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Physicochemical Conditions Modulating the Pigment Profile in Fresh Fruit (*Olea europaea* Var. *Gordal*) and Favoring Interaction between Oxidized Chlorophylls and Endogenous Cu

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The changes in allomerized chlorophyll during the growth and development of the olive fruit as well as during the main operations of its processing as green table olive (alkaline treatment and lactic fermentation) were investigated to study their influence in the color alteration known as green staining (GS). Chlorophyll synthesis coincided in time with the maximum content in allomerized intermediates, weeks before the fruits were harvested for processing. The alkaline treatment caused a subsequent chlorophyll oxidation independent of the high or low initial content of allomerized chlorophylls. However, this oxidation was directly related with the oxidizing capacity of the alkaline solution and the cell deterioration caused. The later maintenance of the fruits in osmotic solutions at different pHs that reproduce the pH reduction caused by the lactic fermentation showed that at pH below 4.5 the insertion of Cu into the chlorophyll molecule was produced in certain fruits; the extent of this reaction was greatest when the prior formation of oxidized chlorophylls exceeded 23%. This apparent relationship between chlorophyll oxidation and copper chlorophyll complexes was investigated in table olives with GS alteration.

KEYWORDS: Allomerized chlorophyll; alkaline treatment; chlorophyll oxidation; copper-chlorophyll; green staining alteration; olive fruit

INTRODUCTION

The olive fruit of the Gordal variety is grown exclusively for alimentary use as table olive. For this type of processing, the fruits are picked during September and October, in the green-yellowish state of ripeness, and are treated first with a dilute NaOH solution to help make the skin permeable and to hydrolyze the bitter glucoside oleuropein. After the alkaline treatment, the fruits are washed with water to remove the remaining NaOH adhered to the skin and are placed in a NaCl solution in which a natural lactic fermentation takes place (*1*).

However, quite often the processed fruits develop an alteration in color, known as 'green staining' (GS). This is seen as bluish-green zones distributed over the skin, contrasting with the natural olive-green color of the rest of the fermented fruit. These zones are initially small spots, the size of a pinhead, but with time the anomalous coloring spreads and can cover a great part of the fruit surface. Earlier works were aimed at identifying all the compounds taking part in this alteration (*2*, *3*). The bluish-green color marking the appearance of the alteration is due to the formation of a new stable chromophore by the insertion of the Cu ion into the porphyrin ring of the chlorophylls. The localized accumulation of these copper-chlorophyll derivatives, in whose formation exogenous copper takes no part, leads to the appearance of the GS. These compounds are also present, although in lower concentration, in the unaltered part of the fruit and in other, apparently healthy, fruits processed simultaneously in the same fermenter (*4*).

The reaction of complexation between divalent metal ions, such as Cu and Zn, and chlorophyll derivatives is well-known, and the literature reports its use for maintaining the green color in canned vegetables, though always with added metal ions (*5*, *6*). To date, the formation of cupro-chlorophyll derivatives in a fruit with endogenous Cu has been described only in table olives of the Gordal variety (*2*, *4*), with the suggestion that the Cu comes from pectin chains acting as reservoir (*7*). The fact that at the industrial level alterated fruits are only found in certain fermenters, when the fruits are processed similarly in all of them, shows that the fruits do not all respond equally to similar physical−chemical treatments. This fact raises the possibility, therefore, that a fruit, depending on its origin or state of development, has a particular predisposition to undergo the cell degradation leading to the GS alteration, in which the endogenous Cu is inserted into the porphyrin ring.

The formation of chlorophyll complexes with the fruit's endogenous Cu must involve a previous cell integrity destroying, allowing contact of the metal with the chlorophyll derivatives,

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which under normal conditions are protected in their natural lipophilic environment. This was confirmed by electron microscopy studies that demonstrated greater cell degradation in fruits where the GS alteration is seen than in healthy fruits (*4*). Complementary studies demonstrated that in the altered zone of the fruits there is a degeneration of the proteins and phospholipids that form the natural pigmen−lipoprotein complexes, together with a lesser interaction between Cu-chlorophyll−lipoprotein complex and thylakoid membrane, confirming the existence of a great loss of cell integrity in that zone (*8*).

Although the identity of the pigments involved in GS is now known, as is their order of appearance in the progress of the alteration (*3*, *4*), the initial cause that triggers the cascade of transformations leading to the formation and accumulation of these pigments in the fruits, and making the GS visible, is not well-known.

Earlier studies carried out in other olive varieties demonstrated the different presence, depending on variety, of oxidized metabolites of chlorophylls, such as 13^2-OH-chlorophyll *a* and 15^1-OH-lactone-chlorophyll *a*, and generically denominated allomerized chlorophylls. They are related with both the turnover and the catabolism of chlorophylls, and their concentration increases both in the final stages of fruit development and at the end of ripening (*9*). These intermediate products in chlorophyll metabolism are also present in olives of the Gordal variety (*10*), although to date their possible involvement in the development of the GS alteration has not been suggested.

The objective of the present study was centered in explaining if the origin of the GS alteration is in the fresh fruit or in the physical−chemical conditions that are inherent to the table olive processing. For it a detailed study of the chlorophyll pigment evolution during the growth and development of the fruit was made as well as during the main operations of the table olive processing.

MATERIALS AND METHODS

Raw Material and Sampling. The study was carried out in olive fruits of the Gordal variety, *Olea europae regalis* (L.) supplied by "Agroaceitunera S.A." company (Utrera, Seville, Spain).

1. Field Trial. These were picked from four trees in an olive plantation property of the mentioned company, during two consecutive harvesting seasons, in order to study the influence of the climatologic changes. Sampling was conducted weekly from the middle of June (at which stage the fruits were so small that no stone could be distinguished) to the end of October (approximate date of fruit harvesting). During this time period the olive color changes from intense green to yellowish green. The fruits were picked from all around the tree, between 9 and 11 in the morning, until an approximately 3 kg sample was collected. All the fruits picked at each sampling were classified according to the size of their equatorial circumference by using manual sizing rings (*11*). The relative abundance and the mean weight of the fruits were calculated for each uniform-sized group. The group with greater relative abundance was selected as representative of the sampling. The selected fruits of this form (approximately 500 g) were destoned and sliced, constituting the raw material for the pigment analysis. Three replicate analysis of each sample were performed.

2. Industrial Trial. The sampling was carried out on three harvesting/processing dates during the months of September and October. In all cases, at each sampling date, the fruits were classified in six groups according to the size of their equatorial circumference, and four fruits from each uniform-sized group were individually weighted and analyzed for chlorophyll pigments.

