JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Pigment−Lipoprotein Complexes in Table Olives (Cv. Gordal) with Green Staining Alteration

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In table olives showing the green staining alteration, extracts of pigment−lipoprotein complexes were obtained from the zone altered and the rest of the fruit. In the altered zone of the olive, the surrounding components of pigments were affected, with the degeneration of proteins and phospholipids forming the pigment−lipoprotein complexes. There was also less interaction between the pigments and the membrane lipids. These results suggested a greater loss of cell integrity in the green-stained zone of the fruit, allowing the migration and local accumulation of Cu-metallochlorophyll complexes, macroscopically visible as the form of green staining alteration.

KEYWORDS: Cu-chlorophyll complex; green staining alteration; pigment−lipoprotein complex; pigment, table olive

INTRODUCTION

The Gordal variety table olive is affected by green staining, seen as small green spots distributed over the surface of the processed fruit. This pigmentary problem has not been described in any other product of vegetable origin. Consequently, it is a specific research matter with no antecedent bibliography. The present authors have been working on the topic for several years. The studies carried out to date have established that during the currently used process for the lactic fermentation of Gordal variety olives, there is a shift in the typical pattern of chlorophyll transformation in a fermented vegetable because, besides pheophytins, pheophorbides, and their respective pyroderivatives, the allomerized compounds 15-glyoxylic acid pheophytins *a* and *b*, 15-formylpheophytin *a*, and 15-hydroxylactone pheophytins *a* and *b* are originated (*1*). It has become known that the pigments responsible for the characteristic color of the green staining alteration are copper complexes of certain oxidized chlorophyll derivatives and of pheophytins and pyropheophytins (*2*, *3*). The copper involved in this alteration has been shown not to have an external origin but to come from the fruit itself (*2*), and it has been suggested recently that the fruit pectins might act as reservoirs of this Cu (*4*). Because metallochlorophyll complexes are also present in the fruit zone not showing green staining, these compounds alone are not sufficient cause for the alteration to appear (*5*).

Earlier electron microscopy studies carried out in olives with green staining have shown that the fruit zone presenting alteration has a greater degree of cell deterioration than the rest of the fruit (*5*). Chlorophylls and carotenoids are localized in the lipid matrix of the thylakoid membranes of the chloroplast, forming pigment−protein macromolecular complexes. Frequently, and particularly in the case of the lipoproteins that solubilize carotenoids, these complexes include phospholipids, although such associations and the types of interaction have not been thoroughly studied. In carotenolipoprotein complexes from sea buckthorn fruits the polar lipids included 61% phospholipids (*6*).

The aim of the work was to elucidate if the greater cell degradation observed by electron microscopy in the fruit zone presenting green staining alteration affected the link between the pigment−lipoprotein complexes and their lipid environment. This study was carried out by comparing in fruit affected by the alteration the nature and extractability of pigment−lipoprotein complexes both in the affected zone and in the rest of the fruit.

MATERIALS AND METHODS

Raw Material. The study was carried out in samples of Gordal variety table olives (*Olea europaea regalis*) showing green staining alteration. The zone with green staining and the remaining zone (the zone without green staining) of the fruits were analyzed separately. Samples were supplied by the industry.

Sampling. Samples of olives with green staining came from three different harvests. For each harvest, the industry supplied 10 kg of altered olives. Analyses were performed in quadruplicate from a homogenate of 100 g of plant material from the zone with green staining or the zone without green staining.

Extraction of Pigment—Lipoprotein Complexes. This was performed according to the method of Milicua et al. (*7*) adapted for the

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10.1021/jf025965b CCC: $25.00 © 2003 American Chemical Society
Published on Web 01/22/2003

olive. Five grams of sample (zone with green staining or zone without green staining) was homogenized in 25 mL of H_2O in an Ultra-Turrax at 4 °C. The homogenate was adjusted to 5% of Triton X-100 in H_2O and extracted with overnight stirring in a cold-storage room, at 4°C, under subdued light. The extract was filtered through four layers of gauze, and the filtrate was centrifuged at 5000*g* for 30 min at 4 °C. The pellet was re-extracted once with 25 mL of 5% Triton X-100 in H_2O until no more color was recovered and then kept at −30 °C for additional extraction of pigments with an organic solvent. The supernatants were pooled, 1.5 g of diatomaceous earth was added to remove the lipid fraction, which was not solubilized in the extraction buffer, and the mixture was centrifuged under the same conditions. The extract was passed through a 1 × 3 cm column of diatomaceous earth to eliminate residual lipids. The pigment−lipoprotein extract was dialyzed for 2 weeks against 1 L of H_2O to remove the detergent. The water was changed daily. The dialysate was used for the analysis of pigments, proteins, and phospholipids.

