Action of Chlorophylls on the Stability of Virgin Olive Oil

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Virgin olive oil was used as substrate to study the influence of chlorophylls on its oxidative stability in light and in darkness. Chlorophylls a and b were added to this substrate, after which oils were stored at 36 ± 2°C for three months under artificial light (1340 lux) or in darkness. The effect of light was greater than that of the additives. The prooxidant action of chlorophylls in the presence of other pigments of the oil was not observed in this assay. During early storage, the rate of peroxide formation was lower in the samples with added chlorophylls, but later it equalled that of the control. In darkness, stability was greater in the samples containing chlorophylls, indicating a slight antioxidant effect, which was more marked for chlorophyll a.

KEY WORDS: Carotenoids, chlorophylls, K_{232}, oxidation, peroxide value, pheophytins, pigment analysis, pigments, virgin olive oil.

Virgin olive oil has a characteristic aroma, taste and color that distinguishes it from other vegetable oils. Its excellent organoleptic and nutritive qualities (1), together with the current tendency of consumers to select the least-processed foods, have caused a re-evaluation of its consumption. It is now often favored over other fats that have more complex processing steps, such as decoloring, deodorizing and refining (2). However, it is a matter of concern for the oil industry to conserve the oil without loss of its positive attributes or deterioration of its quality. Various factors, such as air, heat and light, act as synergists in the autooxidation of an oil by producing hydroperoxides that can seriously and rapidly diminish the original characteristics (3). Hydroperoxides are formed by the action of oxygen on unsaturated fatty acids through free-radical reactions that continue if there are no antioxidants capable of stopping them (4).

A process similar to that of autooxidation, but generated by a different pathway, is photooxidation. Light striking the oil sets off a series of rapidly progressing oxidations. Photooxidation requires the presence of elements known as photosensitizers or chromophores that can capture and concentrate the light energy (4,5). This energy can be transmitted to the oxygen present, converting it to a more active singlet state that reacts directly with the double bonds of fatty acids by a symmetrical addition known as the "ene" reaction. Oxygen is inserted on either of the two carbons of the double bond, giving an allyl hydroperoxide with *trans* configuration (3). Chlorophylls and their derivatives are present in olive oil in variable quantities (6) and can act as photosensitizers. Special attention has been paid to pigments in recent years, to elucidate their influence on oxidation and, hence, on the stability of oils (7–9).

The photooxidative activity of chlorophylls and their derivatives *via* singlet oxygen formation has been demonstrated in experiments on decolored olive oils, seed oils and fatty acid esters, to which these pigments have been added before exposure to light (4,7,8,10,11). In darkness the degradation processes are basically due to autooxidation reactions, and there have been fewer studies reported. The pigments have been postulated to act as protectors, capturing free radicals in a way similar to that of α-tocopherol. Differences in results have been found depending on the types and the proportions of fatty acids present (12, 13). Although the studies carried out up to now indicate a prooxidant role for chlorophylls and their derivatives in oils exposed to light, the data are not conclusive on their antioxidant role in darkness. Almost all of the studies on this topic have used model or near-model systems in oils from which the pigments, peroxides, phenols and other minor constituents had been eliminated. In these simplified systems, the effect of each pigment on the substrate could be controlled without interference from other components. The behavior of a real system may be different, particularly if interactions take place between components.

EXPERIMENTAL PROCEDURES

Materials. Virgin olive oil was obtained from a local supermarket. Three samples of 2,200 mL each were prepared. One was used as control, and 9.30 ppm chlorophyll a and chlorophyll b, respectively, were added to the other two. Storage experiments were performed in vials containing 115 mL of oil at 36 ± 2°C. One series of samples was placed 75 cm under a fluorescent light with a radiation at sample level of 1340 lux. Another series was placed in darkness in an aluminum box. Samples were taken every 24 h during the first week of storage and then at 14, 28, 56 and 90 days.

Standards. Chlorophylls a and b were extracted from fresh spinach leaves with acetone and separated by thin-layer chromatography (TLC) on silica gel (14). Pheophytins a and b were obtained from the respective chlorophyll solutions by acidification with hydrochloric acid (13%). β-Carotene, lutein, violaxanthin, neoxanthin and neochrome were separated and purified by TLC from a pigment extract of green olives saponified with methanolic potassium hydroxide (15).

Pigment identification. The adsorption properties, color in TLC and absorption spectra were used for initial identification of the pigments present in the original virgin olive oil. For confirmation of functional groups of the carotenoids, the usual physicochemical reactions specified in the bibliography were assayed (16). During oil storage, qualitative analysis of the pigments was carried out by TLC according to Mínguez-Mosquera *et al.* (6).

