Distribution of Chlorophylls and Carotenoids in Ripening Olives and Between Oil and Alperujo When Processed Using a Two-Phase Extraction System

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ABSTRACT: The present work has studied the content and type of pigments present in olive fruits and the respective oils and alperujos. The concentration of isochromic pigment fractions—chlorophylls and carotenoids—decreased with fruit ripening, more markedly in the former than in the latter. This implied that the ratio between the two pigment fractions also decreased in parallel in the three products studied. However, the value in the oil was lower than in the fruit, and that in the alperujo was much higher. During the extraction process, the release of acids may have caused pheophytinization reactions in the chlorophyll fraction, increasing pheophytin content in alperujos and oils, whereas the carotenoid fraction was affected only in the alperujo. Chlorophyll *b* derivatives were destroyed in greater proportion than chlorophyll *a* derivatives during transfer to the oil. During processing the destruction of lutein was greater than that of β-carotene. The balance of matter between fruit, alperujo, and oil indicated that not all the fruit pigmentation was released from the structures, and most remained occluded in the alperujo. The rest of the pigmentation, and particularly the chlorophyll fraction, was partly destroyed during its transfer to the oil.

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KEY WORDS: Carotenoid, chlorophyll, olive, olive oil, two-phase olive pomace (alperujo).

Virgin olive oil is the oily juice separated from the other components of the olive fruit. The extraction process comprises the following phases: milling (to destroy the plant tissue structure), beating (to join in a continuous oily phase the oil drops dispersed through the milled paste), and solid–liquid separation (separation of the oil contained in the olive paste). Industrially, this last stage of the process is carried out in different ways. Traditionally, the so-called three-phase centrifugation system has been used, giving three products: oil, olive pomace, and vegetation water. Since the latter is a pollutant and constitutes a serious environmental problem, work has been put into improving centrifugation technology. The two-phase system currently in use has two products: oil and alperujo (two-phase olive pomace—a mixture of solid residue and vegetation water in the form of a semifluid paste).

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The change from the three-phase to the two-phase system has led to the need to characterize the new olive pomaces (alperujos) obtained, to compare them with those obtained formerly. Clemente *et al.* (1) studied the content in fiber, nitrogen, amino acids, soluble sugars, and organic acids in both residues. They concluded that the two-phase system reduces environmental problems by eliminating the vegetation water. However, this novel system does require a strict control of oil loss using new techniques, such as near-infrared spectroscopy for the measurement of oil and moisture content of the alperujo in the two-phase decanter (2).

Little is known about alperujo composition beyond the tests of its suitability for final use. For instance, the alperujo tocopherols have been isolated and separated (3), as their antioxidant activity may be of interest to the food industry. Thus, olive byproducts can be used as a natural source of antioxidants. The carbon/nitrogen (C/N) ratio has also been examined in a feasibility study of using vermicomposting to stabilize dried alperujo for use as soil amendment. The results show that this is a useful medium when combined with nitrogen-rich materials such as cattle manure and sewage sludge in suitable proportions (4). Until now, however, nobody has characterized alperujos for pigment composition. In fact, the only study of the transfer and balance of pigmentation from fruit to oil during the extraction of virgin olive oil is that of Mínguez-Mosquera *et al.* (5). They concluded that during the extraction process, chlorophylls are converted to their respective magnesium-free derivatives. Moreover, there is a considerable loss of chloroplast pigments, which is greater for the chlorophyll fraction than for the carotenoids. Later, Gandul-Rojas and Mínguez-Mosquera (6), comparing the varieties Picual and Arbequina for chlorophyll content in fruits and oils, and for chlorophyllase activity, showed that this enzyme activity is not involved in the loss of chlorophylls between fruit and oil.

