JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Pigment, Physicochemical, and Microbiological Changes Related to the Freshness of Cracked Table Olives

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ABSTRACT: The changes in chloroplastic pigments, mineral nutrients, and characteristics related to freshness were studied during storage and packing of cracked seasoned olives. Cracking produced an initial loss in green pigments and color degradation. Later, storage caused a progressive degradation of chlorophylls and carotenoids, with a slower rate in refrigerated fruits (which preserved the greenish tones better), but after packing (and storage at room temperature), the differential effect disappeared and, at the end of the study, all olives showed similar pigment transformations, which were correlated with CIE a^* and hue. Processing led to a Na content increase in olive flesh (and Ca and Zn, when added) but marked losses in the other mineral nutrients. Sodium metabisulfite and $ZnCl_2$ promoted LAB growth while inhibiting yeast, thus enhancing product stability, and erythorbic acid caused yeast growth and firmness deterioration.

KEYWORDS: *carotenoids, chlorophylls, cracked olives, microbiological characteristics, mineral nutrients, physicochemical characteristics*

INTRODUCTION

The green color, which is related to freshness, is highly valued by consumers and is essential for some directly brined table olive specialties. However, these fruits progressively lose their initial green appearance and develop brownish tones. After packing, these changes continue and lead to a rapid depreciation of the final products, which have a short shelf life. The Protected Denomination of Origin (PDO) "Aceituna Aloreña de Málaga", a seasoned table olive specialty, is a good matrix to study such color transformations. This presentation can be prepared either from fresh fruits or from preserved in brine (11% NaCl) olives that are then packed gradually according to demand.[1] Its changes in appearance have been evaluated only by CIE L^*, a^*, and b^* parameters,[2] but no information on its chloroplastic pigment transformations is available. The color of green fruits (including olives) and vegetables is due to the presence of the chloroplasts of chlorophylls *a* and *b* together with yellow carotenoids. Fruit and vegetable processing produces, to a greater or lesser extent, structural transformations in chlorophylls due to substitution of their central Mg^{2+} ion with H^+. These pigment reactions modify the characteristics of the chromophore groups, with subsequent changes in the hue and/or intensity of the color. The chlorophylls are transformed into pheophytins, pyropheophytins, and pheophorbides,[3] all of them with gray-brownish colors, and the reaction is accelerated under acidic conditions. The carotenoids are also susceptible to some reactions during food processing and storage, such as isomerization and oxidation. These reactions are due to the presence of a long, extensive system of conjugated double bonds (or polyene chain) in their structure and cause a general decrease in the intensity of the yellow color.

Overall, chloroplastic pigment transformations make olives progressively brownish. Ascorbic acid and sodium metabisulfite were used in the previous washing solutions of cracked "Aceituna Aloreña de Málaga" table olives to improve color retention. In addition, the presence of $CaCl_2$ and $MgCl_2$ in the storage brine was also favorable for maintaining appearance.[4] Erythorbic acid had a stronger antioxidant effect in some foods than its diastereoisomer ascorbic acid.[5] Calcium ions decreased the softening rate of fresh and Spanish style green olives[6] and may also be effective for preventing softening and for maintaining the fresh appearance of cracked "Aceituna Aloreña de Málaga" olives. A combination of sulfite and calcium has been used for the preservation of red bell peppers[7] and for preventing the softening of acidified peppers.[8] Hence, sulfite alone or in combination with other compounds may help to maintain the fresh appearance of cracked olives.

Zinc salts have also demonstrated an ability to retain the green color of thermally processed fruits and vegetables,[9] due to the formation of zinc-chlorophyll-derivative complexes with a stable, highly bright green color.[10] The use of zinc salts as additives is not yet accepted for table olives,[11] but $ZnCl_2$ has demonstrated a high antimicrobial activity in olive products.[12]

The aim of this work was to study the changes in chlorophylls, carotenoids, mineral nutrients, and other parameters related to freshness as affected by different processing conditions as well as changes in physicochemical and microbiological characteristics during the storage and packing of cracked Aloreña olives. The results may help to improve the preservation of natural freshness in directly brined olives in general.

MATERIALS AND METHODS

Experimental Design. Fruits from the PDO "Aceituna Aloreña de Málaga" (size 240/260 fruits/kg) harvested at the so-called green maturation degree were processed in the facilities of a local olive

Received: January 18, 2013
Revised: March 21, 2013
Accepted: March 25, 2013
Published: March 25, 2013

industry (Aceitunas Bravo S.A., Málaga, Spain). Olives were mechanically cracked, put in 250 L drums, and covered with 110 g/L NaCl brine (140 kg fruits and 115 L brine). The olives were left to equilibrate for 72 h, and then replicated containers were stored without the addition of any additives (WA): (a) in a cold room (CR (WA)) at 7 ± 2 °C and (b) at room temperature (RT (WA)) (15 ± 7 °C) in a covered yard. In addition, other drums were also stored at RT in a covered yard but with added (c) 1.5 g/L sodium metabisulfite (SMB), (d) 1.5 g/L SMB plus 3 g/L $CaCl_2$ (SMB + $CaCl_2$), (e) 0.5 g/L $ZnCl_2$ ($ZnCl_2$), and (f) 0.64 g/L erythorbic acid (EA). Therefore, the experiment consisted of six different treatments performed in duplicate. The storage phase lasted from the first of November to the end of May, when olives were packed and kept at room temperature (28 ± 5 °C) for another two months. Packing was carried out in plastic containers (0.75 L total capacity) that were filled with 0.4 kg of olives, 0.016 kg of seasoning material, and 0.31 L of cover brine (similar to that used by the industry). The brine composition was 2.63 g/L citric acid, 0.64 g/L ascorbic acid, 1.7 g/L potassium sorbate, and 1.5 g/L lactic acid.

Fruits that were fresh, cracked, and just placed in brine (JBF), as well as stored for 1, 3, 5, and 7 months and packed for 1 and 2 months (and their respective brines, when pertinent), were analyzed.

Analysis of Chloroplastic Pigments. *Pigment Extraction.* Pigment extraction was performed from 10 g of olive sample taken from a homogenized triturate, prepared from 15 to 20 pitted fruits. All procedures were performed under dimmed green light to avoid any photooxidation of chlorophylls and carotenoids. The method of Mínguez-Mosquera and Garrido-Fernández, slightly modified as previously described by Gandul-Rojas, Roca, and Gallardo-Guerrero, was used.[13,14] The technique is based on the selective separation of components between *N*,*N*-dimethylformamide (DMF) and hexane. The hexane phase carried over lipids and carotenes, whereas the DMF phase retained chlorophylls and xanthophylls. The pigments from the DMF phase were later transferred to ethyl ether and concentrated to dryness, and the dry residue was dissolved in 1.5 mL of acetone for pigment analysis by HPLC. β-Carotene was directly quantified in the hexane phase by the absorbance measurement at 450 nm. All analyses were made in triplicate.

