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Optimization of the natural debittering of table olives

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Olives can debitter naturally without the use of NaOH but it is a very slow process. The purpose of this work was to evaluate the influence of both temperature and chemical characteristics of brine on the oleuropein hydrolysis rate in natural table olives. Two different phases were established for natural debittering. During the first 1–2 months of brining, a low concentration of NaCl (60 g/L) and acetic acid (2 g/L) together with a low storage temperature (10 °C) were the processing conditions that promoted a rapid hydrolysis of the bitter phenol because these mild conditions facilitated the action of endogenous enzymes (*β*-glucosidase and esterase). Thereafter, higher concentrations and temperature of storage (140 g/L NaCl, 16 g/L acetic acid and 40 °C) favored the chemical hydrolysis of oleuropein during long term (a few months) storage. These results will contribute to the knowledge of the natural debittering of table olives and they will help processors to accelerate their elaboration methods.

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1. Introduction

The production and consumption of organic and/or natural foods has greatly increased but it has not occurred with table olives. Among table olive elaborations, the most natural trade preparation consists of a direct immersion of the olives in acidified brine for months, during which the bitterness is reduced by the diffusion of the oleuropein from the olive flesh into the surrounding brine (Arroyo-López et al., 2005; Romero, Brenes, García, García, & Garrido, 2004). This process takes a long time and this is one of the reasons why the table olives industry does not produce a large amount of these organic/natural olives.

Oleuropein is a phenolic compound in olives (*Olea europaea* L.) which possesses a strong bitter taste and is present in a high amount in unprocessed olive fruit (Ramírez, Medina, Brenes, & Romero, 2014). During the processing of fruits, enzymatic and chemical reactions can occur on the oleuropein molecule (Ramírez, Brenes, García, Medina, & Romero, 2016). The main objective of any elaboration of table olives is the transformation of this bitter glucoside into non-bitter compounds to obtain a more palatable product. It can be achieved rapidly in a matter of hours by treating the olives with a dilute NaOH solution (Brenes & de Castro, 1998).

Natural green olives are directly placed in brine without any NaOH treatment, the polyphenol compounds are eliminated by diffusion from the pulp into the brine but it takes months and depends on the salt concentration of the medium (Poiana & Romeo, 2006; Fadda, del Caro, Sanguinetti, & Piga, 2014). The acidic conditions of the brine can also favor chemical hydrolysis of oleuropein (Gikas, Papadoloulos, & Tsarbopoulos, 2006; Medina et al., 2008; Servili et al., 2006, pp. 3869–3875). Recently, the involvement of endogenous enzymes such as esterase and *β*-glucosidase in olive debittering has been demonstrated during the first month of brining (Ramírez et al., 2016), and it was also suggested that the hydrolysis of this polyphenol can be achieved by the action of the exogenous hydrolases excreted by the strains of lactic acid bacteria (Ciafardini, Marsilio, Lanza, & Pozzi, 1994; Servili et al., 2008, pp. 6389–6396). A pre-selection of fermentation starters (yeast and lactic acid bacteria strains) for their ability to produce β-glucosidase in model brines of natural black olives has recently been carried out (Bleve et al., 2014; 2015).

Nevertheless, the influence of the components of the brine, sodium chloride and acid concentration, and the storage temperature on the enzymatic hydrolysis of oleuropein has never been studied. The common range of pH and temperature during the processing of natural green olives are 3.9–4.3 and 18–26 °C, respectively in Spain and many other countries worldwide. Maximum *β*-glucosidase activity is reached at a pH close to 5 units (Kara, Sinan, & Turan, 2011; Ramírez et al., 2014; Romero-Segura, Sanz, & Pérez, 2009), and esterase activity decreases to a large extent at a lower pH (Ramírez et al., 2014). The optimal temperature for *β*-glucosidase activity is cultivar dependent and maximum

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activity is reached at 10 and 30 °C in the Gordal cultivar, whereas it is found at 40–45 °C for Picual and Hojiblanca cultivars (Kara et al., 2011; Ramírez et al., 2014). Low temperatures do not favor high esterase activities (Ramírez et al., 2014).

The objective of this study was to determine the influence of the brine components and storage temperature on the enzymatic hydrolysis of oleuropein and to a lesser extent on the chemical hydrolysis of this bitter polyphenol. Better knowledge of these reactions during the elaboration of natural table olives will allow the industry to develop a more effective debittering process.

2. Materials and methods

2.1. Raw material

Experiments were carried out using fruits of Gordal, Manzanilla and Hojiblanca cultivars (*Olea europaea* L.). All olives were harvested at the ripening stage corresponding to a green-yellow surface color from mid-September to mid-October in the province of Seville (Spain) during the 2011/2012, 2012/2013 and 2013/2014 seasons.

2.2. Reagents

Ethylenediaminotetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, *p*-nitrophenol (p-NP), *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG), *p*-nitrophenyl acetate (*p*-NPA), syringic and *p*-cumaric acids, dimethyl sulfoxide (DMSO) and acetic acid were supplied by Sigma Chemical Co. (St. Louis, USA).

