Pigments Present in Virgin Olive Oil

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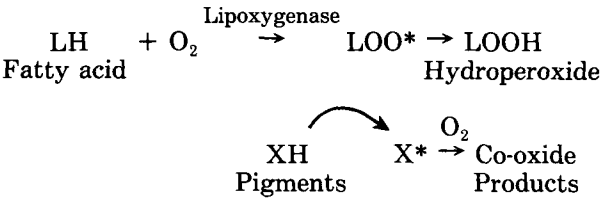
The qualitative and quantitative control of pigments in ripe olives and in extracted virgin olive oil has increased our knowledge of the influence on these compounds in the areas of ripening of the fruit, storage time in the factory and the oil extraction process. As the harvesting time of the fruits increases, pigment content decreases. During storage, the presence of lipoxygenase has been detected, as well as a considerable decrease in chlorophylls and a small decrease in carotenoids. During the extraction process, the chlorophyllic fraction is destroyed in the greater part, and although the carotenoid fraction is also affected, its concentration increases in the oil with respect to that in the fresh fruit. In the pigment degradation, in addition to the acid-catalyzed reaction, the presence of lipoxygenase suggests a role for this enzyme.

During ripening and post-harvesting of the fruit, cellular degradation is accompanied by oxidation of the unsaturated fatty acids that form part of the lipid components of the membranes. This reaction is catalyzed by lipoxygenase (1).

The pigments that color olives and olive oil are chlorophylls and carotenoids (2). These compounds can undergo oxidation under certain conditions, being degraded to uncolored products. The free radicals formed by the action of lipoxygenase on the unsaturated fatty acids with *cis-cis* 1,4 pentadiene system intervene in this reaction.



Although the exact mechanisms of this co-oxidation catalysis are not known, it has been demonstrated that the presence of the specific substrate of lipoxygenase is necessary (3).

Holden (4) found that legume reed and legume extracts bleached chlorophyll in the presence of long-chain fatty acids. Chlorophyll was bleached by extracts which had lipoxygenase activity, but not by purified lipoxygenase preparations. Addition of linoleic acid to seed extracts stimulated bleaching two to three times that of the control, whereas oleic acid had little effect. Thus, chlorophyll appears to be bleached as a secondary substrate during a chain reaction involving peroxidation of fatty acids, and the breakdown of hydroperoxide by a heat-labile factor in that system.

Many reports have shown that the inactivation of enzymes such as peroxidases, lipases and lipoxygenases prevents color changes associated with the conversion of chlorophylls to pheophytins, chlorophyll destruction and the development of off-flavors in the lipid fraction (5–7). The color of olive oil could be affected by the absence or presence of this type of oxidative enzyme during the ripening and storage period of the fruit.

The present work studies the relationship between lipoxygenase enzymatic activity and pigment degradation during the period that fruits are piled up in the so-called "trojes" (or silos), before their oil extraction. This is a very frequent and necessary practice in the olive oil extraction industry, as the amount of fruit received is greater than the daily extraction capacity of the factory. The effect of the extraction process, which combines the effect of ripening throughout the harvesting time and the storage period, on the initial composition of pigments in fruits is also studied. The provitaminic, anticancerogenic and anti-ulcer character that is now assigned to carotenoids adds considerable interest to this study (8,9).

MATERIALS AND METHODS

Materials. With the aim of knowing if there are any degradation processes of pigments during the piling up of fruits, an experimental silo of 2000 kg of ripening fruits of Verdial variety was formed before extraction. Lipoxygenase activity and pigment content were studied periodically during the storage period. Samples were taken every 8 days for a month, from three different zones of the pile: upper, mid and lower.

To study the influence of the industrial extraction process on the initial pigments of fruits, these were controlled both qualitatively and quantitatively in fruits of Hojiblanca variety and in the oil extracted from them throughout the entire season (from December to February). Samples of 1 kg of olives from the feeding line to the mill were taken every 15 days. At the same time, samples of 1 l of virgin olive oil from the extraction of such olives by a Pieralisi centrifugal system were also taken.

Measurement of lipoxygenase activity and preparation of enzyme extracts. Olives were destoned (10 g) and ground with 10 g PVP hydrate (polyvinyl polypyrrolidone, Sigma Chemical Co., St. Louis, MO) and 30 ml of 50 mM sodium phosphate buffer (pH 6.8) containing 0.3 mM DTT (1.4-Dthio+DL-threitol, Fluka AG, Buchs, Switzerland), 0.2 mM EDTA (ethylenediaminetetra-acid, Panreac), and 10 mM sodium metabisulphite (Panreac, Barcelona, Spain), in a homogenizer "Polytron" at 4°C in five portions of 20 seconds (with an interval of one minute between them). The slurry obtained was filtered through four layers of cheesecloth and centrifuged at 27,000 × *g* for 20 min. The supernatant constituted the crude enzymatic extract.