Analysis of Pigments by HPLC. Pigment separation was carried out using a stainless steel column (20 × 0.46 cm i.d.), packed with a multifunctional end-capped deactivated octadecylsilyl (C_{18}) Mediterranea Sea18, 3 μm particle size (Teknokroma, Barcelona, Spain). The column was protected by a precolumn (1 × 0.4 cm i.d.) packed with the same material. Solutions of pigment extract were centrifuged for 10 min at 13000*g* prior to injection (20 μL) into the chromatograph. Separation was performed using an elution gradient (flow rate 1.250 mL min^{-1}) with the mobile phases (A) water/ion pair reagent/methanol (1/1/8, v/v/v) and (B) methanol/acetone (1/1, v/v). The ion pair reagent was 0.05 M tetrabutylammonium and 1 M ammonium acetate in water. The gradient scheme was a modification of that of Mínguez-Mosquera, Gandul-Rojas, Montaño-Asquerino, and Garrido-Fernández[15] and briefly was initially 75% A and 25% B, then changed to 25% A in 8 min, isocratic 2 min, changed to 19% A in 1 min, then to 14% A in 2 min, 11% A in other 2 min, and 10% A in 3 min. Isocratic 3 min and later changed to 8% A in 1 min and 6% A in 0.5 min. Then 100% B in 0.5 min, isocratic 12 min, and returned to initial conditions in 5 min. Spectrophotometric detection of pigments was performed at 410, 430, 450, and 666 nm. The online UV−vis spectra were recorded from 350 to 800 nm with the photodiode-array detector. Data were collected and processed with an LC HP ChemStation (Rev.A.05.04). Pigments were identified by cochromatography with the corresponding standard and from the spectral characteristics as described in detail elsewhere.[15] Pigments were quantified using external standard calibration curves prepared with purified standards of each pigment. Results are reported as means from a triplicate analysis and the standard deviation (SD).

Instrumental Measurement of Color. Surface color analyses were performed on olives using a Cary UV/visible spectrophotometer, equipped with computer software to calculate the CIE coordinates: L^* (lightness), a^* (negative values indicate green, while positive values indicate red), and b^* (negative values indicate blue and positive values indicate yellow), with an illuminant *C* to 10°. Interference by stray light was minimized by covering samples with a box, which had a matt black interior. The data for each treatment were the mean of 20 olive measurements. The green color evolution of vegetables has also been expressed as hue angle (the angular component of the polar representation) and chroma (the radial component).[16] Hue values were estimated from the equation

$$h_{ab} = \arctan\frac{b^*}{a^*} \quad (1)$$

C^* (chroma) values were obtained from the equation

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

Color index (*Ic*) was determined according to Sánchez, Rejano, and Montaño:[17]

$$Ic = \frac{-2R_{560} + R_{590} + 4R_{635}}{3} \quad (3)$$

where the *R*'s stand for the reflectances at 560, 590, and 635 nm, respectively.

Mineral Analysis in Olive Flesh and Brines. The procedure used was similar to that described elsewhere.[18] Cu, Fe, Mn, Ca, Mg, Zn, Na, and K were determined by atomic absorption spectrophotometry. An air acetylene flame was used. Instrumental conditions for each element were fixed according to the equipment manual.[19] Measurements of P were made in a Cary UV/visible spectrophotometer. Analyses were made in triplicate.

Physicochemical and Microbiological Analysis. The analysis of olive brines for pH, chloride concentration (g NaCl/100 mL brine), titratable acidity (expressed as g of lactic acid/100 mL of brine), and combined acidity (as milliequivalents of HCl added to 1 L of brine to reach pH 2.6, mequiv/L) were carried out in triplicate using the standard methods for table olives.[20]

Firmness was measured objectively using a Kramer shear compression cell. The cross-head speed was 200 mm/min. The firmness of the olives was expressed as the mean of 20 measurements, each of which was performed on one cracked, pitted fruit. Shear compression force was expressed as kN/kg of pitted olives.

Brine samples and their decimal dilutions were plated onto the appropriate media. Subsequently, plates were counted and results were expressed as $\log_{10}$ cfu/mL. Enterobacteriaceae were counted on VRBD (Crystal-Violet Neutral-Red bile dextrose) agar, LAB on MRS (de Man, Rogosa, and Sharpe) agar with 0.02% (w/v) sodium azide, and yeasts on YM (yeast-malt-peptone-glucose medium) agar supplemented with oxytetracycline and gentamicin sulfate as selective agents for yeasts. Plates were incubated at 30 °C for 48−72 h.

Apparatus, Reagents, and Standards. *Apparatus.* Equipment included an HP 1100 (Hewlett-Packard, Palo Alto, CA) liquid chromatograph fitted with an automatic injector and diode array detector; a Cary UV/visible spectrophotometer model IE (Varian Australia, Mulgrave, Victoria); a GBC model 932 AA (Victoria, Australia) atomic absorption spectrometer equipped with three hollow multielement cathode lamps, (Cu, Fe, and Mn) (GBC, Victoria, Australia), (Ca, Mg, and Zn) (Photron, Victoria, Australia), and (Na and K) (Photron, Victoria, Australia); a Kramer shear compression cell coupled to an Instron Universal Testing Machine (Canton, MA); and a Spiral System model dwScientific (Don Whitley Scientific Limited, England) in combination with a CounterMat v.3.10 (IUL, Barcelona, Spain).

Reagents. All reagents were of analytical grade and chromatographic grade, according to step.

Standards. Chlorophylls *a* and *b* and β-carotene standards were supplied by Sigma Chemical Co. (St. Louis, MO). All other chlorophyll derivatives were prepared in the laboratory from the related chlorophyll (*a* or *b*) extracted from a pigment extract of fresh spinach, as described in Roca, Gallardo-Guerrero, Mínguez-Mosquera, and Gandúl-Rojas.[21] β-Cryptoxanthin was obtained from papaya, while lutein, violaxanthin, neoxanthin, and anteraxanthin were obtained from

a pigment extract of fresh spinach saponified and separated by TLC. Luteoxanthin and auroxanthin were prepared from violaxanthin by acidification and subsequent separation by TLC. In the same way, neochrome and mutatoxanthin were prepared from neoxanthin and anteraxanthin, respectively.[15]

Statistical Data Analysis. Statistica software version 7.0 (Statsoft Inc., Tulsa, OK) was used for data analysis and graph presentations. Post hoc comparisons were carried out according to Duncan's multiple range test.

RESULTS AND DISCUSSION

Pigment Profile of Olive Fruits and Their Changes during Storage and Packing. The chlorophyll and carotenoid profile in the fresh fruits of green Aloreña olives (Figure 1a) followed a similar profile to that found in other cultivars.[22,23] In the chlorophyll fraction, the main components were chlorophylls *a* and *b* (peaks 15 and 12, respectively), with a small presence of 13^2-OH-chlorophylls *a* and *b* (allomerized derivatives, peaks 13 and 11), pheophytin *a* (peak 20), and a chlorophyll derivative of the *a* series (peak 17); this compound was recently described as an intermediary catabolite of chlorophyll *a* in olives of the Hojiblanca and Arbequina cultivars and provisionally called "oxidized chlorophyll *a*".[24] The carotenoid fraction was composed of lutein (the major one, peaks 10, 10′), *β*-carotene (peak 21, mainly quantified in the hexane phase), and neoxanthin, violaxanthin, antheraxanthin, and *β*-criptoxanthin (peaks 3, 5, 8, and 14, respectively). Peaks with the same number and a prime symbol refer to epimer isomers for chlorophylls and cis isomers for carotenoids.

Cracking markedly modified the fresh fruit chloroplastic pigment profile and led to the appearance of new ones (Figure 1b). In the chlorophyll fraction, the free Mg derivatives pheophytin *b* (peak 19) and pheophorbide *a* (peak 2), which is also a phytol-free derivative, were detected, whereas a significant increase in the proportion of pheophytin *a* (peak 20) stood out. Regarding the carotenoid fraction, three new compounds were detected and identified as luteoxanthin, auroxanthin, and mutatoxanthin (peaks 6, 7 and 9, respectively), all of them with 5,8-furanoxide groups in their molecule. The first two are formed from violaxanthin and the third from antheraxanthin, carotenoids with 5,6-epoxide groups in their molecule, which are converted into 5,8-furanoxide under acidic conditions.[25]

Therefore, the process of cracking the fruits and conditioning them in brine provoked a slight pheophytinization reaction of the chlorophylls and some isomerization of violaxanthin and antheraxanthin. Such transformations are the usual acid-catalyzed reactions of these pigments and might be related to the release of free organic acids during cracking. Furthermore, the detection of pheophorbide *a* in the fruits also implied the de-esterification of the phytol chain of the chlorophyll molecule. This is a specific reaction catalyzed by the endogenous enzyme chlorophyllase, which has been found in diverse olive cultivars.[26,27] The breakdown of cell integrity probably allowed the generalized liberation of acids, which may reach internal 5.5 pH in the case of the olives,[20] and the contact of endogenous enzymatic systems with their substrates, giving rise to a hydrolytic reaction in the chlorophylls.

