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Treatments to inhibit the browning reactions in model solutions of olive fruit extracts

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ABSTRACT

Crude enzymatic and polyphenol extracts from olives were subjected to reaction at diverse buffered pH values. At pH values similar to those found in olive flesh (pH 5.0), the colour of the solution increased with reaction time and, simultaneously, a consumption of phenols and oxygen in the reaction medium was observed, especially under aerated conditions. In alkaline solutions (pH 12.4), a strong initial solution browning was noticed while the phenols from the extract reacted with the oxygen present in the solution and caused a significant decrease in the total phenols and oxygen concentrations. This darkening was due to the appearance of non-enzymatic browning reactions since, at this pH, the polyphenoloxidase is not active. Under acidic conditions (pH 3.0), and in solutions with ascorbic acid added (100 mM), the consumption of oxygen and total phenols was negligible, under both aerated and non-aerated conditions; as a result, no significant changes in the colour of the solutions were observed. So, low pH or ascorbic acid solutions may be useful to prevent browning in the bruised areas of hand- or mechanically-harvested table olives.

1. Introduction

The browning reaction, which results from mechanical injury during post-harvest storage or processing of fruits and vegetables, is a widespread phenomenon. The fruits of the Manzanilla cultivar, which is the most common 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 (Ferguson, 2006; Kouraba, Gil-Ribes, Blanco-Roban, de Jaime-Revuelta, & Barranco-Navero, 2004). As a result, the final product obtained is of poor quality or, in fact, unmarketable because of its unpleasant appearance.

The mechanism of the browning reaction in olives has been demonstrated using *in vitro* models (Segovia-Bravo, Jaren-Galan, García-García, & Garrido-Fernández, 2009). First, there is an enzymatic release of hydroxytyrosol from oleuropein and hydroxytyrosol glucoside due to the action of esterase and β-glucusidase enzymes present in the olive fruit (Briante et al., 2002; Fernández-Bolaños, Rodríguez, Guillén, Jiménez, & Heredia, 1995). Simultaneously, an additional hydroxytyrosol release can also be produced through the chemical hydrolysis of oleuropein (Brenes, García, Durán, & Garrido, 1993; Gikas, Papadopoulos, & Tsarbopoulos, 2006), although this reaction is slower than the enzymatic one. In a second step, the initially present hydroxytyrosol and that released in the enzymatic and chemical reactions, together with verbascoside, are oxidised by the polyphenoloxidase from the fruit itself. The whole process leads to fruit browning. A chemical oxidation of hydroxytyrosol (García, Brenes, Vattan, & Garrido, 1992) may also occur simultaneously but its contribution to deterioration, due to the pH values of the olive flesh (around 5 units) is very limited when compared with the effect caused by the enzymatic action.

This *in vitro* mechanism was in agreement with results obtained in bruised olives. In this case, oleuropein was also the compound that decreased in a higher proportion during the post-harvest period (Segovia-Bravo et al., 2009).

The maximum activity of the PPO of olive Manzanilla cv was at pH 6.0. This activity was completely inhibited at a pH below 3.0, regardless of temperature; however, under alkaline conditions, pH inhibition depended on temperature and was observed at values above 9.0 and 11.0 at 8 and 25 °C, respectively (Segovia-Bravo, Jaren-Galan, García-García, & Garrido-Fernández, 2007).

The beneficial effects of diverse substances (citric, ascorbic acid, and sodium bisulphite) on the phenolic browning reaction in different products, such as lettuces (Altunkaya & Gökmen, 2008), potatoes (Limbo & Piergiovannni, 2006) and pears (Arias, González, Oria, & López-Buesa, 2007), are also well known.

The purpose of this work was to study treatments that were able to inhibit the browning reaction in the Manzanilla cultivar, using *in vitro* model solutions and conditions under which the

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PPO enzyme was not active: that is acidic (pH 3.0) or strong alkaline medium (pH > 11.0) (Segovia-Bravo et al., 2007). Likewise, the use of ascorbic acid in the enzymatic reaction medium, as an antioxidant able to inhibit the action of the enzyme, was also particularly considered.

2. Materials and methods

2.1. 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) in mid-September, during the 2007 season. Only fruits with the optimal green–yellow surface colours (green maturation) were chosen for the experiment, in order to carry it out with homogeneous material.

