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Chlorophyll and Carotenoid Presence in Olive Fruit (*Olea europaea*)

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The high lipid content of olive fruits infers an interference in chlorophyll isolation and identification. The use of phase separation between hexane and *N,N*-dimethylformamide permits obtaining a chloroplast pigment extract free of fatty matter. In fact, *N,N*-dimethylformamide has been proven to be a good solvent for the extraction of these pigments as it does not give rise to alteration in the chlorophyll molecule. The study of chloroplast pigments (chlorophylls, carotenoids) in fruits of Hojiblanca and Manzanilla olive varieties has shown that the qualitative composition is the same for both and does not change with maturation time. During the growth and development stages, a gradual and homogeneous decrease is observed in the individual concentration of both chlorophylls and carotenoids. In all phases, Hojiblanca variety has a greater wealth of pigment than Manzanilla.

The type and amount of pigment in plant tissues depend fundamentally on the species, variety, state of ripeness, development, and cultivation conditions, etc., and, in general, undergo considerable variation during storage and preparation of the products containing them (Haard, 1982).

The olive fruit, *Olea europaea*, a well-known and widespread species of the Oleacea family, is a green drupe that begins to form after floration in May or June and ripens toward the end of autumn. During this process it darkens to purple-black, at the same time as the oil content increases (Vázquez-Roncero, 1963).

Most of the olive production is destined to obtain olive oil. However, a considerable part of it is processed to different types of table olives for direct human consumption (Fernández-Diez et al., 1985). In this case, fruits must be picked at diverse ripeness degrees, according to variety and end use, because the classification of table olives depends on the color of the skin and pulp. This color obviously is a function of the pigment present at each moment. Thus, the study of these compounds is of great interest for a good processing.

Information about the pigments responsible for the green color of olives is very limited. Determinations usually are only of total chlorophyll and carotenoid contents but are always obtained indirectly and, at times, using standards of doubtful purity. Vázquez-Roncero (1963–1965), in a extensive review of olive composition, mentioned only one work on carotenoids in green fruits, because until then the research emphasis had been in relation to olive oil, gave a list of the determinations done, and showed that the xantophyll fraction is higher than the β-carotene. The same author mentions that Macagno (1881) was the first to attribute the olive oil color to chlorophyll, which was later identified by Vitagliano in several oils from different origins. The last study concluded by establishing that the concentration of such substances depended on biological and technological factors but is always low because they are easily degradated to the corresponding pheophytins.

When the fruits are mature, the violet or purple color is due to the formation of anthocyanins, which appear at the end of the maturation process.

Also, studies carried out on olive leaf by Niavis and Kousounis (1981a,b) have shown that the seasonal changes have a great influence on chloroplast pigment counts.

Consequently, the aim of this work was to study in depth the isolation, identification, and evolution of the individual chloroplast pigments present in these fruits during their growth, development, and ripening stages.

MATERIALS AND METHODS

Raw Material Used. The study was carried out on fruits of Manzanilla (*Olea europaea pomiformis*) and Hojiblanca (*Olea europaea arolensis*) varieties, from olive trees of the Agricultural Experimental Station of Cabra (Córdoba), specially dedicated to this study.

Sampling was made at intervals of approximately 10 days from the beginning of September to the middle of February during two consecutive years. The fruits were picked from all around the tree, until an approximately

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1-kg sample was collected. At each date 100 olive fruits were randomly selected in order to evaluate the most representative color at that moment, according to the maturation index introduced by Walali et al. (1984). They were, by the time sequence of evolution, as follows: intense green, green, yellowish green, small reddish spots, and turning color.

For each maturation degree six samples were studied (three each year), and for each analysis, 10 fruits of the most representative color were used.

Analysis of Pigments. The complex composition of olive fruit (Vázquez-Roncero, 1963) creates a serious problem for chlorophyll isolation and quantification, since the characteristic fatty matter of the fruit (15–30%) interferes with the application of the usual techniques recommended in the bibliography. The AOAC (1960) spectrophotometric methods for the determination of mixtures of chlorophylls and their derivatives in solution, taking into account the modifications made by Jones et al. (1962) and White et al. (1972), are not applicable because of emulsion formation, which makes them slow and more difficult, preventing the measurements from being reproducible and thus reliable.

Assays carried out by column chromatography, both with Shepadex LH-20 as indicated by Downey et al. (1970), Iriyama and Yoshiura (1979), and Shimizu (1971) and with Lobar silica gel (Jong and Woodlief, 1978), did not give the expected results, since although the fatty matter is eliminated, the retention times are excessively long, causing pigment alteration.