Substrate preparation. Seventy-five mg of linoleic acid 99% (*cis*-9-*cis*-12-octadecadienoic acid, Sigma) was added to 5 ml of double destilled acid deoxygenated water containing 0.09 ml TWEEN-20 (Sigma). After shaking the mixture, 0.14 ml of sodium hydroxide (2N) was added until total transparency was reached. Different 2 ml aliquots were separated from this solution and frozen at −30°C until use.

Enzyme assay. The reaction medium, in a spectrophotometer cell, consisted of 3 ml sodium phosphate

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buffer (0.2 M, pH 6.2) and sufficient enzyme extract for obtaining an increase of absorbance of 0.09 units per minute. The reaction was initiated by adding 25 μl substrate solution of linoleic acid. The final concentration of linoleic acid in the solution was $4.46 \cdot 10^{-4}$ M. Using a spectrophotometer, lipoxygenase activity was measured by the increase in absorbance at 234 nm resulting from the formation of conjugated diene in the hydroperoxidation of linoleic acid (10).

The unit of enzymatic activity is defined as the quantity of enzyme which catalyzes the formation of 1 μ mol of product in 1 min. Extract preparation and pigment separation for the analysis were made in duplicate at all times.

Chlorophyll and carotenoid evaluation and extract preparation and pigment separation. All pigment extractions were carried out either under green light or in darkness. The sample (15–30 g) was taken from one homogenate of 20–30 destoned fruits. For olive oil, 15 g were taken. The pigment extraction was made with N,N-dimethylformamide. The filtrate was then treated with hexane in a separatory funnel in order to extract and separate the fatty matter from the previous solution. Hexane, in turn, carried over the carotene fraction, while the residue in N,N-dimethylformamide retained chlorophylls, chlorophyll derivatives, and the rest of the carotenoids. Details on the extraction process are referred to in a previous work (2).

Pigment separation was carried out by thin-layer chromatography on silica gel 60 GF_{254}. The plates were dried for 1 hr at 125°C, stored in dessicators and activated for 30 min at 105°C before use. The developing solvent was light petroleum ether/acetone/diethylamine (10:4:1).

Pigment identification. The absorption spectra, as well as the color shown by these substances in TLC under white and UV light, served as a basis for identification of chlorophylls and their derivatives. For carotenoids, the adsorption properties of these pigments in TLC, before and after saponification, absorption spectra in the visible and absorption bands in IR were used. For the confirmation of functional groups, distinct physicochemical reactions, specified by Davis (11), were assayed.

TABLE 1

Scheme of the Characteristic Thin-Layer Chromatogram on Silicagel GF_{254} of Pigments from Fresh Fruit and Virgin Olive Oil (Light Petroleum Ether/Acetone/Diethylamine [10:4:1])

Band no.	Color on plate		R_f values
	Fresh fruit	Virgin olive oil	
1	Orange yellow	Orange yellow	1
2		Grey	0.57
3		Brown	0.53
4	Blue-green		0.51
5	Yellow-green		0.44
6	Light-orange	Light-orange	0.41
7	Yellow-orange	Yellow-orange	0.35
8	Yellow	Yellow	0.28
9		Yellow	0.19
10	Yellow	Yellow	0.15

Pigment quantification. Once the chromatographic development of a known quantity of pigment extract was finished, the corresponding substance was scraped from the plate, eluted with acetone or diethyl ether, and made up to a determinate volume. Next, the respective absorption spectrum was obtained, and the extinction value E_0, at the maximum absorption wavelength, was substituted in the equation $E = E_0 \cdot C$. The results were obtained in milligrams per kilogram of destoned fruit or oil.

Apparatus used. The Buchi Rotavapor, Model R110; DESAGA UV/vis lamp, provided with white light and ultraviolet $UV_{254,366}$; Hewlett Packard UV/vis spectrophotometer, Model 8450, provided with Hewlett-Packard recorder, Model 7225 A; Perkin-Elmer 782 IR spectrophotometer, with computer, Model 3600. Homogenizer "polytron," Ultraturrax T25 Janke Kunker, IKA-Laboratechnik. RC-5 superspeed Refrigerated Centrifuge SORVALL.

