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Characterization of Polyphenol Oxidase from the Manzanilla Cultivar (*Olea europaea pomiformis*) and Prevention of Browning Reactions in Bruised Olive Fruits

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The crude extract of the polyphenol oxidase (PPO) enzyme from the Manzanilla cultivar (*Olea europaea pomiformis*) was obtained, and its properties were characterized. The browning reaction followed a zero-order kinetic model. Its maximum activity was at pH 6.0. This activity was completely inhibited at a pH below 3.0 regardless of temperature; however, in alkaline conditions, pH inhibition depended on temperature and was observed at values above 9.0 and 11.0 at 8 and 25 °C, respectively. The thermodynamic parameters of substrate oxidation depended on pH within the range in which activity was observed. The reaction occurred according to an isokinetic system because pH affected the enzymatic reaction rate but not the energy required to carry out the reaction. In the alkaline pH region, browning was due to a combination of enzymatic and nonenzymatic reactions that occurred in parallel. These results correlated well with the browning behavior observed in intentionally bruised fruits at different temperatures and in different storage solutions. The use of a low temperature (≈8 °C) was very effective for preventing browning regardless of the cover solution used.

KEYWORDS: Browning; bruise; harvest; olive (Manzanilla cultivar)

INTRODUCTION

Worldwide table olive production is about 1 700 000 tons/year. Most of these olives belong to the so-called green "Spanish-style", which is prepared from fruits harvested during the green or yellowish-green maturation stage.

The elaboration process consists of the following steps. (1) The fresh olives are debittered with a diluted NaOH solution (lye) until the chemical reaches two-thirds of the pulp; the alkali hydrolyzes oleuropein (responsible for the natural bitterness of this fruit) into hydroxytyrosol and elenolic acid glycoside. (2) After the alkaline treatment, the olives are washed (one to three times) with tap water to remove any NaOH excess. (3) The fruits are placed in a NaCl brine, where they undergo a lactic fermentation process (*1*).

Traditionally, the harvesting of olives is done by hand using a technique known as "milking the tree", and the cost of this operation accounts for 50−70% of the cost of raw materials (*2*). Currently, mechanical harvesting, using big machines that shake the olive tree or smaller machines that move the branches of the tree, is carried out only on cultivars with low sensitivity to bruising (*2*, *3*).

The fruits of the Manzanilla, which is the most common cultivar for preparing green Spanish-style table olives, cannot be picked using mechanical harvesting because they are very prone to the formation of brown spots due to the blows that the fruits receive during the operation, which remain even after the complete fermentation process. As a result, the final product obtained is of poor quality or, in fact, unmarketable because of its unpleasant appearance.

The browning reaction, which results from mechanical injury during postharvest storage or processing of fruits and vegetables, is a widespread phenomenon. Polyphenol oxidase (PPO) is the main enzyme that causes browning. PPO catalyzes the oxidation of *o*-dihydroxyphenols to *o*-quinones (*4*). The quinones then condense to form darkened pigments (*5*). Some nonenzymatic causes of browning in foods include the Maillard reaction, autoxidation reactions involving phenolic compounds, and the formation of iron−phenol complexes (*6*).

PPO has been studied in several fruits, such as apples (*7*), bananas (*8*), grapes (*9*), raspberries (*10*), and strawberries (*11*). In the olives of Manzanilla and Gordal cultivars the PPO activity profile was established according to pH (*12*, *13*) and monitored during growth (*14*, *15*).

The favorable effect of cold storage to prevent skin color changes in olives has been demonstrated recently (*2*, *16*), and the beneficial effects of solutions of diverse substances (citric, ascorbic acid, and sodium bisulphite) on the phenolic browning reaction in other products such as artichokes (*17*), mushrooms (*18*), and litchi fruits (*19*) are also well-known.

In this work, the olive PPO was kinetically, thermodynamically, and isokinetically characterized. In addition, the inhibition

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of enzyme activity as a function of pH, temperature, and ascorbic acid was studied. The best conditions obtained from the in vitro experiments for the inhibition of the enzymatic and nonenzymatic reactions were validated by applying them to control the appearance of brown spots on bruised fruits after their elaboration as Spanish-style green olives.