Thin-layer chromatography of the initial acetone pigment extract, using various supports and specific eluents, were tried (Choudhury and Bajaj, 1979; Iriyama et al., 1980; Loevenschuss and Wakelyn, 1973), as well as different chromatographic techniques (Jones et al., 1972; Scholz et al., 1981; Sievers and Hynninen, 1977). Pigment separation was achieved, although its quantification still remained very difficult because of lipid presence.

Thus, in order to achieve the above-mentioned objective, it was necessary to develop a method permitting a pigment extract free of fatty substances, making possible the identification and quantification of individual chlorophylls as well as the carotenoids (Minguez-Mosquera and Garrido-Fernández, 1985).

Preparation of Extracts Purified of Fatty Material. From 5 to 30 g of stoned fruit, according to the ripening degree, was weighed and transferred rapidly to a beater vessel containing 100 mL of *N,N*-dimethylformamide saturated with $MgCO_3$ (0.1 g of $MgCO_3$ in 250 mL of *N,N*-dimethylformamide), to prevent pheophytin formation. This was triturated for 1 min with the aid of a beater and filtered on anhydrous Na_2SO_4. The solid residue was collected and the operation repeated until the filtrates were colorless. The extracts were combined and transferred to a separatory funnel in which 70 mL of hexane had previously been placed. After shaking for 1 min, it was left to settle, approximately 10 min, until there was complete phase separation. The upper hexane layer, with light yellow color, retained the lipids. The lower phase, an intense green solution of pigments in *N,N*-dimethylformamide, was transferred to another funnel containing another 70 mL of hexane and extracted again to eliminate any lipid residues.

The green layer was transferred to a 1000-mL funnel containing 400 mL of 2% sodium sulfate solution at approximately 0 °C. A 100-mL portion of hexane was added, and the mixture was left to settle for 5 min. Ethyl ether (60 mL) was added, shaken, and held until phases were separated (about 15 min). The aqueous layer was discarded. The fraction of polyphenols, among other water-soluble compounds, was eliminated in this phase. The organic phase with chloroplast pigments in solution was transferred to a rotavapor flask through anhydrous Na_2SO_4. The residue was finally washed with 40 mL of ethyl ether. The clear, water-free solution was concentrated in a rotavapor at a temperature below 30 °C. The dry residue was dissolved in 5 mL of ethyl ether (solution A).

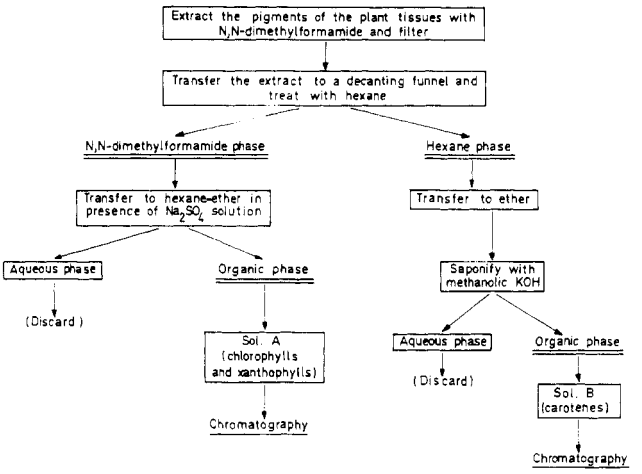

Figure 1. Extraction procedure for purification of chloroplast pigments.

The hexane phases were mixed in a funnel, transferred to 100 mL of ether, and saponified with 100 mL of 20% KOH in methanol, and strongly shaken to hydrolyze the lipids and purify the possible carotenoids. After 1 h, distilled water was added and held until phases separated. The aqueous phase was rejected. The ether phase was washed successively with distilled water to neutral pH, transferred through anhydrous Na_2SO_4, and evaporated to dryness at a temperature below 30 °C at reduced pressure. The dry residue was dissolved in 5 mL of ethyl ether (solution B).

Pigment extraction with acetone was carried out by a standard procedure according to Smith and Benitez (1955) (solution C).

All operations were carried out under diffuse green light to prevent pigment alteration, and all final solutions were held refrigerated at a temperature of about 0 °C. For each sample, two extractions were always made.

The extraction procedure is given in Figure 1.

Separation and Identification of Pigments. Separation of pigments was carried out by TLC using Kieselgel $60GF_{254}$, plates 0.5 mm thick (Merck, Art. No. 5744). The developing solvents used were petroleum ether (65–95 °C)/acetone/diethylamine (10:4:1) and petroleum ether (65–95 °C)/benzene (2:3).

Identification of chlorophylls was made by absorption spectra in both visible and UV light (Smith and Benitez, 1955).

Standards for chlorophyll *a*, No. C-6144 from algae, and chlorophyll *b*, No. C-5878 from spinach, were supplied by Sigma.