2.a. Fresh Fruit. This study was carried out on three batches of fruits (each of 20 kg) received by the company at intervals of one week. A total of 20 fruits for each batch were individually analyzed.

2.b. Fruits from the NaOH Treatment. Three batches of olive fruits, each of 20 kg and with different NaOH treatment (T1, T2, and T3), were supplied by the company. The two first batches were fruits treated with NaOH in water at 1.61% (w/v) (T1) and 1.68% (T2). The third batch was fruits treated with NaOH in recycled alkaline solution at 1.52% (w/v) (T3) instead of water. The use of recycled NaOH solution is a modification of the traditional system of green table olives preparation, aimed at decreasing the volume of wastewaters generating during the process (*1*). A solution that had been previously used for the alkaline treatment of other olive fruits and adjusted in NaOH concentration until 1.52% was used as recycled alkaline solution (T3).

The three NaOH treatments lasted 14 h, and next the fruits were washed with water during 8 h. During the 14 h of the alkaline treatment the penetration of the NaOH solution into the pulp is approximately $^2/_3$ of the distance from skin to pit. Differences in penetration depend mainly on the size of each fruit. In agreement with this, to study how the alkaline treatment could affect of individual form to each fruit, the fruits were classified by size from each NaOH treatment and individually analyzed (20 fruits for each batch).

3. Study of the Effect of pH on Pigment Transformation. The experiment was carried out on two olive batches processed in the industrial plant with different alkaline treatments and supplied after the water washing step. The first olive batch was treated with NaOH solution in water (1.61% w/v) (T4), while the second one was treated with recycled NaOH solution (1.52% w/v) (T5). The fruits were classified by size, and the group with greater fruit relative abundance was selected as the representative to carry out this experiment. The selected fruits from both alkaline treatments were placed in some glass containers (3.5 kg of fruit per each container) with a universal buffer of 0.028 M citric acid, 0.028 M KH_2PO_4, 0.028 M boric acid, and 0.028 M diethylbarbituric acid containing 5% NaCl and 0.01% Merthiolate to avoid undesired fermentations. The buffer:fruit ratio was 1:1 (wt/wt), and the pH was adjusted with 0.2 M NaOH to the experimental pH value. The studied pHs were 3.5, 4.5, 5.5, and 6.5. The buffer solutions were renewed periodically until the fruit pulp reached the predetermined pH. The analysis of the pigment changes began after the fruit−buffer solution equilibrium was reached. From that moment, the olives were maintained for 15 days to the established pH values. The chlorophyll pigments were periodically analyzed each 1 or 2 days. In each control, three fruits by each pH value and type of alkaline treatment were individually analyzed in this experiment until a total of 279 analyzed fruits.

Measurement of Fruit pH. The pH was measured in the homogenized mixture resulting from triturating the plant material in a minimum volume of distilled water with a polytron homogenizer at 24000 rpm/min for 1 min. The water was added to facilitate homogenization.

Pigment Extraction. This was performed with *N*,*N*-dimethylformamide according to the method described in a previous work (*12*). The technique is based on the selective separation of components between *N*,*N*-dimethylformamide and hexane. This system yields a solution of pigments free from fatty matter that is characteristic of these fruits and that would interfere with subsequent separation and quantification of pigments.

Separation, Identification, and Quantification of Pigments. This was carried out by HPLC according to the method described by Mínguez-Mosquera et al. (*13*), using a reverse phased column (25 × 0.46 cm) packed with 5 μm C_{18} Spherisorb ODS2 (Teknokroma, Barcelona, Spain) and an elution gradient with the solvents (A) water/ion-pair reagent/methanol (1:1:8, v/v/v) and (B) acetone/methanol (1:1 v/ v), at a flow-rate of 2 mL/min. The ion-pair reagent was 0.05 M tetrabutylammonium acetate and 1 M ammonium acetate in water. The allomerized chlorophylls and metal chlorophyll complexes of copper were identified by co-chromatography with the corresponding standard, as has been described in detail in previous articles (*3*, *14*). Pigments were detected by absorbance at 410, 430, 450, 650, and 666 nm and quantified from the corresponding calibrate curves.

Reagents. For all purposes, analytical grade (American Chemical Society) reagents were used (Panreac, Barcelona, Spain). The solvents used for chromatography were HPLC grade (Teknokroma, Barcelona, Spain). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corporation, Milford, MA).

Table 1. Changes in Fruit Weight and Total Content of Chlorophyll *a* Derivatives (TChl *a*) and the Percentage of Allomerized Chlorophylls (AChl *a*) during the Stages of Growth and Development of Gordal Variety Olives[a]

		season 1			season 2		
harvest week	month	weight (g)	TChl *a* (nmol/fruit)	AChl *a* (%)[b]	weight (g)	TChl *a* (nmol/fruit)	AChl *a* (%)[b]
1	June	0.64	109.01	2.15	2.61	354.80	6.14
2		1.72	334.73	3.46	3.57	423.02	3.20
3	July	2.60	404.04	4.70	4.19	429.94	2.33
4		3.40	517.25	1.12	4.86	484.63	2.36
5		3.92	550.44	4.00	5.25	578.18	2.26
6		4.93	597.13	1.55	5.87	625.41	1.25
7	August	5.35	631.54	0.91	6.26	599.81	3.16
8		5.84	654.93	1.55	7.23	666.17	2.39
9		6.66	661.46	6.99	7.70	737.39	13.12
10		7.61	707.14	6.78	7.68	670.08	12.22
11		8.37	754.54	2.33	7.95	502.32	9.14
12	September	8.43	769.67	1.32	7.71	462.37	6.65
13		8.71	796.55	2.54	8.34	573.55	1.99
14		9.58	857.36	2.11			
15		9.53	709.07	1.87	9.13	500.83	1.52
16	October	9.51	630.38	2.93	9.17	516.70	1.22
17		9.42	617.10	2.44	9.23	461.26	2.71
18		9.57	585.43	9.14			

[a] Values represent mean for ten determinations for the weight of fruits and three determinations for the pigments analysis (CV < 10%). [b] Percentage with respect to total chlorophyll pigments of the *a* series.

Apparatus. Equipment included a pH meter, model pH 555 (Teknokroma, Barcelona, Spain), an Ultra-Turrax model T-25 polytron homogenizer (Janke Kunker, IKA-Laboratechnik), a Büchi rotavapor, Model R 110 (Laboratoriums-technik AG, Switzerland), and an HP 1100 Hewlett-Packard (Palo Alto, CA) liquid chromatograph fitted with an HP 1100 automatic injector and diode array detector.