In fruits stored for seven months at room temperature (Figure 1c), the pheophytinization reaction (i.e., substitution of Mg^{2+} with H^+ in the porphyrin ring of the chlorophyll molecule) was also the main change detected, a transformation that could have been favored by the low pH prevailing in this processing phase (~4.3). Chlorophylls *a* and *b* were mainly

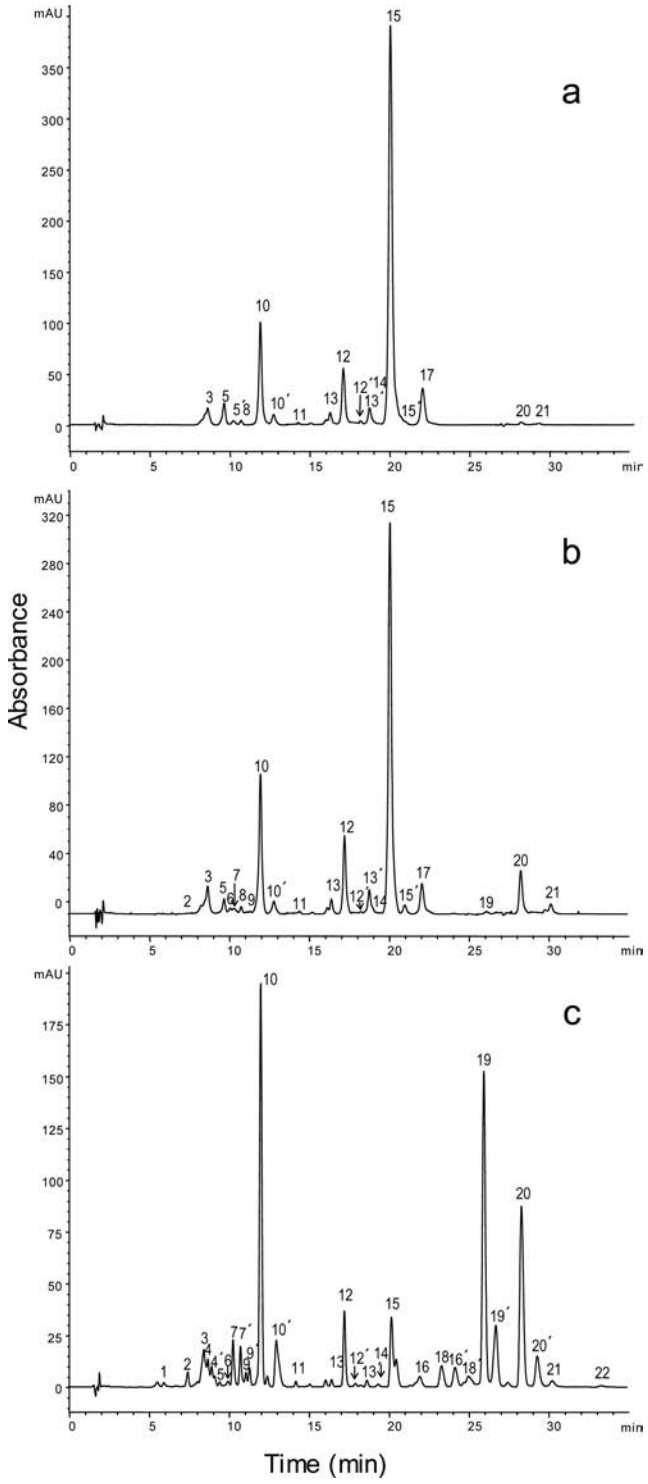

Figure 1. HPLC chromatograms of pigment extracts from green cracked olives: (a) fresh fruits; (b) just brined fruits (JBF); and (c) fruits after seven months of storage at room temperature. Peaks: 1, pheophorbide *b*; 2, pheophorbide *a*; 3, neoxanthin; 4, neochrome; 5, violaxanthin; 6, luteoxanthin; 7, auroxanthin; 8, anteraxanthin; 9, mutatoxanthin; 10, lutein; 11, 13^2-OH-chlorophyll *b*; 12, chlorophyll *b*; 13, 13^2-OH-chlorophyll *a*; 14, *β*-criptoxanthin; 15, chlorophyll *a*; 16, 13^2-OH-pheophytin *b*; 17, oxidized chlorophyll *a*; 18, 13^2-OH-pheophytin *a*; 19, pheophytin *b*; 20, pheophytin *a*; 21, *β*-carotene; 22, pyropheophytin *a*. Peaks with the same number and a prime symbol refer to epimer isomers for chlorophyll pigments and cis isomers for carotenoids.

dx.doi.org/10.1021/jf400240e | J. Agric. Food Chem. 2013, 61, 3737−3747

transformed into pheophytins *a* and *b*, respectively, and 13^2-OH-chlorophylls *a* and *b* into 13^2-OH-pheophytins *a* and *b* (peaks 18 and 16, respectively). Besides the pheophytinization reaction, other minor transformations were detected. Dephytylated derivatives (pheophorbides *a* and *b*, peaks 2 and 1, respectively) increased during the storage process; in addition, a new compound, pyropheophytin *a* (peak 22), revealing a certain degree of decarbomethoxylation at C-13^2 of pheophytin *a*,[3] was found. As a consequence of the increased acid pH of the medium, neochrome (peaks 4 and 4′, the 5,8-furanoxide isomer of neoxanthin) was detected in addition to luteoxanthin, auroxanthin, and mutatoxanthin, in the carotenoid fraction, as a result of the isomerization of the 5,6-epoxide groups of the minor xanthophylls neoxanthin, violaxanthin, and anteraxanthin.[25]

Quantitatively, the first treatment that affected "Aceituna Aloreña de Málaga" fruits was cracking, which significantly reduced the content of green-colored compounds (chlorophylls with Mg in their structure), while it increased the brownish ones (Mg-free chlorophyll derivatives) (Table 1). During the storage period, all the pigment transformations observed in the fruits after the cracking process were increased (Figure 2), although the changes in CR (WA) olives were slower than in those stored at RT. As a consequence, during the storage period, the fruits kept refrigerated were visibly greener in color than those in the rest of the samples.

Table 1. Effect of Cracking on the Pigments, Color, and Texture of Fresh Fruits[a]

parameter	fresh fruits	cracked fruits
green pigments (mg/kg)	31.16 (0.17) a	25.32 (0.96) b
brownish pigments (mg/kg)	1.08 (0.17) a	6.56 (0.26) b
color index	25.94(0.87) a	20.91 (0.80) a
*L**	67.03(0.22) a	59.13(0.72) b
*a**	−10.06(0.19) a	−7.36(0.06) b
*b**	40.06(0.15) a	41.78(0.39) a
chroma	41.30(0.09) a	42.42(0.39) a
hue	104.09(0.30) a	99.99(0.02) b

[a]Row numbers followed by different letters are significantly different at $p < 0.05$.