For identification of carotenoids, visible and IR spectra were determined, before and after saponification (Davies, 1976; Foppen, 1971). For confirmation of functional groups, the different physicochemical reactions were used according to Moss and Weedon (1976).

The standard of β-carotene was supplied by Roche. Standards for the rest of carotenoids were obtained in the laboratory from spinach and alfalfa leaves.

Table I. Characteristics Used To Identify Olive Pigments

band, no.	spectral absorption max, nm: light petroleum ether	spectral absorption max, nm: chloroform	IR band: -OH	IR band: ester C=O	epoxide test (HCl treatment): hypsochromic shift (ethanol), nm	epoxide test (HCl treatment): color on TLC	carotenoid identity
		I. Hydrocarbon					
1	(426), 444, 470, 330, 348, 367, 378, 400, 422	(434), 458, 482, 384, 405, 430	-	-	0	yellow	β-carotene phytofluene ζ-carotene
		II. Xanthophylls					
4	418, 442, 470	430, 452, 482	+	-	0	brown and green	lutein
5		404, 428, 456	+	-	20	blue	luteoxanthin
6	414, 436, 466	422, 446, 476	+	-	40	blue	violaxanthin
7	412, 436, 466	420, 444, 474	+	-	14	blue-green	neoxanthin

band, no.	spectral absorption max, nm: acetone	spectral absorption max, nm: diethyl ether	chlorophyll identity
2	428, 578, 616, 662	426, 576, 614, 660	chlorophyll *a*
3	454, 595, 644	450, 594, 642	chlorophyll *b*

[a] Band 1 is from hexane (solvent B). The others are from *N,N*-dimethylformamide (solvent A). Compounds of band 1 were separated on silica gel 60 GF_{254} with light petroleum ether as solvent. For the others, petroleum ether/acetone/diethylamine (10:4:1) were used.

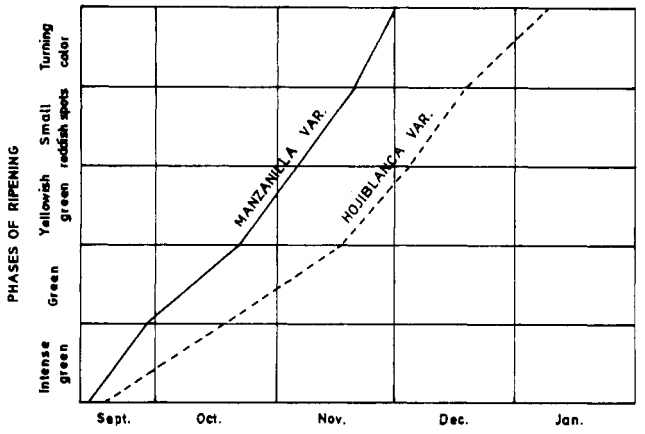

Figure 2. Influence of seasonal changes on the apparent ripening degree of fruits.

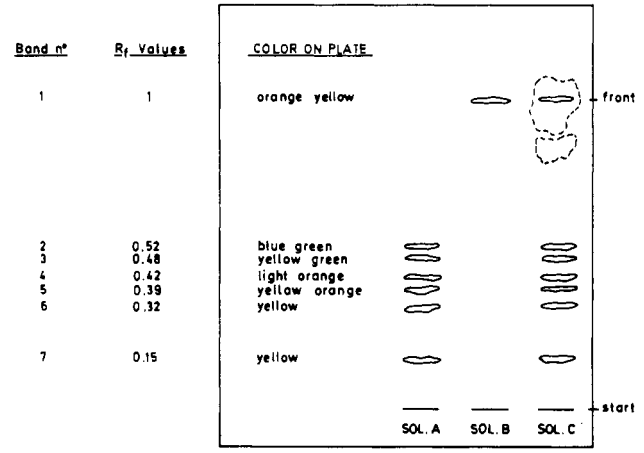

Figure 3. Characteristic thin-layer chromatogram on silica gel 60 GF_{254} of chloroplast pigments from green tissues of olives. Solvent systems: petroleum ether (65–95 °C)/benzene (2:3) for removing the lipids and petroleum ether (65–95 °C)/acetone/diethylamine (10:4:1) for pigment separation. Solutions: A, *N,N*-dimethylformamide phase; B, hexane phase; C, acetone extracts.

Quantification. For quantification, the corresponding substance was scraped from the plate, eluted with the appropriate solvent, and made up to a determinate volume. Next, the respective absorption spectrum was obtained, and the extinction value at the maximum absorption wavelength was substituted in the equation $E = E_0C$. Once the appropriate operations had been carried out, the results were given as milligrams per kilogram of stoned fruit.

