Involvement of Copper and Zinc Ions in Green Staining of Table Olives of the Variety Gordal

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ABSTRACT

The presence of metalochlorophyllic complexes of copper has been detected in table olives showing the alteration known as green staining. These compounds are absent in the healthy fruit. The possible implication of fungicidal treatment of olive trees in this alteration has been studied. No alteration was produced in table olives prepared with fruit from trees with and without fungicidal treatment and the differences found between copper levels in the fruit were not significant. The possibility that the copper involved in this alteration is of extraneous origin was, therefore, discarded. On the other hand, there were no significant differences in the levels of copper in random samples of fruits with and without green staining. Therefore, although the green-staining alteration is the result of the formation of complexes of copper with chlorophyll derivatives, it seems clear that the simple presence in the fruits of copper, by itself, does not lead to the appearance of green-staining.

Key words: Olive, fungicide, color, pigment, metalochlorophyllic complex

Chlorophyll degradation comprises numerous reactions. The olive green color of pickled vegetables is attributed mainly to the formation of pheophytins and, in general and less extensively, to that of pheophorbides, pyropheophytins and other such compounds.

The retention of the bright green color of vegetables during the different treatments which they undergo for the best conservation of their alimentary and organoleptic characteristics has been the object of much research in the pickling industry. Almost all the studies made of the change in the green color of fruits and vegetables have been aimed at preventing these reactions by the combined action of pH, heat and chlorophyllase enzyme control, so that pheophytins are not formed (1,3,15).

However, during the processing of olive fruits for their consumption as Spanish- or Sevillian-style table olives (2), this type of transformation is desirable and necessary. Chlorophylls a and b, initially present in the fresh fruit (12), are totally degraded to pheophytins and pheophorbides by two different and coexisting mechanisms: one enzymatic, provoked by the action of chlorophyllase, and the other resulting from the acid pH of the fermentation medium (9,10,13). The carotenoid fraction is affected only in those components whose molecular structure is sensitive to an acid medium. Pigment degradation in the lactic fermentation process gives the fruits so treated the desired coloration. Nevertheless, during the subsequent period of conservation in brine, the chlorophyllic composition of the recently fermented fruits may undergo another series of oxidative degradations (7).

The varieties of olive most used and considered best for consumption as table olives are Manzanilla and Gordal. However, the use of the latter currently presents certain problems. The occasional appearance of green staining on the surface of the processed fruits during brining is a problem for the industry, because of its potentially negative effect on product marketing. The alteration appears first at localized spots, but may progressively cover the whole skin of the fruit.

It should be noted that although the definition or localization of the green-stained area is quite simple, its shape, distribution, and depth is very diverse. Some fruits are found with green, pin head sized-spots, while others have skin totally covered with the stain. Similarly, the depth of staining is very variable: ranging from fruits where it is hardly below the skin, to others where it occupies several millimeters towards the interior.

This concrete problem is not reflected in the bibliography, although various authors report frequent greening during the conservation or pretreatments of fruits and vegetables, which they relate with the formation of metalochlorophyllic complexes (4,6,16). These compounds may be formed in different ways by chlorophyll degradation products (mainly pheophytins and pheophorbides) in the presence of copper and zinc, with the consequent greening of the vegetable. Copper from the internal covering of the cans used for the conservation of foodstuffs can migrate to the molecules of pheophytins of the vegetable, resulting in its greening. Metalocomplex formation is always from chlorophyll derivatives, never chlorophylls, and takes place above 1–2 ppm of copper or 25 ppm of zinc (16).

As green table olives are processed in polyester and fiberglass fermenters and there is no contact with or intervention of copper or zinc at any stage, the possibility of contamination
from these metals during processing can be excluded. If external copper or zinc is involved in green staining, the only possibility of copper access to the chlorophyll molecule in the olive is through fungicidal treatment of the trees, using complex mixtures that always include copper salts, and frequently those of zinc.

The aim of the present work is to elucidate whether the alteration known as green staining in the Goral olives is related to the presence of external copper or zinc contributed by the products used for fungicidal treatment.

MATERIALS AND METHODS

Raw material

Fruits from the field trial. Three plots of olive trees of the variety Goral were chosen on an estate in the area of Utrera (Seville). The trees of one plot were left as controls, without treatment. The second plot was treated with fungicide A and the third with fungicide B, both of these common fumigants, whose components are basically as follows:

Fungicide A: copper (II) oxychloride 37.5% + zinc ethylenebis(dithiocarbamate), 15%.
Fungicide B: copper-calcium sulphate, 20%.