RESULTS AND DISCUSSION

Pigment identification. Table 1 shows the characteristics of the standard chromatogram obtained by spotting the plate with samples of the extract, purified of fatty matter, corresponding to fresh fruit and virgin olive oil.

In a previous paper (2) we studied chlorophyll and carotenoid changes during fruit growth. This study has shown that the qualitative composition is the same and does not change with ripening time. The data in Table 2 indicate that chlorophylls "a" and "b" present in the fresh fruit are degraded either partially or totally during ripening, giving rise to their corresponding magnesium-free derivatives, pheophytin "a" and pheophytin "b." Of the carotenoid fraction, β-carotene and lutein remain, so that the compounds having 5.6-epoxide groups in their molecule (such as violaxanthin and neoxanthin) have changed partially to their corresponding isomers auroxanthin and neochrome, both with 5.8-furanoid groups. In both cases the pigment transformation detected is a consequence of an acid-catalyzed reaction and might be related with an increase in free fatty acids released in the ripening fruit tissues during storage (1). Phytol-free pigment was not formed in any sample.

Lipoxygenase activity and pigment content during storage. Figure 1 shows the results obtained for the evolution of pigment and enzymatic activity of lipoxygenase in the olives piled up for a month. The carotenoid fraction is the sum of the main components, β-carotene and lutein, and the chlorophyllic fraction includes chlorophylls "a" and "b" and pheophytins "a" and "b." No differences between upper, mid and lower zones have been found, so the values given represent the average of the three levels.

During the storage period, a sudden decrease of pigment concentration was detected about 7 days after the start of the experiment. This coincides with the time of maximum lipoxygenase activity, showing the possible implication of this enzyme in the increase of the degradation rate of chlorophylls and carotenoids. After this first period, the enzymatic activity decreases and, consequently, the pigment remains almost constant.

Effect of the industrial oil olive extraction process on chlorophylls and carotenoids. Table 3 shows the changes in individual content of chlorophylls, chlorophyllic derivatives and principal carotenoids present in the fruit and

TABLE 2

Characteristics Used to Identify Pigments, Separated on Silicagel with Light Petroleum Ether/Acetone/Diethylamine (10:4:1)

Band no.[a]	Spectral abs: λ_{max} nm		IR		Epoxide test (HCl treatment)		Identity
	Light petroleum ether	Chloroform	−OH	Ester C = 0	Hypsochromic shift (EtOH), nm	Color on TLC	Carotenoids
I. Hydrocarbons							
1	(426),444,470	(434),458,476	—	—	0	Yellow	β-carotene
II. Xanthophylls							
6	(418),442,472	(430),454,482	+	—	0	Brown and green	Lutein
6′	380,400,422	388,410,436	+	—	0	Blue	Auroxanthin
7		404,428,454	+	—	20	Blue-green	Luteoxanthin
8	414,436,466	422,446,476	+	—	40	Blue	Violaxanthin
9	399,418,446	402,426,454	+	—	0	Blue	Neochrome
10	410,436,466	420,444,476	+	—	14	Blue-green	Neoxanthin

Band no.	Spectral abs: λ_{max}, nm		Identity
	Acetone	Diethyl ether	Chlorophylls
2	406,470,501,534,558,608,666	406,468,504,532,558,608,666	Pheophytin "a"
3	412,432,522,558,598,654	412,430,522,556,598,654	Pheophytin "b"
4	428,578,516,662	426,576,614,660	Chlorophyll "a"
5	454,595,644	450,594,642	Chlorophyll "b"

[a]Bands 6 and 6′ were separated on silicagel GF_{254} with benzene/ethanol (22:1).

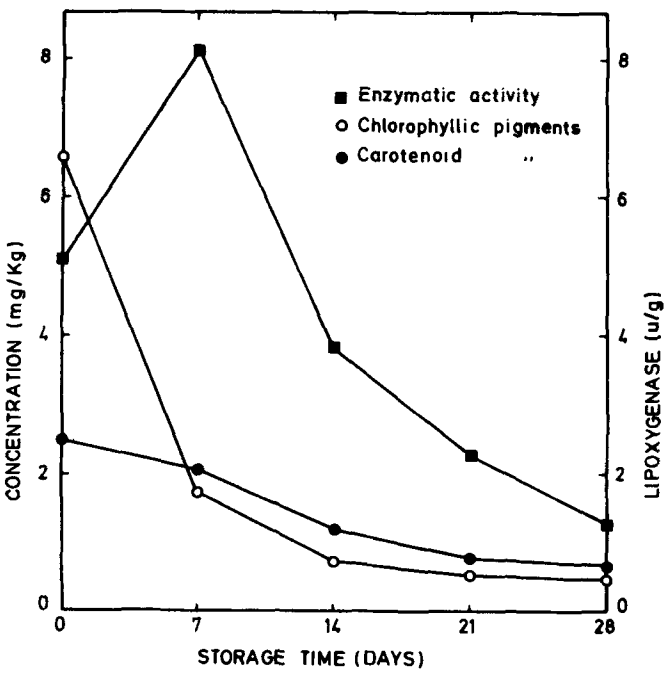

FIG. 1. Evolution of pigments and activity of lipoxygenase in the olives stored for a month.