Apparatus used: Buchi Rotavapor, Model R 110; DESAGA UVIS lamp, provided with white light and ultraviolet $UV_{254,366}$; Hewlett-Packard UV/vis spectrophotometer, Model 8450, provided with Hewlett-Packard recorder, Model 7225A; Perkin-Elmer 782 IR spectrophotometer, with computer, Model 3600.

RESULTS AND DISCUSSION

The different ripening points of fruit, established according to the index mentioned above (Walali et al., 1984), are shown in Figure 2. Manzanilla is an early variety, while Hojiblanca is late. This difference justifies that the research for following the evolution of pigments was planned considering surface color changes instead of just the dates when the samples were collected, since the aim was to be able to verify a comparative study associated with the distinct ripening phases.

Figure 3 shows the chromatogram type obtained by spotting the plate with the lipid-free extracts (solutions A and B) and by the corresponding standard technique (solution C). Visual analysis of the chromatograms by natural and UV light demonstrates the similarity between the pigment composition of both extracts, which is indicative that *N,N*-dimethylformamide is a good solvent for the extraction of chloroplast pigments. The result of research carried out for the characterization of components is shown in Table I. According to the identification study, the *N,N*-dimethylformamide fraction (solution A) retains the chlorophylls (*a*, *b*) and xanthophylls (lutein, luteoxanthin, violaxanthin, neoxanthin). The hexane phase (solution B) has the carotenes, which advance together in the development front.

Solution C needs a double development. The first one, using petroleum ether/benzene (2:3), to eliminate the fatty components that must advance with the front also carry the carotenes. The second development, using the same developer as for solutions A and B, separates the chlorophylls and xanthophylls, as has been explained above. But usually this chromatogram is not good enough for separation and individual quantification, because the fatty substances are often retained along the plate, giving less resolution and disturbing the analysis.

The identification study of bands corresponding to solutions B and C (after saponification) showed that both

