Change in the Natural Ratio Between Chlorophylls and Carotenoids in Olive Fruit During Processing for Virgin Olive Oil

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ABSTRACT: Different varieties of olives from different sources, harvested in a similar ripeness state, have been characterized by their chlorophyll and carotenoid pigment profile and content. Pigment richness is inherent to the variety and enables great differences or similarities to be established between them. In all the fruits, ripening involves pigment loss, with the disappearance of chlorophylls always being slightly greater than that of carotenoids. However, independently of variety and the different pigment content of the fruits, the ratio between chlorophylls and carotenoids tends to remain more or less constant, within a range of 2.5 to 3.7 mg total chlorophyll/mg total carotenoid. Pigment loss caused by the extraction process is more marked for the chlorophyll fraction than for the carotenoids, changing the ratio in the oil to around one unit, whatever the variety of source fruit.

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KEY WORDS: Chlorophylls/carotenoids, degradation, *Olea europaea*, olive fruits, olive varieties, pigments, processing, ripening, virgin olive oils.

During the last century, the study of chlorophylls and carotenoids in different kinds of fruit has been of interest to many researchers (for review see Ref. 1,2).The outstanding and attractive coloration that these compounds confer in foodstuffs has long been valued. Extensive research has shown that these natural pigments have roles in food technology beyond coloring, apart from biological and nutritional properties (3,4). During ripening or as the result of different treatments, chlorophylls and carotenoids in fruits and vegetables undergo qualitative and quantitative changes that are indicators of the quality of the finished product (5,6). Each transformation is the result of a particular cause. Consequently, it is essential to know both the content and the class of pigments in the fresh vegetable material, and the transformations of these compounds associated with the processing system. Only then can color evaluation be completely guaranteed, above all in a manufactured product. The color as a quality, and the quality of this color, must be thought of as inseparable.

Seeking an index of ripeness, Song *et al.* (7) monitored the chlorophyll content in three varieties of apple (Starking Delicious, Golden Delicious, and Law Rome) by using fluorescence spectroscopy. Reay *et al.* (8) analyzed the change in concentration of chlorophylls, carotenoids, and anthocyanins in the skin of Gala apples during ripening; the carotenoid/chlorophyll ratio remained relatively constant until approximately 140 d after flowering, when it increased sharply, coinciding with an increase in anthocyanin synthesis. Mercadante and Rodriguez-Amaya (9) established a single qualitative carotenoid profile for two varieties of mango (Keitt and Tommy Atkins) during ripening and found quantitative differences between the two varieties (up to 25%). Mínguez-Mosquera and Hornero-Méndez (10) studied the effect of processing different varieties of pepper (Bola and Agridulce) for paprika on the initial carotenoid content. They concluded that the different stabilities found and associated with each carotenoid family are due mainly to intrinsic factors of the variety rather than to processing factors.

The fruit of the olive (*Olea europaea* L.) is highly appreciated for its double utility. Certain varieties, because of their characteristics, are processed as green or black table olives (10% of the olive production). Virgin olive oil is extracted from others by a mechanical milling process, beating, and centrifugation to yield virgin olive oil (90% of the production). As olive-growing spread from its origins, outstanding individuals were selected in each particular area for productivity, fruit size, oil content, and adaptation to the environment. The recurrence of the procedure—geographic dispersion, hybridization, breeding, and cloning—has resulted in a great diversity of local cultivars in oil-producing regions of the world.

Color of virgin olive oil has an obvious effect on consumer preference and acceptance. Because olive oil is a natural product, its color is due exclusively to the solubilization in it of the liposoluble chlorophylls and carotenoids present in the fresh fruit.