The chlorophylls of the *a* series were always transformed at a higher rate than the *b* series chlorophylls. In CR (WA), after the first month of fruits in CR, 35% of the initial chlorophyll *a* (Figure 2a) was transformed to pheophytin *a*, while chlorophyll *b* (Figure 2b) remained longer, and only 2% was converted into pheophytin *b*. However, in fruits stored at RT, the above percentages were higher for both chlorophyll *a* (70%) and chlorophyll *b* (17%) because a higher temperature accelerates all reactions. These and other minor transformations progressed with time so that at the end of the storage period chlorophyll *a* and chlorophyll *b* decreased by 70% and 18%, respectively, in CR (WA) fruits, while in olives stored at RT almost all chlorophyll *a* disappeared (~93%) and the decrease in chlorophyll *b* was also very high (~64%).

The pheophytinization reaction was also detected for the allomerized chlorophylls (resulting allomerized pheophytins, Figure 2c), which was again slower in CR (WA) than in fruits maintained at RT. The formation of dephytylated derivatives (pheophorbides *a* and *b*) showed a progressive increase during the storage, reaching values of 2−3% of the chlorophyll fraction, but in this case there was no significant effect of

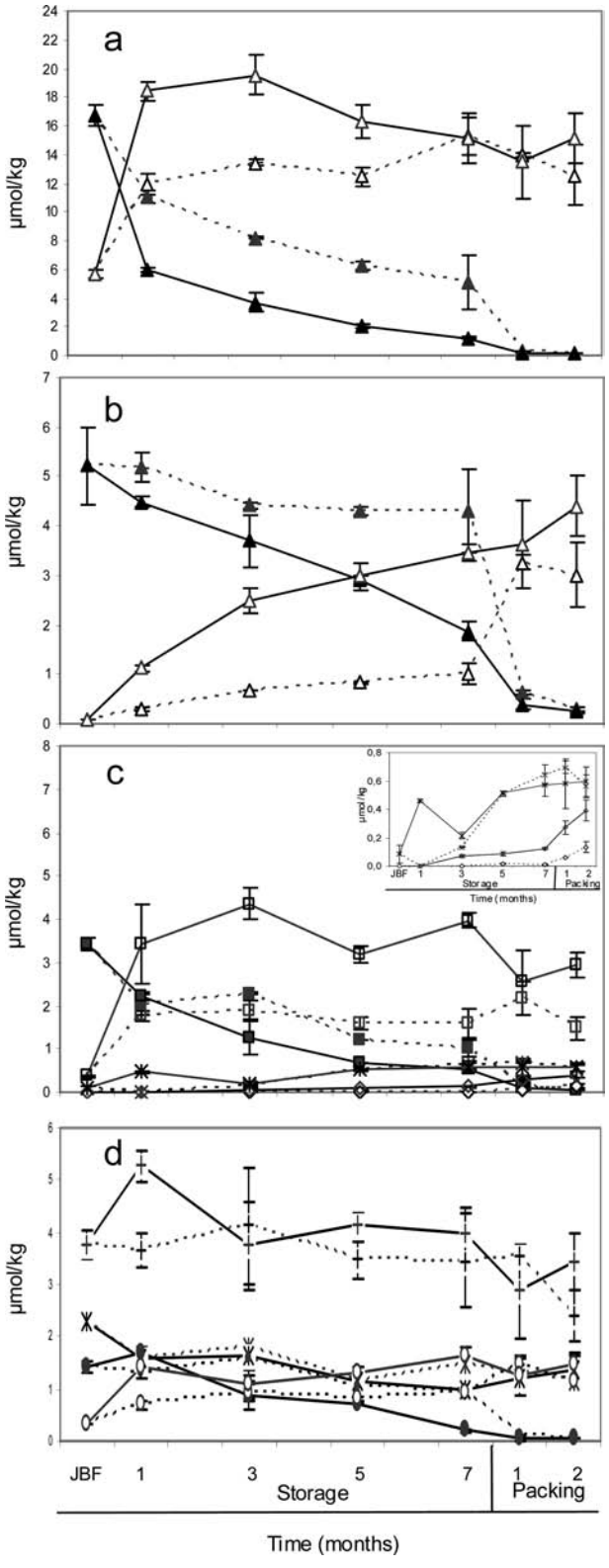

Figure 2. Changes in concentration of pigments during fruit storage, both in CR (WA) (- - -) and at RT (WA) (—), and after being packed: (a) chlorophyll *a* (▲) and pheophytin *a* (Δ); (b) chlorophyll *b* (▲) and pheophytin *b* (Δ); (c) allomerized chlorophylls (■), allomerized pheophytins (□), pheophorbides (∗), and pyropheophytins (◊); (d) lutein (+), *β*-carotene (×), 5,6-epoxide carotenoids (●), and 5,8-epoxide carotenoids (○).

storage temperatures. Pyropheophytin *a* was formed later and in small amounts (0.3−0.5% of the chlorophyll fraction), being first detected in the fruits stored at RT, as this condition favors the formation of pyroderivatives.

Concerning the carotenoid fraction (Figure 2d), the lutein concentration remained constant throughout the storage time. However, *β*-carotene suffered a decrease after the first month of storage, even when fruits were in a CR. The overall carotenoids with 5,6-epoxide groups in their molecule (displayed all together in Figure 2d) were progressively converted into 5,8-furanoxide isomers, as a consequence of the acidic conditions in the media.

All the transformations discussed above continued after packing for the remaining chlorophylls *a* and *b* and for the xanthophylls with 5,6-epoxide groups; the physicochemical and environmental conditions (acidic pH and room temperature storage) increased the degradations of chlorophyll *a* (Figure 2a) and chlorophyll *b* (Figure 2b), particularly in the sample from CR (WA) fruits, which still had high native chlorophyll contents after seven months of storage. Hence, after packing, the fruits had similar compositions of pigments, regardless of the temperature at which they were kept during storage.

To check the effect of the different treatments on the green color stabilization, chlorophyllic pigments were grouped into two fractions: green (Figure 3a) and brownish (Figure 3b). Qualitatively, the pigment profile was the same in all treatments, even in those with added $ZnCl_2$, in which the formation of zinc-chlorophyll-derivative complexes, with a stable, highly bright green color, were not detected. To mitigate slight natural pigment compositional differences and improve comparisons, data are shown as percentages of the total chlorophyll pigments. The carotenoid fraction was excluded from this comparison because green color changes are mainly due to the chlorophyll pigments. The percentage of green pigment significantly decreased in the first month of storage with respect to JBF. The decrease was less marked in CR (WA) followed by RT (WA). Later, during storage, the green pigment decrease in CR (WA) was always slower and its content remained above those of any treatment at RT; however, after packing, green pigments in fruits from CR (WA) decreased dramatically to levels similar to those shown by the rest of the treatments. Therefore, all treatments showed overall similar behavior, and none of them, except temperature, prevented or delayed the conversion of chlorophylls into Mg-free derivatives (Figure 3), and hence the freshness losses in the olive fruits.