Except for these works, the only study determining pigmentation in olive and oil is that of Mousa *et al.* (7), who compared oils extracted from fruits of the variety Mastoides grown at different altitudes (100 and 800 m), monitoring (among many parameters) the total chlorophyll content. Kiritsakis *et al.* (8) also determined, among other factors, the total chlorophyll content in fruits of the variety Koroneiki stored under different conditions and the effect on the quality of the oils extracted. None of these articles evaluated the chlorophyll and carotenoid pigmentation between fruit and

oil. Following a study on criteria of authenticity in 50 single-variety virgin olive oils (9), which established that the ratio between chlorophylls and carotenoids in virgin olive oil remains constant, Roca and Mínguez-Mosquera (10) demonstrated mathematically in five olive varieties that the ratio between the two pigment fractions is altered in the transfer from the fruit to the virgin olive oil.

No data have been found in the literature on pigments in the alperujo. It is not known if the pigment loss during transfer from fruit to oil is because the pigment is destroyed or remains occluded in the alperujo. The aim of this work was to know and explain how this transfer of pigments takes place during the extraction of olive oil. The two-phase system is an improved extraction method for olive oil, but has the drawback of a 60% moisture content in the alperujo. Some olive mills use a second centrifugation for oil extraction from the alperujo. Ranalli *et al.* (11) made an analytical evaluation of first- and second-extraction virgin olive oils, concluding that the qualitative level of the first extraction is higher than that of the second. This new type of oil has arrived on the market. However, because its qualification is disputed, it is currently the object of numerous characterizations and evaluations. Alba-Mendoza *et al.* (12) studied the characteristics of the first- and second-centrifugation oils obtained in a two-phase system. They concluded that the second centrifugation yields oils whose parameters of quality and purity are within the permitted limits. The pentacyclic hydroxy-triterpenic acids in these oils also have been monitored, and the conclusion reached is that their concentration in second-centrifugation oils is higher than in virgin olive oils (13). Therefore, it would be very interesting to know the chlorophyll and carotenoid composition of the raw material from which the second-centrifugation oils are extracted, as this determines the color of the new oils. The distribution of chloroplast pigments in the oil and alperujo is also of great interest to the industrial sector, as chlorophyll and carotenoid content is another attribute in evaluating product quality.

MATERIALS AND METHODS

Raw material and sampling. The study was carried out on fruits, alperujos (two-phase olive pomaces), and oils of the olive variety Hojiblanca. Samples were supplied by the olive mill Cooperativa Sor Angela de la Cruz of Estepa (Seville, Spain). The assays were performed at the end of the 2000–2001 season, during three consecutive weeks (from 22 January to 5 February 2001). In each assay, two samples of each material, taken from the process at two different times on the same day, were analyzed in duplicate.

Extraction of pigments. Samples were taken from a triturate of 50 homogenized destoned fruits 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. For alperujo and oil, samples of 15 g were weighted. Pigment extraction was performed with *N,N*-dimethylformamide according to the method of Mínguez-Mosquera and Garrido-Fernández (14) (for fruits and alperujos) and Mínguez-Mosquera *et al.* (5) (for oils). The technique is based on the selective partition of components between *N,N*-dimethylformamide and hexane. The hexane phase carries over lipids and the carotene fraction, whereas the *N,N*,-dimethylformamide phase retains chlorophylls and xanthophylls. This system yields a pigment extract free of the fatty matter that is characteristic of these fruits and that interferes with subsequent separation and quantification. All analyses were performed under green light.

Separation, identification, and quantification of pigments. This was carried out by high-performance liquid chromatography (HPLC) using an HP 1100 Hewlett-Packard (Palo Alto, CA) liquid chromatograph fitted with an HP 1100 automatic injector and diode array detector. Data were collected and processed with an LC HP ChemStation (Rev. A.05.04). Pigment separation occurred on a stainless steel column (25×0.46 cm), packed with 5 μm C_{18} Spherisorb ODS-2 (Teknokroma, Barcelona, Spain). 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 $13{,}000 \times g$ (MSE Model micro centaur) 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, by 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 (15). Detection was simultaneously performed at 410, 430, 450, and 666 nm. Details about the pigment identification have been described in previous papers (6,15). External standard calibration was used for quantitation. The results were given as milligrams per dry kilogram of destoned fruit without oil.