Interest in the study of chlorophyll and carotenoid composition in edible products from the olive is relatively recent. Analysis of chlorophylls in a lipid matrix required specific adaptation of methodologies (11). However, once the initial problems had been overcome, considerable advances were made in identifying these compounds in the fruits, and in monitoring their changes during ripening and processing (12,13). In the case of virgin olive oil, analysis of the pigment content and class in fruits and oil shows that the extraction process involves a structural transformation of pigments caused by the liberation of acids and a considerable loss of

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pigmentation, particularly from the chlorophyll fraction (14). Working in this field, Papaseit (15) used visible light absorbance measurements to compare an index of carotenoid color with chlorophyll color in oils obtained using different extraction systems and at different temperatures, and related color and quality of the oil. Ranalli (16) used thin-layer chromatography to study the variation in carotenoid composition in virgin olive oils of different qualities, relating it to the oil extraction methods used. More recently, Escolar *et al.* (17) proposed that the chromatic parameters defining the color of a virgin olive oil could be related to its quality. Ranalli and Modesti (18), studying olive oils extracted by different methods from six different varieties, concluded that the effect of genetic or intrinsic factors (olive variety) on olive oil color is more important than that of the technology used.

An earlier work on the characterization of the chlorophyll and carotenoid composition of single-variety virgin olive oils showed that, independently of the source variety and of whether the total pigment content was high or low, the ratio between the two isochromic fractions of pigments (chlorophyll and carotenoid) seemed to remain constant at a value close to unity (19).

Given that the content and class of pigments in a foodstuff of plant origin must be related directly to the pigments present in the raw material and to transformations during processing, the present work attempts to establish the ratio between the chlorophyll and carotenoid in fruits of different olive varieties in different states of ripeness and the pigments transferred to the oils extracted from these fruits.

EXPERIMENTAL PROCEDURES

Raw materials. The study was carried out with fruits of five varieties of olive, *O. europaea* (L.), that are used for oil production. They were selected both as being representative of different Spanish producing-regions and for their importance in crop area and amount of oil produced. The Hojiblanca and Picual varieties are typical of Andalucia (southern Spain) and come from Cabra (Córdoba); the variety Arbequina, originally from Lérida (northeast Spain), has been grown in Andalucia in the last few years, and the fruits for the present study are also from Cabra (Córdoba); the variety Blanqueta originates from Valencia (eastern Spain); the variety Cornicabra is from Toledo. Sampling was carried out during fruit ripening in the harvesting season 1998–1999, beginning when the developing fruit was still green and finishing when it was ripe (detected by the overproduction of anthocyanins). For each sampling, fruits were collected from around the whole perimeter of the tree to make up a 1-kg sample, and 100 fruits were chosen at random to evaluate the most representative color. The sequence of color changes was green, light green, small reddish spots, turning color, purple, and black (20). The light green stage is not the same for all the varieties; for instance Arbequina has a homogeneous yellowish color, whereas Blanqueta is whitish. The morphological characteristics of the fruits are not the same, enabling varieties to be distinguished by direct observation: the average fruit and stone weights in Arbequina and Blanqueta are similar (1.637 and 1.639 g, respectively, for fruit, and 0.352 and 0.300 g, respectively, for stone) and much smaller than in Cornicabra, Picual and Hojiblanca, which are themselves comparable (2.665, 2.939, and 4.080 g, respectively, for the fruit, and 0.737, 0.588, and 0.605 g, respectively for the stone).

Extraction of pigments. Samples were made from a triturate homogenized from 50 de-stoned fruits (*ca.* 40 g) of the most representative color by accurately weighing from 4 to 15 g for each analysis according to the degree of ripeness of the fruits. Pigment extraction was performed with *N*,*N*-dimethylformamide (DMF) according to the method of Mínguez-Mosquera and Garrido-Fernández (11). The technique is based on the selective partition of components between DMF and hexane. The hexane phase carries over lipids and the carotene fraction, while the DMF phase retains chlorophylls and xanthophylls. This system yields a pigment extract that is free of the fatty matter which is characteristic of these fruits and which interferes with subsequent separation and quantification. All analyses were performed in triplicate under a green light. Details about the pigment identification have been described in previous papers (19,21,22).