Changes in the Color Parameters. The first changes suffered by olive surface color were due to the cracking operation (Table 1). After this, *L** and hue angles decreased significantly, *a** values increased significantly, while the color index diminution and increases in *b** and chroma were not significant. Changes in color parameters during storage are shown in Figure 4, in which the first sample on the left, separated from the rest of the data, always corresponds to the JBF and constitutes an initial reference. Storage in brine for just one month produced a significant increase in the color index and in *a** but a decrease in *b**, chroma, and hue, while *L** did not change. During storage, the color indexes in CR (WA) and *a** were mostly below those of any other treatment, whereas hue was above them. In some samplings, average values for *L*, *b**, and chroma for CR (WA) were also the lowest. These changes indicate that CR was fairly appropriate for retaining the green color during storage (Figure 4). No remarkable

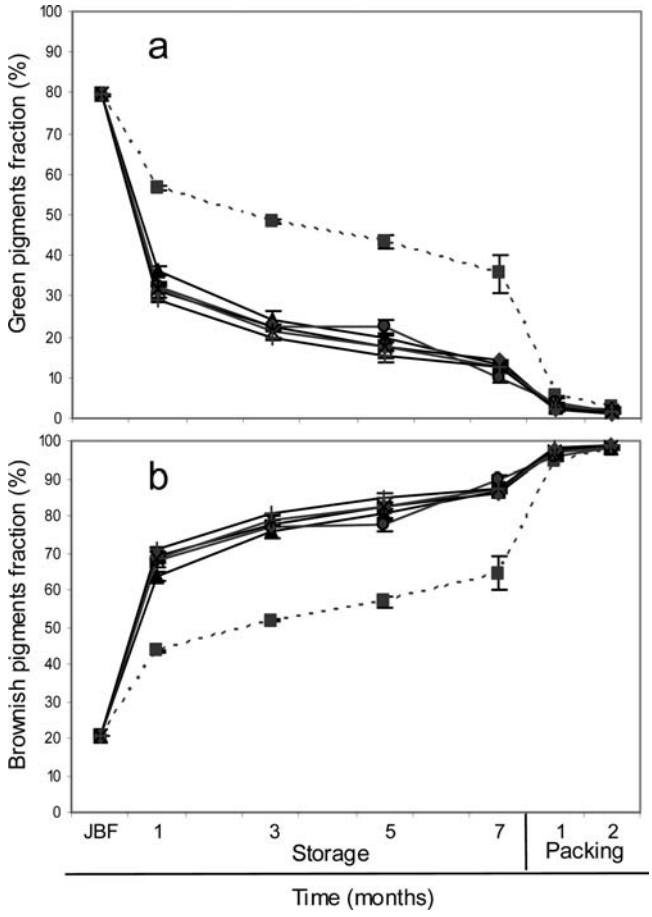

Figure 3. Changes in percentages, with respect to the total chlorophyll pigments, of (a) green pigment fraction, i.e., chlorophylls with Mg in their structure, and (b) brownish pigment fraction, i.e., Mg-free chlorophyll derivatives during olive storage with different treatments and after packing. Symbols for each storage treatment: (■) CR (WA), (▲) RT (WA), (♦) RT (SMB), (∗) RT (SMB + $CaCl_2$), (+) RT ($ZnCl_2$), and (●) RT (EA).

differences in the behavior in other treatments were observed, even in that with added $ZnCl_2$, in which a minor increase in *a** during storage might have been expected if it had formed zinc-chlorophyll-derivative complexes with a bright green color. After packing, there were marked increases in *L**, *b**, and chroma, which recovered again to previous storage values. This homogenization in packed olives was general for all color parameters and treatments (including olives previously stored in CR), indicating a general loss in the greenish tones in this phase. These results were in agreement with the above-mentioned chlorophyll degradations during storage and, particularly, after packing.

The relationship among overall green pigment contents (chlorophylls with Mg) and color parameters showed significant correlation coefficients, but the proportion of variance explained by the regression was fairly limited in the color indexes *L**, *b**, and chroma. Values of *a** and hue (Figure 5) were the two parameters that showed the most reliable linkage, but, even in these cases, there were several points outside the confidence limits of the regression. Thus, the relationships showed that either *a** or hue angle could be the most appropriate parameters to reflect the greenish (freshness) losses during the storage and packing of olives.

dx.doi.org/10.1021/jf400240e | J. Agric. Food Chem. 2013, 61, 3737−3747

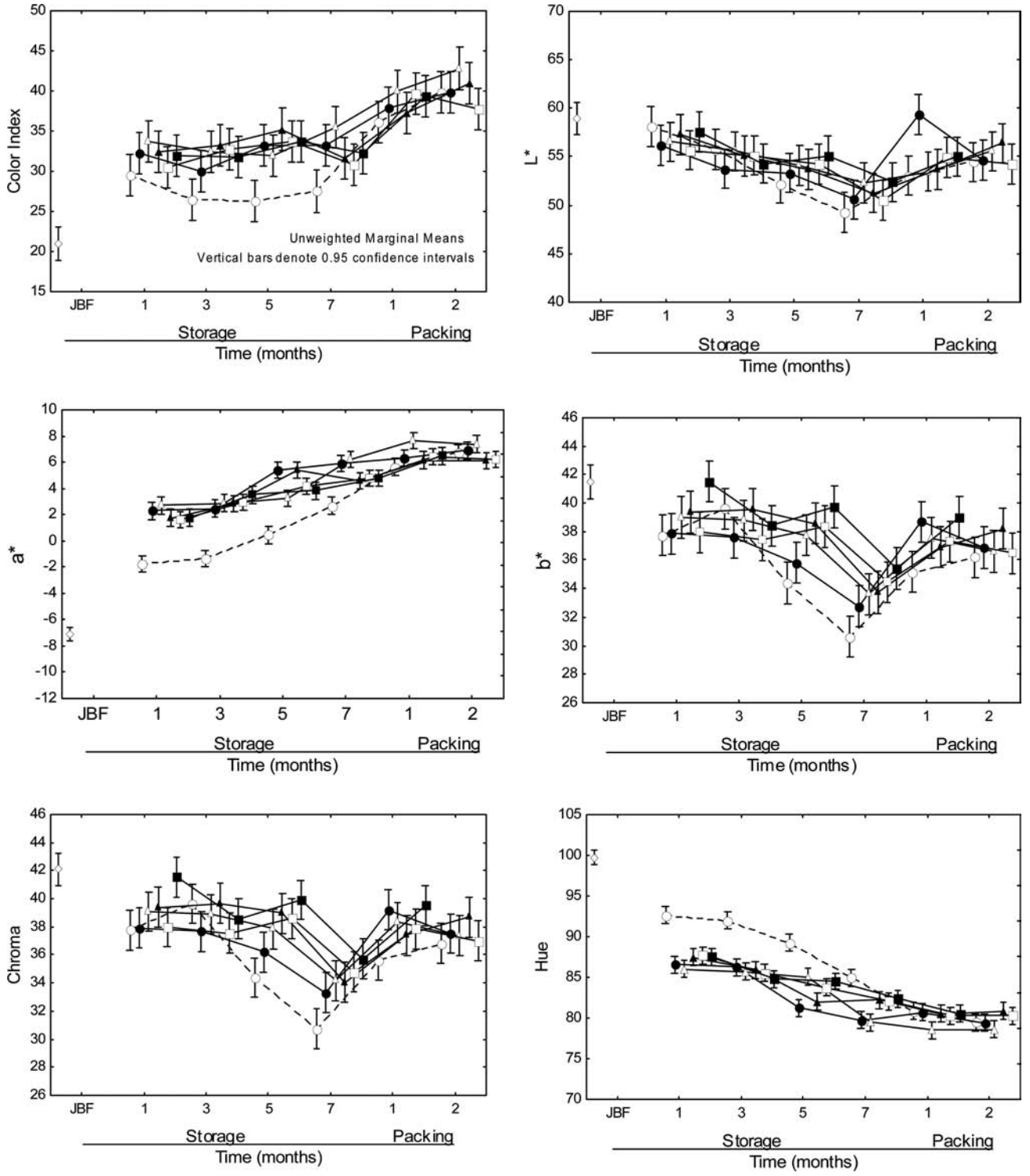

Figure 4. Changes in color index, L^*, a^*, b^*, chroma, and hue during storage in brine and after packing of cracked green olives. (◊) JBF, just brined fruits; (*o*) CR (WA), cold room without additive; (●) RT (WA), room temperature without additive; (Δ) RT (SMB), brine with added sodium metabisulfite; (▲) RT (SMB + $CaCl_2$), brine with added sodium metabisulfite plus calcium chloride; (□) RT ($ZnCl_2$), brine with added zinc chloride; (■) RT (EA), brine with added erythorbic acid.