Quantitation of pigments. Chlorophyll determinations in virgin olive oil is complex because fatty matter must be removed before TLC (6) or high-performance liquid chromatography (HPLC) (15) analysis. Therefore, throughout the study the chlorophylls and carotenoids were analyzed directly from the absorption spectra of each

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TABLE 1

Characteristics of the Virgin Olive Oil Used as Substrate

Quality indices		Composition					
		Fatty acids	%	Pigments	%	Natural antioxidants	ppm
PV (meq/kg)	10.2	16:0	10.97	β-carotene	12.73	Polyphenols (tyrosol)	239
Stability (h)	56.1	16:1	1.04	Lutein	37.36	Tocopherols	350
$E^{1\%}_{1\,cm}$ 232 nm	2.31	18:0	3.49	Minor carotenoids	8.59		
$E^{1\%}_{1\,cm}$ 270 nm	0.16	18:1	78.76	Pheophytin a	41.32		
Acidity (% oleic)	0.38	18:2	4.80				
		18:3	0.94				

virgin olive oil sample (7 g) dissolved in cyclohexane (25 mL), with the help of a multicomponent program for quantitation (17).

Oxidation index. The values of peroxides, acidity and composition of fatty acids and tocopherol were measured according to the methods described by the American Oil Chemists' Society (18–21). Specific extinction values, $E_o^{1\%}$ (K_λ), were determined at 232 and 270 nm in cyclohexane. For calculation, the expression $K_\lambda = (e_\lambda/P) \times 100$ was used, in which K_λ is the specific extinction at wavelength λ; e_λ is the extinction read from the apparatus; and P is the sample weight in mg.

The stability to oxidation was determined by the Rancimat method of Hadorn and Zurcher (22). Polyphenols were determined by the method of Vázquez *et al.* (23).

RESULTS AND DISCUSSION

Substrate characterization. Table 1 shows the initial characteristics of the virgin olive oil used in this study. The peroxide value (PV) and extinction at 232 and 270 nm, respectively, show that oxidation had already begun. Nevertheless, the oil was of extra quality because the values did not exceed the standard limits of 20 meq/kg, 2.40 and 0.20, respectively. The stability of 56.1 h corresponded to a virgin olive oil of stability higher than the mean of 45–46 h. The acidity of 0.38° indicated an oil of extra quality, because the value was below 1°, the standard limit for this category.

The contents in fatty acids and natural antioxidants (polyphenols and tocopherols) corresponded to the normal contents for virgin olive oil, even though the oil was of extra virgin quality. The chlorophyll and carotenoid contents were within the limits for a Spanish virgin olive oil.

Evaluation of the multicomponent analysis method and carotenoid quantitation. To test the accuracy of the method for multicomponent analysis of pigments, standard solutions of chlorophyll a, pheophytin a, chlorophyll b, pheophytin b, β-carotene and lutein were prepared individually in acetone. Their spectra and concentrations based on the $E^{1\%}_{\lambda max}$ values in acetone shown in Table 2, were recorded. Then six different mixtures of known concentrations of these compounds were prepared and analyzed by multicomponent methodology. Pigment content was calculated from the second derivative data in the wavelength range of 350–500 nm. The results are shown in Table 3. Confidence intervals of the differences between the theoretical values and those obtained by the multicomponent program included zero, both for individual pigments and total pigment content. Thus, the proposed method had sufficient accuracy ($P < 0.05$) to be used for the analysis of these pigments throughout the photooxidation study.

TABLE 2

Absorption Maxima and Extinction Coefficients in Acetone

Pigment	λmax (nm)	$E^{1\%}_{\lambda\,max}$
Chlorophyll a	420	840
Chlorophyll b	454	1450
Pheophytin a	406	1290
Pheophytin b	430	2060
Lutein	446	2340
β-carotene	450	2620

Pigment changes throughout storage. Table 4 shows the changes in pigment concentrations for the control sample and those containing added chlorophylls a and b. Exposure to light did not alter the carotenoid composition of the oil during the first 14 d of the experiment. Thereafter, the pigments began to be degraded to non-colored products and β-carotene was destroyed after 90 days. Lutein was the most stable carotenoid, except when chlorophyll a was added in which case it was not detected in the latter stage. However, the effect of light on the chlorophyll fraction of the control sample was evident from the beginning. Pheophytin a was destroyed gradually during the first week in all three samples. The concentration of added chlorophyll a diminished to approximately 10% in one day, and disappeared totally after 48 h. The constant concentration of pheophytin a over the first three days could be due to conversion from chlorophyll a. Degradation followed a similar course in the sample with added chlorophyll b.