Pigment separation and quantification. This was carried out by high-performance liquid chromatography using a Hewlett-Packard (Palo Alto, CA) model HP 1100 liquid chromatograph fitted with an HP 1100 automatic injector and diode array detector. Data were collected and processed with a liquid chromatography HP ChemStation (revision A.05.04). A stainless steel column (25×0.46 cm i.d.), packed with 5 μm C_{18} Spherisorb ODS-2 (Teknokroma, Barcelona, Spain) was used. The column was protected with a precolumn (1×0.4 cm i.d.) packed with the same material. The solution of pigments in acetone was centrifuged at $13,000 \times g$ (Model micro centaur; MSE, Crawley, England) prior to injection into the chromatograph (20 μL). Separation was performed using an elution gradient (flow rate 2 mL min^{-1}) with the mobile phases (A) water/ion pair reagent/methanol (1:1:8, vol/vol/vol) and (B) acetone/methanol (1:1, vol/vol). The ion pair reagent was 0.05 M tetrabutylammonium acetate (Fluka Chemie AG, Buchs, Switzerland) and 1 M ammonium acetate (Fluka) in water. The gradient scheme has been described in detail in a previous work (21). Detection was simultaneously performed at 410, 430, 450, and 666 nm. External standard calibration was used for quantitation. The results were given as milligrams per dry kilogram of de-stoned fruit.

RESULTS AND DISCUSSION

Total pigment content by variety and by state of fruit ripeness. The chlorophyll and carotenoid pigment profiles are similar for the fruits of the five varieties analyzed and include chlorophylls *a* and *b*, lutein, β-carotene, neoxanthin, violaxanthin, antheraxanthin, and β-cryptoxanthin. The sum of chlorophylls *a* and *b* constitutes the chlorophyll fraction and similarly, that of lutein, β-carotene, neoxanthin, violaxanthin, an-

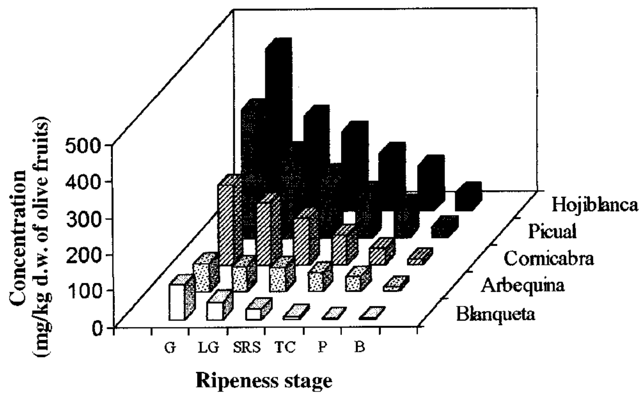

FIG. 1. Changes in the total pigment content (total chlorophylls + total carotenoids) against ripeness stage in the fruits of the different olive varieties. G, green; LG, light green; SRS, small reddish spots; TC, turning color; P, purple; B, black; d.w., dry weight.

theraxanthin, and β-cryptoxanthin constitutes the carotenoid fraction.

Figure 1 shows the relation between the total pigment content (total chlorophylls plus total carotenoids) and the ripening stage in the fruits of the different varieties studied. In general, the pigment concentration decreases gradually with ripening in all varieties, and then decreases sharply in the final stages, when the synthesis of anthocyanins intensifies. The rate of this loss seems to differ with variety. At the same time, the pigment richness of the fruits is apparently inherent to each variety, and the differences enable distinctions to be established between them. The classification made in the green fruit is maintained in all the ripeness states studied. In the final state of fruit ripeness, these differences are maintained or diminished depending on variety. Hojiblanca has the highest pigment content, followed closely by Picual, then Cornicabra, and, with much lower pigment concentration, Arbequina and Blanqueta. For instance, a comparison of pigment in the green stage shows Hojiblanca to have almost fivefold more than Blanqueta (440 mg/kg dry weight of olive fruits against 93 mg/kg), the other varieties being situated within this wide concentration range. These observed differences were maintained to a greater or lesser extent in all the ripeness stages studied.

Up to the purple stage, Duncan's test ($P < 0.05$) indicates that in terms of pigment content Hojiblanca and Picual varieties, both with the highest content, are statistically the same, but different from the others. Similarly, Arbequina and Blanqueta varieties can be grouped as statistically similar to each other but different from Cornicabra.