These products were used in dilute aqueous concentrations (around 0.5%), mixed with other substances (surfactants, coadjuvants, synergists, etc.) to improve their solubility and adhesion to the tree. The treatment consisted in a single application of ten liters of the fungicidal solution per tree. Fruits from the three plots were separately prepared as Spanish-style green table olives using the traditional procedure. Briefly, the process consists of treating the fruits with NaOH solution (approximately 2% w/v) for 8–10 h. Then the fruits are washed with water and kept in water for some 12 hours. Finally they are placed in 10% NaCl solution. Under these conditions, various microorganisms develop, producing the lactic fermentation that gives the fruit its characteristic properties (2).

Fruits supplied by industry. Olive samples were taken from 10,000 kg capacity fermenters in which alteration had been produced. Both fruits with green staining and those which - although they had been in the same fermenter - showed no alteration were analyzed. Fruits from similar fermenters in which this type of alteration had not been produced were analyzed as controls. All samples were from industrial sources.

Pigment extraction

The method used was that proposed by Míguez-Mosquera and Garrido-Fernández (12), which in summary consists of pigment extraction with N,N-dimethylformamide, mixture with hexane to eliminate fat, and transfer of pigments to an ethyl ether-hexane 100:60 mixture. This latter solution, containing xanthophylls, chlorophylls and chlorophyll derivatives, is concentrated and redissolved in acetone for use in thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC).

Preparation of standards

Chlorophylls a and b, pheophytins a and b, chlorophyllides a and b and pheophorbides a and b were obtained from fresh leaves of spinach in accord with procedures described in an earlier work (9). Pheophytins a and b were obtained from the pure standard of the respective pheophytin by reflux-heating at 100°C for 24 h in pyridine or collidine (14).

Copper complexes of pheophytins a and b and pyropheophytins a and b: the procedure used was that of Jones et al. (5). A solution of 20 ml of 1M CuCl₂ was added to 80 ml of pheophytin or pyropheophytin standard dissolved in acetone. Some crystals of ascorbic acid were added to the reaction solution to prevent oxidative changes. The chelation reaction was performed in N₂ atmosphere for 1 h and analyzed by TLC. The metal chelates were transferred from acetone to ethyl ether and dried with anhydrous Na₂SO₄.

Zinc complexes of pheophytins a and b and pheophytins b and pyropheophytins b and b were prepared in a way similar to those of copper, except that 5 g of zinc chloride crystals were added directly to the solution of pigment in acetone.

Separation of metalchlophyllic derivatives

Thin layer chromatography (TLC). Commercial plates of Kieselgel 60 F₂₅₄ (Scharlau, Cà 330) were used, together with others prepared in the laboratory with Kieselgel 60 GF₂₅₄ (Merck, art. 7730). The chromatography was performed in a saturated chamber, using the following mixtures as developers, depending on the object: petroleum ether (40–60°C)/acetone/diethylamine (10:4:1) and petroleum ether (40–60°C)/pyridine/diethylamine (7:1:0.5), all of analytical grade.

High-performance liquid chromatography (HPLC). HPLC analysis was by the method of Míguez-Mosquera et al. (11). The pigment extract (20 μl) was previously dissolved in acetone and filtered through a nylon membrane of 0.45 μm pore size. Separation was performed on a 25 × 0.4 cm C₁₈ Spherisorb ODS2 analytical column of 5 μm particle size. Pigments were separated using a gradient system at a flow rate of 2 ml/min. The eluents used were (A) water/ ion-pair reagent/methanol (1:1:8, v/v/v) and (B) acetonemethanol (1:1, v/v). The ion-pair reagent was 0.05 M tetrabutylammonium acetate and 1 M ammonium acetate in water. The solvents used were HPLC grade. Detection was performed at 410 and 430 nm.

Determination of copper and zinc

The fruits were washed, dried, pitted, and triturated with deionized water in a ratio of 1:1 to give a homogeneous paste. Twenty grams of this, weighed exactly, were dried in a porcelain crucible and 2 ml of a 5% w/v solution of Mg(NO₃)₂ in ethanol added. The resulting mixture was heated at 450°C for 8 h, and the resulting ash was bleached (if necessary) with 2 ml of concentrated HNO₃. The ash obtained was dissolved with two portions of 2 ml of hot 6N HCl made up to 25 ml with deionized water and vacuum filtered.