MATERIALS AND METHODS

Fruits. The olives used in this study were of the Manzanilla cultivar (*Olea europaea pomiformis*). Fruits were harvested by hand in Coria del Rio, Sevilla (Spain), during the 2004/2005 season. Only fruits with the optimal green-yellow surface color (green maturation) were chosen for the experiment in order to carry it out with homogeneous material. The experiment was done with fruits collected in mid-September and mid-October.

The time elapsed from hand harvesting to the beginning of the trials ranged from 1 to 2 h.

To reproduce the bruises caused by mechanical harvesting, a pilot plant scale device was developed. It consisted of a sorting machine with a wooden block of 30 × 20 × 10 cm and weight of 2.5 kg, maintained at a fixed distance above a moving belt. The block was covered on its bottom surface with a piece of metallic mesh formed by 0.6 cm squares of 0.6 mm diameter wire, fixed to the surface so that there was continuous contact between the surface of the wooden block and the mesh. The fruits were passed along the space left between the "floating" wooden block and the belt of the sorting machine. The distance between the sorting machine belt and the wooden block was regulated according to the olive size. The fruits were swept by the movement of the sorting machine belt. The procedure was checked to produce always homogenously distributed bruises on the olives and to cause damages similar to those observed in mechanical harvesting.

Extraction and Measurement of PPO Activity. The method used for the isolation and characterization of PPO was based on those described by Sciancalepore and Longone (*13*) and Hornero et al. (*15*) for olives.

Enzymatic Extract. A protein precipitate (acetone powder) of the fruit was prepared from 50 g of triturated unbruised pitted olive fruit paste extracted once with 100 mL of acetone containing 2.5 g of polyethylene glycol at −30 °C. The residue was re-extracted three times with 100 mL of acetone at −30 °C, dried, and weighed. This acetone powder residue (0.5 g) was resuspended in 25 mL of phosphate buffer 50 mM, pH 6.2 (extraction buffer), containing 1 M KCl. The suspension was stirred at 4 °C for 30 min and then centrifuged at 20000*g* for 20 min at 4 °C. The pellet was discarded and the supernatant divided in two aliquots; one was used as the active enzymatic extract, and the other was boiled for 20 min to obtain the denatured enzymatic extract.

Substrate. A stock solution 0.993% (w/v) of 4-methylcatechol in deionized water was prepared and kept in the dark at 4 °C until use.

Reaction buffer was prepared from a mixture of phosphate and borate, 0.2 M each, and adjusted with HCl or NaOH at pH from 1.0 to 12.0 in an interval of 1 unit.

Reaction Buffer with Ascorbic Acid. Ascorbic acid was added to aliquots of the reaction buffers at all of the studied pH values until a final concentration of 0.2% was reached.

Reaction Conditions. For each studied pH, the reaction was performed at the following temperatures: 8, 12, 16, 20, and 25 °C

The enzymatic reaction was performed in a thermostated spectrophotometric cuvette. The reaction mixture comprised 0.25 mL of enzymatic extract, 0.25 mL of substrate, and 2.5 mL of reaction buffer. The increase in absorbance at 410 nm (ΔAbs) was measured from 12 to 48 s. As a blank of the enzymatic reaction, 0.25 mL of substrate, 0.25 mL of denatured enzymatic extract, and 2.5 mL of reaction buffer was used. The increase in absorbance in this solution was measured simultaneously with that of the solution containing the active enzyme. All reactions were carried out in triplicate.

Before the enzymatic activity was measured, and to prevent any deviation from the conditions fixed for each experiment, the buffers and the reactants were stored for 24 h at the respective temperature of each treatment to allow them to equilibrate at that temperature before being mixed.

Kinetic, Thermodynamic, and Isokinetic Study of PPO. All reactions were carried out using an excess of substrate to guarantee the saturation of the enzyme. The monitoring of the reaction showed that it followed a zero-order kinetic model. The integrated equation of the corresponding reaction rate was

$$C = C_0 + K_v t \tag{1}$$

Plotting k_v/T versus $1/T$ in a semilogarithmic scale, the parameters corresponding to the transition state theory—the enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) (eq 2)—were obtained as follows:

$$\ln\left(\frac{K_v}{T}\right) = \ln\left(\frac{K}{h}\right) + \frac{\Delta S^{\ddagger}}{R} - \frac{\Delta H^{\ddagger}}{RT} \tag{2}$$

The pairs of thermodynamic values obtained were linearly correlated according to the equation

$$\Delta G_{isok} = \Delta H - T_{isok}\Delta S \Rightarrow \Delta H = T_{isok}\Delta S + \Delta G_{isok} \tag{3}$$

which yields the Gibbs free energy of the reaction (ΔG_{isok}) for the isokinetic temperature reaction (T_{isok}) (*20*, *21*).