In terms of ripeness stage, Arbequina and Blanqueta are not different in the green fruit (80–90 mg/kg), but they do differ with ripening, as the rate of pigment loss in Blanqueta is higher than in Arbequina. Although the initial concentrations in the green fruit are similar, in the purple stage the Blanqueta variety presents 3.5 ± 0.6 mg/kg against the 40.5 ± 5.4 mg/kg of Arbequina. Similarly, when the Cornicabra variety reaches advanced ripeness states, such as purple (43.5 mg/kg), it is similar to the Arbequina variety, because the rate of pigment degradation in the former variety is much higher than in the latter. The Hojiblanca and Picual varieties maintain their differences from the others throughout ripening, although in the last stage they also undergo a sharp decrease in pigment concentration. The most outstanding differences between varieties is observed in the Hojiblanca variety, having fruits in the black ripening stage with a similar pigment concentration to those of Blanqueta in the light green stage.

It can be concluded that there are great differences in fruit pigment content between varieties, and that for each variety, this concentration varies with ripeness state. Thus, fruits intended for olive oil extraction can be very different in chlorophyll and carotenoid content.

Disappearance of chlorophylls and carotenoids from fruits during ripening; dependence on the variety. Figure 2 shows the retention of chlorophylls (2A) and carotenoids (2B) with ripeness stage for the five varieties studied. When the percentage of retention of the chlorophyll or carotenoid fraction is plotted semilogarithmically, the negative slope of the line joining two contiguous states of ripening can be considered a measurement of the relative rate of pigment disappearance, allowing varieties to be ordered according to their higher or lower degradation rate and thereby compared.

Since no differences in the qualitative pigment profile have

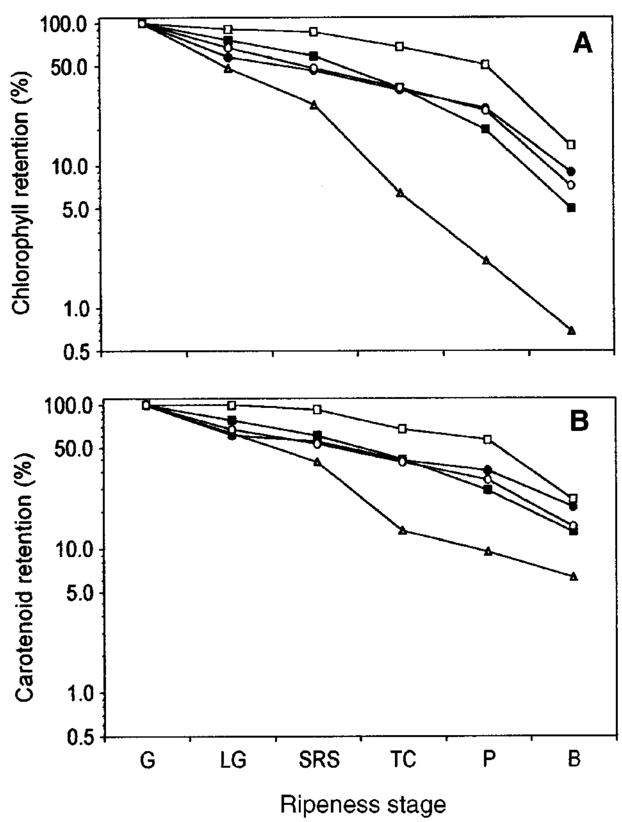

FIG. 2. Changes in (A) chlorophyll and (B) carotenoid pigment retention during ripening of different olive varieties. Arbequina (□), Blanqueta (▲), Cornicabra (■), Picual (○), Hojiblanca (●). For abbreviations see Figure 1.

been detected among varieties during ripening, we can assume that the patterns for catabolism of chlorophylls and carotenoids are identical for all the studied varieties, although the retention of chlorophylls is always slightly lower than that of carotenoids. The plotted parameters, up to the purple stage, in the Arbequina, Hojiblanca, and Picual varieties are linear, enabling calculation of the relative rate that fit best. For this parameter, Arbequina has the lowest disappearance rates for both chlorophylls and carotenoids (0.201 and 0.187, respectively) and thus the highest retention: 53.0% of total pigments up to the purple stage. During the light green stage (yellow for this variety), the carotenoid loss is only 0.53%, against 20–40% in the other varieties. The Hojiblanca and Picual varieties have slopes of higher value (0.620 and 0.630 for chlorophylls and 0.520 and 0.540 for carotenoids, respectively) and show similar behavior, retaining between 25.5 and 27.0% of total pigments up to the purple stage.