Determination of Protein and Phospholipid Concentration. The protein concentration of the pigment−lipoprotein complex was obtained according to the method of Lowry et al. (*8*), using bovine serum albumin (BSA) as standard. Phospholipid concentration was determined according to the method of Böttcher et al. (*9*).

Estimation of the Molecular Weight of the Lipoproteins by Gradient Polyacrylamide Gel Electrophoresis. Estimation was made by electrophoresis on an acrylamide gradient (5−20%) plate (*10*). The gradient was performed with a Bio-Rad gradient maker, using 0.09 M Tris, 0.003 M EDTA, and 0.08 M boric acid, pH 8.3, adjusted with solid boric acid (TBE). The staking gel was 4% acrylamide in TBE. Samples were concentrated 10 times, and 15 μL of concentrated lipoprotein was placed on the electrophoresis gel. Samples of thyroglobulin (669000), ferritin (440000), catalase (232000), lactate dehydrogenase (140000), and BSA (67000) were used as standards. The polyacrylamide gels were silver stained using Bio-Rad silver reagents on the basis of the photochemical method described by Merril et al. (*11*). After electrophoresis, the proteins were fixed on the gel with a solution of methanol (50% v/v) and acetic acid (10% v/v) for 20 min. The gel was then washed three times for 10 min with deionized water. The silver reagent, prepared by mixing three commercial solutions—image developer, silver complex solution, and moderate reducing solution, each of 5%—with an equal volume of 5% (w/v) developing accelerator reagent, was added. The mixture was left to act until the bands were well stained, and then the reaction was halted with a 5% acetic acid solution.

Pigment Analysis. Analysis of the protein-linked pigments was performed by injecting 50 μL of the Triton X-100 extracts directly into the HPLC. For the analysis of the pigments remaining in the pellet, it was necessary to extract them first with *N*,*N*-dimethylformamide (NNDMF) according to the method described in a previous work (*12*). The technique is based on the selective separation of components between NNDMF and hexane. This system yields a solution of pigments free from the fatty matter that is characteristic of these fruits and which interferes with subsequent separation and quantification of pigments. All pigments were separated, identified, and quantified using reversed phase HPLC, following the methodology described by Mínguez et al. (*13*). The metallochlorophyll complexes of copper were identified as described in detail in a previous paper (*14*). Pigments were detected by absorbance at 410, 430, 450, and 666 nm before quantification.

Test for the Pigment—Protein Link. Two milliliters of the pigment−protein extract was treated with 10 mL of diethyl ether in a test tube. Three milliliters of acetone was added, and the mixture was inverted several times and left to settle until complete phase separation. The ether layer became colored and the aqueous one, colorless.

Apparatus. Equipment included the following: an Ultra-Turrax model T-25 polytron homogenizer (Janke Kunker, IKA-Laboratechnik), a Kontron centrifuge model Centrikon T-124, (Kontron Instruments, Milano, Italy), a Hoefer electrophoresis apparatus (Pharmacia Biotech AB, San Francisco, CA), a Bio-Rad gradient maker (Hercules, CA), a source of voltage (Pharmacia Biotech), a Büchi rotavapor model R 110 (Laboratoriums-technik AG, Switzerland), an HP-1100 Hewlett-Packard (Palo Alto, CA) liquid chromatograph fitted with an HP-1100 automatic injector and an HP-1100 diode array detector, and a Hewlett-Packard 8452A UV−visible spectrophotometer.

Table 1. Some Characteristics of Pigment–Lipoprotein Complexes Extracted from the Zone with Green Staining (GS) and from the Zone without Green Staining of Table Olives with Green Staining Alteration[a]

	zone with GS	zone without GS
proteins (mg/mL)	0.66 ± 0.05	0.68 ± 0.05
phospholipids (mM)	0.15 ± 0.02	0.23 ± 0.01
molecular weight (kDa)		
band 1	353 ± 3.1	317 ± 1.1
band 2	514 ± 3.8	460 ± 5.1

[a] Data represent mean and confidence interval for 12 determinations, $p < 0.05\%$.