Study of the Nonenzymatic Browning Reaction. The extension of the nonenzymatic browning reaction was evaluated by comparing the increase in absorbance observed with the mixture of reaction buffer−substrate−denatured enzymatic extract versus a similar one prepared with active enzymatic extract. The concentration of all reactants was the same as those used in the PPO study with a total final volume of 30 mL. The reaction medium was constantly stirred during the period that each measurement lasted. From each solution, for 10 min, a sample of 3 mL was removed every minute to measure the absorbance at 410 nm. The experiment was carried out only under the extreme conditions of temperature (8 and 25 °C) and pH (3.0 and 10.0 units) used in the PPO study, conditions at which the enzyme showed no activity or only a reduced proportion of it. These conditions can improve the estimation of the effects of the variables involved in the oxidation process (temperature and pH). Additionally, the highest temperature (25 °C) and pH (10.0) mimic the conditions of green olive postharvest and some light lye treatments currently applied to mechanically harvested olives, which prevents browning for a limited period of time.

Treatments To Diminish Bruises in Olives. The solutions used for the treatments consisted of (i) tap water, (ii) tap water with the initial pH corrected to 2.0 with HCl (2 N), and (iii) tap water with the pH adjusted to 2.0 with HCl (2 N) with 0.2% (w/v) ascorbic acid added.

Two kilograms of fruits, which had been bruised or not, were placed in PVC cylindrical vessels and covered with 1.5 L of the treatment solutions for 24 h at ambient temperature (25 °C). The same experiment was repeated in a cold room at 8 °C, using liquids that had been previously conditioned to this temperature. To be later used as reference, another 2 kg of bruised, or not, fruits was also stored following the traditional system (*1*), that is, maintaining them open to the air, at ambient temperature (25 °C), for 24 h. All treatments were carried out in duplicate.

Processing of Olives. After the above-mentioned period of 24 h, the olives were put in a 2.5% (w/v) NaOH solution until the chemical reached two-thirds of the distance from the skin to the pit (5.5 h). Then, the olives were washed with tap water for 20 h and, finally, covered with a 9% (p/v) NaCl solution. After 24 h of brining, CO_2 was bubbled for 15 min through all treatments to control the pH to 5.5−6.0. Two days later, the containers were inoculated with a starter culture of *Lactobacillus pentosus* IGLAC01 previously isolated from table olives.

Analysis of Olives and Solutions. Fermentation control (pH, free acidity, and combined acidity) was carried out using the routine methods described by Garrido Fernández et al. (*22*).

The surface color of the fruits was measured using a BYK-Gadner model 9000 color view spectrophotometer (Silver Spring, MD). Interference from stray light was minimized by covering samples with a box, which had a matt black interior. Measurements were carried out using the illuminant "*C*" at 10°. Color was expressed in terms of the CIE *L**, *a**, *b** parameters or as color index, calculated