In contrast, in Blanqueta and Cornicabra the rate of the slope changes in earlier ripeness states. The Cornicabra variety has a total pigment retention (59.0%) that is higher than that of Hojiblanca and Picual (47.0 and 48.0%, respectively) until the small reddish spots stage. From then on, its degradation rate increases, so that in the purple stage the retention is lower (18.0% against the 25.4 and 24.0% for Hojiblanca and Picual). This situation becomes more marked in the transition to the black stage.

The variety Blanqueta always has the largest negative slope values. Until the small reddish spots stage, the relative rate is 0.72 for chlorophylls and 0.40 for carotenoids; in the purple stage it reaches 1.0 for chlorophylls and 0.53 for carotenoids. This variety therefore has the lowest pigment retention in all ripeness states (some 4.0% up to the purple stage). The whitish color characterizing its fruits at the start of ripening is due to the loss of both chlorophylls (52.0%) and carotenoids (37.0%), resulting in the lowest pigment concentration (47 mg/kg). The near-absence of pigments is evident and visible because, as in the case of Arbequina, the synthesis of anthocyanins that could mask this color is very delayed.

Ratio between the isochromic fractions of pigments. The ratio between the chlorophyll and carotenoid pigment fractions as a function of ripeness in the fruits of the varieties studied is shown in Figure 3. In all the varieties, the ratio tends to decrease during ripening, with a period during which it remains more or less constant before decreasing sharply.

In the Hojiblanca and Picual varieties, the changes in this ratio up to the purple stage are parallel and similar in absolute value. The ratio decreases very gradually from the start, until the fruit became purple due to the presence of anthocyanins, when the decrease becomes sharper. Cornicabra and Arbequina follow the same pattern; their ratios are similar in the small reddish spots stage, with the ratio remaining practically constant until the anthocyanin compounds appear in the fruits. Blanqueta behaves differently: in the initial green stage the ratio is higher than for Cornicabra, in the light green stage to the small reddish spots stage is similar to that for Arbequina, and subsequently has the lower values.

Although in principle this ratio seems to have a variety-specific value that changes with ripening, there are points of obvious coincidence. In the present study of pigment behavior during ripening in the different olive varieties, the states of ripeness were uniform. However, in commercial extraction of oil this is not so, because the fruit-picking period varies and is adapted to the characteristics of each variety. Taking into account that, for oil extraction, highly pigmented varieties are not picked green, whereas those of low pigmentation are, Figure 3 details the states of ripeness employed for industrial processing according to variety. Within this frame, the range of the chlorophyll/carotenoid ratio is 2.5 and 3.7, which could be considered narrow, particularly when dealing with vegetable products including varieties of very different characteristics and sources. Irrespective of the greater or lesser pigment content shown by the fruits of different olive varieties and of the different patterns during ripening, the ratio between chlorophylls and carotenoids can be considered to remain more or less constant and independent of variety at around a value of 3 units, with the two parameters having a remarkable correlation (r = 0.989).

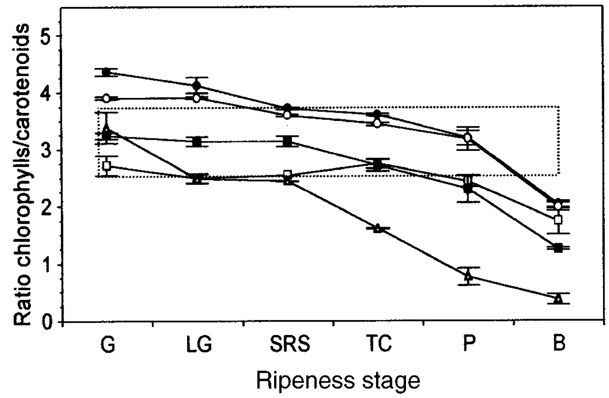

FIG. 3. Chlorophylls/carotenoids ratio vs. ripeness stages of different olive varieties. Symbols as in Figure 2. Rectangle formed by dotted lines indicates the states of ripeness employed for industrial processing. For abbreviations see Figure 1.

With regard to the transfer of the fruit pigment content to the oil, various aspects must be taken into account: olive ripening is not synchronous in all the fruits on the tree, but is phased, so that when anthocyanin synthesis begins, the tree contains fruits that are in ripeness stages of green, small reddish spots, and purple. Their proportions vary with ripening. Depending on variety, this process lasts from 1 to 3 mon.