Determination of moisture and fat content. Moisture was measured by weighing some 15 g of sample that was then oven-dried to constant weight (an average of 4 or 5 d). The determination was performed in triplicate.

Fat content of olives and alperujos was determined according to the UNE standard Spanish method (16). Dry, ground sample (10 g) was extracted with hexane (Panreac, Barcelona, Spain) in a Soxhlet extractor for 5 h (turnover rate: 6 min), and the results were expressed as percentage of oil obtained with respect to the starting material. The analysis was performed in duplicate.

Both determinations are necessary to perform a balance of material.

RESULTS AND DISCUSSION

Chlorophyll and carotenoid profile. Figure 1 shows the content of the chlorophyll and carotenoid fraction of olives, oil, and alperujo during the 3 wk of study. The chlorophyll and carotenoid concentration of the fruits in the first assay was that of ripe black olives (17), while that in the other two assays was of overripe fruits. Nevertheless, it was possible to establish a ripeness sequence between the two, as the date of

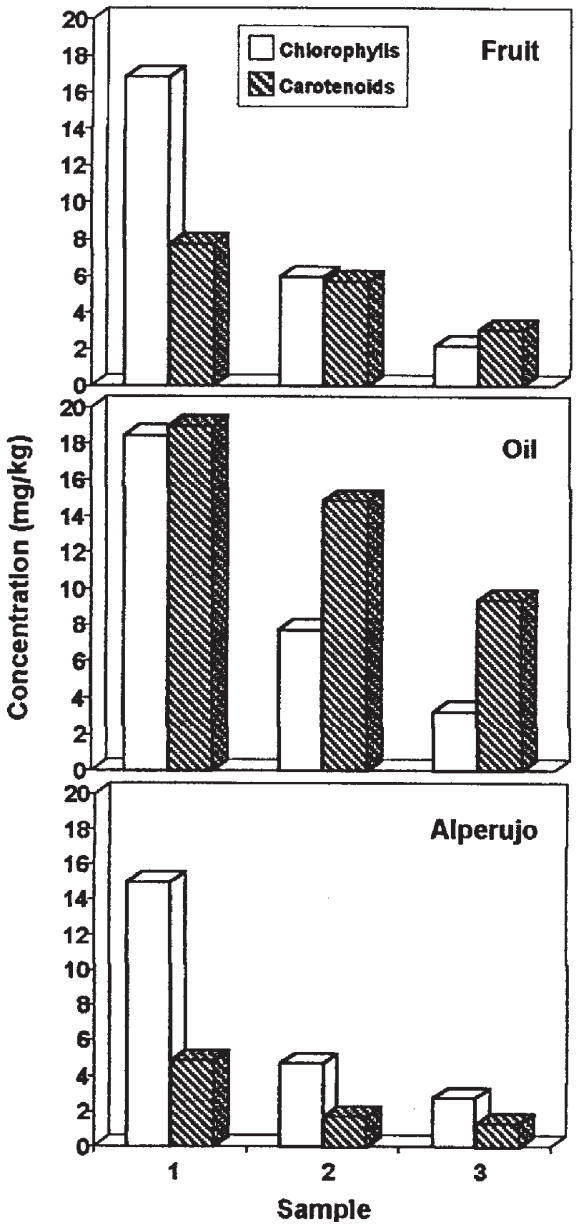

FIG. 1. Content of the chlorophyll and carotenoid fraction of olives and their corresponding oil and alperujo for each studied sample.

sampling coincided in time with a progressive fruit ripening. In general, as the date of picking became later, the concentration of chlorophylls and carotenoids decreased in the different materials studied. In all of them, a greater proportion of the green fraction was lost, seen as a decrease in the ratio between the two pigment fractions. In the fruits, the chlorophyll fraction was predominant in the first assay, the two fractions were equal in the second, and in the last, the yellow fraction was the major one. In the oil, the carotenoid fraction was always greater than that of the chlorophylls (although they were practically equal in the first assay), so that the chlorophyll/carotenoid ratio was lower than in the fruit. In the alperujo, the green fraction was always greater than the carotenoid, and the ratio between the fractions was higher than in the fruit.