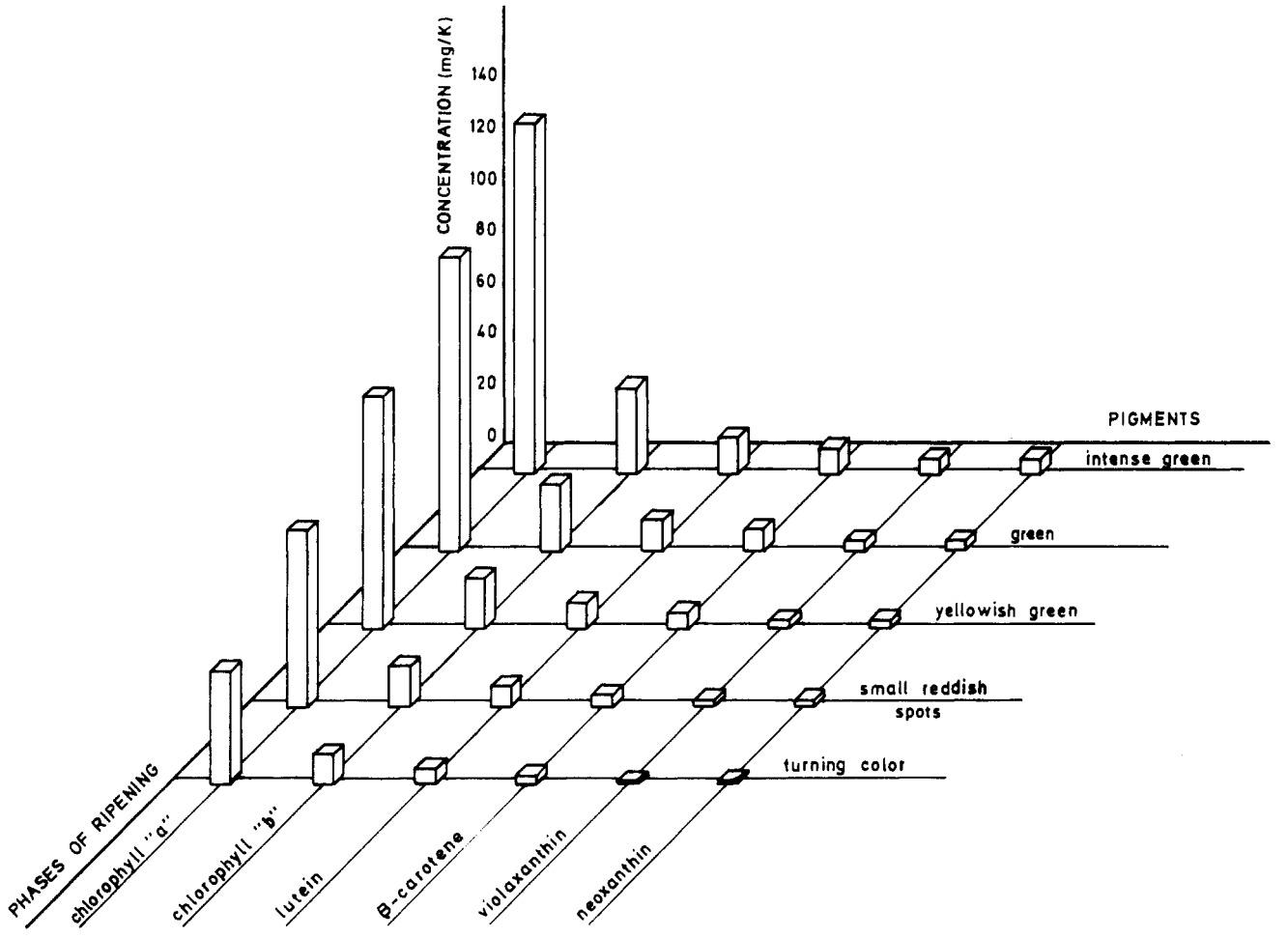

Figure 4. Evolution of individual concentration of chloroplast pigments in the distinct ripening degrees for Manzanilla variety.

Table II. Quantitative Distribution (%)[a] of Chloroplast Pigments of Tissues of Olives from Hojiblanca and Manzanilla Varieties, According to the Different Ripening Degrees

phase of ripening	chlorophyll (±CL)		carotene (±CL):	xanthophyll (±CL)		
	chl *a*	chl *b*	β-carotene	lutein	violaxanthin	neoxanthin
			Hojiblanca Variety			
intense green	66.18 ± 0.35	17.28 ± 0.34	4.04 ± 0.14	6.73 ± 0.38	2.83 ± 0.14	2.88 ± 0.16
green	67.66 ± 0.57	16.27 ± 0.62	3.82 ± 0.20	6.96 ± 0.31	2.87 ± 0.29	2.41 ± 0.25
yellowish green	67.99 ± 1.28	16.83 ± 1.14	3.85 u 0.17	6.68 ± 0.23	2.49 ± 0.22	2.15 ± 0.19
small reddish spots	66.85 ± 1.23	16.75 ± 1.56	4.08 ± 0.29	7.57 ± 0.45	2.42 ± 0.57	2.49 ± 0.36
turning color	62.45 ± 1.20	18.16 ± 0.54	5.19 ± 0.28	9.87 ± 0.57	2.06 ± 0.35	2.61 ± 0.37
			Manzanilla Variety			
Intense green	68.17 ± 0.62	15.95 ± 0.91	3.95 ± 0.23	7.19 ± 0.59	2.42 ± 0.23	2.40 ± 0.26
green	67.24 ± 0.60	17.81 ± 0.88	3.55 ± 0.13	6.47 ± 0.12	2.50 ± 0.10	2.43 ± 0.52
yellowish green	66.80 ± 0.78	17.97 ± 0.47	3.56 ± 0.27	6.63 ± 0.48	2.53 ± 0.30	2.51 ± 0.94
small reddish spots	66.91 ± 2.18	18.06 ± 1.54	3.98 ± 0.38	7.44 ± 0.62	1.86 ± 0.37	2.04 ± 0.27
turning color	62.98 ± 2.48	20.09 ± 1.45	4.57 ± 0.80	8.50 ± 1.98	1.99 ± 0.35	1.88 ± 0.42

[a] Expressed as percentages of total pigments. Average of duplicate analyses of six samples. CL = confidence limits (p = 0.05).

have the same carotenes, β-carotene, phytofluene, and ζ-carotene (Minguez-Mosquera and Garrido-Fernández, 1986).

The chloroplast pigments identified in olives are those of all green plant tissues and do not undergo any change or modification during the stages of ripening studied.

Figure 4 shows the evolution of concentrations of the principal pigments in the distinct ripening states for Manzanilla variety. Hojiblanca variety follows the same pattern. The carotenoids, ζ-carotene, phytofluene, and luteoxanthin, which are present in very small proportion, are not taken into account for this quantification study. A gradual decrease can be seen in the fruit pigment content with time. Both chlorophylls and carotenoids decrease with seasonal changes, almost disappearing at the moment of maturity, giving way to anthocyanic components that begin to appear as reddish surface marks, little by little invading the skin and, later, the whole pulp (Vázquez-Roncero et al., 1974). When the fruits reach complete maturation, these pigments have not been detected, even with the sample weight increasing to over 30 g.

Table II establishes the average percentage distribution of the pigments for both varieties in the distinct stages studied. Obviously, chlorophyll *a* is the majority component at all times, followed by chlorophyll *b*. As minor components, carotenoids have been found, lutein being the main xantophyll and β-carotene the principal carotene. Violaxanthin and neoxanthin are in similar proportions.