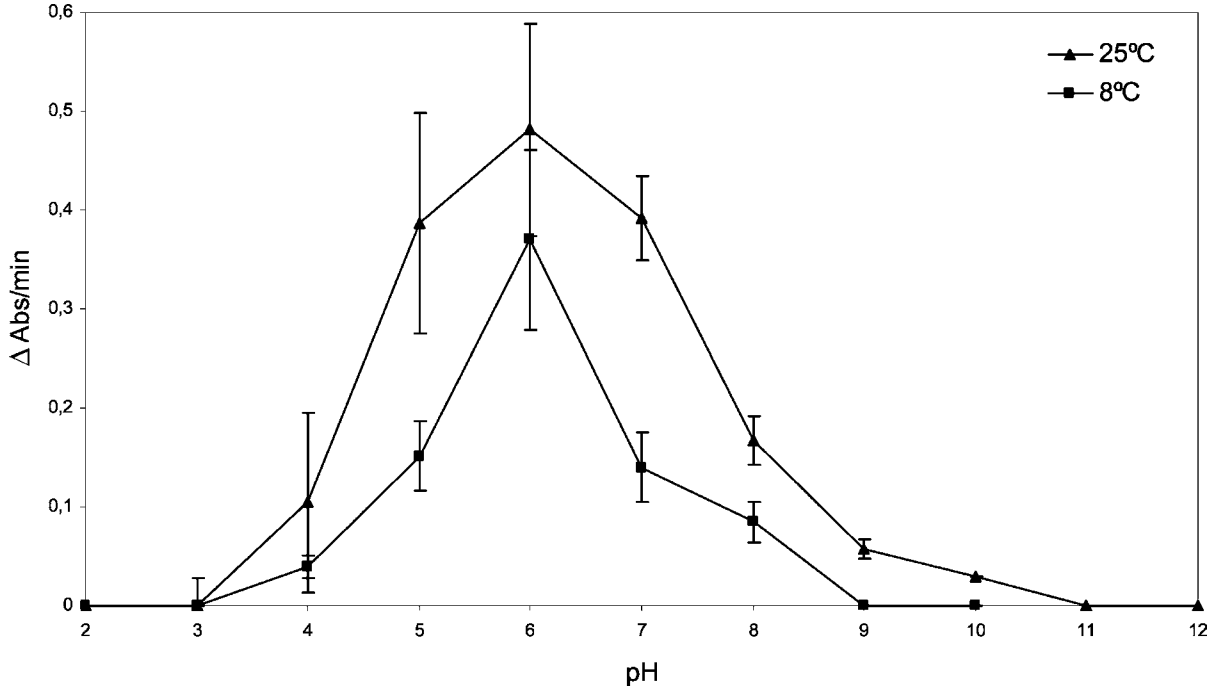

Figure 1. Enzymatic oxidation of 4-methylcatechol by the crude extract of PPO from olives in phosphate–borate buffer at different pH and temperature values.

according to the equation of Sánchez et al. (*23*)

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

where R stands for the reflectance at 560, 590, and 635 nm, respectively. Data were the average of 20 olives in each duplicate experiment.

Color determinations were carried out, at the end of the fermentation period (150 days), by measuring the appropriate parameters on the bruised areas (bruised fruits) or on the whole fruit (unbruised olives).

Olive bruises were also scored at the end of the fermentation period (150 days) on a scale ranging from 1 to 5 points by a six-member trained panel, according to their visual evaluation of brownish intensity. Level 1 was established for fruits in which the bruises on the skin were slightly appreciable and level 5 for those having bruises that were intense and affected the flesh (unmarketable product due to its poor quality). To summarize the evaluation, the following subjective bruise index (D_i) was developed:

$$D_i = \sum_{(n=1...5)} (An \times n/100) \quad (5)$$

where n was the level of bruises, A_n the number of fruits evaluated in the n level, and 100 the total number of evaluated fruits.

Analyses for physicochemical characteristics were always carried out at least in duplicate.

RESULTS AND DISCUSSION

Enzymatic Activity. After measuring the enzymatic activity from pH 1.0 to 12.0 at 8 and 25 °C, it was observed that the maximum activity was systematically found at pH 6.0 (**Figure 1**) as described by Ben-Shalom et al. (*14*). The enzymatic activity increased with temperature (within the range studied) and was completely inhibited at pH values below 3.0 regardless of temperature. In alkaline conditions, the inhibitory pH level depended on temperature; at 8 °C it was observed at pH >9.0, whereas at 25 °C, inhibition occurred at pH values >11.0.

When ascorbic acid was present in the reaction medium, the absorbance did not increase at any pH or temperature studied (data not shown in **Figure 1**). This could be due to (i) the inhibition of the enzymatic reaction in the presence of the ascorbic acid and/or (ii) the ascorbic acid not allowing the quinones formed on the oxidation of the phenols to be condensed to form colored compounds responsible for the absorbance increase at 410 nm (*5*). In any case, the browning reaction apparently did not occur in the presence of ascorbic acid and, as a result, it may, at least theoretically, prevent browning in bruised olives.

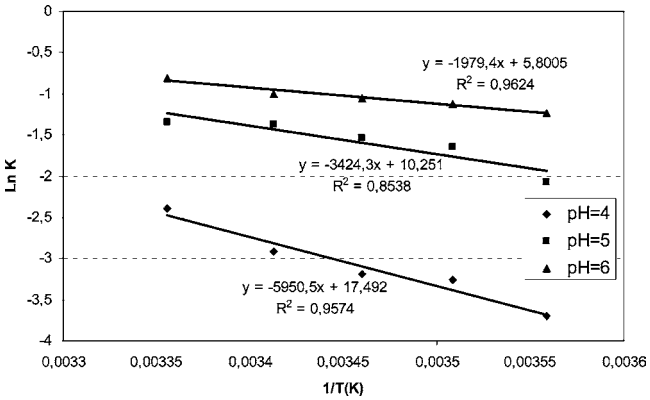

Figure 2. Arrhenius plot of the enzymatic oxidation of 4-methylcatechol by the crude extract of PPO from olives in phosphate–borate buffer at different pH values.