Transformation of pigments during the oil extraction process. Table 1 shows the percentage composition of the chlorophyll and carotenoid compounds (with respect to the total of each fraction) for each of the samples studied. Qualitatively, the fruit contained the typical chloroplast pigments of a fresh fruit—chlorophylls *a* and *b*, β-carotene, lutein, neoxanthin, violaxanthin, antheraxanthin, and β-cryptoxanthin—together with a high percentage of pheophytins *a* and *b*, which increased in the fruits as the date of picking became later, and reached almost 50% of the chlorophyll fraction in the ripest olives. This indicated that the picked fruits had been stored in the mill some time before being processed for oil extraction, because very ripe, recently picked fruits showed only a minute amount of pheophytin *a* (17). Possibly, as the fruits were very ripe, their handling in the mill prior to extraction caused the rupture of intracellular tissues, releasing acids, and leading to a high transformation of chlorophylls into pheophytins. In the alperujo samples, apart from the chlorophyll and carotenoid compounds coming from the fruit, a small amount of auroxanthin and/or mutatoxanthin (probably due to cell rupture and release of acids) was detected. The oil showed no qualitative differences in pigments with respect to those in the olives.

For all the samples of fruits and alperujos, the ratio between the series *a* and series *b* chlorophylls remained constant. In the oils, however, this ratio varied: The presence of the series *a* chlorophylls with respect to *b* chlorophylls increased from three- to sixfold, indicating that series *b* compounds were destroyed in greater proportion (average value 32.30%) than those of series *a* (average value 14.79%) during their transfer to the oil. During the processing of the canola, Endo *et al.* (18) and Suzuki and Nishioka (19) found that in the oil the ratio *a/b* increased with respect to seeds. At the same time, this process favored the transformation of chlorophylls into pheophytins, as the proportion of the latter increased in both oil and alperujo, somewhat more markedly in the oil. Nevertheless, the levels of conversion in the oil were lower than those found in an earlier study (5), when there were samples with up to 84.12% of chlorophyll trans-

TABLE 1
Percentage Composition of the Chlorophyll and Carotenoid Pigments with Respect to the Total of Each Fraction[a]

	Chlorophyll fraction				Carotenoid fraction		
Sample	Chl *a*	Phy *a*	Chl *b*	Phy *b*	β-c	Lut	Minor car[b]
Fruit 1	49.6	31.0	19.0	0.5	11.0	76.5	12.7
Fruit 2	37.0	44.1	18.2	0.7	6.8	85.6	7.6
Fruit 3	35.0	46.6	17.5	0.9	6.4	87.5	6.1
Oil 1	46.2	47.7	5.5	0.6	12.1	75.1	12.8
Oil 2	41.9	53.4	4.4	0.3	5.0	87.7	7.3
Oil 3	32.3	64.4	3.0	0.3	5.3	87.1	7.6
Alperujo 1	27.9	52.6	18.1	1.4	11.0	76.3	12.7
Alperujo 2	31.2	50.6	17.2	1.1	13.0	76.2	10.8
Alperujo 3	29.7	49.6	19.1	1.5	9.1	84.1	6.8

[a]Abbreviations: Chl, chlorophyll; Phy, pheophytin; β-c, β-carotene; Lut, lutein; Minor car, minor carotenoids. 1, 2, and 3 indicate weeks of sampling.
[b]Values are the sum of neoxanthin, violaxanthin, antheraxanthin, and β-criptoxanthin. For alperujo samples auroxanthin and mutatoxanthin are also included.

formation into pheophytins. The differences between the two studies could be due to the state of the raw material and to the physical conditions of the oil extraction, such as temperature, time of beating, or the application of a two- or three-phase system that, within certain limits, can vary between one mill and another. The oil studied earlier (5) was obtained from a three-phase system, whereas that of the present study was from a two-phase system.