RESULTS AND DISCUSSION

Analysis of Lipoproteins. No significant variations were found between the two samples (zone with or without green staining) regarding the amount of proteins (**Table 1**). This indicated that, quantitatively, the peptidic fraction of the pigment−lipoprotein complex remained constant during the process leading to green staining. There were, however, differences in the amounts of phospholipids: the complexes extracted from the zone without green staining contained 1.6-fold more phospholipids than the complexes from the zone with green staining. The amphoteric capacity of the phospholipids enables the stabilization of peptides within a lipid matrix. With the complexes isolated from the green-stained and unaffected zones having similar peptide contents, the differences in phospholipid content must correspond to polarity requirements, depending on the lipid environment in which the lipoprotein is found. Thus, the greater phospholipid content found in the zone without green staining indicated that the protein required large amounts of these compounds to remain stable in the membrane, signifying strongly apolar membrane conditions and, therefore, only a small degree of hydrolytic or oxidative alteration. In contrast, in the case of the pigment−lipoprotein complexes in the green-stained zone, the lower content in phospholipids indicated a lesser requirement of these compounds for stabilizing the complex in the membrane. This suggested conditions of greater polarity in the environment of the lipoprotein complexes, directly related with a greater hydrolytic or oxidative type deterioration of the membrane lipids. The pigment−lipoprotein complex of the green-stained zone being more weakly linked to the membrane, it was more readily liberated and exposed to the external medium, enabling interaction with other cell components. Hence, following the lactic fermentation process in the olives, there was a greater cell degradation in the fruit zones with green staining than in the other zones.

When the extracts of pigment−lipoprotein complexes were subjected to gradient polyacrylamide electrophoresis, two bands separated in each case, with different molecular weights for each sample (**Table 1**). The two bands of the sample for the alteration-affected zone were of higher molecular weight than the corresponding bands separated in the control sample. The explanation for such differences was the formation of larger aggregations of the peptides making up the pigment−lipoprotein complexes in the green-stained zone, due to an increase in environmental polarity as a result of alteration of the membrane and due to its reduced capacity of retention of the pigment−lipoprotein complex.

Analysis of Pigments. In the present study, pigments previously identified (*3*) as typical of olives with green staining were found. However, minor chlorophyll and carotenoid pig-

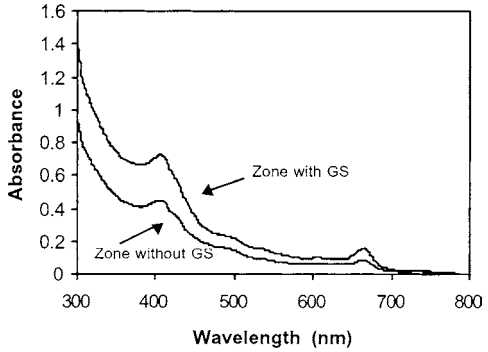

Figure 1. Electronic absorption spectra of the total extract of pigment–lipoprotein complexes obtained from the zone with green staining and the zone without green staining of table olives with green staining alteration.

ments were not considered in the study of results. Chlorophyll derivatives monitored were the Mg-free derivatives (pheophytins *a* and *b*, pyropheophytins *a* and *b*, 15-glyoxylic acid pheophytins *a* and *b*, 15-formylpheophytin *a*, pheophorbides *a* and *b*, and pyropheophorbide *a*) and the metallochlorophyll complexes of copper (Cu−pheophytin *a*, Cu−pyropheophytin *a*, Cu−15-glyoxylic acid pheophytins *a* and *b*, and Cu−15-formylpheophytin *a*). The main pigments in the carotenoid fraction were *β*-carotene and lutein.

In the analysis of pigments, those that had been extracted linked to proteins, as pigment−lipoprotein complexes, were differentiated from those extracted as the free form from the pellet remaining after extraction of the complexes. The pigment extracts used in the earlier studies of green staining alteration (*3*, *4*) were obtained with an organic solvent, which breaks the pigment−protein link. To obtain extracts of olive pigments in their natural state, linked to proteins, it was necessary to use an aqueous system that would not destroy the pigment−lipoprotein complex and which also had to include a detergent to weaken the interaction of the pigments with the lipids of the thylakoid membranes. The use of Triton X-100 in water was appropriate, although pigment extraction was not total. Consequently, the colored residue remaining after extraction of the pigment−lipoprotein complexes had to be later treated with an organic solvent, NNDMF, until the residue was completely colorless. The use of the organic solvent cleaved the pigment−lipoprotein bond but enabled extraction, for the analysis of the pigments, of the remaining compounds not collected with the aqueous system, which were more closely linked to the lipid matrix. In this way, two pigment fractions were obtained for each sample: one extracted as pigment−lipoprotein complexes and the other directly as free pigments.