In darkness, the response of these pigments was markedly different to that in light. No destructive effects on the content of β-carotene and lutein were observed. In the control, the concentration of pheophytin a and the carotenoids remained constant up to 90 days, showing that their destruction was influenced more by light than by temperature. In the oil with added chlorophyll a, it was destroyed gradually, disappearing at 56 days. The increase of pheophytin a during this period was due to its transformation from chlorophyll a. In the sample with added chlorophyll b, pheophytin a remained constant during the

TABLE 3

Pigment Quantitation by Multicomponent Analysis. Comparison Between Theoretical and Experimental Values (mg/kg)

Sample no.	β-carotene		Lutein		Chlorophyll a		Chlorophyll b		Pheophytin a		Pheophytin b	
	T[a]	F[b]	T	F	T	F	T	F	T	F	T	F
1	0.522	0.536	0.618	0.614	1.530	1.514	0.943	0.921	1.042	1.048	0.817	0.809
2	0.269	0.234	0.347	0.355	0.765	0.764	0.530	0.518	0.579	0.562	0.496	0.489
3	0.200	0.192	0.243	0.244	0.562	0.556	0.359	0.338	0.393	0.379	0.320	0.316
4	0.603	0.544	0.650	0.625	1.767	1.647	1.113	1.114	1.315	1.327	0.822	0.874
5	0.235	0.220	0.248	0.240	0.675	0.655	0.423	0.415	0.507	0.514	0.315	0.320
6	0.112	0.120	0.127	0.121	0.335	0.321	0.215	0.212	0.257	0.263	0.155	0.164

[a]T, theoretical.
[b]F, found values.

TABLE 4

Change in Pigment Concentration (ppm) in Virgin Olive Oil with and Without Added Chlorophylls During Storage Under Fluorescent Light and in Darkness at 36 ± 2°C[a]

Time (days)	Control			Chlorophyll added								
				Chlorophyll a				Chlorophyll b				
	β-C	Lut.	Phy a	β-C	Lut.	Chl a	Phy a	β-C	Lut.	Phy a	Chl b	Phy b
						Fluorescent light						
0	2.31	6.78	7.50	2.31	6.78	9.30	7.50	2.31	6.78	7.50	9.30	—
1	2.76	6.92	6.49	2.59	6.63	1.27	7.37	2.69	6.31	6.82	1.05	1.01
2	2.02	6.98	5.35	1.85	6.95	—	7.16	2.03	6.87	6.43	—	0.87
3	2.56	6.74	3.74	2.32	5.88	—	5.97	2.34	6.81	3.89	—	—
4	2.48	6.80	2.22	2.41	6.73	—	4.29	2.41	6.71	2.48	—	—
7	2.41	6.67	—	2.37	6.59	—	0.70	2.34	6.63	0.52	—	—
14	2.05	6.57	—	2.01	6.41	—	—	2.00	6.49	—	—	—
28	1.84	6.23	—	1.73	5.81	—	—	1.98	6.10	—	—	—
56	1.46	6.28	—	1.05	4.77	—	—	1.11	4.63	—	—	—
90	—	4.30	—	—	—	—	—	—	3.60	—	—	—
						Darkness						
0	2.31	6.78	7.50	2.31	6.78	9.30	7.50	2.31	6.78	7.50	9.30	—
1	2.76	6.84	8.13	3.02	6.10	7.49	7.95	2.89	6.91	6.67	6.99	1.87
2	2.15	6.76	7.56	2.32	6.35	6.42	8.52	2.83	6.52	6.60	6.91	2.07
3	2.61	6.76	7.71	2.68	6.26	5.62	8.87	2.58	6.04	6.61	6.95	1.65
4	2.69	6.72	7.71	2.63	6.25	5.03	9.08	2.65	6.12	6.57	7.08	1.43
7	2.80	6.72	7.58	2.80	6.31	4.52	10.03	2.89	6.15	6.38	6.63	1.80
14	2.61	6.78	7.42	2.51	6.49	2.81	11.22	2.78	6.50	6.53	4.83	2.47
28	2.65	6.70	7.48	2.70	6.56	1.03	12.69	2.80	6.40	6.56	4.72	3.20
56	2.54	6.71	6.72	2.52	6.67	3.06	11.70	2.68	6.78	6.90	3.50	3.40
90	3.18	6.37	7.19	3.05	6.43	—	13.90	2.76	6.56	7.00	2.22	3.53

[a]Concentration of chlorophylls added: 9.3 ppm. Chl a, chlorophyll a; Chl b, chlorophyll b; Phy a, pheophytin a; Phy b, pheophytin b; β-c, β-carotene; and Lut., lutein.

whole assay, as in the control, whereas chlorophyll b and pheophytin b followed the pattern shown by chlorophyll a.