In the carotenoid fraction, there was a notable absence in the oils of violaxanthin, neoxanthin, and antheraxanthin isomers with a 5,8-furanoid group, occasionally present in virgin olive oils (5,9). Their formation could have been expected as a consequence of the acid release during oil extraction that favored the chlorophyll pheophytinization reaction mentioned above. However, we must bear in mind that the chlorophyll fraction is more acid-labile than the carotenoid fraction, so that the pheophytinization reaction takes place at a higher rate than isomerization of the 5,6-epoxides to 5,8-furanoids. In contrast, as described previously, auroxanthin and mutatoxanthin—isomers of violaxanthin and antheraxanthin, respectively—were detected in the alperujos because the time of contact with the acid medium was longer. No appreciable differences were found in the ratio between the major carotenoids, lutein and β-carotene, in the fruit, alperujo, and oil for the first sample, whereas for the rest, in which the fruits were riper, the lutein/β-carotene ratio decreased in the alperujo. That is, the proportion of β-carotene retained in the alperujo was greater than that of lutein, probably because lutein is destroyed to a greater extent due to the greater antioxidant capacity of its structure (20). In fact, when the balance of material for each individual pigment was made, a 22.90% average value of destruction was found for lutein, whereas 15.67% was for β-carotene. In the minor carotenoids, which were evaluated jointly in the data study, no well-defined variations were found between the different samples. In some cases, the proportion of pigments was slightly greater in the alperujo or oil than in the fruit, and in others, somewhat lower, without any fixed pattern.

Balance of material. A balance of material was performed to elucidate the distribution of fruit pigments between alperujo and oil during the oil extraction process. In order to express the chlorophyll and carotenoid content of the fruit, alperujo, and oil in the same units, the values of stone weight, moisture, and fat content were corrected in each particular case. Thus, from the previous calculations, the data shown in Table 2 refer to the number of milligrams of chlorophylls and carotenoids retained in the alperujo and that transferred to the oil per kilogram of dry plant material of destoned fruit. This change of units, however, did not alter either the profile or the pattern in pigment content observed before in Figure 1, so that Table 2 also shows a decrease in the concentration of chlorophyll and carotenoid pigments in fruit, alperujo, and oil with ripening. Direct comparison of the three materials by this new estimation of pigments revealed that the chlorophyll and carotenoid content of the fruit was greater than the sum in the corresponding oils and alperujos, implying some destruction of pigments. The chlorophyll fraction in the alperujo was much greater than in the oil, although it tended to decrease with riper fruits. The carotenoid fraction content in the first alperujo sample was similar to that in the oil, but in the other samples it was lower.

TABLE 2
Composition (mg per kg of dry plant material of destoned fruit) of Total Chlorophyll and Carotenoid Pigments in Each Sample[a]

	Total chlorophylls			Total carotenoids		
Sample	Fruit	Oil	Alperujo	Fruit	Oil	Alperujo
1	96.1	18.5	59.0	43.5	19.0	19.2
2	24.4	5.6	14.8	23.5	10.8	5.6
3	13.5	3.0	8.0	16.4	8.8	3.9

[a]Data are means of quadruplicate analysis (coefficient of variation <5% in all cases). 1, 2, and 3 indicate weeks of sampling.

From these data was calculated the percentage of total pigments, chlorophylls and carotenoids of the fruits that were occluded in the alperujo, transferred to the oil, and destroyed during the extraction process (Table 3). The percentage of fruit pigments not transferred to the oil, calculated as the sum of that retained in the alperujo and that destroyed, is also included as losses. In this balance of material, we use the term "losses" when referring to the transfer to the oil, because as these compounds are lipophilic, they would be expected to have been largely solubilized in the oil.