RESULTS AND DISCUSSION

All pigments described for olive fruit of the variety Gordal, before and after being processed as green table olive (*15*), were found in analyzed fruits. However, the present study of results considers only the chlorophyll derivatives of the series *a*—those primarily taking part in the GS alteration.

Oxidized Catabolites Generated in Vivo. The flowering of the olive extends over a period of some 20 days, so that a single tree bears fruits of different sizes as a consequence of the successive pollinations occurring during that period. Thus, and given that olive picking is carried out in massive form on the tree and simultaneously in different groves, fruits are found in different metabolic states at every date of sampling.

These fruits, with different morphological characteristics, such as weight and size, and different content and class of pigments, are nonetheless processed together, not being known if some of these differences cause a particular answer in certain fruits when they are processed. On this assumption, the changes in the content and class of pigments in fruits of the Gordal variety during the stages of fruit growth and development up to picking, according to weight and metabolic state, was studied in two consecutive harvesting seasons. At the same time, with the aim of estimating the variability of these parameters in the raw material being processed as green table olives, three batches of fruits received in the processing plant at intervals of a week were analyzed.

Table 1 shows the changes in fruit weight during the stages of growth and development on the tree. It includes the total content of chlorophyll *a* derivatives (i.e., the sum of chlorophyll *a*, 13^2-OH-chlorophyll *a*, and 15^1-OH-lactone-chlorophyll *a*), and the percentage of allomerized chlorophylls (the sum of 13^2-OH-chlorophyll *a* and 15^1-OH-lactone-chlorophyll *a*). Although in general all the parameters followed a common pattern, certain differences between harvesting seasons could be detected. In the first, the fruit appeared on the tree in mid-June, with a mean weight of 0.64 g, while in the second, it formed earlier, and by the same date had achieved a significantly higher mean weight (2.61 g). However, the rate of growth in the first year was higher, so that after week 8 fruit weights were not statistically different (test *t*-Student, $p > 0.05$), and the maximum weight reached at the end of the study was around 9.5 g in both cases.

Regarding pigment content, it was noteworthy that although fruit weights were statistically different until week 8 (test *t*-Student, $p < 0.05$), this was not the case for the chlorophyll content (test *t*-Student, $p > 0.05$) between weeks 3 and 10 of the study. Later, the changes in chlorophylls indicated small differences between the fruits depending on harvesting. Those of the first harvesting season, whose formation on the tree had been later, synthesized a maximum chlorophyll content of 857 nmol/fruit around mid-September, against the peak of 740, a month earlier, in the second harvesting season. In both cases, the chlorophyll content diminished gradually, after reaching its maximum, to values of around 600 and 500 nmol/fruit, respectively.

The allomerized chlorophyll derivatives are oxidized compounds that participate in the metabolism of chlorophylls, being a study in olive fruits that confirmed for the first time their involvement in turnover and catabolism of chlorophylls (*9*). The results again confirmed that in olives of the Gordal variety, 13^2-OH-chlorophyll *a* and 15^1-OH-lactone-chlorophyll *a* are present during the vegetative cycle of the fruit. In the first harvesting season, the maximum accumulation of oxidized derivatives was around 7%, and this occurred in the fruits before the biosynthetic process finished. In the second harvesting season, the concentration of oxidized chlorophylls in the fruits was almost double, reaching a value of 13%, coinciding in time with the highest level of chlorophylls synthesized. Subsequently, the concentration of the allomerized derivatives fell, more gradually in the second year than in the first, remaining at the proportion found in the growth phase, ranging between approximately 1 and 4%.

It is noteworthy that in both harvesting seasons, these peak levels of oxidized chlorophylls were maintained for two weeks and occurred at the same chronological time (August), in the four or five weeks before picking, in full phase of chlorophyll biosynthesis, when the fruits were in a similar state of development, with a mean weight of between approximately 7 and 8 g. This showed that for a single olive variety, the peak levels of both chlorophyll synthesis and participation of the metabolic intermediates change similarly, independently of the climatic conditions, as had been established in varieties of olives destined to the extraction of oil (*16*).

Table 2 shows the percentage distribution of fruits obtained from the size classification carried out in the consignments of fruits received at the processing plant. It also shows the variability implicit in considering the three batches of fruits jointly, where the range of fruit weight was between 9.5 and 15 g. The variability in fruit weight was because, being picked in massive form from the tree and from different groves, the fruits were in different metabolic states and had synthesized to a greater or lesser degree the different components, such as fatty acids and sugars. In any case, the parameter that essentially determines the weight of the fruit is its uptake of water, and this depends on the climatic changes, in particular the level of rainfall each year (*17*). The moisture content of the olive is

Table 2. Fruit Relative Abundance According to Classification by Their Equatorial Circumference Size (ECS)

ECS (mm)	% fruits batch 1	batch 2	batch 3	overall batches
23.5–24.5	5.56	0	5.56	3.70
24.5–25.5	0	0	38.89	12.96
25.5–26.0	22.22	44.44	27.77	31.48
26.0–26.5	22.22	22.22	11.11	18.52
26.5–27.0	33.33	16.67	16.67	22.22
27.0–27.5	16.67	16.67	0	11.11

between 75 and 80% and determines the development and increase in size of the fruit.

Figure 1 shows, for each batch, the total content of chlorophyll pigments of series *a* (**Figure 1a**) and the percentage of allomerized derivatives (**Figure 1b**) vs the weight of each fruit. The statistical analysis showed that there was no direct correlation between fruit weight and pigment content per fruit ($R = 0.17$ and $R = 0.09$, $p > 0.07$, for total content of chlorophyll pigments and percentage of allomerized derivatives, respectively). Independently of their weight, 74% of the fruits analyzed showed a mean chlorophyll content of between 575 and 800 nmol/fruit, a range that could be considered, dealing with natural products, more or less of the same order as that found in the field study. It was thus confirmed that, in general, chlorophyll synthesis in the fruits culminates before picking and that this occurred at a specific time, independently of the weight reached by the fruit.

The percentage of allomerized chlorophylls was in all the cases inferior to 5%, independently of the size and origin of the fruit. In 77% of the samples, the concentration of oxidized chlorophylls did not exceed 3% of the series *a* chlorophyll compounds. In the remaining 23%, the value was somewhat higher—between 3 and 5% (**Figure 1b**). None of the three batches included fruits in the phase of accumulation of oxidized derivatives, as the percentage of allomerized chlorophylls corresponded, in accord with the prevoius study of growth and development (**Table 1**), to fruits in stages later than total chlorophyll synthesis.