Standards of hydroxytyrosol, tyrosol, oleuropein and rutin were purchased from Extrasynthese S.A. (Genay, France). Hydroxytyrosol-1-glucoside and comselogoside were quantified using the response factors of hydroxytyrosol and *p*-coumaric acid respectively. Salidroside and ligustroside were quantified using the response factor of tyrosol. Hydroxytyrosol-4-glucoside and dialdehydic form of decarboxymethylelenolic acid linked to hydroxytyrosol (HyEDA) were obtained by HPLC preparative system as described elsewhere (Brenes et al., 2000; Romero, Brenes, García, & Garrido, 2002).

2.3. Determination of the hydrolase activity

Acetone powders were obtained from 50 g of olive pulp homogenized with 100 mL of cold acetone (−30 °C) containing 2.5 g of polyethylene glycol (Sciancalepore & Longone, 1984).

The β-glucosidase activity analysis was based on the methodology proposed elsewhere (Ramírez et al., 2014). Briefly, 0.14 g of acetone powder were suspended in 10 mL of a 0.01 mol/L sodium carbonate buffer, containing 0.005 mol/L EDTA, 0.001 mol/L PMSF and 10 g/L of 2-mercaptoethanol. The pH was adjusted to 9.0 and the suspension was stirred at 4 °C for 1 h. After 20 min of centrifugation (15550*g*) at low temperature, the supernatant was used as the active crude enzyme extract. The β-glucosidase activity analysis was determined by monitoring the increase in absorbance at 405 nm for 30 min related to the increasing amount of p-NP liberated from the synthetic glucoside *p*-NPG. The evaluation was performed using a regression curve with p-NP. The enzymatic activity is expressed as nano katal/mL enzyme extract. All reactions were carried out in duplicate.

The esterase activity analysis was carried out on the enzyme extract obtained from the acetone powder as proposed elsewhere (Ramírez et al., 2014). 0.25 g of acetone powder were suspended in 10 mL of a 0.01 mol/L sodium borate buffer containing 0.005 mol/L EDTA and 0.001 mol/L PMSF. The pH was adjusted to 9.0 and the suspension was stirred at 4 °C for 1 h, after 20 min of centrifugation (15550*g*) at low temperature, the supernatant was ready to use as enzyme extract. The esterase activity was determined by continuously monitoring the increase in absorbance at 405 nm for 10 min at 40 °C related to the increasing amount of p-NP liberated from the synthetic ester *p*-NPA. The evaluation was performed using a regression curve with p-NP. The enzymatic activity is expressed as nano katal/mL enzyme extract. All reactions were carried out in duplicate.

2.4. Analysis of phenolic compounds

The extraction of phenolic compounds from the olive pulp was based on the methodology proposed elsewhere (Kumral et al., 2013). 10 g of olive pulp were mixed in an Ultra-Turrax homogenizer with 30 mL of DMSO and 0.25 mL of the supernatant were diluted with 0.5 mL of DMSO plus 0.25 mL of 0.2 mmol/L syringic acid in DMSO (internal standard).

The analysis of phenolic compounds in brines was carried out by mixing 0.25 mL of the brine, 0.5 mL of deionized water and 0.25 mL of internal standard (2 mmol/L syringic acid in water).

All samples were filtered through a 0.22 μm pore size nylon filter and an aliquot (20 μL) was injected into the chromatograph. The chromatographic system was supplied by Waters Corporation (Milford, USA), it consisted of a 717 plus autosampler, a 600 E pump, a column heater module, and a 996 photodiode array detector operated with Empower software. A 25 cm × 4.6 mm i. d., 5 μm, Spherisorb ODS-2 column, at a flow rate of 1 mL/min and a temperature of 35 °C were used in all experiments. Separation was achieved by gradient elution using (A) deionized water (pH 2.5 adjusted with 1.5 mL/L phosphoric acid) and (B) methanol. The initial composition was 90% A and 10% B. Chromatograms were recorded at 280 nm (Medina, Brenes, Romero, García, & de Castro, 2007).

The evaluation of each compound was performed using a regression curve with the corresponding standard. Analyses were performed in duplicate.

2.5. Chemical analyses

The juice of olives was obtained mixing 10 g of olive pulp and 10 mL of distilled water using an Ultra-Turrax homogenizer, the mixture was centrifuged at 6000 g for 5 min and the supernatant was ready to use.

The concentration of sodium chloride was analyzed in olive juice and brine as described elsewhere (Ramírez, Gandul-Rojas, Romero, Brenes, & Gallardo-Guerrero, 2015).

Acetic acid was analyzed in the olive juice and brine by HPLC as described elsewhere (Sánchez, de Castro, Rejano, & Montaño, 2000). The chromatographic system consisted of a 2695 Alliance that includes a quaternary pump, an automatic injector, a column heater module (30 °C), with the detection being performed with a 410 refractive index (40 °C of internal temperature). The entire system was operated with Millenium 32 software (Waters, Milford, USA). A 25 cm × 4.6 mm i. d., 5 μm, Spherisorb ODS-2 column was used and the separation was achieved by isocratic elution using water acidified with phosphoric acid (pH 2.5) as mobile phase. The flow rate was 1.2 mL/min and the volume injected was 20 μL. Quantification of acetic acid was made by using the reference compound obtained from a commercial supplier.

2.6. Effect of temperature on the oleuropein hydrolysis

Crude enzymatic extracts from Manzanilla and Gordal olives were used to study the thermal stability of β-glucosidase and

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