Kinetic, Thermodynamic, and Isokinetic Study. Figure 2 shows an Arrhenius plot of the effect of temperature and pH on PPO activity. At pH 6.0, a value close to optimum for the enzymatic activity of the PPO (**Figure 1**), the rate constants found were the topmost at all temperatures. The rate constant of the oxidation reaction of the 4-methylcatechol, for each studied pH, increased with temperature, as occurs in a reaction of Arrhenius type.

The thermodynamic constants of the enzymatic oxidation of 4-methylcatechol estimated for each studied pH are shown in **Table 1**. Theoretically, the reaction was always the same within the entire range of pH studied and, consequently, the thermodynamic parameters should be the same. However, it was found

Table 1. Thermodynamic Parameters of Enzymatic Reaction of in Vitro Browning at Different pH Values by the Crude Extract of PPO from Olives

	pH 4.0[a]	pH 5.0[a]	pH 6.0[a]
activation entropy ($J\ mol^{-1}\ K^{-1}$)	107.54 (20.7)	171.09 (22.6)	205.89 (6.39)
activation enthalpy ($kJ\ mol^{-1}$)	47.07 (6.1)	25.10 (6.8)	13.73 (1.8)
R^2	0.9532	0.8496	0.9180

[a] Standard deviation is given in parentheses.

that, at each pH, the reaction showed different thermodynamic parameters. These results led to the conclusion that the reaction followed different pathways at each pH, and there were formally three different reactions, or they were just one unique reaction but affected by the compensation effect (*24*).

In a compensated kinetic system, also known as an isokinetic system, the same reaction in different environments modifies at the same time and in a compensated way the energy required and the quantity of substrate available for the reaction. The equation of the curve defined by both thermodynamic values for the same reaction carried out under different conditions defines an isokinetic straight line that describes the reaction thermodynamically (*24*−*29*). This effect explains how changes in environmental factors, which do not formally affect the course of the reaction, can modify the thermodynamic parameters in a compensated way.

Plotting the activation enthalpy versus activation entropy for all levels of pH values studied, a straight line ($R^2 = 0.9997$) of slope 339.78 K and ordinate 84.68 kJ mol^{-1} was obtained. As a consequence, all of these parameters represent the same isokinetic reaction. The pH at which the enzymatic reaction was carried out affected its rate but not the energy required to carry it out.

Nonenzymatic Browning Reaction. The browning reaction in olives due to polyphenol oxidation may have an enzymatic and/or nonenzymatic origin (*6*). To check the contribution of the nonenzymatic reaction to the fruits' browning, the reaction was studied outside the optimal range of enzyme activity: at pH 3.0, at which the enzyme has no activity, and at pH 10.0, at which the detected activity was fairly low (**Figure 1**).

At pH 3.0 and 25 °C, in the presence of active or denatured enzyme extract, no increase in the absorbance at 410 nm during the reaction time was observed (data not shown). This indicated that, under these conditions, both the enzymatic and the nonenzymatic reactions were inhibited.

At pH 10.0 and 25 °C, a marked increase in absorbance with reaction time was found in the presence of the active enzyme (**Figure 3**). However, when the reaction was carried out in the presence of the denatured enzyme, an increase in the absorbance was still observed (**Figure 3**). These results indicate that, at pH 10.0, a remarkable proportion of absorbance increase was due to a nonenzymatic browning (produced in the absence of enzyme activity). Consequently, it is probable that a percentage of the browning reaction observed on bruised fruits at alkaline pH was due to a nonenzymatic reaction.

This effect of pH on the nonenzymatic oxidation rate of the olive phenols could be similar to that described for the darkening process during the elaboration of ripe olives (*30*), which was related to the formation of the characteristic brown-black color of this product (*31*).

