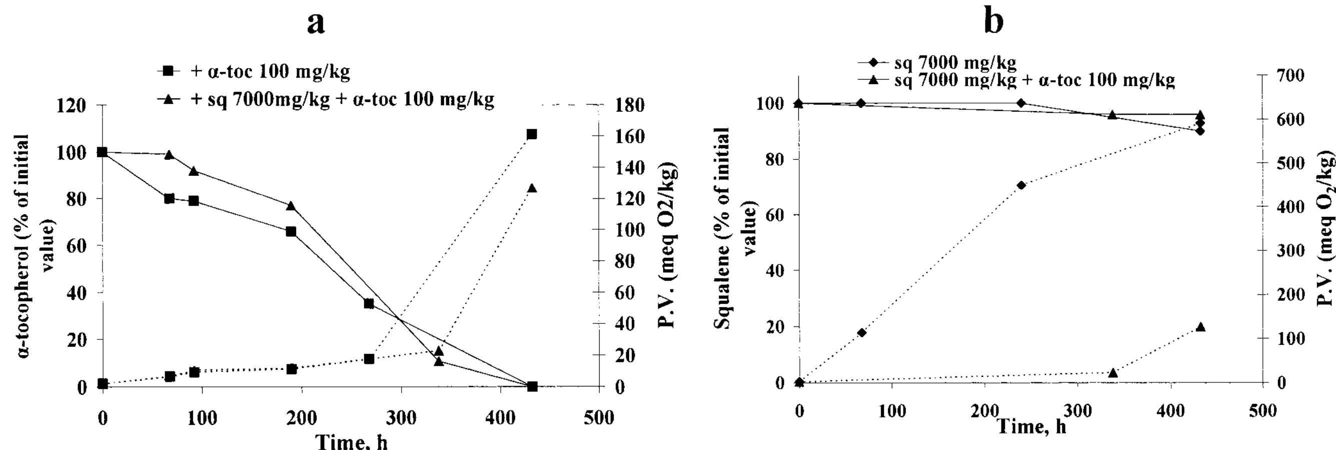

Figure 1. Changes (percent of initial value) in (a) α-tocopherol and (b) squalene during autoxidation of olive oil stripped of antioxidants and pro-oxidants at 60 °C; comparison with the respective changes in PV (—, changes in α-tocopherol, squalene content; - - -, changes in PV).

the long storage period. Squalene was practically stable during storage at 60 °C even for high PVs (>200 mequiv of O_2/kg). The same trend was observed in the samples containing the combination of α-tocopherol and squalene. These findings support the evidence obtained from the storage of VOO samples, confirming that squalene is quite stable during autoxidation. Furthermore, it may be postulated that the products of squalene oxidation are quite stable and would not be further involved in propagation reactions (*23*).

Changes in the Carotenoid Content. *β*-Carotene and lutein destruction patterns were different between samples of series a and b. *β*-Carotene content remained almost unchanged in the samples kept sealed for two years, whereas an appreciable decrease (>20%) was observed in samples of series b. Lutein loss was greater than that of *β*-carotene, in line with previous observations (*16*). The low initial level of individual carotenoids (**Table 1**) makes difficult any discussion about their participation in the autoxidation on the basis of percent loss. However, it seems that due to their high oxidative susceptibility, carotenoids are particularly vulnerable in the presence of oxygen. Although their role as quenchers in photo-oxidation is generally accepted, their function as radical scavengers in autoxidation is perplexing and still not defined (*9*). *β*-Carotene is the most studied member; data on the role of lutein in the autoxidation of lipids are limited (*24*). Both pro-oxidant and antioxidant activities have been attributed to carotenoids depending on the substrate, concentration, and presence of tocopherols. The results of the present study are in accord with comments reported for the contribution of carotenoids to olive oil stability based on statistical treatment of data (*3*).