To interpret the behavior of these pigments more objectively, a regression analysis of their changes was made. Change rates were determined from the slope of the adjusted regression lines. All cases fitted to exponential equations, whose parameters are shown in Table 5.

Changes in PV throughout storage. Figure 1 gives the results of peroxide formation. The influence of the additives was small compared with that of light. Once storage began, the change in PV basically depended on whether it was taking place in light or in darkness. Thus, there was no evidence of a photosensitizing effect of chlorophylls in the presence of light and, consequently, of its prooxidant action. The higher initial PV of the oils with added chlorophylls in relation to the control, due to unknown causes, was compensated by a lower peroxide formation rate during the following days.

The lower oxidation rate during this period may be because the reaction, on being initiated by singlet oxygen,

TABLE 5

Parameters and Correlation Coefficients (r) of the Exponential Equations for the Different Pigment Degradation Curves [general equation $y = e^{(a + bx)}$]

Pigment and degradation time	Control				Chlorophyll added							
					Chlorophyll a				Chlorophyll b			
	a	b	r	Time (days)	a	b	r	Time (days)	a	b	r	Time (days)
					Fluorescent light							
Carotenoids	2.11	−0.01	0.98	(0-91)	2.16	−0.02	0.99	(0-56)	2.19	−0.02	0.99	(0-91)
Pheophytin a	2.04	−0.16	0.95	(0-4)	2.44	−0.34	0.97	(0-7)	2.36	−0.40	0.97	(0-7)
					Darkness							
Chlorophyll a					1.92	−0.05	0.97	(0-56)				
Chlorophyll b									1.98	−0.01	0.96	(0-91)
Pheophytin a					2.18	6×10^{-3}	0.83	(0-91)				

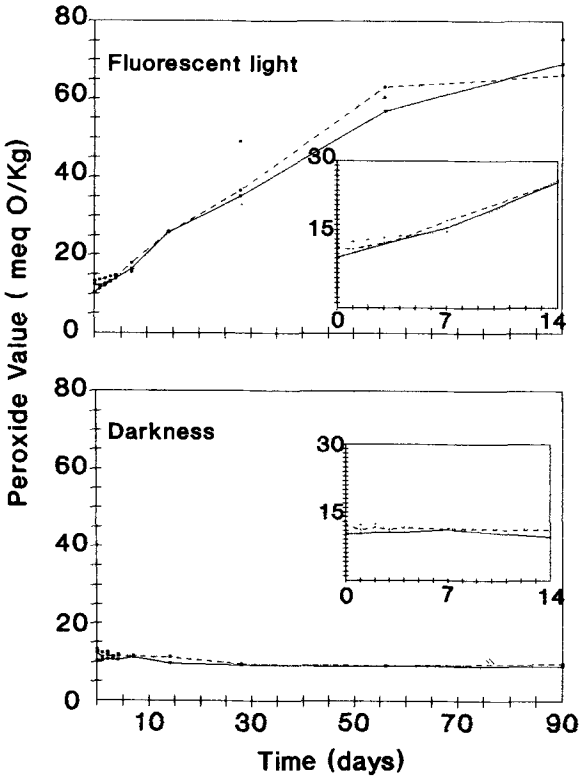

FIG. 1. Peroxide formation in virgin olive oil containing added chlorophyll a (dotted line) and chlorophyll b (dashed line) during illumination under fluorescent light and in darkness at 36 ± 2°C. Concentration of chlorophylls added was 9.3 ppm. Control represented by an unbroken line.