When the same above-described browning reaction was carried out at a temperature of 8 °C, no increase in absorbance was observed, regardless of the pH (3.0 or 10.0) at which the reaction was performed. Thus, as previously demonstrated in this study, under these conditions, neither nonenzymatic nor enzymatic reactions were produced (**Figure 1**).

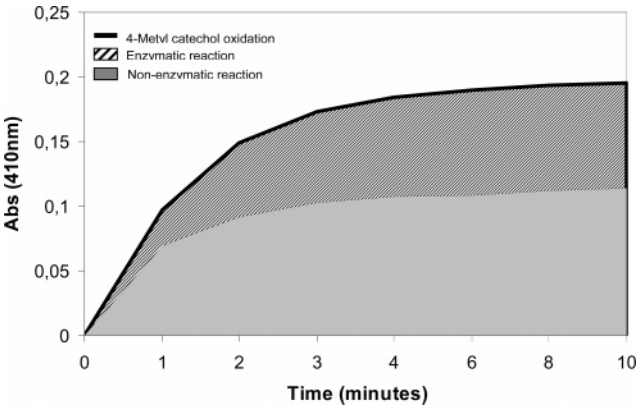

Figure 3. 4-Methylcatechol oxidation at pH 10.0 in the presence of active and denaturalized crude extract of PPO from olives.

Table 2. Color Index (C_i) and Bruise Index (D_i) of Olives from the Different Treatments Designed To Decrease the Browning Effect on Intentionally Bruised Fruits

		$C_i^{a,b}$		
treatment	temp (°C)	unbruised olives	bruised olives	$D_i^{a,b}$
traditional procedure (air)	25	28.89 (1.40)a	19.53 (0.21)c	4.22 (0.20)c
water	25	25.27 (0.94)b	25.02 (1.14)b	3.02 (0.31)b
	8	29.45 (1.02)a	28.79 (1.60)a	2.42 (0.39)a
water (pH 2.0)	25	26.54 (1.02)b	26.54 (1.03)b	3.18 (0.21)b
	8	29.54 (0.48)a	28.51 (0.72)a	2.53 (0.45)a
ascorbic solution	25	26.34 (0.68)b	26.34 (0.79)b	3.19 (0.36)b
	8	28.44 (0.57)a	29.72 (2.01)a	2.34 (0.19)a

[a] Standard deviation is given in parentheses. [b] Column values followed by the same letter do not differ at the 5% level of significance according to Duncan's multiple-range test

Thus, the browning of olive bruises caused by mechanical harvesting or any other handling procedure may be prevented at any temperature by maintaining the fruits in acidic solutions at pH 3.0 or lower because, under these conditions, PPO and nonenzymatic oxidations are inhibited. The protective effect may be stronger when ascorbic acid was added, which prevent quinones' condensation (*5*), and/or the temperature was decreased to 8 °C.

Treatments To Diminish Bruises on Olives. The values of the objective determination of olive color and the subjective visual evaluation of bruises in the different experiments described under Materials and Methods are shown in **Table 2**. Data are the means of the results from olives collected in mid-September and mid-October (two replicates of each experiment × two harvesting dates = four samples).

Keeping unbruised fruits at room temperature (25 °C), regardless of the treatment to which they were subjected, always resulted in a lower color index (C_i) ($p < 0.05$) (**Table 2**) than storing them in open containers before the lye treatment (traditional process) for the same period (24 h). Therefore, the immersion of unbruised fresh fruits in water or in any other of the studied storage solutions always led to a loss of color quality (smaller C_i values). This effect was also appreciated by observing the decrease in luminance values (L^*) and the increase

Table 3. Color of Olives (Expressed as CIE *L**, *a**, *b** Parameters) from the Different Treatments Designed To Decrease the Browning Effect on Intentionally Bruised Fruits