Changes in the Chlorophyll Content. At the end of the storage period all of the samples of series a showed >10% loss in chlorophyll content, whereas in the samples of series b loss was generally lower (5−9%). Meaningful information was collected only when individual chlorophyll pigments were determined by HPLC. In samples stored for two years in sealed bottles the percentage loss in Pheo *a* ranged from 51 to 86%. Pheo *a* loss was greater (~80%) for samples stored in bottles opened periodically during storage. Changes in individual chlorophylls occurred from the first stages of autoxidation. These changes in individual chlorophylls are illustrated in the chromatograms of **Figure 2**. In the chromatographic profile of virgin olive oils at 410 nm two peaks (A and B) often accompany the peak of Pheo *a*, which correspond to Pheo *a* derivatives (*16*). Under the experimental conditions of our study additional oxidation products were formed. In the samples of series a

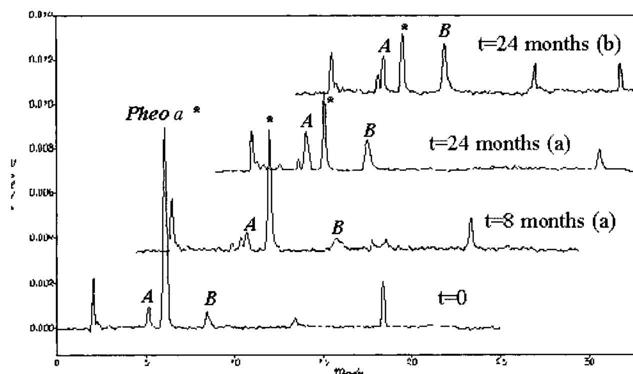

Figure 2. Changes in the chromatographic profile at 410 nm of a virgin olive oil sample kept at room temperature in the dark for 24 months: (a) opened and (b) closed bottles.

derivatives A and B were formed at equal quantities, whereas no consistency was found in the case of samples of series b. The main derivative of Pheo *a* formed during storage is that corresponding to peak A, which may be attributed to pyropheopytin *a* and/or pheophytin *a′* (*10*). If the former is true, then extended storage of the oil in the dark favors the removal of the carboxymethyl group to form the pyro derivative. Indeed, storage of another two extra virgin olive oil samples in the oven at 40 and 60 °C verified that the decrease in Pheo *a* content, as determined by HPLC, was accompanied by a concomitant increase mainly in peak A (**Figure 3**). This further supports the assignment of this peak to pyropheophytin *a*, the formation of which is the result of heat treatment. Peak B, which was mainly formed at low temperature (room temperature, 40 °C), was more difficult to characterize. Peak B may be related to an allomeric derivative of pheophytin *a*. The presence of allomeric forms of chlorophyll pigments has been reported in stored VOO samples (*25*), but no explanation for their formation was given. Although the evidence is limited, allomerization seems to follow a free radical mechanism (*26*). In case peak B was a Pheo *a* allomer, its formation would be ascribed to oxygen availability.

Total Pheo *a* content, defined as the sum of Pheo *a*, peak A, and peak B, decreased during storage, implying, thus, that other reactions may also take place resulting in the formation of components not detected at 410 or 665 nm.

The results obtained from all of the above autoxidation studies revealed that Pheo *a* undergoes significant transformations in contrast to squalene. Taking into account that virgin olive oil contains high amounts of Pheo *a* (*10*), it is important to further