At the same time, although the oil content of the fruits increases during ripening, reaching its maximum when the green fruits disappear from the tree, the organoleptic characteristics of the fruit deteriorate if picking is delayed. The fruity and aromatic olive oils are obtained at the beginning of the ripening period, even when there is an appreciable percentage of green fruit (23).

When lipogenesis (the oil formation process) finishes, the present changes in the acid composition of the fruit have occurred. During ripening, palmitic acid content decreases,

TABLE 1
Relation of Ripeness Index to Coloration of Olive Fruits During the Harvest Period[a]

Hojiblanca variety				Cornicabra variety				Arbequina variety			
I.R.[b]	% Green	% SRS[c]	% Black	I.R.	% Green	% SRS	% Black	I.R.	% Green	% SRS	% Black
3.5	35.85	28.30	35.85	3.64	36	28	36	0	100	0.0	0.0
3.65	43.64	12.72	43.64	4.1	29	29	42	0.46	89.71	10.29	0.0
3.80	37.5	25.0	37.5	4.67	20	31	49	0.49	89.6	7.82	3.12
3.97	28.85	28.85	42.30	5.06	17	25	58	0.73	79.1	20.9	0.0
4.36	30.19	15.09	54.72	5.71	6	29	65	1.05	79.0	20.1	0.9
4.48	20.31	31.25	48.44	6.02	0.0	28	72	1.47	45.0	42.0	13.0
4.71	20.00	25.45	54.55	6.2	0.0	23	77	1.51	57.0	33.0	10.0
4.92	16.95	25.42	57.63	6.05	0.0	27	73	1.65	45.0	53.0	2.0
5.15	18.87	15.09	66.04	6.54	0.0	13	87	3.79	3.0	52.0	45.0
5.30	6.45	29.03	64.52	6.51	0.0	12	88	2.58	49.12	28.07	22.81
5.54	14.55	12.72	72.73	6.47	0.0	15	85				
5.83	12.5	8.33	79.17	6.51	0.0	14	86				
6.05	3.39	20.34	76.27	6.44	0.0	16	84				
6.64	0.00	10.17	89.83	6.3	0.0	20	80				
7.00	0.00	0.00	100.00								

[a]Data provided by Oleoestepa Cooperative (Estepa, Seville, Spain).
[b]I.R., index of ripeness. Ripeness indexes correspond roughly with the ripeness stages of Figures 1–3. Thus, 0 = green or dark green; 4–7 = black epidermis with pulp ranging from white to black; 1–3 = intermediate color.
[c]SRS, small reddish spots.

linoleic acid increases, and oleic acid remains constant. As a result, the monounsaturated/polyunsaturated ratio decreases, with the consequent fall in both nutritive value and oxidative stability of the virgin olive oil. Moreover, the proportion of acids differs with variety: Arbequina and Blanqueta have a greater content of palmitic acid (16:0) and linoleic acid (18:2) than Hojiblanca, Picual, and Cornicabra, while the last three have an oleic acid (18:1) and stearic acid (18:0) content higher than in Arbequina and Blanqueta.

The polyphenol content varies throughout fruit ripening, following a curve with a maximum generally coinciding with the moment when the amount of oil in the fruit reaches the highest value. Oil stability is related to polyphenol content. In the variety Picual, this maximum coincides with a fruit ripeness index of 3.5, while in Arbequina is 2–2.5. (Ripeness indexes correspond roughly to the ripeness stages of Figures 1–3. Thus 0-green, 1-light green, 2-small reddish spots, 3-turning color or purple, 4–7-black epidermis with pulp ranging from white to black.) The oils of Arbequina and Blanqueta are therefore less stable than those of Cornicabra, Hojiblanca, and Picual because they contain lower total polyphenols and less oleic acid, among other components. A delay in picking gives rise to oils that are less fragrant, less brilliant, less bitter, and with a sensation of mildness (23).

From the above, it can be concluded that the Hojiblanca, Picual, and Cornicabra varieties will normally be picked when the predominant color on the tree is not green, but without waiting until the fruits turn completely black, that is, at an intermediate ripening stage.