Copper and zinc were determined by atomic absorption spectrophotometry, atomizing the sample in an air/acetylene flame and using a hollow-cathode lamp of copper and zinc. Absorbance was measured at 324.7 nm for copper and 213.9 nm for zinc with a slit of 0.7 nm in both cases.

Apparatus

Büchi rotavapor, Model R 110; Desaga UV-vis lamp, provided with white and ultraviolet UV 254 nm light; Hewlett-Packard UV-vis spectrophotometer, model 8450, provided with a HewlettPackard recorder Model 7225A; Waters 600E multisolid delivery system fitted with a Waters Model 994 photodiode-array detector and a Waters Model 5200 register-integrator; atomic absorption spectrophotometer Perkin-Elmer mod. 2380.

RESULTS AND DISCUSSION

Pigment analysis

Samples from the field trials. Fruits from trees treated or untreated with fungicides, and destined to processing as table olives, were subjected to periodic visual observation and pigment analysis. After three years of preservation of
the fruits in brine, no anomaly was detected in their pigment composition nor was green staining observed. Samples supplied by industry. Figure 1 shows the characteristics of the TLC chromatogram obtained on developing two samples of fat-free pigment extract, one from healthy fruits and the other from altered fruits, in parallel on the plate. Obvious qualitative differences can be seen between them. The altered fruit gave an outstanding band that was bluish under white light, with an Rf value of 0.68, showed no strawberry fluorescence under ultraviolet light and was absent in the control. It indicated, in principle, a possible copper/chlorophyll derivative complex. Figure 2 shows the electronic absorption spectrum in ethyl ether of this compound. Comparison of its color and Rf value on the plate and the shape and localization of the absorption spectrum maxima with those of standards of metalochlorophyllic complexes obtained in the laboratory showed no similarity (Table 1). It must be a relatively nonpolar chlorophyll derivative not normally present in processed products, as its characteristics have not been found described in the literature. To date it has not been possible to identify this compound.

In the TLC development mentioned, another irregularity seen in the extract of altered fruit is in the zone between Rf values 0.44 and 0.63. Here there is an accumulation of overlapping bands with strawberry fluorescence under UV light which is absent in the sample of healthy fruit. This shows that in the altered fruit the initial pigments of the healthy fruit have given place to a greater number of degradation products, originating compounds that under the assay conditions are masked by those usual in the olive, distorting the chromatogram. It is possible that in olives affected with green stains there are other pigments that, under the analytical conditions used, are not separated clearly from the pigments normally found in healthy olives and which, therefore, remain unresolved. On the other hand, the metalochlorophyllic derivatives show Rf values that coincide with those of the pheophytins native to the fruit.

As a test, and in order to see to what extent such compounds could remain unresolved, a 3:1 mixture of pheophytin a with a complex of pheophytin a and copper was placed on a chromatographic plate. Under these condi-

![Figure 1. Thin-layer chromatogram on silica gel GF_{254} of pigments from healthy and altered fruits (light petroleum ether/acetone/diethylamine (10:4:1)).](image)