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

2.2. Extraction and purification of crude enzymes

A protein precipitate (acetone powder) of the fruit was prepared from 50 g of triturated unbruised pitted olive fruit pastes 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 and the pellet obtained was finally dried and weighed. 0.5 g of this acetone powder was re-suspended in 25 ml of 50 mM phosphate buffer, pH 6.2 (extraction buffer), containing 1 M KCl. The suspension was stirred at 4 °C for 30 min, and then centrifuged at 20,000 *g* for 20 min at 4 °C. The pellet was discarded and the supernatant divided into two aliquots, one was used as the active enzymatic extract (Hornero-Méndez, Gallardo-Guerrero, Jarén-Galán, & Minguéz-Mosquera, 2002; Sciancalepore & Longone, 1984), and the other to obtain denatured enzymatic extract by boiling the aliquot of the supernatant for 20 min. The enzymatic extracts were stored at 4 °C and 2 h before their use were brought to room temperature (25 °C).

2.3. Extraction and purification of phenolic extract

A phenolic extract of olives was obtained, following the procedure described by Romero, Brenes, García, and Garrido (2002). The method consisted of extracting the phenolic compounds with a solution of methanol/water plus 100 mg/l of the sodium salt of diethyldithiocarbamic acid. A C_{18} cartridge was used to purify the phenolic extract. To quantify the different phenols, syringic acid was added as internal standard. The phenolic extract was frozen at −30 °C and 2 h before its use was brought to room temperature (25 °C).

2.4. Reaction medium

Four different media were prepared:

a) *Control* (pH 5.0) this consisted of a buffer solution composed of a mixture of 0.2 M phosphoric and boric acids, adjusted with addition of NaOH solution (10 N) (Segovia-Bravo et al., 2007); this value simulates the pH of the olive flesh (Garrido Fernández, Fernández Díez, & Adams, 1997).
b) *Acidic medium* (pH 3.0): this was prepared similarly to the previous buffer solution but the pH was adjusted to 3.0.
c) *Alkaline medium* (pH 12.4): this consisted of a water solution of NaOH 0.3% (w/v) which was prepared just before the experiment was carried out.
d) *Ascorbic acid* solution (100 mM): this was prepared by directly dissolving the appropriate amount of ascorbic acid 2 h before use. It was maintained in darkness until its utilisation.

All solutions were stored at room temperature (25 °C).

2.5. Experimental protocol

The enzymatic reaction took place in a 50 ml flask at room temperature (25 °C) after mixing 2.5 ml of crude enzyme extract solution and 25 ml of reaction buffer. The reaction was initiated by adding 2.5 ml of phenolic extract.

The solutions with the reactants were left alone (without aeration) or subjected to aeration to mimic the extreme situations (anaerobic and aerobic) that olives may eventually suffer post-harvesting when, after being beaten from the trees, they are transported to industries in aqueous solutions or perforated bins.

Aeration was achieved by placing the flask on a magnetic agitator which vigorously stirred the solution. In all experiments, the control position of the stirring equipment was maintained in the same position.

2.6. Measure of the browning reaction

During the reaction, coloured compounds were formed and the increase of absorbance during the reaction time, at a prefixed wavelength, usually 410 nm (Hornero-Méndez et al., 2002), was used to evaluate the browning progression.

The reaction was monitored for 10 min because, in previous tentative experiments, no changes were observed after this period. Every minute, 1 ml of the reaction solution was taken and placed into a 1 ml cuvette with 1 cm path length and the absorbance of the sample measured in a Cary1E Spectrophotometer (Varian, Mulgrave, Vi, Australia) at 410 nm. Once the analysis was accomplished, the liquid was returned to the flask.

In addition, colour was expressed in terms of the CIE L^* (whiteness or brightness/darkness), a^* (redness/greenness), b^* (yellowness/blueness); from these values, chroma ($C = \sqrt{a^{*2} + b^{*2}}$) and hue angle ($H = \arctan(b^*/a^*)$) were also calculated. In the evaluation of h, the widely accepted international criterion of assigning the angle of 90° to the semi axis $+b^*$ (yellowness) was followed.

All experiences were carried out at least in duplicate.

Statistica software version 6.0 (StatSoft, 2001) was used for data analysis. Comparison between treatments were carried out by Duncan's multiple range tests and differences were considered significant when $p < 0.05$.

2.7. Measure of the products consumed during the browning reaction

During the coloured compounds formation, oxygen and the phenolic compounds, initially present in the reaction medium, were consumed and their changes monitored.

Oxygen uptake in the solution was recorded continuously during the 10 min of reaction, using a Jenway 9200 DO_2 meter (Barloworld Scientific Ltd., Essex, UK).

For the evolution of phenols during the reaction time, periodically 1 ml of the reaction solution was transferred to an Eppendorf tube with 0.1 μl of phosphoric acid and analysed.

2.8. Analysis of phenols

The concentrations of the phenolic compounds were evaluated by an HPLC system, following the method described by Romero et al. (2002). In brief, the phenolic extracts obtained from the olives