From the foregoing it is deduced that the mean level of chlorophylls synthesized is inherent to the variety, that the peak of oxidized derivatives is in the chlorophyll synthesis stage, and that when fruit picking takes place, the metabolic phase of greatest chlorophyll synthesis and highest proportion of oxidized intermediates is over.

Oxidation Originated by NaOH Treatment. The skin of the olive is relatively resistant to penetration of the NaOH solution, so that an initial period is required before the skin is crossed and there is diffusion into the pulp. As mentioned in the previous section, during olive picking fruits coexist with different characteristics of weight (ranging between 9.5 and 15 g) and size (between 23.5 and 27.5 mm equatorial diameter). When the fruits are processed together and in the same way, such differences will have their effect. For a particular concentration of NaOH solution and treatment time, the penetration of this alkaline solution into the fruits will differ depending on fruit size, and, consequently, the effect on the plant tissue may not be uniform.

The present study has considered, in addition to size, the effect on the fruits of three types of alkaline solutions: the first two with fresh solutions of NaOH at different concentration (T1 and T2), and a third using a recycled solution of NaOH (T3). The HPLC chromatograms for pigment extract of fresh fruit and

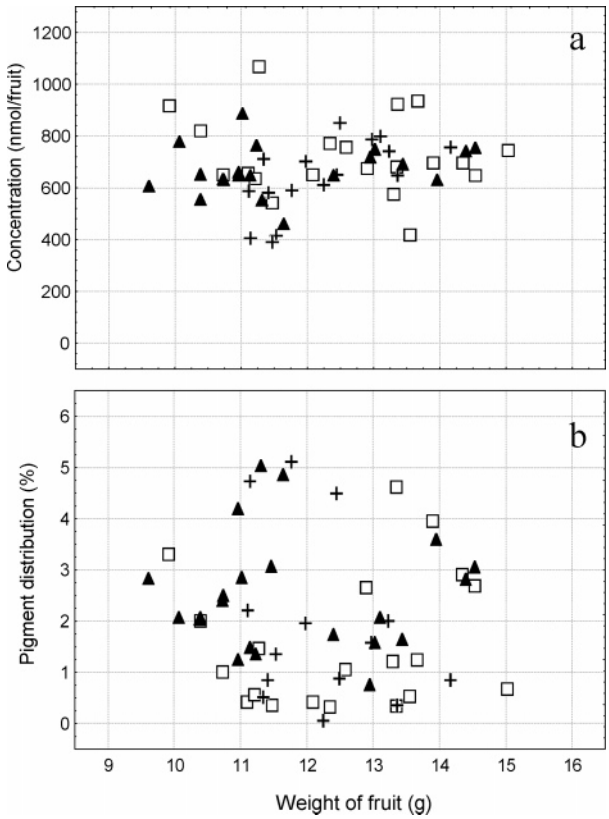

Figure 1. Total content of chlorophyll pigments of *a* series (**a**) and percentage of allomerized chlorophylls (**b**) in olive fresh fruits from three batches. Symbols: (□) batch 1; (+) batch 2, and (▲) batch 3.

fruit treated with NaOH are shown in **Figure 2**. As can be seen, in the fruit treated with NaOH, besides 13^2-OH-chlorophyll and 15^1-OH-lactone-chlorophyll, which are present in the fresh fruit, a new compound was originated: 15-glyoxylic acid chlorophyll *a* (*18*).

Figure 3 shows the effect of the different alkaline solutions on the initial chlorophyll composition of the fresh fruit in function of the fruit weight. In principle, it would be expected that, given the location of the pigments in the fruit (immediately underneath the epidermis), the amount of oxidized chlorophyll derivatives formed is directly related with the concentration of NaOH used. The rest of the alkaline treatment conditions being equal, the greater the NaOH concentration, the more marked would be its action on the skin, and, consequently, on the chlorophylls. For a certain alkaline treatment, there was a great variability between fruits in the proportion of oxidized derivatives that was not related to the size of the fruit. Nevertheless, considering all the fruits altogether, it was seen that the biggest increase in 13^2-OH-chlorophyll (**Figure 3a**) was at the highest concentration of NaOH used (T2), whereas the percentage of 15^1-OH-lactone-chlorophyll (**Figure 3b**) remained practically unaltered from that of the initial fresh fruit. In contrast, when the fruits were treated with a lower concentration of NaOH, but in a recycled solution (T3), besides an effect on both types of allomerized compound, the increases were greater—particularly noteworthy was the increase of 15^1-OH-lactone-chlorophyll, which had not been changed by the treatments with more-concentrated NaOH solution.

Regarding the percentual distribution of the compound 15-glyoxylic acid chlorophyll (**Figure 3c**), in general no differences between treatments were noted, except in four samples from the treatment with recycled NaOH (T3), which showed a greater proportion. In principle, this would be expected for all samples,