Changes in Nutrient Mineral Contents in Flesh. Na, Ca, and Zn were the only minerals whose concentrations increased with olive storage because of salt addition to their brines (Figure 6). Initially, the Na content was only 300 mg/kg flesh but reached 24 000−27 000 mg/kg flesh at the end of the storage period and 16 000−17 000 mg/kg flesh after packing in all samples (Figure 6a); Ca in fresh fruits was about 600 mg/kg flesh and increased to about 1600 mg/kg flesh (~20% recommended daily intake) in SMB + $CaCl_2$ storage olives, but, slightly decreased to 1300 mg/kg flesh (~17% daily intake) after packing (Figure 6b), indicating a strong retention of Ca by the olive flesh, as previously reported.[6,20] Ca also showed a noticeable increment in the rest of the treatments (from 600 to 1200 mg/kg flesh), possibly because of the presence of this

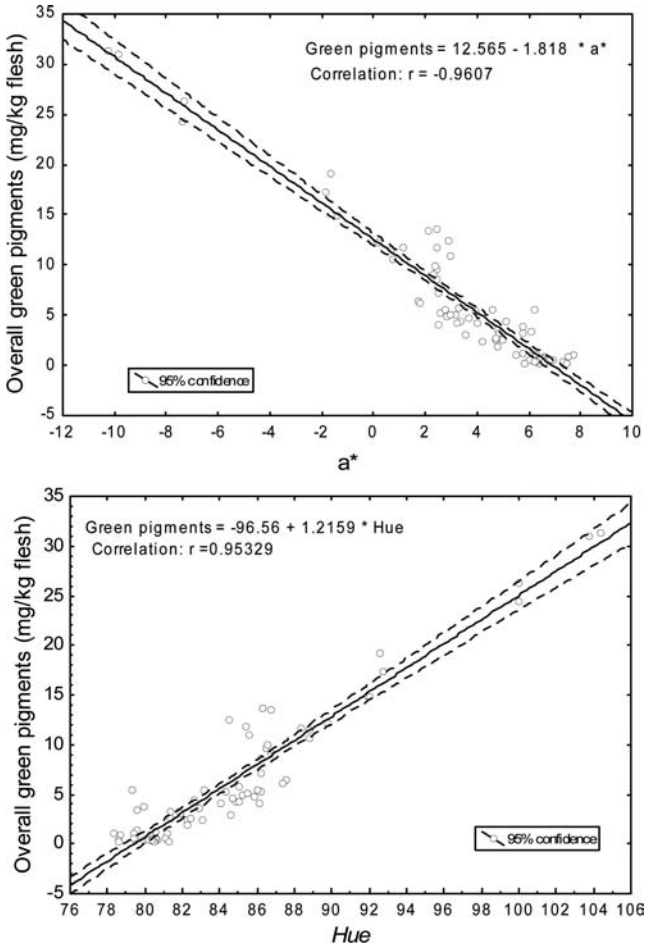

Figure 5. Cracked, green olives. Correlation between overall green pigment contents vs *a** and *hue*.

mineral in the stock brine solution (from flowing waters). Zn content (Figure 6c) was low (~4 mg/kg flesh) in fresh fruits and after storage in all treatments, except in $ZnCl_2$, and was only slightly reduced (~3 mg/kg flesh) after packing, indicating that this element may apparently be retained in the flesh. In treatment with $ZnCl_2$, the average content was 99.6 mg/kg flesh, which was reduced to ~42 mg/kg flesh after packing, a concentration that showed an apparent equilibrium in the distribution between flesh and brine. These results mean that 100 g of packed olive flesh from fruits stored in solutions containing 0.5 g/L $ZnCl_2$ could supply approximately 40% (~4.2 mg) of the recommended Zn daily intake (10 mg/day).[28]

With respect to the other mineral nutrients originally in the olive fruits, there was a general decrease after storage, which was reduced again after packing (Table 2). Reduction was not related to treatments, except in the case of Cu, which experienced a significantly greater leaching in the presence of SMB and $ZnCl_2$, mainly during storage (Table 2).

Therefore, the storage of cracked green table olives in brine enriched with Ca or Zn can lead to improving this element in products; however, Zn presence did not cause the formation of Zn-chlorophyll complexes and thus did not delay the gradual loss in the freshness appearance of the green fruits. With respect to the other mineral nutrients, storage in brine prior to packing always produced a reduction in the mineral nutritional characteristics of the olives, which apparently was not related to changes in appearance.

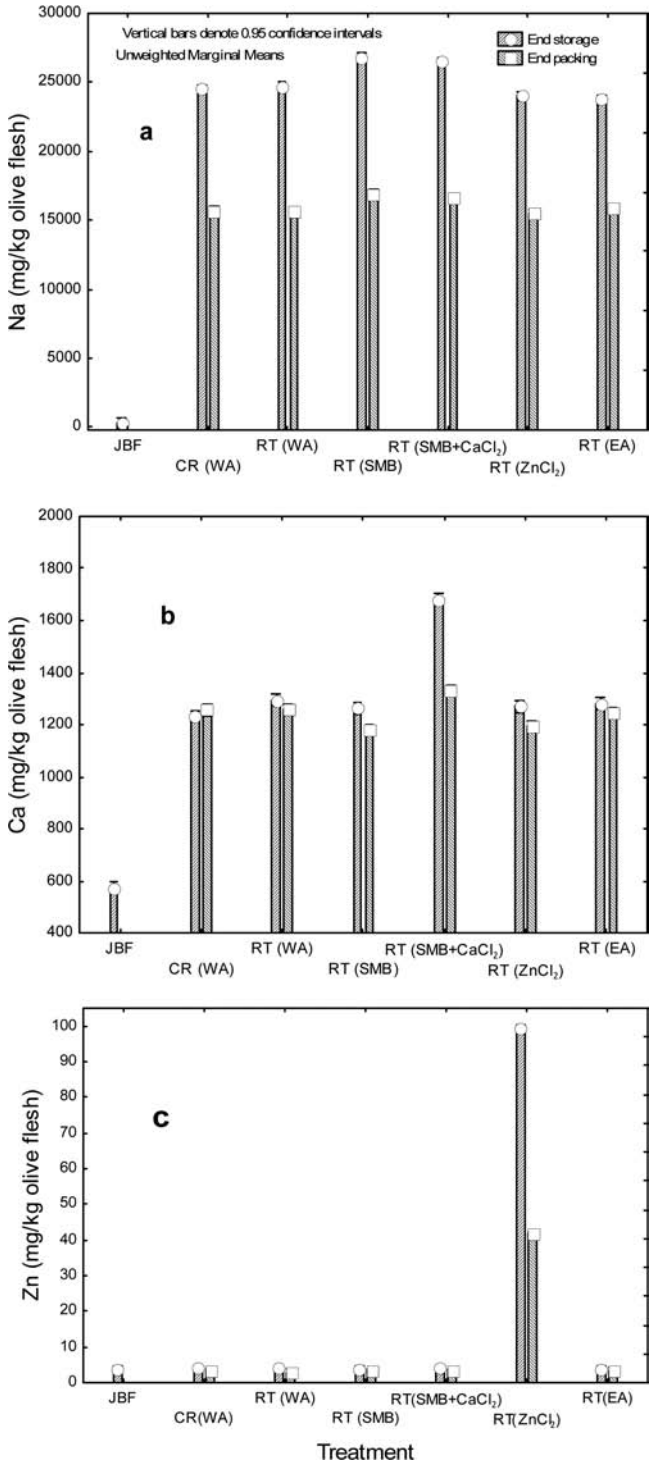

Figure 6. Cracked, green olives. Contents of Na, Ca, and Zn in the olive flesh at the end of the storage and packing phases. JBF, just brined fruits; CR (WA), cold room without additive; RT (WA), room temperature without additive; RT (SMB), brine with added sodium metabisulfite; RT (SMB + $CaCl_2$), brine with added sodium metabisulfite plus calcium chloride; RT ($ZnCl_2$), brine with added zinc chloride; RT (EA), brine with added erythorbic acid. Mineral contents in fresh fruits and just brined fruits were considered similar.