**TABLE 1.** Chromatographic and spectroscopic characteristics of the standards, separated on Silicagel GF_{254}

<table>
<thead>
<tr>
<th>Band No.</th>
<th>White Light</th>
<th>UV Light</th>
<th>Standards</th>
<th>Rf&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rf&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Maxima (nm)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Grey</td>
<td>Red Fl</td>
<td>Pyropheophytin a</td>
<td>0.63</td>
<td>0.67</td>
<td>410, 666</td>
</tr>
<tr>
<td>2</td>
<td>Green</td>
<td>No Fl</td>
<td>Pyropheophytin a–Cu</td>
<td>0.64</td>
<td>0.65</td>
<td>398, 422, 652</td>
</tr>
<tr>
<td>3</td>
<td>Green</td>
<td>Red Fl</td>
<td>Pyropheophytin a–Zn</td>
<td>0.61</td>
<td>0.68</td>
<td>424, 656</td>
</tr>
<tr>
<td>4</td>
<td>Grey</td>
<td>Red Fl</td>
<td>Pheophytin a</td>
<td>0.57</td>
<td>0.48</td>
<td>410, 666</td>
</tr>
<tr>
<td>5</td>
<td>Green</td>
<td>No Fl</td>
<td>Pheophytin a–Cu</td>
<td>0.57</td>
<td>0.43</td>
<td>398, 422, 652</td>
</tr>
<tr>
<td>6</td>
<td>Green</td>
<td>Red Fl</td>
<td>Pheophytin a–Zn</td>
<td>0.56</td>
<td>0.49</td>
<td>424, 656</td>
</tr>
<tr>
<td>7</td>
<td>Brown</td>
<td>Red Fl</td>
<td>Pyropheophytin b</td>
<td>0.51</td>
<td>0.45</td>
<td>432, 650</td>
</tr>
<tr>
<td>8</td>
<td>Green</td>
<td>No Fl</td>
<td>Pyropheophytin b–Cu</td>
<td>0.52</td>
<td>0.40</td>
<td>428, 624</td>
</tr>
<tr>
<td>9</td>
<td>Green</td>
<td>Red Fl</td>
<td>Pyropheophytin b–Zn</td>
<td>0.51</td>
<td>0.46</td>
<td>452, 634</td>
</tr>
<tr>
<td>10</td>
<td>Brown</td>
<td>Red Fl</td>
<td>Pheophytin b</td>
<td>0.44</td>
<td>0.33</td>
<td>432, 650</td>
</tr>
<tr>
<td>11</td>
<td>Green</td>
<td>No Fl</td>
<td>Pheophytin b–Cu</td>
<td>0.44</td>
<td>0.30</td>
<td>428, 628</td>
</tr>
<tr>
<td>12</td>
<td>Green</td>
<td>Red Fl</td>
<td>Pheophytin b–Zn</td>
<td>0.44</td>
<td>0.34</td>
<td>450, 636</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fl: fluorescence.
<sup>b</sup> Developer: light petroleum ether/acetone/diethylamine (10:4:1).
<sup>c</sup> Developer: hexane/acetone/diethylamine (7:4:0.5).
<sup>d</sup> Peaks of maximum absorption in ethyl ether.
tions the green color of the complex was completely masked by the chlorophyll derivative. Furthermore, on developing the mixture using the reference developing fluid, it advanced as one single compound and showed a strawberry-colored fluorescence under UV light. When the amount of complex was increased progressively, no change in coloration occurred until the complex represented more than half of the total mixture.

Since the evidence obtained indicated that these complexes are not detected under the conditions used, special attention was paid to separating them from their pheophytin precursors. Therefore the pigmentation in the $R_r$ 0.44–0.63 range was scraped off, eluted with acetone, and its volume concentrated in a rotavapor. The solution was rechromatographed in TLC, modifying the polarity of the eluent to achieve different mobilities between the problem components. With the mixture hexane/acetone/diethylamine (7:4:0.5) two new bands ($R_r$ 0.65 and 0.43) with green coloration and without strawberry fluorescence under UV light were separated. Comparing the chromatographic and spectroscopic characteristics of these compounds with those shown by the metalochlorophyll complexes (Table 1), the first one corresponded with the copper complex of pyropheophytin a and the second with the copper complex of pheophytin a, respectively. In both cases the shapes and peaks of their absorption spectra were coincident with those of the standards (Fig. 3).

In order to test if these separated pigments were pure and were the only metalochlorophyll complexes present in the green-stained fruit, the solution was studied using HPLC. Figure 4 shows the HPLC chromatograms obtained from the following pigment solutions: a) the total extract of healthy Gordal olives with good color, b) the solution from eluting the zone $R_r$ 0.44–0.63 in TLC in altered fruit, and c) a mixture of metalochlorophyll complexes of copper and zinc. As can be seen clearly in Fig. 4b (chromatogram of the problem zone), the peaks marked I and II are absent in healthy fruit. They had been detected previously in some fruits during brining as rhodin esters (8). These pigments

Figure 3. Electronic absorption spectrum in ethyl ether for the complex pheophytin a-Cu. It is the same for pheophytin a-Cu and pyropheophytin a-Cu.