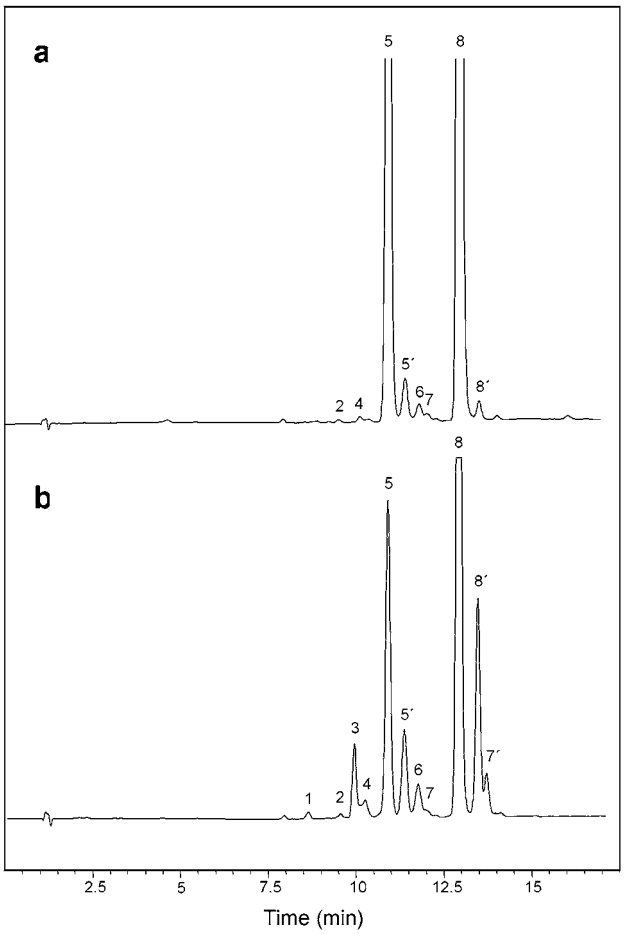

Figure 2. HPLC chromatograms at 650 nm of pigment extracts from (**a**) fresh fruit and (**b**) fruit treated with NaOH. Peaks: 1 = 15-glyoxylic acid chlorophyll *b*; 2 = 15^1-OH-lactone-chlorophyll *b*; 3 = 15-glyoxylic acid chlorophyll *a*; 4 = 13^2-OH-chlorophyll *b*; 5 = chlorophyll *b*; 5′ = chlorophyll *b*′; 6 = 15^1-OH-lactone-chlorophyll *a*; 7 = 13^2-OH-chlorophyll *a*; 7′ = 13^2-OH-chlorophyll *a* isomer; 8 = chlorophyll *a*; 8′ = chlorophyll *a*′.

as this treatment involved the greatest oxidation. This result thus suggested the possibility that the rest of the samples would yield the transformation of 15-glyoxylic acid chlorophyll into 15^1-OH-lactone-chlorophyll (*18*), by the solvation and elimination reactions proposed by Hynninen and Assandri (*19*) (**Figure 4**). With this premise, the sum of the percentages of 15^1-OH-lactone-chlorophyll and 15-glyoxylic acid chlorophyll was calculated in each sample analyzed for all the treatments (**Figure 5a**). The results indicated that the highest values were reached in the fruits treated with recycled NaOH solution, confirming that in this case there had been some transformation of 15-glyoxylic acid chlorophyll into 15^1-OH-lactone-chlorophyll and explaining the increase originated in this latter compound.

Figure 5b includes the total of oxidized chlorophyll derivatives for the three types of alkaline treatment. Fixing the attention to a percentage of oxidized chlorophylls medium of 20%, the number of samples that surpass this value was only 30% in T1, half of the samples in T2, and all samples in T3. Therefore, it was confirmed that at higher concentration of NaOH it tended to have a greater formation of oxidized chlorophylls, but it was seen that the third treatment, using recycled NaOH solution, originated a significant greater formation of such compounds. These results indicated that there must be factors associated with the use of recycled NaOH solution that intensifies its oxidizing capacity, as, despite the fact that the NaOH concentration was lower in T3, the levels of oxidation were higher. Finally, this fruit batch, which included some smaller fruits, showed a certain effect of the fruit size, with the greatest transformation in the smallest ones.

As mentioned in the introduction, although the main aim of treating the fruit with NaOH solution is the hydrolysis of the bitter glucoside oleuropein, during the process some sugars and other fermentable matter, organic and inorganic solids, polyphenols, residues from fungicidal treatments, etc., also pass into the solution (*1*) (*20*).

Therefore, the chemical composition of the recycled NaOH solution is from the start completely different from the fresh solution prepared in water. The difference found in the oxidation of chlorophylls could be ascribed to the presence of other components in this solution, which in some way increase the oxidizing potential of the NaOH, as the characteristics of the raw material were similar in the three treatments studied.

From the foregoing it is deduced that the initial presence of allomerized biosynthetic intermediates in the fresh fruit has no effect in the subsequent oxidation of the chlorophylls that is generated by the alkaline treatment. The level of chlorophyll oxidation tends to increase with the increase in NaOH concentration and is potentiated with the use of recycled NaOH solution. Consequently, the oxidizing power of the medium in which the treatment takes place determines the level of chlorophyll oxidation in the fruit.

Effect of pH on Pigment Transformation. In the green table olive processing, after alkaline treatment and washing, the fruit is transferred to fermentors and covered with a 10% sodium chloride solution (brine). The sugars, vitamins, and amino acids of the fruit pass into the brine by osmosis, and the brine gradually converts into a culture medium suitable for growth of microorganisms. Initially the pH value exceeds 10 units due to the residual lye continuing to come out of the pulp. Through the various stages of fermentation (*1*), the succession of different species of microorganisms reduce the pH to 6 units in the 2−3 first days of brining and to pH values of 4 or less during 30−60 following days, *Lactobacillus plantarum* being the predominant specie.

The influence in the fruit of this pH reduction that results from the natural fermentation process was studied in a model system with this experiment. The aim was to know the individualized effect of each pH value on the chlorophyll composition of the fruits after alkaline treatment. From the results of the previous section, the two extreme alkaline treatments were chosen for this study: the mild and the most severe—that is, the normal industrial treatment with NaOH solution in water, and that using recycled NaOH solution. In both experiments, pigment changes were monitored in the four batches of fruits during the 15 days that each was kept at the determined pH values: 6.5, 5.5, 4.5, and 3.5.

The pigment profile for the two types of alkaline treatment did not undergo any change with time when the fruits were kept at pH 6.5, whereas in those subjected to pH 5.5, the free Mg-derivatives were detected, including pheophytin *a*, 15-glyoxylic acid-pheophytin *a*, 13^2-OH-pheophytin *a*, and 15^1-OH-lactone-pheophytin *a*. A noteworthy feature of the experiments at pH 4.5 and 3.5 was, besides the pheophytinization reaction, the formation of the Cu-15-glyoxylic acid-pheophytin *a* (Cu-G-phy) complex. This compound is the first copper chlorophyll complex detected in olives during the industrial fermentation process when the fruits were developing green staining (*4*). This demonstrated that the precursor compound 15-glyoxylic acid-pheophytin *a*, originated by the alkaline