Physicochemical and Microbiological Changes. The pH values in stored olives ranged from 4.21 to 4.53 and decreased slightly during the storage time to 4.10–4.19 (Table 3), without a noticeable effect of treatments. In the packed

dx.doi.org/10.1021/jf400240e | J. Agric. Food Chem. 2013, 61, 3737–3747

Table 2. Average Mineral Contents (mg/kg flesh) after Brining and at the End of Storage and Packing in Cracked Green Olives

processing phase	treatment[a]	Mg	Fe	Cu	Zn	Mn	P
just brined fruit[b]	JBF	163	7.96	3.80	3.68	1.56	400
end storage	CR (WA)	136	5.90	3.67	4.05	1.23	198
	RT (WA)	131	6.23	3.67	4.03	1.04	184
	RT (SMB)	137	6.11	2.90	3.98	1.22	190
	RT (SMB + $CaCl_2$)	129	6.23	3.66	4.05	1.19	180
	RT ($ZnCl_2$)	134	6.43	2.92	99.62	1.19	195
	RT (EA)	134	6.11	3.31	3.88	1.03	196
end packing	CR (WA)	80	5.41	2.89	3.08	1.03	125
	RT (WA)	79	5.17	2.76	2.95	0.93	118
	RT (SMB)	82	4.61	2.30	3.15	0.96	119
	RT (SMB + $CaCl_2$)	77	4.27	2.53	3.15	0.95	119
	RT ($ZnCl_2$)	78	4.29	2.07	41.54	0.96	117
	RT (EA)	78	4.89	2.28	3.28	0.95	120
pooled SE		2	0.14	0.10	0.39	0.04	2.00

[a]JBF, just brined fruits; CR (WA), cold room without additive; RT (WA), room temperature without additive; RT (SMB), brine with added sodium metabisulfite; RT (SMB + $CaCl_2$), brine with added sodium metabisulfite plus calcium chloride; RT ($ZnCl_2$), brine with added zinc chloride; RT (EA), brine with added erythorbic acid. [b]Mineral contents in fresh fruits and just brined fruits were considered similar.

Table 3. Changes in pH and NaCl Contents (in parentheses and expressed as g/L brine) in Brine during Cracked Green Olive Storage and Packing

	storage			packing	
treatment[a]	1 m[b]	3 m	7 m	1 m	2 m
CR (WA)	4.53[c]	4.37	4.19 (93.1[d])	3.41 (67.2)	3.94 (58.3)
RT (WA)	4.34	4.27	4.16 (105.7)	4.13 (61.7)	3.94 (60.4)
RT (SMB)	4.40	4.30	4.11 (10.63)	3.99 (62.1)	3.94 (61.8)
RT (SMB + $CaCl_2$)	4.21	4.23	4.10 (10.24)	3.79 (64.8)	3.98 (62.0)
RT ($ZnCl_2$)	4.32	4.28	4.13 (9.32)	4.03 (63.8)	4.00 (60.2)
RT (EA)	4.45	4.33	4.12 (9.51)	4.02 (60.8)	4.04 (59.8)

[a]CR (WA), cold room without additive; RT (WA), room temperature without additive; RT (SMB), brine with added sodium metabisulfite; RT (SMB + $CaCl_2$), brine with added sodium metabisulfite plus calcium chloride; RT ($ZnCl_2$), brine with added zinc chloride; RT (EA), brine with added erythorbic acid. [b]m, months. [c]Pooled SD: pH <0.2. [d]NaCl concentration in parentheses (g/L); pooled SD NaCl content <3 g/L brine.

olives, the pH ranged from 3.41 to 4.13, below the 4.3 limit established in the Trade Standard Applying to Table Olives[29] for natural directly brined olives, but without appreciable effect of the previous storage treatments. As mentioned above, the combination of low pH, necessary for safe olive storage, and RT provided the acidic pH and temperature that caused the green pigment transformations during storage. Particularly important was the temperature, because refrigeration considerably delayed the green (freshness) loss rate during storage, but freshness retention in packed products would possibly require distribution at refrigerated temperatures.

The NaCl concentration was stabilized at the end of the storage period between 9.31 and 10.63 g/100 mL brine (Table 3). At least in part, the LAB inhibition, which will be commented on later, could have been due to the high level of salt applied in this phase.[30] Salt concentrations decreased after packing, when new, less covering brine was added, reaching equilibrium at levels between 5.83 and 6.20 (Table 3).

At the initiation of olive storage, the values of titratable acidity ranged from 5.3 to 7.1 g lactic acid/L brine and decreased slightly with time, reaching values from 0.43 to 0.68 g lactic acid/100 mL brine at the end of the storage time (Table 4). After packing, these values increased slightly and were between 4.3 and 6.3 g lactic acid/L brine, which was practically stable up to the end of the packing period. This increase in acid concentration and subsequent pH decrease in storage could have favored, together with room temperature, the acceleration of the pigment transformations and the homogeneous brownish color in the packed fruits, regardless of storage conditions. Combined acidity (Table 4) was not high and ranged from 59.50 to 74.35 mequiv/L; because of the absence of a lye treatment in this processing, combined acidity was due to the organic salts naturally present in olives.[1,2,4] It was measured at the end of storage just to measure it as the maximum once the equilibrium olive flesh/brine was reached. The low combined acidity of this product made important changes in pH levels possible with only limited proportions of added acid. As a result, this low combined acidity was another unfavorable factor to bear in mind when developing procedures for green olive freshness preservation.

Table 4. Changes in Titratable Acidity in Brine during Cracked Green Olive Storage and Packing as Well as Combined Acidity (mM) in Brine at the End of the Storage Period (in parentheses)

	storage			packing	
treatment[a]	1 m[b]	3 m	7 m	1 m	2 m
CR (WA)	7.1[c]	6.3	5.1 (72.16[d])	4.9	5.3
RT (WA)	5.5	5.2	4.5 (62.60)	4.3	4.5
RT (SMB)	5.3	5.0	4.3 (63.40)	5.9	5.7
RT (SMB + $CaCl_2$)	5.3	5.2	3.9 (59.50)	5.7	5.0
RT ($ZnCl_2$)	6.2	6.1	5.2 (74.35)	6.1	5.5
RT (EA)	5.7	5.6	6.8 (73.28)	6.3	6.1

[a]CR (WA), cold room without additive; RT (WA), room temperature without additive; RT (SMB), brine with added sodium metabisulfite; RT (SMB + $CaCl_2$), brine with added sodium metabisulfite plus calcium chloride; RT ($ZnCl_2$), brine with added zinc chloride; RT (EA), brine with added erythorbic acid. [b]m, months. [c]Pooled titratable acidity SD, <0.3 g/L. [d]Combined acidity in parentheses; pooled combined acidity SD <9 equiv/L.

dx.doi.org/10.1021/jf400240e | J. Agric. Food Chem. 2013, 61, 3737−3747

The pooled standard deviation (0.91 kN/100 g flesh) for firmness measurement was high, possibly due to the heterogeneous fruit maturation (Table 5), a fact that made the establishment of significant differences difficult. Nevertheless, at the end of the storage period, those samples treated with SMB, the mixture SMB + $CaCl_2$, and $ZnCl_2$ had average higher values (51.1−53.5 kN/kg flesh) than those WA or treated with EA (41.0−49.4 kN/kg flesh). On the contrary, after packing, the fruits' firmness was modified and the highest average values (46.8−48.0 kN/kg flesh) were observed in olives stored just in brine (WA), regardless of the storage temperature. Packed olives previously stored in brine containing EA were particularly deficient in this attribute (27.2 kN/kg flesh) (Table 5). This firmness (freshness) deterioration may be related to an abundant growth of yeasts during the packing phase (Table 6).