		unbruised olives[a,b]			bruised olives[a,b]		
treatment	temp (°C)	*L**	*a**	*b**	*L**	*a**	*b**
traditional procedure (air)	25	55.0 (0.6)a	3.4 (0.3)a	41.2 (1.4)abc	48.6 (0.7)b	5.5 (0.2)b	33.0 (1.4)b
water	25	50.7 (0.9)c	4.3 (0.4)b	41.5 (1.3)abc	50.7 (0.9)b	4.3 (0.4)a	41.5 (1.3)a
	8	54.9 (0.4)a	4.3 (0.3)b	43.2 (0.8)a	54.0 (0.9)a	4.2 (0.3)a	42.4 (1.1)a
water (pH 2.0)	25	50.1 (0.6)c	5.4 (0.2)c	39.8 (1.2)abc	50.1 (0.6)b	5.4 (0.2)b	39.8 (1.2)a
	8	55.2 (1.5)a	4.1 (0.6)ab	42.5 (1.8)a	53.3 (1.0)a	4.2 (0.1)a	42.8 (1.6)a
ascorbic solution	25	49.4 (0.2)c	5.6 (0.4)c	39.2 (0.2)abc	49.4 (0.2)b	5.6 (0.4)b	33.1 (0.8)b
	8	52.9 (0.8)b	4.5 (0.2)b	42.2 (0.4)ab	53.9 (1.4)a	4.3 (0.4)a	43.6 (0.9)a

[a] Standard deviation is given in parentheses. [b] Column values followed by the same letter do not differ at the 5% level of significance according to Duncan's multiple-range test.

in red tonality (greater values of *a**) but not in *b** because no differences were observed for this parameter (**Table 3**).

However, if the liquids were maintained at 8 °C, no statistically significant differences were observed in the color index (C_i) between olives maintained in solutions with respect to those that followed the traditional procedure (**Table 2**). In fact, only small changes in the CIE parameters were observed with respect to those obtained following the traditional process (**Table 3**): *a** values were higher, but *b** values (yellowness) and luminance (*L**) were statistically the same, except for *L**, which was lower ($p < 0.05$) when the olives were immersed in a solution of ascorbic acid.

When the color evaluation was made on the bruises of intentionally bruised fruits, the worst ($p < 0.05$) color index (C_i) corresponded to those olives that followed the traditional process (**Table 2**). This was due to the loss of luminance because of browning and the changes produced in the other parameters: the values of *a** increased and moved toward a redder region, whereas the *b** values decreased and moved toward a less yellow region. These changes can also be correlated with the appearance of a brown tonality on the bruised surface of olives.

The immersion of the bruised fruits in water or in solutions at room temperature (25 °C) partially prevented such browning, leading to higher C_i values ($p < 0.05$) in the bruised area than those that followed the traditional procedure (**Table 2**). The trend observed in the CIE *L**, *a**, *b** values were in agreement with these observations and presented values that were statistically significantly different in some cases, a fact that confirmed the better evaluation of the color of submerged fruits (**Table 3**).

When the olives were immersed in liquid and kept at 8 °C, they had the same color index (C_i) on the bruised areas (**Table 2**) and CIE *L** and *b** values (**Table 3**) as nonbruised fruits processed according to the traditional system, but values of *a** were slightly higher (around 4.2, which means a more intense red color) on bruised than on nonbruised olives (3.4). As a result, the immersion of the olives in water at 8 °C prevented the browning of the bruised areas.

The visual observation of bruises on the fruits and the calculation of the bruise index (D_i) leads to conclusions similar to those previously described for an objective color analysis (C_i and CIE *L**, *a**, *b** parameters). The highest scores (olives in which the bruises were more evident and, as a result, their quality was worse) were given to the fruits that followed the traditional process (**Table 2**). Bruises were less appreciable (smaller D_i values, $p < 0.05$) when the fruits were kept at 8 °C.

With respect to the effect of the different solutions on the browning of bruises, within each temperature, no statistically significant differences ($p < 0.05$) were observed between those using tap water with the initial pH corrected to 2.0 units by the addition of HCl and those with dissolved ascorbic acid (**Table 2**).

In previous in vitro experiments, the use of solutions with a pH lower than 3.0 or with ascorbic acid added resulted in the inhibition of the enzymatic reaction; furthermore, at this pH or lower, the nonenzymatic oxidation of phenols was also prevented but, in the experiments accomplished with olives, differences between the effect of using tap water or acidic solutions were insignificant. However, when the fruits are handled under real conditions, other factors may also affect the reaction, especially the difficulties that the reactants must overcome to reach the reaction point. The immersion of the olives in solutions apparently decreased the amount of oxygen that came into contact with the bruised areas and prevented enzymatic or nonenzymatic browning. However, during the time elapsed from the moment the liquid was poured off and the lye was added, oxygen freely reached the bruised areas and reactivated the browning reactions. As was demonstrated in vitro, the enzymatic reaction was practically inhibited in cold fruits (8 °C) and, as a consequence, the browning of the blemished areas was markedly less intense than when the fruits were maintained at room temperature.

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