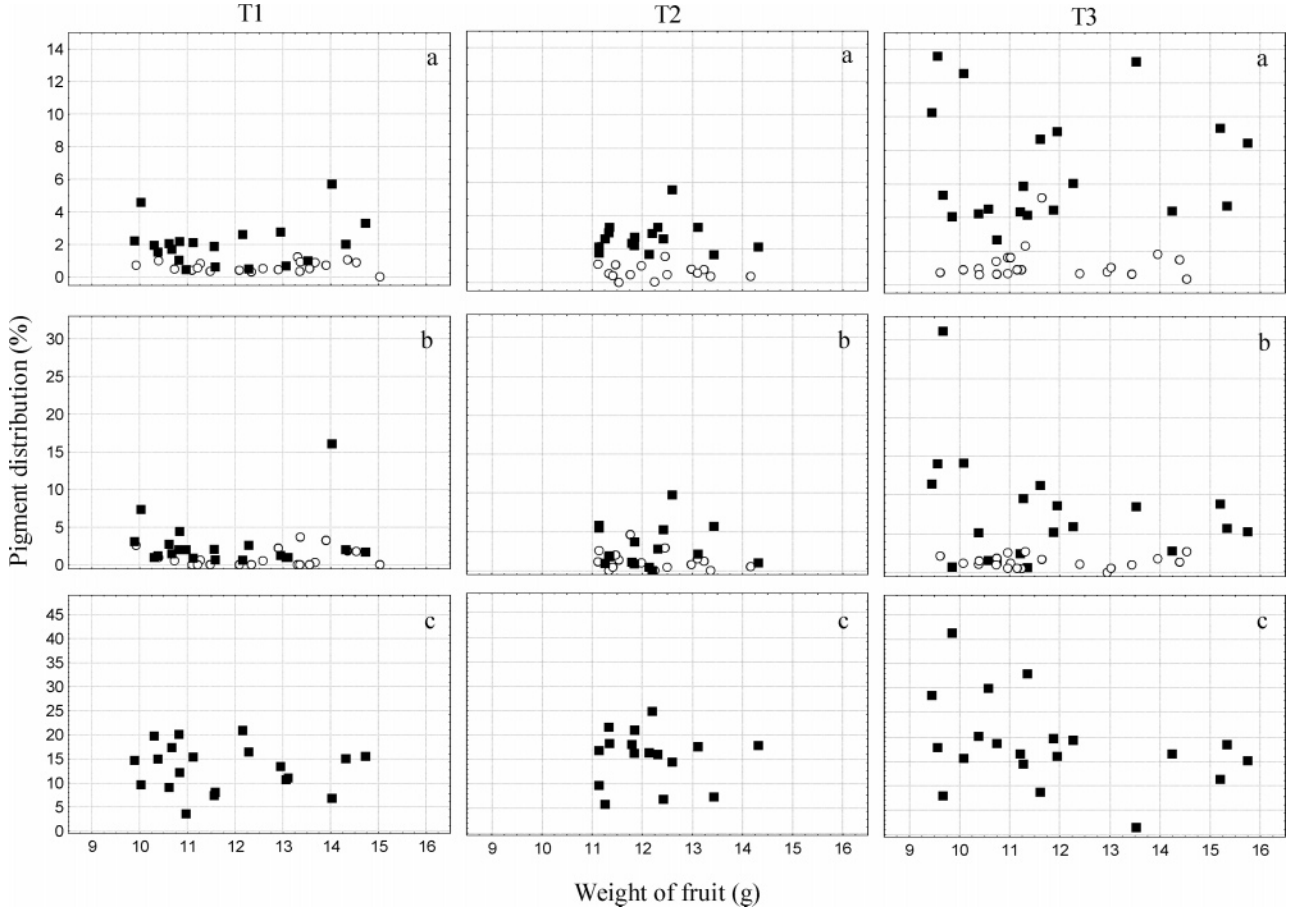

Figure 3. Percentage (with respect to total chlorophyll pigments of *a* series) of 13^2-OH-chlorophyll *a* (**a**), 15^1-OH-lactone-chlorophyll *a* (**b**), and 15-glyoxylic acid chlorophyll *a* (**c**) in olive fruits. Symbols: (○) fresh fruit; (■) fruit treated with NaOH solution at 1.61% w/v in water (T1), 1.68% w/v in water (T2), and 1.52% w/v in recycled alkaline solution (T3).

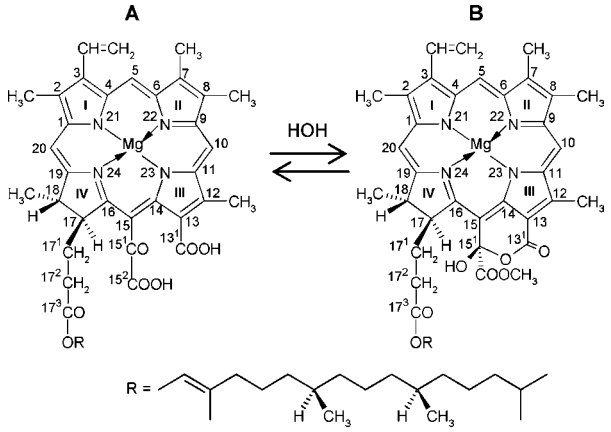

Figure 4. Transformation of 15-glyoxylic acid chlorophyll *a* (**A**) into 15^1-OH-lactone-chlorophyll *a* (**B**).

treatment, has the greatest structural disposition for capturing the endogenous Cu present in the fruit.

Figure 6a shows the changes in total percentage of oxidized chlorophyll derivatives in the fruits kept for 15 days at pH 4.5 and 3.5. It was found that the initial level of oxidation in the chlorophyll fraction, different depending on the alkaline treatment applied, remained unchanged in the time for both pH values. The presence of the Cu-G-phy complex (**Figure 6b**) was detected from the fourth day after starting the experiment for both pH values. In the first experiment, which involved a lower degree of initial oxidation in the fruits, being from the mild alkaline treatment, the concentration of Cu-G-phy *a* was always lower than 0.9%, while in the second experiment, it reached double that value in most of the samples. At the same time, there was a small, but progressive, increase in the formation of Cu-G-phy *a* with time in acid pH, a trend that was more marked at pH 3.5.

It is known that certain chemical reagents, in which NaOH is included, can alter the permeability of membranes and are used in protocols of biochemical studies by its capacity to separate membrane proteins selectively (*21*, *22*). Although studies are not known in this sense, it can be thought that in the processing of green table olives, the intensity of the alkaline treatment can be directly related to separation of the pigment−lipoprotein complexes from thylakoid membranes, favoring the contact with the endogenous Cu. Later, when the fruits are placed in a hypertonic solution to a pH value of 4.5 units or lower, the process of osmotic diffusion allows to reach in chloroplast the necessary concentration of hydrogen ions so that the generalized Mg liberation in chlorophyll pigments takes place and the Cu insertion in those pigment−protein complexes that have been separated of the thylakoid membranes can be produced.