Table 5. Changes in Firmness (kN/kg olive flesh) during Cracked Green Olive Storage and Packing

	storage				packing	
treatment[a]	1 m[b]	3 m	5 m	7 m	1 m	2 m
CR (WA)[c]	53.1[d]	40.8	36.9	41.0	46.8	42.5
RT (WA)	60.0	51.9	37.4	49.4	48.0	49.9
RT (SMB)	50.2	46.8	43.6	51.1	42.8	32.3
RT (SMB + $CaCl_2$)	46.9	46.6	39.9	51.7	39.6	34.0
RT ($ZnCl_2$)	50.0	43.4	43.0	53.5	39.6	41.0
RT (EA)	44.5	36.8	42.3	43.8	39.7	27.2

[a]CR (WA), cold room without additive; RT (WA), room temperature without additive; RT (SMB), brine with added sodium metabisulfite; RT (SMB + $CaCl_2$), brine with added sodium metabisulfite plus calcium chloride; RT ($ZnCl_2$), brine with added zinc chloride; RT (EA), brine with added erythorbic acid. [b]m, months. [c]Fresh fruits firmness, 77.0 kN/kg; just after brining firmness, 44.8 kN/kg olive flesh. [d]Pooled firmness SD = 0.1 kN/kg olive flesh.

No Enterobacteriaceae were found in the brines of any treatment during the olive storage or packing. In the stored fruits (Table 6), LAB were absent in CR (WA), RT (WA), SMB + $CaCl_2$, $ZnCl_2$ brines, and SMB, except in the last sampling. On the contrary, LAB were always found in the EA samples (Table 6). In the packed fruits, LAB were absent in olives stored in just brine (regardless of temperature) but were present in all the containers prepared from olives stored in brine with additives at counts around 6 $\log_{10}$ cfu/mL. Because the raw material used in the experiments was the same for all treatments, it was apparent that, in some way, the presence of additives in the storage brine and/or the lower salt concentration after packing may have favored LAB growth. LAB presence may produce acid and contribute to pH decrease, which can accelerate pigment transformations[3] and freshness loss but improve product safety.[20]

Yeasts were present at similar levels in all treatments during the storage phase (Table 6). Apparently, their growth was not related to the green aspect of the products, which depended mainly on storage temperature. However, a clear effect of the previous storage conditions on the subsequent yeast population in packed olives was noticed. The average highest counts were found in olives previously stored in brines with EA, followed by fruits stored either in CR (WA) or RT (WA). An initial inhibitory action was also observed in packed olives that were previously stored in brine containing SMB, although at the end of the packing period a limited growth (2.79 $\log_{10}$ cfu/mL) was also detected. It can be emphasized that final products from SMB + $CaCl_2$ and $ZnCl_2$ storage olives were free of yeast during the whole packing period. The cause was not apparent, but the residual concentrations of the chemicals added to the storage brine could have acted as a new hurdle to yeast growth.[31] These results were in agreement with the $ZnCl_2$ inhibitory effect against most of the table olive yeasts reported by Baustista-Gallego, Romero-Gil, Garrido-Fernández, and Arroyo-López.[32]

In summary, different alternatives for cracked olive storage were tested. Storage produced marked changes in the fruit pigment composition, degrading the chlorophylls into Mg-free derivatives with gray-brown color. This effect was significantly lower in fruits kept refrigerated, which, during storage, were visibly greener than the rest; however, when these fruits were packed and kept at room temperature, pigments suffered a rapid degradation, which led to similar pigment and freshness degradation in all samples, regardless of the previous storage conditions. Changes in a^* values and hue were the two color parameters that best correlated with the green pigment contents. They changed from green (a^* negative values and >90° hue angle) to slightly red-brown (a^* positive and <90° hue), regardless of the storage or packing phases, with temperature being the only factor that delayed the rate of degradation. The storage and packing of olives produced a marked decrease in the original mineral nutritive value of fresh fruits because of the losses in K, Mg, and P or (in less intensity) Fe, Cu, and Mn, while, regardless of the treatment used, there was a very marked increase in Na concentration in the olive

Table 6. Changes in the LAB and Yeast Populations ($\log_{10}$ cfu/mL) with Time during Cracked Green Olive Storage and Packing

	storage						packing			
	1 m[b]		3 m		7 m		1 m		2 m	
treatment[a]	LAB	yeast	LAB	yeast	LAB	yeast	LAB	yeast	LAB	yeast
CR (WA)	ND[c]	5.76[d]	ND	4.46	ND	4.96	ND	4.11	ND	4.41
RT (WA)	ND	5.42	ND	4.59	ND	5.24	ND	2.96	ND	4.13
RT (SMB)	ND	6.16	ND	5.75	3.85	4.68	6.15	ND	6.10	2.79
RT (SMB + $CaCl_2$)	ND	6.32	ND	5.43	ND	4.58	5.83	ND	6.11	ND
RT ($ZnCl_2$)	ND	5.55	ND	5.40	ND	4.82	6.55	ND	6.27	ND
RT (EA)	5.41	6.31	5.47	5.47	4.00	4.00	5.95	5.95	5.72	5.72

[a]CR (WA), cold room without additive; RT (WA), room temperature without additive; RT (SMB), brine with added sodium metabisulfite; RT (SMB + $CaCl_2$), brine with added sodium metbisulfite plus calcium chloride; RT ($ZnCl_2$), brine with added zinc chloride; RT (EA), brine with added erythorbic acid. [b]m, months. [c]ND, counts below detection limit (1 $\log_{10}$ cfu/mL). [d]Pooled SD <0.5 $\log_{10}$ cfu/mL.

dx.doi.org/10.1021/jf400240e | J. Agric. Food Chem. 2013, 61, 3737−3747

flesh. An addition of $CaCl_2$ or $ZnCl_2$ in the storage brine increased Ca or Zn content, respectively, which were still markedly retained in the olive flesh after packing, although their absorption in the flesh was not accompanied by a better retention of olive freshness. The effect of changes in pH, titratable acidity, combined acidity, and NaCl concentrations during storage and packing was limited, but their levels were in general counterproductive for freshness retention. Firmness was better maintained in RT (WA) traditional storage, while EA treatment led to a marked decrease in this attribute because the EA presence promoted yeast growth, whose excess may be associated with flesh degradation. Thus, application of this antioxidant in cracked olives cannot be recommended because of its unfavorable effect on firmness (and freshness). Although storage treatments other than temperature were minimally linked to freshness evolution in this study, the use of SMB and $ZnCl_2$ during storage was promising for preventing yeast activity and LAB presence in packing, and, hence, for improving freshness retention and stability.

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Funding

This work was supported by the "Comisión Interministerial de Ciencia y Tecnología (CICYT-EU Spanish Government) under projects AGL2009-07436/ALI, AGL2010-15494/ALI, and AGL 2012-39714/ALI, and Junta de Andalucía through financial support to group AGR-125 and project AGR 148-2011, partially financed by European regional development funds, ERDF.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

J. Bautista-Gallego and J. M. Moreno-Baquero thank CSIC for their JAE fellowship. Technical assistance of Sergio Alcañiz García and Veronica Romero Gil is also acknowledged.

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