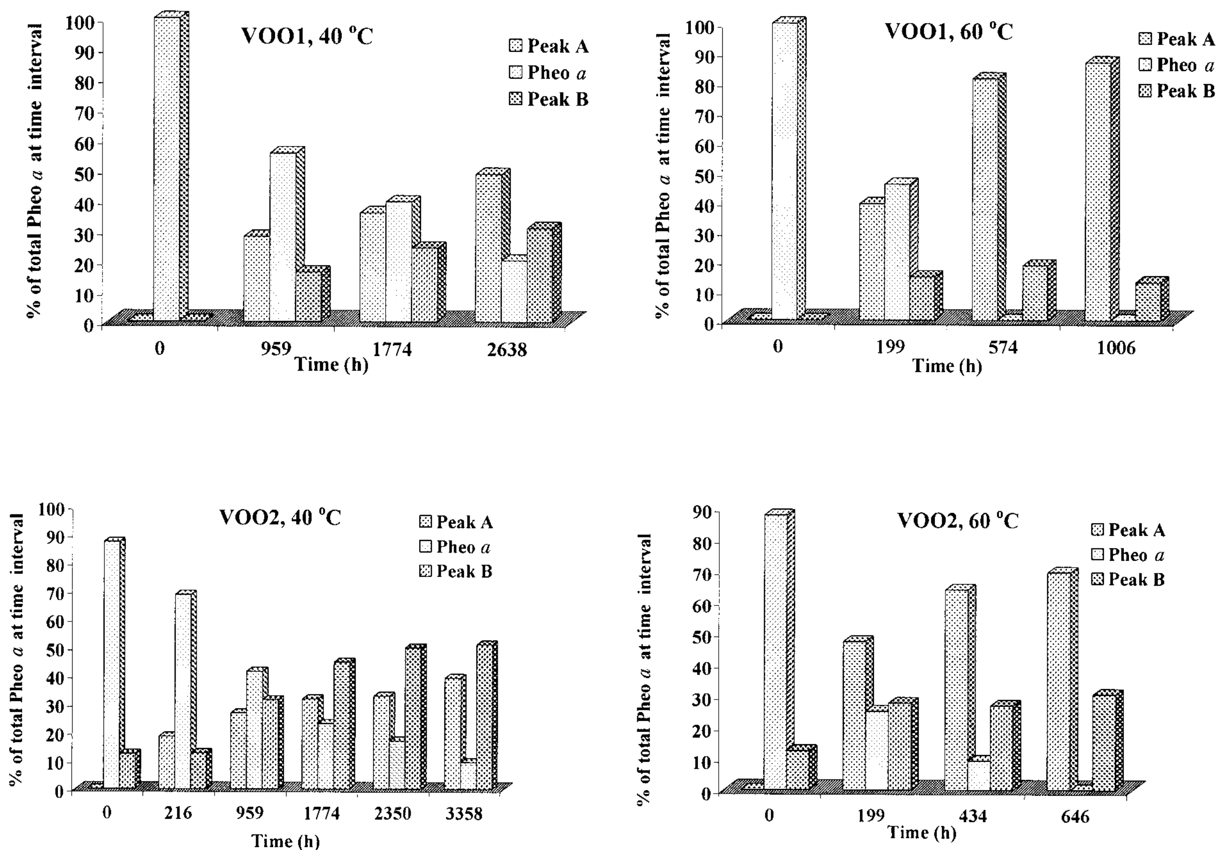

Figure 3. Evolution of chlorophyll pigments during storage of virgin olive oil samples at 40 and 60 °C (total Pheo *a* = Pheo *a* + peak A + peak B, at various time intervals).

investigate its contribution to olive oil stability in the dark. Additional work was then carried out using the olive oil model system. Pheo *a*, at three levels of addition (5, 20, and 40 mg/kg), was examined using a Rancimat test (80 °C, 20 L/h) and also an oven test at 60 °C. Using the first method, the induction period in the presence of Pheo *a* increased substantially, indicating a concentration-dependent antioxidant activity. The induction periods were as follows: 44.2 h (control); 48.4 h (+ 5 mg/kg); 56.0 h (+ 20 mg/kg); 62.0 h (+ 40 mg/kg) (CV% = 1.12, $n = 5$). This activity was also confirmed by an oven test, although the concentration dependence was not so clear. The main product of Pheo *a* transformation under the experimental conditions was that corresponding to peak A. It is probable that the activity of Pheo *a* is enhanced by the presence of pyropheophytin *a*, the formation of which is favored at higher temperatures. A strong antioxidant activity for pyropheophytin *a* has been reported in the literature (*27*). The presence of oxidation products that do not promote further the oxidation seems to be important for VOO stability in the dark. Moreover, our data combined with those produced by Endo et al. (*12*, *13*) add to the knowledge on the activity of chlorophyll pigments as antioxidants.

The results obtained from the parallel study of changes in total polar phenol, α-tocopherol, β-carotene, lutein, chlorophyll, and squalene contents during autoxidation indicate that most interactions may be suppressed if oxygen availability is limited. Pheophytin *a* behavior deviates from this rule. Storage period is a critical factor for the formation of a series of its derivatives. The monitoring of squalene indicated that the role of this compound is limited and that it has a remarkable stability during autoxidation. Virgin olive oil stability in the dark may be striking without significant reduction in the content of antioxidants, providing that a careful control of all relevant parameters was performed before bottling. Precautions for storage in the dark do not, however, ensure the stability of the oil under conditions promoting photo-oxidation. This is addressed in the succeeding paper.

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