The Blanqueta and Arbequina varieties are much appreciated for their early production. The oil from Arbequina has excellent quality and is highly valued commercially for its organoleptic characteristics. As both varieties yield oils of low stability, they are picked early so that their oils have a maximum of total polyphenols, a minimum of linoleic acid, and thus maximum stability. Table 1 shows, in an actual example supplied by the Oleoestepa Cooperative (Estepa, Seville, Spain: an oil-producing company), the changes during the harvest period in fruits of the Hojiblanca variety at an oil mill, indicating for each ripeness index the percentage of corresponding green, small reddish-spotted, and black fruits. It also includes the percentages for the Arbequina and Cornicabra varieties as received in our laboratory. Because of similarity in varieties, the ripeness index of Hojiblanca can be used for Picual, and that of Arbequina for Blanqueta.

Consequently, the fruits entering a mill during the oil production period are not uniformly colored and are not, except at the end of the period, in a particular ripeness state. Thus, to know the chlorophyll and carotenoid content of the raw material entering a mill, we must use Table 1 and calculate the parameters from the percentage of fruits having green, mottled, and black coloring corresponding to each ripeness index.

As the olive has a fat content that ranges between 20 and 30%, depending on variety, and because the pigments are lipophilic, their concentration in the oil should increase three- to five fold with respect to that found in the fruit. For the present study, we assume a mean value of 25% fat content for all the olive varieties, so the pigment concentration of the respective oils should increase some fourfold.

An earlier work (14) monitoring the pigment content of fruits and oils in a mill demonstrated the pigment loss involved in the extraction process. The concentration in the respective oils not only did not increase, but fell with respect to that found in the fruits. The calculated pigment losses were considerable; the main effect was observed on the chlorophyll fraction, with a mean decrease of some 80%, whereas the carotenoid loss was around 50%. These net losses of pigmentation included both those from pigments retained in the solid residue and those associated with co-oxidation reactions involving lipid peroxides and the lipoxygenase enzyme.

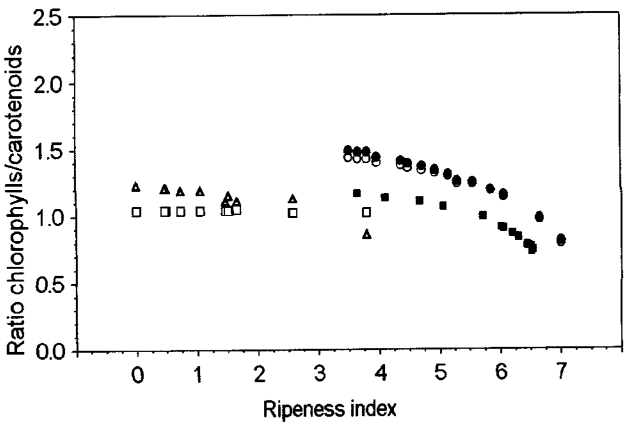

FIG. 4. Chlorophylls/carotenoids ratio vs. ripeness index of the oils theoretically obtained from the mixture of fruits entering the mill. Symbols as in Figure 2. Ripeness indexes corresponds roughly to ripeness stages of Figures 1–3. Thus, 0 = green or dark green; 7 = black epidermis with pulp ranging from white to black; 3 = intermediate color.

From such premises, and accepting the error originated by generalizing both the fat content and the losses for all the varieties and ripeness states, the chlorophyll and carotenoid content of the oils that theoretically would be obtained from the mixture of fruits entering the mill could be calculated. The chlorophyll/carotenoid ratio obtained is shown in Figure 4.

Because of the preferential loss of chlorophylls during the extraction process, the chlorophyll/carotenoid ratio decreases markedly, from 2.5–3.7 in the fruits to 0.8–1.4 in the oils. The greater lability of the chlorophyll fraction means that the final transfer of pigmentation to the oil is much more balanced than the first from the fresh fruit, with a mean value of 1.15 ± 0.24. Consequently, no single- or mixed-variety oil from fruits of the varieties studied can have a chlorophyll/carotenoid value outside the limits found.

These results coincide with and explain those obtained in an earlier study on single-variety oils, in which it is shown that, independently of the variety and of the higher or lower pigment content, the ratio between the two isochromic pigment fractions apparently remains constant, with a value close to unity (19).

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