Figure 4. HPLC chromatogram of pigments extracts from: (a) healthy fruits; (b) altered fruits (only zone $R_r$ 0.44–0.63 in Fig. 1); and (c) complexes. Peaks: 1 = pheophorbide b; 2 = pheophorbide a; 3 = neochrome; 3' = neochrome isomer; 4 = pyropheophorbide a; 5 = auroxanthin; 5' = auroxanthin isomer; 6 = mutatoxanthin; 7 = lutein; 7' = lutein isomer; 8 = rhodin ester; 8' = rhodin ester.
Table 2. Copper and zinc analysis in fresh and processed fruits from fumigated olive trees.

<table>
<thead>
<tr>
<th>Fungicide</th>
<th>Fresh Fruit</th>
<th>Processed Fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copper (mg/kg)</td>
<td>Zinc (mg/kg)</td>
</tr>
<tr>
<td>None</td>
<td>4.89</td>
<td>5.86</td>
</tr>
<tr>
<td>A</td>
<td>4.25</td>
<td>5.62</td>
</tr>
<tr>
<td>B</td>
<td>5.18</td>
<td>5.42</td>
</tr>
</tbody>
</table>

Table 3. Copper analysis in fruits from fermenters with and without green staining (GS).

<table>
<thead>
<tr>
<th>Harvest</th>
<th>Sample</th>
<th>Control</th>
<th>Fruits with GS</th>
<th>Zones with GS</th>
<th>Fruits without GS</th>
<th>Zones without GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1991</td>
<td>S-1</td>
<td>3.47</td>
<td>4.38</td>
<td>3.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S-2</td>
<td>2.07</td>
<td>3.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S-3</td>
<td></td>
<td>2.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1992</td>
<td>S-4</td>
<td>2.46</td>
<td>3.36</td>
<td>2.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S-5</td>
<td></td>
<td>3.11</td>
<td>1.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S-6</td>
<td></td>
<td>3.21</td>
<td></td>
<td>3.90</td>
<td></td>
</tr>
<tr>
<td>1993</td>
<td>S-7</td>
<td>4.98</td>
<td>4.95</td>
<td>4.64</td>
<td>4.42</td>
<td>4.63</td>
</tr>
</tbody>
</table>

Copper and zinc analysis

Fruits from the field trial. Table 2 shows the values of copper and zinc concentrations found in the pulp of fruit with and without fungicidal treatment before and after processing. As can be seen, there is no noticeable tendency in any one direction. Even in the processed fruit the level of concentration of the fresh fruit is not maintained. This demonstrates that the fungicidal treatment, by itself, is not the cause of the alteration.

Samples supplied by industry. Table 3 shows the results found for both the fruits with green staining and those that were unaltered even though they were from the same fermenter. At the same time, as sample controls, the values found in fruits from fermenters that did not show alteration are given. All data are the mean of three replicates. The study was carried out during three consecutive years. In the first two years the results show a tendency to higher copper values in the altered fruit, while in the third year the sequence is inverted, to the point that the zone with green staining shows the lowest value.

However, among the fruits from different harvests, the range of copper levels found, whether there was alteration or not, is very wide, reaching in some cases differences of 100%. Thus, in healthy fruits extreme values in pulp of 2.07 and 4.98 mg/kg are found, and in fruits with green staining, 2.55 and 4.95 mg/kg. These ranges are very wide and very overlapping. Therefore the results obtained up to now do not prove the hypothesis that the formation of green staining is a result of copper contributed by fungicides. There are considerable variations in copper from one sample to another, especially when comparing harvests, and there is not a determinate quantity of copper sufficient by itself to explain the presence of green staining. In fact, samples S-2 and S-3 (harvest 1991) and S-4, S-5, and S-6 (harvest 1992), which present the problem, had less copper than the fruits without staining or the healthy samples S-1 (harvest 1991) and S-7 (harvest 1993).

It must be borne in mind that the composition of the fruit itself includes a certain amount of copper that is necessary for its biological development. The appearance (without apparent cause) of green staining on the surface of processed fruits is due to causes yet unknown, but related to some factor or factors involved in the processes of cultivation, preparation, or conservation.

Due to the very specific circumstances of fermentation, in which associated physicochemical, enzymatic, or microbiological reactions take part, now unknown conditions may originate that cause the localized formation of compounds giving rise to this green coloration.

The fact that the alteration appears in localized spots seems to indicate that pigment modification in concrete zones of the cuticle is caused by the accumulation of a series of factors that initially do not affect other zones.

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