In accord with these results, the quantitative differences in the formation of the Cu-chlorophyll complex would be induced by the different oxidation of chlorophylls which, depending on the type of treatment with NaOH solution, were originated in the fruits before they were subjected to a determined value of acid pH. Higher levels of oxidized chlorophylls corresponded with higher concentrations of cupro-derivatives, for the same value of acid pH. This showed a possible relationship between

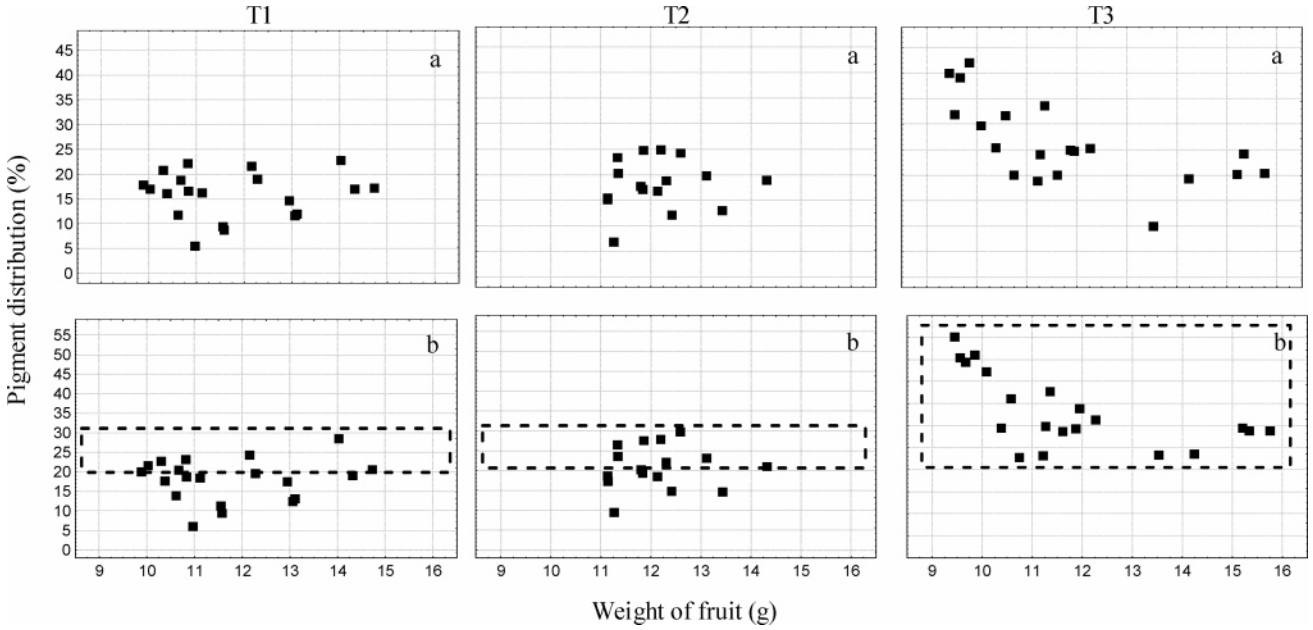

Figure 5. Percentage (with respect to total chlorophyll pigments of *a* series) of the sum of 15[1]-OH-lactone-chlorophyll *a* and 15-glyoxylic acid chlorophyll *a* (**a**) and total allomerized chlorophylls (**b**) in olive fruits treated with three different NaOH solutions (T1, T2, and T3). (See Figure 2 for abbreviations.)

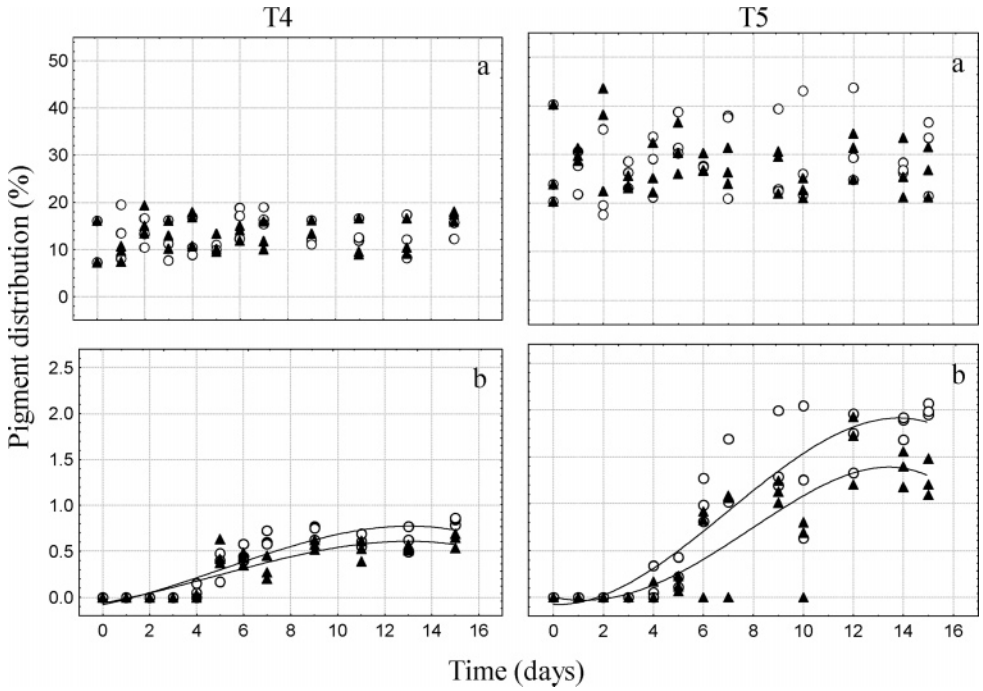

Figure 6. Changes in the percentage (with respect to total chlorophyll pigments of *a* series) of total allomerized chlorophylls (**a**) and Cu-15-glyoxylic acid pheophytin *a* (**b**) in olive fruits subjected in a model system to constant pH after treatment with NaOH solution and washing. Symbols: (○) pH 3.5; (▲) pH 4.5. Abbreviations: T4, treatment with NaOH at 1.61% w/v in water; T5, treatment with NaOH at 1.52% w/v in recycled alkaline solution.

the greater formation of oxidized substrate and a greater degree of cell degeneration, which in acid medium favors the Cu complexation with the chlorophyll molecule of greatest structural affinity for the metal. From then on, it will be a question of time for the accumulation of Cu-chlorophyll pigments and the start of a visible GS.