Under equalized conditions, the electron absorption spectrum obtained by measuring the total extract of pigment−lipoprotein complexes of the green-stained zone of the olive (**Figure 1**) showed greater absorption in the visible region than that of the unaltered zone. Given that the absorption is due to the presence of pigments in the complexes, there was always a greater recovery of pigments as pigment−lipoprotein complexes in the altered zone of the olive. This indicates a greater extractability and thus a lower retention of these complexes by the membrane.

The link between pigments and protein was verified using one of the surest tests to confirm such interaction (*15*, *16*). When the green aqueous solution was treated with ethyl ether, no change was observed in the color of the different phases, whereas on subsequent addition of acetone to the system, the situation changed: the green color passed to the organic phase, leaving the aqueous phase completely colorless. This indicated

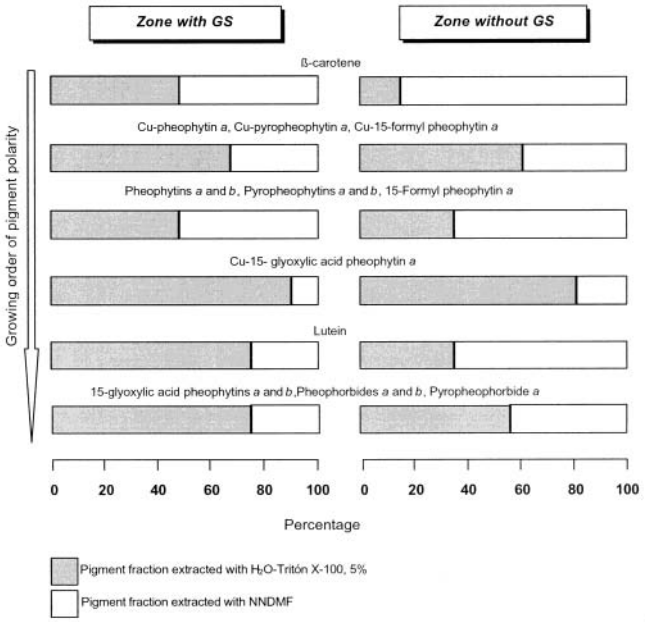

Figure 2. Percentage of each pigment extracted with Triton X-100 (as pigment–lipoprotein complex) or with NNDMF (as free pigment). The zones with and without green staining (GS) of table olives with green staining alteration were analyzed separately.

that the pigment−lipoprotein link had broken and the liberated pigments had transferred to the ether phase, ready for their analysis.

Figure 2 compares the percentage of each pigment extracted with Triton X-100 (as pigment−lipoprotein complex), or with NNDMF (as free pigment); the zone with green staining and the rest of the fruit not showing the alteration were analyzed separately. In general, it could be observed that as the pigments became more polar, they were extracted in higher proportion with the aqueous system. Nevertheless, comparison of results obtained in the two different zones in the same fruit confirmed that the recovery of all the pigments as pigment−lipoprotein complexes was greater from the zone with green staining than from that without it. This result corroborated the assumption that in the altered zone of the olive there was less interaction between the pigments and the membrane lipids, which would be a consequence of the greater loss of cell integrity found after the analysis of phospholipids.

It was also noteworthy that the Cu-chlorophyll derivatives were extracted mainly as pigment−lipoprotein complexes with the detergent in both samples, that is, in both the green-stained zone and the zone without staining. This result indicated that the Cu-chlorophyll−lipoprotein complex formation was due to a minimal or null interaction between the membrane lipids and the pigment−lipoprotein complexes in the fruits, favoring the insertion of Cu ion into the chlorophyll−lipoprotein complex. All of these results coincided with and supported the hypothesis of an earlier work (*5*) based on electron microscopy studies carried out in olives with green staining. This established that for the appearance of the anomalous green color in the Gordal olive, there would have to be a weakening of the interaction between membrane lipid and pigment. However, it did not justify the visualization of the green staining only in certain zones of the fruits. The higher cell deterioration found in the green staining zone of the fruit could be responsible of the Cu-chlorophyll−lipoprotein complex migration and accumulation in a lipid inclusion within the olive, with the result of the green staining visualization.