It is not easy to define the ripening process as a function of the evolution of pigments in plants. According to

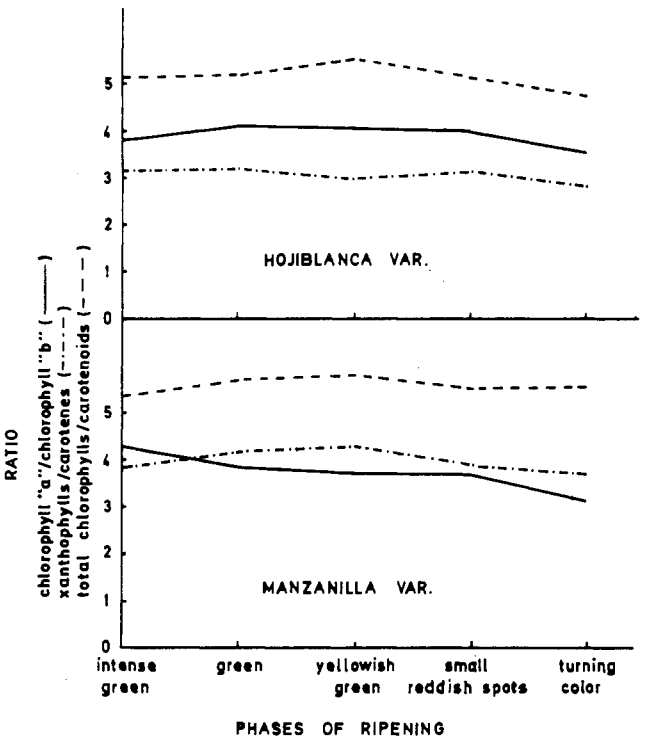

Figure 5. Ratio changes between the main pigment fractions in the course of maturation of olive fruit.

Mackinney (1961), the occurrence of carotenoids is associated with chlorophyll in all photosynthetic tissue. Most fruits are green when unripe. As they ripen, photosynthetic activity decreases and chlorophyll disappears. It is probable that most of the associated carotenoids also disappear. At the same time, the color of the skin changes from green to yellow, reddish, or red.

During this process the concentration of carotenoids and chlorophylls diminishes, while the proportion of xantophylls, which are almost always esterified in these fruits, often increases. The carotenoid pattern is very different from what is was prior to loss of the chlorophylls (Goodwin, 1980).

A sequence of events in the ripening of fruit can certainly be described in which growth is combined with changes in flavor, texture, color, etc. According to Simpson et al. (1976), long before this senescence, many fruits have become devoid of chlorophyll, and chloroplasts have been replaced by chromoplasts. Thus, in a series of complicated events, chlorophyll degradation occurs during the biosynthesis of carotenoids and/or anthocyanins or betalains.

In general, there is agreement among the different authors that there is more than one mechanism to define the ripening process.

Bearing in mind the considerations mentioned above, the yellowish coloration step of olives must be considered as a stage previous to ripening. Chlorophyll degradation is accompanied by the synthesis of other compounds, anthocyanins, because the carotenoids do not produce the final pigmentation of the ripe fruits, which is reddish or purple.

The biological process of ripening implies an analogous sequence for both varieties. The skin color changes are explained by fact that the percentage of each pigment and the ratios between them remain almost invariable with time (Figure 5) in addition to the concentration decrease undergone by all of them. Changes in surface tonality from very green to yellow-green are not due to an increase in the carotenoid fraction—an increase that in the majority of fruits determines and characterizes this coloration—but