In terms of total pigments, it was established that of the initial fruit pigmentation, most was retained by occlusion in the alperujo, some was transferred to the oil, and a smaller amount was destroyed during the process. As the fruits ripened, the proportion of total pigments remaining in the alperujo decreased, and the part transferred to the oil increased. No fixed pattern was found in the amount of pigments destroyed, although the mean values were fairly close. This, together with the decreasing amount of pigments retained in the alperujo, meant that there was an overall decrease in pigment losses as the fruit ripened.

TABLE 3
Percentage of Total Pigments, Chlorophylls, and Carotenoids of the Fruit That Were Transferred to the Oil (Trans-Oil), Occluded in the Alperujo (Occ-Alp), and Destroyed (Destr) during the Extraction Process

	Fruit pigments (%)			
Sample	Trans-Oil	Occ-Alp	Destr	Losses[a]
		Total pigments		
1	26.9	56.0	17.1	73.1
2	34.4	42.5	23.2	65.6
3	39.2	39.9	20.9	60.8
		Total chlorophylls		
1	19.3	61.4	19.4	80.8
2	23.1	60.6	16.3	77.0
3	22.0	59.4	18.6	78.0
		Total carotenoids		
1	43.7	44.1	12.1	56.3
2	46.1	23.7	30.3	53.9
3	53.4	23.9	22.8	46.7

[a]Fruit pigments not transferred to the oil. 1, 2, and 3 indicate weeks of sampling.

Most of the chlorophyll fraction present in the fruit was retained in the alperujo, part was transferred to the oil, and the rest was destroyed, although in amounts that varied with fruit ripening. Thus, in the first assay, of the pigmentation not retained in the alperujo, half was transferred to the oil and the other half was destroyed. In subsequent assays, the pigment destroyed decreased, while that transferred to the oil increased. The mean value of total chlorophyll losses was 78.58%. The behavior of the carotenoid fraction during the olive oil extraction process was completely different from that of the chlorophyll fraction. In the first sample, 88% of the fruit carotenoid pigmentation was distributed equally between alperujo and oil, and the rest was destroyed. This situation changed drastically as the fruits ripened: More and more carotenoids were transferred to the oil, reaching 53%. The rest of the pigmentation was destroyed or retained in the alperujo. The losses from the carotenoid fraction decreased with ripening, from 56 to 47% at the end of the ripeness states studied.

The high percentage of pigments retained in the alperujo led us to deduce that only a part of the fruit pigmentation was released from the intracellular structures during the different stages of the oil extraction process, the rest remaining occluded in the alperujo. Of the chlorophylls released, a greater proportion of chlorophyll *b* was destroyed, due to its greater lability. This was reflected as the change in the chlorophyll *a*/chlorophyll *b* ratio found between fruit and oil, mentioned above. The differences found in the distribution of chlorophylls and carotenoids between alperujo and oil also indicate that the pigmentation released was not transferred equally to the oil, but that the transfer was much greater for the carotenoids, probably because of their lipid nature and solubility, than for the chlorophylls, which, due to their structure of porphyrin linked to a phytol residue, are more amphipathic.

We observed that mean losses for the chlorophyll fraction (79%) exceeded those for the carotenoids (52%). These data are very close to those found earlier by Mínguez-Mosquera *et al.* (5): 82% for chlorophylls and 53% for carotenoids. Given that the losses originating during the oil extraction process depend on the state of ripeness and conditions of the raw material, the mean values will range within set limits for the material used. We can establish a *ca.* 80% loss for the chlorophyll fraction and 50% for the carotenoid fraction during the olive oil extraction process. The consequence of this phenomenon was that the chlorophyll/carotenoid ratio was lower in the oils than in the fruit, as previously reported (10).

The most noteworthy results of this study were the discoveries that the loss of pigmentation, quantified during the olive oil extraction process, was due mainly to retention in the alperujo, rather than to destruction during the process, and that the chlorophyll fraction was essentially occluded in the alperujo, while the carotenoids were transferred in greater proportion to the oil.

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