In addition, for each time and pH value, there is a great variability in the Cu-G-phy content between different fruits. This means that, before an identical alkaline treatment, fruits with the same size respond with a formation of oxidized chlorophylls and Cu complexes that are not homogeneous but differenced for each fruit. This result allows thinking that the ripening stage could have an influence. The optimal moment of the fruit harvesting for the processing as green table olives is when it has initiated the maturation process and its color is yellowish green. As was commented in the materials section, the harvesting of the fruits occurs massively and the ripening stage is not identical in all the fruits. In some of them, therefore, the disintegration process of thylakoid membranes entails that the fruit maturation can be more advanced. In these fruits therefore, the pigment−lipoprotein complexes would be more accessible and susceptible to being affected by the treatment with NaOH.

Interaction of Oxidized Chlorophylls with the Endogenous Cu of the Fruit. The results obtained in the present study revealed that the percentage of oxidized chlorophylls generated in the fruits by the alkaline treatment was especially important.

Table 3. Percentage with Respect to Total Chlorophyll Pigments of the *a* Series of the Allomerized Chlorophylls (AChl) and Total Cupro-Chlorophyll Complexes (TCu-Chl) and Cupro-Allomerized Chlorophylls (Cu-AChl) of the *a* Series, in Olives with Different Degrees of Green Staining Alteration[a]

		cupro-chlorophyll complexes	
sample	AChl *a*	TCu-Chl	Cu-AChl
1	23.49	1.92	1.25
2	23.41	2.06	1.26
3	24.65	2.14	1.28
4	35.40	2.16	1.38
5	33.23	2.33	1.43
6	37.80	2.72	1.71
7	23.09	3.73	2.50
8	24.09	4.00	2.48
9	27.63	4.10	2.52
10	24.07	4.80	2.77
11	27.51	6.29	4.21
12	23.13	6.95	4.97
13	25.03	7.48	5.26
14	25.10	10.49	6.4
15	26.05	11.24	6.46
16	25.68	11.38	7.11

[a] Values represent mean values for four different samples with similar visible degrees of green staining (CV < 10%) and have been ordered in increasing sequence of the total percentage of cupro-chlorophyll complexes.

As this parameter has not been taken into account in previous works related with fruits showing GS alteration, the total percentage of oxidized chlorophylls and of them the fraction that was forming complex with Cu were determined.

From 16 samples of olives with different visible degree of GS—that is, from fruits whose skin visibly shows the alteration as pinhead-sized spots to those with extensive bluish-green coloring—both the total percentage of allomerized series *a* derivatives and that for the sum of the different Cu-complexes, specifying those that included oxidized chlorophylls, were calculated (**Table 3**). The values represent the mean for four different samples with similar visible degree of GS and have been ordered in increasing sequence of the total percentage of cupro-chlorophyll derivatives.

It was verified that the percentage of allomerized was superior in all the cases to 23%, arriving in some cases until 38%, with the total of Cu-chlorophyll complexes varying between 1.92 and 11.38%. The minimum level of allomerized chlorophylls in fruits where alteration GS visualizes (23%) was in accord with the data found in the present work in the second pH experiment where a greater percentage of cupro-chlorophyll derivatives was originated.

In addition it was verified that of the total copper-chlorophylls, around 60% correspond to allomerized cupro-chlorophylls which is related directly to the result of this work. The first copper chlorophyll complex that forms is Cu-G-phy, an allomerized chlorophyll derivative.

The industrial processing of table olives includes a lactic fermentation lasting from 2 to 3 months and involves moderate changes in pH leading to a final value of around 4, or even lower, at the end of the first month. During this period, with the previously mentioned conditions of oxidation, will be formed not only Cu-G-phy *a* but also the other Cu-chlorophyll complexes present in fruits with visible GS (*3*).

From the foregoing, it can be established that the generation of levels of oxidized chlorophylls exceeding 23% during the alkaline treatment is indicative of a certain cell integrity destroying in the fruits. Taken together with other circumstances, this will favor the liberation of both the pigment and the Cu, and—as the consequent first step—the insertion of the latter into the molecule of 15-glyoxylic acid-pheophytin *a*.

Table 4. Distribution of Copper between Metal Chlorophyll Complexes and Pectin Residues in Table Olive with and without GS[a] (*7*)

	fruit without GS	fruit with GS		
		whole fruit	RZ[a]	GSZ[a]
Cu-Chl complexes[b]	0.58 ± 0.02	2.38 ± 0.14	1.89 ± 0.09	6.09 ± 0.37
Cu in AIS[c,d]	14.56 ± 0.71	12.52 ± 0.58	13.08 ± 0.66	9.55 ± 0.49
Cu matter balance	15.14 ± 0.73	14.90 ± 0.71	14.97 ± 0.74	15.64 ± 0.84

[a] GS: green staining. GSZ: green staining affected zone of the fruit with GS alteration. RZ: remaining zone of altered fruit. [b] The data, expressed as μmol/kg of fruit, represent mean values and standard deviation for four determinations. [c] The data, expressed as μmol/kg of fruit, represent mean values and standard deviation for nine determinations. [d] The pectin residue was analyzed in the alcohol-insoluble solids (AIS) fraction.

Correlating this fact with results obtained in previous works on altered fruits (*7*, *8*) leads to the conclusion that the final visualization of the GS alteration is due to the progress of concatenated reactions, in which intervene other constituents of the fruit besides the oxidized chlorophylls, Cu, and acid pH. These reactions begin with the alkaline treatment which not only causes chlorophyll oxidation but also could modify the cell integrity. This fact entails a lesser interaction between pigment−lipoprotein complexes and thylakoid membranes liberating the pigments from their lipid environment (*8*). In addition it modifies the pectin chain, originating first a great decrease in the protopectin fraction and an increase in the formation of calcium pectate. Subsequently, as the lactic fermentation process advances and the fruits remain for 30−60 days in pH acid, the pectin chain undergoes a cationic exchange provoked by the acid pH. The presence of H^+ in the medium, in a first stage, displaces Ca^{2+} and consequently the fraction of calcium pectate also decreases (*7*). Later, H^+ is displaced by Cu^{2+} because the bonding capacity of pectins with this metal increases in acid medium (*23*).

In a previous work (*7*), a matter balance for Cu^{2+} was calculated in table olive as the sum of copper forming the metal chlorophyll complexes and that found in the pectin residues analyzed in the alcohol-insoluble solids (AIS) fraction (**Table 4**). It was verified that the greater the content of copper chlorophyll complexes in the fruits, the smaller the amount of copper in the pectin residue, with the total content of copper remaining practically constant. This result allowed the suggestion that the pectin chain can work as a reservoir of the copper that is going to be captured by chlorophyll pigments separated from the thylakoid membrane to form the copper chlorophyll complexes.

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