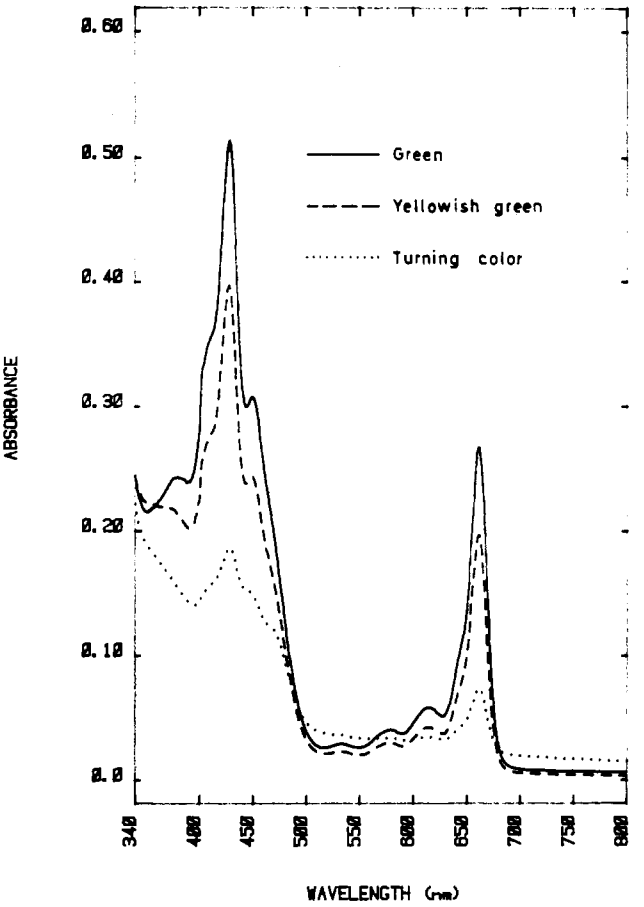

Figure 6. Absorption spectra of crude pigment extracts corresponding to distinct ripening degrees of olive fruit.

Table III. Occurrence of Total Chloroplast Pigments of Tissues from Olives of Manzanilla and Hojiblanca Varieties, According to the Different Ripening Degrees

	av total pigment (±CL),[a] mg/kg	
phase of ripening	Hojiblanca	Manzanilla
intense green	208.25 ± 7.82	185.60 ± 10.62
green	165.79 ± 7.35	137.49 ± 10.36
yellowish green	132.16 ± 11.54	103.49 ± 9.74
small reddish spots	101.93 ± 6.20	69.68 ± 5.22
turning color	70.92 ± 7.66	45.52 ± 5.09

[a] Stoned fresh olives basis, averages of duplicate analyses from six samples. CL = confidence limits (p = 0.05).

to the effect of dilution of all the components in the fruit interior.

The identification study of the group of carotenoids present in the olive (Table I) showed that these are found unesterified at all times. Consequently it can be deduced that both the chlorophylls and the carotenoids remain in the complex photosynthetic apparatus, forming individual balanced cells in fixed proportions, which explains why the relation between these pigments is maintained practically constant throughout ripening. The decrease observed in individual content of all the pigments is due to the fruit's moderating the main function of growth in favor of the transformations the subsequent ripening process implies. In Figure 6 it can be observed how the absorption spectra of crude chloroplast pigment extracts corresponding to distinct ripening states, obtained in analogous conditions, maintain the shape and absorption maxima at the same wavelengths, only the peak heights being different.

Until now, the remarks made are equally applicable to Hojiblanca and Manzanilla varieties. The only difference found between these varieties is the distinct quantitative