Hence, the green staining of the Gordal olive was the final consequence of an alteration in the fruits that originates long before the anomalous color becomes visible in the processed olive. The pigments were not the only compounds of the fruit that were involved—the surrounding molecular environment was also affected, with the degeneration of other components, such as the proteins and phospholipids forming the pigment−lipoprotein complexes. The mechanisms taking part in the alteration remain to be understood so that it can be prevented, even if this means modifying the processing of this type of product.

LITERATURE CITED

(1) Mínguez-Mosquera, M. I.; Gallardo-Guerrero, L. Anomalous transformation of chloroplastic pigments in Gordal variety olives during processing for table olives *J. Food Prot.* **1995**, *58*, 1241−1248.

(2) Mínguez-Mosquera, M. I.; Gallardo-Guerrero, L.; Hornero-Méndez, D.; Garrido-Fernández, J. Involvement of copper and zinc ions in "green staining" of table olives of the variety Gordal. *J. Food Prot.* **1995**, *58*, 564−569.

(3) Gandul-Rojas, B.; Gallardo-Guerrero, L.; Mínguez-Mosquera, M. I. Identification of oxidized chlorophylls and metallochlorophyllic complexes of copper in table olives (cv. Gordal) with green staining alteration. *J. Food Prot.* **1999**, *62*, 1172−1177.

(4) Gallardo-Guerrero, L.; Hornero-Méndez, D.; Mínguez-Mosquera, M. I. Pectins as possible source of the copper involved in the green staining alteration of Gordal cv. table olives. *J. Agric. Food Chem.* **2002**, *50*, 6746−6751.

(5) Gallardo-Guerrero, L.; Gandul-Rojas, B.; Mínguez-Mosquera; M. I. Chlorophyll pigment composition in table olives (cv. Gordal) with green staining alteration. *J. Food Prot.* **1999**, *62*, 1167−1171.

(6) Pintea, A.; Marpeau, A.; Faye, M.; Socaciu, C.; Gleizes, M. Polar lipid and fatty acid distribution in carotenolipoprotein complexes extracted from sea buckthorn fruits. *Phytochem. Anal.* **2001**, *12*, 293−298.

(7) Milicua, J. C. G.; Juarros, J. L.; De las Rivas, J.; Ibarrondo, J.; Gomez, R. Isolation of a yellow carotenoprotein from carrot. *Phytochemistry* **1991**, *30*, 1535−1537.

(8) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(9) Böttcher, G. J. F.; Van Gent, C. M.; Pries, C. A rapid and sensitive sub-micro phosphorous determination. *Anal. Chim. Acta* **1961**, *24*, 203−204.

(10) Anderson, L. O.; Borg, H.; Mikaelsson, M. Molecular weight estimation of proteins by electrophoresis in polyacrylamide gels of graded porosity. *FBBS Lett.* **1972**, *20*, 199−202.

(11) Merril, C. R.; Goldman, D.; Sedman, S. A.; Ebert, M. H. Stain for protein in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **1981**, *211*, 1437−1438.

(12) Mínguez-Mosquera, M. I.; Garrido-Fernández, J. Chlorophyll and carotenoid presence in olive fruit (*Olea europaea* L.). *J. Agric. Food Chem.* **1989**, *37*, 1−7.

(13) Mínguez-Mosquera, M. I.; Gandul-Rojas, B.; Montaño-Asquerino, A.; Garrido-Fernández, J. Determination of chlorophylls and carotenoids by high-performance liquid chromatography during olive lactic fermentation. *J. Chromatogr.* **1991**, *585*, 259−266.

(14) Mínguez-Mosquera, M. I.; Gandul-Rojas, B.; Garrido-Fernández, J. Preparation of Cu(II) complexes of oxidized chlorophylls and their determination by thin-layer and high-performance liquid chromatography. *J. Chromatogr.* **1996**, *731*, 261−271.

(15) Zagalsky, P. F. Invertebrate carotenoproteins. *Methods Enzymol.* **1985**, *111*, 216−247.

(16) Gómez, R.; Milicua, J. C. G. Use of the borohydride reduction methods to probe carotenoid−protein binding. *Methods Enzymol.* **1992**, *213*, 101−110.

Received for review September 18, 2002. Revised manuscript received December 13, 2002. Accepted December 13, 2002. This work was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT, Spanish government) AGL2000-0699 and by Project 1/UPV/EHU 00042.310-EA-8055/2000.

JF025965B