in the oil, both obtained from an industrial oil mill, during a period of controlled harvesting. A gradual decrease in the concentration of the pigments over time is seen in all cases.

In the fruits, appreciable amounts of chlorophyll had been degraded to pheophytin, while other portions had been destroyed during post-harvesting before the oil was extracted. In the same period, β-carotene was degraded to colorless products more rapidly than lutein. The other carotenoids—violaxanthin, neoxanthin, neochrome, etc., whose concentrations were low compared to other pigments—were evaluated together.

Table 4 shows the percentage composition of pigment. It can be seen clearly that chlorophyll "a," the main component of the fresh fruit, loses ground with ripening, and practically disappears in the oil, being transformed into pheophytin "a." Lutein becomes the dominant pigment, followed by pheophytin "a" and β-carotene.

Figure 2 shows the evolution of the different fractions of total chlorophyllic and total carotenoid pigments in fruits as well as in the extracted oil during the period of olive harvesting.

The rapid diminution seen in the chlorophyllic fraction of these fruits is comparable to that observed in the olives that were piled up, the decrease of carotenoids being very much slower. From this similar evolution to that formed in the experimental silo, it can be deduced that the loss or destruction of pigments might also be due to the presence of lipoxygenase during ripening of the fruit.

Table 5 shows that throughout post-harvest time there is a substantial conversion of chlorophylls to the magnesium-free compounds, which is greater in the extracted virgin olive oil than in the fruits. At the same time, a decrease in total pigment concentration was observed. As can be seen, the destruction in chlorophylls is greater than in carotenoids.

As the olive has a fat content of around 20–30%, and the pigments are fat-soluble, the concentration of the latter in the oil should increase some 3–5 times, with respect to the values found in the fruit. The results obtained indicate that this hypothesis is not borne out by the carotenoid fraction, nor by the chlorophylls and their

TABLE 3

Pigment Content in Olives and Oils (mg/kg) from the Industrial Olive Oil Mill Throughout the Season

	Chlorophylls and derivatives				Carotenoids		
Sampling date	Chl "a"	Chl "b"	Phy "a"	Phy "b"	β-C	Lut	Rest of carotenoids
Olive fruit							
12-26-88	9.48	5.16	5.12	1.17	0.87	4.40	0.95
1-9-89	6.81	3.53	9.86	1.27	1.27	3.41	0.72
1-19-89	4.62	1.84	5.37	0.35	0.95	4.33	0.68
1-30-89	1.81	1.15	2.13	0.27	1.00	3.34	0.44
2-20-89	0.43	0.31	0.45	0.10	0.22	0.82	—
Virgin olive oils							
12-26-88	1.91	0.87	11.65	0.42	3.01	8.85	0.85
1-9-89	1.32	1.25	13.48	0.13	4.07	11.15	—
1-19-89	1.27	0.59	8.10	0.39	3.26	8.97	1.01
1-30-89	0.91	0.37	3.84	0.19	1.31	8.44	0.58
2-20-89	0.27	0.18	0.83	0.13	0.22	2.46	—

Chl "a" = chlorophyll "a"; Chl "b" = chlorophyll "b"; Phy "a" = pheophytin "a"; Phy "b" = pheophytin "b"; β-C = β-carotene; and Lut = lutein.

TABLE 4

Pigment Composition Expressed as Percentage of the Total Pigment Recovered from the Industrial Olive Oil Mill Throughout the Season

	Chlorophyll and derivatives				Carotenoids		
Sampling date	Chl "a"	Chl "b"	Phy "a"	Phy "b"	β-C	Lut	Rest of carotenoids
Olive fruits							
12-26-88	34.92	19.00	18.86	4.31	3.20	16.21	3.50
1-9-89	25.34	13.14	36.69	4.73	4.73	12.69	2.68
1-19-89	25.47	10.14	29.60	1.93	5.24	23.87	3.75
1-30-89	17.85	11.34	21.01	2.66	9.86	32.94	4.34
2-20-89	18.45	13.30	19.31	4.29	9.44	35.19	—
Virgin olive oils							
12-26-88	6.93	3.16	42.27	1.52	10.92	32.11	3.08
1-9-89	4.20	3.98	42.93	0.41	12.96	35.51	—
1-19-89	5.39	2.50	34.36	1.65	13.83	38.06	4.28
1-30-89	5.82	2.36	24.55	1.21	8.37	53.96	3.71
2-20-89	6.60	4.40	20.29	3.18	5.38	60.15	—

See Table 2 for abbreviations.