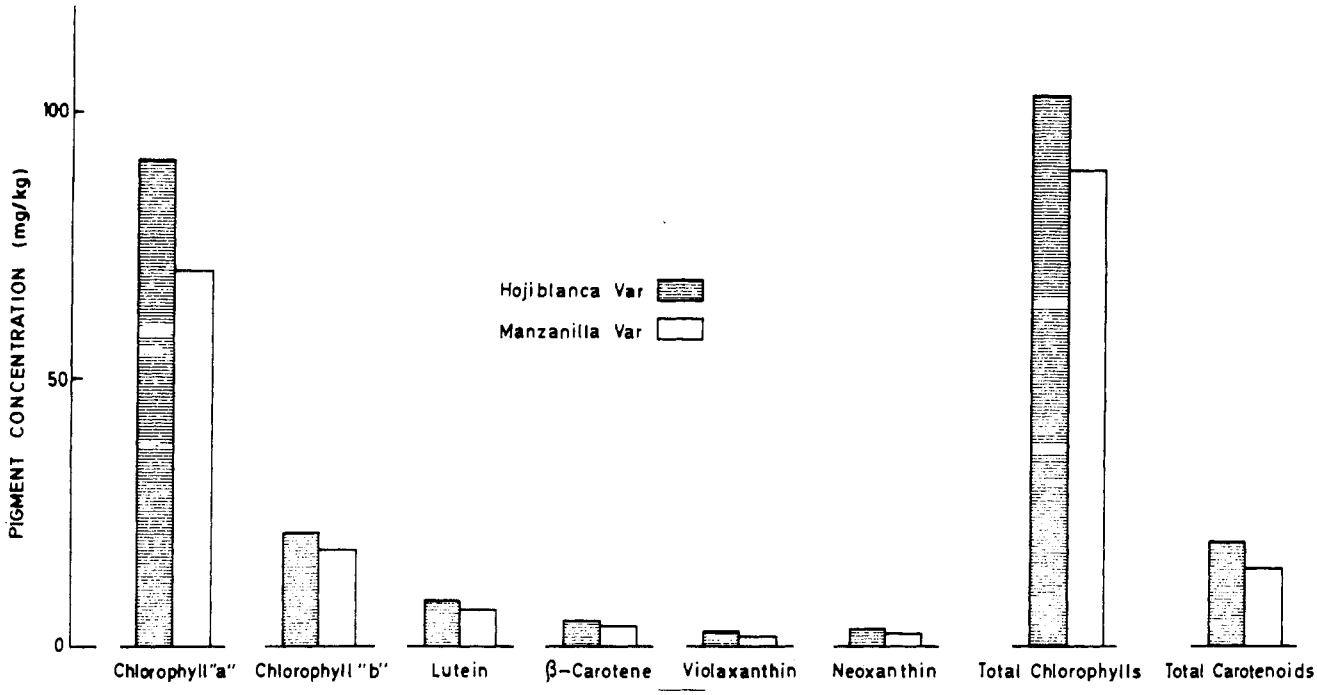

Figure 7. Individual and total pigment concentrations in tissues of olives for the ripening degree yellowish-green, Hojiblanca and Manzanilla varieties.

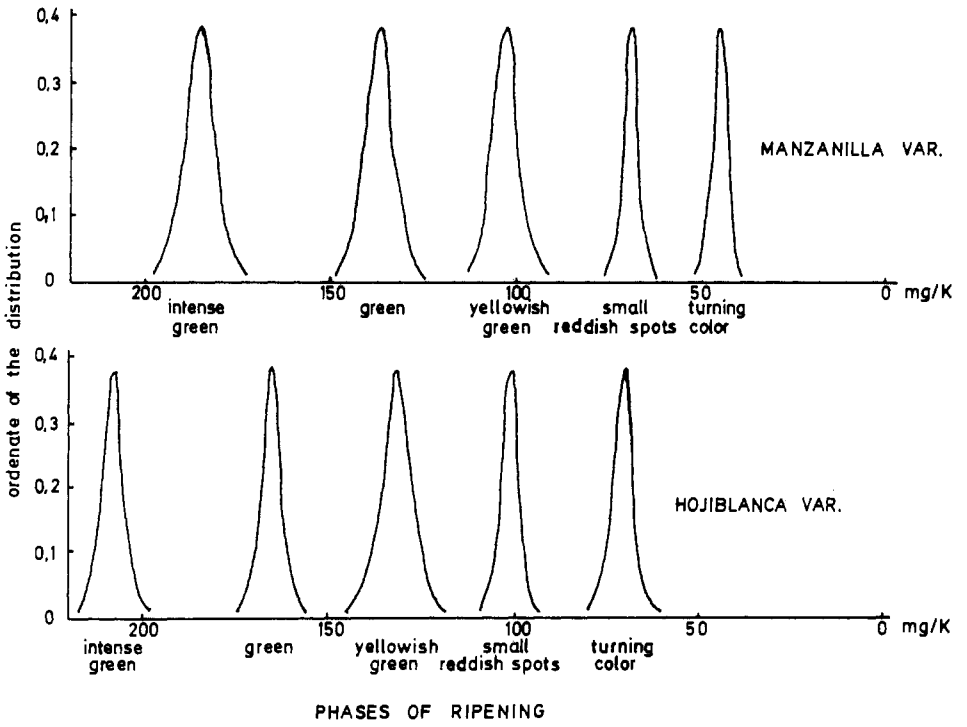

Figure 8. Normal distribution curve for total pigment concentrations according to ripeness degrees.

contribution of pigments during all the phases studied throughout two consecutive seasons. In order to illustrate this, Figure 7 shows the individual mean concentrations of the different pigments for fruits collected with the superficial color corresponding to the ripening degree appropriate for elaboration of green table olives. Hojiblanca variety always maintains higher pigment levels than Manzanilla at all stages.

Bearing in mind this behavior, it is possible to distinguish each ripening degree for both varieties by their total pigment concentration only. Thus, Table III includes the corresponding total means for Hojiblanca and Manzanilla varieties. As can be deduced by observing the confidence limits, the means are significantly different.

Figure 8 is a graphic representation of the distribution of means of these total pigment concentrations for each variety and every color. The normal curves have been drawn with the standard errors of the different ripening degrees (Box et al., 1978).

The fact that there is no overlap between the different curves indicates also that they are of different populations, and the displacement of the population means on comparing homologous states between the varieties proves the pigment difference between both.

From this it can be deduced that, according to present knowledge, the distinct shade in surface coloring shown by fruits from the olive varieties under study is in each case a consequence of the combined effect of the distinct con-

centration of pigments and of the many other components forming the olive, mainly the oil.

Registry No. Chl *a*, 479-61-8; chl *b*, 519-62-0; β-carotene, 7235-40-7; lutein, 127-40-2; violaxanthin, 126-29-4; neoxanthin, 14660-91-4.

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