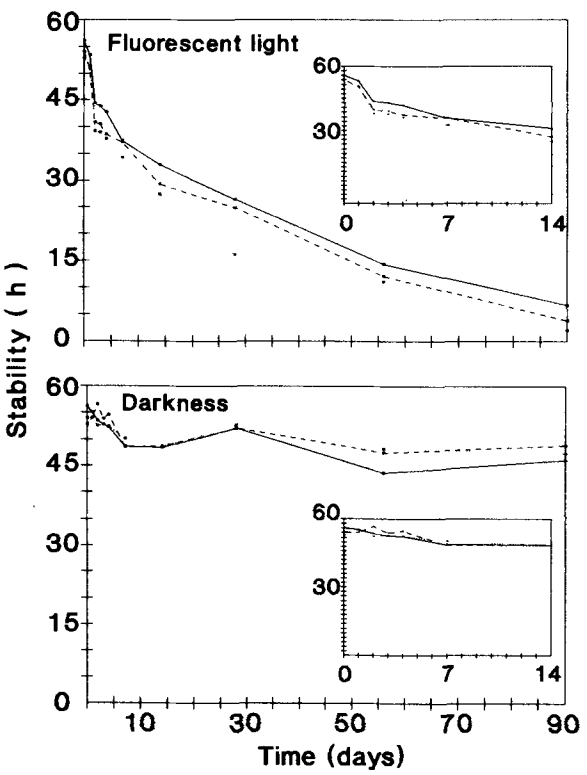

FIG. 2. Change in the stability (induction period) of virgin olive oil with and without added chlorophylls during storage under fluorescent light and in darkness. Concentration of chlorophylls added was 9.3 ppm. Chlorophyll a, dotted line, chlorophyll b, dashed line; and control, unbroken lines.

delayed the formation of free radicals. Later, the rates were similar to those of the control, with a constant value of approximately 0.9 PV/d during the first two months. After that time, the values decreased. The correlation coefficients obtained were significant at a level of 5%.

In the dark, the oxidation rate was so low that the destruction of peroxides was higher than the rate of formation, leading to a total peroxide decrease. Although the decrease was higher in the samples with either chlorophyll, the effect was more marked in the case of chlorophyll a.

TABLE 6

Parameters and Correlation Coefficients (r) of the Equations for the Evaluation of Peroxide Values and Stabilities of the Virgin Olive Oil Samples[a]

	Peroxide value ($y = a + bx$)						Stability ($y = e^{a + bx}$)					
	Fluorescent light			Darkness			Fluorescent light			Darkness		
Sample	a	b	r	a	b	r	a	b	r	a	b	r
Control	12.87	0.69	0.99	10.42	−0.02	0.74	46.07	−0.50	0.93	52.98	−0.10	0.76
Chlorophyll a[b]	12.83	0.74	0.99	11.88	−0.03	0.88	41.45	−0.51	0.90	52.39	−0.06	0.76
Chlorophyll b[b]	12.33	0.68	0.97	11.48	−0.03	0.81	43.38	−0.50	0.93	53.25	−0.07	0.83

[a]Storage time was 90 days.
[b]Samples added with 9.3 ppm of the respective chlorophyll.

This effect may be related to the antioxidant behavior of chlorophylls in darkness.

Table 6 shows the parameters and correlation coefficients of PV. Differences between the slopes of the regression lines in light and darkness were remarkable, however, no differences between samples within the same conditions were observed.

Changes in stability throughout storage. Figure 2 shows the results obtained for stability. Confidence limits for results (average of two replicates) were ± 0.024 SD.

The addition of chlorophylls produced an immediate decrease in stability, of the order of 2–3 h (4–6%) greater for chlorophyll a than for chlorophyll b. This was in agreement with the initial increases in PV (Fig. 1) observed previously. Later, throughout the whole storage time, the influence of light was the most significant effect, and it was practically independent of whether or not any additive was present.

In light, stability decreased exponentially with time in all cases, so that the oils lost stability quickly—of the order of 50% in the first month, 75% in the second and 90–95% in the third—accumulatively. It is noteworthy that during the first four days that the samples were exposed to light, the rate of decrease was higher for the samples with added chlorophylls. It was highest in the samples with chlorophyll a, which is logical because of the initial increase in peroxides. Thereafter, behavior was similar to that of the control.

In the sample stored in darkness, stability also decreased, reaching values of 46–49 h—a decrease of 5–10 h (10–20%) from the initial values—at the end of the period studied. Such values were obtained after only two days of storage when the samples were exposed to light. The degradation rates were significantly lower than those for the samples stored in light. Those for samples with added chlorophyll a were even lower throughout the period studied, which appears to demonstrate its antioxidant capacity. The addition of chlorophyll b gave rise to rates similar to those of the control, particularly in the first four days. The correlation coefficients obtained were significant ($P < 0.05$) in all cases studied.

Parameters and correlation coefficients of the equations for stability are also shown in Table 6. Again, differences in stability changes (slope values) were only observed between samples in light and in dark, while they were not appreciable within the same conditions (light or darkness).

Changes in K_{232} throughout storage. Only a slight increase was observed after 14 days in the samples stored in light, while those stored in darkness remained practically constant.

The action of singlet oxygen in the formation of conjugated dienes was not demonstrated in this experiment, because the values of K_{232} for the samples with added chlorophylls were not different from those of the control. This might be caused by the rapid disappearance of chlorophylls due to their high photolability, or to the particular composition of virgin olive oil.

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