TABLE 5

Effect of the Industrial Extraction Process on Pigment Throughout the Season

	Chlorophyll conversion to magnesium-free derivatives %		Pigment destruction in oil %[a]	
Sample date	Olive	Oil	Chlorophylls	Carotenoids
12-26-88	30.05	81.28	85.81	59.13
1-9-89	51.84	84.12	84.93	43.63
1-19-89	46.96	82.03	83.00	55.57
1-30-89	44.78	75.89	80.19	56.78
2-20-89	42.64	68.09	78.14	48.47

[a]Considering a 20% fat content in all cases.

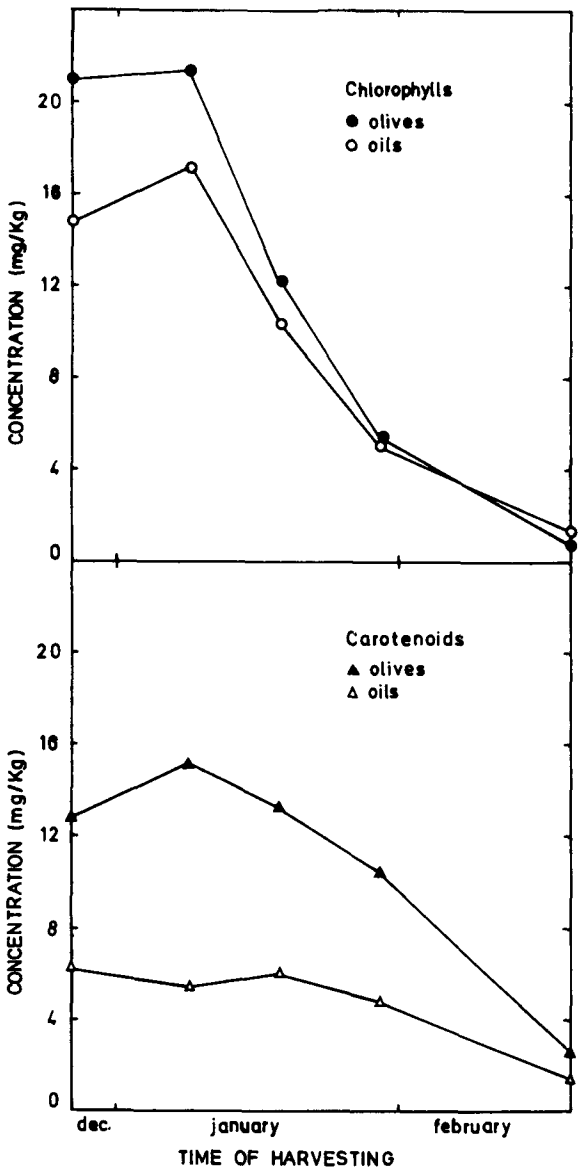

FIG. 2. Chlorophyll and carotenoid evolution during industrial extraction process.

derivatives. However, the different incidence of the extraction process on chlorophylls and carotenoids is clear.

Vitamin A value in virgin olive oil. Considering the provitamin A activity of β-carotene, the vitamin A value was calculated assuming that 0.6 μg of β-carotene is equivalent to 1 I.U. (12). Table 6 shows these data in the extracted oil and the theoretical content that this oil would have had in the case that β-carotene had passed completely to the oil (for a 20% average oil content in olives). As can be observed, there is a considerable destruction of this compound during the extraction, which, added to the destruction caused during storage, means that throughout the extraction process there is an important loss of β-carotene and, consequently, a great decrease in the potential provitamin A capacity.

TABLE 6

Effect of the Industrial Extraction Process on β-Carotene and Vitamin A Throughout the Season

	β-carotene (μg/g)		Vitamin A activity (IU/100 g)	
Date	Found	Theoretical	Found	Theoretical
12-26-88	3.01	4.35	502	725
1-09-89	4.07	6.35	678	1058
1-19-89	3.26	4.75	543	792
1-30-89	1.31	5.00	218	833
2-20-89	0.22	1.10	37	183

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